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Research Article

Defining Genetic Heterogeneity of *Mycobacterium tuberculosis* Isolates by Fingerprinting Technique

Golnar Sami Kashkooli*

Referral Mycobacteriology Lab of Shiraz, Medical University of Shiraz, Iran

***Corresponding Author:** Golnar Sami Kashkooli, Referral Mycobacteriology Lab of Shiraz, Medical University of Shiraz, Iran.

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Abstract

Application of molecular methods in order to identification and isolation of *Mycobacterium tuberculosis* complex from Non tuberculosis Mycobacteria is useful for appropriate treatment of tubercular patients and interruption of transmission chain in endemic areas. This study aimed to assess the PCR-RFLP method for simultaneously identification and isolation of *Mycobacterium tuberculosis* complex of Non tuberculosis *Mycobacteria* and typing of *M. tuberculosis* strains by PCR-based fingerprinting methods.

Methods: This cross - sectional, descriptive study of 120 mycobacterial strains isolated from the Lowenstein – Jenson Media, referring to patients from Fars province and neighboring provinces was conducted in 1391. After differential biochemical tests, PCR-RFLP on *hsp65* gene was performed. *Mycobacterium tuberculosis* strains then subjected to fingerprinting techniques (RAPD, ERIC, GTG5) to find heterogeneity of them.

Results: Of the 120 cases reviewed, 112 cases (93/44%) were *M. tuberculosis* from aspect of yielded pattern of generated bands on polyacrylamide gel and 8 cases (6/66%) belonged to the group of Nontuberculous mycobacteria (3 M. *chelonae* and 5 M. *gordonae*). By usage of fingerprinting techniques, different bands and profiles polymorphism among strains were obtained. RAPD method using primer INS-2, revealed the highest degree of genetic diversity among the three fingerprinting techniques.

Conclusion: The results showed that the RFLP-PCR, is quick, easy method with high sensitivity and specificity which generate unique patterns in Mycobacterium species. Detection of species leads to proper decision for treatment between typing methods, (RAPD) showed more diversity. Therefore utilization of this method with other molecular typing methods is recommended in order to identification and clustering of strains for epidemiological survey.

Keywords: *Mycobacterium tuberculosis* Complex; PCR RFLP; Molecular Fingerprinting

Introduction

Tuberculosis (TB) is a bacterial infection disease cause by acid fast bacilli—Mycobacterium tuberculosis (MTB). This respiratory disease is one of the major treat for public health in the 21st century according to the last World Health Organization report [1]. Based on this report, annually about 9.2 million new cases are added to the TB population. In this report and many other studies in this field it has been clearly mentioned that the most cases of TB and related deaths are occurred in developed countries [1-3]. Due to this high prevalence of TB the main keys control for this disease beside contact tracing for further transmission are include proper case finding, rapid diagnosis, initiation of immediate effective therapy.

One of the major challenges in tuberculosis treatment and control failure is increasing drug resistance in the prevalent strains of MTB [4]. Knowing the resistant profile of prevalent Mycobacterium tuberculosis will help to improve the policy of related disease treatment in the community. Nowadays there are many methods in defining the disease and the sensitive profile of the infectious agent. Recent developments in molecular and genetic technology have led to quick detection and characterization of mycobacterial DNA by nucleic acid amplification [3,5].

Based on that MTB is a slow growth bacterial agent then in case of understanding the dynamics of TB epidemiology in short time, DNA fingerprinting is a time and cost effective method [6,7]. These methods of identification are an important ways of typing in case of tracing, distinguishing between relapse and reinfection by exogenous or endogenous strains and community outbreaks. Furthermore these methods can be applied to detect and confirm the recent TB transmission in a linked population or lab cross contamination [7,8].

Today's several molecular approaches such as restriction fragment length polymorphism (RFLP), direct repeat (DR), the GC rich repetitive sequence (PGRS), variable numbers of tandem repeat loci (VNTR) and single-nucleotide polymorphism (SNP) have been developed for mycobacterial genes fingerprinting [7,9]. Random amplification of polymorphic DNA (RAPD) is another method of genotyping can be assed more simply, quickly and inexpensively in pathogenic bacteria evaluation [10]. This recent method also known as arbitrarily primed PCR, allows the detection of polymorphisms without prior knowledge of the nucleotide sequence. The polymorphisms may be used as genetic markers and the construction of genetic maps [2,11].

