Research Paper

Comparison of Rifampicin resistance in Mycobacterium tuberculosis isolates by Multiplex Allele Specific PCR (MAS-PCR) with Enzyme Linked ImmunoSorbent Assay (PCR-ELISA)

Abstract

Aims: Detection of drug resistance M.tuberculosis isolates is one of the most important strategies to control the disease. Nowadays, with advances in molecular technology, various methods are available to detect drug resistance M.tuberculosis strains such as those based on capture specific probe. In this study, we aimed to investigate the frequency of mutation in rpoB gene of M.tuberculosis by Polymerase Chain Reaction detection based on Enzyme Linked ImmunoSorbent Assay (PCR-ELISA).
Methodology: Thirty-three culture positive isolates were randomly selected for this study. The drug Susceptibility Test (DST) using proportional methods performed on these isolates .Then ability and the efficacy of Multiplex Allele Specific PCR (MAS-PCR) and PCR-ELISA to detect Rifr M.tuberculosis isolates was compared and evaluated, respectively.
Results: Mutation of rpoB gene was detected in 19/33 isolates (57.6%) by PCR-ELISA. Hybridization with specific mutant probe of 516 and 526 codon was occurred in one of 33 cases (3% in each codon). The hybridization with specific mutant probe of 531 was occurred in 13 isolates (39.4%). Three isolates had shown simultaneously mutation in both codon of 516 and 531 (9.2%). The sensitivity and specificity of PCR-ELISA in comparison with MAS-PCR was 75% and 69.2%, respectively. The positive predictive value was 78.9% and negative predictive value was 64.3% and general efficacy of test was 72.7%.
Conclusion: The study showed the sensitivity and specificity of PCR-ELISA to detect mutation in rpoB gene was low. On the other hand, this was a complex and time consuming as well as expensive test. Therefore, this test is not suggested for determining the Rif resistant M.tuberculosis strains.

Key words: M.tuberculosis, PCR-ELISA, MAS-PCR, Rif resistant, rpoB gene.

1. Introduction

Tuberculosis (TB), is one of the most prevalent infectious disease,-causes more than one million death worldwide [1]. The usual short course chemotherapy for TB, includes two months of isoniazid (INH) and rifampicin, ethambutol (ETB) and pyrazinamide (PZA), followed by four month INH & RF. In fact, both INH & RF are highly effective drugs for susceptible M. tuberculosis strains [2, 3]. Although, the emergence and spread of strains that are resistant to both INH and RF, defined as multidrug resistant tuberculosis (MDR-TB) have created new stigma for TB control strategies. MDR-TB requires 18-24 months of treatment with expensive and poorly tolerated second–line TB drugs. Therefore, rapid identification and treatment of TB is the compulsory component of TB control program. During recent years, advances in molecular techniques revealed that mutation in a 81-bp region of rpoB gene, encoded the beta subunit of RNA polymerase, is associated with rifampicin resistant strains of M.tuberculosis [4-6].Resistance to rifampicin is frequently associated with resistance to other drugs like isoniazid. Therefore, RIF resistance (RIFr) can be consider as a powerful marker for determining multi-drug resistant tuberculosis strains also [7].

Recent study in Iran showed that out of 1,139/1742 (65.4%) new cases, 29.9% had resistant to at least one drug and 15.1% were MDR-TB [8]. Furthermore, Tabarsi et al (2009) demonstrated the increase rate of drug resistance up to 43% among Iranian HIV positive patients [9]. Additionally, more dangerous forms of resistant strains i.e., XDR and TDR-TB (Totally drug resistance) were reported in Iran [10]. These observation, underline the need for rapidly detecting resistant TB patients.Today, it is known that solid culture based method for identification and drug susceptibility testing (DST) are time-consuming. Therefore, one of the main strategies for efficient treatment and control of drug-resistant tuberculosis is implementation of rapid susceptibility test [11]. DNA sequencing [12], heteroduplex analysis [13], PCR single-stranded conformational polymorphism [14, 15], line probe assay [16, 17], mismatch analysis [18], and real-time PCR with fluorimetry [19, 20] are some known molecular assays that have been used to screen the rpoB gene mutations. A compatible assay with real-time PCR, a very sensitive method to detect single point mutation (SNP), is PCR- enzyme-linked immunosorbent (ELISA) assay which has been used to detect mutation in the gene. The mechanism of this assay is based on capture probe PCR and detection system by colorimetric assay [21, 22]. In the present study we try to evaluate the sensitivity of PCR-ELISA in comparison to other molecular & classical methods i.e., Multiplex Allele Specific PCR (MAS-PCR) and proportional method, respectively. In additional, we try to identify the rpoB mutations among Iranian pulmonary tuberculosis (PTB) patients.

