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بررسی مولکولی سویه‌های استافیلوکوکوس اورئوس جدا شده از زخم و ادرار در کرمانشاه، ایران.

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چکیده

انسان منبع اصلی استافیلوکوکوس اورئوس در طبیعت است. این باکتری یکی از عوامل مهم عفونت‌های بیمارستانی است. بنابراین، مطالعه بررسی مولکولی این باکتری از اهمیت بالایی برخوردار است. پس از شناسایی و تأیید ۱۰۰ سویه استافیلوکوکوس اورئوس جدا شده از نمونه‌های زخم و ادرار در کرمانشاه با روش‌های استاندارد میکروبیولوژی، آزمایش حساسیت آنتی‌بیوتیکی انجام شد و ژن‌های مقاومت (mecA، aacA-D، tet K، tet M، msrA، ermA) جدایه‌ها با روش Multiplex-PCR بررسی و ژنوتیپ ژن کوآگولاز جدایه‌ها با روش RFLP-PCR بررسی شد. در آزمایش حساسیت، بیشترین مقاومت به پنی‌سیلین (۹۰٪) و کمترین مقاومت به نیتروفورانتوئین (۸٪) گزارش شد. در روش Multiplex-PCR، (۴۹٪) از جدایه‌ها حامل ژن mecA، aacA-D (24%)، (89%) tetM، tetK (14%)، ermA (40%) و msrA (۳۶٪) بودند. در تشخیص پلی‌مورفیسم ژن کوآگولاز از ۶۵ سویه استافیلوکوکوس اورئوس جدا شده از ادرار، پلی‌مورفیسم ژن کوآگولاز در ۵۳ مورد مشاهده شد که ژنوتیپ) I٪۶۱,۵۳) و ژنوتیپ) VIII٪۱۲,۳) و ژنوتیپ) IX٪۷,۶۹) بودند. پلی‌مورفیسم ژن کوآگولاز سویه‌های جدا شده از زخم در ۲۲ نمونه مشاهده شد. ژنوتیپ) I٪۴۲,۸۵) و ژنوتیپ) VIII٪۲۰) گزارش شد. تشخیص و توصیف مولکولی ایزوله‌ها در پیشگیری و کنترل عفونت بسیار مؤثر و کارآمد است.

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کلمات کلیدی: *استافیلوکوکوس اورئوس*، الگوهای مقاومت آنتی‌بیوتیکی، multiplex-PCR، RFLP-PCR، ژن کوآگولاز

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Introduction

Staphylococcus aureus is a major cause of hospital acquired infections, causing high morbidity and mortality throughout the world (1). Rates of *S. aureus* infection have increased during the past 2 decades (2). Antimicrobial resistance has increased drastically in recent years in both developed and developing countries and it has rapidly become a leading public health concern (3). The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infection. However, as early as 1942, penicillin-resistant Staphylococci were recognized, first in hospitals and subsequently in the community. Methicillin, introduced in 1961, was the first of the semisynthetic penicillinase resistant penicillin's. Its introduction was rapidly followed by reports of methicillin-resistant isolates (4). The emergence of antibiotic-resistant strains, particularly MRSA is recognized as very serious health problem because of difficulties in combating these strains (5). Initially, MRSA was limited to hospitals, however it is now increasingly recovered from nursing homes and the community (6). Since the emergence of methicillin-resistant *S. aureus*, the glycopeptide vancomycin has been the only uniformly effective treatment for staphylococcal infections (7,8). In 1997, the first clinical isolate of *S. aureus* with reduced susceptibility to vancomycin was reported from Japan (9). One of the class of antibiotics playing an important role in the therapy of serious staphylococcal infections are aminoglycosides despite reports of increased resistance to these drug in many countries in Europe (10). Coagulase is produced by all strains of *Staphylococcus aureus*. The 3′coding region of the coagulase (*coa*) gene contains varying numbers of 81 bp tandem repeats (11). Molecular typing method PCR is an effective method for control of nosocomial infections, this method can reduce and prevent of epidemic situation nosocomial infections and help in tracing the source of infection. Therefore, the aim of this study was to detect antibiotic-resistant pattern *S. aureus* strains obtained from Imam Reza hospital in Kermanshah by two methods antimicrobial susceptibility test and rapid multiplex PCR and the isolates were typed the *coa* gene by PCR- RFLP. we using one restriction enzyme *AluI* for their typing.

