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Application of Infrequent-Restriction-Site Amplification for Genotyping of *Mycobacterium tuberculosis* and non-tuberculous Mycobacterium

Infrequent restriction site amplification (IRS-PCR) is a method of amplifying DNA sequences, which flank an infrequent restriction site, and produces a strain-specific electrophoretic pattern. We studied the use of IRS-PCR to characterize *Mycobacterium tuberculosis* and non-tuberculous mycobactria (NTM). One-hundred and sixteen *M. tuberculosis* and nine NTM isolated at Hanyang University Hospital in Seoul, Korea were used in this study. IRS-PCR using AH1 and PX-G primers produced unique patterns for reference strains, *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. kansasii*, *M. scrofulaceum*, *M. szulgai*, *M. gordonae*, *M. avium*, *M. intracellulae*, *M. fortuitum*, and *M. chelonae*, respectively. Reference strains *M. tuberculosis* H37Rv, *M. bovis*, *M. africanum*, and all isolates of *M. tuberculosis* showed similar IRS-PCR patterns. The IRS-PCR patterns generated with multiple isolates of *M. tuberculosis* from the same patients were essentially identical. IRS-PCR revealed the greatest difference between electrophoretic DNA patterns from *M. avium*, *M. intracellulae*, and *M. fortuitum* that differed from each other and from the reference strains. We concluded that IRS-PCR is a useful tool for strain typing of NTM, but not for *M. tuberculosis*.

Key Words : *Mycobacterium tuberculosis; Non-tuberculous mycobacteria; Infrequent Restriction Site Amplification*

Tae Yeal Choi, Jung Oak Kang

Department of Clincal Pathology, Hanyang University Medical College, Seoul, Korea

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Address for correspondence
Tae Yeal Choi, M.D.
Department of Clinical Pathology, Hanyang University Hospital, 17 Haengdang-dong, Sungdong-gu, Seoul 133-792, Korea
Tel : +82.2-2290-8974, Fax : +82.2-2298-1735
E-mail : tychoi@hanyang.ac.kr

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INTRODUCTION

The prevalence of culture-positive pulmonary tuberculosis is about 0.2% in Korea (1). Mycobacteria form a heterogeneous group in terms of occurrence in clinical or environmental materials, complex phenotypic and genetic data, and disease association (2, 3). In the clinical mycobacteriology laboratory, strain typing has been essential in the outbreak identification and identification of laboratory cross-contamination (4-6). Tracking a particular strain of *Mycobacterium tuberculosis* as it moves through a susceptible population group has been almost impossible, since in most cases there are not strain-distinguishing characteristics. Antibiotic susceptibility patterns (7), biotyping (8), and bacteriophage typing (9) rarely allow strain identification. With advances in molecular biology, techniques for strain-specific epidemiologic studies of tuberculosis are becoming available (10). Some species have the same sequence or a very high degree of similarity (11). This leads to problems in development of sequence-based analysis methods, such as restriction fragment length polymorphism (RFLP) analysis (12, 13), hybridization with probes (14, 15), pulsed-field gel electrophoresis (PFGE) (16), or DNA sequence analysis (17). All of these methods have inherent experimental difficulties. Recently, a new bacterial typing method known as infrequent restriction site-polymerase chain reaction (IRS-PCR) has been described (19). The IRS-PCR genomic fingerprinting method has been applied to only a few bacterial species (11, 19, 25, 31). We applied IRS-PCR for genotyping of *M. tuberculosis* and non-tuberculous mycobacteria (NTM).

MATERIALS AND METHODS

Organisms

Twelve control strains (*M. tuberculosis* H37Rv, ATCC 272 94; *M. bovis*, ATCC 19210; *M. bovis* BCG, ATCC 27291; *M. africanum*, ATCC 25420; *M. kansasii*, ATCC 12478; *M. scrofulaceum*, ATCC 19981; *M. szulgai*, ATCC 35799; *M. gordonae*, ATCC 14470; *M. avium*, ATCC 25291; *M. intracellulae*, ATCC 13950; *M. fortuitum*, ATCC 6841; *M. chelonae*, ATCC 35749.) were donated from the Korean Institute of Tuberculosis. One-hundred and sixteen *M. tuberculosis* and nine NTM isolated at Hanyang University Hospital in Seoul, Korea were used in this study. These included multiple isolates from the same patients. All Mycobacterium isolates were isolated from sputum. *M. tuberculosis* were subcultured in Ogawa medium and identified using standard biochemical tests such as niacin accumulation test and nitrate reduction test. Identification of NTM was made by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (23).

