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KLİNİK ÇALIŞMA
RESEARCH ARTICLE

Analysis of *rpsL* and *rrs* genes mutations related to streptomycin resistance in Mdr and Xdr clinical isolates of *Mycobacterium tuberculosis*

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SUMMARY

Analysis of *rpsL* and *rrs* genes mutations related to streptomycin resistance in Mdr and Xdr clinical isolates of *Mycobacterium tuberculosis*

Introduction: Streptomycin is a bactericidal and aminoglycoside antibiotic. It is one of the most effective drugs for treatment of multi-drug Tuberculosis disease. Incidence of resistance is increasingly reported. Its action mechanism is by inhibition of binding aminoacyl tRNA to position "A" in elongation phase, which finally it causes to stop bacterial protein synthesis. In this study, resistance rapid investigation to streptomycin was conducted in clinical strains of *Mycobacterium tuberculosis*.

Material and Method: In this study, among 105 strains of phlegm-positive and culture-positive *Mycobacterium tuberculosis*, 45 strains of resistant and sensitive to streptomycin were selected for possible mutations examination in genes *rrs* and *rpsL*. Specific primers that used for PCR were named *rpsL* 1, *rpsL* 2 and *rrsR*, *Frrs*. PCR products were sequenced.

Results: PCR Products represents 504 bp band for gene *rpsL* and 1027 bp for gene *rrs* that shows proper selection of primers and determining an amplification appropriate program. From 26 resistant strains to streptomycin 26 strain have mutation in *rpsL* gene and 1 strain have alteration in *rrs* gene. In this study 19 strains were sensitive to streptomycin that have no mutation in these gene.

Conclusions: Streptomycin resistance is mainly related to mutation at codons 43 and 88 "*rpsL*" gene and to a lesser extent "*rrs*" that are the greatest cause of drug resistance to streptomycin.

Key words: *Mycobacterium tuberculosis*, Streptomycin, drug resistance, *rpsL* gene, *rrs* gene

ÖZET

Mdr ve Xdr *Mycobacterium tuberculosis* örneklerinden streptomisin direncinden sorumlu *rpsL* ve *rrs* gen mutasyonlarının analizi

Giriş: Streptomisin, çok ilaca dirençli tüberküloz tedavisinde en etkili ilaçlardan birisidir. Bakterisidal aminoglikozid bir antibiyotiktir

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ve direnç giderek artmaktadır. Streptomisinin etki mekanizması aminoasit tRNA'nın uzama fazında "A"ya bağlanmasını engeller ve sonuç olarak bakteriyel protein sentezini durdurur. Bu çalışmada *Mycobacterium tuberculosis*'in klinik örneklerinde streptomisin direncinin hızlı incelemesi çalışılmıştır.

Materyal ve Metod: Bu çalışmada balgam ve kültür pozitif 105 *M. tuberculosis*'ten 45 tane streptomisine dirençli ve duyarlı örnek seçilmiş ve *rrs* ve *rpsL* genlerindeki mutasyonlar açısından çalışılmıştır. Polimeraz zincir reaksiyonu (PCR) için kullanılan spesifik primerler *rpsL* 1, *rpsL* 2, *rrsR* ve *Frrs* olarak adlandırılmıştır. PCR ürünleri sekanslanmıştır.

Bulgular: *rpsL* geni için 504 bp bandı ve *rrs* geni için 1027 bp bandı gösteren PCR ürünleri, uygun primerlerin seçimi ve uygun amplifikasyon programını sağlamıştır. Streptomisine dirençli 26 örnekten 26'sında *spsL* geninde mutasyon ve birinde *rrs* geninde değişiklik vardı. Bu çalışmada streptomisine duyarlı 19 örnekte mutasyon saptanmadı.

Sonuç: Streptomisin direncinden *spsL* geninde 43 ve 88 kodonlarda ve nadiren *rrs* genindeki mutasyonlar sorumludur.