2. Material and Methods

2.1. Clinical specimens

In a blind retrospective study thirty five specimens out of sixty five clinical isolates of M.tuberculosis with known DST patterns were randomly selected for investigating the rate of mutation in rpoB gene by PCR-ELISA. These strains were initially identified by IS6110 based PCR and confirmed by PCR-RFLP analysis of the hsp65 gene as M.tuberculosis strains. The existing of mutation in codons 516,526 and 531, as frequent mutant codons of rpoB gene, were detected by a multiplex allele specific PCR (MAS-PCR) assay and PCR-ELISA, simultaneously.

2.2. Drug Susceptibility Test

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 [23].

2.3. DNA extraction from cultures

All procedures were performed in a separate room equipped with biological safety cabinet class II B. To extract the DNA one loop of growth bacteria on culture medium was taken and suspended on 400µl Tris-EDTA buffer, heated at 95℃ for 20 min. To remove R NA contamination 50µg/ml RNase enzyme was added and incubated at 37℃ for 1 hour. Cellular de bris was removed by centrifugation and 5µl of supernatant was used in the PCR.
In order to check the contamination and accuracy of test a negative control consisting of the entire reagent except target DNA and a positive control including H37Rv as a susceptible standard strain and three resistant strains with known mutation at 516,526 and 531 codon of rpoB gene was used as positive controls in each step of assay.

2.4. Multiplex Allele specific PCR (MAS-PCR)

MAS-PCR was performed as described previously[24]. Briefly, the following primers were used for three MAS-PCRs targeting three different codons of the *rpoB* gene: three specific primers R516 (5′-GCTGAGCCAATTCATGGA), R526 (5′-GTCGGGGTTGACCCA), or R531 (5′-ACAAGCGCCGACTGTC) were designed such that their 3′-OH ends, aligned with the second bases of the respective codons in the case of wild-type allele. Consequently, in the absence of mutation in these positions in rpoB531/526/516, wild-type allele-specific fragments (167 or 181 or 214 bp, respectively) were amplified by the reverse primer RIR (5′-TGACCCGCGCGTACAC). Fifty ng of purified DNA sample (5 µl) was added to PCR mixture (final volume of 25 µl) that contained MgCl2 5 mM, 1 U of hotstar *Taq*® DNA polymerase (Qiagene), 200 µM concentrations of each of the deoxynucleoside triphosphates (dNTPs), 30 pmol of outer primers RIR, 10 pmol for *rpoB*516, 20 pmol for *rpoB*526 and 30 pmol for *rpoB*531. the following thermal conditions was performed: initial denaturation at 95℃ for 5 min; 35 cycles of 95℃ for 20 s, 70℃ for 1 min, and 72℃ for 20 s; and final elongation at 72℃ for 10 min. The amplified fragments (10 µl) were electrophoresed in 6% acrylamid/bisacrylamid standard gel and visualized under UV light after staining with ethidium bromide. Absence of specific bands showed the mutation in given allele and the isolate was considered as resistant to Rifampicin.

