

Species Specific DNA Profiling Mycobacterial Genomes Using Polymerase Chain Reaction with Single Universal Primer (UP-PCR)

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ABSTRACT

Three tuberculous, twenty-one non-tuberculous mycobacterial (NTM) reference strains and seventy two isolates classified by biochemical tests were shown to produce specific sets of DNA fragments in a polymerase chain reaction with single universal primer (UP-PCR). A rather wide limit of tolerance for variations in procedure of PCR mixture preparation and thermocycling parameters was found. There was good correlation between UP-PCR pattern obtained in an agarose gel for each strain and the absolute majority of isolates tested. The PCR products of *M. tuberculosis* and *M. bovis* were shown to be very similar and well distinctive from that of all NTM strains tested. When image analysis system was applied to profile the UP-PCR patterns, all *M. tuberculosis* complex isolates were well differentiated from NTM species according to their specific profiles. Thus, UP-PCR profiling could give an efficient means for discriminating mycobacteria that utilizes the least time and applicable to controversial cases of suspected tuberculosis. *Iran. Biomed. J. 2: 1-8, 1998*

Keywords: DNA profiling, polymerase chain reaction, universal primer, mycobacterial, tuberculosis.

INTRODUCTION

Early diagnosis of tuberculous infection remains a major objective in the care of the patients with a view to giving appropriate treatment prior to manifestation of a symptomatic state [1-3]. Although distinguishing within mycobacteria using culture and biochemical methods is accepted as the gold standard, problems arise that can be handled only by well-experienced personnel. On the other hand, similarity between PPD skin reaction and radiological evidences in some cases of tuberculosis or mycobacteriosis can delay establishing the definitive diagnosis [4, 5].

The polymerase chain reaction (PCR) has been recently proved as a potent tool for diagnosis of tuberculosis [6-8]. Modifications of the methodology, random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR) have been applied to discrimination of mycobacterial strains, however, their use for the purpose of species differentiation among isolates is limited [9-12]. Although production of RAPD markers requires appropriate conditions, inconsistent amplification that resulted from permutations in variables of re-

action has been reported [13].

PCR with universal primer (UP-PCR) is a new method of ascertaining genetic markers [14, 15]. It generally utilizes a single primer of an average length (16-22 bases) to generate specific set of DNA fragments. In this study, we optimized the UP-PCR for DNA profiling mycobacteria and for creating computerized library of DNA profiles of reference strains. The library was then used to compare UP-PCR profiles received from isolates with those from the reference strains.

MATERIALS AND METHODS

Bacterial strains, specimen processing and bacteriology. Reference strains of mycobacteria were obtained as follows: *M. avium* (American Type Culture Collection, USA, ATCC 25291), *M. asiaticum* (ATCC 25276), *M. bovis* (ATCC 19210), *M. bovis* BCG (ATCC 35734), *M. chelonae* abscessus (ATCC 19977), *M. chelonae* chelonae (Trudeau Institute Microbial Collection, Canada, TMC 1544), *M. chitae* (Colindale, England, NCTC 10485), *M. fallax* (ATCC 35219), *M. fortuitum* (Borstel

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Institute, Canada, SN 231), *M. gastri* (ATCC 15754), *M. gordonae* (ATCC 14470), *M. intracellulare* (ATCC 13950), *M. kansasii* (ATCC 12478), *M. neoaurum* (ATCC 25795), *M. phlei* (ATCC 11758), *M. scrofulaceum* (ATCC 19981), *M. senegalense* (TMC 806), *M. shimoidei* (ATCC 27962), *M. szulgai* (ATCC 35799), *M. thermoresistibile* (ATCC 19527), *M. triviale* (ATCC 23292), *M. tuberculosis H37Rv* (ATCC 27294), *M. tuberculosis H37Ra* (ATCC 25177), *M. vaccae* (TMC 1526).

