RAPID DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION USING POLYMERASE CHAIN REACTION

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Abstract. Between October 1998 and September 1999, 98 patients with symptomatic exudative lymphocytic pleural effusion were enrolled in our study to evaluated the diagnostic sensitivity of polymerase chain reaction (PCR) assay. The mean age was 53.3 years ranging from 18 to 78 years. There were 61 men and 37 women. Pleural fluid was sent for gram staining, AFB staining, aerobic culture, culture of *Mycobacterium tuberculosis* on LJ media, and cytology. Additional fluid was used for a PCR-assay of the 16 S - 23 S rRNA gene spacer sequences and for a nested PCR of the 16 S rRNA gene as a blind control. In cases of free-flow pleural tapping, histopathological analysis was done on three pleural biopsies. Overall etiologies comprised malignancy 53.1%, tuberculosis 36.7%, lymphoma 2.0% and chronic nonspecific inflammation 8.2%. The sensitivity and specificity of AFB-staining were 6% and 79%, respectively; while cultures on LJ media were 17% and 100%, respectively. The sensitivity of the PCR-assay was 50% (95% CI : 40 to 60%) and the specificity was 61% (95 CI : 52 to 71%). When PCR was nested, the sensitivity was 72% (95% CI : 63 to 81%) and specificity was 53% (95% CI : 43 to 63%). Two thirds (26 of 36) of tuberculous pleural effusion cases underwent pleural biopsy, and 62% were diagnosed by histopathology. There were no complications from thoracocentesis or pleural biopsy in any of the patients. We concluded that PCR assay was more sensitive than AFB staining and mycobacteria culture for diagnosis tuberculous pleural effusion but its specificity was quite low.

INTRODUCTION

Tuberculous pleural effusion is usually a secondary immunologic response to the rupture of bacilli tubercles. It is difficult to diagnose, since only scanty bacilli are contained in the pleural effusion. Recent studies of populations with a high prevalence of tuberculosis report that tuberculous pleural effusion occurs in approximately 30% of patients with tuberculosis, and that there is no difference between HIV- and non-HIV-infected patients (Ferrer-Sancho, 1997). Even though pleural effusion may resolve itself, approximately 65% of cases develop active tuberculosis within 5 years (Light, 1995). Malignancy, melioidosis, fungal infection, sarcoidosis, and connective tissue disease mimic tuberculosis because they also present with exudative lymphocytic pleural effusion.

Traditionally, acid-fast staining and culture have been used to identify *M. tuberculosis*, but both methods have limitations. Staining is rapid, easy and inexpensive, but it is neither sensitive nor specific. Culture requires from six to eight weeks to yield results and sensitivity is poor especially when samples contain only a small number of organisms. Rapid diagnosis of tuberculosis infection would facilitate rapid initiation of an effective treatment, leading to improved clinical management.

Methods for the diagnosis of tuberculosis have been improved in recent years, and several molecular techniques have been introduced for clinical use. Polymerase chain reaction (PCR) yields quick results by amplifying specific DNA sequences, even if only a single copy of a given DNA sequence is available (Kearn *et al*, 1998). Various PCR-assays have been devised for identification of tuberculosis in clinical specimens (Brisson-Noel *et al*, 1991; Eing *et al*, 1998), which may be used for pleural effusions. However, there is no consensus about the usefulness of this method to diagnose tuberculosis (ATS, 1997; Light, 1998). The objective of our study is to evaluate the diagnostic sensitivity of the PCR-assay on tuberculous pleural effusion.

MATERIALS AND METHODS

Patients and clinical samples

We studied each patient with symptomatic pleural effusion prospectively between October 1998 and September 1999. Informed consent was obtained prior to diagnostic thoracocentesis. Patients

with exudative lymphocytic pleural effusion were diagnosed according to Light's criteria (Heffner *et al*, 1997) and then enrolled in our study. Patients with bleeding tendency were excluded.

Pleural fluid was sent for conventional diagnosis including gram staining, AFB staining, aerobic culture, culture for *M. tuberculosis* on Lowenstein-Jensen (LJ)media, and cytology. Additional pleural fluid was used for PCR-assay in a control laboratory; the assessors were not informed of the clinical diagnosis of each patient. For histopathology, three pieces of pleural biopsy using a Abram's needle were taken where free-flow pleural tapping was possible. Tuberculous pleural effusion was diagnosed if: 1) the *M. tuberculosis* culture was positive, 2) the pleural pathology showed caseating granuloma in the absence of other pleural granulomatous diseases, or 3) the clinical symptoms or chest radiograph responded to antituberculous drugs.

