Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 (3) February 2014: 158-165

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Molecular Epidemiology of *Staphylococcus aureus* with ERIC–PCR method

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ABSTRACT
S. aureus is one of the most significant etiological agents responsible for healthcare-associated infections. The aim of this study was to show the genetic relationship in S. aureus isolates and their transmission pattern between hospitals. 90 S.aureus strains, isolated from hospitalized patients in the intensive care unit and infectious wards of Besat and Toohid hospitals, Sanandaj. Antimicrobial susceptibilities were determined by the disc diffusion method, Methicillin resistance was done by agar screen test and the resistance inducible by the D-Test. By ERIC–PCR technique relationship of strains was determined based on the similarities between DNA fingerprints by using Jaccards coefficient in the SAHN program of the NTSYS-pc software. Fourteen different antimicrobial patterns were observed. 46.7% of the strains were susceptible to all antimicrobials tested. The ERIC–PCR profiles allowed typing of the 90 isolates into 75 ERIC-types which were grouped into eleven main clusters (C1–C11). The Fourth group with the largest number was formed 17 strains. Agreement between antibiotic patterns and rep-profiles was not observed for most isolates. The results of our study also showed that most of Staphylococcus isolated produced different genomic fingerprint patterns, therefore , dissemination source of infection is different.
Keywords: S. aureus, ERIC–PCR, resistance

Received 09/12/2013 Accepted 12/01/2014

INTRODUCTION

Although has been estimated that 20–30% of the human population are carriers of *Staphylococcus aureus*, this bacterium is one of the most significant etiological agents responsible for healthcare-associated infections (HAI). The emergence of methicillin resistant *S. aureus* (MRSA) strains has created serious Treatment problems.[1]

Clindamycin is a good substitute to treat soft tissue infections by both methicillin-resistant Staphylococcus and methicillin- sensitive Staphylococcus infections. But, the frequent use of this drug increases the resistance, especially resistance induction, and consequent failure to be treated with clindamycin.[2-4]

Repetitive DNA sequences account more than 5% Microbial genomes. The functions of many of these repetitive sequences elements are not known. These elements have proven to be useful in medical microbiology, epidemiological analyses, molecular diagnostics and environmental microbiology. [5]

Among the many PCR-based genetic typing techniques, repetitive extragenic palindromic elements-polymerase chain reaction (REP-PCR) has attracted much attention. Its stable reproducibility and advantageous discriminatory ability and provided high taxonomic resolution may act as a rapid detector of diversity and evolution of the microbial genomes being studied.[6-7]

The rep-PCR technique is a simple and rapid method that has the necessary resolving power for microbial identification at subspecies or strains level.[5, 8-12] REP has proven to be more discrimination compared to the 16S rRNA PCR methods and limitation fragment length polymorphism, and provides discrimination similar to randomly amplified polymorphic DNA (RAPD). Further, the REP protocol is simple and rapid

Compared with other DNA protocols genomic, such as pulsed field gel electrophoresis (PFGE), for molecular typing.[7]

The aims of this study are to examin PCR-amplified enterobacterial repetitive intergenic consensus (ERIC -PCR) analysis on *Staphylococcus aureus (S .aureus)* andcomparing the genetic relationship of those isolates. These studies are essential to examine the prevalence of Antibiotic-resistant strains, seeking the source of *Staphylococcus aureus* infectious origin and their dissemination, and provide scientific basis for human disease control and warning.

MATERIALS AND METHODS

Collection and culture of isolates. Totally 90 *S. aureus* were isolated from hospitalized patients in the intensive care unit and infectious ward of Besat and Toohid hospitals, Sanandaj. In this study 22 samples were collected in Besat hospital, and 68 samples were from Toohid hospital. Sampling was performed with sterile swab from the throat (73.3%) and nose (26.7%). All the isolates were suspended in 20% glycerol stock and kept at -70 ^{0}C for long-term storage. The isolates were sub cultured on to blood agar and incubated at 37 ^{0}C overnight before they were used. Then, they were identified by using conventional microbiological methodsincluding Gram stain, colony morphology, catalase activity, manitol fermentation and DNase test.[13]

Antibiotic susceptibility test. Susceptibility to antimicrobial agents were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. Antibiotic discs used were erythromycin (15 µg), ciprofloxacin (5 µg), and gentamicin(10 µg) (Figure 1) .