The patients were from geographically distinct regions, i.e., distant provinces of Iran. The spectrum of non-tuberculous mycobacteria (NTM) isolates has been taken with reference to their characteristic scattering in the region. Isolates were grown using specimens obtained from patients suspected of having tuberculosis or mycobacteriosis and referred to Mycobacteriology Department of Pasteur Institute of Iran. The specimens were processed for culture using decontamination by N-acetyl-L-cysteine-NaOH procedure and centrifugation. The cell pellets were suspended and inoculated on Lowenstein-Jensen (L-J) medium containing pyruvate or glycerol and incubated in an atmosphere of 5% CO₂ at 35°C for 8 weeks. Biochemical patterns of isolates of *M. tuberculosis* (n=40), *M. bovis* (n=2), *M. bovis* BCG, and NTM (*M. kansasii*, *M. fortuitum*, *M. scrofulaceum*, *M. triviale*, *M. phlei*, and others) were compared following CDC laboratory procedures manual [16]. Contamination rate for L-J systems was 7.1% for this series of experiments.

Preparation of PCR samples. Mycobacterial DNA was purified from cell colonies grown on a solid medium. Several colonies (10⁶-10⁷ cells) were suspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM Na-EDTA, pH 7.5) and equal volume of freshly prepared lysis buffer (0.25 N NaOH, 1.25% sodium dodecyl sulfate) and half volume of 5 M NaCl solution were added. Mixture was incubated in a water bath at 80° C for 15 min and buffered with 200 µl of 1 M Tris-HCl, pH 8.0. Then, DNA purification was performed by digestion with proteinase K (100 µg/ml, 50°C, 1 h) and extraction with phenol-chloroform mixture (1:1). Two phases were separated by centrifugation at 5000 rpm for 5 min and the upper phase was removed and mixed with two volumes of ice-cold ethanol. Precipitated DNA was collected by centrifugation at 14000 rpm for 10 min, washed with 70% ice-cold ethanol, and dis-

solved in 100 µl of TE buffer. The concentration of DNA was determined spectrophotometrically.

Alternatively, samples for PCR were prepared as described previously [15]. Cells were suspended in 100 µl of TE buffer and 25 µl of a 1 N NaOH solution was added. Mixture was incubated at 70° C for 10 min, frozen at -70° C for 20 min, and thawed. After that, the sample was mixed with 25 µl of a 10% solution of sodium dodecyl sulfate and centrifuged at 5000 rpm for 10 min. DNA was ethanol precipitated from a supernatant fluid, washed with 70% ice-cold ethanol and dissolved in 100 µl of TE buffer.

Polymerase chain reaction. The oligonucleotide primer M-1 (5'-TAAGGTGGCGCCAGT-3') was selected for amplification of mycobacterial DNA from a family of universal primers and used in the PCR as described originally [17]. The mixture was combined of a total volume of 30-100 µl containing 20 mM Tris-HCl, pH 8.5, 10 mM (NH₄)₂SO₄, 0.2 mM each of dNTP, 3.5 mM MgCl₂, 50 µg/ml bovine serum albumin, 0.5 µM primer, 0.05 U/µl Taq DNA polymerase (AmpliTaq[®], Perkin-Elmer Cetus) and 5-10 ng of input DNA under the following thermocycling parameters: denaturation at 94° C for 3 min for the first of 30 cycles and 60 sec for next cycles, primer annealing at 56°C for 30 sec, and elongation at 72°C for 60 sec, final extension for 5 min. Reaction provided PCR product in amount sufficient for visual inspection of DNA bands in an agarose gel stained with ethidium bromide under UV light.

Gel analysis work. The products of amplification were separated by electrophoresis on 1.5 % agarose gels (Sigma) prepared in 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (0.05 µg/ml). A computer assistant image analysis system (Eva ES, Ukraine) was used to collect and profile UP-PCR patterns. In order to evaluate lane-to-lane variability of band location both within and among gels, migration distances of DNA fragments were normalized using the 100 bp DNA ladder (GibcoBRL) in the marker lane and internal size markers (two HindIII-NdeI fragments of pUC18 DNA) in each sample. Variations in band intensities were disregarded.

