

Evaluation of Mupirocin Chromosomal Resistance in *Staphylococcus Aureus* Strains and Mapping of AluI Enzymatic Digestion

R. Yari (PhD)^{*1}, M. Khamsehchi (MSc)², M.R. Mehrabi (PhD)³

1.Department of Biology, Faculty of Sciences, Islamic Azad University of Borujerd , Borujerd, I.R.Iran.

2.Azad Islamic University, Borujerd Branch, Borujerd, I.R.Iran

3.Department of Laboratory Sciences, Medical Faculty, Islamic Azad University of Borujerd, Borujerd, I.R.Iran.

J Babol Univ Med Sci; 18(10); Oct 2016; PP: 46-52

Received: Apr 27th 2016, Revised: Jun 1st 2015, Accepted: Jul 27th 2016.

ABSTRACT

BACKGROUND AND OBJECTIVE: Mupirocin is a secretory antibiotic and a bacterial Isoleucine- tRNA synthetase enzyme inhibitor which is used against impetigo. Mupirocin specifically binds to Isoleucine-tRNA synthetase enzyme and inhibits protein synthesis. The aim of this study was to prepare standard strains of *Staphylococcus aureus* with a designated and approved molecular genetic map from *iles-1* gene using AluI enzyme.

METHODS: In this cross-sectional study, 150 clinical strains of *Staphylococcus aureus* isolated from skin samples of patients and staff in three hospital of Qom, from transfer medium (BHI) (Merck, Germany) on blood agar medium (Merck, Germany) using streak plate method and cultured for 24 to 48 hours and were incubated at 37°C. Using biochemical and PCR methods, srRNA 16 area was validated. Presence of *Iles-1* gene was considered using PCR and mapping of AluI enzymatic digestion was carried out. Also 5ug mupirocin disk was used to investigate bacterial resistance.

FINDINGS: The results showed that from 150 samples, 7 samples are mupirocin resistant strains. The rate of infection with resistant strains in women was 42.58% and men 57.14%, respectively. 100% of resistant isolates to mupirocin had a positive PCR for *iles-1* gene of resistant to chromosomal mupirocin.

CONCLUSION: It is concluded that mupirocin resistant strains of *Staphylococcus aureus* is few.

KEY WORDS: *Staphylococcus Aureus*, *Mupirocin*, *Polymerase Chain Reaction*.

Please cite this article as follows:

Yari R, Khamsehchi M, Mehrabi MR. Evaluation of Mupirocin Chromosomal Resistance in *Staphylococcus Aureus* Strains and Mapping of AluI Enzymatic Digestion. J Babol Univ Med Sci. 2016;18(10):46-52.

* Corresponding Author: R.Yari (PhD)

Address: Department of Biology, Faculty of Sciences, Borujerd Branch of Islamic Azad University, Borujerd, I.R.Iran

Tel: +98 66 42518000

Email: rezayari@yahoo.com

Introduction

Staphylococcus aureus is a gram-positive and anaerobic cocci and the most important species in the *Staphylococcus* genus in the medical sciences (1). Since the bacterium's genome is flexible, drug-resistant strains and pathogenic strains has been expanded (2, 3). In recent years a significant increase has been observed in the incidence of nosocomial infections caused by *Staphylococcus aureus* which are often multi-resistance (4,5).

Local formation of pus Determines *Staphylococcus* infection. *Staphylococcus aureus* with pus formation changes in every organ causes a series of diseases such as pneumonia, endocarditis, gastritis, osteomyelitis, septicemia (6-8). Zeinalinia and colleagues in a study of 70 people carrying the *Staphylococcus aureus* bacteria, the number of 8 isolated bacteria were resistant to the mupirocin antibiotic (9). In 1998, Schmitz and colleagues studied *Staphylococcus aureus* in 19 hospitals of Europe and found that the prevalence of high-level mupirocin resistance in *Staphylococcus aureus* was 1.6% and CONS was 5.6% (10). In a study was done on 100 hospitals staff of Arak in 1995, 34% of staff were nasal carriers of *Staphylococcus aureus* (11). An epidemiologic study on isolated Mupirocin-resistant MRSA showed that high-level mupirocin resistance in Canada hospitals has increased from 1/6% to 7% in the years of 1995-1999 until years of 2000- 2004 (12). Appropriate, sufficient and on time treatment with appropriate antibiotics is an important step in improving of infectious diseases (13). More than fifty years, antibiotics are used to treat infections quickly and effectively, but one of the major problems in the treatment of infectious diseases is antibiotic resistance in pathogenic bacteria (15, 14). At first, antimicrobial materials by *Pseudomonas fluorescens* were reported by Fuller and colleagues, isolated antibiotic was called mupirocin in future studies (16). Mupirocin is used against *Streptococcus pyogenes* and *Staphylococcus aureus* induced impetigo and in the treatment of skin infections (17,18).

