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Nested PCR for the Rapid Detection of TB from Pleural Fluid at HUKM Malaysia

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Abstract: The aim of the present study is rapid detection of tuberculosis from pleural effusion of suspected patients. Molecular technique Nested Polymerase Chain Reaction (PCR) was used for the purpose. A total of 67 pleural fluid collected at Hospital University Kebangsaan Malaysia during May 2005 to October 2006 were sent to Microbiology Laboratory enrolled in the study. Detection rate of *Mycobacterium tuberculosis* in pleural effusion was 0% by acid-fast bacilli (AFB) staining and 1.5% by culture on Lowenstein-Jensen medium. *Mycobacterium tuberculosis* was detected by PCR in 9% of the cases. PCR of pleural fluid had 19% sensitivity and 96% specificity, compared to AFB staining (0% sensitivity and 100% specificity) and culture (4% sensitivity and 100% specificity). PCR also has 67% Positive Predictive Value (PPV) and 72% Negative Predictive Value (NPV) in detecting *Mycobacterium tuberculosis*. Culture of pleural fluid has 100% PPV and 71% NPV while AFB staining has 0% PPV and 31% NPV. This preliminary study showed that PCR is a rapid method for detection of *Mycobacterium tuberculosis* in pleural fluid but its sensitivity is not up the marked.

Key words: *Mycobacterium tuberculosis*, nested PCR, pleural fluid, acid fast stain

INTRODUCTION

Tuberculosis (TB) is the number one infectious disease killer worldwide. The World Health Organization estimates that 2 billion people have latent TB, while another 3 million people worldwide die each year due to TB (Thomas Herchline, 2007). TB can affect almost every organ in a human's body but pulmonary TB is the most common. The high risk of person-to-person transmission, morbidity and mortality makes it one of the major threats to public health worldwide. Early diagnosis followed by adequate treatment is essential to prevent the threat. Tuberculous pleural effusion occurs in 30-60% patients throughout the world (Angeline *et al*., 2007). The conventional laboratory method for detection of Mycobacterium requires weeks for completion. Molecular method promises rapid detection and identification of Mycobacterium directly from clinical samples. Currently, there is no clear guideline for usage of molecular assay in diagnosis of extra pulmonary TB. Therefore the present study is aimed at to determine the sensitivity and specificity of PCR in detecting *M. tuberculosis* from pleural fluid and comparison with other diagnostic methods.

MATERIALS AND METHODS

Study design: The study was performed prospectively in a blinded manner, where the final clinical diagnosis was not available at the time when the specimens were received.

Study population: All pleural fluid sample sent to Microbiology Laboratory, Hospital University Kebangsaan Malaysia for routine culture between May 2005 to October 2006 were eligible for enrollment in this study. Samples collected from other sites such as sputum and bronchi alveolar lavage were also included in this study. Subsequently, the patients were placed under tuberculous (TB) and non-tuberculous (Non-TB) group based on the data collected from patient case files. All patients in the TB group was treated with anti-tuberculous treatment and showed marked improvement.

Sample size: The sample size for this study was calculated by using the Z-test formula. Where Z equals to 1.96 (80% power of study), expected population proportion (prevalence) of 0.04, absolute precision of 0.10 and at 95% confidence interval, the minimum sample size expected 15.

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Sample collection and processing: All pleural fluid received was divided into two parts. One part was centrifuged and processed following the standard operating procedure of laboratory for smear preparation on which Kinyoun stain was done and also cultured on Löwenstein-Jensen (LJ) medium. The inoculated LJ medium was incubated at 37°C for 42 days. The other half was used for Nested PCR.

Clinical data collection: Patient profiles and clinical data were retrieved from the case file of the medical record office. These data were manually entered in a form. The data were finally entered on a master sheet.

Nested PCR assay

Primers: The ABSOLUTE™ MTB II PCR kit by BioSewoom (Seoul, Korea) amplifies the IS6110 fragment of *M. tuberculosis*, a conserved repetitive sequence in the *M. tuberculosis* complex DNA.

Pretreatment of specimens: An amount of 0.5 mL of pleural fluid was centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. Eight hundred microliter of distilled water was added to the pellet. The specimen was again centrifuged at 12,000 rpm for 2 min and the supernatant discarded. Finally, 800 μL of distilled water was added to the remaining pellet. The specimen was again centrifuged at 12,000 rpm for 3 min and the supernatant discarded.

