OVERCOMING THE ERRORS OF IN-HOUSE PCR USED IN THE CLINICAL LABORATORY FOR THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS

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Abstract. Our experiences from 1993 to 1997 in the development and use of IS*6110* base PCR for the diagnosis of extrapulmonary tuberculosis in a routine clinical setting revealed that error-correcting processes can improve existing diagnostic methodology. The reamplification method initially used had a sensitivity of 90.91% and a specificity of 93.75%. The concern was focused on the false positive results of this method caused by product-carryover contamination. This method was changed to single round PCR with carryover prevention by uracil DNA glycosylase (UDG), resulting in a 100% specificity but only 63% sensitivity. Dot blot hybridization was added after the single round PCR, increasing the sensitivity to 87.50%. However, false positivity resulted from the nonspecific dot blot hybridization signal, reducing the specificity to 89.47%. The hybridization of PCR was changed to a Southern blot with a new oligonucleotide probe giving the sensitivity of 85.71% and raising the specificity to 99.52%. We conclude that the PCR protocol for routine clinical use should include UDG for carryover prevention and hybridization with specific probes to optimize diagnostic sensitivity and specificity in extrapulmonary tuberculosis testing.

INTRODUCTION

There has been a concomitant increase in tuberculosis cases with the acquired immunodeficiency syndrome (AIDS) epidemic (Ellner *et al,* 1993; Murray, 1995). The incidence of extrapulmonary tuberculosis is high in the AIDS patients (Castilla *et al,* 1995; Hill *et al,* 1991; Labarga Echeverria *et al,* 1995; Poznansky *et at,* 1995). However, because of the paucibacillary nature of extrapulmonary tuberculosis, diagnosis is more difficult than pulmonary tuberculosis. Acid fast bacilli by Zeihl-Neelsen staining are scarce in the body fluids from extrapulmonary tuberculosis patients. The standard culture technic requires month to complete and the new rapid culture systems take at least 2-3 weeks (Carbonnelle *et al,* 1995; Hanna *et al,* 1995). Even the nucleic acids amplification methods for testing pulmonary tuberculosis require some modification to be used in extrapulmonary samples.

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Among the many nucleic acids amplification technics applied toward the detection of *Mycobacterium tuberculosis* such as PCR (Beavis *et al,* 1995; Eisenach *et al,* 1990; Kox *et al,* 1994; Shankar *et al,* 1991; Tevere *et al,* 1996), ligase chain reaction (Winn-Deen *et al,* 1993), strand displacement amplification (Walker *et al,* 1994), Q-beta replicase-amplified assay (Shah *et al,* 1995), transcription based amplification (Miller *et al,* 1994), or nucleic acid sequence-based amplification (van der Vliet *et al,* 1993), the in-house PCR is still the assay of choice. Although commercial kits based on PCR (Beavis *et al,* 1995) or other nucleic acids amplification methods (Miller *et al,* 1994; Shah *et al,* 1995;) are now available in the United States and other countries, the kit availability is limited in many countries by high cost and distribution infrastructure. The in-house PCR technic is still needed for the diagnosis of extrapulmonary tuberculosis as commercial kits were designed and licensed for testing only respiratory specimens. In addition, this commercial PCR kit has been found to have lower sensitivity than in-house PCR (Schirm *et al,* 1995). This article presents the authors' experiences in identifying and reducing the errors of in-house PCR for testing extrapulmonary tuberculosis specimens in a routine clinical laboratory environment.

METERIALS AND METHODS

Clinical specimens

In 1992, the Clinical Immunology Laboratory began using PCR for the detection of *M. tuberculosis* in extrapulmonary specimens. To be clinically practical, we conducted the PCR testing in a diagnostic laboratory setting in conjunction with routine clinical laboratory work. From 1993-1997, 800 clinical specimens of patients, whom were suspected of extrapulmonary tuberculosis, were sent to the laboratory. DNA from the specimens was extracted by the guanidine isothiocyanate-diatom method (Boom *et al,* 1990) and tested weekly by PCR. PCR results were compared with culture results. Clinical history was reviewed in cases of ambiguous results. The cases were considered 'clinical tuberculosis case' if at least 2 of the following findings were found: acid fast bacilli staining positive in clinical specimens, an X-ray or computerized tomographic scan suggestive of tuberculosis, tissue pathology showing granulomatous inflammation, body fluids profiles compatible with tuberculosis, response to anti-tuberculous drugs, or having a past history of tuberculosis.

