Original Article

Detection of *Mycobacterium tuberculosis complex* by *gyrB* PCR in patients with clinical suspicious of tuberculosis in Mazandaran, Iran

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Abstract

Introduction: *Mycobacterium tuberculosis* complex (MTBC) are causative agents of human and animal tuberculosis. Differentiation of MTBC members is required for appropriate treatment of individual patients and for epidemiological purposes and reduction in drug resistances.

Material & Methods: 1345 patients were collected with clinical suspicions of tuberculosis who referred to the Health Care Center of Mazandaran province from July 2010 to June 2011. The specimens were stained by the Ziehl-Neelsen staining technique and were cultured on Lowenstein-Jensen medium to detect the mycobacteria. For recognition of *Mycobacterium tuberculosis* complex MTUB-f and MTUB-r primer (gyrB-PCR1) were used. For differentiation of *Mycobacterium tuberculosis* complex members MTUB-756-Gf and MTUB- 1450Cr (gyrB-PCR2) and RFLP PCR using *RsaI* restriction enzymewere used.

Results: Of 1345 specimens, only 65(4.83%) isolates were positive culture of which59 (90.76%) were MTBC and 6 (9.24%) identified as Mycobacteria other than tuberculosis. All of 59 isolates were *M. tuberculosis*.

Conclusion: The gyrB-RFLP PCR and using the *RsaI* restriction enzyme is a rapid and easy technique to perform for differentiation of the member of *M. tuberculosis* complex.

Key words: gyrB- RFLP PCR- RsaI - Mycobacterium tuberculosis complex

Introduction

The genus mycobacterium includes 70 species, that causing serious human and animal disease and is considered as a major source of morbidity and mortality worldwide(1, 2). It is estimated that one-third of the world´s population is currently infected with the tuberculosis bacillus in their lungs (3) 8-12 million new cases are adds every year (4, 5). The mycobacterial species that occur in humans and belong to the *Mycobacterium tuberculosis* complex include *M. Tuberculosis, M. bovis, M. microti*, *M. canetti* and *M. africanum.*

Identification of a clinical isolate is primarily based on conventional methods such as culture characteristics, acid fast staining, as well as biochemical tests (6). These methods take about 4 to 8 weeks to identify the species of mycobacterium and often are inconclusive (7, 8). Various PCR-mediated methods have been developed for the rapid detection and differentiation of mycobacterial species.One of these methods is PCR amplification of a fragment of *gyrB* gene followed by restriction digest of the PCR products. This method is proved to be useful not only for mycobacterium detection directly from mycobacterial DNA in clinical samples but also for its species identification(1, 9, 10, 11, 12). It is a rapid, easy to perform and cost effective method that does not involve radioisotopes (7, 10, 13, 14). The aim of this study is to differentiate between pathogenic mycobacteria with specific concern on *gyrB* RFLP PCR in patients who referred to the Health Care Center in Mazandaran province. By the help of this study we can determine the prevalence rate of pathogenic mycobacteria by identification of their species in order to provide less danger during therapy and also to reduce drug resistance and decrease economic losses in families and society.

Material and methods

Patients & clinical specimens

1345 samples were collected from patients with clinical suspicion of tuberculosis who were referred to Health Care Center of Mazandaran Province from July 2010 to June 2011. The samples comprised 1222 (90.8%) sputa, 12 (0.9%) urine, 15 (1.11%) pleural effusion, 1 (0.07%) aspirate cerebrospinal fluid (CSF), 8 (0.6%) asit fluid, 2 (0.14%) blood samples, 63 (4.68%) bronchial washes, 14 (1.04%) gastric washes, 1 (0.07%) skin biopsy, 1(0.07%) abscesses, 1 (0.07%) wound, 2 (0.14%) liver cystic fluids and 3 (0.22%) tissue.

Pre-processing of Samples

Samples were treated with 4% NaOH and 4% HCl, both for liquefaction and decontamination, followed by centrifugation at 12, 000×g for 15 min to sediment bacterial cells.