RESULTS

In order to differentiate mycobacterial species ac-

cording to banding patterns of amplified DNA in agarose gel, we optimized PCR variables and defined a framework where results were reproducible.

Optimization of UP-PCR for identification of mycobacterial species. First, PCR products obtained at two different annealing temperatures, 56°C and 60°C, were compared. As shown in Fig. 1 (A,B), four-five bands from about 300 bp to 1000 bp were most obvious in UP-PCR pattern of *M. tuberculosis*. In addition, several minor bands could be seen. UP-PCR replicates produced from the same species at both regimens appeared to be almost identical by the spectrum of the major bands, demonstrating minor differences in their intensity. However, the 56° C was chosen over the 60° C as annealing temperature considering that the former was closer to value predicted on the base of the nucleotide composition of the primer. In order to maximize consistency of PCR products, the variability of DNA banding patterns depending on duration of annealing was also studied. Annealing times of 30 and 60 sec were compared giving practically indistinguishable DNA patterns for each of all above organisms (Fig. 1B,C). When influence of MgCl₂ concentration was evaluated, 2.5-4.2 mM encompassed range in which UP-PCR patterns were consistent. Thus, the 3.3 mM MgCl₂ was chosen as the optimal concentration for UP-PCR of mycobacteria.

The utility of the UP-PCR was also determined with DNA samples prepared from the same culture by two different methods (see, Materials and Methods). The banding patterns of samples prepared in both ways were very similar (data not shown). However, phenol-chloroform extraction of DNA resulted in higher reproducibility of amplification patterns received and minimized an effect of prolonged storage of samples on the yield of PCR product.

The reproducibility of amplification was also examined by employing various template and primer concentrations. To find out a level of confidence to these parameters, amplification with amount of template ranging from 1 to 50 ng of *M. tuberculosis* and *M. kansasii* DNA was accomplished. In this range, 5-10 ng of template produced the most reproducible bands giving admissible interval where assay did not get out of hand (Fig. 2A). Interestingly, as primer concentration increased from 0.3 µM to 0.9 µM, there were minimal changes in the banding pattern from two examined mycobacteria

(Fig. 2B). However, a remarkable shift from larger to smaller DNA fragments came out of 2.7 µM primer concentration in PCR mixture. The increase in primer concentration was also noticed to contribute to the greater yield of PCR product. For this reason, we used 0.5 µM primer rather than 0.3 µM in standard procedure.

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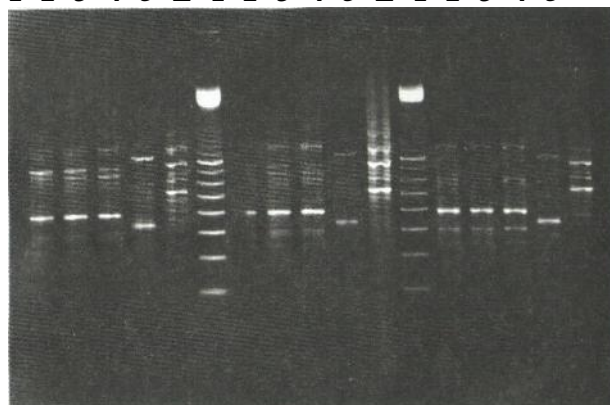


Fig. 1. Effect of various annealing temperature and time intervals on the UP-PCR patterns of mycobacteria in agarose gel. DNA samples were amplified using 0.3 µM primer M-1 with annealing step of 30 sec at 60°C (A), 30 sec at 56°C (B) or 60 sec at 56°C (C). Lanes: (1), *M. tuberculosis* H37Rv ATCC 27294; (2), *M. tuberculosis* H37Ra 25177; (3), *M. tuberculosis* 404; (4), *M. fortuitum* SN 231; (5) *M. kansasii* 12478; (L) marker (100 bp DNA ladder, GibcoBRL).

