

Differentiation of *Mycobacterium tuberculosis* Strains by Arbitrarily Primed Polymerase Chain Reaction-based DNA Fingerprinting

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Arbitrarily primed polymerase chain reaction (AP-PCR) method was applied to the differentiation of clinical isolates of M. tuberculosis strains. The primer which is specific to human papilloma virus (HPV) type 18 was found to be appropriate for the AP-PCR-based differentiation of M. tuberculosis isolates, since AP-PCR produced multiple polymorphic DNA bands when M. tuberculosis DNA was used as template. AP-PCR was performed using either one of the HPV type 18 primer and IS6110-specific primer (half-specific PCR, HS-PCR) or HPV type 18 primer pair only (nonspecific PCR, NS-PCR). The usefulness of these two methods in differentiating M. tuberculosis isolates, was measured by calculating dissimilarity values of 16 isolates using Cluster Analysis software. The highest dissimilarity values by HS-PCR and NS-PCR methods were 0.38 and 0.59, respectively. This suggested that NS-PCR method is better than HS-PCR method in strain differentiation. Although the dissimilarity value calculated by Cluster analysis of the standard restriction fragment length polymorphism method, in which IS6110 was used as a probe, was much more higher than the NS-PCR method, NS-PCR method using HPV18 primers was quite useful for the differentiation of M. tuberculosis strains due to its rapidity and simplicity.

Key Words: AP-PCR, tuberculosis, strain differentiation, DNA fingerprinting

Tuberculosis is a chronic infectious disease which causes high morbidity and mortality in human and, therefore, still remains to be a severe health problem in developing countries. The morbidity of mycobacterial diseases including tuberculosis in developed countries is also being increased dramatically as a complication of AIDS which has become pandemic (Daley *et al.* 1992; Edlin *et al.* 1992; Kiehn *et al.* 1987). Polymerase chain reaction (PCR) (Saiki *et al.* 1988; Saiki *et al.* 1985) has been reported

to be helpful in shortening the time required for diagnosis of tuberculosis compare to conventional diagnostic tools such as culture and identification of the *M. tuberculosis* (Eisenach *et al.* 1989; Pao *et al.* 1990; Thierry *et al.* 1990). The prevention of tuberculosis transmission by epidemiological tools is as important as early diagnosis in the control of tuberculosis. The differentiation of *M. tuberculosis* isolates is very helpful for the epidemiological study of tuberculosis. Differentiation of *M. tuberculosis* isolates is important in tuberculosis control in many aspects; it helps tracing the sources of tuberculosis infection and identifying specific strains which may have characteristic phenotype, such as drug resistance. It can differentiate reinfection from reactivation of the tuberculosis in the same patient. Restriction fragment length polymorphism (RFLP) (Cave *et al.* 1991; Mazurek *et al.* 1991; Otal *et al.* 1991;

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van Soolingen *et al.* 1991; van Embden *et al.* 1992) analysis, multiplex PCR (Haas *et al.* 1993), and pulsed-field gel electrophoresis (PFGE) (Zhang *et al.* 1992) methods have been reported to be useful in differentiation of *M. tuberculosis* strains. Arbitrarily primed (AP)-PCR is based on the production of polymorphic PCR products when short oligomer was used for PCR with low annealing temperature (Welsh *et al.* 1990). Although AP-PCR was reported to be useful in differentiating many bacterial strains, it was not applied to the differentiation of *M. tuberculosis* strains using primers which are not related to *M. tuberculosis* DNA sequences.

In this paper, we examined the usefulness of the AP-PCR method in differentiating 16 Korean *M. tuberculosis* isolates, which showed different IS6110-based RFLP patterns from each other, using primers which were specific to human papilloma virus (HPV) type 18. Our results suggested that AP-PCR method was quite useful in the differentiation of *M. tuberculosis* strains since it could be performed rapidly using a simple procedure, although it was

not so discriminative as RFLP analysis.

