Direct Identification of *Mycobacterium Tuberculosis* from Sputum on Ziehl-Neelsen Acid Fast Stained Slides by Use of Silica-Based Filter Combined with Polymerase Chain Reaction Assay

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Abstract

This paper describes a method for isolation of deoxyribonucleic acid (DNA) from Ziehl-Neelsen stained sputum smears on glass slides; and isolated DNA was used for the IS*6110* polymerase chain reaction (PCR)-based identification of *M. tuberculosis*. A total of 221 samples from newly diagnosed suspected tuberculosis cases were first examined by microscopic examination. For DNA extraction by silica-based filter, a home-made modified spin column gave the efficacy as did the nucleospin tissue reagent kit and therefore was selected for PCR template preparation. The extracted DNA was amplified by the IS*6110* PCR using a primer pair that amplifies a 377-bp target, and the product was analyzed by agarose gel electrophoresis with confirmation by Southern blot hybridization. In comparison with culture, PCR with template prepared by the silica based filter showed overall sensitivity and specificity of 91.7 and 100 per cent, respectively. This study used the over one year and less than one year slides samples to study the effect of storage time. In the more than one year storage group, PCR assay gave a sensitivity and specificity of 83.3 and 100 per cent, respectively. In conclusion, the applicability of the PCR directly to DNA extracted from Ziehl-Neelsen stained smears could become a valuable alternative approach for rapid identification of *M. tuberculosis*, and could be used to evaluate quality of the control of local laboratories in tuberculosis (TB) screening and solve the problem of specimen transportation. In addition, the method could be used in retrospective studies involving a wide range of PCR-based analyses, such as detection of rifampicin resistant gene in multidrug-resistant tuberculosis (MDR-TB) study.

Key word : *Mycobacterium Tuberculosis*, PCR, Sputum, Ziehl-Neelsen Acid Fast Stained Slides, DNA Isolation, Silica-Based Filter

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Tuberculosis (TB) has re-emerged worldwide as an important public health problem. One-third of the world's population has been infected with *Mycobacterium tuberculosis*. Much concern has been expressed about the dramatic outbreaks of multidrug-resistant tuberculosis (MDR-TB) in HIV-infected TB patients in various parts of the world[1-3]. Nowadays, TB control programs are very important in public health policies. The quality evaluation of laboratory diagnosis has become a hot issue in TB management. Most laboratories in Thailand have been using Ziehl-Neelsen (ZN) acid fast staining method in TB first screening because this method is rapid, easy, and cheap. Sputum specimens collected in peripheral health centers, where only direct microscopy is performed, must often be sent to central laboratories for culture and quality evaluating. However, the transport of sputum specimens might be dangerous for spreading, and also the delay time or contamination of sputum specimens may affect the culture recovery. The transport of fixed and stained smears was another way to solve these problems, since the slides after staining can be stored for a long time and provide a simple, inexpensive and convenient way to transport with less contamination than sputum specimens.

Several reports on the successful use of the PCR for the detection of *M. tuberculosis* in a variety of clinical specimens have been published[4-6]. In the present study, the authors combined the advantages of both PCR technology and the classical sputum examination by ZN staining for the diagnosis of *M. tuberculosis* infection. However, a high degree of nucleic acid purity is essential to ensure reliable PCR from sputum samples. The purification system based on silica membrane technology is fast and convenient and can be used for a wide variety of sample sources [7]. The authors present a protocol for removal of sputum from the ZN stained slides, and for purification of DNA using the silica membrane based method combined with IS*6110* PCR-based identification of *M. tuberculosis*. The efficacy of PCR assay from the fixed, stained sputum film slides was evaluated in comparison with conventional culture for detection of *M. tuberculosis* from sputum specimens. The benefit of the developed protocol together with the PCR assay for the identification of *M. tuberculosis* in fixed, ZN stained slides with a long storage time was also determined.

MATERIAL AND METHOD

Specimen collection, microscopic examination and culture

Either spot or collection sputum was collected from 221 newly suspected TB patients at the Tuberculosis Division, Ministry of Public Health, Bangkok from December 2001 to August 2002. These patients were over 15 years old with chest symptoms suggestive of suspected tuberculosis, and had never received anti-tuberculosis drugs before or for not more than 2 weeks. All specimens were examined first in stained smears by ZN staining and scored according to the IUATLD scale[8]. Sputa were decontaminated with an equal volume of N-acetyl-L-cysteine (NALC)-4 per cent NaOH, and centrifuged before inoculating the sediment onto two slopes of Lowenstein-Jensen (L-J) media and incubated at 37°C for 8 weeks. Positive cultures were examined for growth rate, colony morphology, and identified as *M. tuberculosis* by conventional methods[9].

