Amplification of *Mycobacterium tuberculosis* in clinical samples and detection using the EIA format

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Abstract

A test based on the polymerase chain reaction was used for the detection of *Mycobacterium tuberculosis* in clinical samples. A 400 bp sequence of the *mtp*40 that was specific for *M. tuberculosis* was amplified and detected using the enzyme-linked immunosorbent assay (EIA) detection format. We tested 61 clinical samples of various types from 44 patients with suspected tuberculosis. These samples included cerebrospinal fluids, pleural tap/fluids, sputum, nasopharyngeal aspirates, gastric lavage, peritoneal fluids, urine, tracheal aspirates, serum and blood. Only sputum samples were first decontaminated. All samples were heated to lyse the cells and the extracted DNA was cleaned with phenol-chloroform. The specificity of the probe was tested with amplified products of primers for IS*986*. In this study, 41 samples were positive with the EIA format whereas 32 samples were positive based on analysis by gel electrophoresis.

Key words: PCR; *M. tuberculosis*; EIA detection

Introduction

Mycobacterium tuberculosis and *M. bovis* are important mycobacteria causing tuberculosis in human. The development of PCR-based tests specific for mycobacteria has been shown to improve rapid diagnosis of tuberculosis (Pao *et al.*, 1990; Cousins *et al.*, 1992; Brisson-Noel *et al.*, 1991). The laboratory diagnosis of extrapulmunory tuberculosis, especially those affecting bones, joints, and brain, can be challenging as samples such as pleural exudates and CSF contain only few mycobacteria, resulting in low sensitivity of the acid-fast staining technique.

Several research groups have described the PCR assay for direct detection of mycobacteria in respiratory samples (Bearis *et al.*, 1995; Cho *et al.*, 1995; Maher *et al.*, 1996). Furthermore, commercial kits are for use with respiratory samples only. Nevertheless, these kits could be used to establish early diagnosis of extrapulmunory tuberculosis (Ehler *et al.*, 1996; Abe *et al.*, 1993; Vlaspolder *et al.*, 1995).

In this study, the Digene system based on sandwich capture molecular hybridization assay was used. An aliquot of a PCR reaction containing 5'-biotinylated products was hybridized with a specific single-stranded RNA probe. The resultant RNA:DNA hybrids were captured through biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids were reacted with an anti-hybrid antibody conjugated to alkaline phosphate and was detected with a colourimetric substrate (*para*-nitrophenyphenol, PNPP). The amount of biotinylated PCR product in the each reaction was proportional to the intensity of the colour generated. Clinical samples included cerebrospinal fluids, pleural tap/fluids, sputum, serum, nasopharyngeal aspirates, gastric lavage, peritoneal fluids, urine, tracheal aspirates, and blood.

Material and Methods

Clinical samples

Sixty-one samples from 44 patients were obtained from participating hospitals in Malaysia. Of the 61 samples, there were 15 body fluids and aspirates, 21 CSF samples, 8 gastric lavage and sputum, and 17 miscellaneous samples. Some of the samples were sent more than once from the same patient.

Treatment of samples for bacterial lysis

For the release of *M. tuberculosis* DNA from clinical samples, we used a simple lysis procedure using heat treatment. Only sputum samples were decontaminated. Samples (300µl) were centrifuged at 12,000 rpm for 5 min. The pellets were resuspended in 200µl lysis buffer (2M NaCl, 0.1M NaOH, 0.5% SDS) and heated at 95°C for 15 min. Samples were again centrifuged at 4°C for 15 min. The supernatants were collected and diluted in 0.1M Tris-pH 8.0 to a final volume of 400µl. The samples were vortex and suspended in 600µl phenol solution, vortexed and centrifuged again at 15,000 rpm for 15 min at 4°C. The supernatants were collected and 700µl of chloroform/isoamyl alcohol solution were added. The samples were vortexed and centrifuged at 15,000 rpm for 15 min

at 4°C. The supernatants were precipitated in 2 volumes of ice cold isopropanol and stored at -20°C overnight. The samples were centrifuged at 12,000 rpm for 15 min at 4°C. The pellets were resuspended in 70% ethanol and centrifuged at 15,000 rpm for 5 min at 4°C. The pellets were then centrifuged dry in a concentrator and resuspended in 50µl sterile water.

