Brief Report

DOI: 10.5582/bst.2011.v5.4.159

The advantage of using IS6110-PCR *vs*. BACTEC culture for rapid detection of *Mycobacterium tuberculosis* from pleural fluid in northern India

Anand K. Maurya[1], Surya Kant[1,*], Ram Awadh Singh Kushwaha[1], Vijaya Lakshmi Nag[2], Manoj Kumar[2], T. N. Dhole[2]

[1] *Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University (Erstwhile King George Medical College), U.P., Lucknow, India;*
[2] *Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.*

Summary **Pleural tuberculosis is an extra-pulmonary disease which poses a diagnostic dilemma. The detection of mycobacterial DNA by IS6110 polymerase chain reaction (PCR) in clinical samples is a promising approach for the rapid diagnosis of pleural tuberculosis infections. The aim of the present study is to evaluate the advantage of using IS6110 PCR for rapid detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) from pleural fluid. 102 clinically suspected cases of pleural tuberculosis cases were enrolled from inwards and outwards of the Department of Pulmonary Medicine at Chattrapati Shahuji Maharaj Medical University, Lucknow from April 2007 to April 2010. The pleural fluids were processed at the Mycobacteriology Laboratory of Department of Microbiology at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Pleural fluid samples were processed and examined by Ziehl Neelsen (ZN) staining for acid fast bacilli and detection of *M. tuberculosis* by BACTEC culture. We applied IS6110 PCR to detect specific *M. tuberculosis* complex in pleural fluid samples. We found a significant difference in sensitivity of different tests, acid fast bacilli were detected in 17 (16.6%) samples by ZN Staining , 47 (46.1%) by BACTEC culture and using IS6110 PCR, 62 (60.7%) were positive for IS6110 PCR for *M. tuberculosis*. We found IS6110 PCR was much more sensitive than ZN staining and BACTEC culture. IS6110 PCR detection of *M. tuberculosis* may be very useful in cases that are highly suspect as pleural tuberculosis and those that are negative for AFB and culture. IS6110 PCR may gain an immense prospective to better clinicians ability to improve diagnosis of pleural tuberculosis.**

Keywords: Tuberculosis, pleural fluids, *Mycobacterium tuberculosis (M. tuberculosis)*, polymerase chain reaction

1. Introduction

Pleural tuberculosis is responsible for 30-80% of all pleural effusions encountered and may complicate tuberculosis in 31% of all cases (*1*). Thus, tuberculous pleuritis remains a major contributor to worldwide morbidity and mortality. It poses diagnostic and therapeutic problems due to the low sensitivity of the diagnostic tools. Conventional culture is time consuming and lacks sensitivity; smears for acid-fast bacilli (AFB) is rapid but the sensitivity has not been evaluated in pleural fluid. However, it has been reported to be positive in less than 10-37% of patients and mycobacterial cultures in variable proportions (12-80%) in different body fluids (*2*). The diagnostic dilemma can affect treatment by either delaying it or causing inappropriate empiric therapy for tuberculosis (TB) to subjects without mycobacterial infections or

*Address correspondence to:
Dr. Surya Kant, Department of Pulmonary Medicine Chhatrapati Shahuji Maharaj Medical University (Erstwhile King George Medical College), U. P., Lucknow, India.
e-mail: dr.kantskt@rediffmail.com

with atypical mycobacteria (*3*). The development of a diagnostic method capable of rapidly identifying *Mycobacterium tuberculosis* (*M. tuberculosis*) in pleural fluid from patients remains a worthwhile aim. Several studies have been reported on PCR to detect *M. tuberculosis* (*4-8*). The detection of the IS6110 insertion element present in multiple copies (*9*) can be used to detect *M. tuberculosis* complex, but no other mycobacterial species. The aim of the present study is to evaluate the advantage of IS6110 PCR *vs*. BACTEC culture for rapid detection of *M. tuberculosis* from pleural fluid in Northern India.

2. Materials and Methods

2.1. *Study design*

The study was performed prospectively in a blinded manner. Study setting was Referral Medical Institutions in Northern India.

