EVALUATION OF POLYMERASE CHAIN REACTION FOR RAPID DIAGNOSIS OF CLINICALLY SUSPECTED TUBERCULOUS PLEURISY

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ABSTRACT

Pleural effusion is one of the commonest presentations of tuberculosis, the clinical manifestations being typically abrupt resembling bacterial pneumonia. Since delayed hypersensitivity is the underlying immune response, bacterial load is very low. Owing to these facts, tuberculous pleurisy as an extra-pulmonary disease poses a diagnostic dilemma. The conventional bacteriological methods rarely detect Mycobacterium tuberculosis in pleural fluid and are of limited use in diagnosis of tuberculous pleurisy. We evaluated the efficacy of polymerase chain reaction (PCR) in the diagnosis of tuberculous pleurisy by targeting the gene segment coding for MPB64 protein specific for Mycobacterium tuberculosis. Based on the clinical criteria, 82 patients with lymphocytic exudative pleural effusion were included in the study. Patients were analyzed in two groups; one group consisting of 48 patients of tubercular pleural effusion confirmed by various diagnostic procedures and another group of 34 patients comprising of non-tubercular pleural effusion. There were no false positive results by PCR and the specificity worked out to be 100%. Twenty two patients tested positive for Mantoux with a sensitivity of 45%. ZN-staining for AFB was found in samples from 15 patients (20% sensitivity). ADA was positive for 28 patients with a sensitivity of 53%. PCR was positive for 32/48 patients (67% sensitivity). Thus, PCR was found to be more sensitive than any other conventional method in diagnosis of clinically suspected tubercular pleurisy.

KEY WORDS

Polymerase chain reaction, Mycobacterium tuberculosis, MPB64, tubercular pleurisy

INTRODUCTION

A recent WHO survey has estimated that tuberculosis has emerged as the leading cause of death from a single infectious agent in the world and 7 to 8 million new tuberculosis cases add up each year. India alone comprises nearly 30% of the global burden of tuberculosis and approximately 2 million people die from the disease (2). In many parts of the world, tuberculosis remains the most common cause of pleural effusion (3) and involves nearly one in 30 patients. Except for tubercular emphysema, the bacterial load in the pleural effusion of tubercular pleurisy is very low. Owing to this fact, the

reliability on conventional methods of laboratory diagnosis like AFB smear is very low (4, 5). In such clinical dilemma, a more rapid, sensitive and specific tool like the polymerase chain reaction is an added windfall, which yields results even with low microbial load (6).

The sensitivity and specificity of PCR as a diagnostic tool for timely diagnosis of clinically suspected tuberculous pleurisy has been varying in different studies done previously. Sritharan *et al* (1994) reported the sensitivity and specificity of PCR for the diagnosis of tuberculosis as 97% and 100% respectively (7). However, study done by Villen *et al* in 1998 reported that PCR was much less accurate. In their study PCR was positive in 42% of patients (8). The present study was conducted by targeting the *MPB64* gene (1) specific for *Mycobacterium tuberculosis* to evaluate the efficacy of PCR as a diagnostic tool by determining the sensitivity and specificity of the detection of tubercular pleural effusion cases in the Kashmir valley.

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MATERIALS AND METHODS

Clinical specimens

Based on the clinical criteria, patients with lymphocytic exudative pleural effusion admitted in the tertiary care cum referral centre at Sher-I-Kashmir Institute of Medical Sciences, Srinagar were recruited for the study which was carried out over a period of two years from May, 2002 to April, 2004. Ethical clearance from the IEC of the institute and patients' consent was sought before their inclusion in the study. Detailed history was taken and physical examination was performed according to the prescribed proforma. Routine laboratory investigations including Hemogram, ESR, KFT, LFT, Blood glucose, X-ray chest, ECG and Mantoux test were done for all the patients. Diagnostic thoracocentesis was also done for all the patients. Eighty-two patients (age 13-72 years) with lymphocytic exudative pleural effusion were included in the study. Patients were analyzed in two groups; one group consisting of 48 patients of tubercular pleural effusion with a strong clinical, radiological and histopathological evidence of tuberculosis including clinical response to antitubercular treatment and another group of 34 patients comprising of non-tubercular pleural effusion. 3-4 ml of pleural effusion samples were collected from the eighty-two patients enlisted for this study. Pleural fluid analysis was done for presence of malignant cells and AFB staining. ADA activity of pleural fluid and CT scan chest were also carried out for the patients to establish diagnosis.

Bacteriological examination of the pleural effusion samples

Ziehl-Neelsen (Z-N) staining of smears was performed for all pleural effusion samples.

Polymerase chain reaction

PCR was performed in three different areas of the laboratory physically separated from each other as a precaution to avert cross-contamination.

Sample processing for DNA

DNA was extracted from pleural effusion samples following a modification of the protocol of Seth *et al* (9) by centrifugation at 3000 rpm for 30 min. followed by treating 200 µl of the sediment with equal volume of lysis buffer (consisting of 0.2M NaOH, 2M NaCl and 1% SDS) and 100µg proteinase-K at 60°C for 1hour followed by 95°C for 15min. to inactivate proteinase -K. The lysate was extracted successively with

chloroform. The aqueous phase was adjusted to 0.3M sodium acetate (pH 5.2), precipitated with ethanol and dissolved in double distilled water.

