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DIAGNOSTIC EVALUATION OF 38 kDa GENE BASED PCR ASSAY IN DIAGNOSING SMEAR NEGATIVE PULMONARY TUBERCULOSIS

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ABSTRACT: The impact of smear negative tuberculosis presentation on transmission dynamics of the disease has long been ignored and is finally getting its due attention. For more than two decades, polymerase chain reaction (PCR) assays have been shown to supplant the conventional methods for the precise detection of tubercle bacilli in microscopically positive respiratory specimens. However, in smear negative disease, its utility is less clear. Therefore the present study is designed to explore the clinical significance of 38 kDa gene-based PCR assay in diagnosing smear negative pulmonary disease, a total of 214 sputum samples (142 from smear negative pulmonary tuberculosis patients and 72 from non-tuberculous subjects) were analyzed by culture and PCR. The sensitivity and negative predictive value of PCR ranged from 91.70% and 94.50% in bacteriologically negative cases to 100% in culture positive pulmonary specimens in contrast to 66.20% and 60% of culture. The positive predictive value of PCR test among smear negative culture positive and culture negative sputum samples was 96.90% and 93.60%, respectively when compared to culture (100%). Although, the specificity of PCR assay remained same irrespective of the culture status (95.80%) but below culture (100%), however, the diagnostic efficiency of PCR assay is 98.20% and 94.20% in culture positive and negative samples respectively, against culture (77.60%). Given that PCR showed high sensitivity as well as negative predictive value, it has the ability to offer accelerated case confirmation in suspected tuberculosis patients when clinical and microbiological diagnoses are not conclusive.

KEYWORDS: Culture, PCR, predictive values, pulmonary, smear negative.

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1.INTRODUCTION

Tuberculosis (TB) remains a global pandemic that still takes a terrible toll despite the introduction of inexpensive and effective chemotherapy for more than five decades. According to WHO, *Mycobacterium tuberculosis* kills nearly 1.7 million people a year with an annual addition of 10.4 million new cases [1]. TB has a profoundly damaging impact on developing countries, particularly India, where more than 6000 people develop disease and nearly 740 die of TB per day leading to 0.27 million causalities and 2.3 million new cases annually [2]. The traditional diagnostic approaches to TB include acid-fast bacilli (AFB) microscopy and culture. AFB smear microscopy, although simple and cost effective, has low sensitivity and specificity. Consequently, 30 – 60% of culture proven TB cases are missed with this conventional method [3, 4]. The gold standard for the diagnostic confirmation of TB is culture; however, it takes 6 – 8 weeks to assign a result to be positive or negative and gives frequent negative results in paucibacillary disease. Despite having modest sensitivity and specificity, sputum smear microscopy is still the hallmark for establishing the presumptive diagnosis of pulmonary tuberculosis in national and international TB control programmes. The underlying reason is that it is inexpensive and technically simple to accomplish under field conditions. In addition, the smear positive cases are considered to be highly contagious contributing to the majority of transmission of infection. Therefore, the identification and management of such cases would help to break the chain of transmission of this deadly disease. However, epidemiological studies reported that patients with smear negative presentation are also responsible for substantial transmission of infection [5, 6]. Although such patients are less infectious than smear positive cases, their overall contribution to the disease transmission is considerable because nearly half of TB patients present negative findings [7, 8]. Given that a substantial number of suspected TB patients fail to give a positive microscopy test, the onus of TB diagnosis, therefore, relies on the clinical acumen of a physician. However, the situation happens to be more complicated in patients with smear negative disease as they present varying clinical and radiological findings due to the smaller mycobacterial load [9, 10]. In this scenario, the decision of the clinicians to start or stop anti tuberculosis therapy (ATT) based on clinical grounds can affect treatment by either delaying it in patients who actually have TB or causing unnecessary empiric therapy to subjects without mycobacterial infection.This issue of diagnostic challenge can be addressed with the help of rapid and non-conventional tools like polymerase chain reaction which offer better sensitivity than microscopy and greater speed than culture [11, 12]. PCR has been successfully employed in the early detection and identification of tuberculosis patients, but most of these studies are attempted on microscopically confirmed specimens and revealed a high degree of specificity and sensitivity [13-15]. The available information is precious but is not adequate for showing their efficacy in the diagnosis of smear negative TB patients. Furthermore, since the endorsement of first point of care assay Xpert MTB/RIF by WHO with the expectation of robust diagnosis and timely institution of

ATT, around 23 million Xpert tests have been performed in 130 countries [16]. Although Xpert showed overall high sensitivity and specificity with microscopically positive pulmonary samples, however, its sensitivity has been lower & vacillating with paucibacilliary pulmonary involvement. The objective of the present investigation was to evaluate the clinical relevance of PCR for positive identification of smear negative tuberculosis disease where clinical diagnosis is ambiguous and thus if remain under diagnosed can contribute to the ongoing transmission of infection.

