Original Article

Comparison of polymerase chain reaction single-strand conformation polymorphism with DNA sequencing to detect drug resistance of mycobacterium tuberculosis isolates

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Abstract

Objectives: Advancements in molecular technology increased our understanding of genetic mechanism of drug resistance. Nowadays, the chance of rapid detection of resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) strains is increased. In the present study, we aimed to investigate the sensitivity and specificity of PCR-SSCP for detecting susceptible and resistant strains of *M. tuberculosis* compared with DNA sequencing.

Patients and Methods: To calculate the sensitivity and specificity of PCR-SSCP assay to detect drug resistance in *M. tuberculosis,* respiratory samples were collected from suspected patients referred to Mycobacteriology Research Center (Masieh Daneshvary Hosptial) since 2002. Susceptibility testing against first line drugs was performed on 74 culture-positive specimens. Consequently, PCR-SSCP and DNA sequencing were performed on katG, inhA, ahpC and rpoB genes.

Results: Drug-susceptibility testing by the proportional method in selected samples revealed 16 MDR (21.6%), 23 mono-drug resistant (31%) and 35 susceptible strains (47.3%). In comparison with DNA sequencing as a gold standard for molecular methods, the sensitivity of PCR-SSCP assay for detecting of mutation in 315 codon of katG gene was 94.74% (CI=73.97%-99.87%) with 100% (CI=93.51%-100%) specificity. In contrast, the sensitivity and specificity of this assay in detecting of rpoB gene were 70.8% (CI=48.91%-87.38%) and 88% (CI=75.69%-95.47%), respectively.

Conclusion: PCR-SSCP in combination with DNA sequencing can be used as screening method to detect MDR-TB and mono-drug resistant cases.

Keywords: polymerase chain reaction, Mycobacterium tuberculosis, drug resistance.

Introduction

Tuberculosis (TB) remains a major public health problem, particularly in developing countries [1]. The major factors which resulted in sustaining TB epidemics include expanding human immunodeficiency virus (HIV) infection and the spread of multidrug-resistant TB (MDR-TB), which defined as resistance to isoniazid (INH) and rifampin (RIF). In addition, recent arrival of extensively drug- resistant tuberculosis (XDR-TB) and totally or extremely drug resistant tuberculosis (TDR or XXDR-TB) has further complicated TB control program in these conditions [2, 3]. Therefore, there is an urgent need to rapidly detect MDR-TB strains which may in turns ensure effective treatment of TB patients and limit further development of resistance to additional drugs. Recent advancement in molecular biology revealed methods that open up a highly sensitive and specific for MDR-TB strains detection within short time. They have shown that mutations in katG encoded Katalase Peroxidase and rpoB encoded RNA polymerase β subunit can cause resistance to INH and RMP in *M. tuberculosis* strains, respectively [4]. Mutations in katG gene were found in 42-58% of INH resistant *M. tuberculosis* strains [5]. Although it has been demonstrated that resistance to INH is controlled by other genes such as inhA, KasA and ahpC, the inhA encoded enoyl-ACP reductase enzyme that synthesizes mycolic acids, ahpC encoded Iron-regulated alkyl hydroperoxidase and kasA encoded b-ketoacyl-ACP synthase [5-8]. On the other hand, it has been observed that 96% of RMP resistance cases possess mutations within 81 bp region of rpoB gene called rifampin resistance determine region (RRDR) [9-12].

Generally, increase knowledge about genetic and molecular aspects of drug resistance has accompanied by concomitant molecular strategies to rapidly diagnose MDR-TB. One such method is single stranded conformation polymorphism (SSCP) analysis, which involves amplification by PCR of a segment of the gene encoding for the specific drug target, in which mutations result in an altered pattern. This technique is relatively simple and was promising initially, however, recent studies have questioned its sensitivity and specificity.

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Received for Publication: October 28, 2010
Revision Received: January 14, 2011
Revision Accepted: February 7, 2011

Here, we investigated the usefulness of PCR-SSCP to detect mutation in katG, inhA, ahpC and rpoB genes. The results were compared with DNA sequencing and classical method.

