

Performance Assessment of the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method for Rapid Detection of Susceptibility to Ethambutol and Molecular Prediction of Extensively Drug-resistant Tuberculosis in Clinical Isolates of *Mycobacterium tuberculosis*

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ABSTRACT

Introduction: The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was employed for rapid detection of ethambutol (EMB) resistant clinical isolates of *Mycobacterium tuberculosis*.

Materials and Methods: From 182 clinical isolates of *M tuberculosis* collected from different regions, 103 strains were entered in the investigation. DNA was extracted by Chelex 100 method and PCR was performed using specific primers for embB gene. Polymerase chain reaction products were digested with HaeIII and NlaII restriction endonucleases and the patterns of restriction fragments were analysed. Some randomly selected samples were sequenced.

Results: Out of 103 studied strains, 52 were resistant to EMB. The cases of secondary tuberculosis were 53 (51.50 ± 1.77%), and primary cases 50 (48.50 ± 1.77%; $p > 0.05$). From 63 extensively drug-resistant (XDR), pre-XDR and multidrug-resistant (MDR) isolates, 27 (87%), 18 (81.8%) and 7 (70%) strains were resistant to EMB, respectively. Results of PCR-RFLP method showed that from 27^R EMB XDR isolates, 13 (sensitivity 48% with CI: 0.307, 0.66 and specificity 100%), from 18^R EMB pre-XDR strains, 4 (sensitivity 22% with CI: 0.09, 0.45 and specificity 100%) and of 7^R EMB MDR, 2 (sensitivity 28% with CI: 0.082, 0.64 and specificity 100%) had mutation in ATG-Met codon 306. Results of sequencing were concordant with RFLP method. Overall, sensitivity of the molecular method was 36.5% (CI: 0.09, 0.45) and specificity 100%. None of the 40 pansusceptible strains was embB306 mutants. Extensively drug-resistant strains had a higher proportion of embB306 mutants (43%) than pre-XDR and MDR isolates (odds ratio 6.78; $p < 0.001$).

Conclusion: Fast detection of susceptibility to EMB drug is possible by PCR-RFLP. The embB306 locus is a candidate marker for rapid prediction of high resistance of MDR and XDR forms to anti-tuberculosis drugs using this method.

Keywords: Ethambutol, *Mycobacterium tuberculosis*, PCR-RFLP, susceptibility

Evaluación del Rendimiento del Método de Polimorfismo de la Longitud de los Fragmentos de Restricción Amplificados por Reacción en Cadena de la Polimerasa (PCR-RFLP) para la Rápida Detección de la Susceptibilidad al Etambutol y la Predicción Molecular de la Tuberculosis Extremadamente Resistente a los Fármacos en los Aislados Clínicos de *Mycobacterium tuberculosis*

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RESUMEN

Introducción: El método de polimorfismo de la longitud de los fragmentos de restricción amplificados por reacción en cadena de la polimerasa (PCR-RFLP) fue empleado para la detección rápida de aislados clínicos de *Mycobacterium tuberculosis* resistentes al etambutol (EMB).

Materiales y métodos: De 182 aislados clínicos de *M. tuberculosis* recogidos de diferentes regiones, 103 cepas fueron tomadas para la investigación. Se extrajo el ADN por el método de Chelex100 y se realizó el PCR usando iniciadores específicos para el gene *embB*. Los productos de la reacción en cadena de la polimerasa fueron digeridos con endonucleasas de restricción HaeIII y NlaII, y se analizaron los patrones de fragmentos de restricción. Algunas muestras seleccionadas al azar fueron secuenciadas.