Materials and Methods

Bacterial isolates

Totally 100 strains of *Staphylococcus aureus*, 65 samples from urine infections and 35 samples of wound infections, were collected from patients of Imam Reza

Hospital in krmanshah in 2012. The samples were inoculated onto 5 percent sheep blood agar plates and incubated at 37°C for 48 hStandard microbiological methods and biochemical tests for identification of *S. aureus* included gram staining, catalase, coagulase, oxidase, hypersensitivity to novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test, carbohydrate (xylose, sucrose, terhalose and maltose, fructose, lactose, mannose) fermentation tests (12).

Antimicrobial agents and susceptibility testing.

Antimicrobial susceptibility testing was performed by using disk diffusion method on Mueller-Hinton agar (Merck, Germany) plates that were inoculated with 0.5 McFarland. Antibiotic discs were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each growth zone was measured in millimeters. The antibacterial agents tested were: gentamicin (10µg), neomycin (30µg), penicillin (10µg), erythromycin (15µg), tetracycline (30µg) , amoxicillin-clavulanic acid (30 µg), clindamycin (2µg), vancomycin (30µg), trimethoprim sulfamethoxazole (25µg), vancomycin (5µg), ciprofloxacin (5µg), and ceftriaxone (30 µg), tetracycline (30µg), azithromycin (15µg), norfloxacin (10µg), nalidixic acid(30µg), nitrofurantoin (50 µg) and methicillin (5µg). CLSI guidelines for susceptibility testing and qualitative interpretation were used throughout(13). Standard strains of *S. aureus* ATCC 25923 as a negative control and ATCC 33591asa positive control for *mec A* gene (14).

Antibiotic sensitivity of the isolates initially demonstrating resistance to methicillin was confirmed using BD phoenix TM (System, Becton, Dickinson Company, Shannon, Ireland) according to the recommendations given by the national reference center in Saudi Arabia.to methicillin was confirmed using BD phoenix TM (System, Becton, Dickinson Company, Shannon, Ireland) according to the recommendations given by the national reference center in Saudi Arabia (15).

Determination of minimum inhibitory concentrations (MIC)

MIC values of antibiotics were determined by the broth microdilution test. All isolates were subcultured on blood agar and incubated for 24 h at 37 °C. Then, two-fold serial dilutions of each antibiotic were made in Mueller-Hinton broth to achieve a concentration range from 0 to 256 µg/ml. After incubation at 37° for 24 h, the MIC was defined as the lowest concentration of

antibiotics that produced no growth. The broth microdilution tests were performed according to the CLSI guidelines (16)

DNA extraction

Strains were cultured on blood agar. One colony was suspended in 1 ml LB broth (Merck, Germany) for 24 h at 37°C. Genomic DNA as a template for PCR assay was extracted by DNA extraction kit DNA (DNP^{TM} - Iran) and according to the manufacturer's instructions.

Multiplex PCR for detection of antibiotic resistance genes

The antibiotic resistance determinants investigated were the *aac-aphD* (aminoglycoside resistance) *mecA* (methicillin resistance) *ermA, msrA* (erythromycin resistance) and *tetK, tetM* (tetracycline resistance) Genes. PCR was performed in a PCR thermocycler (Eppendrof Mastercycler, Germany). The PCR primers used to detect the different loci in a multiplex PCR approach are listed in Table 1Multiplex PCR assays were performed in 25µL PCR mixtures 1 and 2. The mixture 1 contained 1 U of Taq DNA polymerase (Fermentas, Germany), 2.5µL PCR buffer (10×), 1µM each forward and reverse primers of *mecA, tet K* and *tet M* gene, 150 µmol/L of each dNTP and DNA template (50 ng). Using thermal cycling, the target genes were amplified (94°C 5 min, 30 cycles of 1 min at 95°C for the denaturation step and 1 min at 55°Cfor the annealing-extension step and 90 s at 72°C for the extension step). In mixture 2, the forward and reverse primers of the genes *aacA-D*, *msrA* and *ermA* (2.5µM) were used amplification products were analyzed on a 1/5% agarose gel for 30 min (17).