Patients and Methods

Clinical isolates and susceptibility test: seventy four positive cultures from different patients were used in this study. The samples were isolated from suspected TB-resistance patients referred to National Reference TB–Laboratory (NRL) of Iran. The studied strains characterized as *M. tuberculosis* by PCR based on IS6110 (Fig 1) as previously described by Soolingen et al. [13]. Drug susceptibility test against isoniazid (INH), rifampicin (RF), streptomycin (SM) and ethambutol (ETB) were performed by the proportional method on Löwenstein-Jensen media at a concentration of 0.2, 40, 4.0 and 2.0µg/ml, respectively. Isolates were considered resistant if more than 1% of the bacteria in the inoculums grew in the presence of drugs [14].

DNA extraction: DNA was extracted by standard procedure according to a previously described method [13]. Briefly, one loop of colony growth of Lowenstein-Jensen media was suspended on TE buffer. After heat killing of bacteria in 80°C for 20 minutes (min), 50µg lysozyme was added and incubated at 37°C for at least 1 hour. After adding 10% SDS/proteinase K and incubation in 65°C for 10 min, 100µl 5M NaCl and 100µl CTAB/NaCl solution were added. The DNA was purified by chloroform/isoamyl alcohol and was precipitated by isopropanol. The pellet was washed by 70% ethanol and dried in air temperature. Then they were resuspended in TE buffer (50mM Tris, 100mM EDTA, pH 8.0) and stored in 4°C.

PCR: PCR reactions (50 µl) contained target DNA (10 ng), 20 pmol primers, 2 mM dNTP (Fermantase), 2.5 U Taq DNA polymerase (Qiagene), 2 mM MgCl2, and 5 µL 10X buffer. To amplify kat G, rpoB, inhA and ahpC genes different primers were used as described by telenti and et al [15]. The sequence of oligonucleotides is shown in table 1.

Thereafter, samples were subjected to one cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 65°C for 30 sec, 72°C for 45 sec, and one final cycle of 72°C for 5 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels made up in 0.5X Tris-Borate-EDTA (TBE) buffer and contained ethidium bromide and examined for the presence of the desirable bands (Fig 2).

SSCP: the PCR-SSCP products were analyzed by electrophoresis on 12% acrylamid gels (sigma), which was prepared according to the manufacturer's instructions. Six µl of PCR products were mixed with 4 µl loading buffer (95% formamide, 20mM EDTA, and 0.05% bromophenol blue). The mixture were denatured by heating at 95°C for 10 min and cooled immediately on ice for 5 min, and loaded on the gel at low voltage for 14-16 hours. The gel was stained by silver nitrate. Briefly, after fixing the gel by glacial acetic acid solution for 10-30 min, silver nitrate solution (1 gr AgNO3 in 1 L ultrapure water and 1.5 ml 37% formaldehyde) as stain solution was added. The gel was developed by solution contains 30 gr sodium carbonate, 1 L ultrapure water, and 1.5 ml 37% formaldehyde and 200 µl sodium thiosulfate as developing solution (Fig 3,4). The pattern of bands movement was compared with the standard H37Rv laboratory strains. All of samples, which had both similar and different patterns in comparing with H37Rv strain, were selected and sequenced for katG and rpoB genes[15].

DNA sequencing: The PCR products of five samples were randomly selected, purified and sequenced for inhA and ahpC genes by TB90 and TB92 primers. Seventy four samples were sequenced for rpoB and katG genes based on singer assay by TB86 and TR8 primers with ABI automated sequencer machine [16]. All post runs analysis was performed using Clustalw, BioEdit version 7.0.0. Each sequence was compared with the control strain sequence (H37Rv). All sequences of isolates were recorded in gene bank with accession number HM178967-HM179098.

Statistical analyses: the chi square results were analyzed by SPSS software version 19 and statsdirect version 2.7.2.

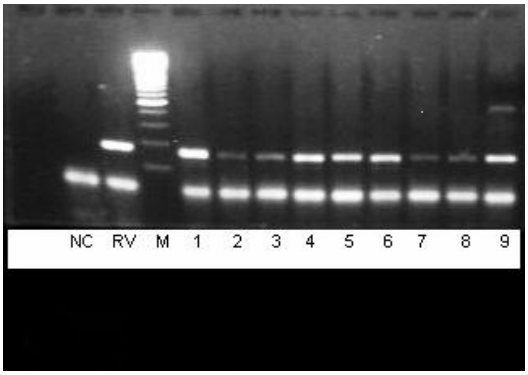

Fig 1- Result of PCR based on IS6110. Line 1: negative control, line2: H37Rv as a laboratory standard strain, lane 3: Marker 100bp, line 4-12: isolated strains.