Removal and isolation of DNA from the fixed, ZN stained slides

The whole film of the fixed, stained smear on each glass slide was scraped off with a sterile scalpel blade into a microtube containing 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.3) and then centrifuged at 10,000 x g for 15 min before discarding the supernatant. The resuspended sediment (250 µl) was then treated with an equal volume of NALC-2 per cent NaOH, and then incubated at room temperature for 15 min with shaking. The volume of the mixture was adjusted to 25 ml with sterile water and centrifuged for 30 min at 10,000 x g. The supernatant was discarded and the sediment was washed again with sterile water. Pellets were resuspended in 250 µl TE buffer and used for DNA isolation.

Two different approaches were used to isolate DNA for PCR including silica membrane based and boiling methods. For silica membrane based spin-column method, the authors first used both the commercial kit test (Nucleospin tissue column, Macherey-Nagel, Germany) and a home-made modified spin column for isolation of DNA from 35 stained slides containing different acid fast (AF) smear grading as classified by the results on ZN stain for AF negative (-), AF1+, 2+ and 3+. The duplicate stained smear from each sputum samples were prepared; and

all the materials on the stained smear from duplicate glass slides were removed. Finally, the pellets were resuspended in TE buffer and divided into 2 aliquots. The DNA from the first aliquot was extracted using the Nucleospin tissue column, following the protocol in the accompanying handbook. The DNA from the second aliquot was extracted using a home-made spin column method. Briefly, after resuspending the pellets in lysis bufer (20 mg/ml lysozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton X) at 37°C for 30 min, the sample was digested with proteinase K (final concentration of 5mg/ml) at 56°C for 1 h. Then, 200 µl of guanidium buffer (4 M Guanidium isothiocyanate, 50 mM Tris HCl [pH 7.0], 20 mM EDTA) was added and incubated at 70°C for 10 min, then 210 µl of absolute ethanol was added and mixed by vortex. All the sample was applied to the silica spin filter column and centrifuged for 1 min at 10,000 x g. The column was washed twice with 500 µl of washed buffer (50% ethanol containing 200 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl [pH 7.4]). The DNA was eluted with 200 µl of prewarmed (70°C) Tris- HCl, pH 9, and 10 µl of the eluate were used directly in the PCR reaction.

All of the samples were also extracted using the boiling method. Briefly, after removal of all the materials on stained smear, the pellets were resuspended in 20 µl TE buffer and boiled in water bath at 95°C for 10 min and then placed on wet ice for at least 5 min. The pellets were removed by centrifugation at 12,000 x g for 5 min, and 10 µl of the supernatant were used directly in the PCR reaction.

Polymerase chain reaction

The PCR amplification to the IS*6110* fragment of the *M. tuberculosis* complex was performed as described previously[10], using one pair of primers (TB1, 5'-CCA ACA AGA AGG CGT ACT CG-3'; TB2, 5'-GGA GAC TCT CTG ATC TGA GAC C-3') which anneal to a sequence in the repetative DNA element IS*6110*[11]. The PCR reaction mixture, the amplification time, and temperature followed the optimized PCR condition as described previously[10]. The PCR mixture (30 µl) consisted of 1X PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM (each) dNTP, 0.3 µm each primer, 0.6 units *Taq* DNA polymerase (Promega) with 10 µl of extracted DNA. PCR was performed in an automated thermal cycler (Perkin-Elmer Cetus) by using 1 cycle at 94°C for 5 min, 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1 min at 72°C and 1 cycle at 72°C for 10 min. The amplified products were run on 2 per cent agarose gel, stained with ethidium bromide, and visualized under UV light. The expected size of the amplified DNA fragment was 377 bp.