2.5. PCR amplification

A 437bp fragment of rpoB gene include the 81bp region associated with drug resistance (RRDR) was amplified by two oligonucleotides defined by previous study [25]:
Forward primer, RpoB-for1:"TGG TCC GCT TGC ACG AGG GTC AGA" and
Reverse primer, RpoB-rev1:" CTC AGG GGT TTC GAT CGG GCA CAT"
In our experience the PCR product was DIG labeled by using a commercial kit of digoxigenin (PCR DIG Labeling Mix plus Roche). Briefly, a 50µl reaction volume of PCR mixture was consisted of 1X PCR buffer, 1U of HotStarTaq DNA polymerase (Qiagene), 3mM MgCl2, 50 pmol each of primers, 1X mix of PCR dig labeling kit containing 2mM of each dATP, dCTP, dGTP, and 5.7 mM dUTP plus 0.3mM of digoxigenin-11-dUTP. Five microliter of DNA (50-100ng) template was added to PCR mix in a working station of PCR in separate room to prevent the contamination. The thermal PCR conditions was followed as: initial denaturation for 5 min at 95℃, 40 cycl es comprising denaturation 95℃, annealing 70℃ and extension 72℃ each 30 sec. Final extension at 72℃ for 10 min.

2.6. Design of oligonucleotide probes

The 5'-biotinylated oligonucleotide probes were designed and used based on the sequence of rpoB gene described before (Fig 1) [25]. Five wild type probes (Wt1-Wt5) were specific for the wild type of M.tuberculosis rpoB sequence encoding amino acid codons 509-534. Seven 15-20 oligonucleotides, with melting temperature at 55-60℃ were designed as mut ant probes (Mt). Varying amounts of concentration of probes ranging from 10 to 60 pmol were used to determine the optimal probe concentrations in such a way that all the probes resulted in equally intense signals. The hybridization temperature (56℃) was optimized by hybridizing the PCR product with oligonucleotide in 3 different temperatures (50, 56 and 60℃).

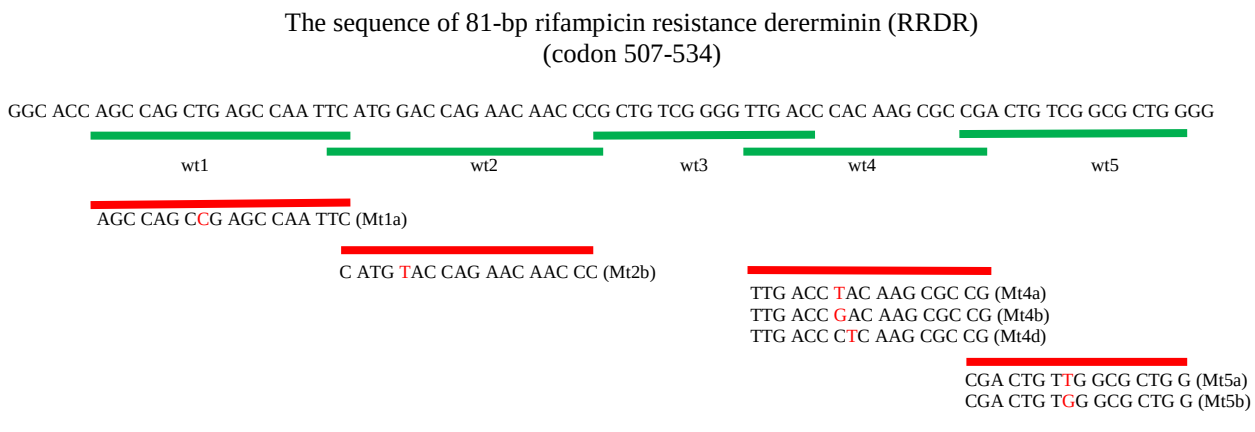

Fig. 1- Schematic representation of the Wt and Mt probes. There are 5 Wild type probes (Wt1-Wt5) and 7 Mt probes carrying specific mutant alleles (red color). The sequences of probes were described by Suresh [25].