For the first time in 1986, mupirocin was administered for patients by doctors and subsequent clinical use, resistant strains were immediately reported (19). There are two chromosomal and plasmid mupirocin resistance and phenotypes can be divided into 3 groups: low-level resistance with MIC 256-8 µg/ml and with point mutations in chromosomal genes *iles-1* = Isoleucine-tRNA ligase1. high level of resistance with MIC=Minimum Inhibitory Concentration,

equal and higher than the 512 µg/ml is created with *iles-2* (*mupA*) plasmid gene and very high level of resistance with MIC above 1024 µg/ml is generated by *mupB* plasmid gene (20-22).

In the past, several phenotypic features such as bio typing, serotyping, bacteriophage or bacteriocin typing and antibiotic susceptibility profiles have been used for typing of germs and to classify infectious agents. Some molecular techniques that are used for microorganisms typing include: PFGE= Pulse Field Gel Electrophoresis, RFLP, Plasmid analysis, ribotyping, Spoligotyping, RAPD-PCR= Random Amplified Polymorphic DNA PD, Rep-PCR= Repeated sequences -PCR and ERIC-PCR= Enterobacterial Repetitive Intragenic Consensus Sequence PCR. Recently, some researchers have reported that DNA-based techniques, molecular typing methods, PFGE and MLST= Multi Locus Sequence Typing are efficient method for typing of resistant bacteria to methicillin (28-23).

In this study the presence of *iles-1* gene in isolated *Staphylococcus aureus* from skin was studied by PCR to determine mupirocin resistant strains. The aim of this study was to prepare standard strains of *Staphylococcus aureus* with a designated and approved molecular genetic map from *iles-1* gene using *AluI* enzyme to record in Iran collection center of microorganisms (PTCC) for usage of other internal and foreign researchers.

Methods

Isolation: In this cross-sectional study, 150 clinical strains of *Staphylococcus aureus* isolated from skin samples of patients and staff in three hospital of Qom, from transfer medium (BHI) (Merck, Germany) on blood agar medium (Merck, Germany) using streak plate method and cultured for 24 to 48 hours and were incubated at 37 °C. *Staphylococcus aureus* suspected colonies was initially diagnosed by gram staining and catalase test. Then, by using coagulase test, differential biochemical (DNA Agar, Mannitol Salt Agar and Urea Broth) (Kinda-Madrid) (29) and PCR to identify the exact 16s rRNA region were isolated.

Disk diffusion method: To determine the resistance patterns of *Staphylococcus aureus* strains, sensitivity to mupirocin according to diffusion method (30) and by using 5µg antibiotic disk for mupirocin (Haymdya-India) and by standard guidelines of CLSI = Clinical and Laboratory Standards Institute was done. Samples were cultured in Mueller Hinton broth media for 4

hours at 37°C. After growing the bacteria, a suspension with a concentration of 0.5 McFarland was prepared. The suspension was cultured using a sterile swab on Mueller Hinton agar media as trips and mupirocin antibiotic disks were placed on the media. After 24 hours of incubation at 37°C, the diameter of inhibition were measured around the disc. The criteria for mupirocin disc were as follows: if there is no halo around the mupirocin disc, the strains were considered as mupirocin resistant strains and if there is a halo around the mupirocin disc was considered as mupirocin sensitive strains (31).

Molecular method of PCR: DNA extraction kit (SinaClon) was used to extract DNA from Gram-positive bacteria. After purification of the bacterial genome, to identify *iles-1A* gene, *iles-1* primers were used (table 1). To perform PCR, thermocycler device (Biorad USA PCR T100) was used (tables 2,3).

Table 1. The sequence of target genes and primers used in this study, and the molecular weight of the PCR product resulting from this primer

Primer	Sequences from 5' to 3'	Amplicon size	Source
iles1-A	F: gtaaatcttttagtgtaattgtgtac	541 bp	21
	R: tcttcttaacatgtggtgtatgaga		

Table 2. Temperature-time cycle of *iles1-A* gene amplification

Step	Temperature(°C)	Time	Cycle
Initial Denaturation	95	5(min)	1
Denaturation	95	50(Sec)	34
Annealing	52	50(Sec)	
Extension	72	120(Sec)	
Final extension	72	10(min)	1

Table 3. The amount of materials needed to perform the PCR reaction in a volume of 25 ml

Compounds	Concentration
ddH ₂ O(DW)	—
Buffer 10X	1x
MgCl ₂	1.5 mM
dNTP	0.2 mM
Primer	0.6 mM
Taq polymerase	0.4 U
DNA	10-40 ng

After completion of the reaction, 4 ml of PCR mix was electrophoresed on 1.5 percent agarose gel containing DNA Safe Stain color using x 0.5 TBE buffer for 45 minutes with 70 voltage and the created

bands were studied by Gel Doc device (E-BOX VILBER). To estimate the size, the 100 bp marker (Cat. No. PR911653) (Sina clone) was used.

