

Research Paper 12

3 Comparison of Rifampicin resistance in 4 Mycobacterium tuberculosis isolates by Multiplex 5 Allele Specific PCR (MAS-PCR) with Enzyme Linked ImmunoSorbent Assay (PCR-ELISA)67

8 Abstract

9 Aims: Detection of drug resistance *M.tuberculosis* isolates is one of the most important strategies
to 10 control the disease. Nowadays, with advances in molecular technology, various methods are
available to 11 detect drug resistance *M.tuberculosis* strains such as those based on capture specific
probes. In this study 12, we aimed to investigate the frequency of mutation in *the M. tuberculosis -*
rpoB gene of *M.tuberculosis* by Polymerase Chain 13 Reaction *detection* based on Enzyme Linked
ImmunoSorbent Assay (PCR-ELISA) *detection*. 14 Methodology: Thirty-three culture positive
isolates were randomly selected for this study. *All the isolates were subjected to a* The drug 15
Susceptibility Test (DST) using *the* proportional methods *performed on these isolates*. Then *the*
ability and the 16 *efficacy of* Multiplex Allele Specific PCR (MAS-PCR) and PCR-ELISA to detect Rif^r
M.tuberculosis 17 isolates was compared and evaluated, *respectively*. 18 Results: Mutation of *rpoB*
gene was detected in 19/33 isolates (57.6%) by PCR-ELISA. Hybridization 19 *withwith the* specific
mutant probes of 516 and 526 codon *was* occurred in *1/one of 33 cases-isolates each* (3%
respectively in each codon). 20 *The h* Hybridization with *the* specific mutant probe of 531 *was*
occurred in 13/33 isolates (39.4%). Three isolates (9.2%) 21 *showedhad shown* simultaneously
mutation in *both* codons of 516 and 531 (9.2%). The sensitivity and specificity 22 of *MAS-PCR in*
comparison to the Proportion Method was 100%. On the other hand, PCR-ELISA showed in
comparison with MAS-PCR was 75% *sensitivity* and 69.2% *specificity, respectively*. The positive
predictive 23 value *for the PCR-ELISA method* was 78.9% and *the* negative predictive value was
64.3%. *% and The* general efficacy of test was 72.7%. 24 Conclusion: The study showed that *thee*
sensitivity and specificity of PCR-ELISA to detect mutations in *the* *rpoB gene in Drug Resistant*
strains 25 *gene* was low. *On the other hand Furthermore, this proved to be was* a complex, and time
consuming *and as well as* expensive test. 26 Therefore, this test is not *suggested-recommended* for
determining *the* Rifampicin resistance in *M.tuberculosis* strains. 27 28 Key words: *M.tuberculosis,*
PCR-ELISA, MAS-PCR, Rif resistant, *rpoB* gene.

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35 1. Introduction

36 Tuberculosis (TB) is one of the most dangerous infectious disease-causing death of more than
one 37 million patients annually (1). A combined of first line anti-TB drugs including isoniazid and

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rifampicin are 38 used against tuberculosis. Emerging and increasing drug resistance TB strains have created new 39 problems for public health. Studies showed that mutation in a 81-bp region of rpoB gene, encoded the 40 beta subunit of RNA polymerase, is associated with rifampicin resistant strains of M.tuberculosis in more 41 than 96% of isolates (2-4). Resistance to rifampicin is associated with resistance to other drugs, 42 especially isoniazid. Therefore, detecting of resistance to rifampicin is critical because it is a powerful 43 marker for determining of multi-drug resistant tuberculosis strains (5).