One of the methods which nowadays used in genotyping of Gram negative bacteria is ERIC-PCR. ERIC is an enterobacterial repetitive intergenic consensus (ERIC) sequences which are repetitive elements of 126bp and seems to be restricted to transcribed regions of the chromosome [6,11,12]. According to that the position of these sequences are different in different species then these sequences are a good candidates for typing to such pathogens [12]. In some reports it has been mentioned that ERIC sequences can be used to differentiate different strains and either related clones of MTB with identical IS6110 fingerprints [6,11,13].

Another suggested and accurate tool can be used in epidemiological study of NTM is (GTG)5 test which a simple oligonucleotide repeat is evaluated [14]. According to the important of typing methods in microbiological outbreak investigations in this study we highlight common available molecular techniques in genotyping of Mycobacterium isolates from patients referred to Shiraz TB center, Fars, Iran.

Materials and Methods

Mycobacterial isolates and strains

From January 2013 to June 2014, a total of 108 Mycobacterium tuberculosis strains were isolated as monocultures from single patients. Isolates were taken from sputum samples collected from patients with pulmonary TB. Tuberculosis criteria were confirmed before in patients with clinical symptoms by TB center clinicians. Mycobacterium tuberculosis strains were isolated from Lowenstein-Jensen media after about 6 to 8 weeks incubation and confirmed by phenotypic and molecular methods (IS6110 detection) [15]. According to the study purpose to find the heterogeneity of Iranian prevalent strains of Mycobacterium tuberculosis, we aimed to perform random amplification of polymorphic DNA (RAPD), enterobacterial repetitive intergenic sequence-based polymerase chain reaction (ERIC-PCR) and GTG5 PCR analysis as three less complicated molecular typing methods.

Genomic DNA extraction

Chromosomal DNA of isolates was extracted from suspension of harvested colonies in normal saline. DNA extraction was performed with QIAamp DNA mini kit (QIAgen, Inc., Valencia, California, and USA) according to the manufacturer's instruction. The extracted DNAs were stored at -70ºC. Furthermore to find the integrity of extracted DNA five microliters of each DNA was analyzed by electrophoresis.

hsp65 amplification and restriction analysis

For differential identification of *M. tuberculosis* complex from NTM strains *hsp65* fragments were analyzed by Restriction Fragment Length Polymorphism (RFLP) method. *hsp65* is a gene that have a single copy in *Mycobacterium tuberculosis* genome which is not easily transferred from one bacterium to another and can be used in phylogenic approaches [16].

In this step the partial DNA sequence of hsp65 with about 439 bp was amplified using primers Tb11(5/-ACCAACGATGGTGTGTCCAT-3/), and Tb12(5/-CTTGTCGAACCGCATACCCT-3/) as previously described [7].

Specific amplified fragments were digested separately with restriction enzymes *Bst EII* and *Hae III* (Biolabs, New England) according to the enzyme instruction. Digested fragments were separated by electrophoresis in 8% polyacrylamide gel. Fifty–base pair DNA ladder (Invitrogen-USA) was used as molecular ladder standard.

Typing of strains

The extracted DNA from confirmed isolates were subjected to fingerprinting techniques (RAPD, ERIC, GTG5) to find heterogeneity of them.

RAPD PCR: the PCR amplifications for RAPD fingerprinting were carried out with 3 arbitary primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1525R (5´-AAGGAGGTGATCCAGCC-3´), 3') and INS-2 (5´-GCGTAGGCGTCGGTGACAAA-3´) in a low-stringency PCR amplification.

ERIC and GTG5analysis: Enterobacterial repetitive intergenic consensus PCR was performed with primers ERIC1R-5V ATGTAAGCTCCTGGGGATTCAC and ERIC2-5V AAGTAAGTGACTGGGGTGAGCG. Reactions were performed in a 50 ml volume in Eppendorf thermo cycler (Gene Amp PCR System 2,400). Reaction mixtures contained 1 mM of each primer, 50 - 100 ng of genomic DNA, 1.5 mM MgCl2; each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP; Promega, USA) at a concentration of 0.2 mM, and 1 U of Taq DNA polymerase (Promega, USA). amplifications were made with

1 cycle at 95ºC for 5 min, 30 cycles at 90°C for 30 s, 50°C for 30 s, 52°C for 1 min, and 1 cycle at 72°C for 8 min.

