Acta Microbiologica et Immunologica Hungarica 65 (1), pp. 93–106 (2018)
DOI: 10.1556/030.64.2017.032
First published online August 31, 2017

PREVALENCE AND MOLECULAR TYPING OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* CARRYING PANTON–VALENTINE LEUKOCIDIN GENE

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(Received: 18 November 2016; accepted: 26 May 2017)

Panton–Valentine leukocidin (*pvl*) toxin is an important virulence factor of *Staphylococcus aureus*. The main genes are *coa* and *spa* for distinguishing and typing of *S. aureus* isolates. The aim of this study was to investigate antibiotic resistance, presence of *mec*A and *pvl* genes, as well as epidemiological typing of these isolates according to polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method in clinical sample isolated from Rasht city, Iran. A total of 250 clinical samples have been isolated from different hospitals. First, isolates of *S. aureus* were identified through microbiological methods and their antibiotic sensitivity was determined by disk diffusion agar based on a standard method of Clinical and Laboratory Standards Institute. DNA was extracted by boiling and presence of *pvl* and *mec*A genes was investigated by PCR using specific primers. To type these isolates, amplification of fragments of *coa* and *spa* genes was done and restriction enzyme digestion pattern was determined by PCR-RFLP method. Among the 250 samples, 50 isolates belonged to *S. aureus* and results of antibiotic sensitivity showed that 68% (34 samples) of isolates were methicillin resistant. Frequency of *mec*A and *pvl* genes among *S. aureus* isolates were 60% (30 samples) and 20% (10 samples). The PCR of *coa* gene showed three patterns whereas that of *spa* gene showed two patterns for enzyme digestion. Result of PCR-RFLP using *Hae*III enzymes for *coa* gene and *Bsp*1431 for *spa* gene showed three patterns for enzyme digestion. Recent studies indicated increase in the resistance of *S. aureus* to different antibiotics, which is a serious problem in the treatment of infections resulting from *S. aureus* in this region. The result of PCR of *pvl* showed high frequency of this gene in this region, and *coa* and *spa* typing by PCR-RFLP was a useful tool for typing of *S. aureus* isolates.

Keywords: *Staphylococcus aureus*, *mec*A, *pvl*, *coa*, *spa*, PCR-RFLP

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Introduction

Staphylococcus aureus is a Gram-positive, facultative anaerobic bacterium, and is a common pathogen in hospital cases. Studies show that it can exist in 20% of people constantly, while 60% of people are considered as alternative carriers of this bacterium. This bacterium causes an extensive spectrum of diseases, such as endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, boils or abscesses, and the most important way of delivery, this bacterium is caused through polluted hands, specifically in health-care centers [1]. The resistance of *S. aureus* to antibiotic has special importance, because this bacterium shows drug resistance as compared with other bacteria. Based on geographical region, it has faced significant changes in the pattern of antibiotic sensitivity in previous years. One main problem in the treatment and prevention of infections by *S. aureus* is the resistance of this bacterium to different antibiotics namely, β-lactams, aminoglycosides, and macrolides. Infections caused by multiresistant *S. aureus* leads to higher mortality, longer hospital stay, increased cost of treatment, and possible further dissemination of resistant strains [2]. Studies showed that about 30% to more than 50% of *S. aureus* isolates are resistant to methicillin and the reason is related to the presence of *mec*A gene [3]. The pathogenicity of *S. aureus* infections is related to various compounds of bacteria level and extracellular protein, such as Panton–Valentine leukocidin (*pvl*) [4]. *pvl* is one of the important virulence factors in *S. aureus* and is composed of two protein components S (38 KDa) and F (32 KDa), which are controlled by the *Luks/pv* and *Lukf/pv* genes. The product of this gene can cause opening of calcium channels, necrosis, and apoptosis of human leukocytes [5–7]. Positive *pvl* strains of *S. aureus* have high virulence and are more accompanied by furuncle, skin abscesses, and infections with severe necrosis [8]. Molecular typing of *S. aureus* is a useful tool to discriminate isolates during tracing source of infection and to strengthen infection control. It is important to note that a variety of *Staphylococcus* coagulase enzyme causes difference in their antigenicity. *Coa* gene is the main gene that discriminates isolates of *S. aureus*. The end of 3′ repetitive short frequency with the size of 81 bp and their number is different among different strains. *Coa* gene was observed in all the *Staphylococcus* strains that shows capability of typing for this gene [9]. Protein A is the surface protein and coded by *spa* gene, a region which in a repetitive district, is identified at the end of 3′ X region. Repeating region of district X includes 12 sections with length of 24 nucleotides and these 24 nucleotides are a polymorphism region with repetitive and short frequency, and variation of protein A is due to variation in X region [10]. Some types of *S. aureus* isolates do not have the ability to retain protein A in their wall, so they release all the proteins produced. This phenomenon

is mainly observed in all methicillin-resistant *S. aureus* (MRSA) [11]. Regarding the importance of these strains, the aim of this research was to study antibiotic resistance and consider the presence of *mec*A gene for confirming MRSA strains and also consider the rate of distribution of *pvl* gene among clinical strains of *S. aureus* isolated from hospitals of Rasht, Iran and molecular typing of these isolates based on *coa* and *spa* genes through polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method.