Synthetic oligonucleotide primers and probes

Oligodeoxyribonucleotides used as primers and hybridization probe in the Digene test kit were purchased from Digene Diagnostics Inc., Beltville, USA. The kit was supplied with the primers (P-1 and P-2) and the expected size of amplified fragment was 400bp on analysis with gel electrophoresis. The hybrid DNA/RNA probe in the kit was specific to the 400 bp region of the *mtp*40 gene in *M. tuberculosis*. Another set of primers (INS1 and INS2) was used in this study. The sequences for the primers were: INS1-5'CGTGAGGGCATCGAGGTGGC3', corresponding to the IS*986* insertion elements at 631 to 650 and INS2-5'GCGTAGGCGTCGGTGACAAA3' , position at 856 to 875 (Hermans *et al.*, 1990). The primer INS1 was biotin labeled at the 5' end. The size of the expected amplified product using the INS1 and INS2 primers was 240bp on analysis with gel electrophoresis.

Polymerase Chain Reaction (PCR)

The PCR was performed with Taq polymerase in the reaction buffer mix (BRL, USA). The final composition of the PCR mix contained 0.2mM (each) deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP). Two sets of primers were used in the PCR mix. The mixture for the test kit (Digene Diagnostics Inc., Beltville, USA) contained 0.4µM (each) primers P-1 and P-2, while the other test contained 0.4µM (each) primers INS1 and INS2 (i.e., 132ng/50µl reaction volume). The reaction mixtures were overlaid with mineral oil and subjected to denaturation of 10-min. and addition of 1.25U of Taq polymerase (BRL, USA) per 50µl reaction volume. Two different amplification procedures were used. Amplification procedure for primers (P-1 and P-2) consisted of a 1-min denaturation at 94°C, a 2-min annealing step at 70°C, and a 3-min extension step at 72°C, and these steps were repeated for 40 cycles in a programmable temperature cycler (Perkin-Elmer Corp., USA). An additional cycle for extension step at 72°C for 7 min was done. Subsequent amplification procedure for primers (INS-1 and INS-2) consisted of a 1-min denaturation at 94°C, a 1-min annealing step at 64°C, and a 1-min extension step at 72°C, and these steps were repeated for 30 cycles. An additional cycle for extension step at 72°C for 10 min was done.

Visualization and detection of amplified products

For visualization of amplified products by UV fluorescence, 20µl of amplified mixture and 100bp molecular marker (BRL, USA) was subjected to electrophoresis through 2% agarose gel.

Five microliters of PCR products were transferred into clean tubes and denatured with 0.4N NaOH. The tubes were vortexed and incubated for 10 min at 25°C. The initial purple colour of the mixture changed to yellow, after addition of 25µl of probe mixture to the tubes. The tubes were vortexed and incubated in a water bath for 30 min at 65°C. The contents were transferred into corresponding capture plate wells and incubated on a rotary shaker for 30 min at 35°C. Capture plates were blotted and 100µl of detection reagent was added to each well. The plates were incubated on rotary shaker for 30 min at 30°C. The plates were washed five times with wash buffer and once with deionized water. 100µl of signal reagent was added to each well. The plates were incubated for 1 to 24h at 37°C, and then read with an EIA plate reader set at 410nm. Positive cut-off optical density value was recommended as 0.126.

Results

Specificity of DNA/RNA hybrid as probe

The specificity of this PCR assay involved amplification of the particular region in IS*986*. Only one of oligonucleotide primers that was complementary to the region flanking the IS*986* sequence was biotinylated. In the reaction, these primers bound to the target region creating biotinylated amplicons. These amplicons were detected on gel electrophoresis (Fig.1), but not in the microwell plate.

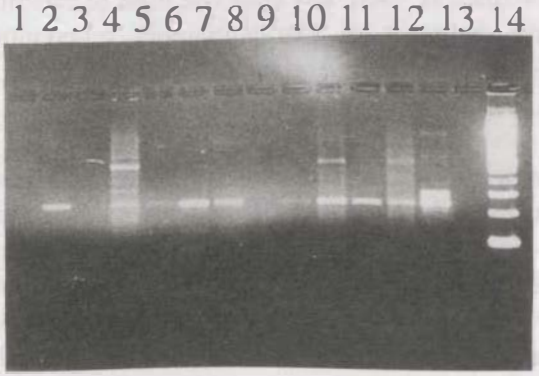

Fig.1. Analysis of PCR amplification products using IS*986* primers by gel electrophoresis. Lanes: lanes 1 to 11, amplification of clinical samples; lanes 12 & 13, positive & negative control; lane 14, 100bp molecular weight marker (BRL).

Oligonucleotide probe RNA:DNA hybrid was only specific for the *mtp*40 amplicons. The probe and detection molecule used in the assay (anti-RNA-DNA-alkaline phosphate conjugate) could not bind to the nonspecific biotinylated amplicons.