Southern blot hybridization was performed on appropriate samples which gave a positive result after ethidium bromide staining so that the specificity of the PCR could be confirmed. The PCR products in the gel were transferred to a positively charged nylon membrane (Hybond N^+; Amersham) and hybridized with the DNA probe by the method of Southern[12]. Probe for hybridization was synthesized by PCR using *M. tuberculosis* H37Rv reference strain as template for amplification and the PCR product was purified and labeled by random priming with fluorescein-dUTP, using the random Primer Fluorescein Labeling Kit (Renaissance®, PerkinElmer Life Sciences, Inc., MA, USA). The protocol for hybridization followed the instructions in a Renaissance® using 1-h prehybridization and a further 2-h hybridization with fluorescein-labeled probe (final concentration, 20 ng/ml) at 60°C. Immunological detection with antifluorescein-alkaline phosphatase conjugate and chemiluminescent detection with CDP Star® substrate (Tropix, Inc., USA) were followed the instructions in the manufacturer's protocol. The membranes were exposed to Kodak XAR5 photographic film for 1-4 min.

Effect of time storage on ZN stained slides for PCR-based detection assay

Two groups of sputum samples on the ZN stained slides with different collection time periods were used in the present study. The ZN stained slides in the first group were prepared more than one year before the PCR assay was performed; and the second group was prepared less than one year. The modified spin column DNA extraction method was used for template preparation for PCR assay.

Control procedures

Stringent contamination control procedures followed those recommended by Kwok and Higuchi [13]. All PCR reactions were performed using a negative reagent control, containing all PCR components and sterile distilled water instead of DNA, a negative control containing DNA was extracted from *M. flavescens* ATCC 23035 (100 pg/reaction), and a positive control containing DNA was extracted from *M. tuberculosis* H37RV (100 pg/reaction). Inhibition of the PCR was checked by spiking duplicates of the tested

DNA extracts with approximately 50 fg of H37Rv DNA. Samples showing inhibition were retested at 1 : 10 dilution.

Statistical analysis

Validation of the silica-based filter combined with PCR assay for the identification of *M. tuberculosis* from all ZN stained slides were determined by using culture results as the "gold standard". Statistical comparison was performed by using McNemar χ^2-test; and p of < 0.05 was considered significant. The Kappa statistic was used to determine agreement between the tests.

RESULTS

AF microscopy and conventional culture results

The 221 examined sputum samples were classified into 4 groups according to AF staining and conventional culture; 116 (52.48%) were AF positive and culture positive, 5 (2.26%) were AF negative and culture positive and 100 (45.24%) were AF negative and culture negative. Of 116 samples with smear positive, 38, 38 and 40 samples were graded as 1+, 2+, and 3+, respectively. If cultures which were biochemically confirmed are regarded as the most reliable diagnostic method, then 121 samples were TB positive and 100 samples were TB negative.

Silica membrane based spin-column for isolation of DNA for PCR assay

Comparison between two silica membrane based spin-column methods for isolation of DNA from AF stained slides is presented in Table 1. In the present study, a total of 35 stained slides comprising 20 samples of culture positive (AF3+ = 6, AF2+ = 5, AF1+ = 5, and AF negative = 4) and 15 samples of culture negative and AF negative were studied. Both DNA extraction methods yielded the PCR products with molecular size of 377 bp that were nearly the same brightness and sharp. Mostly, stained slides containing high AF smear grading (i.e., AF3+ and AF2+) showed more bright sharp bands than those of low AF smear grading i.e., AF1+ and AF (-). The correlation between the commercial kit test and the modified spin column DNA extraction method was statistically significantly different ($p < 0.001$) with the Kappa agreement rate at 0.994.

Comparison between PCR-based identification from AF stained slides and conventional culture for detection of *M. tuberculosis* from sputum specimens

Table 2 demonstrates the PCR results for identification of *M. tuberculosis* from AF stained slides prepared from 221 sputum specimens. PCR results with templates prepared by the modified spin column showed all 40 samples (100%) with AF3+ produced positive 377-bp bands the same as 36 (94.74%) of 38 samples with AF2+, and 33 (86.84%) of 38 samples with AF1+. For AF negative and culture positive samples, 2 (40%) of 5 samples produced a positive PCR band. All 100 ZN stained slides prepared from AF negative and culture negative samples yielded negative PCR bands. Most samples with AF3+ showed more bright sharp bands, but some 2+ or 1+ grading samples showed positive PCR bands that were not different from those of 3+ grading samples (data not shown).