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A retrospective study conducted in Iran showed that out of 1,139 44 (65.4%) new cases 29.9% had showed 45 at least one drug resistance and 15.1% were MDR-TB (6). The other study showed 28 (43%) of Iranian 46 HIV positive patients were suffered from any drug resistance (7). In a recent study, it was also determined 47 that among 146 Iranian MDR-TB patients 8 patients (5.4%) were XDR and 15 patients (10.3%) were TDR 48(Totally drug resistance) (8). These studies showed the high rate of drug resistance in Iranian TB patients 49 and the necessity of rapid detection of it especially MDR-TB isolates to control the disease. 50 Culture based method for detecting of *M.tuberculosis* infection and drug susceptibility test (DST) are time- 51 consuming. Rapid detection of Rif susceptibility is one of the most important strategies for efficient 52 treatment and control of drug-resistant tuberculosis (9). DNA sequencing (10), heteroduplex analysis (11), 53 PCR single-stranded conformational polymorphism (12, 13), line probe assay (14, 15), mismatch analysis 54 (16), and real-time PCR with fluorimetry (17, 18) are some known molecular assays that have been used 55 to screen the rpoB gene mutations. A compatible assay with real-time PCR, a very sensitive method to 56 detect single point mutation (SNP), is PCR-ELISA which has been used to detect mutation in the gene. 57 The mechanism of this assay is based on capture probe PCR and detection system by colorimetric assay 58 (ELISA) (19, 20). In the current study two objectives was followed: 1) Set up and evaluate a PCR system 59 based on capture specific probe method and detection of mutations in rpoB gene using an enzyme-linked 60 immunosorbent assay (ELISA) and 2) Identify the rate of rpoB mutation associated with drug resistance in 61 M.tuberculosis complex strains isolated from Iranian patients. 62 63 2.material and methods

64 2.1.Clinical specimens

65 In a blind retrospective study thirty five specimens from sixty five clinical isolates of M.tuberculosis 66 complex growth on Löwenstein-Jensen media with known DST patterns were randomly selected for 67 investigating the rate of mutation in rpoB gene by PCR-ELISA. These strains were initially identified by 68 IS6110 based PCR and confirmed by PCR-RFLP analysis of the hsp65 gene as M.tuberculosis complex 69 strains and the existing of mutation in codons 516,526 and 531, as frequent mutant codons of rpoB gene, 70 were detected by a multiplex allele specific PCR (MAS-PCR) assay and PCR-ELISA, simultaneously.

71 2.2.DNA extraction from cultures

72 All procedures were performed in a separate room equipped with biological safety cabinet. To extract the 73 DNA one loop of growth bacteria on culture medium was taken and suspended on

Comment [LF1]: What is this percentage? 65.4% of what? The total number of TB-diagnosed patients?.

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400µl Tris-EDTA 74 buffer, heated at 95°C for 20 min. To remove RNA contamination 50µg/ml RNase enzyme was added and 75 incubated at 37°C for 1 hour. Cellular debris was removed by centrifugation and 5µl of supernatant was 76 used in the PCR.

77 In order to check the contamination and accuracy of test a negative control consisting of the entire 78 reagent except target DNA and a positive control including H37Rv as a susceptible standard strain and 79 three resistant strains with known mutation at 516,526 and 531 codon of rpoB gene was used as positive 80 controls in each step of assay.

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82 2.3.PCR amplification

83 A 437bp fragment of rpoB gene include the 81bp region associated with drug resistance (RRDR) was 84 amplified by two oligonucleotides defined by previous study (21): 85 Forward primer, RpoB-for1: "TGG TCC GCT TGC ACG AGG GTC AGA" and 86 Reverse primer, RpoB-rev1: "CTC AGG GGT TTC GAT CGG GCA CAT" 87 In our experience the PCR product was DIG labeled by using a commercial kit of digoxigenin (PCR DIG 88 Labeling Mix plus Roche). Briefly, a 50µl reaction volume of PCR mixture was consisted of 1X PCR 89 buffer, 1U of HotStarTaq DNA polymerase (Qiagene), 3mM MgCl₂, 50 pmol each of primers, 1X mix of 90 PCR dig labeling kit containing 2mM of each dATP, dCTP, dGTP, and 5.7 mM dUTP plus 0.3mM of

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digoxigenin-11-dUTP. Five microliter of DNA (50-100ng) template 91 was added to PCR mix in a working 92 station of PCR in separate room to prevent the contamination. The thermal PCR conditions was followed 93 as: initial denaturation for 5 min at 95°C, 40 cycles comprising denaturation 95°C, annealing 70°C and 94 extension 72°C each 30 sec. Final extension at 72°C for 10 min.

