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Utility of polymerase chain reaction using two probes for rapid diagnosis of tubercular pleuritis in comparison to conventional methods

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We have used polymerase chain reaction (PCR) with IS6110 and a new set of primers from an insertion element like repetitive sequence, (TRC4) to detect *Mycobacterium tuberculosis* in pleural effusion samples from 50 patients having pleuritis. The results of PCR were compared with the results of conventional methods like smear, culture and adenosine deaminase activity. Thirty six specimens were positive and 14 were negative by PCR. Among the 36 samples, 33 were from patients with clinical evidence of tuberculosis including response to anti-tuberculosis therapy. Only six samples were positive by the gold standard which is culture, and three were positive by smear. The measurement of adenosine deaminase activity classified 19 samples as positives. The overall sensitivity and specificity of PCR was 100 and 85 per cent respectively. PCR using IS6110 and TRC4 primers is a sensitive test as compared to conventional tests for detection of *M. tuberculosis* from pleural fluid samples of patients with tubercular pleuritis.

Key words *Mycobacterium tuberculosis* - polymerase chain reaction - repetitive sequence - conventional methods

Early diagnosis of tuberculosis is an important arm in the control of tuberculosis. Conventional methods of detecting mycobacteria in clinical samples, especially in extrapulmonary tuberculosis are either low in sensitivity and specificity or are time consuming. There is an urgent need for developing newer tools for the rapid diagnosis of tuberculosis. Numerous diagnostic approaches based on immunological[1], biochemical[2] and molecular biological method[3-5] have been reported.

Among these techniques, the polymerase chain reaction (PCR) has been found to be useful in the early diagnosis of extrapulmonary and pulmonary tuberculosis. Many investigators have utilised this principle for diagnosis of TB, the only difference being the target site in the genome. Some of the targets used to amplify *Mycobacterium tuberculosis* for detection include sequences from 6.5 kDa antigen[6], 32 kDa antigen[7], 38 kDa antigen[8], *dnaJ* gene[9] and insertion sequence IS6110[10-11]. Of all the primer pairs designed so far IS6110 is reported to be used world-wide. Repetitive DNA sequences[12,13] are better targets for detection systems because of manifold repetition of the sequences within the genome thus conferring a high sensitivity to the assay system.

We have cloned and sequenced an insertion element-like repetitive sequence, TRC4. which IS specific for *M. tuberculosis* complex[14]. A new set of primers has been designed from this repetitive sequence which is an ideal target for PCR to identify *M. tuberculosis* especially in strains carrying no copy

of IS6110[15]. The present study was undertaken to evaluate this new set of primers and study -its usefulness in detection of *M. tuberculosis* in pleural effusion along with the IS6110 primer pair described by Eisenach *et al* [16], which amplifies a 123-bp fragment. The PCR results have been compared with culture, smear and biochemical tests like adenosine deaminase activity.

Material & Methods

Samples of pleural fluid were drawn from 50 patients of pleuritis. Fifty ml of pleural fluid was initially centrifuged at 3,800 rpm for 20 min. The supernatant was stored for adenosine deaminase activity (ADA) assay. Sediment was divided into three parts to be used for smear, culture and PCR.

Smear : Using a 5 mm loop, one loopful of sediment of the pleural fluid was smeared on the slide for examination.

Culture : Sediment was decontaminated using 5 per cent sulphuric acid and inoculated on three solid media : Lowenstein-Jensen medium; Lowenstein-Jensen medium with 0.5 per cent sodium pyruvate; and 7H11 Middlebrook medium.

A portion of the decontaminated sediment was transferred to Kirchner's medium, using a sterile pipette. The remaining portion was used for extraction of DNA.

Extraction of DNA : Initially, two DNA extraction methods were tested. During sample processing a blank consisting of double distilled water was used to check cross contamination.

(i) Simple boiling method– A portion of the pellet was boiled at 100°C with equal volume of TE (10mM Tris; 1mM EDTA) for 10 min. The resulting solution was stored at –20°C until use.

(ii) STET method– The pellet was boiled with 100 µl of STET [8% sucrose; 5% triton X-100; 50mM EDTA; 50mM Tris (pH 8)] for 40 sec, centrifuged and the supernatant was collected. DNA was precipitated with isopropanol. Washing was carried out with 70 per cent ethanol. Finally the precipitate was resuspended in TE and stored at –20°C until use.

In a preliminary experiment 10 samples were processed by these two methods. STET was found to be better compared to the simple boiling method as the former helped in getting rid of inhibitors present in the sample (data not shown). Hence, STET method of DNA extraction was used for all samples.

Adenosine deaminase activity (ADA) : ADA activity was assayed in the pleural fluid by following the calorimetric method of Giusti[17] which is based on indirectly measuring the formation of ammonia produced when adenosine deaminase acts with excess of adenosine. The cut-off level taken for considering a patient to be positive for *M. tuberculosis* using ADA levels is 47.3 IU/l[18].

Amplification of mycobacterial DNA : Precautions were taken to avoid false positivity in each step. Preparation of PCR reagents, addition of template DNA and analysis of amplified products was done in three different rooms to avoid carry over contamination. Reagents were aliquoted and each aliquot was used only once. DNA hot start method with wax beads was used to minimize non specific amplification (according to the protocol supplied by Promega Inc., USA).