MATERIALS AND METHODS

Bacterial strains and Media

Twenty-seven mycobacterial strains isolated from patients who were suspected to have tuberculosis were used. Three strains were obtained from Taegu branch of Korean Association of Tuberculosis. Thirteen strains were obtained from Dr. Y. Chong of Yonsei University College of Medicine, Seoul Korea. All of the clinical isolates were confirmed to be *M. tuberculosis* by their colony morphologies, growth characteristics, Niacin test, and by detecting the 123-bp *M. tuberculosis* complex-specific IS6110 sequence using PCR (Eisenach *et al.* 1989, Yoon *et al.* 1991). *M. tuberculosis* strains were cultured in Sauton liquid media at 37°C without shaking.

DNA preparation from *M. tuberculosis* strains

DNA was prepared by bead-beating method

Table 1. Primer sequences used in this study

Primer sets	Specific to	Primer sequences
P1	<i>M. tuberculosis</i>	5'-CCTGCGAGCGTAGGCGTCGGT-3'
P2		5'-CTCCTCCAGCCCCCCTTCCC-3'
INS1	<i>M. tuberculosis</i>	5'-CGTGAGGGCATCGAGGTGGC-3'
INS2		5'-GCGTAGGCGTCGGTGACAAA-3'
TC60	Epstein-Barrvirus	5'-CCAGAGGTAAGTGGACTT-3'
TC61		5'-GACCGGTGCCTTCTTAGG-3'
HPV-A	human papilloma virus type 6	5'-AGACCAGTTGTGCAAGACGTTTAA-3'
HPV-B		5'-GCACGTCTAAGATGTCTTGTTTAG-3'
HPV-C	human papilloma virus type 11	5'-AGACCAGTTGTGCAAGACGTTTAA-3'
HPV-D		5'-AAGGGAAAGTTGTCTCGCCACACA-3'
HPV-E	human papilloma virus type 16	5'-ACCGAAACCGGTTAGTATAAAAGC-3'
HPV-F		5'-GATCAGTTGTCTCTGGTTGCAAA-3'
HPV-G	human papilloma virus type 18	5'-CACACCACAATACTATGGCGCGCT-3'
HPV-H		5'-CTGCTGGATTCAACGGTTTCTGGC-3'

^aIndicates bacteria or viruses to which primers produce specific DNA amplifications by polymerase chain reaction.

as described before (Yoon *et al.* 1991). One loop of bacterial cells were suspended with Tris-EDTA-NaCl (TEN, pH 7.4) buffer. Each 100 μ l of zirconium bead and phenol/chloroform/isoamylalcohol (25:24:1, v/v) was added to the bacterial suspension. The mixture was beaten by mini-beadbeater (Biospec Products, Bartlesville, Ok.) for 1 minute. After centrifugation the DNA in the upper aqueous phase was precipitated by ethanol and used for PCR analysis.

Primers

The primers used in this study, their sequences, and sources are listed in Table 1.

Identification of *M. tuberculosis* by PCR.

Since the isolated strains were not identified to be *M. tuberculosis* by standard biochemical test, we examined whether the strains contained IS6110 which was specific to *M. tuberculosis* complex by PCR. *M. tuberculosis*-specific PCR was performed as described by Eisenach *et al.* (1989). In short, the reaction condition was as described below; the reaction was performed in a volume of 50 μ l containing 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Nor-

walk, Conn.), 10 μ M of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.5 μ M of each of the two primers, 200 μ M of deoxynucleoside triphosphate mixtures (dATP, dCTP, dGTP, and dTTP). Fifty microgram of mycobacterial DNA was added in a volume of 5 μ l. Three steps were repeated with 30 times in the thermal cycler (Easy Cycler, Ericomp, Inc., San Diego, Calif.); denaturation at 95°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. PCR products were examined by 8% polyacrylamide gel electrophoresis (PAGE) (Maniatis *et al.* 1982), visualized, and photographed on the ultraviolet transilluminator after ethidium bromide staining.