DNA extraction: The pellet from the pretreated sample was mixed with 50 μL of DNA extraction buffer. The mixture was placed in a heat block at 56°C for 15 min, then vortexed at high speed for 10 sec. Next, the mixture was placed in a heat block at 100°C for 8 min. Then again vortexed at high speed for 10 sec and followed by centrifugation at 12,000 rpm for 3 min and the supernatant was used for the amplification step.

Amplification of *M. tuberculosis* DNA: Nested PCR kit was used for amplification of DNA. The first PCR master mixture was prepared by adding 15.0 μL of first PCR mixture with 0.5 μL of first PCR enzyme. A 4.5 μL DNA extract was added to the first PCR master mixture and mixed well. The samples were subjected to first PCR condition as described in Table 1.

The second PCR master mixture was prepared by adding 18.0 μL of second PCR mixture with 0.5 μL of second PCR enzyme. This master mixture was then added with 1.5 μL of the first PCR product and mixed well. The samples were subjected to second PCR condition as described in Table 2.

Table 1: Step of amplification for first PCR condition

Step	Temperature	Duration	Cycle
Initial denaturation	95°C	2 min	1
Denaturation	94°C	20 sec	
Annealing	56°C	35 sec	35
Extension	72°C	40 sec	
Final extension	72°C	5 min	1

Table 2: Step of amplification for second PCR condition

Step	Temperature	Duration	Cycle
Initial penetration	95°C	5 min	1
Penetration	94°C	30 sec	
Annealing	65°C	30 sec	25
Extension	72°C	30 sec	
Final extension	72°C	5 min	1

Positive and negative controls were included in each run of PCR. Distilled water was used to replace the DNA extract as a negative control. Positive control was provided with the kit.

Detection of nested PCR product: A 5 μL portion of second PCR product was added to 1 μL of loading dye and subsequently was electrophoresed on a 2% agarose gel (Promega Corporation, USA, catalogue number V3121) in Ultra Pure™ TBE buffer (GIBCO BRL, USA, catalogue number 15546-013) at 100 volt for 30 min. The gel was stained with ethidium bromide and the 183 bp specific amplified bands were visualized on an ultraviolet transilluminator. GeneRuler™ 100 bp Ladder Plus (MBI Fermentas, USA, catalogue number SM0328) was used to identify the specified amplified fragment.

RESULTS AND DISCUSSION

Patients's profile: During 18 months study period, 67 pleural fluid samples were included in the study. Based on data collected from patient's clinical record 21 patients were grouped into final diagnosis of TB and 46 were diagnosed other than TB. The latter group was regarded as control group (Table 3).

In the tuberculous group there were 7 females and 14 males. They were placed into this group after final diagnosis of pulmonary TB on the basis of histological study, staining and cultural characteristics of the organisms, radiological findings and/or clinical improvement (Table 3). Age ranges of the patients were between from 17 to 84 years with a mean age of 48.8 years (Fig. 1). Ten Chinese, 8 Malays and 3 non-Malaysian were recorded in this group (Fig. 2).

The ages of the patients in non- tuberculous group ranged between 14 and 87 years with a mean age of 54.4 years. Eighteen females and 28 males were included in this group (Fig. 1). Four Indians, 20 Malays, 21 Chinese and 1 non-Malaysian were in this group (Fig. 2).

Table 3: Profile of the patients included in the study

Patient group	Number of patients
Tuberculosis	21
Sputum AFB (+)	1
Sputum culture (+)	8
Both stain and culture (+)	1
Clinical diagnosis*	11
Non-tuberculous#	46
Total	67

*: Group with suggestive chest radiographic findings and marked improvement after antituberculosis treatment

#: Control group: Systemic lupus erythematosus, congestive cardiac failure, liver disease, lymphoma, carcinoma and others

