DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM, PLEURAL, AND BRONCHOALVEOLAR LAVAGE FLUID USING DNA AMPLIFICATION OF THE MPB 64 PROTEIN CODING GENE AND IS6110 INSERTION ELEMENT

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Abstract. Two gene sequences specific for *Mycobacterium tuberculosis* were evaluated for the diagnosis of pulmonary tuberculous (PTB) in pleural fluid (PF), bronchoalveolar lavage fluid (BAL) and sputum (Sp). The 240 bp sequence (nts 460-700) coding for the MPB 64 protein coding gene and the 123 bp IS6110 insertion element present in multiple copies in the mycobacterial genome were amplified using the polymerase chain reaction. Fifty-nine clinical specimens were studied. The diagnosis of PTB was confirmed by positive *M. tuberculosis* cultures in 14 specimens, and by the presence of characteristic histological features of granuloma and Langerhan's giant cells on pleural biopsy in 3 PF specimens though cultures for *M. tuberculosis* were negative. The remaining 42 specimens were obtained from patients with non-tuberculous pulmonary infections or malignancy, and these served as negative controls. Our results showed that the IS6110 insertion element and MPB 64 gene sequence were detected in all 14 culture positive PTB cases, although detection of the latter sequence required both DNA amplification and oligonucleotide hybridization. There was however one false positive specimen with the MPB 64 detection protocol. More importantly, both the MPB 64 sequence and IS6110 insertion element protocols were unable to detect *M. tuberculosis* DNA in the 3 PF samples diagnosed by histological characteristics on pleural biopsy and culture negative. We conclude that DNA amplification for *M. tuberculosis*-specific sequences is a useful method for rapid diagnosis of PTB in culture positive specimens. However, the false negative results with TB culture negative cases of tuberculous pleurisy, limits its usefulness for the diagnosis of tuberculous pleurisy.

INTRODUCTION

In Southeast Asia, tuberculosis is a major health problem with an annual risk of infection of 2% (Lin, 1986). In a special study undertaken in 1989/1990 by the WHO Tuberculosis Unit, the Southeast Asian region recorded an incidence of 2.5 million new cases (31% of the global toll) with the largest number of deaths (Kochi, 1991).

DNA amplification using the polymerase chain reaction has recently been widely utilized for the detection of *Mycobacterium tuberculosis* DNA in clinical specimens. DNA can now be efficiently extracted from cerebrospinal fluid (Shankar *et al*, 1991), urine (Manjunath *et al*, 1991), sputum (Altamirano *et al*, 1992; Soini *et al*, 1992), bronchoalveolar lavage fluid (Walker *et al*, 1992), biopsied tissues (Narita *et al*, 1992), gastric aspirate and pleural effusions (De Witt *et al*, 1990), and purified for DNA amplification by the polymerase chain reaction.

Amplification of gene sequences specific for *M. tuberculosis* genome has offered a sensitive and specific means for diagnosis of tuberculosis. The rapidity of diagnosis using DNA amplification is in sharp contrast to culture identification which requires 6-8 weeks. Smear examination for the detection of acid-fast bacilli has its disadvantages in that it does not differentiate pathogenic from contaminant mycobacteria, and a minimum of about 10,000 organisms/ml is required for detectability (Bates, 1979).

Amplification assays for diagnosis of TB have utilized gene sequences specific to *M. tuberculosis*. To date, the MPB 64 protein coding gene (Kaneko *et al*, 1990), the 65 kDa (Hance *et al*, 1989) and 32 kDa (Cormican *et al*, 1992) antigens, the 318 bp cloned

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fragment of *M. tuberculosis* (Altamirano *et al*, 1992), the 369 bp species-specific *M. tuberculosis* DNA fragment (Del Portillo *et al*, 1991) and the IS6110 repeat sequence (Eisenach *et al*, 1991) have been evaluated for the specific diagnosis of tuberculous meningitis (TBM).

We have previously evaluated three *M. tuberculosis* specific DNA sequences - MPB 64 protein coding gene, 65 kDa antigen and the IS6110 insertion element - for diagnosis of TBM in cerebrospinal fluid specimens (Lee *et al*, 1994). Our results indicated that although all 3 sequences detected TB DNA in the six positive cases, false positive results especially with the 65 kDa antigen and the IS6110 insertion element were encountered. Subsequently, the IS6110 insertion element protocol was changed by reducing the number of amplification cycles from 30 to 25.