Methicillin resistance. To identify strains of MRSA strains were cultured on Mueller Hinton agar medium supplemented with 4% NaCl and 6 mg / L oxacillin. Isolates were incubated at temperatures of 35°C for 18 to 24 hours. The growth of more than one colony was showed resistance to methicillin(Figure 2) [14] .

Detection of inducible Clindamycin resistance. Inducible Clindamycin resistance was performed according to CLSI guidelines by using D -Test method [14] A 0.5 McFarland equivalent suspension of organisms was inoculated onto a Mueller - Hinton agar (MHA) plate, the ER (15 µg) disk was placed 15-26 mm (edge to edge) apart from CL (2 µg) disk on MHA. Plates were surveyed after 18 hours of incubation at 35°C (Figure 3) **[2].**

DNA extraction. Genomic DNA extraction of *S. aureus* strains were performed by use of DNA Cinna Pure kit (Cinagen, Iran).

REP-PCR amplification. The Rep primers were Rep1-R: 5′-ATG TAA GCT CCT GGG GAT TCA C-3′and Rep2: 5′-AAG TAA GTG ACT GGG GTG AGC G -3′ .

Rep-PCR was performed in final volume of 25 µl by DFS Master Mix Kit PCR (BIORON) including Taq polymerase enzyme, $Mgcl_2$, dNTP , $(NH_4)_2SO_4$, TrisHCl , Tween – 20. Reaction mixtures consisted of 12.5 µl Master Mix, 1 µl $Mgcl_2$, 1 µl of primer F, 1 µl of primer R, 7.5 µl Distilled water and 2µl DNA template. Cycling conditions were Primary denaturation 95°c for 2 min , Denaturation 92°c for 30 s, Annealing 50 °c for 1 min, Extension 65 °c for 8 min, then 35 cycle , followed by a Final Extension 65 °c for 8 min.[15]

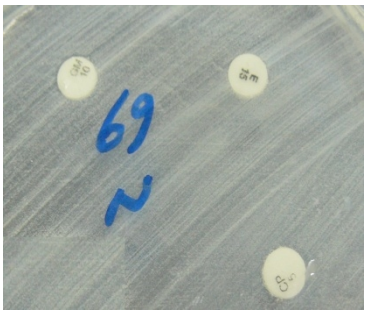
Electrophoresis. Agarose gel electrophoresis were done by 1.5% (0.6 g of agarose (Neda Fan Rah, Iran) in 40 cc of 0.5X TAE buffer). After solidification and removing of the comb, 4µl pcr products with 2µl buffer loading loaded onto the gel and electrophoresis were performed for 1 h at 50 voltage. After staining with ethidium bromide (BIORON 10 $^{mg}/_{ml}$), gels were scanned using device UV translluminator and Marker of Standard Molecular Marker 100 bp DNA Ladder (BIORON) was used to determine the size.[16]

Computer-Assisted ERIC-PCR DNA Fingerprint Analysis. Data matrix was formed based on presence or absence of bands and analyzed using the NTSYS-pc software (version 2.02 K, Applied Biostatistics, Inc., NY, USA). Dendrograms of dissimilarity were produced for each case. The similarity between the strains was determined on the basis of the Jaccard similarity. The dendrogram was produced on the basis of the averaged similarity of the matrix with the use of the algorithm of the Unweighted Pair-Group Method (UPGMA) in the SAHN program of the NTSYS-pc software. The nearest neighbor-joining clustering method has been used to show relations between similar groups.[17]