Table1. Oligonucleotide primers for amplification of antibiotic resistance genes in *Staphylococcus aureus*.(Zmantar et al., 2008)

Primer	Nucleotide sequence (5-3)	Product Size (bp)
mecA	F :AAAA G A GG AAGG GG	532
	R : AG C G AG A GGA TTG C	
aac-	F :A A CA AGAG GA AAGGG C	227
aphD	R :G GA A CA A AA GA CA	
tetK	F :G AG GA GA AGG AA AG T	360
	R :G AG GA GA AAA CC C A	

tetM	F :AG GGAG GA AA AGAA	158
	R :A AG GC GG G G C A	
msrA	F :GG A AA AAGAG G TTA AAGG	940
	R AAG TA A A GAA AGA TG C C T	
ermA	F :AAG GG AAA CCCC C G	190
	R TG AAA CCC TC A C	
16s-	F :G AGG GG AAG G TA CC	228
rDNA	R :G A A AG G AG	

Statistical analysis

The data were analyzed using SPSS ver. 16.0 statistical software and a Chi-square test analysis was performed. Also, differences were considered significant at values of $p<0.05$.

Restriction fragment length polymorphism

PCR was performed in a 25 µl reaction mixture containing 1µl of template DNA, 12.5 µl of masterkit, 10 µll of H2O, and 0.5 µl of each primer COAG2*:* CGA GAC CAA GAT TCA ACA AG, *COAG*3: AAA GAA AAC CAC TCA CAT CA. The PCR conditions were as follows: Initial denaturation at 95°C for 2 minutes followed by 30 cycles of amplification with 94°C for 30 seconds, annealing at 58°C for 2 minutes, extension at 72°C for 4 minutes and final extension at 72°C for 7 minutes. So for RFLP reaction mixture containing 12.5µl of PCR products, 2.5µlof enzyme, 3 µl of H2O and 2µl restriction buffer, and so incubated at 37°C overnight (14). The PCR products were analyzed on a 1.5% agarose gel five micro liters of the PCR products were loaded into 1.5% agarose and electrophoresis was performed in .5x TBE buffer at 90 V for 75 min. The gels were subsequently stained with 1µg/ml ethidium bromide for 30 min, visualized under UV and photographed (15).

Results

Antibiotic susceptibility test results

Antibiotic resistance patterns of 100 strains of *S. aureus* isolated from urine infections and wound infections are shown in Table 2. The rate of antibiotic resistant strains of *S. aureus* following nitrofurantoin (92%), vancomycin (86%) , gentamicin (80%), neomycin (80%), ciprofloxacin (79%), rifampin (72%), clindamycin (64%), azithromycin (53%), ciprofloxacin, norfloxacin and trimethoprim sulfamethoxazole (52%), cefixime and nalidixic acid (50%), erythromycin (48%),

ampicillin (45%), methicillin (36%), tetracycline (24%) and penicillin (10%). Most antibiotic resistance was observed in penicillin (90%), tetracycline (76%) and the least resistance innitrofurantoin (8%) and vancomycin (14%).In this method, antibiotic resistance were reported 44/6% in isolated from wound and 42.7% from urine. In *S. aureus* strains isolated from two different sources of wound infection and urine infection between the rate of resistance to penicillin antibiotic with ((P=0.045, ceftriaxone (P=0.003), nalidixic acid ((P<0.000, trimethoprim sulfamethoxazole (P=0.049), ampicillin (P=0.01), nitrofurantoin (P<0.000), ciprofloxacin (P=0.001), neomycin (P=0.03), clindamycin (P=0.01, gentamicin ((P=<0.000, Cefixime (P=0.012), rifampin (P=0.048), erythromycin (P=0/023), tetracycline ((P=0.038 azithromycin P=<0.000)) and the source of infection there is significant association. But there is no Significant association between resistant antibiotics norfloxacin with (P=0.098), methicillin (P=0/064) and vancomycin (P=0.31) and the source of infection.