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Staining and culture of samples

Smears were prepared from all samples and stained by Ziehl-Nelssen method (carbon fuchsine for acid fast technique). The smears were then examined under oil immersion objective. The samples were then cultured in Lj. medium and the growth rate, pigment production by colonies, colony morphology, nitrate reduction and niacin were determined.

DNA extraction and amplification of the gyrB gene

For direct PCR, DNA was extracted from the samples. Clinical samples were incubated with 20 µl of 10 mg/ml proteinase K, overnight at 37 °C and then heated at 90 °C for 10 min. DNA of mycobacteria was extracted using DNG Plus DNA extraction kits (Cinnagen; Tehran, Iran). DNA concentration was determined by spectrophotometer.

2.4.1. gyrB-based species specific PCR for Mycobacterium tuberculosis complex:

The primers MTUB-F (5´-TCGGACGCGTATGCGATA TC-3´) and MTUB-R (5´-ACATACAGTTCGGACTT GCG-3´) (CinnaGen Co., Iran) were used. The 20 µl PCR mixture contained 12.5 µl of PCR Master Mix (2x) (Fermentas, Canada, #K0171), 1.3 µl of each primer, 2 µl of template DNA and 7.9 µl of nuclease free water. PCR amplification was performed in an automated PROGENE thermal cycler by the protocol described by Niemann *et al* (15) except the first extension for 10 min. A positive control containing chromosomal DNA of *M. tuberculosis* H37Rv (National Reference Laboratory) and a negative control without template DNA were included in each run. The amplified fragments were analyzed by 1% agarose gel electrophoresis at a constant voltage of 95v for 40min. The target DNA for amplification was 1, 020 bp fragment of the gyrB gene, which was used to identify members of the target DNA for amplification was 1, 020 bp fragment of the gyrB gene, which was used to identify members of the *M. tuberculosis* complex.

2.4.2. gyrB-based species specific PCR for Mycobacterium tuberculosis:

The primers MTUB-756-Gf (5´-GAAGACGGGGTCA ACGGTG-3´) and MTUB-1450-Cr (5´-CCTTGTTCAC AACGACTTTCGC-3´) (CinnaGen Co., Iran) were used. The 20 µl PCR mixture contained 12.5 µl of PCR Master Mix (2x) (Fermentas, Canada, #K0171), 1.3 µl of each primer, 2 µl of template DNA and 7.9 µl of nuclease free water. The thermal cycling profiles were as follows: 5 min incubation at 95°C, followed by 30 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 1 min and final step at 72°C for 10 min. *M. tuberculosis* H37Rv was used as the positive controls and sterile distilled water without PCR product was as negative control. The amplified products were analyzed by 1% agarose gel electrophoresis at a constant voltage discharge mode (95 V) for 40 min. The target DNA for amplification was 734 bp.

2.4.3. Restriction Fragment Length Polymorphism:

The DNA polymorphism was analyzed by restriction with *RsaI* as indicated by the manufacture (Fermentas, Canada, #ER1121). Samples for electrophoresis were prepared by adding 2 µl of the restriction enzyme, 2 µl of 10X Buffer Tango, 18 µl nuclease free water and 10 µl PCR reaction mixtures. The mixture was mixed gently and spun down for a few seconds, then incubated at 37°C for 16 hours. The mixture was separated in 2% agarose gel by electrophoresis at a constant voltage discharge mode (100 V) for 30 min.

Statistical Analysis

The $\chi 2$ and Fisher exact tests were used to compare the frequency of discrete variables. Mean, Central tendency and Dispersion were used for analyzing data by SPSS18.

Results

Of the 1345 samples of patients with clinical suspicion of tuberculosis referred to Health Care Center of Mazandaran, 65 (4.83%) were positive culture and 57 (4.23%) were acid-fast bacillus (AFB) positive (Table1). positive samples were from patients in age range between 10 to 80 years old, of which 24 (≈37%) and 41 (≈63%) samples were male and female, respectively. The average of age of patients was 45.5±17.93. The minimum of age was 15 years old and the maximum of age was 79 years old. The prevalence of tuberculosis was 3-5% with confidence interval 95% (CI95%).

Based on *gyrB* gene region amplification, 65 (4.83%) clinical isolates were identified as *Mycobacterium tuberculosis* complex (Figure 1).