AP-PCR based DNA fingerprinting

Five different primer pairs were examined for their ability to produce multiple DNA bands by AP-PCR. The annealing temperature for AP-PCR was 37°C. Other PCR conditions were same as described above. AP-PCR was performed using either the combination of HPV-G/H (human papilloma virus [HPV] type 18-specific primers, nonpecific PCR [NS-PCR]) or INS1 (*M. tuberculosis*-specific primer) and

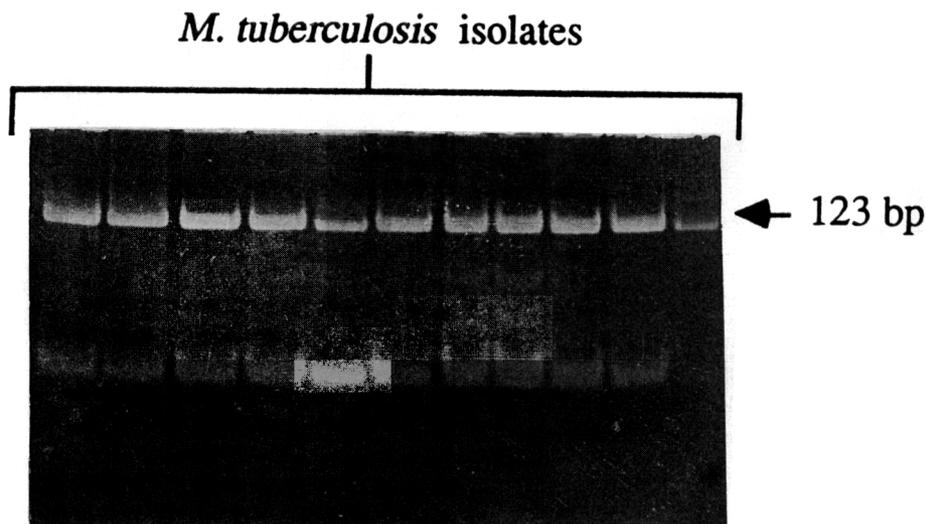


Fig. 1. The isolated mycobacteria were identified to be *M. tuberculosis* by polymerase chain reaction using IS6110-specific primers (P1/P2). The target sequence of the P1/P2 PCR was 123 bp in size (as indicated by arrow).

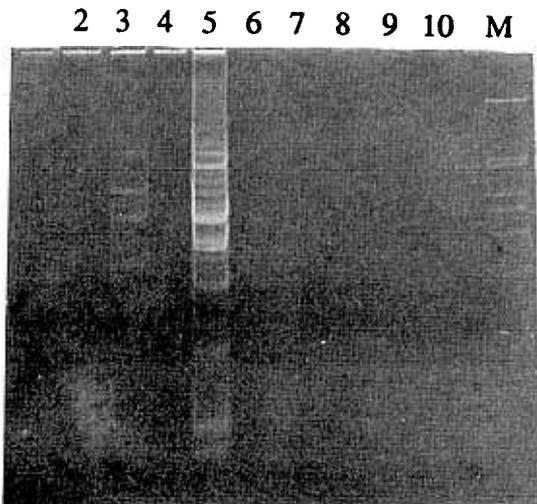


Fig. 2. Selection of primers for differentiation of *M. tuberculosis* isolates by AP-PCR. *M. tuberculosis* H37Rv DNA was used as templates in lanes 1 to 5. Distilled water was added, instead of template DNA, in lanes 6 to 10. Lanes 1 and 6, Epstein-Barr virus specific primer pair (TC60 and TC61); lanes 2 and 7, HPV type 6 specific primer pair (HPV-A and HPV-B); lanes 3 and 8, HPV type 11 specific primer pair (HPV-C and HPV-D); lanes 4 and 9, HPV type 16 specific primer pair (HPV-E and HPV-F); lanes 5 and 9, HPV type 18 specific primer pair (HPV-G and HPV-H); lane M, λ BR322 DNA digested with *Hinf*I (1632 bp, 517 bp, 504 bp, 396 bp, 344 bp, 298 bp, 220 bp, and 154 bp, from top to bottom).

