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Comparison of Conventional and Molecular Methods in the Detection of *Mycobacterium tuberculosis* in Clinically Suspected Samples of Tuberculosis

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Abstract

Impeded diagnosis in Tuberculosis may be a major cause of morbidity and mortality among particular group of patients, hence better methods are needed for the accurate detection of tuberculosis (TB) among both smear positive and smear negative cases. This study was aimed to compare conventional and molecular methods in detecting *Mycobacterium tuberculosis* among the clinically suspected cases of TB. A total of 100 clinically diagnosed TB patients were incorporated in this study. All the patients were either admitted or attending JSS Hospital, Mysore during the study period i.e., from January 2018- December 2018. Sputum, Gastric aspirate, Pleural fluid, Ascitic fluid, Pus discharge, CSF and Tissue samples were gathered for smear microscopy, culture (Lowenstein – Jensen medium) and PCR testing. The sensitivity of smear and PCR were compared to that of culture considering as gold standard. 50 of 100 patients were positive on smear microscopy. 51 specimens yielded the growth of Mycobacterium *tuberculosis* on Lowenstein-Jensen's medium and PCR detected the presence of MTB specific gene in 77 specimens. In clinical diagnosis of tuberculosis, molecular methods are probably a useful adjunct certainly in smear negative paucibacillary cases. Early diagnosis of TB is cornerstone for proper treatment and control of this deadly disease. Polymerase chain reaction testing is the most rapid and sensitive method for the diagnosis of tuberculosis while culture is specific but it takes 4 to 8 weeks to provide results and smear testing is the cheapest but least sensitive test.

Keywords: *Mycobacterium tuberculosis,* Tuberculosis, patients, PCR, Mysore.

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INTRODUCTION

Tuberculosis (TB) is the major air-borne infectious bacterial disease caused by *Mycobacterium tuberculosis* (MTB) remains a major worldwide health problem. In the South-East Asian region (SEAR), India is amongst the high tuberculosis burden countries in the South-East Asian region(SEAR)[1]. India alone harbours one fourth of the global burden of tuberculosis patients[2]. There has been an increase in notification of new tuberculosis cases from India leading to an increase in notified cases by 37% in 2016 in comparison to that in 2013[3]. TB is a disease for which the use of vaccines has shown no efficacy, hence the best control mechanisms to reduce transmission rates are early diagnosis and early treatment. Diagnostic procedure of tuberculosis begins with a high clinical suspicion, and is reinforced through the use of different diagnostics[4,5]. In spite of the fact that acid fast bacilli (AFB) microscopy, and conventional Lowenstein Jensen (L-J) culture remain the keystone of the diagnosis of TB, these traditional bacteriological methods are either slow or their sensitivity is fairly low, mainly with clinical samples that contain less number of organisms[6]. This can influence treatment by either delaying it or giving inappropriate empiric therapy for TB to subjects without mycobacterial infections or with atypical mycobacteria[7]. Microscopic analysis of Ziehl-Neelsen (ZN) stained smear preparations and sputum culture are the gold standard method for diagnosing pulmonary tuberculosis (TB) in spite of many advances in laboratory diagnostic techniques[8]. Although it has a low sensitivity, the ZN technique remains the principal tool for detecting pulmonary TB[9]. The sputum culture can delay diagnosis by 3-6 weeks and has a biosafety hazard[10]. Along, culture may give false-negative results in 10-20% of cases[11,12]. Despite these shortcomings, ZN is often employed in low-income countries because it is low-cost and easy to conduct and culture is not common due to a lack of laboratory facilities, materials and trained professionals. Therefore, new diagnostic methods have been developed with the goal of replacing direct testing and culture. The classic test would be one that has high sensitivity, provides quick results and is inexpensive. Molecular techniques, such as polymerase chain reaction (PCR), reduce the time necessary for detection and identification of *M. tuberculosis*. Various studies have been done to detect *M. tuberculosis* in respiratory and other clinical samples by amplifying different DNA sequences of *M. tuberculosis* by polymerase chain reaction (PCR) test with encouraging results[13,14]. These studies have mainly focused on IS6110 sequence of mycobacterial genome and partly on 38 kDa protein antigen b (Pab), and 65 kDa antigen encoding gene. The present study was carried out targeting the 225bp gene coding for IS6110 sequence which is specific for *M. tuberculosis* complex. The study was aimed to compare the results of PCR with those of conventional techniques such as smear microscopy and culture by LJ media.