DNA samples were subjected to rep-PCR using the (GTG)5 primer (5′-GTGGTGGTGGTGGTG-3′) (gtg2013). The PCR assays were performed in a 50-µl reaction volume containing 10 ng of genomic DNA, one unit of Taq DNA polymerase (Roche, Mannheim, Germany), 0.8 mM of the (GTG)5 primer (Sigma-Aldrich, St. Louis, MO), 1.5 mM $MgCl_2$ and 200 µM of each deoxynucleotide triphosphate (Roche, Mannheim, Germany). PCR cycling conditions consisted of initial activation at 94°C for five min, followed by 35 -cycles of a three-step PCR program (94°C for 45 seconds,40°C for one min. and 65°C for 10 min.) and a final extension at 65°C for 20 min.

The PCR products were separated by electrophoresis on 1.5% agarose gel and photographed on a Uvidoc gel documentation system. The 100bp DNA Ladder (Promega) was used as molecular size marker. DNA fragment sizes in the RAPD, ERIC and GTG5 patterns were calculated using DNA frag 3.03 package (Nash, 1991). The NTSYS-pc software ver. 2.02 is used to estimate genetic similarities with the Jaccard's coefficient. The matrix of generated similarities is analyzed by the unweighted pair group method with arithmetic average (UPGMA.

The index of similarity (Fxy) between samples was calculated using the formula (Nei and Li, 1979).
Fxy= 2nxy/nx+ny.

where nxy is the number of RAPD bands shared by the two samples and nx and ny are the number of RAPD bands scored in each sample. The genetic distance (d) was calculated using the equation d. 1ÿFxy. given by Hillis and Moritz (1990).

Results

Neasted PCR for all 120 isolates confirmed them as Mycobacterium species as 439 bp fragmant was present in all strains.

Figure a

Electrophoresis of Neasted-PCR products: 439 bp fragments on agarose gel. 1-ladder, 2-8-Mycobacterium species, 9- H37RV (standard species), 10-NC.

Enzyme digestion results with *BstEII* revealed 85.120.235 fragments and with *Hae III* showed 0.70.130.150 fragments for 112 isolates which have been detected as MTB through conventional methods. It was compatible with *Mycobacterium tuberculosis* complex pattern on Mycobacteriom identification site(app.chuv.ch/parasite/index.html).

8 isolates which has been detected as NTM with phenotypic methods after enzyme digestion method were detected as 3 *Mycobacterium chelonae* and 5 *M. gordonae*.

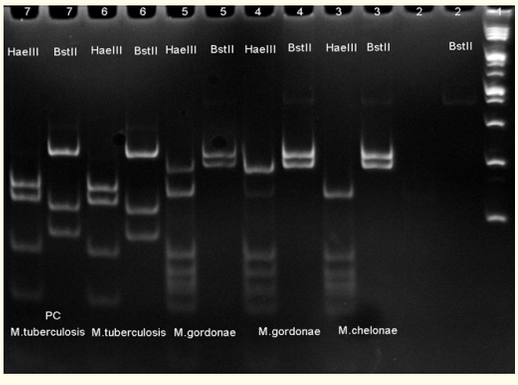

Figure b

Enzyme digested patterns with BsteII and HaeIII: 3-M. chelonae, 4 and 5-M. gordonae, 6- M. tuberculosis, 7- H37RV.

Typing results

Application of RAPD method on 100 isolates which were detected as M. TB By the use of 3 primers revealed different bands (table 1).

Total of producted bands	primer	row
17	INS-2	1
16	1525R	2
10	27F	3

Table 1: Total of produced bands for RAPD primers.

Results of RAPD with INS-2 primer: 17 different bands were obtained. The resulting band size was measured from 400 bp to 3000 bp. 700,1000,1500 bp bands were identified as common to most strains and were considered as main bands. The rest of the bands were considered as sub- bands.

Figure c

Electrophoresis of RAPD-PCR with primer INS-2: 1-Ladder100 bp, 2- 16: M. TB strains, 17- PC, 18-NC.