Adaptors and Primers

The design of our adaptors was same with that described by Mazurek et al. (19). The adaptors were constructed to ligate specifically to the CG-3′ two-base overhang generated by *Hha*I digestion or to the 5′-CTAG four-base overhang generated by *Xba*I digestion. The *Hha*I adaptor (AH) consists of a 22-base oligonucleotide (AH1) with a 7-base oligonucleotide (AH2) annealed to bases 14 through 20 from the 5′ end leaving a CG-3′ overhang (Table 1). To prepare the adaptor, AH1 and AH2 were mixed in equal molar amounts in 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0,001% [w/v] gelatin) and were allowed to anneal as the mixture cooled from 80℃ to 4℃ over 1 hr in a thermal cycler. The stock adaptor was stored at -20℃ at a concentration of 20 μM. The *Xba*I adaptor consists of an 18-base oligonucleotide (AX1) with a 7-base oligonucleotide (AX2) annealed to bases 5 through 11 from the 5′ end leaving a 5′-CTAG overhang. The 5′ end of AX1 was phosphorylated with T4 polynucleotide kinase as specified by the manufacturer (BIONEER Co., Cheongwon, Chungbuk, Korea). The kinase was subsequently inactivated by heating the mixture to 65℃ for 10 min. AX2 and phosphorylated AX1 were mixed and annealed as described above. An oligonucleotide primer (PX) was constructed to complement AX1 and the one base left on the 3′ end of the native DNA following *Xba*I digestion (Table 1). The oligonucleotides were purchased from BIONEER Co.

Preparation of template DNA

Bacterial suspensions (10^5-10^8 cells, total) were made using McFarland turbidity standards. DNA was purified by InstaGene (BioRad, Hercules, CA, U.S.A.). A portion of lysate (2.5 μL) was digested with 20 U of *Hha*I and 20 U of *Xba*I in a mixed buffer (final volume 12.5 μL) for 2 hr at 37℃. All enzymes were obtained from BioRad (Hercules, CA, U.S.A.). T4 DNA ligase (1.5 U), ATP (12.6 pmol), 10 × ligase buffer (2 μL), the *Xba*I adaptor AX (20 pm), the *Hha*I adaptor AH (20 pmol), and water were added to a total volume of 20 μL. The mixture was incubated at 4℃ overnight to ligate the adaptors to the digested DNA and then at 65℃ for 20 min to inactivate T4 DNA ligase. The sample was digested with 5 U of *Xba*I and 5 U of *Hha*I at 37℃ for 20 min to cleave any restriction sites reformed by ligation.

Table 1. Adapter and primer oligonucleotides

*Hha*I adapter	
AH1	5′-AGA ACT GAC CTC GAC TCG CAC G-3′*
AH2	3′-TG AGC GT-5′
*Xba*I adapter	
AX1	5′-PO4-CTA GTA CTG GCA GAC TCT-3′
AX2	3′-AT GAC CG-5′
*Xba*I primers, PX-N†	5′-AGA GTC TGC CAG TAC TAG AN-3′

*AH1 serves as part of the adapter and as the *Hha*I primer; †N denotes any of the four nucleotides A, T, G, or C. Four kinds of primers were actually synthesized.