Resultados: De 103 cepas estudiadas, 52 fueron resistentes al EMB. Los casos de tuberculosis secundaria fueron 53 ($51.50 \pm 1.77\%$), y los casos de tuberculosis primaria 50 ($48.50 \pm 1.77\%$; $p > 0.05$). De 63 aislados (XDR), pre-XDR y multirresistentes (MDR) extremadamente resistentes a los medicamentos, 27 (87%), 18 (81.8%) y 7 (70%) cepas fueron resistentes al EMB, respectivamente. Los resultados del método PCR-RFLP mostraron que de 27 aislados XDR^{EMB} 13 (sensibilidad 48% con IC: 0.307, 0.66 y especificidad 100%); 18 cepas pre-XDR^{EMB}, 4 (sensibilidad 22% con IC: 0.09, 0.45 y especificidad 100%) y de 7 MDR^{EMB}, 2 (sensibilidad 28% con IC: 0,082, 0.64 y especificidad 100%) presentaron mutación en el codón 306ATG-Met. Los resultados de la secuenciación estuvieron en concordancia con el método RFLP. En general, la sensibilidad del método molecular fue 36.5% (IC: 0.09, 0.45), y la especificidad 100%. Ninguna de las 40 cepas pansusceptibles fue mutante *embB306*. Las cepas extremadamente fármacorresistentes tuvieron una mayor proporción de mutantes *embB306* (43%) que los aislados pre-XDR y MDR (odds-ratio 6.78; $p < 0.001$).

Conclusión: Mediante el PCR-RFLP, es posible la detección rápida de susceptibilidad al fármaco EMB. El locus de *embB306* es un marcador candidato para la predicción rápida de alta resistencia de formas MDR y XDR a medicamentos contra la tuberculosis utilizando este método.

Palabras claves: Etambutol, *Mycobacterium tuberculosis*, PCR-RFLP, susceptibilidad

West Indian Med J 2015; 64 (4): 326

INTRODUCTION

Mycobacterium tuberculosis was identified and described in 1882 by Koch. After more than 50 years since the first anti-tuberculosis drug, almost one-third of the world's population is thought to have been infected with *Mycobacterium tuberculosis*, and about two million people die from tuberculosis (TB) annually. During recent years, due to the incidence and spread of drug resistance, in 1993, the World Health Organization (WHO) declared TB to be a global health emergency (1, 2).

Ethambutol (EMB) is the frontline anti-TB drug used in combination with other drugs. Ethambutol targets the Mycobacterial cell wall through interaction with arabinosyl transferases involved in arabinogalactan (AG) biosynthesis. It specifically inhibits the polymerization of cell-wall arabinan of arabinogalactan, induces the accumulation of mycolic acid, prevents mycolic acid from entering the Mycobacterium cell wall, and finally triggers cell death. Mycobacterium uses different mechanisms to escape being killed by a drug. One of these mechanisms is to create mutation in the genes which encode the target proteins of a drug. Mycobacterium mb CAB operon contains three joined genes of *embA*, *embB* and *embC* that encode three arabinosyl transferases homologous. The *embB* gene encodes arabinosyl transferase, which is the target protein of EMB (3–5). Ethambutol resistance is thought to occur due to mutations in *embB* gene of *Mycobacterium tu-*

berculosis. The most frequent mutation occurs at the 306 amino acid position (codon Met 306) in *embB* gene (6, 7).

Mutations lead to replacement of amino acid residues of methionine, by three amino acids: valine, leucine and isoleucine. Five different novel mutations found in this codon that occur in the first and third bases of codon 306 in the *embB* gene include ATG to GTG, CTG, ATA, ATC or ATT (8).

The aim of this work was application of a rapid molecular method, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), to determine the susceptibility to EMB by detection of mutations in the first and third bases of codon 306 in the *embB* gene.

MATERIALS AND METHODS

One hundred and eighty-two clinical isolates of *M tuberculosis* from the bank of Tuberculosis and Pediatric Infections Research Center, Arak University of Medical Sciences, Arak, Iran, were evaluated and 103 isolates were selected for the study. Drug susceptibility testing for EMB was determined by culture method (proportion).

DNA Extraction

Bacterial DNA was extracted by Chelex 100 as described in a previous study (9). Briefly, 0/07 g of Chelex 100 was placed in 270 µl buffer TAEIX; some new colonies of bacteria were

added and then centrifuged strongly and kept at 95 °C for 45 minutes. During this process, the cell wall is destroyed and DNA is released and intense centrifugation is then performed again. During the third stage, the Chelex particles are fully separated. The remaining Chelex particles could be inhibitors for PCR. Thus, after heating, centrifugation was performed for 10 minutes at 14 000 rpm; then the supernatant was removed and centrifuged again twice. The final supernatant was used as DNA for PCR.

PCR reaction

In order to investigate the mutation in *embB* gene, the extracted DNA by specific primers EMB306A, EMB306B was used in the PCR reaction. Amplification of the target gene was conducted in thermal device by touchdown programme [Table 1] (8).