The RAPD analysis performed with DNA from the Iranian M. tuberculosis isolates, and primers27F, 1525R was found to generate less informative banding patterns. However, the RAPD analysis using primer INS-2 generated altogether 25 different patterns for the isolates studied here. These profiles designated RAPD types 1 to 25 (Figure 1).

Figure 1

- Cluster analysis with UPGMA method and using NTSYS-PC software by primer INS-2.
- The clusters revealed that the diversity of strains belonging to cities seen in most groups.
- Number of clusters that contained only unique strain were seen.

Calculate the genetic distance between strains for instance:

a15 and a37

F_{xy}= 2*3/11=0.54

d=1-0.54=0.46

If genetic distance (d) is closer to zero, the two strains are genetically more closely and place in closer clusters. If reach zero strains are in same cluster.

RAPD result with 27F primer: 10 different bands were obtained. The resulting band size was measured from 400 bp to 3000 bp., 1000, 3000 bp bands were identified as common to most strains and were considered as main bands. The rest of the bands were considered as sub- bands.

Figure d

Electrophoresis of RAPD-PCR with primer 27F: M- Ladder100, 1-12-M. TB isolates, PC, NC.

Figure e

Cluster analysis with UPGMA method and using NTSYS-PC software by primer 27F

17 clusters were obtained and the largest cluster with 24 strains revealed diversity in jeographical area.

RAPD results with primer 1525R: 16 different bands were obtained. The resulting band size was measured from 300 bp to 3000 bp.,1000,2500,3000 bp bands were identified as common to most strains and were considered as main bands. The rest of the bands were considered as sub- bands.

Figure f

Elecelectrophoresis of RAPD-PCR with primer 1525R: M- Ladder100 bp, 1-16-M. TB isolates, PC, NC

Figure g

Cluster analysis with UPGMA method and using NTSYS-PC software by primer 1525R.

By using of 1525R primer isolates were classified into 15 different groups.the largest group contain 21 isolates which revealed diversity in geographical location.

Results of ERIC PCR for 100 M. TB strains were 11 different bands between 500 to 3000 base pair. Common bands were 1100 and 1500 base pair.

Figure h

Electrophoresis of ERIC PCR: M-100 bp ladder,1-11-M. TB strains, PC

In the resulted clusters we found diversity of strains which belonged to different locations. This method didn't show high degree of polymorphism in the resulted patterns.

Rsults of GTG5: 7 different bands were obtained. The resulting band size was measured from 1000 bp to 3200 bp. 1000,1500 bp bands were identified as common to most strains and were considered as main bands.

Figure i

Electrophoresis of GTG5 PCR: M-100 bp ladder,1-17-M. TB strains, PC, NC.

By using of GTG5primer isolates were classified into 9 different groups.

We couldn't draw dendrogram for resulting profiles in ERIC and GTG5 method because 11 and 2 strains didn't reveal any detectable pattern respectively by ERIC and GTG5 PCR.

Figure j

Combined dendrogram for 3 RAPD primers: Reaveled 40 RAPD groups and more diversity in compare to each primer.

Largest cluster with 10 strains and 24 unique cluster with single strain resulted.

By analyzing of all three methods results together for 100 strains in this survey we got 64 clusters which showed direct correlation between number of yielded clusters and number of fingerprinting methods and it gives groups with genetically similar strains.

Figure k

Dendrogram with 3 typing method

PIC calculating

primer	PIC
INS-2	0.88
1525R	0.81
27F	0.87
ERIC	0.82
GTG_5	0.83
3 RAPD primers	0.93
All methods Primers	0.95

Table 2

Among RAPD primers INS-2 revealed the highest level of polymorphism and 1525R showed the least level of polymorphism and compared to the overall. Simultaneous use of all three methods, revealed the greatest amount of overall typing polymorphism.

Discussion

The three molecular methods (RAPD, ERIC and GTG5) used in this study enabled typing of independent and related *M. tuberculosis*. This is the first effort to gain epidemiological information concerning the *M. tuberculosis* isolates within our geographic are. The molecular typing of the isolated strains was successfully achieved with all three molecular methods, especially RAPD.100, independent isolates showed considerable polymorphism, with the three methods. Among the three typing methods, RAPD analysis showed the highest degree of polymorphism. Generally there was good correlation between the groups identified by the three typing techniques, but we found less diversity in studied strains by ERIC and GTG5 methods by the 13 and 9 distinct profile respectively and some of strains with these two typing methods were non-typable and revealed any distinct profiles.