UP-PCR banding patterns of mycobacterial species. To determine the feasibility of using the optimized UP-PCR for distinguishing within mycobacterial species, a wide variety of mycobacteria were tested. Comparison of UP-PCR products from mycobacterial reference strains, revealed that different species formed clearly discernible patterns, except for *M. tuberculosis* and *M. bovis* (Fig. 3 A, B). The strains representing both species have produced almost identical DNA patterns. Noteworthy also were the differences between species of each of Runyon groups, including mycobacteria hardly discriminated by standard methods.

Application of the UP-PCR to different isolates of *M. tuberculosis* and *M. bovis* has almost demonstrated no differences in the patterns received, except for few minor bands (Fig. 4). Repeated subculturing of isolates did not alter the UP-PCR pattern,

Profiling mycobacterial genomes. To analyze species-specific fingerprints of mycobacteria, we performed DNA profiling on PCR products prepared

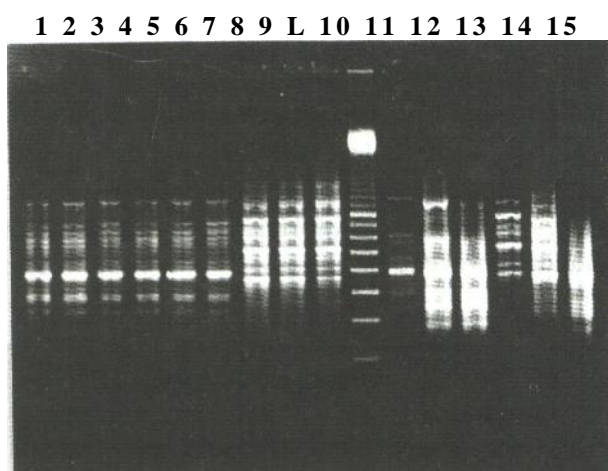


Fig. 2. Increasing template and primer concentration in UP-PCR with DNA samples of *M. tuberculosis* and *M. kansasii*. DNA samples from *M. tuberculosis* H37Rv 27294 (lanes 1-3, 10-12), *M. tuberculosis* 404 (lanes 4-6), and *M. kansasii* 12478 (lanes 7-9, 13-15) were proceeded to UP-PCR with (A) increasing amount of DNA template, lanes: (1,4,7), 1 ng DNA; (2,5,8), 7 ng DNA; (3,6,9), 50 ng DNA; or (B) increasing concentration of primer, lanes: (10,13), 0.3 μ M primer; (11, 14), 0.9 μ M of primer; (12,15), 2.7 μ M of primer; (L) marker (100 bp DNA ladder).

from 24 reference strains and 72 isolates using image analysis system. All gel images were collected to a computerized library and lanes were profiled (Figs. 4 and 5). Alignment of profiles has enabled to find reference pattern to which experimental one is matched, or almost matched. Then, discrepancy values between UP-PCR profiles were analyzed for isolates of *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, and other NTM isolates compared to corresponding reference strains.

Profiling *M. tuberculosis* isolates from different geographical regions gained coinciding characteristic bands even though variation in minor bands could be seen (Fig. 4). While UP-PCR patterns were similar for 39 of 40 biochemically identified *M. tuberculosis* isolates, one of them developed different, "mixed" pattern containing some bands that are peculiar to *M. tuberculosis* complex and others of unknown origin (data not shown). Because of this discrepancy, the above isolate and reference *M. tuberculosis* strains were additionally compared using biochemical procedures. It was found that the isolate under suspicion did not perform well some biochemical tests, including the niacin test, as reference strains did.