Table 2. Antibiotic resistance pattern in *Staphylococcus aureus* strains isolated from urine and wound

S amples	Antibiotic																															
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Percent	3		2		3		1		3			6		4				3			2		4		4		2		6		2	
		8		0		9		2		8	0		8		3	2	7		0	8		8		0		8				3		
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U		8		3		6		6		5	5			:		3		2	1	4			2		:	4		8			:	/027
rine													8		1		6					4		5						4		

Total	7	2	2	3	2		3	3			4		2	3	3	2	1	3	
		4	0	3	0	7	2	1	7	2	3	6	2	8	2	0	8	0	
Percent	7	2	2	3	2		3	3			4		2	3	3	2	1	3	
		4	0	3	0	7	2	1	7	2	3	6	2	8	2	0	8	0	
W	3	1	2	2	2		0	5			0		1	6	7	2	3	8	
ound	5	9	0	4	2	8	8	1		1	2	0	1	8	2	0	3	3	
U	5	2	3	2	2			6	8		0		3		1	3	4	9	0/000
rine	5	9	0	4	6	7				5		4	9			2	3		
Total	9	4	5	4	4		8	2			2		5	1	2	5	7	4	
	0	8	0	8	8	5		1	0	6	0	4	0	4	8	2	6	7	
Percent	9	4	5	4	4		8	2			2		5	1	2	5	7	4	
	0	8	0	8	8	5		1	0	6	0	4	0	4	8	2	6	7	
p-value	0	0	0	0	0		0	0			0		0	0	0	0	0	0	
	.45	.003	,000	.98	.049	.01	,001	.001	.03	.01	,000	.064	.012	.31	.048	.023	.038	,000	

Multiplex PCR antibiotic resistance genes

Amplification of *16s-rDNA* confirmed all the 100 staphylococcal isolates as *S. aureus*. The distribution of methicillin resistance gene (*mecA*) were in 58% strains, that 29 samples (82.9%) from wound infections and 29 samples (42%) from urine infections. The prevalence aminoglycoside resistance genes (*aacA-D*) Was just in 24% of isolates in urine infections. This resistance gene was not found in strains isolated from wound infections. The distribution of tetracycline resistance gene (*tetK*) was detected in 13% of strains, 9 strains (7.25%) isolates of wound, and 8 strains (3.12% isolates of urine. *tet M* were known in 89% of the isolates, 54 isolates (84%) from urine and 35 isolates (100%) of wounds. Macrolide resistance genes (*ermA* and *msrA*) were detected, respectively 40% and 36% of the isolates. Results of molecular detection of antibiotic resistance genes is presented in table 3. There is statistically significant association between the rate of resistance to aminoglycoside, macrolides, tetracycline and and sources infections. But there is no significant association between *mecA* with P=0.141 between the source of infection.

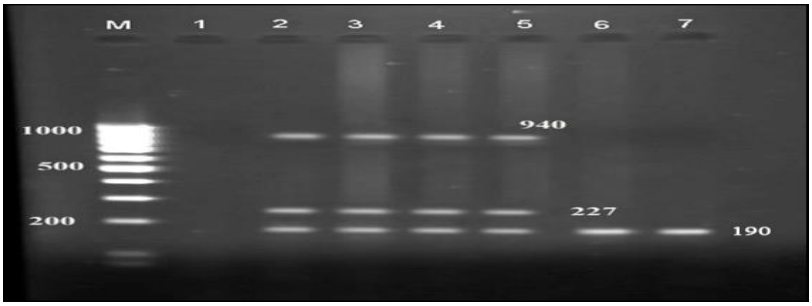

Figure (1): Multiplex PCR amplifications of *S. aureu*s strains isolated from

wounds. Lane M: 100 bp marker. Lane 1: negative control. Lanes 2-7: 227 bp fragment of *aacA-D*, 940 bp fragment *msrA* and 190 bp fragment of *ermA*.