HPV-G primer (half-specific PCR, HS-PCR). Sixteen *M. tuberculosis* isolates were analyzed by both HS-PCR and NS-PCR.

Comparison of fingerprinting methods by Cluster Analysis

The usefulness of these 2 different fingerprinting methods, HS-PCR and NS-PCR, were compared by drawing dendrograms. Dendrograms were based on the dissimilarity values of the *M. tuberculosis* strains which were calculated using Cluster Analysis software (Ludwig and Reynolds, 1988) and IBM computer. The results with higher top

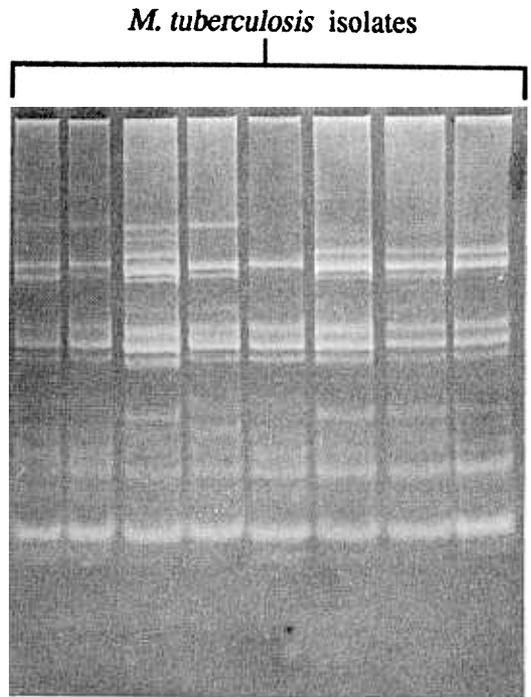


Fig. 3. The HS-PCR patterns of *M. tuberculosis* isolates are shown. *INS1* and *HPV-G* primers were used.

dissimilarity value was interpreted to be more discriminative method for the strain differentiation.

RESULTS

Identification of *Mycobacterium tuberculosis* isolates by polymerase chain reaction analysis

Clinical isolates of 16 mycobacterial strains were examined whether they contained *M. tuberculosis*-specific *IS6110* DNA by PCR analysis. All of the isolates showed the *M. tuberculosis*-specific 123-bp DNA bands (Fig. 1).

HPV type 18-specific primers were selected for AP-PCR-based differentiation of *M. tuberculosis* strains

Several primers were examined whether they produced multiple DNA bands by AP-

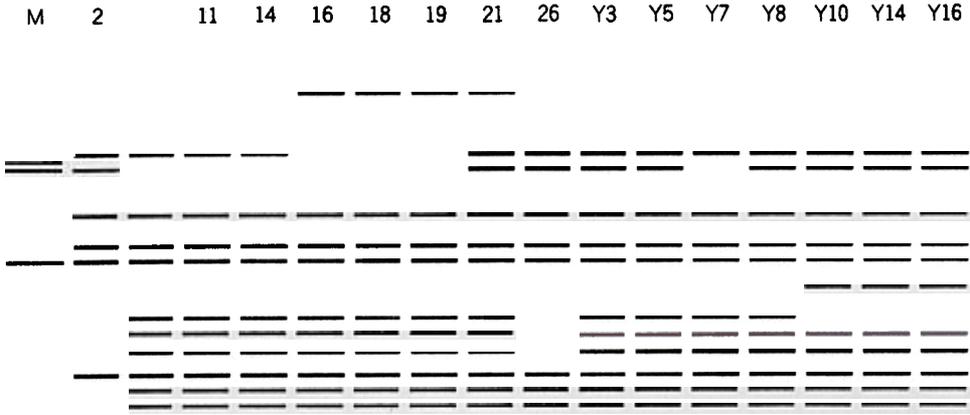


Fig. 4. Summary of the HS-PCR patterns of 16 isolates of *M. tuberculosis*. Lane M, same as described in Fig. 2.