PCR results with templates prepared by the boiling method showed only 22 of 121 specimens were positive by PCR (sensitivity; 18.18%). These positive PCR results were from AF3+ mostly; i.e. 11 (27.5%) of 40 samples with AF3+ produced visible bands the same as 4 (10.53%) of 38 samples with AF2+ and 7 (18.42%) of 38 samples with AF1+. For the AF nega-

Table 1. Measurement of agreement rate between two silica membrane based spin-column methods for isolation of DNA for PCR-based identification of *M. tuberculosis* from 35 fixed Ziehl-Neelsen stained slides.

Nucleospin tissue column method	Modified spin column DNA extraction method		
	PCR positive	PCR negative	Total
PCR positive	15	2	17
PCR negative	0	18	18
Total	15	20	35

$p < 0.001$, $K = 0.994$

Table 2. Percentage of *M. tuberculosis* identification by PCR from 221 AF stained slides with different microscopic grading and culture. The templates for PCR were prepared by the modified spin column DNA extraction and boiling methods.

Acid-fast (AF) and culture (Cul) results	No. sample	PCR results with templates prepared by					
		Modified spin column			Boiling		
		PCR positive	PCR negative	% of identification	PCR positive	PCR negative	% of identification
Culture positive (n = 121)							
AF-/Cul+	5	2	3	40.00	0	5	0.00
AF1+/Cul+	38	33	5	86.84	7	31	18.42
AF2+/Cul+	38	36	2	94.74	4	34	10.53
AF3+/Cul+	40	40	0	100.00	11	29	27.50
Culture negative (n = 100)							
AF-/Cul-	100	0	100	0.00	0	100	0.00

tive and culture positive samples, none produced a positive band. All 100 AF negative and culture negative samples were also negative by PCR assay (specificity; 100%). However, most visible bands with a molecular size of 377 bp from templates prepared by the boiling method were not as bright as those prepared by the silica-based spin column method.

Statistical comparison using Mc Nemar χ^2 test showed significant different in the performances of PCR from AF stained slides and the conventional culture for detection of *M. tuberculosis* from sputum specimens ($p < 0.001$). The sensitivity, specificity and efficiency of the PCR-based assay with template prepared by the modified spin column DNA extraction method are 91.7, 100 and 95.5 per cent, respectively; the same figures with template prepared by the boiling method are 18.2, 100 and 22.5 per cent, respectively (Table 3).

Effect of time storage on AF stained slides used for PCR assay

The first group of AF stained slides was prepared more than one year before the PCR assay was performed (range 1-2 years); and the second group was prepared less than one year (range 2 weeks to 6 months). Stained slides of the first group were from 30 TB cases with different smear grading results (AF3+ = 10, 2+ = 10, and 1+ = 10) and 10 non TB-cases; and the second group was from 25 TB cases (AF3+ = 10, 2+ = 10, and 1+ = 5) and 10 non-TB cases. For the first group, 25 of 30 culture positive specimens were positive by PCR (sensitivity; 83.3%). These positive PCR results were from AF3+ mostly; i.e. 10 (100%) of 10 samples with AF3+ produced visible PCR positive bands the same as 9 (90%) of 10 samples with AF2+ and 6 (60%) of 10 samples with AF1+. In comparison with the less than one year group, all (100%) of the 25 culture positive samples produced visible PCR positive bands (sensitivity; 100%) and more brightness than the more than one year storaged slides group (Fig. 1). Of all the negative samples in both groups, none produced positive PCR band (specificity; 100%).

Analysis of PCR product by hybridization assay

The PCR products were confirmed by hybridization assay. The results of the film derived from hybridization assay clearly showed dark bands without non-specific bands. Each of 32 PCR positive samples (AF1+ = 10, AF2+ = 10, AF3+ = 10, and AF-Cul+ = 2) from the total specimens presented positive results and 8 of PCR negative did not produce dark bands on the film (data not shown).

DISCUSSION

Nowadays, TB cases are still increasing and the problem of MDR-TB remains enhancing. Delayed diagnosis of active TB is an important problem that results in greater morbidity and mortality and intra-institutional spread of TB. There is a need for a high efficiency diagnosis method for early case finding. Microscopic examination for AFB has been the mainstay of the diagnosis of pulmonary TB while the results of sputum cultures were pending. It is the most cost-effective method and used throughout the country for case finding and the assessment of treatment: the method is quick and simple. Of the available staining techniques for direct microscopy, the Ziehl-Neelsen acid-fast stain is the most widely used. Here, the authors describe a reliable method for removing and

Table 3. Comparison between the PCR assay from AF stained slides and the conventional culture for identification of *M. tuberculosis* from 221 sputum specimens. The templates for PCR were prepared by the modified spin column DNA extraction and boiling methods.