RESULTS

The antimicrobial susceptibility of 90 *S. aureus* isolates was determined in vitro against 5 commonly used antibiotics in hospitals of Sanandaj. Table 1 indicates in both hospitals the most of resistance isolates to Methicillin. The strains were tested to antimicrobial resistance and fourteen different drugs patterns were observed (Table 2), designated A to N descending in order of antibiotic resistance. Only one of the antibiotypes (H) were present amongst the isolates Toohid hospital but the Beast hospital isolates were observed 10 anti-biotype (B, C, D, E, F, G, I, J, L and M). It is important to remark that different

antimicrobial patterns were found within each hospital. 46.7% of the strains antibiotype **N** was susceptible to all drugs tested and 23.3% of the strains antibiotype **A** was resistance to all drugs tested.



Figur1. Antibiotic susceptibility test
Antibiotic discs used were Erythromycin(15µg), Ciprofloxacin(5µg), and Gentamicin(10 µg)

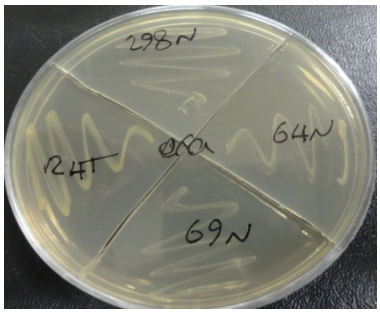


Figure 2. Methicillin resistance test
growth more than one colony was showed resistance to methicillin

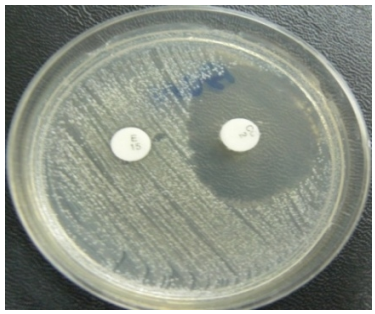


Figure 3. inducible Clindamycin resistance
E(15µg); erythromycin, CC(2µg); clindamycin. Phenotype D (D form) : $iMLS_B$

Table 1. Percentage of resistant *S.aureus* against antibiotics in hospitals of Sanandaj

Ward of hospital / Antibiotics	Percentage of resistant isolates in Toohid hospital			Percentage of resistant isolates in Besat hospital		
	CCU Ward	Infectious Ward	Total	CCU Ward	Infectious Ward	Total
Gentamicin	5.9%(4)	39.7%(27)	45.6%(31)	0.0%(0)	9.1%(2)	9.1%(2)
Ciprofloxacin	4.4%(3)	33.8%(23)	38.2%(26)	0.0%(0)	9.1%(2)	9.1%(2)
Erythromycin	5.9%(4)	38.2%(26)	44.1%(30)	0.0%(0)	9.1%(2)	9.1%(2)
Methicillin	8.8%(6)	38.2%(26)	47.1%(32)	0.0%(0)	22.7%(5)	22.7%(5)
Clindamycin	5.9%(4)	38.2%(26)	44.1%(30)	0.0%(0)	9.1%(2)	9.1%(2)
$cMLS_B$	5.9%(4)	33.8%(23)	39.7%(27)	0.0%(0)	9.1%(2)	9.1%(2)
$iMLS_B$	0.0%(0)	4.4%(3)	4.4%(3)	0.0%(0)	0.0%(0)	0.0%(0)

$cMLS_B$: constitutive resistant Macrolide- Lincosamide –StreptograminB
$iMLS_B$: inducible resistant Macrolide- Lincosamide –StreptograminB

Table 2. Antibiotic resistance profiles of *S.aureus* isolates at hospitals of Sanandaj , and percentage of resistant isolates