Table 1: Touchdown programme for amplification of *embB* gene

Periods	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 minutes	1 cycle
	97 °C	1 minutes	1 cycle
Denaturation	95 °C	1 minutes	2 cycles
Annealing	64	30 seconds	
Extension	72 °C	1 minute	
Denaturation	95 °C	1 minute	2 cycles
Annealing	60	30 seconds	
Extension	72 °C	1 minute	
Denaturation	95 °C	1 minute	2 cycles
Annealing	58	30 seconds	
Extension	72 °C	1 minute	
Denaturation	95 °C	1 minute	20 cycles
Annealing	56	30 seconds	
Extension	72 °C	1 minute	
Final extension	72 °C	10 minutes	1 cycle

PCR-RFLP

In order to identify mutations in the *embB* gene and differentiation of susceptible strains from EMB-resistant forms, the PCR product was used in two ways for enzyme restriction.

A) *HaeIII* enzyme

HaeIII enzyme can cut the fragment and be used to determine mutations in codon 306, as shown in Fig. 1.

B) *NlaIII* enzyme

Incision created by this enzyme is visible in Fig. 2. The occurrence of mutations in codon 306 causes non-recognition of site by enzymes and failure to make incision.

Restriction fragment length polymorphism products were used to determine the size of the obtained digested fragments electrophoresed on agarose gel 1/8% with low voltage (70 V) for one hour and also with polyacrylamide gel 8% simultaneously. After electrophoresis, the respective bands

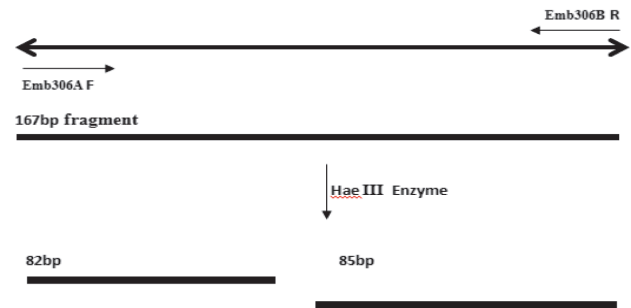


Fig. 1: Cutting action of *HaeIII* enzyme on a 167 bp polymerase chain reaction product of *embB* gene.

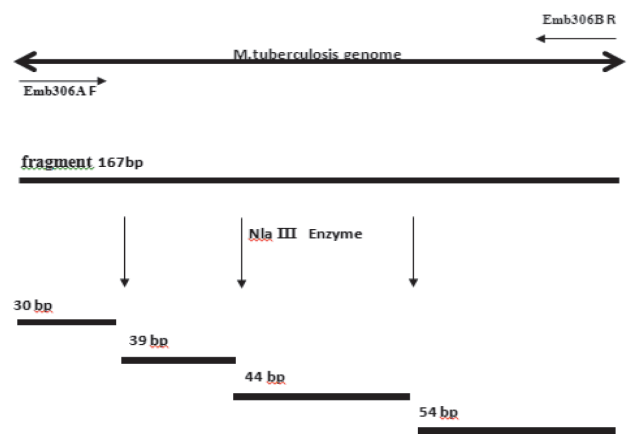


Fig. 2: Endonuclease activity of *NlaIII* enzyme on a 167 bp polymerase chain reaction product of *embB* gene.

were observed and photographed. To ensure accurate results, some steps were repeated two to three times.

Sequencing

To evaluate mutation determination by PCR-RFLP, sequencing method as the gold standard was accomplished by an Applied Biosystem apparatus. Moreover, standard strain H37Rv was studied in the same way.

RESULTS

M tuberculosis isolates

From 103 isolates examined, 52 strains were phenotypically resistant to EMB and 40 strains were pansusceptible to all drugs. Furthermore, 11 resistant strains were susceptible to EMB. Secondary TB cases were 53 (51.50 ± 1.77%) and primary cases, 50 [48.50 ± 1.77%] ($p > 0.05$).

All isolates based on resistance behaviour were divided into four groups as extensively drug-resistant (XDR), pre-XDR, multidrug-resistant (MDR) and susceptible to EMB. Totally, from 52 EMB-resistant strains, 27 (87%) were XDR, 18 (81.8%) were pre-XDR and seven (70%) were MDR strains.