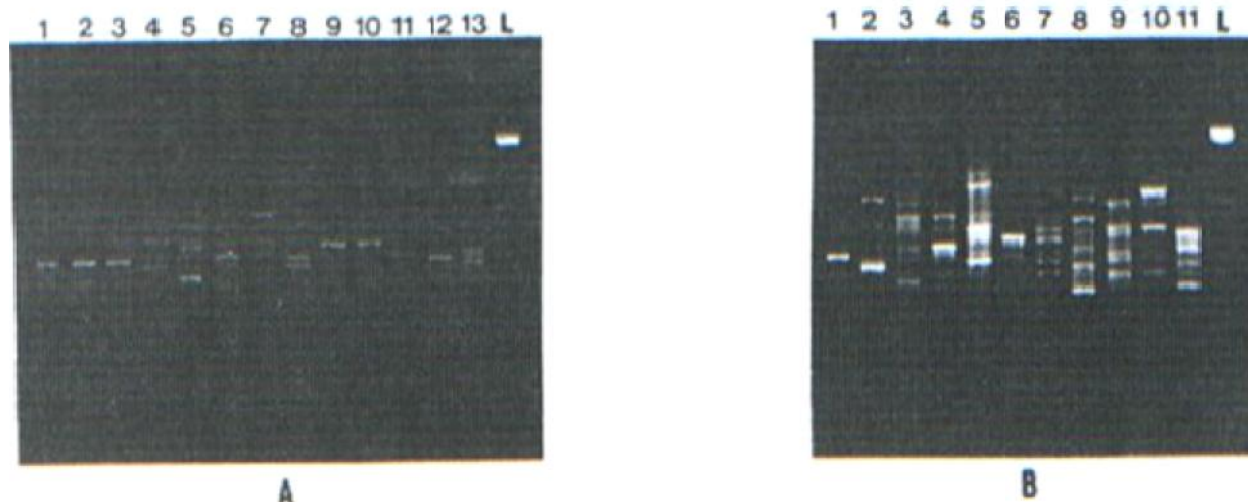


Fig. 3. UP PCR banding patterns of reference mycobacterial strains grouped according to Runyon classification. DNA samples were amplified using optimized procedure. (A) Lines: (1), *M. tuberculosis* H37Rv 27294; (2), *M. bovis* 19210; (3), *M. bovis* BCG 35734; (4), *M. kansasii* 12478; (5), *M. asiaticum* 25276; (6), *M. scroffilaceum* 19981; (7), *M. szulgai*; 35799; (8), *M. gordonae* 14470; (9), *M. avium* 25291; (10), *M. intracellulare* 13950; (11), *M. gastri* 15754; (12), *M. shimoidei* 27962; (13), *M. triviale* 23292; (B) Lines: (1), *M. tuberculosis* H37Ra 25177; (2), *M. fortuitum* 231; (3), *M. chelonae chelonae* 1544; (4), *M. chelonae abscessus* 19977; (5), *M. phlei* 11758; (6), *M. vaccae* 1526; (7), *M. chitae* 10485; (8), *M. senegalense* 806; (9), *M. neoaurum* 25795; (10), *M. fallax* 35219; (11), *M. thermoresistibile* 19527; (L) marker (100 by DNA ladder).

When NTM isolates were analyzed, alignment of DNA profiles obtained from *M. fortuitum* and *M. kansasii*, most frequent NTM causing mycobacteriosis in the region, to corresponded profiles from the reference lane library showed good correlation between them. Differences in the intensity of the bands and appearance of few bands in some of UP-PCR products did not interfere with characteristic patterns (Fig. 5). All other NTM isolates examined also produced patterns resembling corresponded reference strains in their main characteristic bands.



Fig 4. Comparative UP-PCR analysis of *M. tuberculosis* isolates from different provinces of Iran. UP-PCR products from mycobacterial DNA were compared on agarose gel, and processed with image analysis system. Lanes: (1), *M. tuberculosis* 15GD; (2), *M. tuberculosis* 11 GD; (3), *M. tuberculosis* 12GD; (4), *M. tuberculosis* 91; (5), *M. tuberculosis* 93; (6), *M. tuberculosis* 87; (7), *M. tuberculosis* 304; (8), *M. tuberculosis* 317; (9), *M. tuberculosis* 232; (10) *M. tuberculosis* 23; (11), *M. bovis* 12; (12), *M. bovis* 19; (13) *M. tuberculosis* H37Rv 27294.