DNA extraction

All pleural samples were centrifuged at 10,000 rpm for 10 minutes, the supernatant was discarded and the pellets were re-suspended in 300 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA). Lysozyme was then added at a concentration of 1 mg/ml and incubated for 90 minutes at 37ºC. Next, proteinase K and sodium dodecyl sulfate (SDS) solutions were added at 1 mg/ml and 3%, respectively, and the mixture was further incubated for 30 minutes at 60ºC. DNA was then extracted with phenol and chloroform and re-covered with ethanol for precipitation. The DNA extracted was re-suspended in 50 μl of dextrose water, of which 10 μl was used for PCR. This method of DNA extraction was described by Miyazaki *et al* (1993).

PCR using 16 S – 23 S ribosomal DNA spacer primers

PCR was done with primers 16 SA and 23 SA. The sequence of the primers were 16 SA (5′-TCG AAG GTG GGA TCG GC-3′) and 23 SA (5′-GCG CCC TTA AAC ACT TAC-3′) which is identical to the primer 16 SC and 23 SG previously described by Sansila *et al* (1998), except for the 14th base of 16 SA and for the 13th base of 23 SA, which became A instead of C and G instead of A, respectively. The primer sequences selected are shared by most mycobacteria but are different from those of most other bacteria in the 3′ end of the 16 S rRNA gene and 5′ end of the 23 S rRNA gene, respectively. The sequence of primer 16 SC is identical to a sequence in the 16 S rRNA gene 63 bp upstream of the spacers of most mycobacteria except *M. asiaticum*, which has the base A instead of C at the 14th position of the primer (this sequence is also shared by *Nocardia asteroides*). The sequence of 23 SG is complementary to a sequence in the 23 S rRNA gene 2 bp downstream from the spacers of *M. tuberculosis* complex, *M. kansaii,* and *M. gastri.* It has a single-base mismatch (G instead of T) with the corresponding sequences of *M. avium, M. paratuberculosis,* and *M. phlei* at the 10th base of the primer.

Amplification was done in a total volume of 50 μl with 10 μl of extracted DNA sample, 1 μl of each primer, 200 μM of each deoxynucleoside triphosphate and 1.25 units of *Taq* DAN polymerase (GIBCO BRL) in a PCR buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$. The reaction mixtures were amplified in a thermocycle (Gene Amp PCR system 2400, PE Applied Biosystems) as follows: a 3 minutes incubation at 94ºC for five cycles each cycle consisting of 30 seconds at 94ºC, 45 seconds at 55ºC and 40 seconds at 72ºC. This was followed by 25 cycles, each cycle consisting of 30 seconds at 94ºC, 45 seconds at 62ºC and 40 seconds at 72ºC for a total of 30 cycles. A final incubation at 72ºC for 7 minutes was included to allow complete strand synthesis. The amplified products were visualized by ethidium bromide-staining after electrophoresis in 2% agarose.

Nested PCR using 16 S rRNA

The nested PCR was based on selective amplification of the genes coding for 16 S rRNA. The primers were designed by using Oligo 5.0 software (National Biosciences, Inc, USA). The primers VL14 (5′- CAC ATG CAA GTC GAA CGG AAA GG -3′) and VL537 (5′- TTC ACG AAC AAC GCG ACA AAC CA -3′) were used in the primary PCR. It was found that the sequence VL14 was identical to the primer KY18 previously described by Tevere *et al* (1996). For the secondary PCR, primer GP50 (5′- TAC TCG AGT GGC GAA CGG GTG -3′) and primer GP397 (5′- CGG ACC TTC GTC GAT GGT GAA -3′) were used. In the primary PCR, the reaction tubes were subjected to a 2 minutes incubation at 50ºC and 2 cycles of 98ºC for 20 seconds, 55ºC for 20 seconds and 72ºC for 45 seconds. This was followed by 37 cycles of 94ºC for 20 seconds, 60ºC for 20 seconds and 72ºC for 45 seconds. A final incubation at 72ºC for 7 minutes to complete the extension of the primers. For the secondary PCR,

1 µl of the primary PCR product was mixed with 49 µl of a freshly prepared reaction mixture. This was followed by the same procedures used to obtain the primary PCR product.

Ethics

This research was approved by the Ethics Committee of the Faculty of Medicine, Khon Kaen University.

Statistical analysis

Descriptive statistics were used. Etiology of the exudative lymphocytic pleural effusion was given as number and percentage. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the conventional diagnostic tests (AFB staining and culture), PCR and nested PCR were calculated on a 2 x 2 table. The 95% confidence interval (CI) for the sensitivity and specificity of each diagnostic test were also calculated.