Specimen type and PCR with template prepared by	No. of specimens with the following culture result [c]		Sensitivity (%)	Specificity (%)	Predictive value (%)		Efficiency (%)
	Positive	Negative			Positive	Negative	
PCR [a]							
All specimens							
Positive	111	0	91.7	100.0	100.0	90.9	95.5
Negative	10	100					
Smear-positive specimens							
Positive	109	0					
Negative	7	0					
Smear-negative specimens							
Positive	2	0					
Negative	3	100					
PCR [b]							
All specimens			18.2	100.0	100.0	50.3	22.5
Positive	22	0					
Negative	99	100					
Smear-positive specimens							
Positive	22	0					
Negative	94	0					
Smear-negative specimens							
Positive	0	0					
Negative	5	100					

[a] PCR with template prepared by the modified spin column DNA extraction method.
[b] PCR with template prepared by the boiling method.
[c] Using culture as the 'gold standard'.

isolating DNA from fixed, ZN stained slides; and DNA isolated by this method was used for PCR-based identification of *M. tuberculosis.*

In the present study, the results of AF smear correlated well with culture results. The sensitivity of AF staining (97.7%) was considerably higher than those reported by others(14,15). The high sensitivity in the present study reflects the low proportion of specimens showing culture positive with AF negative. This situation arises from the fact that the study population were new cases; 132 males (59.72%) and 89 females (40.28%) who came to the TB Division that is the reference central diagnoses work office. Most patients who come to this out patient department must have relative signs and symptoms of TB infection. These samples were first examined by microscopic examination, thus most of the AF positive smears together with the evaluation by chest film data may be suggestive of diagnosis for TB infection.

There are several methods to remove the smear from the slides for DNA extraction, i.e. cotton swab, and scraping with blade(16-19). In a pilot study, the authors tried to use a cotton swab to remove the stained sputum smear from each glass slide. Although the method was simple and rapid, the bacilli were likely to be lost during the process especially the AF1+ slide that contains less bacilli. The scraping was another interesting choice that was selected for the present study. The blade (Germino no.10) was used and it gave good efficiency for this process. The glass slides, after the removal procedure, were re-stained and evaluated by microscopic examination. The scraping method could remove most of the sputum.

For the reasons of being easy, rapid, effective and non-induced more inhibitors, the scraping method was used for removal of the sputum specimens from the slides. The physical characteristics of sputum samples may vary from containing copious amounts of mucus to being purulent, haemoptyic or tenacious. The common DNA extraction methods involve the use of enzymes such as lysozyme and proteinase K treatment and extraction with organic solvents, detergent-induced lysis or lysis using guanidium isothiocyanate. Firstly, the digestive processed by *N*-acetyl-*L*-

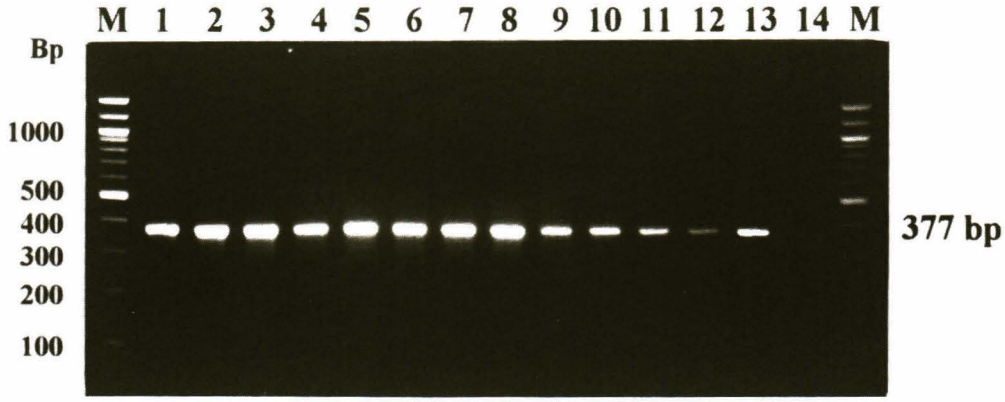

Fig. 1. Representative results of the PCR-based identification of *M. tuberculosis* from some AF stained slides with different times storage (2 weeks, 6 months, and two years). The templates for PCR were prepared by the modified spin column DNA extraction method. Lanes: M, 100-bp molecular weight marker; 1 to 4, samples with 2 weeks period; 5 to 8, 6 months period; 9 to 12, 2 years period; 13, H37Rv DNA positive control; and 14, negative reagent control.

cysteine was used in sample preparation. After decontamination, the samples were washed with distilled water instead of phosphate buffer since phosphate may induce inhibitors of PCR[20]. In cases of DNA extraction by boiling method, the pellets were washed with distilled water twice for reducing the other inhibitors.