Anahtar kelimeler: *Mycobacterium tuberculosis*, streptomisin, ilaç direnci, *rpsL* geni, *rrs* geni

INTRODUCTION

Tuberculosis was associated with mankind throughout history. With formation of societies and crowding of increased population, tuberculosis was marked as a cause for one fourth of mortality in 17th and 18th centuries in Europe, so that in industrialization in Europe, it was called white plague. Despite extending and developing technology science, especially medical science as well as relatively improvement of living standards, it remains as one of the most important and common factors of people mortality (1). It is an infectious disease with chronic nature and highly contagious that in most cases resulted from *Mycobacterium tuberculosis*. Frequent site of its involvement is lung, but it infects other members in one third of cases (2). This disease is a health problem for human societies, especially in developing societies. Within these countries, between 0.1% to 0.3% population are infected this disease each year, although disease prevalence was going to fall in early 20th century, but since 80s, disease outbreak were raised. It is very dangerous for human being so that World Health Organization (WHO) has been suggested this problem as a "global EMS" in 1991 (3).

Drug resistance to tuberculosis bacillus occurs mainly when there is a mutation in bacillus and the reason of resistance is incomplete or incorrect treatment that allows resistant bacilli mutant turn into dominated strains. The incidence of multi-drug resistant tuberculosis (MDR-TB) is rapidly increasing and its incidence estimated at 460.000 worldwide in 2005 (4). If a person who is infected with TB is not treated, on average, he/she infects 10 to 15 people each year, because TB transmission is through respiratory tract. Drug-resistant incidence caused by increasing anti-tuberculosis drugs consumption and other factors are among major

concerns. Streptomycin is from aminoglycosides family. Aminoglycosides inhibit protein synthesis by binding to ribosome, thus they have a bactericid effect (5). Streptomycin operation mechanism is that this drug acts on 16S rRNA and S12 ribosomal protein and this leads to ribosomal changes induction. These changes cause to make a mistake in reading mRNA and finally inhibition of protein synthesis (3,6,7)

Streptomycin resistance is mainly related to mutation in gene *rpsL* and in lesser amount to gene *rrs*, other factors such as changes in permeability of the wall and membrane and modifying enzymes of aminoglycosides are slightly effective. Mutation in gene *rpsL* depends on changes in codons 43 in 70% cases and codon 88 in much less cases. Streptomycin resistance occurs because of occurrence of mutation in genes such as *rpsL* and *rrs* that are respectively coding of 16SrRNA and ribosomal protein S12. They are responsible for high levels of resistance to Streptomycin (6,8-10).

MATERIALS and METHODS

DNA Extraction

CHELEX100 kit (Sigma) is used to DNA extraction. According its manufacture, 0/07 gr of CHELEX powder is saluted in 270 µl TAE1X buffer and after adding a few new colonies of bacteria and severe vertex are incubated at 95°C for 45 minutes. CHELEX particles were fully separated during 3 stages. After heating and vortex at 14.000 rpm centrifuge for 10 minutes and final supernatant as DNA is used in PCR.

Primers Design

The used primers in the present study designed by OLIGO6, MEGA4, IDT software. Sequences the designed primer and PCR conditions to amplify target genes is presented in Table 1.

PCR

For *rpsL* gene PCR mixture containing 2 mL DNA (40 ng), 2.5 mL MgCl₂, 2.6 mL *rpsL* F, *rpsL* R 0.7 macro liter of fourfold nucleotides desoxy and 0.7 mL of enzyme taq polymerase were used in a final volume of 25 mL.

For *rrs* gene, as above mentioned its desired primers was designed and PCR mixture containing 2 mL DNA (40 ng), 2.5 mL MgCl₂, 2 mL *rpsL* F, *rpsL* R, 0.7 macro liter of fourfold nucleotides desoxy mixture and 1 mL of enzyme taq polymerase were used in a final volume of 25 mL.

