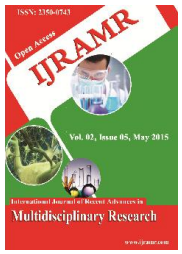

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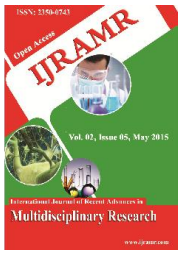

Research Article

A COMPREHENSIVE COMPARISION OF MPB64 BASED PCR ASSAY VERSUS MICROSCOPY AND CULTURE IN THE DIAGNOSIS OF CLINICALLY SUSPECTED CASES OF EXTRAPULMONARY TUBERCULOSIS

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ABSTRACT

Background: Extrapulmonary tuberculosis (EPTB) often possesses a diagnostic dilemma in comparison to pulmonary tuberculosis which can be easily diagnosed by simple microscopy. Paucibacillary nature of specimens giving negative smear for acid fast bacilli, lack of granulomas on histopathology and failure to culture Mycobacterium tuberculosis do not exclude the diagnosis of EPTB. To overcome these limitations novel diagnostic methods of nucleic acid amplification like Polymerase Chain Reaction have been reported with good sensitivity and rapidity for diagnosis of EPTB. Polymerase Chain Reaction PCR could have a significant advantage over the conventional methods for early diagnosis of clinically suspected cases of EPTB.

Objectives: 1) The present study was conducted to evaluate the role of PCR using MPB64 species specific primer in early diagnosis of extrapulmonary tuberculosis. 2) To compare the results of PCR v/s microscopy and culture.

Material and Methods: A total of 100 clinical specimens comprising pleural fluid, cerebrospinal fluid, ascitic fluid, fine needle aspiration biopsy, pus and biopsy from clinically suspected EPTB cases were included in the present study. These specimens were processed by conventional diagnostic methods i.e Microscopy by Ziehl Neelsen (ZN) stain and culture on Lowenstein Jensen (LJ) medium. The PCR was performed by using species specific MPB64 primer.

Results: In the present study tuberculous pleural effusion (39%) followed by tubercular meningitis (31%) was found to be the commonest clinical presentation of EPTB. The overall positivity of PCR was 53% in patients with EPTB. Microscopy and culture could detect only 12% of these. On histopathological examination 100% positivity by PCR was seen in tissue samples suggestive of tuberculosis. Of the 77 EPTB patients who responded to antituberculosis treatment (ATT), 53 patients were PCR positive. Comparing the results of PCR visa `a vis conventional technique using response to the treatment as a gold standard the sensitivity and specificity of PCR was found to be 68.8% and 100% respectively.

Conclusion: This study shows that PCR can serve as a useful complement when used along with conventional diagnostic methods in rapid diagnosis of EPTB.

INTRODUCTION

Tuberculosis (TB) remains one of the leading infectious diseases throughout the world accounting for about 8.8 million incident cases in 2010 (Griffiths, 2010). According to National Tuberculosis Control Programmes (NTPs), 2.6 million new cases of sputum smear-positive pulmonary TB (PTB), 2.0 million new cases of sputum smear-negative PTB and 0.8 million new cases of extrapulmonary tuberculosis (EPTB) were reported in 2010 worldwide (Griffiths, 2010). EPTB has become more common since the advent of human immunodeficiency virus (HIV) infection (Cabandugama, 2011). EPTB constitutes about 15–20% of TB cases and can cause up to 50% of TB cases in HIV-infected individuals. India has high burden of TB cases thus proportionately more EPTB cases are also observed in this country (Noussair, 2009). But the cases of EPTB are underreported due to difficulty in diagnosis of EPTB, particularly with unavailability of advanced technology in resource limited set up. In developing countries laboratory diagnosis of EPTB depends upon the conventional methods like microscopy and culture. Microscopy is an easy technique for diagnosis of pulmonary tuberculosis but it lacks in sensitivity for diagnosis of EPTB. Isolation of *M.tuberculosis* on culture is gold standard for diagnosis but it takes long period for growth. Routine hematological and biochemical investigations are not always suggestive of EPTB. To overcome these limitations

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with conventional methods many gene amplification techniques for the direct detection of *M.tuberculosis* have been reported with good sensitivity and rapidity. The polymerase chain reaction is one of them. For detection of *M.tuberculosis* by PCR various species specific primers are available .MPB64 antigen coding gene based primer is one of them. Literatures have reported PCR as a diagnostic tool for early diagnosis of EPTB but very few studies are reported from resource limited set up. So the present study was conducted in Department of Microbiology, B.J Government Medical College to find out the role of PCR using MPB64 primer in early diagnosis of EPTB.