PCR and detection

Four PCR protocols were developed during the course of this study. All methods amplified the insertion sequence IS*6110* (Thierry *et al,* 1990) of the *M. tuberculosis* complex. In method A, PCR using primer rt1 (5′-CCT GCG AGC GTA GGC GTC GG, position 894-875 of IS*6110*) and rt2 (5′-CTC GTC CAG CGC CGC TTC GG, position 762-781) was done as described (Eisenach *et al,* 1990). For reamplification. 1 µl of the first round of PCR products was put in the second round reaction with the same protocol. The products of reamplification were visualized in ethidium bromide stained gel electrophoresis as decribed (Eisenach *et al,* 1990). Method B was the PCR with carryover prevention by uracil DNA glycosylase (UDG)/dUTP using primers pt8 and pt9 (Kox *et al,* 1994). Method C was method B plus dot blot hybridization (Kox *et al,* 1994). The probe used in dot-blot hybridization was generated by nested PCR using primer mt3 (5′-GAT GGT TTG CGG TGG GGT GT, position 426-445) and rt1 nested inside the pt8/9 products, with digozigenin-dUTP in the reaction as described (Kunakorn and Markham, 1995).

In method D, the one-tube seminested PCR (Kunakorn and Markham, 1995) was used. The reaction mixture consisted of 10 mM Tris-HCl pH8.4; 50 mM KCl; 3 mM $MgCl_2$; 0.1 mM each of dATP, dGTP, dCTP, dUTP; 0.05 µM of pt8; 0.24 µM of pt9; 0.2 µM of mt3; 1 unit of *Taq* DNA polymerase (Boehringer Mannheim); and 0.1 unit of UDG (Gibco BRL); in a total volume of 50 µl. The reaction began at 50°C for 5 minutes, then 95°C for 5 minutes and continued with a cycling phase of 60 cycles, each of which were 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. The reaction ended at 72°C for 10 minutes and was kept at 4°C until detection. The PCR was detected by Southern blot hybridization using a digoxigenin-dUTP tail-labeled oligonucleotide probe mt4 (5′-CGT AGT TGC CGG CGT GGA, position 594-577). Hybridization was done at 50°C. The probe labeling, capillary bloting, hybridization and chemiluminescence detection were done according to digoxigenin manufacturer's protocol (Boehringer Mannheim).

The strategy for controlling the performance of the PCR (Kox *et al,* 1994) was adopted with some modifications. *M. tuberculosis* H37Rv DNA at concentrations of 1 pg, 100 fg, and 10 fg were included as positive control templates in each run. Only results with a sensitivity of at least 100 fg would be acceptable. PCR products containing uracil bases were also included as templates in each run to control for the decontamination activity of UDG in the reaction. Each DNA sample was split and one aliquot was spiked with 100 fg of *M. tuberculosis* DNA to control for inhibitors in the samples.

Statistical analysis

Chi-square of contingency table and z-test of observed proportion in the Sigma Stat Software (Jandel Scientific, San Rafael, California) were used for statistically comparison of the sensitivity and specificity of the four PCR methods.

RESULTS

The 800 clinical specimens consisted of 458 samples of cerebrospinal fluid (CSF), 149 samples of tissue specimens (From suspicious cases of spinal tuberculosis, joint tuberculosis, and lymph node tuberculosis), 156 samples of body fluids other than CSF (pleural fluid, pericardial fluid and ascites), and 37 other samples (bone marrow, pus, and bronchoalveolar lavage) were evaluated.