PCR products were loaded on 1% agarose gel (Cinagen) and suitable molecular marker 100 bp (Vivantis) were used for band measurement.

Sequencing

PCR Products were sent to Source BioScience Company in order to DNA purification and sequencing process using Applied Biosystem device. The results of sequencing were analyzed by Mega 4, Chromas and Bioedite software.

Mutation occurrence determination results were compared to resistance phenotypic results.

RESULTS

From a total of 45 studied *Mycobacterium tuberculosis* strains, 26 strain were streptomycin resistant and 19 of them were sensitive to drug. Running PCR on clinical samples, 504 bp band for *rpsL* gene and 1027 band for *rrs* gene were provided in electrophoresis gel 1% that show correct choice of primers and determining an appropriate program for amplification (Figure 1 and 2).

Sequencing result showed that from 45 samples that were sent for sequencing of *rpsL* gene, all of 26 resistant samples had mutation in *rpsL* gene and 19 sensitive samples had no mutation. Change in amino acid 121 included the highest amount of mutation. Change in position of Wobble site (third nucleotide) of codon occurred (AAA → AAG) i.e. (K-121-K). Therefore, a lysine (K) amino acid turned into another of the same amino acid, so there is no mutation in protein surface and it is only on nucleotide level.

Table 1. The information about the primers used for amplification of the target sequence in each gene, the fragment length, and the annealing temperature are summarized

Name of primer	Sequence (5-3)	Protocol of PCR	PCR product size
F- <i>rpsL</i>	5'-GGCCGACAAACAGAACGT-3'	94....5 min	502 bp
R- <i>rpsL</i>	5'-GTTACCAACTGGGTGAC-3'	94....1 min 59....1min 72....45 sec/35 cycle 72....10 min	
F- <i>rrs</i>	5'-GAGAGTTTGATCCTGGCT-3'	94....5 min	1027 bp
R- <i>rrs</i>	5'-TGCACACAGGCCACAAGG-3'	94....1 min 60...1 min 72....55 sec/40 cycle 72...7 min	



Figure 1. 504 bp bands of PCR products from *rps* gene.

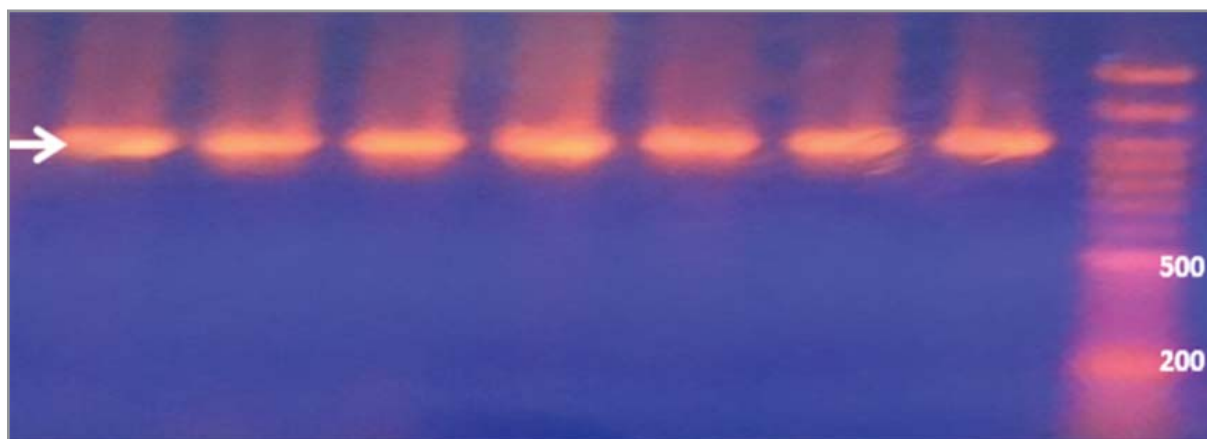


Figure 2. 1027 bp bands of PCR products from *rrs* gene.