RESULTS

During the study period, 98 patients presented with symptomatic exudative lymphocytic pleural effusion. The mean age was 53.3 years (ranging from 18 to 78 years). The male to female ratio was 1.65:1. Tuberculous pleural effusion was diagnosed in 36 patients (36.7%). Other causes of exudative lymphocytic effusion were non-hematologic malignancy (53.1%), lymphoma (2.0%) and chronic nonspecific inflammation (8.2%) (Table 1). Of the patients diagnosed with tuberculosis, Ziehl-Neelsen-staining was positive in 2 patients (6%), pleural fluid culture grew *M. tuberculosis* in 6 patients (17%), and all patients responded to antituberculous drugs. Pleural biopsy, performed on 26 of 36 patients, revealed granulomas with caseous necrosis in 16 patients (62%), confirming tuberculosis.

The sensitivity and specificity (both at 95% CI) of AFB staining, culture, PCR-TB and nested PCR-TB are presented in Table 2. The sensitivity and specificity of AFB staining were 6% (95% CI : 1 to 10%) and 79% (95% CI : 71 to 87%), respectively. The sensitivity and specificity of culture tests were 17% (95% CI : 9 to 24%) and 100% (95% CI : 91 to 100%). For PCR using 16 S – 23 S ribosomal DNA spacer primers, the sensitivity was 50% (95% CI:40% to 60%), greater than AFB staining and culture, however, specificity was only 61% (95%CI:52 to 71%). When the 16S rRNA primers were used for nested PCR, the sensitivity was 72% (95% CI:63 to 81%) and the specificity was 53% (95% CI:43 to 63%). The PPV for PCR-TB and nested PCR-TB were 43% and 47%, respectively (Table 3), less than AFB staining and culture.

DISCUSSION

Pleural tuberculosis remains an important treatable cause of exudative lymphocytic pleural effusion (Epstein *et al*, 1987; Ferrer-Sancho, 1996; Seibert *et al*, 1991). Recent studies have investigated the usefulness of measuring adenosine deaminase (Valdes *et al*, 1998), interferon gamma (Villena *et al*, 1996), and PCR (Querol *et al*, 1995) as tools for early diagnosis.

Table 1
Etiology of exudative lymphocytic pleural effusion.

Etiology	Number	%
Non-hematologic malignancy	52	53.1
Tuberculosis	36	36.7
Lymphoma	2	2.0
Chronic nonspecific inflammation	8	8.2

Table 2
Sensitivity and specificity of each diagnostic test.

Tests	Sensitivity (95% CI)	Specificity (95% CI)
AFB staining	6% (1-10%)	79% (71-87%)
CultureTB	17% (9-24%)	100% (91-100%)
PCR-TB	50% (40-60%)	61% (52-71%)
Nested PCR-TB	72% (63-81%)	53% (43-63%)

Table 3
Positive predictive value (PPV) and negative predictive value (NPV) of each diagnostic test.

Tests	PPV	NPV
AFB staining	13%	59%
Culture TB	100%	67%
PCR-TB	43%	68%
Nested PCR-TB	47%	77%

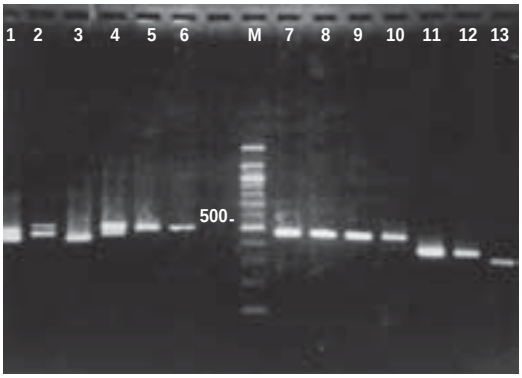

Fig 1–The amplified products of various *Mycobacterium* species using 16 S - 23 S rRNA primers with reference strains: 1-3 = *M. fortuitum*, 4 = *M. flavescens*, 5 = *M. duvatii*, 6 = *M. vaccae*, 7 = *M. smegmatis*, 8 = *M. chelonae*, 9 = *M. pheli*, 10 = *M. neolactis*, 11 = *M. tuberculosis* H37Rv, 12 = *M. avium*, 13 = *M. xenopi*, M=100 bp marker.