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96 2.4.Design of oligonucleotide probes

97 The 5'-biotinylated oligonucleotide probes were designed and used based on the sequence of rpoB gene 98 described before (Fig 1) (21). Five wild type probes (Wt1-Wt5) were specific for the wild type of 99 M.tuberculosis rpoB sequence encoding amino acid codons 509-534. Seven 15-20 oligonucleotides, with 100 melting temperature at 55-60°C were designed as mutant probes (Mt). Varying amounts of concentration 101 of probes ranging from 10 to 60 pmol were used to determine the optimal probe concentrations in such a 102 way that all the probes resulted in equally intense signals. The hybridization temperature (56°C) was 103 optimized by hybridizing the PCR product with oligonucleotide in 3 different temperatures (50, 56 and 104 60°C).

105 The sequence of 81-bp rifampicin resistance dererminin (RRDR) 110067 (codon 507-534)

108 GGC ACC AGC CAG CTG AGC CAA TTC ATG GAC CAG AAC AAC CCG CTG TCG GGG TTG ACC CAC AAG CGC CGA CTG TCG GCG CTG GGG

109 110 wt1 wt2 wt3 wt4 wt5

111 AGC CAG CCG AGC CAA TTC (Mt1a)

112 C ATG TAC CAG AAC AAC CC (Mt2b) 113 TTG ACC TAC AAG CGC CG (Mt4a) 114 TTG ACC GAC AAG CGC CG (Mt4b)

111156 TTG ACC CTC AAG CGC CG (Mt4d) 117 CGA CTG TTG GCG CTG G (Mt5a) 111189 CGA CTG TGG GCG CTG G (Mt5b)

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 (21). 2.5.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°C. 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°C) for 3 hours. The wells were washed 5 times with washing solution of detection kit to remove non hybridized PCR products. The plates were incubated at 37°C with 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°C on a microplate 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.

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2.6.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

Forty three Rif^r (66.15%) and 22 Rif^s (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 drug susceptibility testing completely. It means that all of Rif^r clinical isolates of M.tuberculosis complex had shown mutation in the 81bp region of rpoB gene, while no such mutations were observed in the rifampicin-susceptible (Rif^s) strains. The MAS-PCR revealed that the frequency of mutation at codons 516, 526 and 531 was 7/65 (10.77%), 6/65 (9.23%) and 29/65 (44.62%), respectively. One of isolates showed simultaneous mutation at codon 516 and 531 (1.53%).

Thirty-five isolates was randomly selected among above isolates and introduced to PCR-ELISA. The investigator who performed the laboratory work was blinded to the resistance status of

the isolates. 158 In spite of repeating the test, the Optical Density of two samples was not determined, i.e. these two 159 samples showed a signal with both wild type and mutant probes, so these two samples were excluded 160 from study. The PCR-ELISA could detect mutation in RRDR of the rpoB gene in 19 from 33 samples 161 (57.6%). The most frequent codon containing the mutation was 531 (39.4%), 526 and 516 (each 3%). 162 There was no any non-specific mutation in these isolates. Simultaneous mutation in two codons 516+531 163 was seen in 3 cases (9.2%) (Fig 2).164

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166 Fig. 2- The frequency of mutation in three codons of rpoB gene associated with anti-TB drug resistance 167 detected by two different molecular assays 168 Five from 19 Rif resistant isolates (26.3%) were detected as wild type by PCR-ELISA. It was determined 169 by MAS-PCR that three of them had mutation in 526 and one isolate had shown mutation in 516 and the 170 other one had mutation in 531 codon. On the other hand, 4 from 14 Rif susceptible isolate (28.6%) 171 showed mutation in 531 codon of rpoB gene by using this assay. The sensitivity and specificity of PCR- 172 ELISA in comparison with MAS-PCR to detect Rifr isolates was 75% and 69.2%, respectively. The 173 positive predictive value was 78.9% and negative predictive value was 64.3%. General efficacy of PCR- 174 ELISA was calculated 72.7% by chi square test (Table 1). 175 176 Table 1- comparison of PCR-ELISA and MAS-PCR to detect mutation of rpoB gene in M.tuberculosis 177 isolates

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178 Result MAS-PCR Total

Resistance Susceptible

PCR-ELISA

Resistance 15 4 19

Susceptible 5 9 14

Total 20 13 33

117809 Sensitivity: $15/20=75\%$ 181 Specificity: $9/13=69.2\%$ 182 P.P.V: $15/19=78.9\%$

183 N.P.V: $9/14=64.3\%$ 184 General Efficacy: $24/33=72.7\%$ 185 186 Rifampicin is one of the

most important first line drugs used against tuberculosis and it is a main marker 187 for detecting MDR-TB, so rapid detection of Rif resistant strains is very important in terms of disease 188 control