Some changes were recorded in codon 43 that is related to nucleotide 128 and lead to the conversion of AAG to AGG, that is, a change occurred in second nucleotide that lead to conversion of amino acid lysine to amino acid arginine (R). Thus for this change, there is a mutation in protein surface.

Some changes were recorded in codon 88 that is little but it is related to nucleotide 263 and lead to the conversion of AAG to AGG and this change occur in second nucleotide that leads to conversion of amino acid lysine to amino acid arginine. Thus for this change, there is a mutation in protein surface. In samples of 725.571 (AAG → A GG) and actually the K88R i.e. lysine is transformed into arginine. It means there are a mutation in codon 88 and a mutation in codon 43 (K43R), so these samples have mutations both in 43 of 88.

In Figure 3, a result of sequencing performed on a sample that is resistant to streptomycin is shown with Chromas and Mega-4 software. Results analysis of sequencing on resistant bacteria is shown that is done by using primers R sequence *rpsL* with Bioedit software in Figure 4.

18 resistant strains were sequenced for *rrs* gene. Among 18 samples, just a resistant sample (No. 84p) has mutation in *rrs* gene. 14 sensitive strains were reported without mutation.

DISCUSSION

Tuberculosis is one of global problems and 3 million people lost their life due to this disease (11). In this study, mutations existed in *rpsL* and *rrs* genes in drug sensitive and resistant clinical strains were determined and compared with each other. Mutation in codons 43 (AAG → AGG) - 88(AAA → AAG) were reported

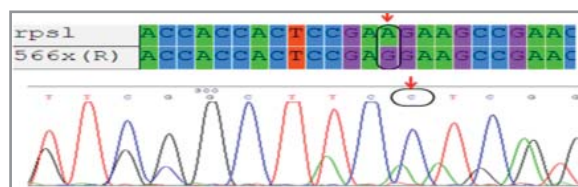


Figure 3. Sequencing results that analysis with Chromas and Mega-4 softwares from a resistance strain to streptomycin (arrow shows modified nucleotide).

as the most frequent mutations of *rpsL* gene worldwide. In present study, the most frequent mutated codons are codons 121 and 43 and 88.

Srinand sreevatsa was conducted a research to indentify mutations in *rpsL* and *rrs* in resistant mycobacterium to streptomycin, in this study, *Tuberculosis mycobacterium* 139 strains of patients in South America, Europe, Africa and Asia were examined that used sequence method and it was observed that 78 strains were resistant and 61 strains were susceptible that 54% of resistant strains had missense mutation in codon 43 of *rpsL* (k-43-R) also in codon 88, 4 Asian samples were detected. 4 strains from 8 strains that were got from Japan had mutations in (k-43-R) and had 2 strains in (k-88-Q), in addition just one strain isolated in New York among 78 resistant strains with mutations in *rpsL* and *rrs* were together as mutations in *rpsL* was 56.8% and in *rrs* was 15.6% (6,12).

In a study that was conducted by Miho Fukuda in 1999, sequencing method was used. From 121 strains isolated, 26 strains were resistant to streptomycin, 78% mutation in codon 43 and 22% in codon 88 was from *rpsL* gene and no change was reported in *rrs* gene (7). In a study that was conducted by Ryan in 2004, sequencing method was used and