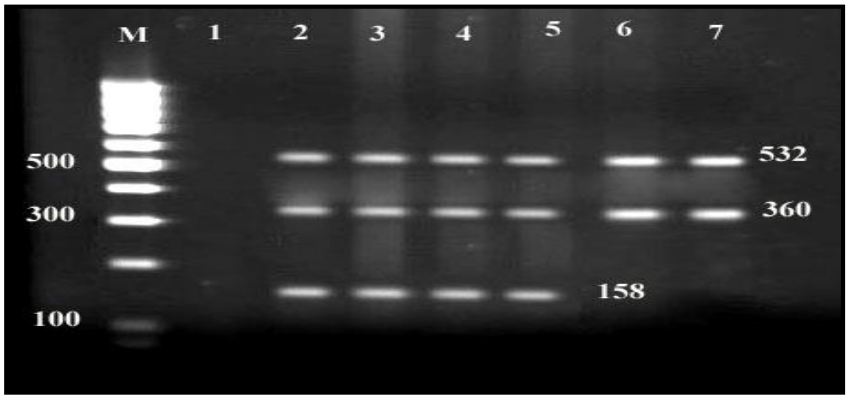

Figure (1): Multiplex PCR amplifications on *S. aureus* strains isolated from wounds and urine. Lane M: 100 bp marker. Lane1: negative control with distilled water, Lanes 2-7: 532 bp fragment of *mecA*, 360 bp fragments indicated *tet K* and 158 bp fragment of *tet M*.

Table 3. Distribution of genes encoding the antibiotic resistance of *S. aureus* strains isolated from urine and wound

Gene	Wound	Urine	Total	Percent	P-value
mecA	29	29	58	58%	0.141
aacA-D	۰	24	24	24%	0<000
tetK	9	4	13	13%	0.004
tetM	35	54	89	89%	0.001
msrA	16	20	36	36%	0.038
ermA	15	25	40	40%	0.0001

Table 4.Comparison of antibiotic resistance of *S. aureus* strains by disk diffusion and multiplex PCR

antibiotic resistance by multiplex PCR method	antibiotic resistance by disk diffusion method	antibiotic
Tetk 13% *tetM* 89%	76%	tetracycline
aacA-D 24%	Neomycin 20% Gentamicin 20%	aminoglycoside
ermA 40% *msrA* 36%	Erythromycin 52% Azithromycin 47%	macrolides
mecA 58%	64%	methicillin

Coagulase gene RFLP results:

In the polymorphism of coagulasegene, PCR products were analyzed by using enzyme Alu1. By using this enzyme based on the size of the band, 9 genotypes of coagulase was observed. The band sizes in genotypes 1-9 are as follows: I, II, III, IV, V, VI, VII, VIII, and IX, 970, 810, 810, 810, 890, 810-1050, 890, 730, and 730 bp. Coagulase gene was found in all of the samples. In this study, from 65 *S. aureus* strains isolated from urine infections, polymorphism of coagulase gene was observed in 53 samples, The genotype in 40 isolates (53/61%) (25 samples isolated from male and 15 samples from women) and genotype VIII in 8 isolates (3/12%) (5 samples from male and 3 samples from women) and genotype IX in 5 isolates (69/7%) (3 samples isolated from male and 2 samples from women) were observed .In the

strains isolated from wound infections, polymorphism of coagulase gene was observed in 22 samples. The genotype in 15 isolates (85.42%) (8 samples isolated from male and 7 samples from women) and genotype VIII in 7 isolates (20%) (5 samples isolated from male and 2 samples from women) was observed. The results in tables 5 and 6 are shown. There was no association significant between genotype and sex in the statistical analysis software SPSS 16.