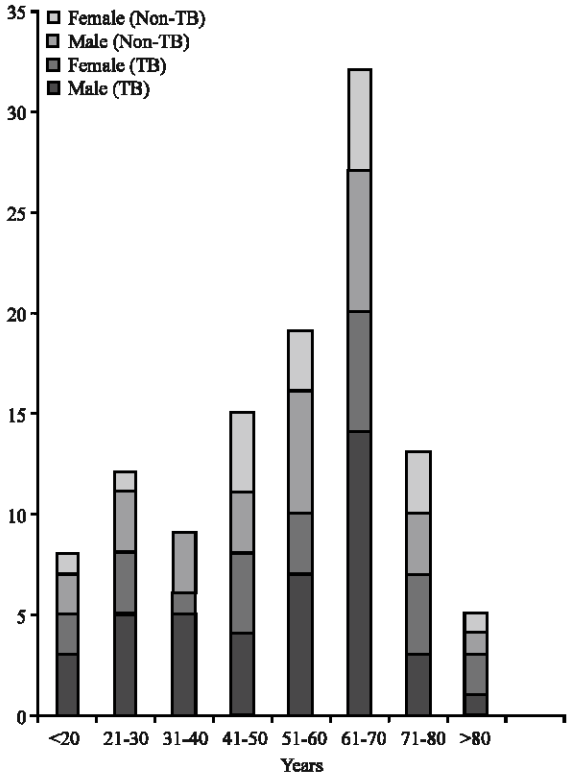

Fig. 1: Distribution of patients by age

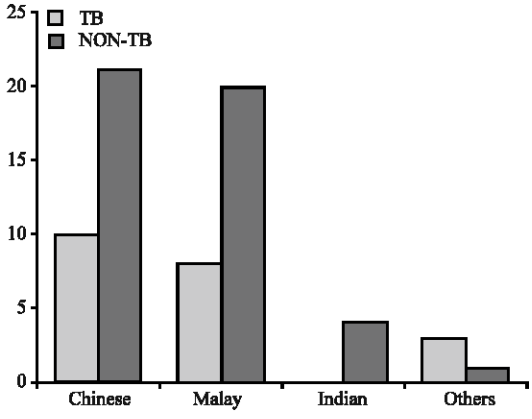

Fig. 2: Distribution of patients by race

Table 4: Results of acid fast staining, culture and nested PCR

Test	Final diagnosis TB	Final diagnosis Non-TB	Total
PF AFB (+)	0	0	0
PF AFB (-)	21	46	67
PF culture (+)	1	0	1
PF culture (-)*	19	46	65
PF TB-PCR (+)	4	2	6
PF TB-PCR (-)	17	44	61

*Note: One sample for culture was contaminated

Acid-fast staining of pleural fluid: All pleural fluid samples in the TB group were found to be AFB negative. None of the samples from the non-TB group was AFB positive (Table 4). Those samples taken from other sites, 2 (sputum samples) were AFB positive. Thus, AFB staining method was not able to detect any bacteria from pleural fluid even in case of 100% positive samples. Thus the sensitivity of AFB stain on pleural fluid was 0% with a specificity of 100%. The detection rate of *M. tuberculosis* by AFB staining was 0%.

Culture of pleural fluid: Only one pleural fluid produced typical colonies in the specialized media *M. tuberculosis*. Another sample was found to be contaminated. All samples in the non-tuberculous group were cultured negative (Table 4). The detection rate of *M. tuberculosis* in pleural fluid by culture was 1.5%. The sensitivity of culture in this study showed 4% with a specificity of 100%. The positive predictive value was 100% with 71% negative predictive value.

Nine patients exhibited positive cultured results from sputum samples, including the patient with the positive culture from pleural fluid. Thus, pleural fluid culture could not detect 89% of positive samples.

Figure 3 showed gel documentation of electrophoresis for the detection of the nested PCR product of TB. The size of *M. tuberculosis* specific amplified product is 183 bp. Four samples showed positive band at the 183 bp position. Distilled water was used as the negative control. Positive control was observed as a broad band at position 183 bp.

Using IS6110 insertion sequence, *M. tuberculosis* complex DNA was detected in 6 patients. Four of them were in the tuberculous group and the remaining 2 were in the non-tuberculous group (Table 4). The detection rate of *M. tuberculosis* in pleural fluid by TB PCR was 9%. The sensitivity of PCR was 19% with 96% specificity. PCR results exhibited 67% positive predictive value and 72% negative predictive value.