2.2. *Study population*

A total of 102 clinically suspected cases of pleural tuberculosis were enrolled from inwards and outwards of the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, from April 2007 to April 2010.

2.3. *Clinical information and data collection*

The clinical history regarding disease, present and past history of TB and anti-tuberculosis treatment (ATT) taken along with information regarding family history of tuberculosis was obtained in prescribed proforma. Clinical examination and pleural fluid (approximately 2-5 mL) was aspirated, after informed consent, by the clinician and stored at 4°C and transferred to the Mycobacteriology Section of Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India (within 4 h of collection). Patient profiles and clinical data were retrieved from the medical record case file.

2.4. *Processing and microbiological test of pleural fluid*

Pleural aspirate was divided into two parts, one part was kept at −20ºC for PCR processing and the other part was processed for mycobacterial smear preparation and BACTEC culture. Smears were stained using the ZN method and examined for AFB (*10*). BACTEC vials were incubated and interpreted as per Becton Dickinson (BD, Sparks, MD, USA) manual instructions (*11*). The p-nitro-α-acetylamino-β-hydroxy propiophenone (NAP) identification was done to differentiate *M. tuberculosis* complex from non tuberculous mycobacteria (*11*).

2.5. *Extraction of DNA*

Extraction of DNA was done by the CTAB (cetyl-tri-methyl-ammonium bromide) -phenol chloroform extraction method (*12*). First the pleural aspirate was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 567 µL of TE (Tris EDTA, pH 7.4) buffer, 30 µL 10% SDS (sodium dodecyl sulfate) and 3 µL proteinase K (20 mg/mL), mixed and incubated at 37°C for 1 h. After incubation, 100 µL of 5 M NaCl and 80 µL of high-salt CTAB buffer (containing 4 M NaCl), 1.8% CTAB was added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (0.7-0.8 µL) of chloroform-isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged for 4-5 min in a microcentrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform-isoamyl alcohol (1:1) was added followed by a 5 min spin at 12,000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 100 µL of TE buffer.

2.6. *Primers and PCR*

The amplification reaction was performed in a final volume of 20 µL. The reaction mixture contained 10 µL Pyrostart Fast PCR Master Mix 2X (dNTP, Taq polymerase with $MgCl_2$, Fermentas, India), 1 µL (10 pmole) of each primer, 3 µL water (nuclease free) and 5 µL of extracted DNA. The oligonucleotide primers (*13*) used were P1 and P2, and are: 5'-CCT GCG AGC GTA GGC GTC GG3' and 5' CTC GTC CAG CGC CGC TTC GG 3' respectively (SBS Gentech Co., Ltd.). These primers amplified a target fragment 123 base pairs (bp) from the insertion of the *M. tuberculosis* sequence element IS6110. The PCR amplification was done in a thermal cycler (MJ Research ,PTC-100, GMI, Inc, USA), which involved 40 cycles of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 1 min. The amplified products were separated on 2% agarose gels and visualized on a UV-light transilluminator (Bangalore Genei, Bangalore, India). The presence of the 123 bp fragment indicated a positive test (*M. tuberculosis* complex). The positive controls included the DNA of the H37Rv strain. Negative control included PCR grade water.

2.7. *Statistical analysis*

We assumed statistical significance at $p < 0.05$. The sensitivity, specificity, positive predictive value and negative predictive values were calculated with a 95% confidence interval (95% CI) using the standard formulas

considering BACTEC as the gold standard (*14*).

3. Results and Discussion

3.1. *Patients characterization*

A total of 102 patients were enrolled in our study. Of these 102 patients, 77 (75%) patients were males and 25 (25%) were females. The mean age of all patients was 30.4 ± 13.2 years. Patients 25-44 years of age accounted for 42.2% of the total cases. Among all cases, 70 were newly detected cases (68.6%), 25 were previous treated cases (24.5%), 5 were on treatment (4.9%) and 2 were unknown (1.9%). The history of contact with TB patients was determined in 20 cases (19.6%), 19(18.2%) had a history of diabetic mellitus and a past history was present in 11 (10.7%). In the case of HIV presentation, 2 cases were HIV positive (2.5%) and they had an antiretroviral therapy (ART) and ATT taken and 100 were HIV negative (97.5%). We found common symptoms of pleural tuberculosis, 17 patients had hempotosis (16.6%), 62 cough (60.7%), 75 fever (75.9%), 71 anorexia and/or weight loss (70%), 53 chest pain (51.9%), 58 night sweat and or chills (56.5%) and 21 dyspnoea (20.5%).