For the PCR based assay, the pair of primers designed by Tansuphasiri et al[10] permitted the amplification of 377-bp DNA fragment located at the end region position +961 to +1377 bp of IS*6110* sequence. The sensitivity, specificity, and efficiency of this pair of primers in *M. tuberculosis* detection were 96.27, 99.48, and 98.17 per cent, respectively. However, diagnostic techniques based on PCR have two major problems: false-positive reactions due to contamination with DNA fragments from previous amplicons and false-negative reactions caused by inhibitors that interfere with the PCR. For this reason, the extraction of DNA from clinical specimens is very important. The phenol-chloroform is one of the extraction methods that has been most widely used for *M. tuberculosis*. In recent years, many new technologies have been developed to reduce the use of hazard reagents. These new methods are easy and rapid. One of the DNA extraction methods is based on silica membrane and lysis buffer containing enzymes.

The short and easy protocol was applied. This study used the DNA extraction method with silica membranes spin column because this method is rapidly performed and has high efficiency in the reduction of inhibitors. All reagent buffers used in the extraction protocol can be prepared. By this procedure, the authors could save the budget to buy all commercial reagent kits except the silica membrane filter column. The silica membrane was recommended to replace silica particle in order to solve the inhibitor effects that might occur by the pellets of reagent contamination to PCR reaction[21].

The correlation between the home-made modified spin column and the nucleospin tissue reagent kit were statistically significantly different ($p < 0.001$) with the Kappa agreement rate of 0.994. For this reason, modified spin column DNA extraction method was selected in the present study. The boiling method was also used in comparison with modified spin column DNA extraction because this method is easy, rapid and the different results of both methods may explain the benefit of removal inhibitors by spin column and lysis buffer too.

The correlation between PCR assay using modified spin column for DNA extraction and culture method were statistically significantly different ($p < 0.001$) and the sensitivity, specificity, and efficiency of test were 91.7, 100 and 95.5 per cent, respectively. The other group that used the boiling method for template preparation, the sensitivity, specificity, and

efficiency of test were 18.2, 100 and 22.5 per cent, respectively. The difference of the DNA extraction method showed that the modified spin column method was highly effective in reduction of inhibitors and gave a high sensitivity and high specificity of PCR assay.

Due to the advantage of PCR assay that can be used to detect long stored specimens[22], the authors applied PCR to detect the sputum sample on ZN stained slides which were stored for over or less than a one year period. The correlation between PCR assay and culture of both the over or less than one year groups were statistically significantly different ($p < 0.001$). The sensitivity, specificity and efficiency of the test in the more than one year storage group were 83.3, 100 and 87.5 per cent, respectively. In the less than one year slide group, a high sensitivity was presented. The sensitivity, specificity and efficiency of the test in this group were all 100 per cent. The present study indicated that storage did not influence the quality of DNA for PCR, however, the efficiency of the test may be decreased with longer storage times. However, the occasional false positive or false negative of PCR may occur. The inhibitors in specimens, the medication treated, or process of specimens can reduce the PCR efficiency. The nested PCR or hybridization assay may be applied to increase the sensitivity and specificity of PCR in reliability of TB detection.

In conclusion, PCR amplification of DNA isolated from ZN acid fast stained slides using the silica membrane based technique can be used for reliable and sensitive identification of low levels of *M. tuberculosis* with an AF smear grading of 1+. The sensitivity and specificity of PCR assay compared with culture were 91.7 and 100 per cent, respectively ($p < 0.001$). To increase the sensitivity of detection, the authors recommend to scraping multiple slides from each specimen. This method can be a choice for rapid identification of TB in combination with the conventional method, and will facilitate a variety of retrospective studies involving a wide range of PCR-based analyses, such as detection of the rifampicin resistant gene from specimens which are available only as fixed, stained microscope slides prepared several years earlier.