None of the pleural fluid samples in the tuberculous group were both PCR and culture positive. Five of the PCR positive samples were cultured negative and 1 was contaminated. Nine patients in the tuberculous group grew *M. tuberculosis* in samples taken from other sites, i.e., sputum. Among the 4 true positive PCR, one patient's

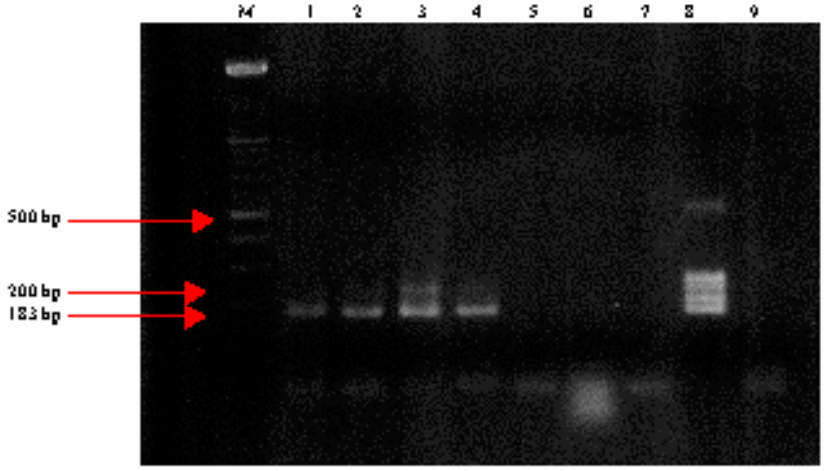

Fig. 3: Detection of PCR product of *Mycobacterium tuberculosis*. M: 100 bp Marker, 1: Patient 45, 2: Patient 46, 3: Patient 47, 4: Patient 48, 5: Patient 49, 6: Patient 50, 7: Patient 51, 8: Positive control, 9: Negative water

sputum was AFB positive but cultured as negative. Another patient had sputum AFB negative but culture gave positive results. The remaining two patients were sputum AFB and culture proved negative. They were both clinically diagnosed as TB. One patient had family history of TB and the other had radiological evidence of pulmonary TB. Both patients respond to therapeutic treatments.

The high number of false negative result in this study is a matter of great concern. There are few possible reasons for false negative result in pleural effusion samples. The pleural effusion is a result of possible hypersensitivity reaction as mentioned earlier (Nagesh *et al*., 2001). The paucibacillary nature of the disease, the availability of small amount or volume of sample and non-uniform distribution of microorganism during aliquoting of samples further contributes to the number of false negative result.

False negative result causes reduction in sensitivity of any test. A false negative result in any molecular technique may be due inhibitor present in sample. Improving the extraction technique and the use of internal control to determine efficiency may prevent this. Another reason is the specific infecting organism that was detected by the primer was not probably present in the samples. This problem may be overcomed by increasing the sample number from each patient to cover more types and time points (Huggett *et al*., 2003). The absence of IS6110 might have also caused a false positive result. The occurrence of *M. tuberculosis* strain deficient of IS6110 has been reported worldwide such as 1% in San Francisco, Vietnam (2%) and Chennai (4%) (Radhakrishnan *et al*., 2001). The author mentioned that 62.5% of the study population in Kerala was not typeable by IS6110- based fingerprinting.

The diagnosis of extra pulmonary tuberculosis is still challenging for a number of reasons. The lack of adequate sample volumes, non- uniform distribution of microorganism contributes to this problem. The paucibiliary nature of the specimen and the presence of inhibitors undermine the performance of molecular techniques (Pfyffer *et al*., 1996).

The result of this study shows a detection rate of 9% by PCR compared to 0% by AFB staining and 1.5% by culture. This suggests that PCR is a more sensitive method for detection of TB from pleural fluid (19%) compared to AFB stain (0%) and culture (4%). However, the test singly is not enough to identify all cases of TB. However, it can be used as an adjunct to the traditional methods of laboratory diagnosis of TB.

In conclusion, TB PCR is a rapid method in the diagnosis of tuberculous pleural effusion. Molecular detection of *M. tuberculosis* is very useful in cases that are highly suspected as pleural tuberculosis those are negative for AFB and culture. However, because of the relatively low sensitivity of TB PCR in pleural effusion, clinical judgment remains the ultimate decision in the management of tuberculosis.

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