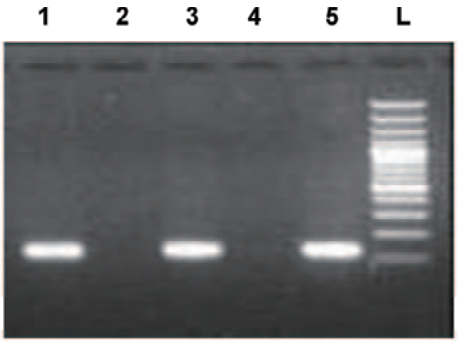

Figure 1. Gel documentation of electrophoresis separation of the amplicon into 2% agarose gel is documented across Lanes 1-5. The presence of a 123 bp. Amplicon in the **Lanes 1** and **3** indicated the presence of the target while the absence of the amplicon in the **Lane 2** pointed towards the absence of the target. **Lane 4** was Negative Control (PCR water) and **Lane 5** was Positive Control (H37Rv). **Lane L** was 100 bp Ladder well shown.

3.2. *Detection rate of M. tuberculosis by smear, BACTEC culture and IS6110 PCR*

All pleural fluid samples in the suspected cases of pleural TB were found to be AFB positive in 17 (16.6%). The sensitivity of AFB staining on pleural fluid was 16.6 % and its detection rate for *M. tuberculosis* by AFB staining was 16.6%. The detection of *M. tuberculosis* by BACTEC culture was 47 (46.1%). Using IS6110 PCR, 62 (60.7%) were positive for IS6110 PCR for *M. tuberculosis*, and results are shown (Figure 1).

3.3. *Comparison of sensitivity of PCR test vis-a-vis other tests*

Seventeen patients were positive with AFB and PCR was positive (100%) for *M. tuberculosis*, 85 patients were negative with AFB and PCR was positive for 45 (52.9%) but culture was subsequently positive in 32 (31.3%) patients. The sensitivity of PCR testing was 100% for 15 patients positive for both AFB and culture, where we found low sensitivity for 1 (50%) of 2 patients were AFB positive with negative cultures. 32 patients (93.7%) had PCR sensitivity with smears negative and cultures positive. In other words, 30 of the 32 *M. tuberculosis* complex culture positives were positive for IS6110 sequences. Therefore, given the sensitivity of 93.7 % and 53 negatives by all other tests used (smear negative and culture negative) samples which were positive by PCR were 16 (30.1%). These were not likely to represent false positive results because repeated PCR tests were positive for these samples and these samples belonged to highly suspected cases of pleural tuberculosis which responded to antitubercular treatment (Table 1).

3.4. *Comparison of sensitivity and specificity of PCR tests and smear microscopy vs. BACTEC as the gold standard*

On taking BACTEC culture as the gold standard the sensitivity, specificity, positive predictive value and negative predictive value of microscopy and PCR are given in Table 2. The sensitivity, specificity, positive

Table 1. Comparison of sensitivity of PCR test via a via others tests

Test	No. (%)	PCR Results (*n*)		Sensitivity of PCR test (%)
		Positive	Negative	
Smear positive	17 (16.6)	17	0	100
Smear negative	85 (83.4%)	45	40	52.9
BACTEC Positive	47 (46.1)	45	2	95.7
BACTEC negative	55 (53.9)	17	38	30.9
Smear positive BACTEC positive	15 (14.8)	15	0	100
Smear Negative BACTEC Positive	32 (31.4)	30	2	93.7
Smear Positive BACTEC Negative	2 (1.9)	1	1	50
Smear Negative and BACTEC negative	53 (51.9)	16	37	30.1