2.7. Detection of capture probes by ELISA

Detection of the digoxigenin-labeled amplified DNA was carried out by PCR ELISA DIG Detection kit (Roche) as instruction of manufacturer. Briefly, after thermocycling was complete, the amplicons including controls and reagent blank were denatured by incubating in a microtube with denaturation solution for 10 min at 15-25℃. Twelve wells were used for each PCR product sample, one well for each capture probe. After adding hybridization solution and 5'-biotinylated oligonucleotide probes to each microtube contain denatured PCR products and vortexing, the mixtures immobilized on a streptavidin-coated microplate. Hybridization was done by incubating the streptavidin-coated microplate at optimal temperature (56℃) for 3 hours. The wells were washed 5 times with washing solution of detection kit to remove no hybridized PCR products. The plates were incubated at 37℃ wit h peroxidase-conjugated anti-digoxigenin antibody for 30 min. Wells were washed again for 5 times with washing solution and 200µl of colorimetric substrate ABTS was added and incubated at 37℃ on a microplat e shaker until color becomes visible in all wells. The absorbance of each well was read at 405nm (reference wavelength 492nm) by a microtiter plate reader. A clearly visible, distinguishable, and interpretable signal from a given probe was considered as positive for hybridization. When hybridization signal from all Wt probes (Wt1+–Wt5+) were positive and the Mt probes reacted negative, the M. tuberculosis isolate was considered susceptible to RIF. Absence of hybridization signal from one of the Wt probes (Wt-) indicated the presence of a mutation in that probe region. Absence of a signal from one of the Wt probes (Wt), no signal from an Mt probe in these isolates indicates the presence of a mutation for which there was no specific Mt probe designed.

2.8.Statistical analysis.

Sensitivity and specificity of PCR-ELISA in comparison with MAS-PCR to detect Rif resistant M.tuberculosis complex isolates was calculated by Chi square analysis.

3.Results AND DISCUSSION

3.1.rpoB mutations

In total, forty three rifampicin-resistant (66.15%) and 22 susceptible (33.85%) isolates with known DST patterns were evaluated for RIF susceptibility test by MAS-PCR assay. The result of MAS-PCR was compatible with Proportional susceptibility testing completely. That means all of Rifr *M.tuberculosis* isolates had mutation in the 81bp region of rpoB gene, while no such mutations were observed in the rifampicin-susceptible (Rifs) strains. The MAS-PCR revealed the high frequency of mutation at codon 516

(7/65; 10.77%) followed by codons 526(6/65 ;9.23%) and 531 (29/65 ;44.62%), respectively. One of isolates showed simultaneous mutation at codon 516 and 531 (1.53%).

The PCR-ELISA assay was performed on thirty-five strains; these strains were randomly selected from identified *M. tuberculosis* isolates by MAS-PCR. The investigator who performed the laboratory work was blinded to the resistance status of the isolates. Two samples were excluded from study because in spite of repeating the test, the optical density of two samples was not determined, i.e. these two samples showed a signal with both wild type and mutant probes. The PCR-ELISA could detect mutation in RRDR of the rpoB gene in 19 from 33 samples (57.6%). The most frequent codon containing the mutation was 531 (39.4%), 526 and 516 (each 3%). There was no any non-specific mutation in these isolates. Simultaneous mutation in two codons 516+531 was seen in 3 cases (9.2%) (Fig 2).