Antibiotic profile							Percentage of resistant isolates		
Me	Er	Gn	Cp	Cl	MLS_B	Anti- biotype	Toohid.H (N=68)	Besat.H (N=22)	All isolates (N=90)
R	R	R	R	R	$cMLS_B$	A	21.1%(19)	2.2%(2)	23.3%(21)
R	R	R	R	R	$iMLS_B$	B	2.2%(2)	0.0%(0)	2.2%(2)
S	R	R	R	R	$cMLS_B$	C	5.6%(5)	0.0%(0)	5.6%(5)
R	R	R	I	R	$iMLS_B$	D	1.1%(1)	0.0%(0)	1.1%(1)
R	R	S	S	R	$cMLS_B$	E	1.1%(1)	0.0%(0)	1.1%(1)
S	R	S	S	R	$cMLS_B$	F	2.2%(2)	0.0%(0)	2.2%(2)
R	S	R	S	S	S	G	1.1%(1)	0.0%(0)	1.1%(1)
R	S	I	S	S	S	H	0.0%(0)	2.2%(2)	2.2%(2)
R	S	S	I	S	S	I	1.1%(1)	0.0%(0)	1.1%(1)
S	S	R	I	S	S	J	1.1%(1)	0.0%(0)	1.1%(1)
R	S	S	S	S	S	K	7.8%(7)	1.1%(1)	8.9%(8)
S	S	R	S	S	S	L	2.2%(2)	0.0%(0)	2.2%(2)
S	S	I	S	S	S	M	1.1%(1)	0.0%(0)	1.1%(1)
S	S	S	S	S	S	N	27.8%(25)	18.9%(17)	46.7%(42)

Me : Methicillin, Er : Erythromycin, Gn : Gentamicin, Cp : Ciprofloxacin, Cl : Clindamycin, R: resistant, S: susceptible.

Table 3. Clustering and similarity of *S. aureus* isolates and Number of strains in each cluster

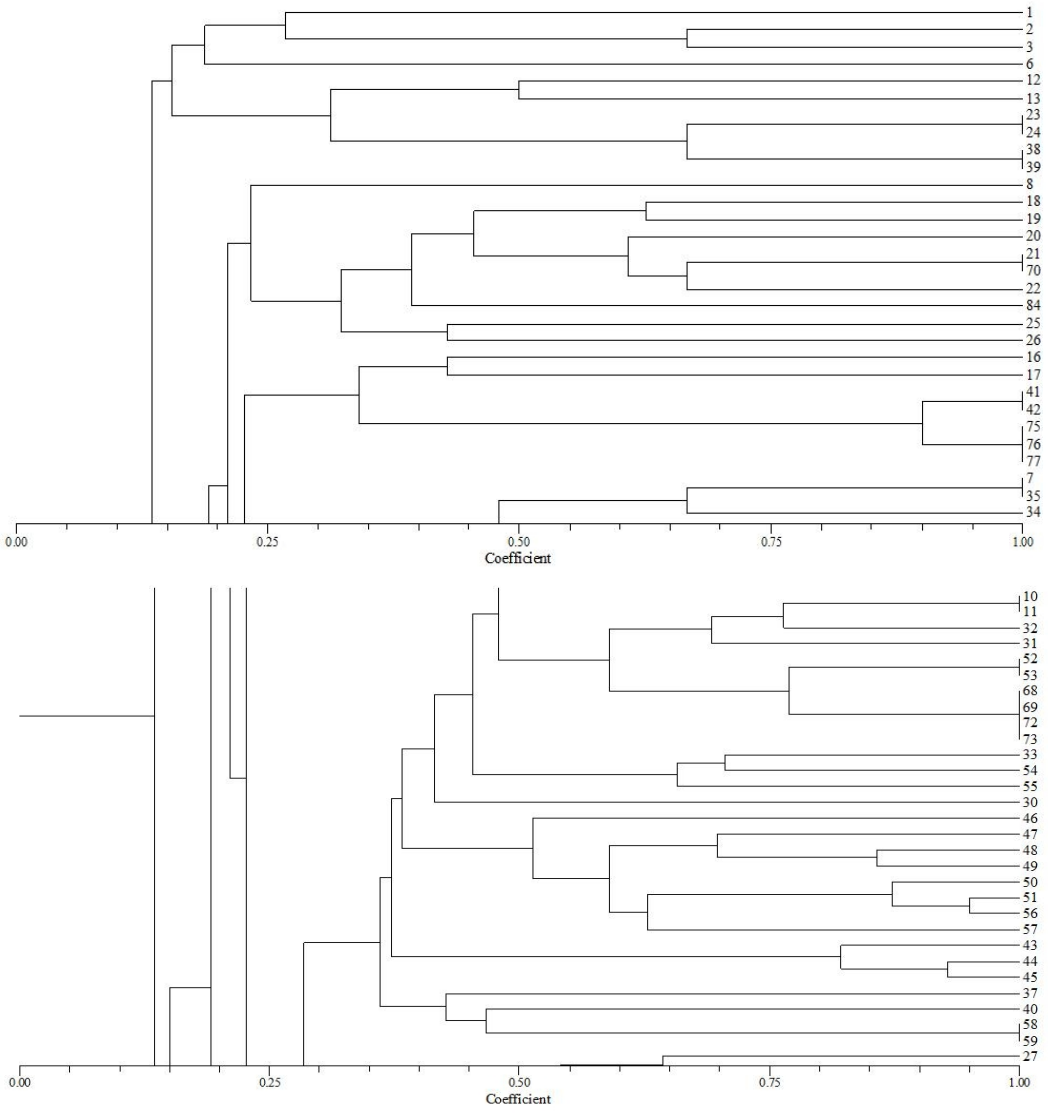
Clusters	Dendrogram similarity in clusters	Number of strains in each cluster		
		Toohid .H	Besat .H	Total
C1	16%	7	3	10
C2	23%	8	2	10
C3	34%	4	3	7
C4	43%	10	7	17
C5	52%	8	0	8
C6	83%	2	1	3
C7	44%	3	1	4
C8	37%	9	2	11
C9	32%	8	2	10
C10	58%	4	1	5
C11	24%	5	0	5