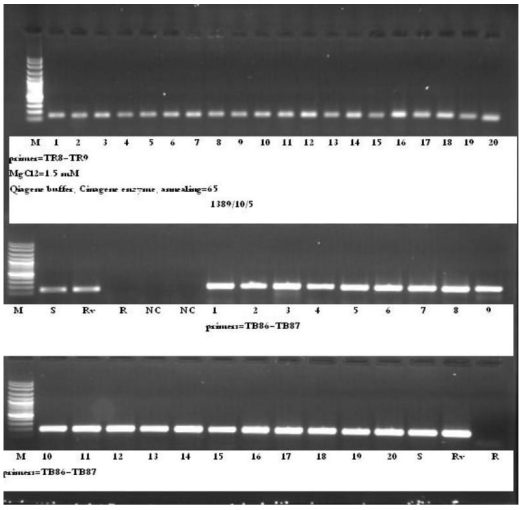

Fig 2- The amplified product of katG and rpoB genes.
Row 1; Lane 1: Marker 100bp, lane 2-21: isolates no.1-20 amplified for rpoB gene.
Row 2; lane 1 Marker 100bp, Lane 2-3: The H37Rv was used as laboratory standard strain for rpoB gene. Lane 4-6: negative controls. Lane 7-15: isolates no. 1-9 amplified for katG gene.
Row 3; Lane 1: Marker 100bp, Lane 2-11: isolates no. 10-20 amplified for katG gene, Lane 12-13: The H37Rv was used as laboratory standard strain for katG gene, lane 14: negative control

Results

All isolates were identified as *M. tuberculosis* by PCR using IS6110 primer (Fig. 1).

Drug–susceptibility testing by the proportional method revealed 16 MDR (21.6%), 23 mono-drug resistant (31%) and 35 susceptible strains (47.3%). Out of 16 MDR-TB cases, we could detect 4 (25%) by PCR-SSCP. Of 17 and 7, INH and RF mono-drug resistant cases, 10 (58.82%) and 4 (57.1%) were detected by PCR-SSCP, respectively. In overall, the agreement of PCR-SSCP with classical method was 54.05% (40/74).

Comparison of PCR-SSCP with DNA sequencing showed the sensitivity of the PCR-SSCP assay for detecting mutation in 315 codon of katG gene was 94.74% (CI= 73.97%-99.87%) with 100% CI= (93.51%-100%0 specificity. In contrast, the sensitivity and specificity of this assay in detection of rpoB gene were 70.8% (CI= 48.91%-87.38%) and 88% (CI= 75.69%-95.47%), respectively.

Positive and negative predictive values in addition to general efficacy of PCR-SSCP to detect mutation in katG gene in comparison with DNA sequencing were 98.21% (CI= 90.45%-99.95%), 100% (CI= 81.47%-100%), 98.64% (CI= 90.40%-100%), respectively. On the other hand, the positive and negative predictive values of PCR-SSCP to detect the mutation in rpoB gene in comparison with DNA sequencing were 73.91% CI= [51.59%-89.77%] and 86.27% CI= [73.74%-94.3%], respectively, and its general efficacy was 82.43% (CI= 65.8%-85.2%) (tables 2, 3).

The findings represent no mutation in inhA and ahpC gene of INH resistant strains, using PCR-SSCP and DNA sequencing. Totally, 51.5% (17/33) of the INH resistance strains had mutation in 315 KatG and 65.22% (15/23) of RIF resistance strains had mutation in 516,526 and 531 codons of rpoB gene.