PCR using *M. tuberculosis* DNA as template. Low annealing temperature (37°C) was used to induce nonspecific annealing and subsequent DNA amplification. PCR using primers specific to Epstein-Barr virus (EBV), human papilloma virus (HPV) type 6, and HPV type 16 showed no DNA amplification with *M. tuberculosis* DNA. PCR using HPV type 11-specific primers showed several DNA bands, however, they were faint and small in numbers. PCR using HPV type 18 primers showed discrete multiple DNA amplifications, which suggested that the primers could be examined for AP-PCR-based DNA fingerprinting of *M. tuberculosis* strains (Fig. 2).

HS-PCR using half-specific primer set

M. tuberculosis-specific primer (INS-1) and HPV type 18-specific primer (HPV 18-G) were used in HS-PCR. HS-PCR with 4 different annealing temperatures were examined to find the appropriate annealing temperature in differentiation of *M. tuberculosis* strains. When the primers were annealed at 37°C, the *M. tuberculosis* strains could be differentiated, though many bands were shared by several strains (Fig. 3). With the increase in annealing temperatures to 42°C or 48°C, several bands disappeared. This suggested that these bands were produced from the annealing sites by the HPV 18-G primer, which was not specific

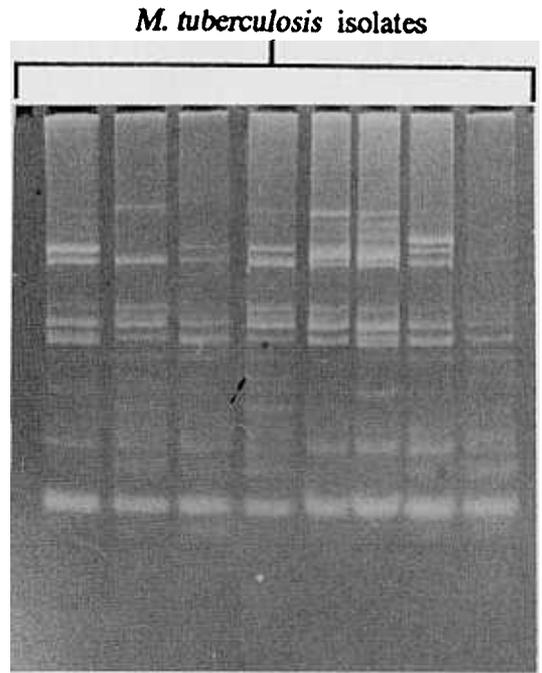


Fig. 5. The NS-PCR patterns of *M. tuberculosis* isolates are shown. HPV-G and HPV-H primers were used.

to *M. tuberculosis*. When the primers were annealed at 55°C, the HS-PCR showed almost uniform patterns (data not shown). This sug-