DISCUSSION

The methodology of using single oligonucleotide primer to generate specific pattern of PCR product from genomic DNA has been in practice under a number of terms (RAPD, AP-PCR, DNA amplification fingerprinting, UP-PCR) for several years [9-12, 14, 15]. It has been applied for various purposes, especially for epidemiological investigations [18]. In general, the methods derived are capable of discriminating species, sub-species and strains.

Their resolving ability greatly depends on the sequence of chosen primer and conditions of amplification. Relatively short primer and "mild" thermal conditions for annealing to template in RAPD and AP-PCR result in variable number of genomic sites to which primer is targeted. Despite the apparent ease of these methods, reproducibility of DNA pat-

terns can be problematic in view of the number of variables to reproduce.

UP-PCR is the special modification of the methodology differing in important features, namely, specificity and length of oligonucleotide primer and annealing temperature. The discriminatory ability of UP-PCR is mainly based on the "core" nucleotide sequences located in 3'-part and central region of universal primer. Previously, nonrandom oligonucleotide 3-1 (universal primer M-1) was found to be efficient for distinguishing within mycobacteria species and their discrimination from other bacteria [15]. The primer showed no homology to conservative loci of genomes priming DNA synthesis from variable sites to which it was matched.

The peculiarities of UP-PCR place a wide tolerance on the conditions necessary for DNA patterns to be reproducible. In this study, we improved the assay by the introduction of a modified conditions under which profiles were not affected by changing annealing temperature and time and $MgCl_2$ concentration over wide range of the parameters. Furthermore, the feasible area of primer and template concentration where it did not interfere with receiving characteristic profiles was extended. These results demonstrated the advantages which UP-PCR offered over RAPD that required a narrower range of the variables.

Then, it was shown that UP-PCR patterns of mycobacterial species clearly differed from each other with exception for *M. tuberculosis* and *M. bovis*. Interestingly, the characteristic bands of these two species coincided, supporting a close relatedness between them at the genome level. Additional molecular techniques may unravel genomic base for specificity of UP-PCR patterns. Although some minor variability among the UP-PCR patterns of tubercle bacilli has been demonstrated, it was restricted to relative intensity and appearance of minor bands. However, the difference between DNA banding patterns of *M. tuberculosis* complex isolates and other mycobacteria tested was importantly distinctive.

When the methodology was further attempted to available NTM isolates, each of them had shown species characteristic UP-PCR pattern unequivocally distinguishable from that of other mycobacteria. There was no any important mismatching between profiles of isolate and corresponded reference strain. Thus, UP-PCR profiling could potentially allow an identification of mycobacteria and earlier confirmation of diagnosis. The very similar UP-PCR patterns found for *M. tuberculosis* isolates