In this study, we compared the single copy MPB 64 protein sequence with the IS6110 repeat sequence (at 25 cycles) for the detection of *M. tuberculosis* DNA in pleural fluid (PF), bronchoalveolar lavage (BAL) fluid and sputum (Sp) specimens, as a rapid means for the diagnosis of PTB.

MATERIALS AND METHODS

Bacterial cultures

M. tuberculosis cultures were kindly provided by the TB laboratory, Tan Tock Seng Hospital, Singapore. Non-mycobacterial cultures of *Escherichia coli, Pseudomonas* species, *Klebsiella* species, *Streptococcus* A, B and G were obtained from the Microbiology Department, National University Hospital (NUH).

Clinical specimens

A total of 59 clinical specimens from patients hospitalized at the NUH were evaluated. Fourteen of these specimens (7 PF, 4 BAL, and 3 Sp specimens) were obtained from patients diagnosed as PTB by a positive *M. tuberculosis* culture. In addition, 3 PF specimens were diagnosed as PTB positive by characteristic histological features of granuloma and Langerhan's giant cells on pleural biopsy, although TB cultures were negative. The remaining 42 non-tuberculous specimens served as negative controls.

DNA extraction

Bacteria strains: Bacteria cultures were resuspended in Tris-EDTA (10/1) and DNA was extracted using sodium dodecyl sulphate (SDS) and proteinase K at final concentrations of 0.5% and 100 μg/ml respectively. The mixture was incubated for 1 hour at 37°C followed by a 10 minute incubation at 65°C in cetyltrimethyl-ammonium bromide (CTAB, Aldrich, Milwaukee, USA) to remove cell wall debris and proteins.

Pleural fluid and bronchoalveolar lavage fluid: DNA was extracted from 1 ml of PF and BAL fluid using SDS and proteinase K at the same concentrations as described. The mixture was incubated at 65°C for 1 hour followed by 5 hours at 37°C.

Sputum: One ml of sputum was first liquefied with 10 mg Sputasol (OXOID Limited, Hamshire, England) and 5 mg lysozyme (Sigma Chemical Co, St Louis, MO, USA). DNA was then extracted following the same protocol of PF and BAL.

All extracted DNA from bacteria strains, PF, BAL and Sp were purified using phenol-chloroform-isoamyl alcohol extractions. DNA was precipitated in 3 mol/l sodium acetate and 100% cold ethanol, washed, dried and then solubilized in double distilled water. DNA concentration was calibrated at 260 nm using a Philips UV spectrophotometer.

DNA amplification by the polymerase chain reaction

One microgram of DNA from each specimen was amplified. The PCR reaction was carried out in 50 μl reaction volume containing 50 mM KCl, 10mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 20 pmol of primers. Enzyme Taq polymerase was used at a final concentration of 2.5 Units.

MPB 64 protein coding gene: The 240 bp gene sequence coding for the MPB 64 protein specific to *M. tuberculosis* was amplified using two primers - 5'-TCCGCTGCCAGTCGTCTTCC-3' and 5'-GTCCTCGCGAGTCTAGGCCA-3'. Amplification was carried out for 40 cycles at 94°C for 1 minute, 60°C for 2 minutes and 71°C for 2 minutes (Kanedo *et al*, 1990).

IS6110 insertion element: The IS6110 repeat sequence present in *M. tuberculosis* was amplified

using 5'-CCTGCGAGCGTAGGCGTCGG-3' and 5'-CTCGTCCAGCGCCGCTTCGG-3'. The PCR conditions involved 25 amplifications at 94°C for 2 minutes, 68°C for 2 minutes and 72°C for 2 minutes. An additional 5 seconds extension was included in every cycle at the 72°C step.

Gel electrophoresis of amplified DNA

Amplified DNA products were electrophoresed in 1% agarose and observed under UV illumination. MPB 64 amplified DNA was then transferred to HyBond-N membranes (Amersham International plc, England) by the Southern blotting technique (Southern, 1987) and hybridized to an oligonucleotide probe.

Oligonucleotide hybridization

The oligoprobe 5'-CTTCAACCCGGGGAGT-3' from the midportion of the MPB 64 amplified sequence (Yamaguchi *et al*, 1989) was end-labeled using T4 polynucleotide kinase (Amersham International plc, England). Hybridization, washing of Hy-Bond-N membranes and autoradiography was carried out as previously described (Lee *et al*, 1994).