MATERIALS AND METHODS

In the present study total 100 specimens from clinically suspected cases of EPTB were included. Samples were collected before starting antituberculosis treatment (ATT) to the patient. Total 100 specimens were collected from patients of tuberculous pleuritis, tuberculous meningitis, abdominal kochs, tuberculous lymphadenopathy, cold abscess and tuberculosis of breast and joints. Specimens collected were pleural fluid, cerebrospinal fluid (CSF), ascitic fluid fine needle aspiration biopsy (FNAB), biopsy sample from tuberculoma breast and joint and pus sample from cold abscess. Each sample was divided into two parts. First part was processed for microbiological investigations i.e microscopy by Ziehl Neelsen (ZN) Stain and culture on Lowenstein Jensen (LJ) Medium. The second part was stored at -20^{0}C in Deep freezer until processed for PCR within 24 hours. Part of tissue sample was sent to pathology laboratory for relevant cytopathological and histopathological investigations. The samples were processed as per standard microbiological procedure. Smears were prepared and stained by Ziehl Neelsen (ZN) stain. Centrifuged deposit of CSF was inoculated on Lowenstein Jensen (L-J) medium directly. The pleural fluid, ascitic fluid and FNAC/Pus/ homogenized tissue specimen were decontaminated by modified Petroff's method and then cultured on L-J medium. For diagnosis of EPTB, isolation on L-J medium was considered as gold standard. L-J medium after inoculation was incubated at 37^{0}C up to 8 weeks. The slow growing, dry, non-pigmented and buff coloured colonies were further subjected to niacin, and nitrate reduction test. Niacin accumulating and reducing nitrate to nitrite were labeled as *M.tuberculosis*.

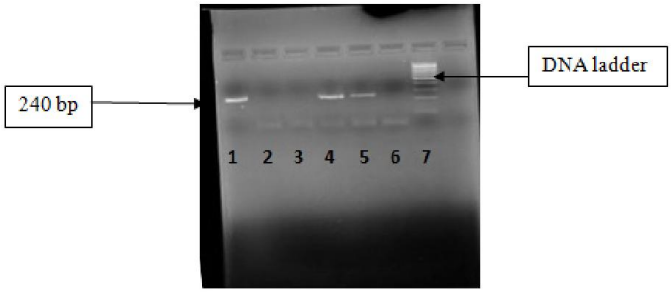

Fig. 1. Gel Electrophoresis showing 240bp amplified product of *M.Tuberculosis* from specimens of EPTB cases

Well no.1- Positive control, 2- Negative control, 3- CSF sample, 4 - FNAB sample, 5- CSF sample, 6- Pleural fluid sample, 7- Molecular weight marker (1000bp). Well no. 4 & 5 samples showing positive PCR giving 240bp product (using MPB64 gene based primer)

DNA was extracted from 100 µl of the pellet from centrifuged deposit/100 µl of pus and FNAB samples by using silica gel method. After DNA extraction the samples were subjected to PCR. MPB64 gene based primer (Sigma Genosys) giving 240 bp product was used for the amplification. An initial denaturation at 95^0 C for 3 minutes was performed to ensure complete separation of two strands of template DNA. The thermal cycler was set for 30 cycles. Each cycle consisted of a step of denaturation at 95^0 C for 40 sec, annealing at 65^0 C for 45 sec followed by a step of extension at 72^0 C for 40 sec. A final extension cycle at 72^0 C for 4 min was performed to ensure complete extension of partially extended PCR product. PCR products were detected by using 2% agarose gel electrophoresis. Band at 240 bp was considered as positive PCR reaction with no band in negative control (Fig 1). The results obtained from conventional method and PCR were compared and evaluated statistically by applying test of sensitivity and specificity, positive predictive value, negative predictive value and Z test.