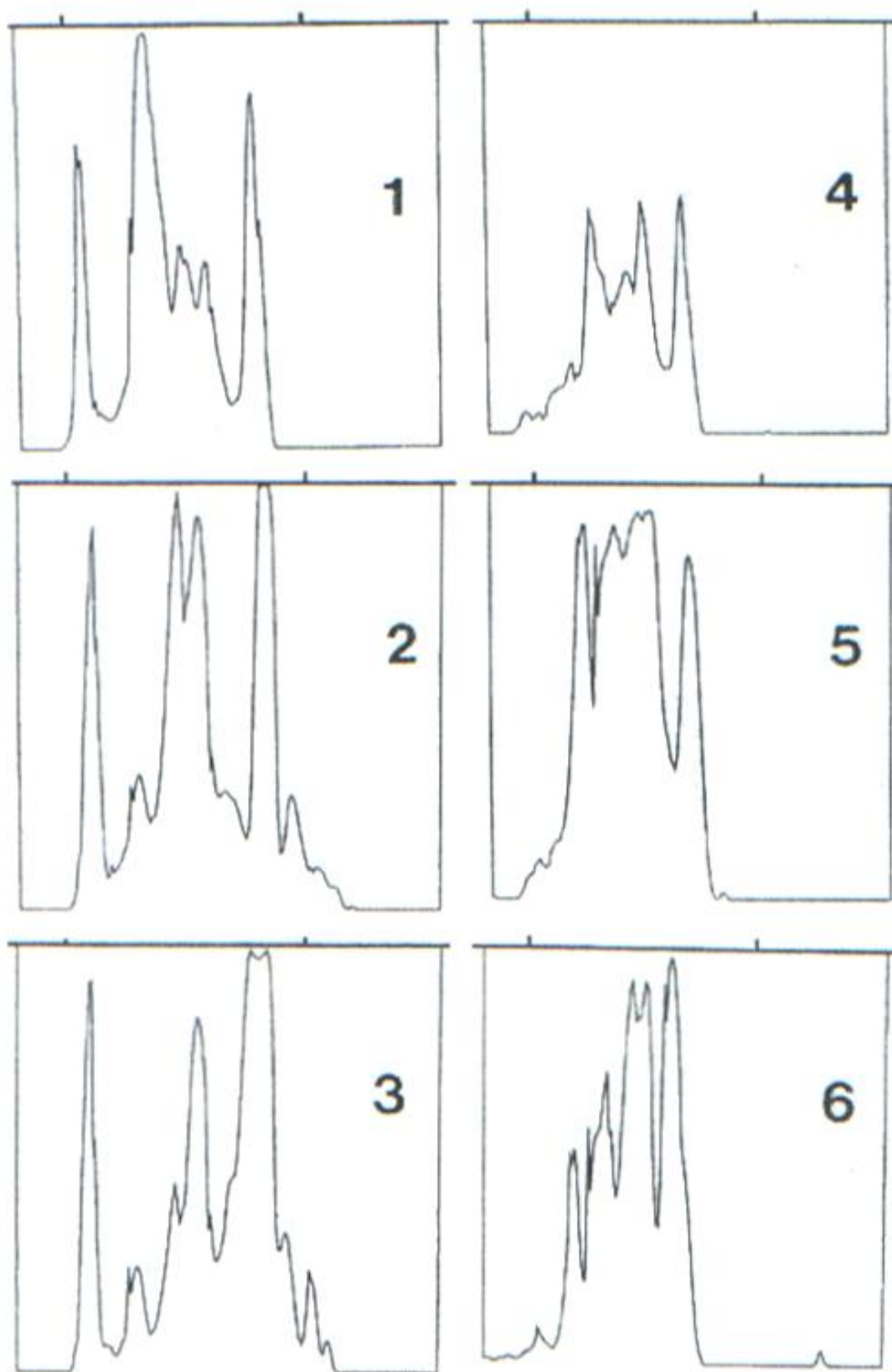


Fig. 5. Comparative UP-PCR profiling of NTM isolates. UP-PCR banding patterns from mycobacterial DNA were profiled and compared by using image analysis system. Profiles: (1), *M. fortuitum* SN231; (2), *M. fortuitum* 245; (3), *M. fortuitum* 372; (4), *M. kansasii* 12478; (5), *M. kansasii* 129; (6), *M. kansasii* 130.

from different geographical regions of Iran suggests that this assay may be complementary to conventional methods for discrimination of mycobacterial species. This feature of UP-PCR points to an application of the assay for diagnosis of tuberculosis, particularly when culture method fails to do it. The library of DNA profiles can be applied to identify those patterns from samples of unknown origin that closely match *M. tuberculosis* reference profile.

There were many attempts to apply PCR technique for discriminating tuberculosis and mycobacteriosis [19-21]. The proposed UP-PCR assay combines the main advantages of conventional PCR and PCR "fingerprinting", namely, the species-specific DNA product and one universal primer applicable to many species. It appears to be a potential test for proving the identity of *M. tuberculosis* complex and can give an efficient means for discrimination between mycobacteria.

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