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REFERENCES

1. American Thoracic Society and Centers for Disease Control. Nosocomial transmission of multidrug resistant tuberculosis among HIV-infected persons-Florida and New York 1988-1991. Morbid Mortal Wkly Rep 1991; 40: 585-91.
2. Edlin BR, Tokars JI, Grieco MH. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N Eng J.Med 1992; 326: 1514-21.
3. Fischi MA, Uttamchandani RB, Daikos GL, et al. An outbreak of tuberculosis caused by multiple-drug-resistant tubercle bacilli among patients with HIV infection. Ann Intern Med 1992; 117: 177-83.
4. Forbes BA, Hicks KES. Direct detection of *Mycobacterium tuberculosis* in respiratory specimen in a clinical laboratory by polymerase chain reaction. J Clin Microbiol 1993; 31: 1688-94.
5. Bascunana CR, Belak K. Detection and identification of Mycobacteria in formalin-fixed, paraffin-embedded tissues by nested PCR and restriction enzyme analysis. J Clin Microbiol 1996; 34: 2351-5.
6. Caldarelli-Stefano R, Vago L, Bonetto S, et al. Use of magnetic beads for tissue DNA extraction and IS*6110 Mycobacterium tuberculosis* PCR. J Clin Pathol: Mol Pathol 1999; 52: 158-60.
7. Boddinghaus B, Wichelhaus TA, Brad V, Bittner T. Removal of PCR inhibitors by silica membranes: Evaluating the Amplicor *Mycobacterium tuberculosis* kit. J Clin Microbiol 2001; 39: 3750-2.
8. The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network. IUATLD; 1998: 12-5.
9. Kent PT, Kubica GP. Public Health Mycobacteriology. A guide for the level III laboratory Atlanta: Centers for Disease Control; 1985: 71-80.
10. Tansuphasiri U, Vathanophas K, Pariyanonda A, Chanyasanha CH. Detection of *Mycobacterium tuberculosis* in respiratory specimens using IS *6110*-PCR based method with new designed primers. Mahidol J 1999; 6: 71-6.
11. Thierry D, Cave MD, Eisenach KD, et al. IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. Nucleic Acids Res 1990; 18: 188.
12. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98: 503-17.
13. Kwok S, Higuchi R. Avoiding false positive with PCR. Nature (London) 1989; 339: 237-8.
14. Tan TH. Simple and rapid cold stain method for acid fast bacteria. Am Rev Respir Dis 1962; 85: 753-4.
15. Tansuphasiri U, Kladphuang B. Evaluation of sputum staining by modified cold method and comparison with Ziehl-Neelsen and fluorochrome methods for the primary diagnosis of tuberculosis. Southeast Asian J Trop Med Public Health 2002; 33: 128-35.
16. Fukunaga H, Murakami T, Gondo T, et al. Sensitivity of acid-fast staining for *Mycobacterium tuberculosis* in formalin-fixed tissue. Am J Respir Crit Care Med 2002; 166: 994-7.
17. Amar C, Pedraza-Diaz S, McLauchlin J. Extraction and genotyping of *Cryptosporidium parvum* DNA from fecal smears on glass slides stained conventionally for direct microscope examination. J Clin Microbiol 2001; 39: 401-3.
18. Jethwa HS, Schmitz JL, Dallabetta G, et al. Comparison of molecular and microscopic techniques for detection of *Treponema pallidum* in genital ulcers. J Clin Microbiol 1995; 33: 180-3.
19. Rossi MC, Gori A, Zehender G, et al. A PCR-colorimetric microwell plate hybridization assay for detection of *Mycobacterium tuberculosis* and *M. avium* from culture samples and Ziehl-Neelsen-positive smears. J Clin Microbiol 2000; 38: 1772-6.
20. Leckie GW, Erickson DD, He Q, et al. Method for reduction of inhibition in a *Mycobacterium tuberculosis*-specific ligase chain reaction DNA amplification assay. J Clin Microbiol 1998; 36: 764-7.
21. Suffys P, Vanderborght PR, Santos PBD. Inhibition of the polymerase chain reaction by sputum samples from tuberculosis patients after processing using a silica-guanidium thiocyanate DNA isolation procedure. Mem Inst Oswaldo Cruz, Rio de Janeiro 2001; 96: 1137-9.
22. Konomi N, Lebwohl E, Mowbray K, et al. Detection of mycobacterial DNA in Andean mummies. J Clin Microbiol 2002; 40: 4738-40.