PCR results

Polymerase chain reaction of *embB* gene on all isolates and standard strain H37Rv showed bp 167 band. Figure 3 shows the electrophoretic pattern of gene *embB*.

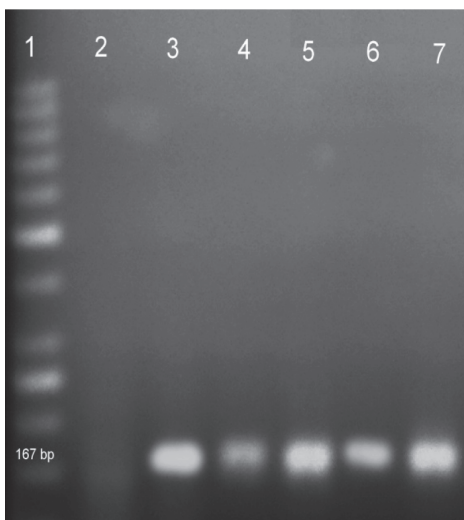


Fig. 3: Electrophoretic pattern of gene *embB*.
1: size marker (50 bp); 2: negative control; 3–7: 167 bp band from clinical isolates

PCR-RFLP results

A) Digestion by *NlaIII* enzymes

In this method, cutting enzyme products in RFLP, as anticipated by the Genetyx software, created 39, 74, 54 bp bands for resistant strains and 30, 39, 44, 54 bp bands for susceptible strains. This method was conducted for 23 strains, but was excluded considering lightness of bands and probability of error in the procedure (Fig. 4).

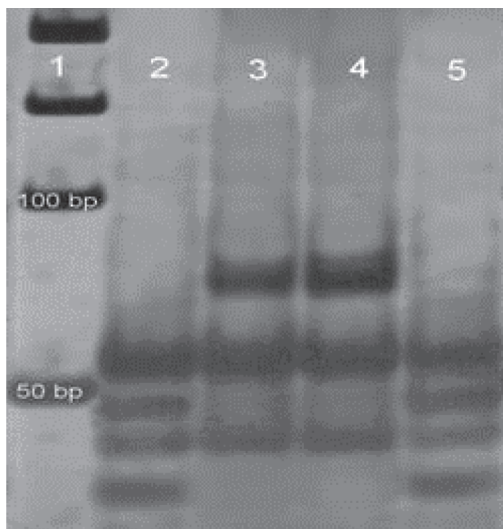


Fig. 4: Restriction fragment length polymorphism patterns on polyacrylamide gel 8% *embB* gene after enzymatic digestion by *NlaIII*.

1: size marker (50 bp); 3, 4: ethambutol-resistant strains with the band (39, 74, 54 bp); 2, 5: ethambutol-susceptible strains with the band (30, 39, 44, 54 bp)

B) Digestion with *HaeIII* enzyme

The results demonstrated the feasibility of adequately distinguishing between susceptible and resistant strains pattern by *HaeIII* enzyme in routine work. Thereby, susceptible strains constituted 85 and 82 bp bands and resistant strains formed the 167 bp (Fig. 5).

In this way, 82, 85 bp bands are considered to be practically one band. So, the agarose method can easily be used to check the results instead of the acrylamide method that is time consuming and laborious.

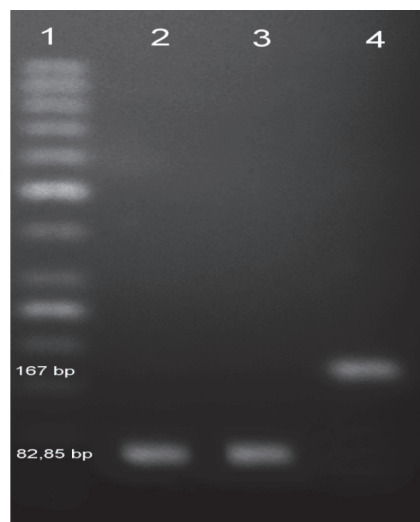


Fig. 5: Restriction fragment length polymorphism patterns *embB* gene on agarose gel after digestion with enzyme *HaeIII*.

1: size marker (50 bp); 2, 3: ethambutol-resistant strains with two bands (85, 82 bp); 4: ethambutol-susceptible strains with the band (167 bp).

