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# Performance Assessment of IS1081-PCR for Direct Detection of Tuberculous Pleural Effusion:Compared to rpoB-PCR

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**Abstract:** Pleural fluid samples of 78 tuberculous pleuritis were examined by IS1081-PCR and rpoB-PCR. The results were compared with the culture of the samples in the Loewenstein-Jensen (LJ) medium and Ziehl-Neelsen (ZN) staining. Of the 78 tuberculous pleuritis, 66(84.6%) were positive in IS1081-PCR, 43(55.1%) in rpoB-PCR, 17(21.7%) in the LJ and 3(3.8%) in ZN. It is concluded that IS1081-PCR seems to be the most sensitive method than the others.

**Key words:** tuberculous pleuritis, Pleural fluid, IS1081-PCR, rpoB-PCR, culture

## INTRODUCTION

Pleural tuberculosis (tuberculous pleuritis) is a major treatable cause of exudative pleural effusions[1]. The frequency of pleural effusion in tuberculosis (TB) patients was approximately 31%[2]. Pleural reaction in HIV patients with TB is seen in up to 37% of cases[3].Pleural involvement may be primary, secondary to pulmonary TB (e.g., miliary TB) or post primary (reactivation) TB pleurisy. Among the extrapulmonary presentations of TB, pleural TB is second only after TB lymphadenitis[4]. The diagnosis of pleural TB is generally established by the presence of tubercle bacilli in the sputum, pleural fluid, or pleural biopsy specimen, laboratory culture of the causative organism or the demonstration of granuloma in the pleura by histopathologic examination. Analysis of pleural fluid and pleural biopsy, adenosine deaminase (ADA), INFp and other lymphokines can be improved diagnostic efficiency[5].TB pleural effusions are not always easy to diagnose, because the typical features such as presence of a lymphocyte rich exudative pleural effusion associated with caseous necrotic granuloma in pleural biopsy, positive Ziehl-Neelsen (ZN) stains or lowenstein-Jensen (LJ) cultures of effusions or tissue samples, and cutaneous sensitivity to PPD are not invariably present but diagnosis is still sometimes difficult[6]. Diagnostic assays based on polymerase chain reaction (PCR) dramatically decrease the time required to organism detection[7] This method has been tested extensively for extrapulmonary tuberculosis[7-11]. PCR also has been used to detect mycobacterium tuberculosis (MTB) in pleural fluid samples, with highly variable sensitivity (11 to 81%) in different studies[12-14]. Comparatively little work has focused on the utility of different type of PCR and culture. In this study, we examined whether IS1081- PCR of pleural specimens would be superior to that of pleural fluid in detecting MTB. We applied PCR to detect DNA specific for MTB in pleural fluid specimens using a multiple copies (five to seven) of IS1081-based primer that was specific for mycobacterium complex[15-16] and compared it to rpoB-PCR assay, the results of pleural fluid cultures performed on both LJ medium and ZN staining.

## MATERIALS AND METHODS

**Processing of Pleural Fluid Samples:** 78 tuberculous pleural fluid samples that were confirmed by histopathology of pleural biopsy specimens, supported by laboratory(sputum culture), biochemical markers like ADA, gamma interferon and tumor necrosis factor, radiographic, and clinical data, were subjected to decontamination with *N*-acetyl-L-cisteine-NaOH (NALC)

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method and then were concentrated by centrifugation for 15 min at 3,000*g*[17]. Then the sediments were resuspended in sterile normal saline solution, and used for ZN smear, culture, and PCR studies.

**Loewenstein-Jensen Culture of Pleural Fluid Samples:** Loewenstein-Jensen (LJ) medium were inoculated with 0.1 mL concentrated pleural fluid specimen and were kept in an incubator for 8 weeks. Bottles were inspected twice a week for visible colonies, and suspicious growth was subjected to ZN staining. Negative culture was discarded after 8 weeks. Resulting growth was left in light for 2 h and was examined for yellow pigments to identify photochromogen species.

**DNA Extraction:** DNA extraction was performed by SDS/Lysozyme method. Two hundred and fifty microlitres of concentrated pleural fluid specimens were resuspended in a final volume of 1 mL of Tris-EDTA, pH 7.6 containing 10 mg of lysozyme mL. Samples were then incubated at 37°C for 1 h; 30 µL of proteinase K (14 mg/mL) and 3% SDS were added, followed by incubation for 2 to 3 h at 56°C or overnight at 37°C. Proteinase K was inactivated by 15 min of incubation at 95°C. To monitor for cross-contamination, one water-containing negative control tube was used[17].

**IS1081-PCR:** A set of IS1081 Primers (BW-6 [5' CGA CAC CGA GCA GCT TCT GGC TG 3'] and BW-7 [5' GTC GGC ACC ACG CTG GCT AGT G 3']) was used to amplify IS1081 DNA (306-bp) region of the multicopy insertion sequence IS1081. The amplification parameters included an initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 68°C for 1.5 min, and extension at 72°C for 2 min. The extension step in the 35th cycle was held for 10 min before the samples were shifted to 4°C for storage[18].