Tabel 1- the sequence of oligonucleotides, target genes and the size of their products

primer sequence	Target	size
TR8: tgcacgtcgcggacctcca TR9: tcgccgcgatcaaggagt	rpoB	157
TB86: gaaacagcggcgctggatcgt TB87: gttgtcccatttcgtcgggg	katG	209
TB92: cctcgctgcccagaaaggga TB93: atcccccggtttcctccggt	inhA	248
TB90: ccgatgagagcggtgagctg TB91: accactgctttgccgccacc	ahpC	236

Table 2- Result of PCR-SSCP in comparison with DNA sequencing for katG gene

Genotype		DNA sequencing of katG		Total
		Has mutation in 315 codon	Has no mutation in 315 codon	
PCR-SSCP of katG	With different migration pattern	18 24.3%	0 0%	18 24.3%
	Without different migration pattern	1 1.4%	55 74.3%	56 ץ5.7%
Total		19 25.7%	55 74.3%	74 100%

Sensitivity=94.74%
Specificity=100%
Positive predictive value=100%
Negative predictive value=98.2%
General efficacy=98.64%

Table 3- result of PCR-SSCP in comparison with DNA sequencing for rpoB gene

Genotype		*DNA sequencing of rpoB*		*Total*
		Has mutation in RDRR	*No mutation in RDRR*	
PCR-SSCP of rpoB	*With different migration pattern*	*17* *23%*	*6* *8 %*	*23* *31%*
	Without different migration pattern	*7* *9.5%*	*44* *59.5%*	*51* *69%*
Total		*24* *32.5%*	*50* *67.5%*	*81* *100%*

Sensitivity=70.83%
Specificity=88%
Positive predictive value=73.9%
Negative predictive value=82.27%
General efficacy=75.31%

Table 4- result of PCR-SSCP in comparison with DNA sequencing for katG and rpoB genes

Genotype		DNA sequencing of katG+rpoB		Total
		Has mutation	Has no mutation	
PCR-SSCP of katG+rpoB	With different migration pattern	7 9.45%	0 0%	7 9.45%
	Without different migration pattern	4 5.4%	63 85.13%	67 90.54%
Total		11 14.86%	63 85.13%	74 100%

Sensitivity=63.63%
Specificity=100%
Positive predictive value=100%
Negative predictive value=94%
General efficacy=94.59%

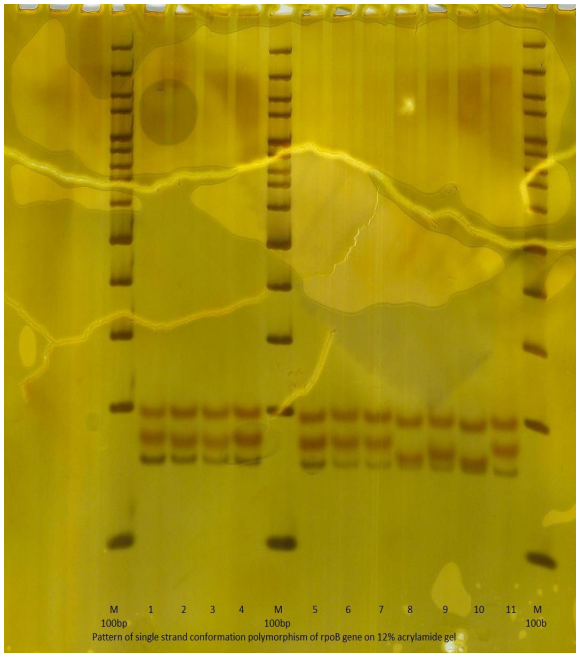

Fig 3- Migration pattern of rpoB gene on 12% acryl amid gel stained with silver nitrate. M: Marker 100bp. Lanes 1-7: strains without any probably mutation in rpoB gene, lanes 8-10: strains with probably mutation in rpoB gene. Lane 11: H37Rv as standard strain for rpoB gene.