As shown in Table 2, overall, 103 strains consisting of 63 resistant and 40 pansusceptible isolates were entered in the study. From 31 XDR isolates, four were susceptible to EMB and 27 were resistant to the drug. From 27^R EMB XDR isolates, 13 strains had mutation in ATG-Met codon 306 (sensitivity 48% with CI: 0.307, 0.66 and specificity 100%). From 18^R EMB pre-XDR strains, 4 (sensitivity 22% with CI: 0.09, 0.45 and specificity 100%) and of 7^R EMB MDR, 2 (sensitivity 28% with CI: 0.082, 0.64 and specificity 100%) were mutant in codon 306.

Overall, sensitivity of the molecular method was 36.5% (CI: 0.09, 0.45) and specificity was 100%. Results of sequencing were concordant with the RFLP method. None of the 40 pansusceptible strains was *embB306* mutants, and non-mutant H37Rv strains were detected with this method.

Extensively drug-resistant strains had a higher proportion of *embB306* mutants (43%) than pre-XDR and MDR (odds ratio 6.78; $p < 0.001$). Therefore, this method has the sensitivity and specificity of 100% for determining susceptible strains. Thus, the test is capable of 100% accuracy in determining the critical strain that is clinically very valuable.

Sequencing results

To evaluate the accuracy of the PCR-RFLP results, sequencing, as the gold standard method, was used for the molecular phase. Complete conformity (100%) of the sequencing method with PCR-RFLP results reflects the accuracy of the used technique (Table 2).

test requires bacterial culture (one month) and resistance assessment (one other month). Thus, a procedure which could determine resistance or sensitivity to antibiotics in a few hours would be extremely worthwhile.

Given the relatively high number of TB patients and the necessity to determine drug resistance, especially in the early

Table 2: Results of molecular and phenotypical methods

Resistance	Ethambutol resistance					
	Phenotypical (proportion method)			Molecular method		
	Total tested	R	S	Total tested	R	S
XDR	31	27 (87%)	4 (13%) 4 ^S	27 ^R -	13 (48%) 4 (100%)	14 (52%)
Pre-XDR	22	18 (81.8%)	4 (18.2%) 3 ^S	18 ^R -	5 (27.7%) 3 (100%)	13 (72.2%)
MDR	10	7 (70%)	3 (30%) 3 ^S	7 ^R -	1 (14.3%) 3 (100%)	6 (85.7%)
Pan-Susc	40	-	40 (100%)	40 ^S	-	40 (100%)

R: resistant; S: susceptible; XDR: extensively drug-resistant; MDR: multidrug-resistant; Pan-Susc: pansusceptible

DISCUSSION

This study presents a fast method for determining susceptible strains by an in-house PCR-RFLP method instead of sequencing procedures and commercial kits. In this way, simplification of the technique, speeding up of the process using agarose gel instead of polyacrylamide, and the enzyme *HaeIII* (rather than *NlaIII*) have been tried to help the fast determination of susceptibility to EMB in routine work.

It was found that by molecular detection of mutation in *embB306*, it may predict high resistance, consisting of MDR and XDR forms, to anti-tuberculosis drugs. By the increase of resistance due to mutations, the chance of resistance to EMB would be increased. Mutation in *embB306* was increased in MDR, pre-XDR and XDR, respectively. Extensively drug-resistant and pre-XDR isolates had a higher proportion of mutants (43%) than MDR. None of the 40 pansusceptible strains harboured an *embB306* mutation.

We hypothesized that by molecular detection of mutation in *embB306*, it might predict high resistance, consisting of MDR and XDR forms, to anti-tuberculosis drugs. The theory would be supported by absence of mutations in susceptible strains. Furthermore, it was shown that discrepancy between the results of phenotypic EMB resistance method and molecular tests in this study was restricted to the isolates already resistant to other anti-TB drugs.

Given the increasing proliferation of drug-resistant tuberculosis, and the therapeutic need for TB control, providing methods for rapid detection of drug sensitivity or resistance of *M tuberculosis* clinical strains is necessary.

According to the treatment protocol for TB, after four months of first-line prescription drugs, in case of the probability of resistance (positive being on monthly sputum smear tests), tests are performed to determine drug resistance. This

days, further simplification of the test and reduction of error in each method would be crucial in terms of preventing the spread of resistant strains in the community. Thus, providing a quick and convenient method for the identification of such resistance will reduce the spread of drug-resistant strains.