Amplification

Each 50 μL PCR mixture included 2 μL of template DNA, 0.5 U of *Taq* DNA polymerase, deoxynucleoside triphosphates (200 μM each), and two oligonucleotide primers (1.0 μM) in 10 × PCR buffer. Typically, the oligonucleotides AH1 and PX-G were used together as primers. Amplification was performed in a GeneAmp PCR system 9600 (Perkin-Elmer, Branchburg, NJ, U.S.A.) with an amplification profile that consisted of an initial denaturation step at 94℃ for 5 min and then 40 cycles of denaturation at 94℃ for 30 sec, primer annealing at 62℃ for 30 sec, and extension at 72℃ for 90 sec. The PCR products were loaded into wells of a 7% polyacrylamide gel (BioRad Laboratories, Hercules, CA, U.S.A.) in 1 × TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). After electrophoreses for 2 hr at 200 V, the gel was stained with ethidium bromide (0.5 μg/mL) for 10 min, destained in water 25 min, and photographed with UV illumination.

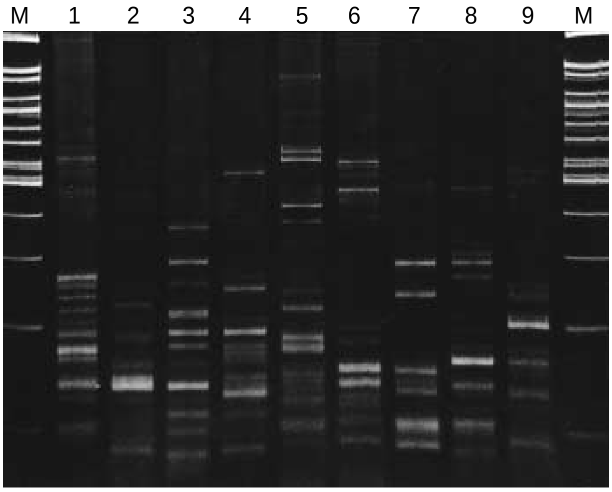

Fig. 1. IRS-PCR electrophoretic patterns of *Mycobacterium* control strains; M, 100-bp ladder; lane 1, *M. tuberculosis* H37Rv ATCC 27294; lane 2, *M. kansasii,* ATCC 12478; lane 3, *M. scrofulaceum*, ATCC 19981; lane 4, *M. szulgai*, ATCC 35799; lane 5, *M. gordonae* ATCC 14470; lane 6, *M. avium*, ATCC 25291; lane 7, *M. intracellulae*, ATCC 13950; lane 8, *M. fortuitum*, ATCC 6841; lane 9, *M. chelonae*, ATCC 35749. The primers used were AH1 and PX-G.

RESULTS

IRS-PCR using AH1 and PX-G as primers produced a unique pattern for *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. kansasii*, *M. scrofulaceum*, *M. szulgai*, *M. gordonae*, *M. avium*, *M. intracellulae*, *M. fortuitum*, and *M. chelonae*, respectively (Fig. 1). It was also possible to generate other similar unique patterns in each primer.

M. tuberculosis H37Rv, *M. bovis*, and *M. africanum*, which are all *M. tuberculosis* complex, showed similar patterns using AH1 and PX-G as primer. The IRS-PCR patterns of *M. tuberculosis* complex were also similar when other PX-N primers were used. There were only one- or two-band differences among those strains. Except *M. bovis* BCG, we could not differentiate the species even through the results using four sets of primers (AH1 and PX-N) were combined (Fig. 2). The IRS-PCR of clinically isolated *M. tuberculosis* using AH1 and PX-G showed very similar patterns to those of the reference strain *M. tuberculosis* H37Rv. There were also only one- or two-band differences among those isolates (Fig. 3). Other similar patterns of IRS-PCR were also produced when PX-G was replaced with PX-C, PX-A, or PX-T (data not shown). Three strains isolated from each of two patients within a period of 1 week produced same patterns respectively (Fig. 4).

IRS-PCR using AH1 and PX-G produced different patterns for each of the three *M. avium*, four *M. intracellulae*, and