MATERIALS AND METHODS

A sum of 100 samples received at the DMC, department of Microbiology, JSS Hospital, Mysore with clinical suspicion of tuberculosis were included in the study. Of which 12 samples were received from patients with suspected extra-pulmonary tuberculosis and 88 from patients with suspected pulmonary tuberculosis. The samples collected were processed for smear examination, culture on Lowensten-Jensen's media and detection of MTB specific gene using PCR. The results of all different diagnostics were compared and analysed. The sensitivity of PCR test, smear microscopy was evaluated considering culture as gold standard.

Processing of samples

Sample handling and all laboratory procedures were conducted following safety regulations and standardized handling measure. Smears were made from the mucopurulent part of sputa and other appropriate methods depending on the nature of sample. Zeihl-Neelsen (ZN) staining was done on these smears using standard techniques and observed for the presence of acid fast bacilli (AFB). AFB was graded using IUALTD recommendations. All the specimens were further subjected to digestion and decontamination using standard N-acetyl L-cysteine – NaoH (NALC-NAOH) method. Sediments obtained after processing the samples were inoculated into two bottles of LJ-Medium. One bottle was incubated at room temperature and other at 37°C. Culture readings were taken on weekly basis until eight weeks. The

Mycobacterial isolates obtained were subjected to MPT64Ag ICT test for confirmation of isolates as MTBC.

DNA Extraction and amplification

Samples such as sputum, pus and tissue were priorly treated with NALC-NAOH. Tissue specimens were transferred into centrifugation tubes. Lysis buffer, Proteinase K was added and homogenized and the tubes are centrifuged at 6000rpm for 5 minutes and binding buffer is added and purification steps are followed. Sterile body fluids were directly added with proteinase K and purification steps were followed as per manufacturers protocol.

DNA extraction and amplification of 225bp of *M.tuberculosis* was done by using commercially available HELINI™ pure fast bacterial genomic DNA minispin prep kit and HELINI MTB PCR kit. The samples were processed as per the guidelines of the manufacturer of the kit to obtain DNA. A 225bp region of IS6110 gene coding for *M. tuberculosis* was choosen as primer target for DNA amplification. DNA amplification by PCR was performed with a total reaction volume of 20µl by using Biorad PCR systems. The amplification reaction contained, Red dye PCR Master mix, Endogenous primer mix, MTB Primer mix and purified DNA sample at a final volume of 20µl. MTB positive template provided by the manufacturer was used as positive control and sterile water was used as negative control for the amplification. Amplification protocol was set according to protocol, initial denaturation at 95c for 5minutes followed by denaturation at 95c for 30 seconds, Annealing at 58° for 30 seconds, Extension at 72° for 30seconds. After final extension the samples were immediately kept at -20°. PCR products were using 1% agarose gel in 0.5X TAE buffer containing ethidium bromide at 10µg/ml concentration and the samples showing the presence of band at 225bp under gel doc were considered as positive for *M. tuberculosis.*

Statistical analysis such as sensitivity and diagnostic accuracy for various diagnostic tests was calculated in this study.

RESULTS

Of 100 samples included, 50 were smear positive and 50 were smear negative (Table 1). Among 100 patients, 59 (59%) of the patients were males and 41 (41%) were females. The mean age of subjects among males and females were 44.63 and 40.15. Considering 19.28 as standard deviation for males and 22.32 for females, overall age of the total subjects was 42.79±20.59.

A total of 100 samples were subjected to microscopy, culture on LJ medium and PCR. The sensitivity of each test is compared (Table 2).

Table 2. Sensitivity of different tests conducted

Tests performed	No of samples tested	Result		Sensiti-vity
		Positive	Negative	
ZN smear	100	50	50	50%
PCR	100	77	33	77%
Culture on LJ-Media	100	51	49	51%

Sensitivity of PCR test with two different tests was compared for pulmonary and extrapulmonary samples. The PCR test gave a higher sensitivity compared to ZN smear and LJ medium in both pulmonary and extra pulmonary samples (Table 3).

Table 1.

Smear positive (n=50)	Smear negative (n=50)
49- Sputum	39- Sputum
1-Pus discharge	5- Gastric aspirate
	3- Pleural fluid
	1-Tissue
	1-Ascitic fluid
	1-CSF

Table 3. Sensitivity of different tests in pulmonary and extra pulmonary samples

Nature of Clinical Samples	Identification rate in percentage, (number) by different tests		
	ZN (Positive)	LJ (Positive)	PCR
Pulmonary	55.90(49)	54.50(48)	81.80(72)
Extrapulmonary	8.33(1)	25(3)	41.60(5)

The diagnostic accuracy of PCR was calculated by considering culture as gold standard and the diagnostic accuracy of PCR is 74% (Table 4).