In this test using the Digene kit, amplification with primers located in the *mtp*40 gene produced an amplified product of 400 bp on analysis with gel electrophoresis (Fig. 2). The EIA format was also used to detect the amplified product.

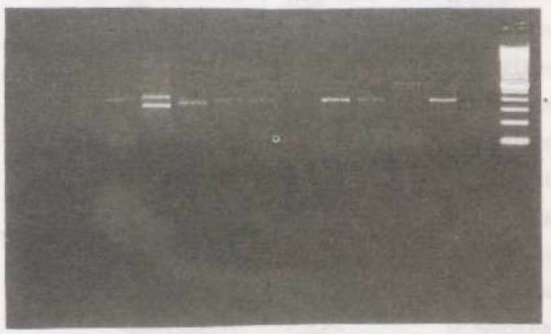

Fig. 2. Analysis of PCR products using *mtp*40 primers by gel electrophoresis. Lanes: lanes 1 to 11, amplification of clinical samples; lanes 12 & 13, positive & negative control; lane 14, 100bp molecular weight marker (BRL).

Detection of M. tuberculosis DNA from clinical samples

A total of 61 samples from 44 patients were assayed using Digene with EIA format (Tables 1 & 2). Of the 41 samples positive by EIA format, 9 samples showed specificity of the probe detection technique since they were not detected on gel electrophoresis (Table 1).

Samples Nos. 1 and 46, were from patients undergoing anti-tuberculosis therapy, the latter being diagnosed as having tuberculosis from a positive culture. Samples of both patients were positive on gel electrophoresis and EIA analysis. Different types [tracheal aspirates (TA), blood (Bld) and urine]of samples were received at one time from the same patient (Nos. 38, 39, & 40 in Table 2). The EIA was positive for all 3 samples but these were not detected on gel electrophoresis. The sensitivity of the probe could be particularly relevant in this case as the concentration of the amplified fragment was too low to be detected on gel electrophoresis.

Different sample types [nasopharyngeal aspirate (NPA), gastric lavage (GL) and cerebrospinal fluids (CSF)] were received on different occassions from a patient (Nos. 18,19, 24 & 29 in Table 2). The EIA was positive for the CSF and second GL specimen but negative for NPA and the first GL specimen. Different sample types were also sent at various times from another patient (Nos. 7,17,22 & 26 in Table 2). The EIA was positive for blood, and first the CSF specimen but negative for the NPA and second CSF specimen. Both CFS and blood samples from a patient (Nos. 44 & 45 in Table 2) were positive.

Of the 15 body fluids and aspirates, which included 2 pleural tap, 6 pleural fluids, peritoneal fluids, 1 tracheal aspirates and 5 nasopharyngeal aspirates, 9 were positive with the EIA compared to 6 positive with gel electrophoresis. Both pleural taps (PT) (Nos. 8 & 9 in Table 2) of the same patient, which were sent on different occassions, were negative on gel electrophoresis and EIA.

Of 21 CSF samples examined, 16 were positive with the EIA. One sample negative on EIA was positive by gel electrophoresis. Gastric aspirates are often submitted for diagnosis of pulmonary TB when sputum samples are not available. Of the 8 specimens (5 gastric aspirates and 3 sputum) received, 5 were positive with EIA, whereas 4 were positive with gel electrophoresis. Gastric lavage specimens (Nos. 20 & 31 in Table 2), sent at different times from the same patient, was only EIA positive for the second sample. Of 17 miscellaneous samples (2 urine, 11 blood and 4 serum), 11 were positive with EIA whereas 9 were positive with gel electrophoresis.

Table 1. Detection of *Mycobacterium tuberculosis* in clinical samples by EIA format and gel electrophoresis

Sample	No. of samples	No. of samples positive by	
		EIA	Gel analysis
Body fluids and aspirates	15	9	6
CSF samples	21	16	13
Gastric aspirates and sputum	8	5	4
Other samples	17	11	9
Total	61	41	32