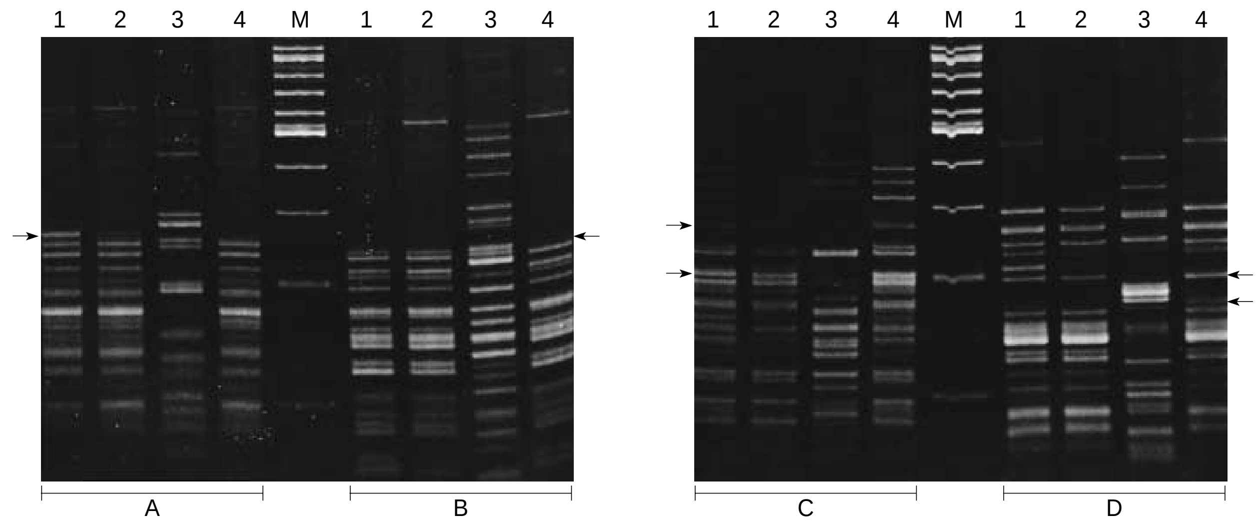

Fig. 2. IRS-PCR electrophoretic patterns of *Mycobacterium* control strains; M, 100-bp ladder; lane 1, *M. tuberculosis* H37Rv ATCC 27294; lane 2, *M. bovis*, ATCC 19210; lane 3; *M. bovis* BCG ATCC 27291; lane 4, *M. africanum* ATCC 25420. The primers used were AH1 and PX-G (A part), PX-A (B part), PX-C (C part), and PX-T (D part). Arrows indicate main band difference.

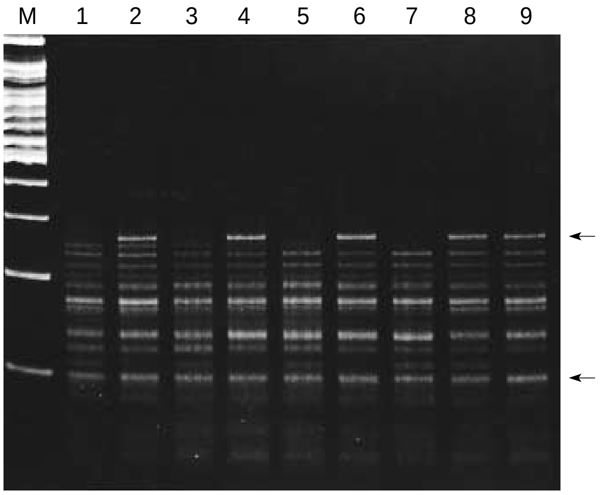

Fig. 3. IRS-PCR electrophoretic patterns of *Mycobacterium tuberculosis* isolates using AH1 and PX-G as primers.; M, 100-bp ladder; lanes 1 through 9, nine random clinical *M. tuberculosis* isolates from different patients.

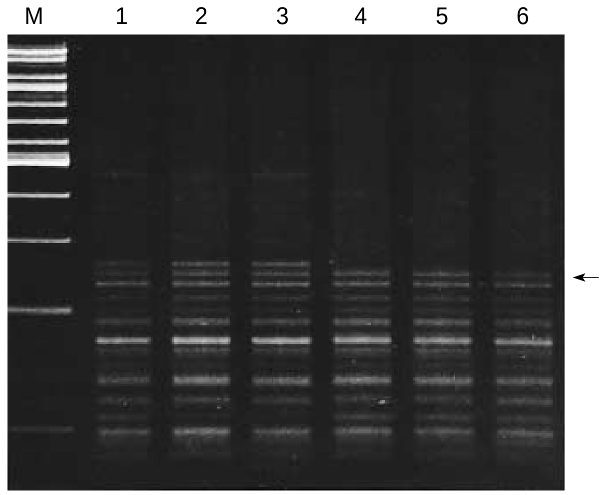

Fig. 4. IRS-PCR electrophoretic patterns of *M. tuberculosis* isolates from multiple clinical specimens from same patients using AH1 and PX-G as primers. lane M, 100-bp ladder: lanes 1-3, samples from one patient: lanes 4-6, samples from another patient.