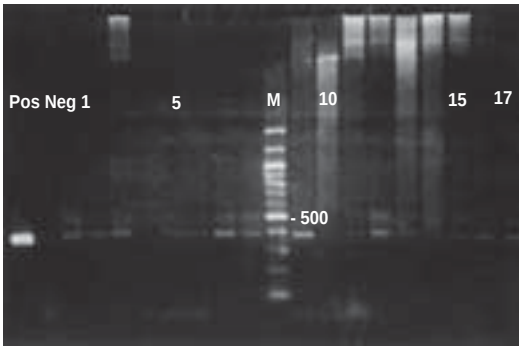

Fig 2–Amplification of pleural effusions using 16 S - 23 S rRNA spacer primers. Pos, Neg = Positive and negative control, 1-17 = pleural effusion specimens, M = 100 bp marker.

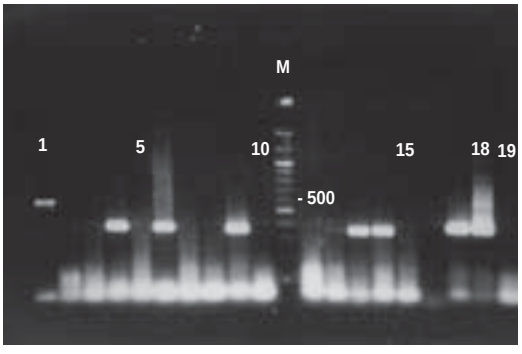

Fig 3–Nested PCR of pleural effusions using our in-house 16 S rRNA nested primers. 1 and 18 = 1st and 2nd positive PCR control; respectively, 2-17 = pleural effusions, 19 = negative control, M = 100 bp marker.

PCR is a sensitive and useful technique that has been used for rapid diagnosis of infectious diseases, especially mycobacterial infections (Young, 1994).The sensitivity of PCR in diagnosis of tuberculous pleurisy ranges from 20 to 81%, depending on the genomic sequence amplified and the procedure used during the extraction of the DNA. The specificity ranges from 78 to 100%(de Lassence *et al*, 1992; de Wit *et al*, 1992; Kuwano *et al*, 1995; Querol *et al*, 1995; Villena *et al*, 1998). In this study, we primarily used 16 S – 23 S ribosomal DNA spacer primers as has been described by Lappayawichit *et al*. (1996). By amplification with this pair of primers and restriction enzyme analysis of the amplified products can be used for species differentiation of some commonly isolated pathogenic mycobacteria (Fig 1). This test, however, has not been used for the direct detection and identification of mycobacteria in clinical samples. Our study is the first in which a 16 S – 23 S ribosomal DNA spacer-based PCR assay was used directly on clinical sample. By using these primers, the sensivity was higher than AFB staining and culture (50% *vs* 6% and 17%) (Table 2), however, its specificity was less (61% *vs* 79% and 100%). The less specificity of these primers may be due to the large amount of cellular DNA presented in the pleural fluid which caused many non-specific amplifications leading to false positive interpretation (Fig 2). Unlike pleural fluid, we have used these primers for detection of mycobacteria in sputum and found that non-specific amplification was not seen. Thus, we suggested that in case of samples with large amount of cells like pleural effusion, amplification with these primers is not suitable. Due to non-specific amplification of these primers in case of pleural effusion and to enhances sensitivity of the test, we have adopted a nested PCR method with the use of 16 S rRNA primers for amplification. This method had already been used to detect *M. tuberculosis* in various clinical specimens including pleural effusion (Pierre *et al*, 1991; Miyazaki *et al*, 1993). The nested PCR using 16 S rRNA (Fig 3) improved sensitivity, but did not appreciably improve specificity (Table 2). The parameter that determines the sensitivity is probably the number of bacilli in the pleural fluid sample analysed. It has been reported that PCR is positive in 66 to 100% of culture-positive tuberculous pleural fluids. In this study, the culture positive for *M. tuberculosis* was 17% (6 of 36 cases). The most of our cases, the diagnosis of tuberculous pleural effusion was made by histopathology (62%) and clinical response to anti-

tuberculous drugs (100%).

We also experienced false positive and false negative results. False positive results were caused by contamination of the system despite using negative controls. False positive results were also caused by nonviable mycobacteria in the pleural fluid (Trinker *et al*, 1996). Inhibitors that interfere with the PCR can cause false negative reactions. Many techniques have been developed to overcome these errors (Kox *et al*, 1994; Kunakorn *et al*, 1999).

Although the PCR-assay for diagnosis of tuberculous pleural effusion has been widely studied in recent years, its clinical value has not yet been fully determined. In clinical practice rapid diagnosis of tuberculosis is desirable, and pleural histopathology by pleural biopsy is usually the most useful test. We agree with other authors that PCR is more sensitive than AFB staining or culture of pleural fluid with use of LJ media. We conclude that PCR-assay in combination with culture may be useful if pleural biopsy is not feasible. Interpretation of PCR-results must be compared to overall clinical observations.

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