Table 2. Comparison of sensitivity and specificity of PCR test , smear microscopy and BACTEC culture in 102 patients

Test	BACTEC Culture *M. tuberculosis*		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
PCR						
Positive	45	17	95.7 %	69.1 %	72.5%	95.0%
Negative	2	38				
Smear						
Positive	15	2	31.9%	96.3 %	88.2 %	62.3 %
Negative	33	52				

Sensitivity, specificity, positive and negative predictive values of PCR and microscopy were calculated using BACTEC culture results as the gold standard. PPV: positive predictive value; NPV: negative predictive value.

predictive value and negative predictive values were calculated with a 95% confidence interval (95% CI) using standard formulas. Among 102 patients, 62 were positive with IS6110 PCR and 17 were positive with AFB smear microscopy. By defining BACTEC culture as the gold standard for comparative usefulness of the PCR assay, the sensitivity and specificity of the assay were 95.7% (95% CI 0.85-0.98) and 69.1% (95% CI 0.55-0.79) shown in Table 2. We found that sensitivity of PCR (95.7%) was very high in comparison to smear microscopy but specificity of PCR (68.5%) was lower than smear microscopy (96.3%). The variation in the specificity of IS6110 PCR may be due to the varied methods of extraction of DNA, use of different sets of PCR primers designed to amplify IS6110 nucleotides and expertise in performing the PCR technique.

Pleural tuberculosis is a major, treatable cause of exudative pleural effusion. Chakrabati *et al*. (2006) stated that the epidemiology and demographics of tuberculous pleurisy are changing due to the impact of HIV co-infection and the increasing amount of pleural effusion seen as part of reactive diseases (*15*). The diagnosis of pleural fluid is still a challenge for number of reasons. The lack of adequate sample volumes and the non uniform distribution of microorganisms contribute to this problem. Escudero BC *et al.* (1990) suggested that the diagnosis of TB pleurisy is usually accomplished using radiological and clinical findings, pathology of pleural tissue from biopsy, and several laboratory methods (*16*). Conventional methods include direct examination of pleural fluid with ZN staining of acid-fast bacilli and culture. ZN staining is rapid and inexpensive but requires a bacilli concentration of 10,000/mL and has a low sensitivity of approximately 0-1% (*16*). Earlier studies suggested that culture was more sensitive (11-50%); where only 10-100 bacilli yield the diagnosis, but required 2-6 weeks to grow *M. tuberculosis* (*17*). Pleural biopsy studies have high sensitivity (70-80%), but the procedure is not free of risk (*18,19*). At the present time, the most reliable method for diagnosis of pleural TB is the detection of *M. tuberculosis* in pleural specimens. Rapid diagnosis of pleural TB is critical in order to reduce morbidity and mortality. Study data showed PCR was a molecular biology technique that can detect *M. tuberculosis* genome in pleural fluid or tissue specimens (*20,21*). The sensitivity (31.3-81%) and specificity (96.6-100%) were variable. Studies conducted by Parandaman V *et al*. (2000), Tan J *et al*. (1995), Takagi N *et al*. (1998) and Jatana SK *et al*. (2000) showed disparate results where sensitivity is 100% and specificity varied from 70-90% (*22-25*). But these studies have been carried out on a small sample size; all of them have targeted the IS6110 insertion sequences and have engaged different sets of primers. The IS6110 PCR protocol used to detect the IS6110 insertion sequence was more sensitive than other PCR protocols (*26*). Previous studies found the IS6110 sequence present in 10-15 copies in each mycobacterial genome (*9,27*), and increased sensitivity afforded by the detection of the IS6110 insertion sequence considerably improves the yield for the detection of mycobacterial DNA in pleural fluid. Because this insertion sequence is present only in mycobacteria of the *M. tuberculosis* complex, positive results were not observed for other species of mycobacteria (*9*). Our study results suggest that IS6110 PCR is more sensitive than conventional methods, but still not absolute to identify all cases. In cases where ZN positive samples were found IS6110 PCR amplification was positive. Studies revealed that the insertion element of IS6110 primers for detection of TB, PCR directed towards the IS6110 sequence of *M. tuberculosis* have been evaluated by Villegas *et al*. (2000) (*28*). Parandaman *et al.* (2000) found 100% positive PCR of positive culture samples, but PCR was positive in 30-60% of culture negative pleural fluids (*22*). Our results have shown that PCR was positive in 15/15 (100%) from positive cultures but PCR was positive in 17/55 (30.9%) from negative cultures. Reechaipichitkul *et al*. (2000) revealed that the sensitivity and specificity of pleural fluids on culture were 17% but we found sensitivity of pleural fluid on culture were 46.1% (*29*).