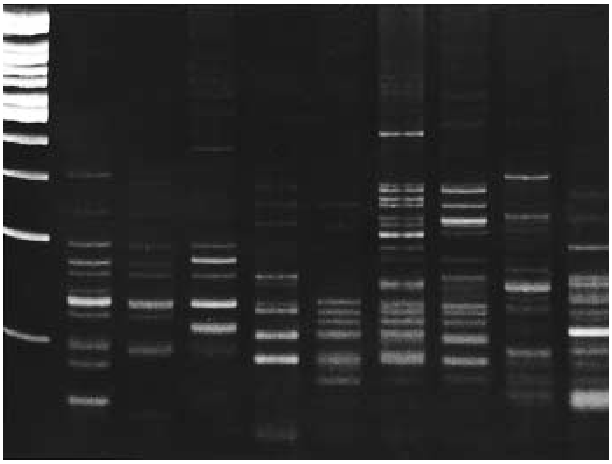

Fig. 5. IRS-PCR electrophoretic patterns of non-tuberculous mycobacterium isolates using AH1 and PX-G as primers. M, 100-bp ladder: lanes 1-3, *M. avium* isolates: lanes 4-7, *M. intracellulae* isolates: lanes 8 and 9, *M. fortuitum* isolates.

two *M. fortuitum* isolates when compared to the reference strains, respectively (Fig. 5). Other different patterns of IRS-PCR for NTM were also produced when PX-G was replaced with PX-C, PX-A, or PX-T (data not shown).

Totally, the fragments between 50 and 300 bp were found to be the most reproducible in this study. Fragments above or below this range were ignored in further analysis. Some of the IRS-PCR were run in duplicate (the reactions were completely separate, DNA extraction, restriction enzyme digestion, adaptor ligation, PCR amplification, and electrophoresis). There was little variability in the band patterns of duplicated reactions, and only minor variations were observed in band intensity.

In conclusion, the pattern variation in genotyping of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) by the IRS-PCR was relatively low, while that of NTM was high.

DISCUSSION

Identification of bacterial strains by DNA fingerprinting facilitates epidemiologic studies and disease control. IRS-PCR has been shown to be a robust method for the molecular characterization of bacteria (19-22). This technique allows for a high level of flexibility and can produce several different genomic fingerprints of varying complexity for each sample analyzed, depending on enzyme combination and primer modification. Furthermore, the restriction endonuclease digestion is sequence-specific, the primers used in the PCR are specific to the previously ligated sequence, and only limited information about the target DNA is needed. Compared to other typing techniques such as PFGE, this is more efficient using minimal amounts of genomic DNA (19, 20).

We used the *Hha*I-*Xba*I endonuclease combination to cut the bacterial DNA. *Hha*I is a frequently cutting restriction enzyme and *Xba*I is an infrequently cutting restriction enzyme. Selection of restriction endonuclease pairs can be simplified if the basic genomic organization is known. Several restriction endonuclease combinations can be utilized to generate optimized, unique, and easy to interpret patterns (20). The designs of this *Xba*I adaptor and the corresponding primers allow initiation of amplification and additional selectivity. The use of primers that are longer than the oligonucleotides in the adaptor prevents the amplification of primer-dimers. Further increase in the primer length (as done for primer PX-G, PX-C, PX-A, and PX-T) promotes an added degree of selectivity and facilitates the production of four sets of electrophoretic patterns. Thus, the relationship between isolates can be confirmed by examining the patterns produced with different primer sets (19).