Table 1: Results of positive culture and smear according to sex and age obtained from patients of tuberculosis

Range of age	Positive culture				Positive smear			
	Male		Female		Male		Female	
	No	Per (%)	No	Per (%)	No	Per (%)	No	Per (%)
10-19	3	4.61	2	3.07	3	5.2	2	3.5
20-29	3	4.61	8	12.3	3	5.2	7	12.28
30-39	3	4.61	5	7.7	2	3.5	5	8.77
40-49	4	6.15	13	20	4	7.01	11	19.3
50-59	2	3.07	8	12.3	2	3.5	7	12.28
60-69	2	3.07	3	4.61	1	1.75	3	5.2
70-79	7	10.76	2	3.07	5	8.77	2	3.5
Total	65 (4.83%)				57 (4.23%)			

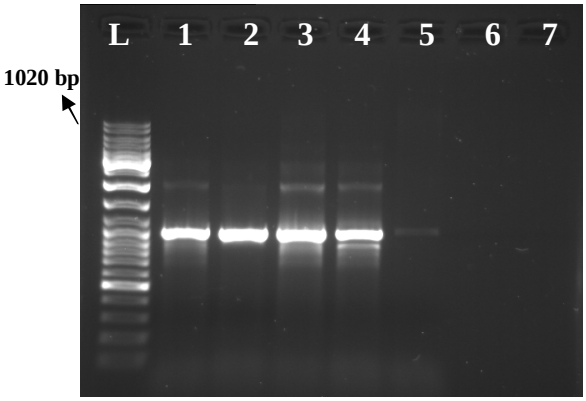

Figure 1: PCR amplified 1, 020 bp fragment of the *gyrB* gene. L: 100 bp ladder, Lane 1: positive control amplified from *Mycobacterium tuberculosis* H37Rv. Lane 2-5: DNAs from clinical isolates identified as *Mycobacterium tuberculosis* complex. Lane 6: Negative control. Lane 7: Non Mycobacterium tuberculosis complex.

The 59 MTBC isolates confirmed with *gyrB* PCR were further differentiated by species specific PCR using specific set of the primers MTUB-756-Gf and MTUB-1450-Cr that allowed selective amplification of the *gyrB* fragments from *M. tuberculosis.* All tested isolates produced a band of 734 bp, which is specific to *M. tuberculosis* (Figure 2).

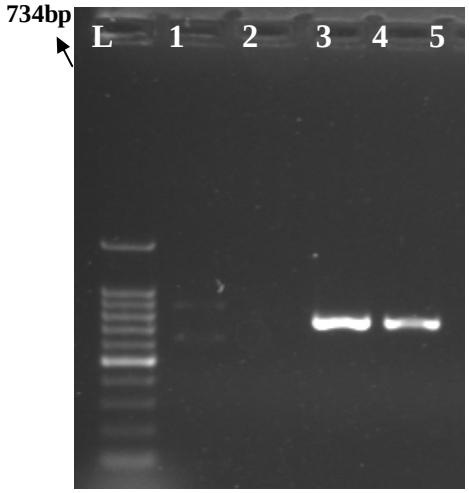

Figure 2: Analysis of PCR amplified 734 bp fragment of the *gyrB* gene from *M. tuberculosis*. L: 100 bp ladder, Lane 1-2: Negative control. 3: positive control amplified from *M. tuberculosis* H37Rv. Lane 4: clinical isolates identified as *Mycobacterium tuberculosis*.

The further confirmation of the differentiation system used, reference strains *M. tuberculosis* H37Rv as well as all of 59 *M. tuberculosis* isolates confirmed by gyrB PCR were analyzed RFLP PCR. All *M. tuberculosis* isolates showed the typical *RsaI* pattern (480 and 230 bp) compared to reference strain (Figure 3).