Table 4. Distribution of genetic and characterization of 9 cluster of 90 *S.aureus* clinical isolates.

Cluster	number of Profile	percentage of strain	number of bands	Anti- biotype	hospital	ward
C1	8	11.1% (10)	3 - 7	A (5.6%) , N (2.2%) B, H, K (1.1%), C- G, I, J, L, M (0%)	T. H 7.8% B. H 3.3%	INFEC 10.0% CCU 1.1%
C2	9	11.1% (10)	3 - 9	N (6.7%), A (2.2%), F, G (1.1%), B- E, H- M (0%)	T. H 8.9% B. H 2.2%	INFEC 10.0% CCU 1.1%
C3	4	7.8% (7)	8 - 12	N (3.3%), C (2.2%), A, K (1.1%) B, D-J, L, M (0%)	T. H 4.4% B. H 3.3%	INFEC 5.6% CCU 2.2%
C4	11	18.9% (17)	6 - 17	N (12.2%), A (3.3%), C, K, L (1.1%) B, D- J, M (0%)	T. H 11.1% B. H 7.8%	INFEC10.0% CCU 8.9%
C5	8	8.9% (8)	12 - 20	N, K (3.3%), A (2.2%), B-J, L, M (0%)	T. H 8.9% B. H 0%	INFEC 4.4% CCU 4.4%
C6	3	3.3% (3)	12 - 14	N (2.2%), I (1.1%), B-H, J-M (0%)	T. H 2.2% B. H 1.1%	INFEC 0 % CCU 3.3%
C7	3	4.4% (4)	8 - 11	C (2.2%) , A, N (1.1%) B, D-M (0%)	T. H 3.3% B. H 1.1%	INFEC 3.3% CCU 1.1%
C8	11	12.2% (11)	10 - 18	N (4.4%), A (2.2%) D, E, F, K, M (1.1%), B, C, G-J, L (0%)	T. H 10.0% B. H 2.2%	INFEC 8.9% CCU 3.3%
C9	9	11.1% (10)	6 - 11	N (6.7%), A (3.3%), H (1.1%) B-G, I-M (0%)	T. H 8.9% B. H 2.2%	INFEC 8.9% CCU 2.2%
C10	5	5.6% (5)	8 - 10	N (2.2%), A, J, L (1.1%) B-I, K, M (0%)	T. H 4.4% B. H 1.1%	INFEC 3.3% CCU 2.2%
C11	3	5.6% (5)	3 - 7	N (2.2%), A, B, K (1.1%) C-J, L, M (0%)	T. H 5.6% B. H 0 %	INFEC 5.6% CCU 0%