In our study RAPD showed more polymorphism in *M. tuberculosis* strains. Molecular typing by these fingerprinting methods is an attractive option in clinical microbiology. The method is applicable to any organism containing DNA, it is more rapid and less technically demanding than most other molecular typing methods, and no DNA sequence information is necessary [11,13,14].

Random amplified polymorphic DNA analysis is a less specific method of producing DNA "fingerprints". This technique employs the polymerase chain reaction (PCR) to amplify DNA fragments but instead of the usual pair of primers directed at a specific target a single randomly selected primer is used.it binds at multiple sites along the genome at the low annealing temperatures used; products are produced between primers binding in close proximity to opposite DNA strands. Compared with other recently described molecular typing methods, RAPD is more rapid and less technically demanding to perform. RAPD has been used successfully to type a variety of different bacteria but its potential for typing *M tuberculosis* has only recently been reported. RAPD can be performed rapidly on primary isolates of *M tuberculosis* and is a technique well suited for preliminary epidemiological studies of strains [17].

It is noteworthy that the reproducibility of RAPD bands was affected by DNA purity. Before purification of the DNA, we observed slight variation in banding pattern for the same MTB strain that came from different DNA extraction methods, but after we purified the DNA, from either extraction method and ran RAPD again, we obtained an identical and reproducible banding pattern. We suggest that purifcation of DNA is needed, and that using an inexpensive method such as phenol chloroform extraction is sufficient.

In summary, our results demonstrate that RAPD analysis is a reproducible method for identifying MTB strains and RAPD is simple, cheap and fast and also that large numbers of samples can easily be handled. along with further study of large scale MTB typing, using more extensive primer screening and comparing results with other typing techniques is recommended [18].

For *M. tuberculosis*, we found that fingerprints generated by using more than one primer (separately) were necessary to render the method sufficiently differential. The methods outlined readily differentiated geographically distinct isolates. Further work will involve trying new primers for RAPD with the aim of finding more discriminatory primers for *M. tuberculosis.* Five or six primers could

routinely be used, and the RAPD profiles generated with all of these primers for each strain of *M. tuberculosis* under investigation could be compared, increasing the discriminatory power of the results.

This procedure yields greater polymorphism, is technically simpler and faster, and requires no radioactive tracers. It has been reported that the degree of polymorphism obtained by RAPD is almost the same as that obtained by RFLP [11].

In addition, a smaller amount of purified DNA (<50ng) is required than for methods such as RFLP. Although RAPD is relatively simple and useful for epidemiological analysis, standardization of the PCR conditions is very important for reproducibility (12). For instance in our experiment it was necessary to standardize the amount of DNA in each RAPD reaction mixture (50 ng of DNA) to ensure that non-specific bands were not present. And last but not least, even with this standardization, reproducible patterns were difficult to acquire, and duplicate analyses were unavoidable for the true profile differences to be evaluated.

In the current study the RAPD analysis based on the primer INS-2, resulted in 25 RAPD types for 100isolates. We classified M. tuberculosis strains as either clustered (containing ≥3 members), small group (2 members) or unique. Of 100 M. tuberculosis isolates, 68 (70.8%) grouped into clusters, the largest cluster involved 14 strains which showed diversity in geographical locations. 12 (12.5%) distributed into small groups and 16 (16.7%) had unique RAPD profiles. Thus, we might infer that the cases of tuberculosis with identical RAPD fingerprints are suspected to be due to epidemiologically related strains while the cases with unique RAPD finger prints are likely due to remote infection or recurrent tuberculosis.

Survey of combined dendrogram of three RAPD primers resulted 40 group with high polymorphism compare to each primer dendrogram and 24 unique groups with single strain were identified. That revealed very close correlation between strains and geographical area.

Final dendrogram of combining results of all three typing methods showed 64 group with higher polymorphism and strains with very close genetic were in same group. It demonstrate that using of more typing methods together will lead to reliable fingerprints and better understanding of molecular epidemiology of tuberculosis.

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