2. MATERIALS AND METHODS

Clinical samples

Two hundred and fourteen sputum samples were obtained from as many individuals visiting DOTS centers at Gulab Devi TB hospital, Jalandhar; Yadav Chest Clinic, Jalandhar; Sri Guru Ram Das University of Health Sciences, Amritsar; TB and Chest Hospital, Government Medical College, Amritsar; Civil hospital, Gurdaspur and Batala.; before the start of ATT. Name, age, sex, history of ATT, family history of ATT, clinical presentation, clinician's final diagnosis and subsequently ATT response were recorded of each patient. Informed consent was obtained in writing from all the participants and the study was approved by the Ethics committee of the institution. The samples were processed for analyzing the presence of *M. tuberculosis* by conventional and PCR methods.

Microbiological processing and analysis of clinical samples

The sputum samples were decontaminated using standard mycobacteriological guidelines and concentrated by centrifugation at 10,000 rpm for 15 minutes. The pellet was washed with equal volume of sterile double distilled water, vortexed and recentrifuged at 10,000 rpm for 15 minutes [17]. The sediment obtained was collected for further use. A small aliquot of the decontaminated sediment was inoculated onto Lowenstein-Jensen (L-J) slants, in duplicate, and incubated at 37°C for at least 8 weeks. Date of inoculation and date of appearance of mycobacterial colony was recorded.

PCR analysis

Isolation of mycobacterial DNA from another aliquot of processed specimens was achieved by modified freezing and thawing protocol [18]. PCR assay was performed using in house forward primer 5'-CTACCCGCTGTTCAACCTGT-3' and reverse primer 5'-CTTTTCCGTTCAGCTTGAGG-3' (Sigma-Aldrich Inc., MO, USA) to amplify a 263 bp fragment of the species specific 38 kDa antigen coding gene of *M. tuberculosis*. The amplification reaction mixture contained 10mM Tris (pH 9.0), 50mM KCl, 0.01% gelatin, 1.5mM $MgCl_2$, 50µM of each dNTP, 200nM of each primer and 0.5U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a total reaction volume of 25µl. The reaction mixture was initially denatured at 94°C for 3 min and then subjected to 35 cycles comprising denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 30s followed by a final extension at 72°C for 3 min. Finally, the PCR products were detected on 2% agarose gel stained with 0.5µg/ml of ethidium bromide.

Data analysis

Statistical analysis was carried out employing SPSS ver. 10 for windows software (SPSS Inc. Chicago, IL, USA). The diagnostic methods were compared in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficiency.

3. RESULTS AND DISCUSSION

A total of 214 clinical samples were examined for the presence of *M. tuberculosis* by conventional culture and PCR methods. On the basis of ATT response, 142 of the clinical specimens were tuberculous and 72 were from non-TB subjects. The distribution of subjects based on age, sex, history of TB and family history of disease is shown in Table 1. In the studied groups, population was dominated by males. Irrespective of the disease and sex, 50% of the patients were in the age group of 21-40 years. None of the non-TB patients had prior exposure to tubercle bacilli.

Table 1: Demographic characteristics of subjects

Demographic features	Subjects (n = 214)	
	PTB patients (n = 142)	Non-TB controls (n=72)
Age in years (Mean ± S.D)	12 – 70 (34.75 ± 14.08)	16 - 70 (37.14 ± 12.62)
Sex; M/F	88 / 54	40 / 32
ATT history; Yes/No	11 / 131	00 / 72
Contact history; Yes/No	35 / 107	00 / 72

PCR Vs Culture in smear negative tuberculosis disease

Table 2 summarizes culture and PCR results in patient population. Evidently, In addition to the AFB microscopy, the direct detection of *M. tuberculosis* was missed by culture in 34% of the sputum samples. In house PCR assay, however, besides detecting positively all the culture positive cases, could detect the mycobacterial genome in 92% of bacteriologically negative samples, which could not be detected with either conventional microbiological assay. Additionally, positive PCR amplification was found in 4% of smear and culture negative individuals who were declared not to have any clinical form of tuberculosis as per the diagnostic paradigm of DOTS. However the intensity of PCR products from non TB subjects was significantly lower than those of the active disease cases (Figure 1).