Enzyme cutting: AluI enzyme from SinaClone company was used for enzymatic digestion of the PCR samples (35-32). For this purpose, two solutions of 10X RapidDigest Universal Buffer and 10X RapidDigest Blue Buffer were mixed together for amounts of 300 ml and 120 ml, respectively. 10 microliter of PCR product with 3 ml Rapid Digest and 17 ml distilled water poured in a micro tubes and gently mix. Micro tube was incubated for 30 min at 37°C in order to activate enzymes. After the activation of the enzyme, in order to deactivate enzymes, micro tube was incubated for 20 min at 65°C and after cutting, pieces were electrophoresed by using 2% agarose gel.

Results

Using phenotypic confirmatory tests and PCR for 16s rRNA (Fig 1), the 150 strains as *Staphylococcus aureus* were identified and confirmed. Among 150 strains of *Staphylococcus aureus*, 7 mupirocin resistant strains were found by disk diffusion method and no halo around the disc mupirocin was observed (Fig 2).

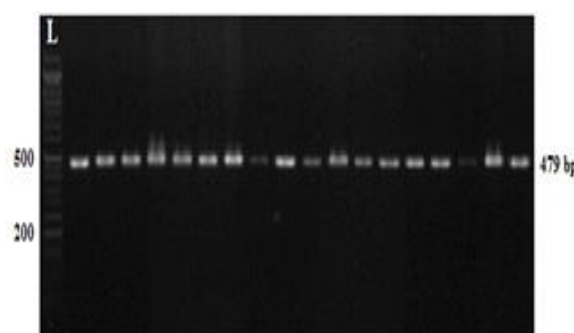


Figure 1. The gel electrophoresis of PCR product for 16s rRNA, L: DNA markers (Cat. No. PR901633)

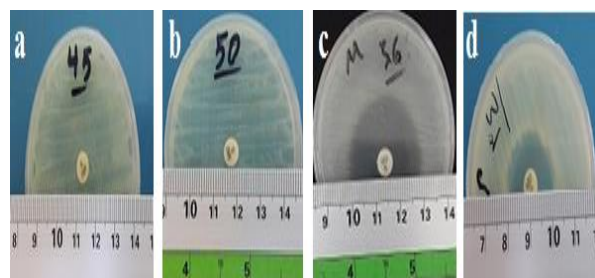


Figure 2. Resistant isolated samples (a and b) and non-durable (c and d) of *Staphylococcus aureus* to mupirocin antibiotic evaluated by disk diffusion method

On the other hand, it was found that mupirocin resistance in men was more than women. 100% of resistant isolated strains to mupirocin had a positive PCR (chromosomal) of resistance mupirocin gene and in all samples resistant to mupirocin, *iles-1A* gene amplification with primers *iles-1* was detected (Fig 3). After PCR, samples were cut using the *AluI* enzyme. After cutting with the *AluI* enzyme, generated data for matrix 1 and 0 were analyzed with SPSS software. Data was transferred 3.22 to MVSP=Multi Variate Statistical Package and 2.02 NTSYS = Numerical Taxonomy and Multivariate Analysis System and dendrogram and similarity matrix were draw using them (Fig 4,5).

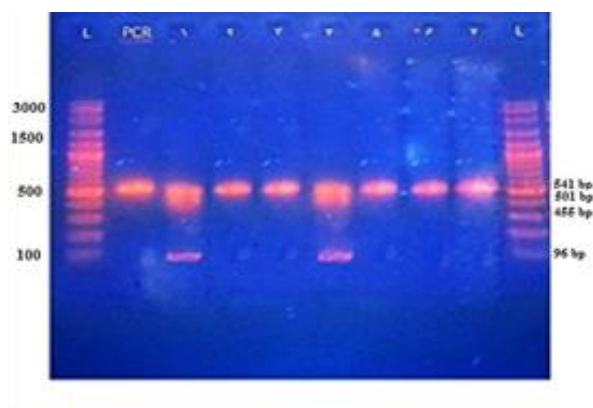


Figure 3. Gel electrophoresis of PCR product cutting with *AluI* enzyme, 2% agarose, products in wells 1 and 4 were cut by enzyme and in bp96, bp 455 and bp 501, band were formed. L: DNA marker (Cat. No. FER SM1153).