T. H : Toohid hospital , B. H : Beast hospital
INFEC : Infectious ward , CCU : CCU ward

In this study, rep-PCR typing could successfully differentiate *S. aureus* strains of hospitals Sanandaj. Seventy-five different genetic profiles were obtained using ERIC-PCR in the range size from slightly less than 100 bp to about 1400 bp were identified after rep-PCR analysis. Figure 4, a dendrogram that included all profiles was constructed on the basis of the levels of similarity. The 75 ERIC–PCR profiles grouped into nine main clusters (C1–C11) (Table 3). The Fourth group with the largest number was formed by 10 *S. aureus* strains isolated from Toohid hospital and 7 each isolated from the Beast hospital. The Sixth group with the lowest number contained 2 strains isolated from Toohid hospital and 1 isolated from the Beast hospital. The Eleventh group contained only 5 isolated from Toohid Hospital. Complex patterns of fingerprints have been obtained for all strains. Generally, the electrophoretic analysis of the PCR reaction products has revealed that the number of bands in particular electrophoretic paths ranged from 3-20 (Table 4).



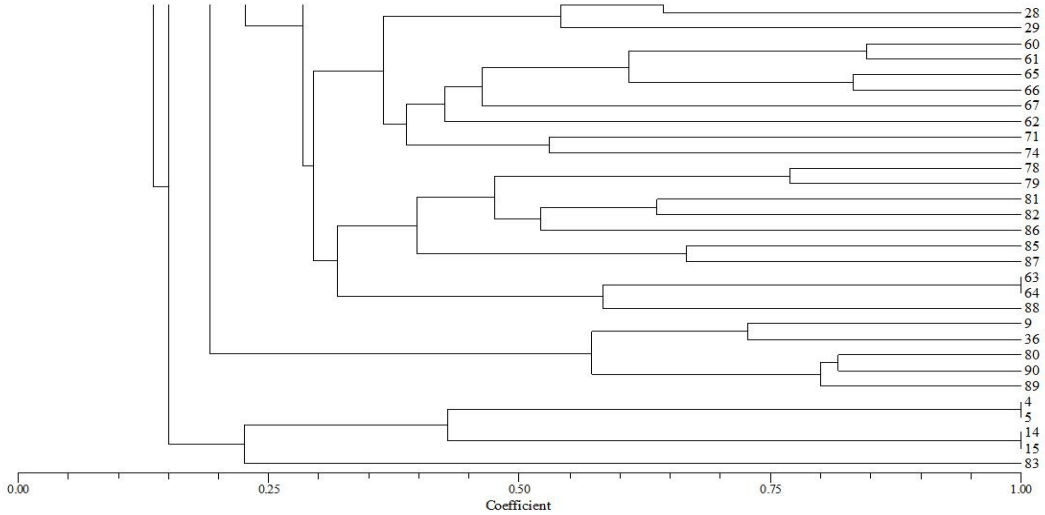


Figure 4. Dendrograms of genomic similarity of 90 *S.aureus* strains in Sanandaj hospitals

DISCUSSION

Antibiogram results showed 41.1% strains resistance to Methicillin, 35.6 % resistant to Erythromycin, 36.7% resistant to Gentamicin, 33.1% resistant to Ciprofloxacin, 35.6% resistant to Clindamycin and 3.3% inducible resistant to Clindamycin that similar to reported by Fiebelkorn et al., Gadepalli et al., and Jorgensen et al [18-20]. Many researchers have reported a higher incidence [21-23] while others showed a lower incidence [24-25].