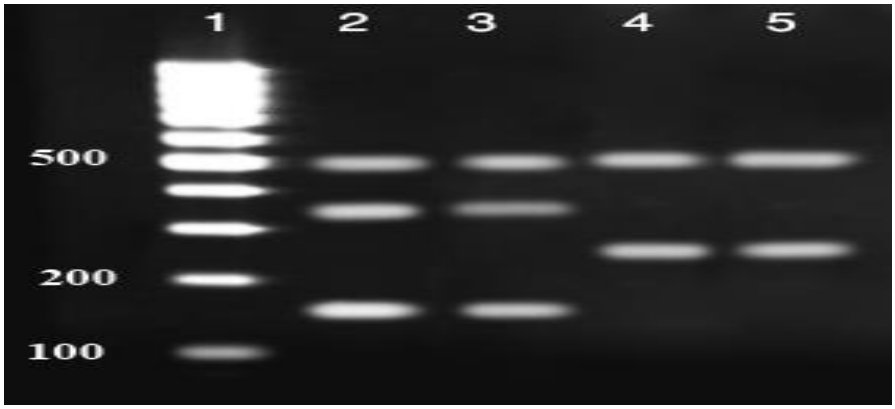

Figure 3. RFLP Patterns of the *coa* amplicon after digestion with endonuclease *Alu*. Lane M: 100 bp marker. Lanes 1–4: approximately 490-320-160-240bp *S. aureus* PCR product.

Table5: coagulase gene genotyping strains isolated from urine

Type code	PCR product (bp)	RFLP (bp)	N %
I	970	490-320-160	40 (61.53%)
II	810	410-240-160	-
III	810	490-240-80	-
IV	810	490-240-160	-
V	890	410-240-160-80	-
VI	810+1050	490-410-320-160	-
VII	890	490-410	-
VIII	730	490-240	8 (12.3%)
IX	730	410-320	5 (7.69%)

Table 6: coagulase gene genotyping strains isolated from wound

Type code	PCR product (bp)	RFLP (bp)	N %
I	970	490-320-160	15(42.85%)
II	810	410-240-160	-
III	810	490-240-80	-
IV	810	490-240-160	-
V	890	410-240-160-80	-
VI	810+1050	490-410-320-160	-
VII	890	490-410	-
VIII	730	490-240	7(20%)
IX	730	410-320	-

Discussion:

Molecular characterization 100 strains of *S. aureus* isolated from urine and wound were studied by using two methods Multiplex PCR and RFLP-PCR. Resistant gene *mecA, msrA, ermA, aacA-D, tetK* and *tetM* isolated were identificated by Multiplex PCR method. In this study, 58% of isolated were carrying *mecA*.The presence of *mec*A gene complex which specifies the production of an abnormal penicillin binding protein PBP2a that has a decreased affinity for binding β-lactam antibiotics results in resistance to methicillin and also to all β-lactams including penicillins and cephalosporins (13). Aket et al., were observed, 40 % of the isolates carried the *mecA* gene in north India in 2012 (19). High antibiotic resistance rates in wound infections is worrying. In this research, 89% of *S. aureus* strains isolated from wound were carrying *mecA.* Akapka et al., were reported that most cases of MRSA was in wound swab specimens (20). Nature of methicillin resistance in strains of *S. aureus* is heterogeneous .Identification of methicillin-resistant *S. aureus*, sometimes due to the heterogeneous expression of resistance is complex and influenced by variables such as pH, temperature and salt concentration (21). In this study ,24% of the isolates carried the *aacA-D gene* for resistant to aminoglycoside, that only were detected in the urine. Italy, France, Portugal and Spain have shown high levels of resistant to aminoglycoside. Level of resistance to aminoglycosides in *S. aureus* change from