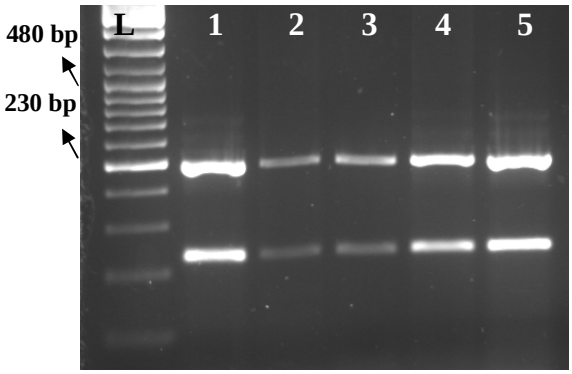

Figure 3: RFLP pattern of PCR products obtained by *RsaI* digection.
L: 100 bp ladder, Lane 1: positive control amplified from *M. tuberculosis*. Lane 2-5: *Mycobacterium tuberculosis* clinical pattern.

Discussion

Mycobacterial infection is considered as a threat to human health, worldwide. Identification and detection of mycobacterial species need rapid, reliable, specific and cost effective techniques (16, 17). Conventional biochemical tests such as AFB staining and culture are not only time consuming but also unable to differentiate *M. tuberculosis* from Non- Tuberculosis Mycobacterium in many cases(16, 18, 19). Our results as well as those obtained by some other researchers show that PCR based methods could be more reliable in this regard. In a study by Banavaliker(16) and colleagues, the sensitivity of PCR was reported to be nearly 100% in both smear and culture positive samples. In our study, the sensitivity of PCR was 100% in case of specimens determined positive by both smear and culture methods. Of 1345 clinical specimens which included sputa, urine, pleural liquid, cerebrospinal fluid, bronchial washes, asit fluid, blood, gastric washes, skin biopsy, abscesses, wound, liver cystic fluids and tissue, 65 specimens were positive for *M. tuberculosis* by culture and smear methods, which *M. tuberculosis* DNA was detected in all of them by PCR. Out of 65, 59 (90.76%) were MTBC and 6 (9.24%) were NTM[3]. All of 59 isolates were *M. tuberculosis* and showed the typical *RsaI* pattern.

PCR-restriction enzyme analysis is a rapid reliable method for differentiation between MT and NTM and identifying *Mycobacterium tuberculosis* complex members. In our study, the prevalence of MT in samples with positive PCR was 4.83%. This result is similar to that reported by Abass NA et al., 2010 (20). In their study, out of 79, 77 (97.5%) isolates were positive and identified as members of *M. tuberculosis* complex and 2 (2.6%) isolates were negative and identified as a Mycobacteria other than tuberculosis (NTM). All of 77 specimens were *M. tuberculosis*(20). In a study done by Bannalikar and Verma 2006(16), 3 (10%) out of 30 samples were shown to be *M. avium*, while others were *M. tuberculosis.* In another study (21), out of 88 clinical samples, 16 *M. tuberculosis* and 72 *M. bovis* were detected by Paraso LM et al., 2002. In a study done by Chimara E. 2004, 306 (99.6%) out of 307 samples were

None-tuberculosis [1]

shown to be *M. tuberculosis*, while only 1 samples (0.4%) was *M. bovis*(22).

The comparison between our results and those of the studies quoted above reveals that the prevalence of *M. tuberculosis* in patients referred to the Health Care Center of Mazandaran, located in north of Iran, is 100%. The prevalence of tuberculosis was 3-5% with confidence interval 95% (CI95%).

Based on the results obtained in this study and those previously published (23) PCR RFLP of the *gyrB* gene using the commonly used enzyme *RsaI*, permits one to easily separate the MTBC species from other atypical *Mycobacterium*. However, once this basic differentiation between MTBC and atypical mycobacteria is achieved, further differentiation among the MTBC member species is not feasible by PCR RFLP detection of *gyrB* variability. By early diagnosis of TB, we can help clinicians to select appropriate drugs and reduce drug resistance and MDR[4].

Conclusion

In conclusion, findings of this study suggest that PCR-RFLP is a useful method for rapid detection of Mycobacteria directly from clinical specimens, and differentiation of Mycobacterium tuberculosis from NTM. Application of this method will be helpful for early diagnosis and treatment of Mycobacteria infections. Furthermore, Our results are alarming for the local health system of the sampling area to pay more attention to the new species of Mycobacteria as a potential source of human infection.

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Multiple Drug Resistance[1]