Oligonucleotide primers

The target for the PCR assay was *MPB64* gene (1) which codes for an immunogenic protein specific to *Mycobacterium tuberculosis* complex. The sequences of the two primers used were:

Forward primer (460-479)	5'-TCCGCTGCCAGTCGTCTTCC-3'
Reverse primer (700-681)	5'-GTCCTCGCGAGTCTAGGCCA-3'

Amplification reaction

DNA amplification by PCR was performed in the total reaction volume of 25µl with 5µl of extracted DNA, 10mM Tris-Cl (pH 8.3), 1.5mM MgCl₂, 50mM NaCl, gelatin 0.01% (w/v), 100µM of each dNTP (Genei-India), 0.5µM of each primer & 0.5 Units of *Taq polymerase* (Genei-India). Amplification was carried out on a programmable Minicycler™ (MJ Research, USA). Initial denaturation at 94°C for 5 min. was proceeded by 30 cycles each of denaturation (94°C for 30sec.), annealing (60°C for 1min) and extension (72°C for 2min) followed by a final extension at 72°C for 7min.

Detection and identification of amplified product

The amplified product was electrophoresed into 2% agarose gels. The gels were stained with ethidium bromide and visualized in a UV-transilluminator (Vilber Lourmat, France). The presence of a 240 bp fragment indicated a positive test (Fig. 1).

RESULTS AND DISCUSSION

In our study, based on the clinical criteria, 82 patients with lymphocytic exudative pleural effusion included in the study were analysed for tubercular pleural effusion. Twenty-two patients tested positive for Mantoux with a sensitivity of 45% (Table No.1). ZN-staining for AFB (Table No.1) was found in samples from 15 patients (20% sensitivity). ADA was positive for 28 patients with a sensitivity of 53% (Table No.1).

Mycobacterium tuberculosis specific *MPB64* targeted sequence was detected in pleural effusion samples from 32 out of 48 patients, remaining 16 cases were negative. There were no false positive results by PCR, out of 34 control cases none was tested positive for PCR. Thus, sensitivity of PCR

Table No. 1
Diagnostic yield for pleural tuberculosis

DIAGNOSTIC VARIABLES	ZN-STAINING	MANTOUX TEST	ADA	PCR
POSITIVE	15	22	28	32
SENSITIVITY (%)	20	45	53	67

for detection of tubercular pleurisy worked out to be 67% while specificity for the same was 100%. These results suggest that the PCR is more sensitive than other co-existing conventional methods, but still not absolute to identify all cases. However, all ZN positive samples were found positive on PCR amplification. Our results were comparable with those of Delassence *et al* (5) who found PCR sensitivity of 60% in their patients of pleural tuberculosis. Our results were also consistent in respect of sensitivity and specificity with those of Querol *et al* (10) who had shown sensitivity and specificity of PCR in the diagnosis of pleural tuberculosis to be 81% and 100% respectively.

In our report, negative PCR results seen in 16 patients with pleural tuberculosis were either due to presence of inhibitors that are particularly high in pleural fluids (11) or due to sampling difficulties. The chance of detecting mycobacterium becomes higher with a bigger sample volume. Since, we had taken only 3-4 ml per patients, part had to be utilized for ZN-staining and ADA, only 1-2ml was available for PCR. In view of the fact that delayed hypersensitivity is the underlying immune response in tubercular pleurisy, the pauci-bacillary state could be accounted for the false-negative results to some degree. However, whenever the result was positive, the mycobacterial load must have been sufficiently high.

Studies conducted by Parandaman V *et al* (12), Tan J *et al* (13), Takagi N *et al* (14) and Jatana SK *et al* (15) show disparate results where sensitivity is 100% and specificity

Table No. 2
2 Specificity and Sensitivity of PCR in tubercular and other pleural effusions.

S. No.	PLEURAL EFFUSION	No. OF PATIENTS	PCR (+)	PCR (-)	SPECIFICITY (%)	SENSITIVITY (%)
1	TUBERCULAR	48	32	16	100	67
2	NON-TUBERCULAR	34	none	34

Fig. 1 PCR-based detection of *Mycobacterium tuberculosis* targeting *MPB64* gene. Electrophoretic separation of the amplicon into 2% agarose gel is documented across Lanes 1-7. Lanes 1-3 represent the clinical pleural fluid samples, Lanes 4 & 5 exemplify the colony-PCR from atypical *Mycobacterium* (*Mycobacterium bovis*) and lanes 6 & 7 stand for the colony-PCR from *Mycobacterium tuberculosis* culture of the clinical samples of tubercular pleurisy. The presence of a 240 bp. amplicon in the Lanes 1, 2, 3, 6 & 7 indicated the presence of the target while the absence of the amplicon in the Lanes 4 & 5 pointed towards the absence of the target.

varied from 75-90%. These studies have been carried out on a smaller sample size; all of them have targeted the insertion sequence IS6110 and have employed different set of primers. Moreover, a sensitivity of 100% and a low specificity means that all mycobacteria that are picked up by PCR are not necessarily *Mycobacterium tuberculosis*. This further lends credit to our study where the specificity is 100% and sensitivity is 67% implying that all the mycobacteria picked up by PCR targeting *MPB64* gene are *Mycobacterium tuberculosis*; these results were further substantiated by colony PCR of various *Mycobacterium* species (Fig.1). Hasaneen *et al* (16) have reported that 93% sensitivity of PCR for pleural biopsy samples but the underlying invasive procedure makes it intricate to comply with. However, to further enhance sensitivity of PCR-*MPB64* together with the IS6110 targeted primers in a multiplex PCR- possibly will be the answer, where 100% sensitivity and specificity could be accomplished.

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