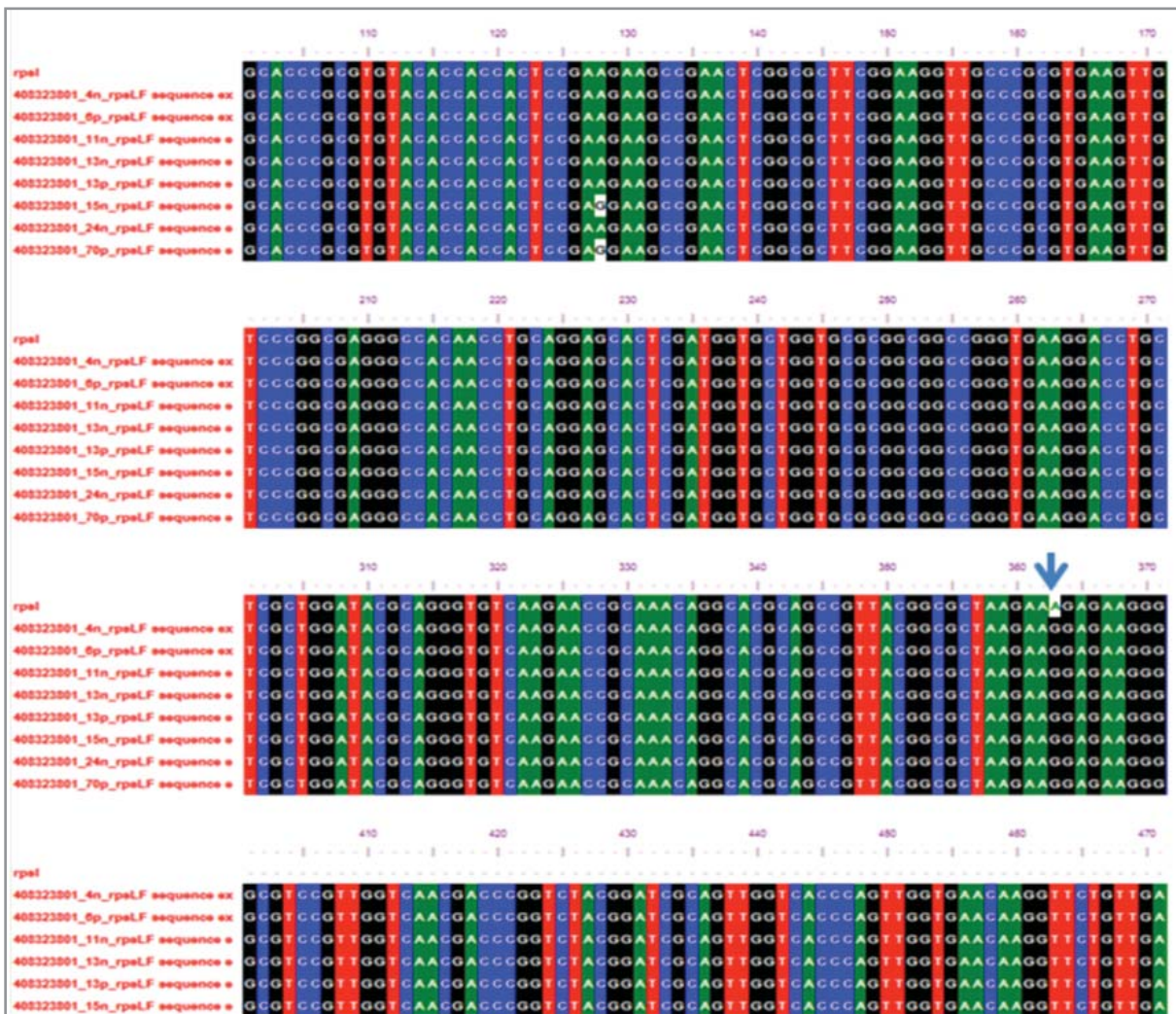


Figure 4. Sequencing results that analysis with Bio Edit Sequence Alignment Editor BLOSUM62 softwares from 6 R and S strains to streptomycin (arrows show modified nucleotide).

it was shown that two *rpsL* and *rrs* genes are responsible for tuberculosis *Mycobacterium* resistance to streptomycin, results analysis showed that in 61% cases, *rpsL* gene and in 24% cases, *rrs* gene are responsible for resistance. Using former reports that are conducted as an alternative in *rrs* and *rpsL* genes, their results show that (52-57%) in *rpsL* and (8-15%) in *rrs* had mutations and most highest mutation codon is codon 43 (Lys-Arg) (13).