Table 2: Amplification of 38 kDa gene of *M. tuberculosis* in patient population

Patient group (n)	Culture status (%)	PCR status	
		PCR Positive (%)	PCR Negative (%)
Smear negative P TB (142)	Culture positive 94 (66.20)	94 (100)	00 (0.00)
	Culture negative 48 (33.80)	44 (91.70)	04 (08.30)
Non TB (72)	Culture positive 00 (0.00)	00 (0.00)	00 (0.00)
	Culture negative (Sputum) 72 (100)	03 (04.20)	69 (95.80)

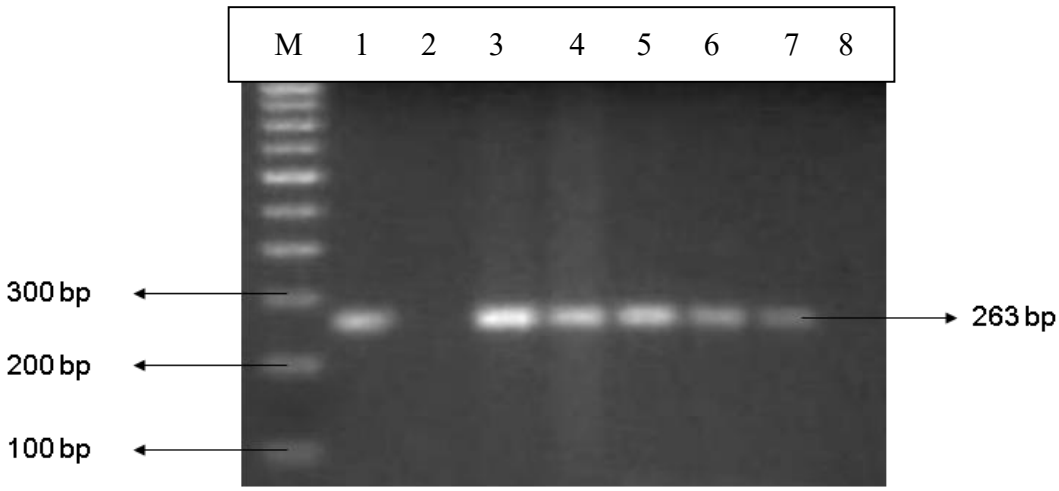

Figure 1: Amplification of 38 kDa gene in pulmonary samples from patient population. Lane M: 100 bp ladder; Lanes 1 and 2: Positive and negative controls, respectively; Lanes 3 and 5: Neat DNA samples from smear negative culture positive and culture negative pulmonary TB patients, respectively; Lanes 4 and 6: Twenty-fold diluted DNA samples from smear negative culture positive and culture negative pulmonary TB patients, respectively; Lanes 7 and 8: Neat and twenty-fold diluted DNA samples, respectively from non-tuberculous patient.

Table 3 lists the statistical analysis of PCR in relation to culture. Although the culture exhibited relatively higher specificity as well as PPV in diagnosing tuberculosis, however, PCR was shown to have an overall unprecedented sensitivity of 97.20% versus 66.20% of culture in detecting tubercle bacilli in pulmonary specimens with remarkable exclusion potential owing to highest NPV (94.50% vs 60% with culture). Furthermore, the overall diagnostic efficiency of PCR assay was higher (~97%) when compared to culture (77.60%).

Table 3: Comparison of sensitivity, specificity, predictive values (PPV and NPV) and diagnostic efficiency of PCR to culture in smear negative TB patients

Diagnostic method		**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Diagnostic Efficiency (%)**
Culture		66.20	100	100	60.00	77.60
PCR assay	Culture positive PTB (n = 94)	100.00	95.80	96.90	100	98.20
	Culture negative PTB (n = 48)	91.70	95.80	93.60	94.50	94.20
	Overall	97.20	95.80	97.90	94.50	96.70

Considering the highly contagious status of tuberculosis, identification and cure of smear positive tuberculosis patients remain the cardinal objective of the TB control programmes. However, studies reported that 17% - 20% of *M. tuberculosis* transmission occurs before the level of bacilli reach 10^4 per ml in the sputum i.e. threshold for a smear to show the presence of AFB with microscopy [5, 19]. Similarly, it has been documented that TB patients with smear negative presentation are the cause of transmission for at least one in six patients with pulmonary involvement[6]. Given that 50% of the TB patients present negative findings, so despite having low infective potential, their overall contribution to the transmission of infection cannot be overlooked. Although culture isolation of etiological agent remains the final laboratory tool in the diagnostic paradigm of tuberculosis, it requires 6-8 weeks for meaningful results and frequently shows negative results in paucibacillary specimens. In the present study, 66.20% of the smear negative pulmonary samples grew on L-J slants (Table 2). The observed recovery rate of *M. tuberculosis* from paucibaciliary form of disease on L-J medium was found to be higher than previously reported studies [20, 21], but lower than observed by some other investigators [22, 23]. Furthermore, the average incubation time for detection of *M. tuberculosis* in smear negative sputum samples is 42.2 days as compared to 20.1 days for smear positive specimens. Similar findings were reported by other studies [24]. This enhanced time window for paucibacillary specimens to show positive culture growth may be attributed to the fact that the burden and metabolic activity of mycobacteria in these specimens are very low [25]. Given that infection is a stochastic process, the delay in getting culture results and atypical clinical manifestations in smear negative disease will lead to progression of disease to advanced stages as well as transmission of infection to social and household contacts.