**RpoB-PCR**: A set of mycobacterium-specific primers (MF, 5 ' C G A C C A C T T C G G C A A C C G 3 ' ; M R 5'TCGATCGGGCACATCCGG3') was used to amplify rpoB DNA (342 bp) encompassing the Rif region, which is associated with rifampin resistance in *M. tuberculosis*. The primers were selected from the highly conserved regions on the basis of known rpoB sequences. Template DNA and 20 pmol of each primer were added to a PCR mixture tube which contained 1U of Taq DNA polymerase, 250 µM each deoxynucleoside triphosphate, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$ and gel loading dye and the volume was adjusted to 20µL with distilled water. The reaction mixture was subjected 30 cycles of amplification (30s at 95°C, 30s at 60°C and 45s at 72°C) followed by a 5 min extension at 72°C [17].

The amplified products were detected by gel electrophoresis using 1.5% agarose gel with ethidium bromide.

## RESULS AND DISCUSSIONS

Rapid diagnosis and treatment of tuberculous pleuritis is crucial to reduce morbidity and mortality from untreated tuberculosis. Because the differential diagnosis of exudative pleural effusion is broad, the rapid diagnosis of tuberculous pleural effusion would greatly facilitate the management of these patients. At present, the most reliable method for the diagnosis of tuberculous pleuritis is the identification of MTB in the pleural specimens[19]. Theoretically; PCR for the MTB genome should be highly effective and rapid in diagnosing tuberculous pleuritis. Using different genomic DNA sequences and extraction methods, MTB-PCR studies of pleural fluid from patients with tuberculosis pleuritis have produced very variable outcomes, with diagnostic sensitivity ranging from 20 to 81%[20-24].This probably reflects the importance of experience, technique and sensitivity in the utilization of this test. Most researchers have reported using insertion element IS6110 primers for direct detection of tuberculosis. Villegas *et al* have evaluated PCR directed to the IS6110 sequence of MTB. They reported that IS6110-PCR had a false-negative result in two patients with tuberculous effusion. [25] In the study of Parandaman *et al*, there were 22% false-negative results by PCR with IS-6110 primers. The higher false negativity of PCR with IS6110 may in part be due to the absence of IS6110 copies in MTB[26].PCR is positive in 100% of culture positive TB pleural fluids and only in 30-60% of culture negative pleural fluids[2]. Study of Reechaipichitkul *et al* reveal that the sensitivity and specificity of pleural fluid cultures on LJ medium were 17% and 100%, respectively and the sensitivity of the pleural fluid PCR-assay of the 16 S-23 S rRNA gene spacer sequences was 50% and the specificity was 61%[27]. We compared the performance of the IS1081-PCR assay with that of the rpoB-PCR assay for the detection of tuberculosis pleuritis. Our study showed that the IS1081- PCR assay was positive in 66 of 78(84.6%) samples and did not show a single false-positive result. The rpoB-assay was positive in 43 of 78(55.1%) samples (Table 1). The sensitivities of the IS1081-PCR and rpoB-PCR assays differed significantly (84.6 versus 55.1, respectively, $P < 0.05$). ). The inferior sensitivity of IS1081-PCR could be due to multiple copies (five to seven) of IS1081 within the chromosome of all strains belonging to

**Table 1:** Comparison of Culture, ZN, IS1081-PCR and rpoB-PCR assay

| Result of pleural fluid Culture | Result of pleural fluid ZN smear | No. of pleural fluid samples with indicated result by PCR with: | | | |
|---|---|---|---|---|---|
| | | IS1081 | | rpoB | |
| | | Positive | Negative | Positive | Negative |
| Positive | 3 | 17 | 0 | 16 | 1 |
| Negative | 0 | 49 | 12 | 27 | 34 |
| Total | 3 | 66 | 12 | 43 | 35 |

**Table 2:** Results of IS1081-PCR and rpoB-PCR assay

| Result by IS1081- PCR | No. of pleural fluid samples with indicated result by rpoB- PCR | | Total no. of pleural fluid samples |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 43 | 23 | 66 |
| Negative | 0 | 12 | 12 |
| Total | 43 | 35 | 78 |

M. tuberculosis complex. The ZN staining results of pleural fluid specimens were positive in only 3 of 78 patients (3.8%) in the tuberculous pleural effusion(Table 1) .Of the 78 samples, only 17(21.7%) led to a confirmed diagnosis of tuberculous pleuritis based on culture (Table 1). The mean detection time of MTB was 28 days for LJ medium. Of the 61 culture negative samples, 49(80.3%) were positive by PCR using IS1081and 27 (44.2%) were positive by PCR using rpoB-PCR. 23 IS1081- PCR assay positive sample tested negative in the rpoB-PCR assay(Table 2) indicated that the rpoB-PCR assay has a lower sensitivity and this could be due to the one copy number of rpoB in MTB. One false negatives result of rpoB-PCR assay in our study from pleural fluid may related to high content of inhibitor (blood) in pleural fluid. The presence of multiple copies (five to seven) of IS1081 within the chromosome of all strains belonging to M.tuberculosis complex should be allowed the easy detection of MTB in tuberculous patient by PCR. In conclusion, PCR assays proved to be suitable for detecting pleural tuberculosis. The IS1081-PCR assay was found to be more sensitive than the rpoB-PCR assay. The main limitation of our study is that currently there is no diagnostic test to serve as an adequate "gold standard" to evaluate PCR. While culture has low sensitivity, clinical assessment may be subjective.

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