RESULTS

The overall results are summarized in Table 1. The 14 PTB specimens diagnosed by positive culture consisted of 3 Sp, 7 PF and 4 BAL specimens. All the 14 PTB specimens gave positive results with both DNA amplification protocols (Table 1, Fig 1, 2). Using the MPB 64 protocol, 11 of the 14 PTB specimens clearly amplified the 240 bp specific *M. tuberculosis* fragment, while the remaining three showed positive bands after autoradiography. In contrast, the 123 bp *M. tuberculosis* fragment of the IS6110 insertion element was amplified by all 14 PTB specimens and oligonucleotide hybridization was not required.

The three PF specimens, which were negative culture results but had histological features highly suggestive of tuberculosis on pleural biopsy, were negative with both MPB 64 and IS6110 detection protocols. Oligonucleotide hybridization with the MPB 64 specific probe also did not produce any bands after autoradiography.

A total of forty-two non-tuberculous specimens were included as negative controls, of which 12 were Sp specimens, 13 PF and 16 BAL specimens. Forty-one of the non-tuberculous specimens gave negative

Table 1

Overall summary of the results for the diagnosis of PTB.

Sample type	Diagnosis	TB culture	IS6110	MPB64	
			PCR+	PCR+	Oligoprobe+
Sputum (n = 16)	Non-tuberculous infections	0/10	0/10	1/10	1/10
	Malignancy	0/3	0/3	0/3	0/3
	Tuberculosis	3/3	3/3	2/3	3/3
Pleural fluid (n = 23)	Non-tuberculous infections	0/10	0/10	0/10	0/10
	Malignancy	0/3	0/3	0/3	0/3
	Tuberculosis	7/10*	7/10	5/10	7/10
BAL (n = 20)	Non-tuberculous infections	0/13	0/13	0/13	0/13
	Malignancy	0/3	0/3	0/3	0/3
	Tuberculosis	4/4	4/4	4/4	4/4

* In 3 cases with pleural effusion and negative TB culture, the diagnosis of tuberculosis was made by characteristic histological features of granuloma and Langerhan's giant cells on pleural biopsy.

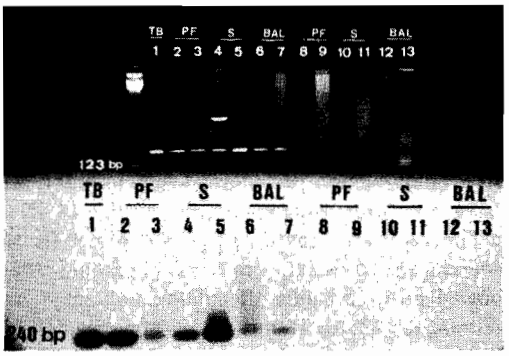

Fig 1–Detection of *M. tuberculosis* DNA in pleural fluid (PF), sputum (Sp) and bronchoaveolar lavage (BAL) specimens using the MPB 64 protein coding gene. Upper panel-gel electrophoresis of PCR products, and Lower panel - oligonucleotide hybridization with the midportion of the MPB 64 sequence and autoradiography. Lane 1: DNA from *M. tuberculosis*; lanes 2-3: PTB-positive PF; lanes 4-5: PTB positive Sp; lanes 6-7: PTB-positive BAL; lanes 8-9: PTB-negative PF; lanes 10-11: PTB-negative Sp and lanes 12-13: PTB-negative BAL.

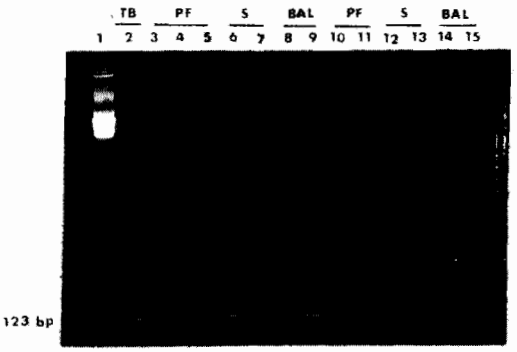

Fig 2–Detection of *M. tuberculosis* DNA in pleural fluid (PF), sputum (Sp) and bronchoalveolar lavage (BAL) specimens using the IS6110 sequence by gel electrophoresis of PCR products. Lane 1: 123 bp ladder; lane 2: DNA from *M. tuberculosis*; lanes 3-5: PTB-positive PF; lanes 6-7: PTB-positive Sp; lanes 8-9: PTB-positive BAL; lanes 10-11: PTB-negative PF; lanes 12-13: PTB-negative Sp and lanes 14-15: PTB-negative BAL.

results with both protocols. There was one false positive result with a sputum sample as the MPB 64 protocol consistently amplified the 240 bp fragment and produced a positive band after autoradiography. However, this sputum sample was repeatedly negative with the IS6110 protocol (Table 1). DNA from all the non-mycobacterial cultures of *E. coli*, *Pseudomonas* species, *Klebsiella* species, *Streptococus* A, B and G gave negative results with both PCR protocols (results not shown).