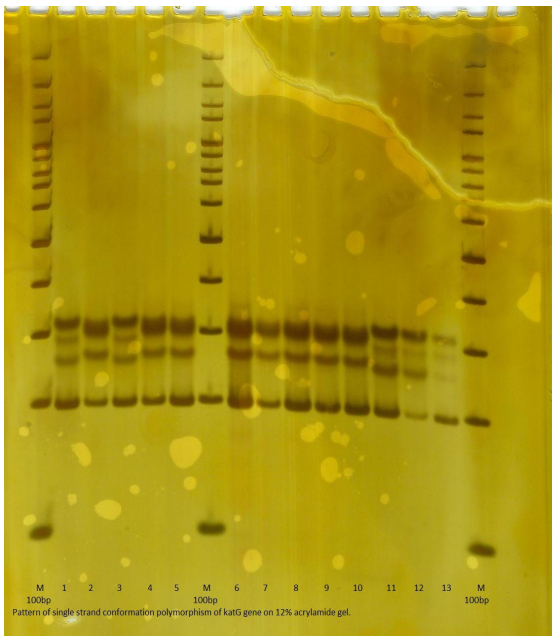

Fig 4- Migration pattern of katG gene on 12% acryl amid gel stained with silver nitrate. M: Marker 100bp. Lane 1, 3, 11-13: strains with probably mutation on katG 315. Lane 2, 4, 6-10: strains without any probably mutation on katG 315. Lane 5: H37Rv as standard strain for katG 315.

Discussion

MDR-TB cases are threatening the efforts of any national TB (NTP) program. Generally, the rapid availability of information concerning drug susceptibility patterns not only facilitates rapid selection of regimens, but also it minimizes the spread of drug–resistant strains. Here, we showed PCR-SSCP a useful tool to detect drug resistance TB cases. The sensitivity of this assay to detect the INH and RIF resistance TB cases was 58.82% and 57.1%, respectively. Therefore, PCR-SSCP can be used just as a screening test for drug resistance TB detection. However, present investigation demonstrated lower sensitivity of PCR-SSCP, when detecting MDR-TB cases.

The gold standard for drug–susceptibility testing is classical method which is time consuming and exposing health personnel to the risk of acquiring infection. Thereby, it is better to include other complementary tests i.e. DNA–sequencing when detecting single drug mutation by PCR-SSCP. Usually, the strain resistant to INH has mutation, insertion or deletions in multiple sites of katG or in the regulatory region of inhA, or ahpC [15]. It has also been revealed that certain mutation in katG i.e. deletions in this gene or substitution S315T is associated with a high level of resistance, whereas others may not confer in vivo-resistance (e.g. R436L) [15]. The sensitivity of PCR-SSCP for the detection of mutations in KatG in comparison with DNA-sequencing was 94.74%, but the positive predictive value of SSCP was 98.21%. With regard to inhA and ahpC, we did not find any mutations in these regions by both studied methods. Studies from other researchers, documented 42-58% of mutations on codon 315 in katG of INH–resistance strains [5, 9, 17]. In our study, the frequency of mutation in katG was 51.5%, which is similar to other reports [5, 9, 17]. Since the positive predictive value of PCR-SSCP for detecting mutation in katG gene is 98.21%, this method can be used as a predictive screening test for INH–resistant cases. However, sensitivity of PCR-SSCP to detect mutations in rpoB gene was 70.8% and its positive predictive value was 73.91%. The reported sensitivity was differed from other reports in literatures [9-12, 19]. The result of DNA sequencing in 81-bp core of rpoB gene showed mutations in 24 samples (32.4%). Eight RIF resistant–strains by Proportional method, showed no mutations in RDRR by DNA sequencing. This means, the studied isolates might harbor mutations in other codons, which were not analyzed in our investigation. Here, the codons (531,526 and 516) were studied while mutations outside of RRDR region may cause resistance to RIF.

Generally, drug resistance of *M. tuberculosis* develops by selective growth of resistant mutants (2, 3). The incidence of drug–resistant cases depends on the number of bacilli and the probability of drug-mutant in the lesion. It has been proposed that the probability of INH resistance (10^6) is 100 times more than RIF resistance mutations (10^8). Our findings provided higher frequency of INH resistance in comparison to RIF as 33 samples (44.5%) had probably mutation in katG gene *vs.* 23 samples (31.1%) in rpoB gene among studied cases. Similarly, Mohajeri P *et al* [18], reported the high frequency of INH resistance in strains isolated from Markazi province of Iran. The PCR-SSCP can be used for INH mono-drug resistant cases.