In a study that was carried by WU Xue-Qiong in 2004, method of sequencing was used that 98 strains were studied, results from sequencing included 78 (79.6%) had mutation in codon 43 or 88 of *rpsL* gene (Lys>Arg), (6.1%) 6 in *rrs* gene were in (A-C 513) or (C-T 516) and (14.3%) 14 had no mutations (14).

Ozturk was conducted a research in 2005 as resistant molecular analysis to isoniazid, rifampin, and streptomycin and he used sequence method in which from total 52 samples that function of 5 were related to streptomycin and all had mutations in codon 43, therefore mutation in *rpsL* and *rrs* are responsible for over 70% of resistance to streptomycin which this result is consistent with present study (9,15).

In 2009, Shubhada identified rapid resistance to isoniazid, rifampin and streptomycin among clinical isolates of *Mycobacterium* with mutation. Among them, 75% had mutations in *rpsL* gene in codon 43. PCR multiplexing and sequence methods were referred in this study that mutations are seen in codons (43,88) *rpsL* gene and (491.513.516) in *Mycobacterium tuberculosis*. The largest mutation

Table 2. The frequency of changes codons and amino acids in 26 mutant samples that have mutation at *rpsL* gene

Row	No. modified codon	Modified Nu.	Name of sample	Modified sequence	Modified a.a.
1.	363	(A→G)	692XDR	AAA→AAG	K-121-K
2.	363	(A→G)	571XDR	AAG→AG	K-121-K
	263	(A→G)		AAG→AGG	K-88-R
3.	363	(A→G)	124XDR	AAA→AAG	K-121-K
4.	128	(A→G)	665XDR	AAG→AGG	K-43-R
5.	363	(A→G)	725XDR	AAA→AAG	K-121-K
6.	363	(A→G)	568XDR	AAA→AAG	K-121-K
7.	128	(A→G)	566XDR	AAG→AGG	K-43-R
8.	363	(A→G)	535XDR	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
9.	363	(A→G)	662XDR	AAA→AAG	K-121-K
10.	363	(A→G)	570XDR	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
11.	363	(A→G)	2n	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
12.	363	(A→G)	4n	AAA→AAG	K-121-K
13.	363	(A→G)	5n	AAA→AAG	K-121-K
14.	363	(A→G)	21n	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
15.	363	(A→G)	25n	AAA→AAG	K-121-K
16.	363	(A→G)	27n	AAA→AAG	K-121-K
17.	160	(A→G)	15n	AAA→AAG	K-121-K
18.	160	(A→G)	24 n	AAA→AAG	K-121-K
19.	363	(A→G)	26n	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
20.	363	(A→G)	7P	AAA→AAG	K-121-K
21.	363	(A→G)	130P	AAA→AAG	K-121-K
22.	363	(A→G)	6P	AAA→AAG	K-121-K
23.	363	(A→G)	9n	AAA→AAG	K-121-K
24.	128	(A→G)	734	AAG→AGG	K-43-R
25.	363	(A→G)	229XDR	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R
26.	363	(A→G)	604 XDR	AAA→AAG	K-121-K
	128	(A→G)		AAG→AGG	K-43-R

was in *rpsL* Lys43Arg that is 75% and in *rrs* gene was 12%, 2% had no mutation and 6% had mutation in position 516. 98% had phenotype DST compliance.

In his study, Pablo Bifani reviewed 26 strains using gene sequences, that 14 strains of them showed an amount of high level resistance to streptomycin and 6 sample were poly resistant, an analyze was performed to confirm that all isolated strains have a mutation in codon 43 of *rpsL* gene and change includes the conversion of lysine to threonine and no change was observed in *rrs* gene (16).

Tatjana Tracevska was conducted a research in 2004 that 66 strains were from TB patients that were resistant to Rif, NH, SM and 33 were resistant to EMB. (61%) 40 strains from 66 Isolated strain were resistant to streptomycin. From 26 remaining strains, (24%) 16 had a mutation at position 513 (A → C), 516 (C → T) of *rrs* gene and remained (15%) 10 of them were wild (17).