The main method for studying mutations in genes associated with resistance is to determine possible mutations using sequencing. This technique is expensive and laborious and it is impossible to purchase equipment and supply professional staff in all centres. Samples would need to be sent to specially equipped centres. This process is often time consuming; therefore this technique cannot be used routinely.

Using kits often requires costly equipment and laboratories are perpetually dependent on these kits. Moreover, the cost of implementing the test also increases. Liquid culture-based methods (*eg* MGIT) are too expensive, and there is no possibility of using them routinely; therefore, simpler methods are preferred in routine use.

In routine molecular technique methods, called in-house, a variety of PCR methods are easily modified according to the changing circumstances.

According to our findings, PCR-RFLP technique presented can be used as a rapid and simple method for determining the sensitivity to EMB in *M tuberculosis* strains.

According to Ahmad *et al*, *EmbB497* codon covers a low percentage of resistant strains (8). Shen *et al* (6), Plinke *et al* (3) and numerous authors have exclusively evaluated mutations of this gene in the clinical strains by examining codon *embB306*. Therefore, study of codon *embB497* is neglected with the aim of reducing further simplification of the procedure, and making it possible to perform routinely. Codon 306 has been the focus in this research.

Two endonuclease enzymes can be used to implement the method: *NlaIII* and *HaeIII*, both used in this study.

The *NlaIII* method was abandoned for two reasons: 1) creating very light and a large number of bands (30, 39, 44, 54, bp) and 2) the necessity of using polyacrylamide gels for electrophoresis.

The *HaeIII* method is based on the study of two bands, 85 and 82 bp, in susceptible strains and detection of only one bp167 band for resistant strains. Thus, the *HaeIII* technique is preferred due to ease of separation of susceptible and resistant strains and the possibility of using agarose gel, and it is recommended as a routine technique.

During the literature review, no study on these genes in clinical strains of *Mycobacterium tuberculosis* was found inside the country using the PCR-RFLP technique. Ahmad *et al* in 2004 presented the PCR-RFLP method to identify the mutations, in which out of seven studied resistant strains to EMB, four strains were identified with mutations in these genes (8).

In the present study, this technique was used successfully on the 63 resistant strains.

No exact compliance of culture results (*ie* phenotype) with the results of molecular (*ie* genotype – to determine occurrence of *embB* gene mutations in different codons) in determining drug resistance to EMB has been proven by other investigators (Table 3). However, given the importance of this drug and the need for rapid detection of resistance, providing results with any degree of accuracy is preferred to the lack of results in the first few months of treatment.

In their research, Huang *et al* studied 162 strains resistant to EMB and 72 susceptible strains using kits; 9.3% of resistant strains had no mutations in *embB* gene. Among the 72 sensitive strains without mutations in codon 306, three isolates had a mutation in codons 297 and 328 (10). Adaptation rate between phenotypic assessment of culture and two genotypic assays for resistance to EMB was 68.4% by GenoType MTBDRs1 kit and 92.3% with sequencing. In the 162 resistant strains, 56.2% of mutations associated with resistance to EMB were detected by the kit and 90.8% with sequencing (10). Our findings confirmed their results that case study using kits and also in-house technique covers only some codons while the sequencing procedure explores whole mutations in the entire gene. The main issue in the rapid detection tests of drug resistance in TB is speed and low cost. Given the emphasis of different research, in-house methods and kits were found to meet physician needs of determining the resistant strains isolated from patients; thus, the emphasis of this study was using the PCR-RFLP method.

There are several limitations of using the GenoType tests in clinical detection, including high costs and low sensitivity in detecting resistance. In addition, GenoType kits require DNA replication and the colouring process may provide different interpretations of the results due to different devices of replication and various colouring time. In addition, the banding patterns of the genotype tests are not always obvious and

trained technicians are needed to interpret the test results (10).

Brossier *et al* investigated 52 clinical isolates of *M tuberculosis* using MTBDRs1 techniques and DNA sequencing. Sensitivity and specificity of MTBDRs1 test for EMB was 57% and 92%, respectively (11). Analysis showed conflicting results; among the 28 strains resistant to EMB, 16 strains were reported mutations in the *embB* gene. In contrast, the remaining 12 strains resistant to EMB (43%) showed no mutation in *embB* by DNA sequencing and wild-type pattern obtained from the MTBDRs1 measurement. Totally, MTBDRs1 test (57%) identified strains resistant to EMB. Also, among the 24 strains susceptible to EMB, 22 cases had no mutation in codon 306 *embB* gene and two strains susceptible to EMB were associated with mutations (11).