(5). The conventional methods for detecting of drug resistance such as proportional drug 189 susceptibility test are time consuming and have limited sensitivity, despite high specificity as well as its 190 dangerous for laboratory personnel (22). Nowadays, in developed countries rapid, accurate and sensitive 191 molecular methods to detect mutation in genes associated with drug resistance

in M.tuberculosis have 192 become developed (23-26). Since Iran is known as an endemic region for Tuberculosis along with the 193 high rate of MDR-TB (8), it is critical for us to detect Rif

resistant TB strains by rapid, sensitive and 194 accurate tests such as molecular assays. For this reason, we tried to set up a captured probe based PCR 195 method and detection of amplicons

based on colorimetric assay (ELISA) to detect Rif resistant strains in 196 our laboratory. This assay was compatible with real-time PCR, a new rapid, sensitive, accurate and 197 specific method to

detect SNP. In this study, 65 clinical samples of *M.tuberculosis* complex isolated from 198 Iranian patients growth on culture media with known pattern of DST was evaluated by two molecular 199 assays in a blinded study. A multiplex allele specific priming of PCR (MAS-PCR) had been set up in our 200 laboratory lately. Additionally, our study displayed the outcome of this assay was absolutely in 201 accordance with DST pattern; hence the results of new assay, PCR-ELISA, were compared with that. 202 Based on our experience, *rpoB* mutations were identified in all clinical isolates of Rif^r *M.tuberculosis* by 203 MAS-PCR. That means the mutation in *rpoB* gene could be detected in all of Rif^r strains by MAS-PCR 204 assay. The result of this test was agree with previous studies showed that >96% of Rif^r *M.tuberculosis* 205 strains contain mutations within the RRDR and >70% of these mutations occur in codons 531 and 526 (3, 206 13, 14, 27-29). Our study showed the rate of mutation in 531 codon (29 from 65 isolates 44.6%) was 207 accordance with the other studies (30-33). In contrast, based on our result, the second frequent mutant 208 codon was 516 (7/65 about 10.8%). It seemed that the lower rate of mutation in 526 in comparison with 209 516 codon was due to the low quantities of studied samples. If the study has been lasted and more many 210 isolates analyzed, the rate of mutation in codon 526 would be higher than above. Since the main aim of 211 the study was to set up a new test and to evaluate the efficacy of that in comparison with previous assay 212 in one side and on the other side, previous studies showed the frequency of mutation of different codons 213 of *rpoB* gene of Iranian *M.tuberculosis* isolates (data was not shown), it was not necessary to analyze 214 more isolates for this purpose. 215 The frequency of point mutation detected by PCR-ELISA in 516, 526 and 531 codons of *rpoB* gene in 216 *M.tuberculosis* was differed with MAS-PCR result. PCR-ELISA could be detected mutation at 531codon in 217 91% of isolates, accurately. On the other hand, this assay was also able to identify mutation at 516 codon 218 in 80% of isolates, correctly. But the ability of PCR-ELISA to detect point mutation in 526 codon was weak 219 since it was lower than MAS-PCR (1 versus 6 isolates). In spite of these differences and based on our 220 experience the reproducibility of PCR-ELISA was higher than MAS-PCR because in all experiments the 221 known mutant strains, used as control, were detected correctly by PCR-ELISA. 222 It seems that PCR-ELISA was able to detect point mutation more accurate than MAS-PCR because of its 223 mechanism. The mechanism of MAS-PCR is based on utilizing a primer with a 3' mismatch at the position 224 of the variable nucleotide together with another primer complementary to both alleles. In this case, when 225 the mismatch occurs, 3' elongation by Taq DNA polymerase is prevented. To ensure high specificity in 226 this assay, careful optimization of the concentrations of magnesium, oligonucleotide, dNTP, Taq DNA 227 polymerase, and DNA concentration, as well as the PCR cycling conditions, and the design of the Allele