21.1% of isolates of the Toohid hospital and 2.2% of Besat hospital were resistance to all drugs tested and indicates that resistance against 5 commonly used antibiotics is a problem in hospitals of Sanandaj. 10.0% of the strains isolated from Toohid hospital and 1.1% Besat hospital showed resistance to one antimicrobials and 1.1%, 2.2% , 1.1% , 5.6% of the strains isolated from Toohid hospital showed resistance to tow, three, four and five drugs, respectively. 1.1% of the strains isolated from Besat hospital showed resistance to one drugs. The different resistance to antimicrobials was found within isolates collected from Besat and Toohid hospitals that these results may indicate differences in therapeutic measures between these hospitals, particularly, differences in the type of drugs and the frequency of their use, and so exposure of patients to antimicrobials.

Rep-PCR determine the genetic diversity and also transmission trace of *S. aureus* clinical isolates in the community and hospitals. This is the first study carried out in hospitals of Sanandaj which describes the genetic relationships among *S. aureus* isolates from hospitalized patients in the intensive care unit and infectious ward of Besat and Toohid hospitals by rep- PCR. As shown in previous reports, rep- PCR was proven to be a highly discriminate and rapid screening method to classify a large number of isolates into clusters [15-16, 26-27] Most of the strains (18.9%) were grouped in cluster 4. Genetic heterogeneity among *S. aureus* isolates was observed within hospitals. The Sixty-eight *S. aureus* strains from Toohid hospital were divided into 57 profiles belonged to all clusters and the 22 isolates from Besat hospital were divided into 18 profiles belonged to all clusters except cluster 5,11. Sixty-one (67.8%) of the isolates displayed a single profile; whereas, twenty-nine (32.2%) of them showed shared patterns. This result may suggest that genotypes were similar in the different hospitals which are indicative of similar origin of dissemination.

Predominant profiles were found within each hospital. Nine predominant profiles were found in Toohid hospital (strains 38-39; 21- 70; 75-76-77; 10-11; 52-53; 58-59; 63-64; 4-5; 14-15), two predominant profiles were identified in Besat hospital (strains 23-24; 41-42). Furthermore, isolates obtained from the different hospital strains 7, 68, 69 and 35,72,73 , showed similar genotype which indicate clonal transmission of *S. aureus* in hospitals.

Antimicrobial patterns alone were found to be of limited value in differentiating closely related strains. However, rep-PCR was able to differentiate among many isolates that were indistinguishable by drug susceptibility testing, as reported previously.[26, 28] Agreement between antimicrobial patterns and rep-profiles was not observed for most isolates. Some isolates from different hospitals that had the same rep-profile had different drug patterns. A low percentage of isolates (4.4%) shared rep-profiles and antimicrobial patterns. isolates 38, 39 from Toohid hospital and 41, 42 from Besat hospital had the same rep-profile and antimicrobial pattern. This finding is in contrast with the results by Rivas et al. in 1997 [29].

CONCLUSION

In conclusion, this study was to show the genetic relationship in isolates *S. aureus* and their transmission pattern between hospitals. Most of the isolates show unique patterns which indicate that the rate of transmission resistant strains are very low in Sanandaj hospitals. The remaining patterns showed similarity in most characteristics which is indicative of similar origin of dissemination.

ACKNOWLEDGEMENTS

Authors wish to extend their gratitude to the Kurdistan University of Medical Sciences for supporting the project. We thank the Microbiology laboratory staff of the Beasat and Toohid Hospitals for their technical support.

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Citation of this article
Shadieh A , Rashid R , Enayat K , Serveh Z. Molecular Epidemiology of *Staphylococcus aureus* with ERIC–PCR method. Bull. Env. Pharmacol. Life Sci., Vol 3 (3) February2014: 158-165