In conclusion, IS6110 PCR assays can successfully be used to detect *M. tuberculosis* DNA in pleural fluid samples for a more rapid, specific and reliable TB diagnosis than the BACTEC culture and ZN staining methods. IS6110 based PCR detection of *M.*

tuberculosis may be very useful in cases that are highly suspect for pleural TB and those that are negative for AFB and culture, and it may better clinicians ability to improve diagnosis in pleural TB.

Acknowledgements

This work was supported by a grant from Indian Council of Medical Research, New Delhi (Extramural ICMR Project Sanction No. 5/8/5/4/2007-ECD-I). Authors would like to thank the Technical Members of Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, India for technical support during research work. We would like to give special thanks to residents of the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Uttar Pradesh Lucknow, India for their support during sample collection.

References

1. Lazarus AA, McKay S, Gilbert R. Pleural tuberculosis. Dis Mon. 2007; 53:16-21.
2. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian J Med Res. 2004; 120:316-353.
3. Montenegro SH, Gilman RH, Sheen P, Cama R, Caviedes L, Hopper T, Chambers R, Oberhelman RA. Improved detection of Mycobacterium tuberculosis in Peruvian children by use of heminested IS6110 polymerase chain reaction assay. Clin Infec Dis. 2003; 36:16-23.
4. Pao CC, Yen TS, You JB, Maa JS, Fiss EH, Chang CH. Detection and identification of Mycobacterium tuberculosis by DNA amplification. J Clin Microbiol. 1990; 28:1877-1880.
5. De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of mycobacterium tuberculosis in clinical specimens by DNA amplification. J Clin Microbiol. 1990; 28:2437-2441.
6. Hermans PW, Schuitema AR, Van Soolingen D, Verstynen CP, Bik EM, Thole JE, Kolk AH, van Embden JD. Specific detection of mycobacterium tuberculosis complex stains by polymerase chain reaction. J Clin Microbiol. 1990; 28:1204-1213.
7. Kuwano K, Minamide W, Kusunoki S, Igimi H, Fujiki T, Matsuba K, Hara N. Evaluation of nested polymerase chain reaction for detecting mycobacterial DNA in pleural fluid. Kansenshogaku Zasshi. 1995; 69:175-180.
8. Aslanzadeh J, de la Viuda M, Fille M, Smith WB, Namdari H. Comparison of culture and acid-fast bacilli stain to PCR for detection of Mycobacterium tuberculosis in clinical samples. Mol Cell Probes. 1998; 12:207-211.
9. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for Mycobacterium tuberculosis. J Infect Dis. 1990; 161:977-981.
10. Baron EJ, Finagold SM. (Eds) Mycobacteria, chapter 13. In: Bailey and Scott's Diagnostic Microbiology, 9th ed. (The CV Mosby Company, St Luis). 1994; 590-633.
11. Siddiqi SH. BACTEC 460 TB System. Product and procedure manual, revision D. Sparks, Md: Becton Dickinson Microbiology Systems; 1996.
12. Somerville W, Thibert L, Schwartzman K, Behr MA. Extraction of Mycobacterium tuberculosis DNA: A question of containment. J Clin Microbiol. 2005; 43:2996-2997.
13. Hasaneen NA, Zaki ME, Shalaby HM, El-Morsi AS. Polymerase chain reaction of pleural biopsy is a rapid and sensitive method for the diagnosis of tuberculous pleural effusion. Chest. 2003; 124:2105-2111.
14. Kolk AH, Kox LF, van Leeuwen J, Kuijper S, Jansen HM. Clinical utility of the polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis. Eur Respir J. 1998; 11:1222-1226.