In conclusion, PCR-SSCP can be used as a screening method to detected RIF and INH mono-drug resistant cases, however, sensitivity of PCR-SSCP to detect MDR-TB cases was low (25%). On the other hand, agreement of this method with DNA-sequencing to detect mutation in rpoB gene was 82.4%. Therefore, as RIF resistance serves as a surrogate marker for estimation of MDR-TB, it would be suggested to combine PCR-SSCP with DNA–sequencing.

Acknowledgment

The authors express their gratitude Mycobacteriology Research Center of Tehran, Iran, for their supports.

Conflict of interest

None declared.

References

1. World Health Organization. Global Tuberculosis control-Surveillance, Planning, financing, vol. Report 2007. WHO, Geneva, Switzerland.
2. Velayati A.A., Masjedi M.R., Farnia P., Tabarsi P, Ghanavi J, Ziazarifi AH, et al. multidrug and extensively drug-resistance tuberculosis bacill: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. Chest 2009;136(2):119-28
3. Caminero J.A., Sotgiu G., Zumla A., Migliori G.B.; Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect Dis. 2010;10(9):621-9.
4. Zimhony O., J.S.Cox, J.T.Welch, C.Vilcheze, W.R. Jacobs, Jr. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. Nat. Med. 2000; 6(9):1043-7.
5. Somoskovi A., L.M.Parsons, and M. Salfinger. The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis.* Respir. Res. 2001; 2(3):164-8.

6. Sherman D.R., K. Mdluti, M.J. Hickey, T.M. Arain, S.L. Morris, Barry CE 3rd, et al. Compensatory ahpC gene expression in isoniazid-resistant *Mycobacterium tuberculosis.* Science. 1996;272(5268):1641-3.
7. Gagneux S, Burgos MV, DeRiemer K, Encisco A, Muñoz S, Hopewell PC, et al. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis.* PLoS Pathog. 2006;2(6):e61.
8. Riska PF, Jacobs WR Jr, Alland D. Molecular determinants of drug resistance in tuberculosis. Int. J. Tuberc. Lung Dis. 2000;4(2 Suppl 1):S4-10.
9. Ramaswamy S., Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber. Lung Dis. 1998;79(1):3-29.
10. Heep M, Brandstätter B, Rieger U, Lehn N, Richter E, Rüsch-Gerdes S, et al. Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. J.Clin. Microbiol. 2001; 39(1):107-10.
11. Jin DJ, Gross CA. Mapping and sequencing of mutations in the Escherchia coli rpoB gene that lead to rifampicin resistance. J. Mol. Biol. 1998; 202(1):45-58.
12. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis.* Lancet 1993;341(8846):647-50.
13. Van Soolingen D., P.E.W. de Haas, P.W.M. Hermans, and et al. RFLP analysis of Mycobacteria. 1995. National Institute of Public Health and Environmental Protection, Bilthoven. The Netherlands.
14. Kent PT, Kubica GP. Public health mycobacteriology, a guide for the level III laboratory, CDC, U.S. Department of Health and Human service publication no. (CDC) 86-216546, Atlanta 1985; 21–30.
15. Telenti A, Persing DH Novel strategies for the detection of drug resistance in *Mycobacterium tuberculosis.* Res. Microbiol. 1996;147(1-2):73-9.
16. Senger F., S.Nicklen, and A.R.Coulson. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 1997;74(12):5463-7.
17. Zhang Y, Telenti A. Genetics of drug resistance in *Mycobacterium tuberculosis.* Molecular Genetics of Mycobacteria. Edited by Hatful GF, Jacobs WR Jr. Washington DC: ASM Press; 2000; 235–254.
18. Mohajeri P, Tavakoli A, Moghim Sh, Nasresfahani B. Detection of Mutation in Codon 315 katG Gene as a Gene Marker Associated with Isoniazid Resistance, in *Mycobacterium tuberculosis* Strains Isolated from Patients in Isfahan and Tehran by PCR-RFLP Method. ZUMS J. 2008; 52:29-40.
19. Nasr Isfahani B, Tavakoli A, Salehi M, Tazhibi M. Detection of rifampin resistance patterns in *Mycobacterium tuberculosis* strains isolated in Iran by polymerase chain reaction-single-strand conformation polymorphism and direct sequencing methods. Mem. Inst. Oswaldo Cruz .2006. vol.101 no.6