In this study, this technique had high sensitivity and specificity for determining the susceptible strain. Thus, culture results do not comply fully with the molecular results (determine the occurrence of mutations with low sensitivity, besides high specificity). The precision of determining resistant strains by molecular method has been always debated in previous research (3–6, 11). In most of the studies conducted by other researchers, no mutation in sensitive strains was found (10).

Ahmad *et al* in two studies in 2004 and 2007 conducted molecular analysis of sensitive strains and reported no mutation (8, 12). The same result can be observed in other studies presented in Table 3 (13, 14).

In the present study, of 40 studied strains susceptible to EMB, none had *embB* gene mutations. The sequencing results also confirmed lack of mutations in the whole gene, corroborating the results of other investigators.

According to the scientific resource reviews outlined in Table 3, the vast majority of studies were performed with a large number of samples, with several authors finding no sensitive phenotype of strains with mutations in codon 306 (15, 16). These results are quite compatible with the results of this research. Other researchers found mutation in codon 306 in less than 10 sensitive strains (11, 17).

According to research conducted by Ahmad *et al*, the relationship of resistance to EMB with resistance to multiple drugs as well as isoniazid has been proven in *M tuberculosis* (8, 12). In their study, analysis of 157 strains selected from a collection of more than 2000 clinical isolates of *Mycobacterium tuberculosis* revealed that isoniazid resistance can be seen in all strains resistant to EMB. In strains with a high resistance to EMB, high resistance to isoniazid was also observed. Despite low phenotypic and genotypic matching in resistance to EMB, rate of resistance determining and identifying resistant strains (with the possibility of multiple resistances) is very important, and can completely justify implementation of rapid molecular detection technique (with a sensitivity of about 50%) [12]. The authors suggested that isolates of *M tuberculosis* with mutation in *katG315* are very similar to mutant strains in *embB306* with high resistance to EMB (12).

Results of the present study confirmed the results of Ahmad *et al* (8, 12). A strain with a mutation in *embB* gene is

Table 3: Evaluation of other researchers' results in using molecular methods. Phenotypic and genotypic noncompliance and specificity being variables as well as mutations in the susceptible strains are visible in all molecular approaches

Researchers	Mutation at 306 codon in sensitive strains	No. of sensitive strains	Total sample	Methods	Per cent compliance	Sensitivity	Specificity
Kiet, 2010 (18)	–	–	53	MTBDRsl test	69.4 %	64.2%	100%
Wyant, 2011 (17)	14	160	314	DNA sequencing	–	78.6%	93.1%
Nam, 2010 (16)	–	–	113	Direct DNA sequencing	–	69.2%	97.5%
Shimizu, 2008 (19)	–	–	48	DNA microarray	–	90%	89.0%
Huang, 2011 (10)	3 mutations at other codons	72	243	GenoType MTBDRsl test	68.4%	95.8 %	56.2%
		72	243	DNA sequencing	92.3%	–	90.7%
Palomino, 1999 (13)	–	75	101	MGIT	–	96.1%	100%
Belay, 2012 (15)	–	–	260	GenoType® MTBDRsl	–	42%	100%
Mubayi, 2011 (20)	–	–	286	Nitrate reductase	96.6%	94.2%	99%
Sienkiewicz, 2008 (21)	–	–	90	Nitrate reductase	–	75%	100%
Brossier, 2010 (11)	2	24	52	MTBDRsl	–	57%	92%
				and DNA sequencing	–		
Hillemann, 2009 (22)	–	43	106	MTBDRsl	–	59%	100%
				and DNA sequencing	–		

resistant to EMB, and there is the possibility of resistance to other drugs in this strain; therefore, the epidemiology of *embB* mutant strains should be considered.

CONCLUSION

Results showed that PCR-RFLP can be used as a simple and rapid method for detection of EMB susceptibility in TB strains. Our results confirmed that *embB306* mutation was a useful marker for predicting MDR and XDR forms. *embB306* mutations may predispose isolates to the development of increasing resistance to antibiotics. Association between mutation in *embB306* and resistance to drugs was highly significant ($p < 0.001$).

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