The IS6110 primer pairs used in this process were synthesized based on the published sequences from Bangalore Genei Pvt Ltd, India.

IS6110 (a) 5' CCTGCGAGCGTAGGCGTCGG 3'

(b) 5' CTCGTCCAGCGCCGCTTCGG 3'

TRC4 primers have been sent for patenting. The 18 mer primers amplify a fragment of length 173 bp.

Amplification was carried out in a final volume of 25 µl containing 200 μM each of dNTPs (Pharmacia, USA) 300 µM of primers, 1.25 units of Taq polymerase (Amersham Plc., UK) and 5 µl of the sample. A positive control was included in each test which was a DNA sample from standard strain of *M. tuberculosis.* A negative test had distilled water substituted for DNA.

Amplification cycles were performed on a automated thermal cycler (MJ Research Corp, USA).

Each cycle consisted of three steps comprising denaturation at 94°C, annealing at 58°C and primer extension at 72°C. Each step had a duration of one minute. Thirty five amplification cycles were carried out with a final primer extension step at 72°C for 10 min.

Detection of amplified products was carried out in a 2 per cent agarose gel electrophoresis stained with ethidium bromide and subsequently visualized under UV light. Positivity was recorded only when a well defined DNA band was observed in the agarose gel run along with the controls and molecular weight marker (Bangalore Genei Pvt. Ltd., Bangalore).

Results

All the 50 pleural effusion samples were analysed by smear, culture, adenosine deaminase activity and PCR using IS6110 and TRC4 primers for the presence of the *M. tuberculosis* DNA. Samples which had an amplified product of 123 bp for IS6110 primers and 173 bp product for TRC4 were considered positive. A test result was recorded only when the blank maintained during sample preparation and the negative control included during the test remained negative.

Of the total samples analysed, 14 samples gave a negative result by PCR and 36 samples were positive. None of the patients who were pleural fluid PCR negative had clinical features of active TB and no patient had positive sputum smear for acid fast bacilli (AFB), or a positive mycobacterial culture or radiological evidence of TB. Among the 36 patients with a positive PCR result for *M. tuberculosis,* 30 patients had active TB, as judged by one or more of the following parameters, a positive pleural fluid examination for AFB (smear or culture), radiological evidence of TB and response to anti-tuberculosis therapy. Of the remaining 6 PCR positive patients, 3 had no evidence of active tuberculosis while 3 had history of contact with tuberculosis patients. Of the 30 patients identified as having active TB by PCR and other parameters tested, only 3 were positive by smear examinations and 6 were positive by the gold standard, i.e., culture. Pleural fluid of 25 of these 30 patients was tested for ADA and 19 were positive. All these patients had hilar or mediastinal lymphadenopathy as the predominant abnormality on chest radiograph. All these patients responded well to the anti tuberculosis treatment. Based on the above results, the sensitivity and specificity of the PCR assay were calculated for the diagnosis of active TB; the overall sensitivity of the PCR assay was 100 per cent and specificity was 85 per cent. The 3 PCR positive patients with history of contact were also tested for ADA and one of them had a positive result. Nine of the 14 PCR negative patients were tested for ADA levels and 4 were positive.

Discussion

The incidence of tubercular pleuritis is relatively high in patients with AIDS[19]. The differential diagnosis of effusive pleuritis is broad and the ability to promptly diagnose tubercular pleuritis would greatly facilitate the management, of many patients with pleuritis. Smear examination for AFB is a fast method, but this method is insensitive and does not differentiate the different mycobacteria. Diagnosis by culture, the gold standard, is specific but less sensitive for extrapulmonary tuberculosis and also takes a long time. Diagnosis by PCR took two days in our study. The use of PCR on pleural fluid for the diagnosis of TB has been reported previously but the sensitivity of PCR is variable ranging from 11 to 81 per cent[20-22]. We have used primers from the widely known insertion element IS6110 as well as primers from a repetitive element of *M. tuberculosis* cloned by us. Both probes gave a sensitivity of 100 per cent and specificity of 85 per cent.

As smear and culture have shown very. low sensitivity, we have used clinical evidence of the disease and the positive response to anti-tuberculosis therapy (ATT) as the parameter to calculate the sensitivity and specificity of PCR. Among the 50 samples, only 6 were positive by culture. All the six had clinical evidence of disease and also positive response to ATT. These 6 samples were positive by PCR using the 2 probes. But among these 6 specimens only 3 were positive by adenosine deaminase assay and 3 were negative. PCR has been better in sensitivity and specificity compared to ADA levels. Similarly among the 14 PCR negative samples, 4 of the 9

samples tested were positive by ADA levels. Three samples from patients with no clinical evidence of tuberculosis were positive by PCR which accounts for 17 per cent false positivity and reduces the specificity to 85 per cent.

Among the 3 patients who were false positive by PCR, one patient had an ovarian tumour with metastasis and the other two had congestive heart failure. The probability of tuberculous infection in these three patients is low but cannot be ruled out. Since PCR is extremely sensitive, it may pick up positive cases earlier *i.e.*, much before the appearance of the other clinical symptoms. This false positivity was not on account of technical inadequacy because the processing blank and PCR blank were negative for *M. tuberculosis.*

In conclusion, PCR can be used as an additional test for detecting *M. tuberculosis* in patients with tubercular pleuritis because conventional methods have low sensitivity.

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