AP-PCR Differentiation of *M. tuberculosis* Isolates

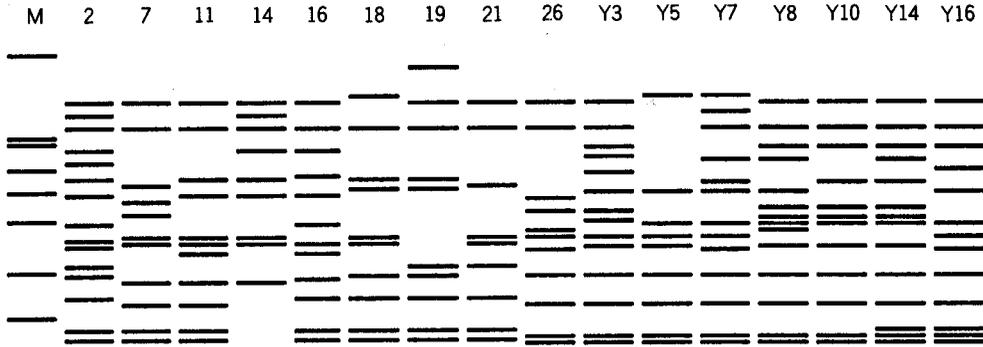


Fig. 6. Summary of the NS-PCR patterns of 16 isolates of *M. tuberculosis*. Lane M, same as described in Fig. 2.

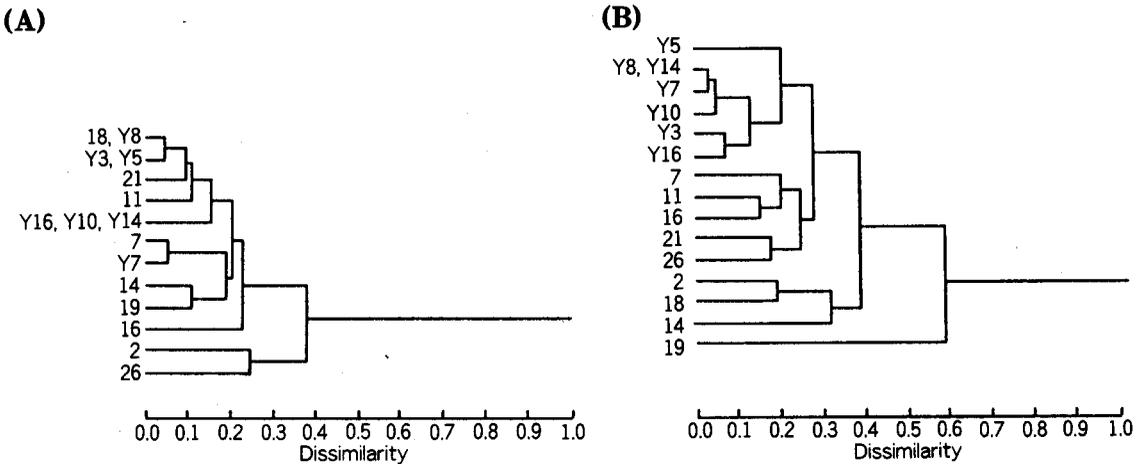


Fig. 7. Dendrograms of the 16 isolates of *M. tuberculosis* based on 2 different methods are shown, HS-PCR (A), and NS-PCR (B). The dissimilarity values were calculated by Cluster Analysis software.

gested that the DNA bands were specifically amplified by *M. tuberculosis*-specific INS-1 primer. The HS-PCR pattern of *M. tuberculosis* performed with the annealing temperature of 37°C was the most polymorphic and it was summarized in Fig. 4. HS-PCR was not so useful in differentiating *M. tuberculosis* strain since many strains showed the same banding patterns.

NS-PCR using HPV type 18-specific primers

NS-PCR was performed using HPV type 18-specific primers (G and H). More bands were

amplified in NS-PCR than in HS-PCR (Fig. 5). This suggested that both of the nonspecific primers annealed with *M. tuberculosis* DNA template more randomly than the primers for HS-PCR did. The NS-PCR patterns of *M. tuberculosis* isolates were summarized in Fig. 6. The NS-PCR was more discriminative than HS-PCR for *M. tuberculosis* strain differentiation.