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Specific Oligonucleotide (ASO) is needed. Theoretically, the possibility 228 of spurious binding could be 229 higher in this method, especially when there was an asymmetric concentration of mutant and wild type 230 bacteria in a sample. But in PCR-ELISA a set of biotinylated allele-specific capture probes was used to 231 detect a specific point mutation. As described above, when an isolate was announced as resistant that it 232 had shown signal with mutant probe while hadn't

any visible signal with wild type probes, so it could 233 reduce the amount of background signal generated from the abundant wild-type template. The result of 234 another study was showed the sensitivity of PCR-ELISA in comparison with DNA sequencing to detect 235 SNP was higher than Allele specific priming PCR (34). It seems that this theory is also true in our 236 experience, so that PCR-ELISA was detected simultaneous mutation at 531 and 516 codon in 3 isolates 237 in which mutation was detected at just 516 codon by MAS-PCR. As you know synthetic DNA probes, 238 under high stringency conditions, will only anneal to their complementary target sequences (35, 36). This 239 experiment was set up in a very stringent condition, so it seemed that the PCR-ELISA was more 240 accurate to identify a specific mutation and to indicate the region in which the mutations was located as 241 well as to detect the simultaneous mutations in the rpoB gene. To determine the accuracy of PCR-ELISA 242 in detection of point mutation in rpoB gene, the result of two molecular assays should be compared with 243 DNA sequencing as a gold standard of molecular assays. It should be noted that knowledge of the 244 specific mutation conferred resistance is not necessary for efficient patient management. Therefore, we 245 ignored to determine the accurate location of point mutation and its type of mutation. 246 The second aim of this study was to evaluate the ability and efficiency of PCR-ELISA to determine the 247 rifampicin resistant strains. Since the result of MAS-PCR was completely concordance with the result of 248 DST, we compare two molecular assays with each other. To determine the point mutation of rpoB gene, 249 the amplified dig-labeled fragments were hybridized by specific oligonucleotide probe including wild type 250 and mutant, separately. A series of probes were used that they were complementary to wild type or 251 mutant sequence of rpoB gene. They were designed so that the mutant base was placed in the middle of 252 their sequences. So, the specificity of probes to align to their complementary sequences was become 253 higher. On the contrary, allele specific primers were designed so that the mutant bases were placed at 254 the 3' end of their sequences. Therefore, absence of a visible band on the agarose gel indicated the 255 mutation. We expected PCR-ELISA was more sensitive and specific and by applying this accurate 256 method not only the rifampicin susceptibility M.tuberculosis status could be predicted in clinical isolates 257 but also it could be predicted the kind of mutation in the rpoB gene. The results showed that in spite of 258 our imagination the new test was not too sensitive to detect mutations in rpoB gene. Overall, the 259 sensitivity and specificity of PCR-ELISA in comparison with MAS-PCR was low (75% and 69.2%, 260 respectively). Although the positive predictive value was relatively high but it could not be applied for 261 detecting susceptibility M.tuberculosis because of the importance of Rifampicin as a marker to detect 262 MDR-TB. Our result was in contrast with the other study showed that PCR-ELISA system was a good 263 assay in identifying Rifr strains (37). 264 On the other hand, based on our calculating the cost of PCR-ELISA was too expensive in our country. In 265 spite of implementing in house PCR in our laboratory the detection procedure was depending on an 266 ELISA detection kit. This kit was so expensive and it was not available all of the time in our country. 267 Therefore, this assay cannot be suggested as an alternative molecular method for detecting Rifr strains.

268 4.Conclusion

269 The study showed the sensitivity and specificity of PCR-ELISA to detect mutation in rpoB gene

was low. 270 On the other hand, this was a complex and relatively time consuming as well as expensive test. 271 Therefore, this test is not suggested for determining the Rif resistant M.tuberculosis strains. The authors 272 suggest performing DNA sequencing as a gold standard for molecular assays to determine the accurate 273 kind of point mutation in each method.

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275 COMPETING INTERESTS

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

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AUTHORS' 277 CONTRIBUTIONS

278 'Author A' and 'Author C' managed the analyses of the study. 'Author C' managed the literature searches. 279 'Author B' designed the study, performed the statistical analysis, wrote the protocol, and wrote the first 280 draft of the manuscript. All authors read and approved the final manuscript."

281 282 Ethical approval

283 The work has been approved by the ethical committees of Mycobacteriology Research Center. A Written 284 informed consent has been obtained from all subjects.

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