PCR, more sensitive and specific than AFB microscopy and more rapid than culture, can be a promising tool in diagnosing TB patients with early stage disease having low burden of infection and minimal symptoms. In the present investigation, we evaluated the performance of an in-house PCR test in smear negative specimens using 263 bp region of 38 kDa gene of *M. tuberculosis* as a target to be amplified. The PCR test was found to be more sensitive than culture as the overall sensitivity of detection of tubercle bacilli among smear negative pulmonary was 97.20% with a specificity of 95.80% (Table 3). In contrast to this, the studies employing the new and robust point of care testing Xpert MTB/RIF Ultra, has reported a sensitivity & specificity of 78.9% and 98.7% respectively, on sputum smear-negative samples [16]. Similarly, in another report the investigator developed an an in-house multiplex PCR test targeting the 38 kDa gene and *IS6110* insertion sequence, specific to *Mycobacterium tuberculosis*to further increase the sensitivity of a TB-PCR kit targeting only 38 kDa gene developed earlier in the same laboratory documented 67.9 % of the sensitivity and 97.3% of specificity in smear negative samples. They also observed that sensitivity of 77.1 % observed for the detection of *M. tuberculosis* with single target PCR increased to 89.2 % with multiplex PCR in culture positive samples [26].The most probable explanation for these variations in sensitivity of PCR assay includes differences in processing of samples, different DNA purification and PCR protocol. Although the specificity obtained in the present study is within the published range but this could be easily increased, if the criterion for selecting patients or specimens on which PCR assays were carried out be made more stringent. Similar views were also echoed by other mycobacteriologists [27]. The criterion followed for sample collection in this study was TB suspicion in any of its clinical / symptomatic form.Of the smear negative TB cases missed by culture, 92% of PTB episodes were picked by PCR (Table 2). It can be partially attributed to the presence of non-viable bacilli pertaining to the harsh treatment during liquefaction and disinfection of the clinical samples [28]. Additionally, the AFB content in clinical specimens is non-uniform, scanty and some of the mycobacteria rendered uncultivable by the immune system of the host. Furthermore, we ruled out the possibility of contamination with the inclusion of negative control in each batch of amplification reaction. In addition to this, 8% of smear negative culture negative sputum samples were found to be negative with PCR protocol (Table 2). Although, the possibility of presence of PCR inhibitors in the clinical samples was further discarded by amplifying the appropriate dilution (1:20) of DNA samples along with their undiluted stocks, however, the low quality of clinical samples with high percentage of epithelial cells along with the respiratory tract microbiota may interfere with the optimal performance of in house PCR [29]. The sensitivity was evidently influenced by the high PCR positive rate among TB patients and low specificity was due at least to 4% positive PCR results among non TB patients (Table 2). However, the intensity of PCR products in non-tuberculous cases was significantly lower than that obtained from bacteriologically negative TB cases (Figure 1). A positive PCR result may not be an indicator for active TB disease because it

is difficult with this technique to distinguish former from clinically insignificant mycobacterial load which is not sufficient for commencing anti tubercle chemotherapy. Pertaining to the significantly prevalent latent form of TB in our endemic setting, the limiting factor of the clinical applicability of PCR is its inherent inability to differentiate latent form of the disease from the active infectious case. As compared to culture the PPV of PCR test was low (97.90% vs 100%) in pulmonary TB patients. However, the NPV varied from 94.50% - 100% in smear negative culture negative and culture positive respectively, much higher than culture (60%) (Table 3). Moreover, the PCR test detected tubercle bacilli in less than a day as compared to an average of 42.2 days on L-J medium.The sensitivity and specificity parameters directly assess the utility of the diagnostic test but these values remain unaffected by prevalence of the disease. In contrast to this, the predictive values (PPV and NPV) are although clinically significant, but strongly depend on the prevalence [30] and generally, the sensitivity of a test directly correlates with its NPV [31].

4. CONCLUSION

Given NPV is the probability that an individual with a negative test result does not have disease, therefore, PCR with higher NPV might be a good decisive factor for diagnosing an individual with tuberculosis in combination with other clinical information. These findings will be of immense help especially in smear negative form of the disease where conventional tests are of little help.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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