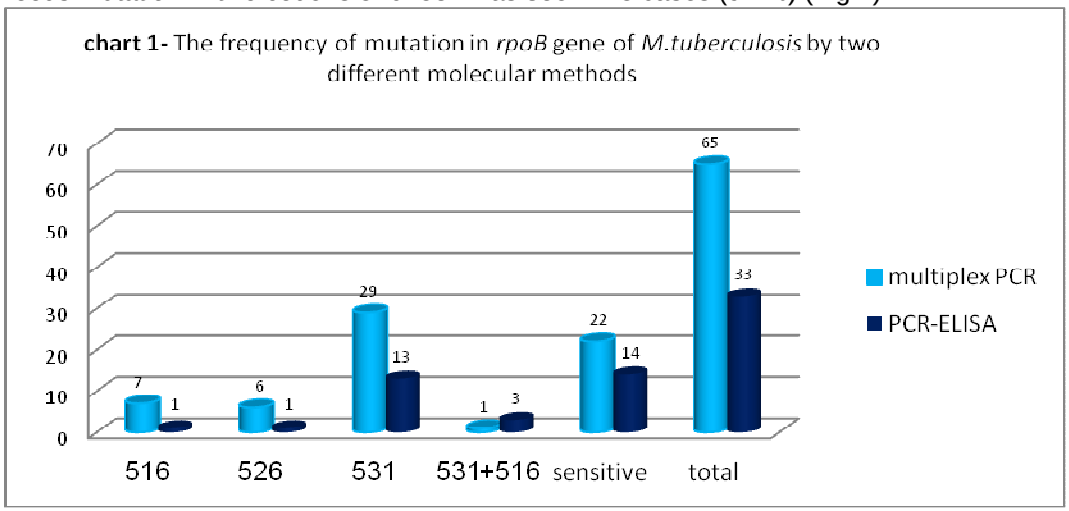

Fig. 2- The frequency of mutation in three codons of rpoB gene associated with anti-TB drug resistance detected by two different molecular assays

Five from previously reported Rif resistant isolates (26.3%), were reported as a wild type by PCR-ELISA. Three of these isolates had mutation in 526, one isolate had shown mutation in 516, and the other one had mutation in 531 codon by MAS-PCR. On the other hand, 4 from 14 Rif susceptible isolate (28.6%) also showed mutation in 531 codon of rpoB gene by PCR-ELISA. Thereby, the sensitivity and specificity of PCR-ELISA in comparison with MAS-PCR to detect Rifr isolates was 75% and 69.2%, respectively. The positive predictive value was 78.9% and negative predictive value was 64.3%. General efficacy of PCR-ELISA was calculated 72.7% by chi square test (Table 1).

Table 1- comparison of PCR-ELISA and MAS-PCR to detect mutation of rpoB gene in M.tuberculosis isolates

Result		MAS-PCR		Total
		Resistance	Susceptible	
PCR-ELISA	Resistance	15	4	19
	Susceptible	5	9	14
Total		20	13	33

Sensitivity: 15/20=75%
Specificity: 9/13= 69.2%
P.P.V: 15/19=78.9%
N.P.V: 9/14=64.3%
General Efficacy: 24/33=72.7%

Rifampicin is one of the most effective drugs that used against tuberculosis. Generally, the loss of rifampin from treatment regimen has considerable implication for any national tuberculosis program

(NTP). The patients with Rifampicin resistant bacilli need longer and more expensive treatment. Therefore, rapid detection of Rifr strains is very important in terms of disease control [7]. The conventional methods for detecting of drug resistance such as proportional drug susceptibility test are time consuming with high risk of infection for laboratory personnel [26]. Today with advancement in molecular techniques, laboratories adapted more rapid, accurate and sensitive methods for detecting drug resistance isolates of *M.tuberculosis* [27-30]. Previously, we reported the high rate of MDR-TB within the country. Therefore, it was mandatory for the National Reference TB laboratory of Iran to evaluate and implement a rapid and sensitive test to detect Rifr TB strains. In this study, 65 clinical samples of *M.tuberculosis* isolated from Iranian patients with known pattern of DST were evaluated by two molecular assays in a blinded study. All the selected Rifr *M.tuberculosis* isolates by MAS-PCR had rpoB mutations. The result are in agreement with previous studies that showed >96% of Rifr *M.tuberculosis* strains contain mutations within the RRDR and >70% of these mutations occur in codons 531 and 526 [5, 15, 16, 31-33]. Our study showed the rate of mutation in 531 codon (29 from 65 isolates 44.6%) was accordance with the other studies [34-37]. Although, in contrast to other studies, the second frequent codon was 516 (7/65 about 10.8%) and not the 526. The low number of studied samples might explain the results discrepancy with other reports. In additional, the frequency of detected point mutation (516, 526 and 531 codons of rpoB gene) was different between PCR-ELISA and MAS-PCR . On the other hand, MAS-PCR assay could identify mutation at 516 codon in 80% of isolates, correctly. But the ability of PCR-ELISA to detect point mutation in 526 codon was weak (1 versus 6 isolates). In spite of these differences the reproducibility of PCR-ELISA was higher than MAS-PCR because in all experiments the known mutant strains, used as control, were detected correctly by PCR-ELISA.