The PCR protocol has been improved many times during the study period (Table 1). In 1993, reamplification PCR (method A) was used. This method gave the sensitivity of 90.91% and a specificity of 93.7%. The false positive results in method A was investigated by amplified the sample again by primers from another gene of *M. tuberculosis* (Shankar *et al,* 1991) which showed negative results. This investigation suggested that amplicon carryover might be the cause of the false positive. Therefore, in 1994, the dUTP/UDG carryover prevention protocol was used in our single PCR (method B). This method showed no false positives and resulted in 100% specificity ($p<0.01$, compared with method A). However, method B had many false negative outcomes reducing the sensitivity to 62.96%. False negatives by method B suggested the inadequacy of the single round PCR without hybridization. The false negative samples showed no bands on the agarose gel but gave specific bands after Southern blot hybridization. In August 1995, dot blot hybridization was added to method B to become method C and the sensitivity increased to 87.50%. However, the false positive results resumed, reducing the specificity to 89.4% ($p<0.01$, compared with method B). To investigate the cause(s) of false positive results by method C, PCR products from false positive samples available were reexamined and showed no band when visualized on agarose gel. The gel was Southern transferred and hybridized with the probe used in the dot blot. The Southern blot results showed non specific bands, which were of different base lengths from the positive samples. Therefore, from January 1996, the PCR protocol was adjusted by changing the dot blot to Southern blot to avoid any nonspecific hybridization signal. The probe used was also changed from a PCR generated one to the oligonucleotide mt4. This final protocol, method D improved the specificity to 99.52% ($p<0.01$, compared with either method A or C) with a sensitivity of 85.71% ($p<0.01$, compared with method B).

DISCUSSION

Conditions in research and routine clinical laboratories are different from each other. In the typical reseach laboratory, tests are performed in an optimally controlled environment, which in the typical routine laboratory, day to day variations are common. This study details the development of in-house PCR for the diagnosis of extrapulmonary tuberculosis in a routine clinical setting over a period of five years. The laboratory continuously improved the diagnostic protocol overtime to reduce two kinds of error, false positive and false negative. As the methods evolved, the results improved in one aspect but could worsen in anther, Ultimately, the laboratory established a method which optimized PCR for routine diagnosis.

False positive

The false positive PCR cases were investigated to see whether the false positive by the reamplification method could give positive results by other PCR primers. The false positive samples by reamplification were retested with another set of PCR primers and showed negative results. Thus the false positives were likely the result of carryover of PCR products used during the reamplification period. The reamplification or nested amplification method is more susceptible to false positive by amplicon carryover because of the transferring step from first amplification to second amplification (Young *et al,* 1995). The protocol was changed to single round PCR incorporated with UDG and dUTP to prevent amplification of any carryover product. The uracil

Table 1

PCR results analyzed according to the methods used.

Methods	True positive	True negative	False positive	False negative	Total	Sensitivity (%)	Specificity (%)
A	10	75	5	1	91	90.91	93.75
B	17	158	0	10	185	62.96	100.00
C	14	34	4	2	54	87.50	89.47
D	48	412	2	8	470	85.71	99.52
Total	89	679	11	21	800	80.91	98.41

base of the PCR products could be destroyed by UDG at the beginning of the cycle; hence, this method could not be applied with nested PCR or reamplification. In starting the UDG protocol, new primers were used to avoid carryover of the old PCR products with thymine bases which could not be destroyed by UDG. Finally, the PCR method that used UDG should include a proper dilution of the uracil products as a template to control for the deterioration of the UDG activity. Users of UDG should be aware that UDG cannot prevent a false positive result from contamination by the *M. tuberculosis* natural DNA. Therefore, it is necessary to clean the working space after DNA extraction. *M. tuberculosis* DNA contamination usually comes from *M. tuberculosis* culture, typically found in the laboratory that performs the restriction fragment length polymorphism (RFLP) analysis (Noordhoek *et al,* 1994). The place that is used for DNA extraction from culture and RFLP should be separated from the place used for diagnostic PCR.

False positive were not found in the PCR products of the UDG method until dot blot hybridization was added. The false positive results of method C were analyzed and showed non specific bands different from the expected base length on the Southern blot hybridization, a finding which had also been found with other IS*6110* based PCR (Kent *et al,* 1995) using a PCR generated probe. From these results, the PCR generated probe was changed to an oligonucleotide probe and dot blot hybridization was replaced by Southern blotting.