country to country and year to year. High levels of resistance to aminoglycosides in *S. aureus* was reported 48% in Greece in 2012 (22). In our study 76% of strains were resistant to tetracycline by disk diffusion method but in the multiplex-PCR method, 89% of strains have *tetM* and 13% have *tetK* for resistance to tetracycline. This result confirms the high sensitivity of the multiplex-PCR method in compared to disk diffusion. All strains isolated from wound could carry *tetM* for resistance to tetracycline.In our study, *ermA* and *msrA* genes for erythromycin resistance were identified respectively of 40 % and 36 %.Duran et al. reported only13 % of strains carrying *msrA* in the hospitals in Turkey and Hatary in 2012 (23).We did report a high level of resistance to vancomycin and rifampin, that is worrying. Classified the *S. aureus* on the basis of coagulase gene by molecular typing method is simple and accurate. This method can study epidemiological of *S. aureus* isolated. Scientists have demonstrated that special genotypes of coagulase gene is dominant in each region that is resistant to the action of neutrophil phagocytosis (24). The results of this research shows 55% of all cases of wound and urine have genotype I. This result shows that the type I can be responsible for infections in many parts of the body. This type is able to adapt to the host tissue that is responsible for virulence in skin and urinary tract infections. Most strains were isolated from urine and wound have similar pattern. Most strains were isolated from urine and wound similar pattern existence of genotype XI in 69/7% of the strains isolated from urine is indicating different type's indifferent locations. There are several virulence factors such as adhesion and antimicrobial peptides and fatty acids suggest increasing stability of this type in the urinary tract, and skin (25). Montesinos et al., observed polymorphism of coagulase gene in four patterns. patternCoa1 (89%) was the most common pattern that cause all of epidemic cases and other patterns were observed sporadic (26).With regard that the prevention and control of Staphylococcal infection depends on the identification of risk factors for infection, more studies on these bacteria should be done so as to obtain information about the prevalence of *S. aureus* infections in hospitals.

Our study showed that *S. aureus* is increasingly resistant to various antibiotics, which is a serious warning for the treatment of infections caused by *S. aur*eus in the region. In principle, the results of antibiograms in different geographical areas differ from each other, because this is

influenced by factors such as how antibiotics are used in hospitals and in the community. To prevent the increase in resistance to other antibiotics, avoid the unnecessary and unnecessary prescription of available antibiotics. Also, avoid prescribing antibiotics that are highly resistant to this bacteria.

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Molecular characterization of *Staphylococcus aureus* strains isolated from wound and urine in Kermanshah, Iran.

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Abstract:

Human is the main source of *Staphylococcus aureus* in the nature. This bacterium is one of the important agents of nosocomial infections. Therefore, is study molecular characterization of this bacteria of high important. After identification and confirmation of 100 strains of *Staphylococcus aureus* isolated from wounds and urine samples in Kermanshah with microbiology standard methods, the antibiotic susceptibility testing was done and resistance genes (*mecA, aacA-D, tet K, tet M, msrA, ermA*) of isolates were studied with multiplex-PCR method and genotyping of coagulase gene of isolates was studied by RFLP-PCR method. In susceptibility testing were reported greatest resistance to penicillin (90%) and lowest resistance nitrofurantoin (8%). In multiplex-PCR method, (49%) of the isolates carry *mecA* gene, (24%) *aacA-D*, (89%) *tetM*, (14%) *tetK*, (40%) *ermA* and (36%) *msrA*. In the detection polymorphism of coagulase gene from 65 strains of *Staphylococcus aureus* isolated from urine, coagulase gene polymorphism was observed in 53 cases, genotype I (61.53%) and genotype VIII (12.3%) and genotype IX (7.69%). The coagulase gene polymorphism of strains isolated from wound were observed in 22 samples. Genotype I was reported (42.85%) and genotype VIII (20%) was observed. Detection molecular characterization of isolates is very effective and efficient in prevention and control infection.

Keywords: *Staphylococcus aureus*, antibiotic resistance patterns, multiplex-PCR, RFLP-PCR, coagulase gene