DISCUSSION

DNA from PF and BAL specimens was easily extracted using SDS and proteinase K. In the case of sputum specimens, the sputum had to be liquefied first because of its high viscosity. The commercially available Sputusol (OXOID Limited, Hamshire, England) together with lysozyme liquefied sputum sufficiently for DNA extraction to be carried out using the normal SDS and proteinase K procedures. DNA extracted and purified from sputum specimens using the above protocols produced distinct amplified DNA fragments. There was no necessity for further DNA purification using the more tedious techniques of sucrose purification (Victor *et al*, 1992) and powdered glass (Eisenach *et al*, 1991).

The current diagnosis of pulmonary tuberculosis by light microscopy of acid-fast organisms in sputum produces positive smears in only about 25%-75% of cases (Schluger *et al*, 1994). In this study, we evaluated the PCR technique for the diagnosis of PTB using Sp, PF and BAL specimens. Both the MPB 64 and IS6110 insertion element protocols proved to be sensitive techniques as *M. tuberculosis* DNA was detected in the 14 specimens obtained from patients diagnosed with PTB by positive culture.

The MPB 64 protein coding gene exists as a single copy sequence within the genome of *M. tuberculosis* (Shinnick, 1987). DNA amplification alone was insufficient to produce positive results and additional probing with an oligonucleotide from the mid-portion of MPB 64 amplified sequence (Yamaguchi *et al*, 1979) increased the sensitivity of this technique to 100% for the 14 specimens (Fig 1). The IS6110 insertion element, present in repeat copies in *M. tuberculosis* genome, allowed detection of the 123 bp sequence by PCR alone without further need for oligonucleotide hybridization (Fig 2).

However, the MPB 64 and IS6110 insertion element protocols could not detect *M. tuberculosis* DNA in the 3 PF specimens, which were *M. tuberculosis* culture negative but had characteristic histological features of tuberculosis with granuloma

and Langerhan's giant cells on pleural biopsy. The inability of both MPB 64 and IS6110 insertion element protocols to detect *M. tuberculosis* in the PF could be due to the absence of the bacilli in these specimens. Although tuberculous pleurisy with effusion results from rupture of a pulmonary lesion into the pleural cavity (Stead *et al*, 1955), however, a hypersensitivity reaction ensues with outpouring of fluid inflammatory cells into the pleural cavity (Berger and Majia, 1973). This immense dilution of the bacilli is therefore the most likely reason for the lowered sensitivity of the PCR protocols in these specimens. This has been substantiated by the low frequency of positive cultures in PF of tuberculous pleurisy, which was reported at less than 30% (Falk, 1965).

The IS6110 insertion element appeared to be a more specific sequence for the detection of *M. tuberculosis* as there were no false positive results with this protocol. The MPB 64 protocol gave a false positive result with a non-tuberculous Sp specimen. The 240 bp MPB 64 protein coding gene has recently been reported to be cross-reactive with DNA from Mycobacteria *avium intracellulare* complex (MAIC) (Bull and Shanson, 1992). In a recent study of cross-reactivity of the MPB 64 sequence in our laboratory, we found the sequence to cross-react with strains of *M. avium*, MAIC, MAIS complex (*M. avium, M. intracellulare* and *M. srofulaceum*) and *M. smegmatis* while the IS6110 protocol did not cross react with any of the above (manuscript submitted for publication). It is therefore possible that the false positive result detected by the MPB 64 protocol was a result of DNA from a cross-reacting Mycobacterial strain other than *M. tuberculosis*. This was, however, not supported by the culture result which was negative for the Sp sample in question.

In conclusion, we have found that DNA amplification techniques were useful for the rapid detection of *M. tuberculosis* DNA in Sp, PF and BAL specimens. The IS6110 insertion element sequence was found to be more specific compared to the MPB 64 sequence. This technique, however, was not found to be useful for the diagnosis of TB culture negative tuberculous pleurisy.

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