False negative

Although the PCR was optimized to its highest sensitivity to detect 100 fg of *M. tuberculosis* DNA, the false negative results by method B suggested the inadequacy of single round PCR without hybridization. In contrast to the western isolates of *M. tuberculosis* which carry multiple IS*6110* copies, up to one fifth of *M. tuberculosis* in Thailand carry only one copy of IS*6110* (Palittapolgarnpim *et al,* 1997). Hybridization was added after PCR to increase the sensitivity. Dot blot hybridization was used initially but because of the non specificity of the PCR-generated probe, oligonucleotide probe and Southern blot were used instead to ensure specificity. With the specificity of the probe assured, it was used in the microtiter plate hybridization with enzyme immunoassay detection (Kox *et al,* 1996; Kunakorn and Markham, 1995) for more convenience.

PCR in known to be susceptible to the presence of reaction inhibitors in DNA extracts from clinical specimens. These inhibitors can lower the test sensitivity or cause false negative results, and could be checked by spiking the PCR mixture with either diluted *M. tuberculosis* DNA (Kox *et al,* 1994) or modified control DNA (Kolk *et al,* 1994). Another cause of false negatives might be that the samples did not truly represent the infection. In the specimens that had a low number of mycobacteria, one part of a sample might contain the mycobacteria while another part might not. This was demonstrated by the discrepancy between PCR and culture in the samples of 3 patients (Table 2). In patient A, the first

Table 2

Discrepancy between PCR and culture due to nonrandom distribution of *M. tuberculosis* in the culture.

Patient	Specimen taken date	Specimen	TB-PCR result	Culture result
A	7 August 93	CSF	+	no growth
	27 August 93	CSF	+	no growth
	7 September 93	CSF	+	*M. tuberculosis*
	11 October 93	CSF	+	no growth
B	14 September 93	tissue	+	*M. tuberculosis*
	18 October 93	tissue	+	no growth
C	29 February 96	CSF	+	*C. neoformans*
	4 March 96	CSF	+	*M. tuberculosis*

Table 3

Causes and remedies of the errors of PCR for diagnosis of tuberculosis.

Errors	Causes	Remedies
False positive	Product-carryover contamination	1. Change primers 2. Use UDG and dUTP 3. Control for UDG activity
	M. tuberculosis DNA contamination	1. Do not extract DNA from *M. tuberculosis* culture in the same place that use for clinical samples. 2. Clean bench after extraction of DNA
	Non specific hybridization signal	1. Use specific oligonucleotide probe in dot blot hybridization 2. Use Southern blot instead of dot blot when unsure of the probe's specificity
False negative	Inhibitors in samples	1. Spike the samples with weak positive control *M. tuberculosis* DNA or use internal DNA control 2. Reextract of the samples with inhibitors
	Sample is not representative of the infection	1. Multiple samples from same patient required
	Systemic errors	1. Systemic weak positive DNA control tube must be positive
	PCR is not sensitive enough	1. Improve sample extraction 2. Optimize the PCR reaction 3. Add hybridization

and second dates' samples were positive by PCR while there was no growth by culture. Later when the PCR result was negative, the culture yielded *M. tuberculosis*. At 3 months after treatment both PCR and culture in patient A became negative. In patient B, the samples from the first date were both positive by PCR and culture. While the second date's samples, PCR remained positive while culture gave no organism. In patient C who had AIDS, culture of first date' s sample yielded *M. tuberculosis*. PCR of both first and second dates'samples were positive. This showed the overgrowth effect of another organism co-infected with *M. tuberculosis* in and AIDS patient. The cause of the false negative by uneven distribution of mycobacteria in the samples indicated that multiple samples might be required for accurate diagnosis by PCR. However, unlike hemoculture, obtaining samples such as CSF could not be done too frequently.

The causes and remedies of the PCR errors discussed above were summarized in Table 3. The evaluation of PCR in a routine situation has enabled us to identify the errors that might not be encountered in a well controlled research setting. Despite all of the prevention measures taken, it is not uncommon that human error could affect outcomes. To this end, automated PCR machines have been built to correct this influence (DiDomenico *et al,* 1996; Jungkind *et al,* 1996; Wilke *et al,* 1995). The future application of PCR in infectious disease diagnosis will depend on how well the errors inherent to PCR have been realized and handled.

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