In a study in 2009 by Chaoui and colleagues using sequences method, from 58 strains isolated resistant to streptomycin, it was confirmed that most amount of mutations were in codon 43 of *rpsL* gene and

there was rare mutation in codon 512 of *rrs* gene. In present study also the most significant mutations were in codon 43 of *rpsL* gene (18).

In 2012, Arch Orthop Trauma Surg was done a comparison study between cultures L_J and absolute concentration with DNA sequencing for detection of drug resistant tuberculosis SPINAL/that only 7 drug resistant samples from 50 samples were recognized by culture and 34 days lasted, but by sequencing of 11 drug resistance, it was found that 6 days lasted. Majority of mutations in *rpsL* genes were in codons 43 and 88 according to previous reports (19).

In 2012, in a research, Silke Feuerriegel conducted 97 strains to identify mutations in *rpsL* and *rrs* genes and so on by sequencing method, it results showed that none of strains resistant to streptomycin in *rrs* gene had mutation but 47.5% had resistant in *rpsL* gene (20).

90-95% of mutations in mutants resistant to streptomycin had mutation in *rpsL* and *rrs* that most of them happened in bp 43 88 bp. The largest mutation in *rpsL* in codon 43 is associated with a stop in protein synthesis in bacteria. Its consequences are fully consistent with DNA sequencing. There was no mutation in susceptible isolates and 84% of isolates showed resistant to streptomycin of mutation in *rrs* and *rpsL* genes. In this study, similar to other studies *rpsL* played a more important role than *rrs* in resistant to streptomycin, mutation in *rpsL* occurs frequently in codon 43 and less in codon 88. 0.14% of isolates resistant in *rrs*, *rpsL* genes had no mutation. Findings in this study is consistent with results obtained in present study (3,15,21).

We sequenced 45 samples that were given to resistant streptomycin drug after PCR and electrophoresis on agarose gel. From 45 samples that were sent for sequencing of *rpsL* gene, all of 26 resistant samples had mutation in *rpsL* gene and 19 sensitive samples had no mutation. Also 18 resistant strains were sequenced for *rrs* gene. Among 18 samples, just a resistant sample has mutation in *rrs* gene. Number of 14 sensitive strains were reported without mutation.

Among 45 of samples resistant to streptomycin that were sequenced, 17 samples had no any mutation in bp 43 88 bp region of *rpsL* gene. Also, 26 samples had mutations in this area that are listed in the table.

Mutations at codon AAA → AAG121, AAG → AGG43, 511 and 516 were reported as the most

frequent mutations of gene I around the world. In present study, the most frequent mutated codons are codons 121 (40%) and codon 43 (20%) that in codon 121 as no change happened in slip position i.e. third nucleotide so amino acid does not change and lysine turn into lysine (AAA → AAG) thus no mutation happen in protein surface and there is only mutation in nucleotides surface. And actually, one codon of amino acid lysine is converted to another codon of the same amino acid.

Frequency of codon 43 is compatible with these results but frequency of codon 121 is inconsistent with these reports. Probably this is due to geographical differences between under study samples and this shows plenty of this mutation in samples. From 8 observed mutations in codon 43, all mutations are made under the effect of (AGG → AAG Arg) → AGG change and in codon 88 also a mutation as result of (Lys → AAG Arg) → AGG change and in codon 121 because of (Lys → AAA >> AAG) Lys) change.

But in 725.571 samples (AAG >> AGG) and actually K88R i.e. lysine is transformed to arginine. It means that there is a mutation in codon 88 and one in codon 43 (K43R) so these samples have mutations in both 43 and 88. And they are valuable to us for assessment mutations. Sequencing method as a genotypic gold standard of molecular techniques were used. In this study, sequencing method was used to determine mutation in *rpsL* gene as well as *rrs* gene.

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