Comparison of HS-PCR and NS-PCR by Cluster Analysis

The usefulness of these 2 methods in strain

differentiation were compared by dendrogram which was based on the Cluster Analysis results (Fig. 7). The top dissimilarity value of NS-PCR was quite high (0.59) compared with that of HS-PCR method (0.38). Only 2 strains showed identical NS-PCR patterns, while 2 pairs and 1 triple strains showed identical HS-PCR patterns. This showed that NS-PCR is more useful for the differentiation of *M. tuberculosis* strains than HS-PCR. Since it can be performed easily with a simple procedure, NS-PCR may be used as an alternative method in differentiation of *M. tuberculosis* strains to RFLP analysis.

DISCUSSION

The epidemiologic study of tuberculosis is important in the control of the disease. Recently, molecular techniques has been applied to the epidemiologic study of infectious disease. One of the most popularly used molecular epidemiological tools is RFLP analysis (Cave *et al.* 1991; Mazurek *et al.* 1991; Otal *et al.* 1991; van Soolingen *et al.* 1991). In case of *M. tuberculosis*, insertion sequence (IS) 6110 (Thierry *et al.* 1990; McAdam *et al.* 1990) has been reported to be the appropriate probe for RFLP differentiation of *M. tuberculosis* strains, since the sites and numbers of insertions are different among strains and since they are stable after several animal passages. But RFLP analysis has several limitations. (1) It needs quite pure DNA which can be digested by restriction endonuclease. (2) It needs Southern hybridization which takes about 2 days, and cumbersome. (3) Many laboratories are still using radioisotopes (RI) for probe labeling, although many of them replaced RI with nonRI labelling procedures (Ross *et al.* 1991). Therefore, it is necessary to find another method which can be performed more conveniently and in a short time. One of such methods is AP-PCR, which originally uses a short oligomer and low annealing temperatures (Welsh *et al.* 1990; Williams *et al.* 1990). AP-PCR has several advantages; it is not necessary to purify DNA, to blot or to perform hybridization procedures and to use RI.

In this study, we showed that the primers which were specific to HPV 18 were suitable for differentiation of *M. tuberculosis* strains by AP-PCR. In general, AP-PCR is performed using short (10 to 15mers) primers. Although, we did not perform extensive screening to find the appropriate primers which produce multiple and polymorphic bands, we could find that HPV type 18 primers produce multiple bands with *M. tuberculosis* DNA as template. We performed AP-PCR using pairs of primers. In HS-PCR, we used a primer pair, one of which (INS1) was specific to *M. tuberculosis*, while the other was HPV-G primer. Several annealing temperatures were examined to decide the most suitable temperature. By increasing the annealing temperature, the numbers of amplified DNA bands were decreased until 48°C. When the annealing temperature was over 55°C, the number of amplified DNA bands were increased, while the patterns were almost identical to each other. This suggested that only the *M. tuberculosis*-specific primer annealed to template DNA at 55°C. In NS-PCR, only HPV type 18-specific primer pair (HPV-G/H) was used. NS-PCR using HPV 18 primers produced more DNA bands than HS-PCR and the patterns were more polymorphic than those of HS-PCR. The HS-PCR and NS-PCR patterns were reproducible when the concentrations of template DNA and primers were not changed. HS-PCR or NS-PCR based DNA fingerprinting method is very simple and can be performed in a short time. This method also has several limitations including DNA carryover. This disadvantage can be overcome by using carryover prevention kits which destruct the amplicons produced by PCR.

Sixteen *M. tuberculosis* strains were differentiated by HS-PCR and NS-PCR. Though many of the HS-PCR and NS-PCR bands were shared in many strains, only two strains showed identical PCR patterns by NS-PCR, while other 14 strains could be differentiated each other. These two strains showed different patterns by RFLP analysis. The Cluster Analysis results also suggested that NS-PCR could differentiate *M. tuberculosis* isolates quite well with the highest dissimilarity value of 0.59. Though this was lower than the highest

dissimilarity value of RFLP analysis (0.97), this suggested that NS-PCR based on HPV type 18 primers could be used as alternative tool for differentiation of *M. tuberculosis* strains to RFLP analysis.

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