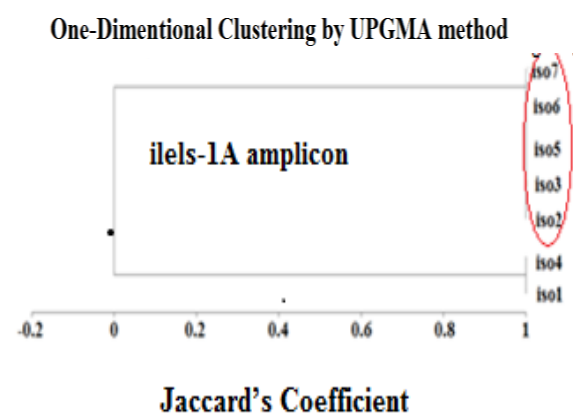


Figure 4. Cluster analysis of 7 isolated strains resistant to mupirocin using UPGMA (UPGMA: Unweighted Pair Group Method With Arithmetic Mean Averages) and Jaccard coefficient of *iles-1A* gene.

Similarity Matrix

	iso1	iso2	iso3	iso4	iso5	iso6	iso7
iso1	1.000						
iso2	0.316	1.000					
iso3	0.316	1.000	1.000				
iso4	1.000	0.316	0.316	1.000			
iso5	0.316	1.000	1.000	0.316	1.000		
iso6	0.316	1.000	1.000	0.316	1.000	1.000	
iso7	0.316	1.000	1.000	0.316	1.000	1.000	1.000
	iso1	iso2	iso3	iso4	iso5	iso6	iso7

Figure 5. Similarity matrix of 7 isolated strains in evaluation of cutting pattern of bp 541 amplicon, *iles-1* amplicon, *iles-1* gene by using *AluI* enzyme.

Discussion

According to the results of cluster analysis, it was found that genotyping of *iles-1* gene using the cutting map of *iles-1A* amplicon using *AluI* enzyme had separated clinical samples obtained from patients (7-5V 3 and 2), except 3 samples obtained from hospital staff. The samples collected from male members of the study (2 and 5-7) were sorted in the separated cluster from other samples and samples of female (1, 3 and 4) were sorted in a separated cluster from other samples except for 3 sample. Samples 1 and 4, which are located in a cluster, had a high genetic similarity to each other and because sample 1 and sample 4 had been isolated from the Kamkar and Beheshti hospitals respectively, the cause of pollution spread are probably the same.

On the other hand, Samples 2, 3, 5, 6 and 7 have high genetic similarity and since this samples were obtained from Kamkar and Nekouei (Hedayati) hospitals, the cause of pollution spread are probably the same. In the study of Zeinalinia and colleagues, 261 samples were collected and found that 70 people are carriers of *Staphylococcus aureus*, so that 8 strains of them were resistant to mupirocin (9).

The prevalence of resistance was lower compared to this study, because resistance percentage of 150 isolated strains in the present study was reported 66.4%. In study of Sadari and colleagues, 94 strains were confirmed as *Staphylococcus aureus* and also by using phenotypic methods 6 mupirocin-resistant *Staphylococcus aureus* strains were detected (36).

In the present study, chromosomal *iles-1* gene which is responsible for low resistance was identified in 7 isolated strains. In the study of Sadari, among the resistant strains, strains with high resistance had higher

percentage compared to a low resistance strains. But in the study of Fitzroy and colleagues, because of chromosomal mutations in the *iles-1* gene in their study, low resistance strains had higher percentage compared to high resistance strains (37). There are 3 modes of resistance to mupirocin: 1) in the first state, resistance gene is located in the *iles-1* gene (low resistance), 2) the second, resistance gene is in the *mupA* plasmid (high resistance) and 3) in the third state, resistance gene is located in *mupB* plasmid (very high resistance). However, the bacteria may be has none of the resistance genes to mupirocin, but is still resistance to mupirocin due to the genes coding Efflux pump, change in membrane permeability, inactivation of the enzyme or chemical change (38). In this study, *AluI* enzyme was used for cutting, because the *AluI* enzyme leads to lower cut less than other enzymes, therefore identifies areas that are some distance apart which makes the diagnosis of created bands more

precise and easier. Obtained results demonstrated that isolated samples were properly typed using enzyme digestion with isolated samples of healthy people and patient as well as males than females. It is recommended that more mupirocin resistant samples should be used in genotyping studies, also other enzymes should be used for cutting to obtain more precise results. The prevalence of *Staphylococcus aureus* which are resistant to mupirocin is relatively low in Iran, so there is no reason for serious concern. But hygiene is necessary. *Iles-1* gene genotyping could properly separate isolated samples from patients and staff as well as male and female.

Acknowledgments

Hereby, we would like to thank from respected official of Molecular Cell Biology Research Laboratory of Islamic Azad University of Borujerd.

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