Table 1. Distribution of EPTB cases and the nature of specimen collected

Clinical Diagnosis	Nature of specimen	Total No.(%)
Tuberculous pleuritis	Pleural fluid	39
Tuberculous meningitis (TBM)	CSF	31
Abdominal Kochs	Ascitic fluid	15
Cervical lymphadenopathy	FNAB	5
Cold abscess	pus	3
Osteomyelitis	Knee aspirate	2
Pott's spine	Biopsy	2
TB hip joint	Biopsy	1
Tubercular Pericarditis	Pericardial fluid	1
Tuberculoma of Breast	Biopsy	1
Total (n)		100

RESULTS

100 clinically suspected cases of EPTB were included in the study. Majority of the patients were in the age group of 31-40 years. Male constituted 62% of cases while 38% of the patients were female. The clinical specimens included in this study were obtained from patients of different clinical conditions of EPTB. The commonest presentation of EPTB observed in the present study was Tuberculous pleural effusion (39%). The Overall positivity of PCR in clinically suspected EPTB cases was found to be 53%. PCR showed 100% positivity for FNAB and Biopsy specimens. All samples were subjected to ZN stain. The overall positivity rate for microscopy was 12% (Table 2).

Table 2. Sample wise distribution of results of PCR, Microscopy and culture in EPTB cases

Specimen	Total no.of specimens received(n)	PCR Positive (%)	Microscopy positive (%)	Culture positive (%)
Pleural Fluid	39	24 (61)	6(15.3)	8(20.5)
CSF	31	12(38.7)	2(6.4)	1(3.2)
Ascitic Fluid	15	06 (40)	1(6.6)	1(6.6)
Biopsy	09	08 (88)	01 (22.2)	1 (11.1)
Pus	05	03 (60)	02 (40)	1(20%)
Pericardial fluid	01	0 (0)	0 (0)	0 (0)
Total	100	53(53)	12(12)	12(12)

Table 3. Comparison of PCR and Microscopy

PCR	Microscopy		Total
	Positive	Negative	
Positive	10	43	53
Negative	02	45	47
Total	12	88	100

Table 4. Comparison of PCR and culture in diagnosis of EPTB cases

PCR	Culture		Total
	Positive	Negative	
Positive	11	42	53
Negative	1	46	47
Total	12	88	100

Z = 4.58, P<0.0001
Sensitivity: 91.7 %
Specificity: 52.2%

The results of PCR were compared with microscopy. It is seen from table 3 that, PCR could detect additional 43 cases which were negative by microscopy. Isolation of *M.tuberculosis* was done on L-J medium. The colonies on L-J medium were identified as *M.tuberculosis* by doing niacin and nitrate tests. The overall culture positivity was 12%. Maximum culture positivity was seen in pleural fluid (20.5%), pus (20%) followed by biopsy samples (11.1%). As shown in table No.4, 42 of more cases were detected by PCR than culture (Table 4). On comparison with culture as a gold standard (Table 4), sensitivity and specificity of PCR was found to be 91.7% and 52.2% respectively. In PCR positive cases, difference between the culture positive and negative group was found to be statistically significant. (Z= 4.58, P< 0.0001). Out of total 100 samples received, five samples were of FNAB and four samples were of biopsy. These samples were subjected to histopathological examination. PCR could detect *M.tuberculosis* from all the tissue specimens showing caseating granuloma on histopathological examination. The treating physician started antituberculosis treatment (ATT) to the patient on the basis of clinical and laboratory diagnosis. These patients were reviewed for the response to the treatment with respect to clinical improvement. A total of 77 patients responded to the ATT. Using response to the therapy as the gold standard, PCR technique was evaluated against conventional technique (Table 5). Conventional techniques were taken to be positive when either smear or culture was positive. As shown in table 5, PCR gave a sensitivity of 68.8% for diagnosis of EPTB. Whereas conventional technique gave a low sensitivity of 15.6%. The Specificity of both the technique was found to be equivalent. Thus, PCR performed much better than the conventional technique for diagnosis of EPTB.