Table 2. Comparison of EIA detection system with agarose gel electrophoresis

Sample no.	Types of sample	EIA detection A410nm	EIA detection Interpretation of assay	Agarose gel electrophoresis 400bp fragment	Agarose gel electrophoresis Interpretation of assay
1	CSF	1.161	Positive	+	Positive
2	NPA	0.013	Negative	-	Negative
3	Urine	1.196	Positive	+	Positive
4	CSF	1.175	Positive	+	Positive
5	Bld	1.084	Positive	+	Positive
6	CSF	1.291	Positive	+	Positive
7	Bld	1.069	Positive	+	Positive
8	PT	1.228	Positive	+	Positive
9	PT	1.148	Positive	+	Positive
10	Bld	1.056	Positive	+	Positive
11	PF	1.176	Positive	+	Positive
12	Serum	0.019	Negative	-	Negative
13	Bld	0.039	Negative	-	Negative
14	CSF	0.493	Positive	+	Positive
15	CSF	0.449	Positive	+	Positive
16	NPA	0.081	Negative	-	Negative
17	NPA	0.005	Negative	-	Negative
18	NPA	0.005	Negative	-	Negative
19	GL	0.017	Negative	-	Negative
20	Serum	0.370	Positive	+	Positive
21	GL	0.470	Positive	+	Positive
22	CSF	0.515	Positive	+	Positive
23	GL	0.427	Positive	+	Positive
24	CSF	0.479	Positive	+	Positive
25	NPA	0.307	Positive	+	Positive
26	CSF	0.029	Negative	-	Negative
27	SPT	0.004	Negative	-	Negative
28	CSF	0.337	Positive	+	Positive
29	GL	0.372	Positive	+	Positive
30	PF	0.595	Positive	+	Positive

CSF: cerebrospinal fluid; PF: pleural fluid; PT: pleural tap; P: peritoneal fluid: Bld: blood; GL: gastric lavage; SPT: sputum; TA: tracheal aspirate; NPA: nasopharyngeal aspirate; ND: not done

Continuation...

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Sample no.	Types of sample	EIA detection A_{410nm}	Interpretation of assay	Agarose gel electrophoresis 400bp fragment	Interpretation of assay
31	CSF	0.030	Negative	+	Positive
32	Bld	0.028	Negative	-	Negative
33	CSF	0.413	Positive	-	Negative
34	Bld	0.166	Negative	-	Negative
35	Bld	0.349	Positive	-	Negative
36	CSF	0.288	Positive	-	Negative
37	PF	0.059	Negative	-	Negative
38	TA	0.327	Positive	-	Negative
39	Bld	0.388	Positive	-	Negative
40	Urine	0.429	Positive	-	Negative
41	CSF	0.438	Positive	+	Positive
42	CSF	0.556	Positive	+	Positive
43	PF	0.491	Positive	-	Negative
44	CSF	0.588	Positive	+	Positive
45	Bld	0.502	Positive	+	Positive
46	Spt	0.488	Positive	+	Positive
47	Spt	0.446	Positive	-	Negative
48	Serum	0.558	Positive	+	Positive
49	CSF	0.371	Positive	+	Positive
50	PF	0.466	Positive	+	Positive
51	CSF	0.008	Negative	-	Negative
52	Bld	0.006	Negative	-	Negative
53	CSF	0.105	Negative	-	Negative
54	GL	0.010	Negative	-	Negative
55	CSF	0.022	Negative	-	Negative
56	Bld	0.020	Negative	-	Negative
57	CSF	0.337	Positive	-	Negative
58	P	0.028	Negative	-	Negative
59	PF	0.437	Positive	ND	ND
60	CSF	0.565	Positive	+	Positive
61	Serum	0.272	Positive	+	Positive

CSF: cerebrospinal fluid; PF: pleural fluids; PT: pleural tap; P: peritoneal fluid; Bld: blood; GL: gastric lavage; SPT: sputum; TA: tracheal aspirate; NPA: nasopharyngeal aspirate; ND: not done

Discussion

We tested a PCR system for rapid direct detection of *M. tuberculosis* in various clinical samples. Although some researchers stated that the presence of inhibitors yielded false-negative results, the inclusion of a positive control in the isolation step in all samples and also during the PCR process, allowed us to monitor inhibition, thus detecting false-negative results. We also carried out phenol extraction to remove all possible inhibitors in the various clinical samples. A 400 bp amplification product was detected on gel electrophoresis when primers from the Digene kit were used in the PCR; a 240 bp product was obtained using primers based on IN*S986*. The EIA only detected amplified products using primers from the Digene kit. Hermans *et al.* (1990) and Kolk *et al.* (1992) used primers and probes based on IS*6110* and IS*986* insertion sequences in their evaluation of the PCR for the diagnosis of *M. tuberculosis*. Kox *et al.* (1992) and Ehler et al. (1996) used the Gen-Probe and Amplified *Mycobacterium Tuberculosis* Direct test (AMTD) for the detection of extrapulmunory tuberculosis. In this study, we used primers and probes based on the 40-kDA protein antigen of *M. tuberculosis* (Hermans *et al.*, 1990), to evaluate the specificity of the Digene kit.

Based on our results, a combination of gel electrophoresis and EIA with the Digene kit might be very useful for detecting *M. tuberculosis* in various clinical samples.

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