Table 4. Diagnostic accuracy of PCR v/s culture

		Culture		Total
		Positive	Negative	
PCR	Positive	17	10	37
	Negative	0	23	23
Total		17	33	50

DISCUSSION

World health organization on the motto of end TB strategy and with the goal of reducing the spread of TB has been recommending for the development of newer and reliable TB diagnostic tools which would be more sensitive and simpler[15,16]. Scientists and researchers around the world have been working on these strategies of ending TB and reducing the spread of infection. Efforts for development of rapid and low cost tests with high sensitivity and specificity which can be used in limited resource settings as a point of care diagnostics are still on its way with the goal of decreasing TB. Evidence based diagnosis like smear microscopy is very much needed for the efficient diagnosis of TB. But it has its own limitations such as low sensitivity[17]. Hence, this is the primary disadvantage and there is a need for developing new tools to diagnose tuberculosis[18]. Sensitivity of Microscopy in our study is 50%, was moderately high than compared to other studies. This might be due to in appropriate selection of samples where among a total of 100 samples, 50 smear positive and 50 smear negative samples. A wide range of smear positivity between 0-75% has been reported in earlier studies[19]. Prompt and accurate diagnosis of tuberculosis is still a dilemma in developing countries where LJ culture is still used as the gold standard for its diagnosis. The culture is time consuming (4-8 weeks) therefore, evidences like histology/cytology along with clinical evaluation are still being used to treat the patient with a full course of anti-tubercular treatment[20]. In the present study, the sensitivity and yield of culture positivity was almost equivalent when compared with the smear positivity. Our study showed sensitivity of 51% which has varied results when compared with the studies by Dunlap *et al.* and Yeager *et al*. They showed a culture sensitivity of 80-85%[21,22]. The higher sensitivity towards culture is because, detection of *Mycobacterium tuberculosis* using culture on LJ medium is very specific and can detect as few as 10 bacteria/ml of specimen as compared to smear that requires about 5000 to 10000 acid-fast bacilli / ml of specimen. Diagnostic techniques based on amplification have the potential to increase the sensitivity for detecting mycobacterium which can also reduce the turnaround time that is usually necessary to isolate and to identify these organisms using biochemical reactions[23,24]. A sensitivity of 77% for PCR test was seen in our study, this is concordance with a study who reported a sensitivity of 74.4%[25]. Various studies showed varied results for sensitivity for PCR. In a study conducted by Muhammad Kashif Munir *et al.,* sensitivity of 92% was reported. The sensitivity of PCR was remarkably high when compared to smear and culture in this study and PCR took much shorter time (1-3 days) as compared to culture (6-8 weeks). In a pooled analysis of 125 studies[26], the overall sensitivity of PCR was 85%. The results of this study states that smear is cheap and rapid method of detecting mycobacterium tuberculosis but it has a very low sensitivity. Culture is more sensitive but it takes a longer time to give results while PCR is specific, rapid, more sensitive but expensive technique, and can be used in difficult cases where diagnosis become a challenge.

CONCLUSION

The rate of infection with Tuberculosis, it's mortality and morbidity rates are in the line of increasing in recent years, hence, rapid and accurate diagnosis and treatment plays an important role in reducing the risk of increasing the rates of infection including the morbidity and mortality. Staining techniques such as ZN and Auramine staining for acid fast bacilli is fast and inexpensive but has its own limitation of less sensitivity and also cannot differentiate between typical and atypical *Mycobacteria*. Diagnosis by culture, even though being gold standard, relatively specific and sensitive in comparison with smear microscopy but slow and time taking process. A quicker and yet accurate diagnosis

of *Mycobacterium tuberculosis* is pivotal in the management of TB. PCR is specific, rapid, more sensitive but expensive technique. The sensitivity of PCR was remarkably high when compared to smear and culture in this study i.e., out of 100 test samples 77 were positive for PCR, 51 were positive for culture and 50 were smear positive and PCR took much shorter time (1-3 days) as compared to culture (6-8 weeks). Hence, from our study we conclude that for timely detection and management of tuberculosis PCR can be used, which can help the physicians for timely decisions for patient's management. PCR can also be used in smear negative cases where diagnosis becomes a challenge.

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CONFLICT OF INTEREST
The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION
All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

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DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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