Table 5. Comparison of PCR, Microscopy and Culture with response to ATT

TEST	PCR (%)	MICROSCOPY(%)	CULTURE(%)
SENSITIVITY	68.8	15.6	15.6
SPECIFICITY	100	100	100
PPV	100	100	100
NPV	51	26.1	26.1

PPV-Positive predictive value
NPV-Negative predictive value

DISCUSSION

Tuberculosis, "the captain of all the men of death", a re-emergent killer, is a major world health problem. Global mortality due to TB is ranging from 1.6 to 2.2 million lives per year (Kalaiselvi, 2013). The problem has become more severe by the emergence of multi-drug resistant strain and coinfection with HIV. Extra Pulmonary tuberculosis is increasing worldwide. The diagnosis of EPTB is always challenging. A rapid, sensitive and cost effective diagnostic test would aid in early diagnosis and prompt initiation of appropriate treatment. This will help in limiting the spread of infection especially in the developing countries where the numbers of cases are more. The present study was conducted in tertiary care hospital to evaluate the PCR using MPB64 primer for early diagnosis of EPTB against conventional methods of microscopy and culture. In the present study, male predominance was observed in the patients of EPTB (62% Vs 38%). The most common exudative pleural effusion present in India in contest to the West is tubercular pleural effusion, where malignant effusions are more frequent (Chakravorty, 2005). Same observations were noted in the present study where tuberculous pleural effusion (39%) was found to be the commonest presentation of EPTB followed by tubercular meningitis (31), abdominal kochs (15%) and cervical lymphadenopathy (5%). Arvind *et al.* (2014) have reported 27.3%, 14.7% and 4.7% incidence of tuberculous pleuritis, tubercular meningitis and lymphadenitis respectively. Laboratory diagnosis of tuberculosis is usually based on conventional methods i.e microscopy by Ziehl Neelsen or Fluorescent staining, and on isolation of Mycobacteria as a gold standard. However, in case of extrapulmonary tuberculosis, the Z-N stain, though rapid and inexpensive, lacks sensitivity in clinical specimens. The laboratory culture of *M.tuberculosis* requires a long duration and so, clinical and therapeutic decisions have to be made before the laboratory diagnosis becomes available. Since the conventional microbiological techniques proved to be unsatisfactory for diagnosis of EPTB, there is an urgent need of rapid and sensitive diagnostic test. Molecular methods have been used for the diagnosis of various infectious diseases. These techniques are reported to be more useful for the detection of the microorganisms, which takes long period for the growth on culture. So the present study was conducted to find out the role of PCR using MPB64 as a primer in early diagnosis of clinically suspected cases of EPTB.

Different sets of primers have been used for *M.tuberculosis* PCR, of which the most commonly used primers are IS6110 and MPB64. However, there have been recent reports that isolates from some geographical areas like the Indian subcontinent contains less copies of the insertion sequence compared with the 8 to 15 copies usually found in strains from most developed countries. Of 124 strains of *M.tuberculosis* from South India, 42.7% showed single to no copies of IS6110. As the number of copies of the target sequence is an important determinant of PCR sensitivity, it would be lower for the strains having only a few copies of IS6110. A 240 bp region from the gene, coding MPB64 species-specific cell wall protein antigen has been reported to be highly specific for *M. tuberculosis* complex (Lalit, 2005). Therefore in the present study, MPB64 gene based primer giving 240 bp products was used for the amplification. The overall positivity of PCR using MPB64 as a primer was found to be 53% among EPTB cases in this study (Table 2). Lilly *et al.* (2005) and Seth *et al.* (1996) have reported 35.2% and 85% positivity of PCR with MPB64 in diagnosis of EPTB respectively. In the present study, specimen-wise analysis of results of PCR showed higher percent of positivity in FNAB and biopsy samples (88%), followed by pleural fluid (61%), pus (60%), ascitic fluid (40%) and CSF