The mechanism of point mutation by MAS-PCR is based on utilizing of primer with a 3′ mismatch at the position of the variable nucleotide together with another primer complementary to both alleles. In this case, when the mismatch occurs, 3′ elongation by Taq DNA polymerase is prevented. To ensure high specificity in this assay, careful optimization of the concentrations of magnesium, oligonucleotide, dNTP, Taq DNA polymerase, and DNA concentration, as well as the PCR cycling conditions, and the design of the Allele Specific Oligonucleotide (ASO) is needed. Theoretically, the possibility of spurious binding could be higher in this method, especially when there was an asymmetric concentration of mutant and wild type bacteria in a sample. But in PCR-ELISA a set of biotinylated allele-specific capture probes was used to detect a specific point mutation. As described above, when an isolate was announced as resistant that it had shown signal with mutant probe while hadn't any visible signal with wild type probes, so it could reduce the amount of background signal generated from the abundant wild-type template. The result of another study was showed the sensitivity of PCR-ELISA in comparison with DNA sequencing to detect SNP was higher than Allele specific priming PCR [38]. It seems that this theory is also true in our experience, so that PCR-ELISA was detected simultaneous mutation at 531 and 516 codon in 3 isolates in which mutation was detected at just 516 codon by MAS-PCR. As it is known synthetic DNA probes, under high stringency conditions, will only anneal to their complementary target sequences [39, 40]. This experiment was set up in a very stringent condition, so it seemed that the PCR-ELISA was more accurate to identify a specific mutation and to indicate the region in which the mutations was located as well as to detect the simultaneous mutations in the rpoB gene. To determine the accuracy of PCR-ELISA in detection of point mutation in rpoB gene, the result of two molecular assays should be compared with DNA sequencing as a gold standard of molecular assays. It should be noted that knowledge of the specific mutation conferred resistance is not necessary for efficient patient management. Therefore, the accurate location of point mutation can be ignored.

The present study was also aimed to evaluate the ability and efficiency of PCR-ELISA to determine the RIFr strains. Since the result of MAS-PCR was completely concordance with the result of DST, we compare two molecular assays with each other. To determine the point mutation of rpoB gene, the amplified dig-labeled fragments were hybridized by specific oligonucleotide probe including wild type and mutant, separately. A series of probes were used that they were complementary to wild type or mutant sequence of rpoB gene. They were designed so that the mutant base was placed in the middle of their sequences. So, the specificity of probes to align to their complementary sequences was become higher. On the contrary, allele specific primers were designed so that the mutant bases were placed at the 3' end of their sequences. Therefore, absence of a visible band on the agarose gel indicated the mutation. We expected PCR-ELISA was more sensitive and specific and by applying this accurate method we could be

predicted the susceptibility and/or kind of mutation in the rpoB gene. But, the sensitivity and specificity of PCR-ELISA in comparison with MAS-PCR was low (75% and 69.2%, respectively). Although the positive predictive value was relatively high but it could not be applied for detecting susceptibility *M.tuberculosis* because of the importance of Rifampicin as a marker to detect MDR-TB. Our result was in contrast with the other study that showed PCR-ELISA system as a good assay in identifying Rifr strains [41]. Furthermore, PCR-ELISA test is a very cost-effective technique and the use of this technique in many laboratories are not applicable. Thereby, the assay cannot be suggested as an alternative molecular method for detecting Rifr strains.

4. Conclusion

The study showed the sensitivity and specificity of PCR-ELISA to detect mutation in *rpoB* gene was low. On the other hand, this was a complex and relatively time consuming as well as expensive test. Therefore, this test is not recommended for determining the Rifr *M.tuberculosis* strains. The authors suggest performing DNA sequencing as a gold standard for molecular assays to determine the accurate kind of point mutation.

COMPETING INTERESTS

The authors report no conflicts of interest and accept the responsibility of the content of the paper.

AUTHORS' CONTRIBUTIONS

'Author A' and 'Author C' managed the analyses of the study. 'Author C' managed the literature searches. 'Author B' designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. All authors read and approved the final manuscript."

Ethical approval

The work has been approved by the ethical committees of Mycobacteriology Research Center. A Written informed consent has been obtained from all subjects.

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