15. Chakrabarti B, Davies PD. Pleural tuberculosis. Monaldi Arch Chest Dis. 2006; 65:26-33.
16. Escudero Bueno C, García Clemente M, Cuesta Castro B, Molinos Martín L, Rodríguez Ramos S, González Panizo A, Martínez Glez-Río J. Cytologic and bacteriologic analysis of fluid and pleural biopsy specimens with Cope's needle: Study of 414 patients. Arch Intern Med. 1990; 150:1190-1194.
17. de Wit D, Maartens G, Steyn L. A comparative study of the polymerase chain reaction and conventional procedures for the diagnosis of tuberculous pleural effusion. Tuber Lung Dis. 1992; 73:262-267.
18. Seibert AF, Haynes J Jr, Middleton R, Bass JB Jr. Tuberculous pleural effusion. Twenty-year experience. Chest. 1991; 99:883-886.
19. Liu KT, Su WJ, Perng RP. Clinical utility of polymerase chain reaction for diagnosis of smear-negative pleural tuberculosis. J Chin Med Assoc. 2007; 70:146-151.
20. Lima DM, Colares JK, da Fonseca BA. Combined use of the polymerase chain reaction and detection of adenosine deaminase activity on pleural fluid improves the rate of diagnosis of pleural tuberculosis. Chest. 2003; 124:909-914.
21. Villena V, Rebollo MJ, Aguado JM, Galán A, López Encuentra A, Palenque E. Polymerase chain reaction for the diagnosis of pleural tuberculosis in immunocompromised and immunocompetent patients. Clin Infect Dis. 1998; 26:212-214.
22. Parandaman V, Narayanan S, Narayanan PR. Utility of polymerase chain reaction using two probes for rapid diagnosis of tuberculosis pleuritis in comparison to conventional methods. Indian J Med Res. 2000; 112:47-51.
23. Tan J, Lee BW, Lim TK, Chin NK, Tan CB, Xia JR, Yap HK, Kumarasinghe G. Detection of Mycobacterium tuberculosis in sputum, pleural and brocheoalveolar lavage fluid using DNA amplification of the MPB 64 protein coding gene and IS6110 insertion element. Southeast Asian J Trop Med Public Health. 1995; 26:247-252.
24. Takagi N, Hasegawa Y, Ichiyama S, Shibagaki T, Shimokata K. Polymerase chain reaction of pleural biopsy specimens for rapid diagnosis of tuberculous pleurisy. Int J Tuberc Lung Dis. 1998; 2:338-341.
25. Jatana SK, Nair MN, Lahiri KK, Sarin NP. Polymerase chain reaction in the diagnosis of tuberculosis. Indian Pediatr. 2000; 37:375-382.
26. de Lassence A, Lecossier D, Pierre C, Cadranel J, Stern M, Hance AJ. Detection of mycobacterial DNA in pleural fluid from patients with tuberculous pleurisy by means of the polymerase chain reaction: Comparison of

two protocols. Thorax. 1992; 47:265-269.
27. Hermans PW, van Soolingen D, Dale JW, Schuitema AR, McAdam RA, Catty D, van Embden JD. Insertion element IS986 from Mycobacterium tuberculosis: A useful tool for diagnosis and epidemiology of tuberculosis. J Clin Microbiol. 1990; 28:2051-2058.
28. Villegas MV, Labrada LA, Saravia NG. Evaluation of polymerase chain reaction, adenosine deaminase, and interferon-gamma in pleural fluid for the differential diagnosis of pleural tuberculosis. Chest. 2000; 118:1355-1364.
29. Reechaipichitkul W, Lulitanond V, Sungkeeree S, Patjanasoontorn B. Rapid diagnosis of tuberculous pleural effusion using polymerase chain reaction. Southeast Asian J Trop Med Public Health. 2000; 31:509-514.

(Received April 14, 2011; Revised May 30, 2011; Accepted June 6, 2011)