IRS-PCR using AH1 and PX-G as primers produced different patterns for ten reference strains. We could not use this pattern variation for differentiation of species of NTM, because there are variations in the same NTM species. The reference strain of *M. tuberculosis* complex except *M. bovis* BCG showed similar patterns using AH1 and PX-N as primers. Using this approach, we found only 1- or 2-band difference between each strain of *M. tuberculosis* complex. We could not easily differentiate these similar patterns by combination of results using four kinds of primer, which means the DNA sequence of *M. tuberculosis* complex is distinctly simple with limited genetic diversity in agreement with previous report (17). Conventional DNA restriction endonuclease analysis showed an almost complete similarity between the species in *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*) (24, 25). The poor discrimination found with other typing methods is most readily explained by the intrinsic lack of genetic diversity in genomic sequences of the *M. tuberculosis* complex (25). Our results support the suggestion that they should be considered as subspecies of a single species (24, 26). *M. bovis* BCG, an attenuated culture of *M. bovis*, shows morphological, biochemical and immunological differences (27). The pattern of IRS-PCR of the *M. bovis* BCG reference strain is quite different from those of other *M. tuberculosis* complex such as *M. tuberculosis*, *M. bovis*, and *M. africanum*. The pattern of *M. bovis* BCG strain by pulsed-field gel electrophoresis and other methods was substantially different from those of other members of *M. tuberculosis* complex such as *M. tuberculosis*, *M. bovis*, and *M. africanum* (12, 28-31).

The patterns of IRS-PCR of *M. tuberculosis* isolates were simple in each primer set and has only one- to two-band difference between clusters. We could not easily differentiate these similar patterns by combination of results from using other primers (PX-N). Although *M. tuberculosis* isolated from each country had comparative similar characteristics depending on the classification factor, each country's isolates showed a characteristic fingerprinting and differed slightly from the isolates from other countries (32). *Mycobacterium avium* complex (MAC) is the most common NTM associated with human disease. MAC consists of the two species *M. avium* and *M. intracellulae*, with each species being subdivided further into distinctive serovar groups. *M. avium* was classified into many kinds of patterns by PFGE (3). Mazurek et al. reported that the use of *Hha*I-*Xba*I produced informative patterns for *M. avium* and *M. intracellulae* isolates (19). In our study, the IRS-PCR of *M. avium*, *M. intracellulae*, and *M. fortuitum* isolates also revealed the greatest differences in electrophoretic DNA patterns between strains, and differed from each other and from the reference strains by IRS-PCR. This result suggests *M. avium*, *M. intracellulae*, and *M. fortuitum* need to be further divided into distinctive serovar groups.

The IRS-PCR patterns of multiple *M. tuberculosis* isolates from the same patients were identical. The patterns generated by IRS-PCR were reproducible when we used four kinds of primers for the same specimens. This means IRS-PCR is a highly reproducible tool for epidemiologic study in clinical microbiology. The fragments amplified by IRS-PCR are small (50-300 bp), facilitating separation within 2 to 3 hr by polyacrylamide gel electrophoresis. This method of strain identification can be completed within a day from the time of receipt of an isolate. This procedure can be performed with equipment that is readily available in many clinical laboratories. This method of DNA fingerprinting may be potentially applicable to a wide array of organisms in clinical microbiology laboratory. In this study, small variations in temperature, target DNA concentration, or PCR buffer could have significantly influenced the pattern produced by the same primer (19). All IRS-PCR genomic fingerprints of NTM isolates resolved by polyacrylamide gel were run in duplicate (reactions were completed separately from start to finish, i.e., DNA extraction, restriction endonuclease digestion, adapter ligation, PCR amplification, and electrophoresis). There was little to no pattern variability between duplicate reactions, only minor variations in band intensity.

The use of fluorescent-labeled primers has been described for the resolution of amplified fragment length polymorphism (AFLP) analysis (20). Resulting patterns are highly resolved and accurately sized with internally labeled DNA fragment size standards (13, 20). We do not need the fluorescent-labeled primers for further resolution of the amplified fragments in IRS-PCR.

In conclusion, IRS-PCR is a useful tool for epidemiologic studies of NTM without the need for radioactive or specific probes, but not for *M. tuberculosis*.

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