(33%) (Table 2). Kesarwani *et al.* (2004) have reported 92% positivity of PCR on different tissues while Nagesh *et al.* (1998) have reported 73.6% positivity of PCR on tissue specimens from EPTB cases. Microscopy for demonstration of acid fast bacilli remains the cost effective and rapid diagnostic method for tuberculosis. In the present study, Ziehl-Neelsen technique was used for diagnosis. The overall positivity of microscopy was found to be 12% (Table 2). Chan *et al.* (1996) reported 15% positivity of direct microscopy for detection of EPTB cases. In the present study, on comparison of results of PCR with microscopy it was found that, PCR could detect extra 43 cases, which were negative by microscopy (Table 3). This shows that PCR is more sensitive than routine microscopy in detecting EPTB cases. Same observations were made by other workers (Chan, 1996) (Martins, 2000). There should be 10,000 bacilli present in the fluid specimen to be seen on direct microscopy. Low sensitivity of microscopy may be due to paucibacillary nature of infection in case of EPTB. Though culture is considered the gold standard in diagnosis of EPTB, its overall positivity was found to be 12% in this study (Table 2). Mycobacteria could not be isolated from remaining 88% of specimens, though they were incubated for up to 8 weeks. Lilly (2005) has reported 4% culture positivity in EPTB cases. In the present study, culture was found to be 20.5%, 20% and 11.1% positive in specimens of, pleural fluid, pus and biopsy respectively (Table 2). As evident from table 4, PCR picked up an additional 42 cases that were culture negative. There should be 10 to 100 viable bacilli present in sample for culture to become positive (Boom, 1990). In this study, low positivity of culture could be because of paucibacillary nature of infection, presence of non-viable bacteria due to underlying treatment with antituberculosis treatment (ATT), or use of only the solid L-J media for cultivation. In three cases where conventional technique was positive the PCR could not detect *M.tuberculosis*. This false negative PCR could be due to insufficient lyses of cells or loss of DNA during purification.

Granulomatous inflammation has extensive differential diagnosis like sarcoidosis, histoplasmosis and chronic fungal infections. Therefore PCR has advantage of confirming the diagnosis of tuberculosis rather than relying on histopathology only. In the present study out of nine biopsy samples received PCR could detect eight cases (87.5%) as EPTB cases. Kesarwani *et al.* (2004) have reported 92.1% positivity of histopathology in diagnosis of tuberculosis from different tissue samples. In the same study PCR was found to be 97.8% sensitive. In the present study, many patients were empirically started on ATT by the clinicians on the basis of clinical diagnosis. Taking this into consideration the present blind study was conducted to prospectively evaluate the role of PCR in early diagnosis of clinically suspected cases of EPTB. PCR was performed on the specimens of these EPTB cases and the patients were also reviewed for response to the treatment with respect to clinical improvement. Seventy seven out of hundred EPTB patients responded to ATT. Of which, PCR could detect 53 cases. However PCR could not pickup 24 cases of EPTB who had responded to ATT. Chakravorty *et al.* (2005) reported 17% and 33% false negative PCR among pleural effusion and lymph node biopsies. The various reasons for PCR negativity in the microscopy/culture positive cases and the clinically suspected cases which responded to antituberculosis drug therapy could be small volume of specimen , low number of bacteria in samples, poor lyses of bacteria in the samples or the presence of some PCR inhibitors like bacterial contaminants, phenol etc in the samples. Similar factors have also been reported to be responsible for PCR negativity in culture confirmed as well as clinically suspected cases of TB in other studies. Sometimes the tough cell wall of *M. tuberculosis* makes the isolation of target DNA difficult. The false negative PCR results could be because of 1) Sampling error 2) Presence of PCR inhibitors like blood, host proteins and eukaryotic DNA, proteases etc. and 3) Inefficient extraction of DNA from the specimen. Comparing the results of PCR visa `a vis conventional technique using response to the treatment as a gold standard the sensitivity and specificity of PCR was found to be 68.8% and 100% respectively (Table 7). PCR performed significantly better as far as sensitivity was considered. Comparison of PCR using MPB64 primer with conventional technique Martins *et al.* (2000) found 70% and 88% of sensitivity and specificity respectively for diagnosis of EPTB.

Conclusion

MPB-64 PCR can play potentially important role in strengthening the diagnosis of EPTB. This can be applied where there is strong clinical suspicion but conventional techniques are negative. PCR also offered the advantage of speed in obtaining results rather than waiting for culture. The rapidity, high sensitivity of PCR may even compensate the higher cost of the test compared with less sensitive conventional tests in the diagnosis of EPTB.

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