การพิสูจน์เชื้อวัณโรคโดยตรงจากเสมหะบนแผ่นสไลด์ที่ย้อมสีทนกรดวิธีซีล–เนลเซ่น ด้วยการใช้เยื่อกรองซิลิการ่วมกับการวิเคราะห์โดยวิธีพีซีอาร์

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การศึกษานี้ได้กล่าวถึงการพิสูจน์เชื้อวัณโรคโดยตรงจากเสมหะบนแผ่นสไลด์ที่ได้ผ่านการย้อมสีทนกรดด้วยวิธีซีล–เนลเซ่นมาก่อน โดยอาศัยการขูดลอกเสมหะออกจากแผ่นสไลด์ การย่อยและการสกัดแยกดีเอ็นเอให้บริสุทธิ์ด้วยเยื่อกรองซิลิกา ดีเอ็นเอที่ได้นำมาใช้เป็นตัวตั้งต้นในการวิเคราะห์ด้วยวิธีพีซีอาร์และหาประสิทธิภาพของวิธีพีซีอาร์ โดยการศึกษาเปรียบเทียบกับวิธีมาตรฐานการเพาะแยกเชื้อวัณโรคจากเสมหะของผู้ป่วยรายใหม่ที่สงสัยว่าเป็นวัณโรค จำนวน 221 ราย ซึ่งเสมหะของผู้ป่วยได้ผ่านการย้อมสีทนกรดด้วยวิธีซีล–เนลเซ่นและตรวจขั้นต้นด้วยกล้องจุลทรรศน์ ในการศึกษาเปรียบเทียบวิธีการสกัดดีเอ็นเอด้วยเยื่อกรองซิลิกา ระหว่าง Modified spin column ที่เตรียมน้ำยาขึ้นเอง กับชุดตรวจสำเร็จรูป nucleospin ที่ซื้อมา พบว่ามีประสิทธิภาพในการวินิจฉัยไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ จึงใช้ชุดน้ำยาที่เตรียมขึ้นเองเพื่อสกัดแยกดีเอ็นเอจากตัวอย่างที่ศึกษาทั้งหมด ดีเอ็นเอที่ได้นำมาวิเคราะห์ด้วยวิธีพีซีอาร์โดยอาศัยการเพิ่มปริมาณของดีเอ็นเอขนาด 377 คู่เบส จากดีเอ็นเอเป้าหมายในกลุ่ม IS*6110* และวิเคราะห์ผลผลิตพีซีอาร์ด้วยเทคนิค Agarose gel electrophoresis และยืนยันความจำเพาะของผลผลิตดังกล่าวด้วยวิธี Southern blot hybridization ผลการศึกษาพบว่า วิธีพีซีอาร์ มีความไวและความจำเพาะร้อยละ 91.7 และ 100 ตามลำดับ ในการศึกษาผลการเก็บสไลด์เสมหะติดสีทนกรดซีล–เนลเซ่นไว้นาน ๆ ต่อประสิทธิภาพของการพิสูจน์หาเชื้อวัณโรค พบว่า สไลด์ที่เก็บไว้นานกว่า 1 ปี วิธีพีซีอาร์ให้ผลความไว และความจำเพาะ ร้อยละ 83.3 และ 100 ตามลำดับ โดยสรุปการนำวิธีการด้านอณูชีววิทยามาประยุกต์ใช้เพื่อพิสูจน์เชื้อโดยตรงจากเสมหะบนแผ่นสไลด์ที่ติดสีย้อมซีล–เนลเซ่นมาก่อน จะเป็นแนวทางเลือกอีกอันหนึ่งที่มีประโยชน์ในการวินิจฉัยวัณโรคได้อย่างรวดเร็วขึ้น ตลอดจนนำไปสู่การพัฒนาการวินิจฉัยเพื่อประเมินคุณภาพของห้องปฏิบัติการวัณโรคระดับท้องถิ่นด้านงานคัดกรองโรค หรือแก้ปัญหาอันเนื่องจากการขนส่งสิ่งส่งตรวจ รวมทั้งประโยชน์ของการศึกษาย้อนหลัง ซึ่งสามารถนำวิธีการพีซีอาร์มาประยุกต์เพื่อศึกษาเกี่ยวกับเชื้อวัณโรคดื้อยาต่อไป

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