Materials and Methods

Bacterial isolates

In this study which lasted over a year and was approved by the committee of research ethics, written informed consent of patients, 250 clinical samples (injury, skin, blood, and urine) from different hospitals (Razi, Poursina, Ghaem, Alzahra, and Ashtiani's lab) were collected during 2014–2015. Phenotypic identification of strains of *S. aureus* was carried out by test of Gram-staining, catalase, coagulase, DNase, and cultivation on the environment of Baird–Parker agar and Mannitol Salt agar (Merck, Germany) [12].

Antibiotic susceptibility

Microbial sensitivity test was carried out using Kirby–Bauer method and according to the instruction of the Clinical and Laboratory Standards Institute [13]. Sensitivity of isolates of *S. aureus* against antibiotic discs of cefoxitin (10 μg), neomycin (10 μg), ciprofloxacin (5 μg), penicillin (10 units), gentamicin (10 μg), amoxicillin (10 μg), chloramphenicol (30 μg), and clindamycin (2 μg) (MAST, UK) was done in Mueller–Hinton agar (Merck, Germany). In all experiments, standard strain of *S. aureus* (ATCC 33591) was used as positive control resistant against methicillin (having *mec*A gene) and standard strain of *S. aureus* (ATCC 49775) as positive control having *pvl* gene and *S. epidermidis* (ATCC 12228) was used as negative control.

DNA extraction and PCR

DNA extraction carried out using DNA Extraction Kit (CinnaGen, Tehran, Iran) and consequently PCR method was done. PCR was consistently performed

Table I. Sequence of primers used in PCR [14–17]

Gene	Primer sequence (5′-3′)
mecA-F	TCCAGATTACAACTTCACCAGG
mecA-R	CCACTTCATATCTTGTAACG
pvl-F	AGAAGATACAAGTAGCGATAAGTG
pvl-R	AAGGATTGAAACCACTGTGTAC
coa-F	CGAGACCAAGATTCAACAAG
coa-R	AAAGAAAACCACTCACATCA
spa-F	ATCTGGTGGCGTAACACCTG
spa-R	CGCTGCACCTAACGCTAATG

in a 20 µL reaction volume, with each reaction mixture containing 1.0 µL of DNA template, 10 mM of each primer, 2.0 µL of Taq buffer, 2.5 mM of deoxynucleotide triphosphates, and 2.5 mM of Taq polymerase (CinnaGen, Tehran, Iran). Sequence of primers used in PCR is shown in Table I.

Thermal cycling was performed in a Prime Thermal Cycler (Techne, Germany) under the following conditions: denaturation for 5 min at 94 °C, 1-min amplification cycles at 94 °C, and additional amplification cycles for 50 s at 55 °C, 1 min at 72 °C, and a final extension cycle for 10 min at 72 °C. The PCR products were detected by electrophoresis on agarose gels, stained with power load stain, and photographed using a UV transillumination imaging system.

RFLP of coa *gene PCR products*

Depending on 81 bp repeats, a strain analysis of PCR-RFLP products was performed with *Hae*III restriction enzyme (Thermo Scientific, USA), where 10 µL of PCR product of *coa* gene was incubated with 6 U of the enzyme at 37 °C for 45 min in a water bath.

RFLP of spa *gene PCR products*

Five µL of each *spa* gene amplicon and 10 units of *Bsp*1431 restriction enzyme (Thermo Scientific, USA) were incubated at 37 °C for 3 h. The PCR products and restriction digest fragments were detected by electrophoresis in 2% agarose gel. The interpretation criteria for identifying different strains were a single band difference. Unique PCR-RFLP patterns were assigned a genotype. It should be noted that in this step of studying, strain of standard *S. aureus* ATCC 8325/4 was used as control. Statistical analysis was carried out through statistical software of SPSS 22 and χ^2 test.

Results

Samples were separated from urine, blood, skin, and wound and using Gram stain, mannitol salt agar, catalase test, coagulase test, 50 isolates of *S. aureus* were separated (Table II).

The sensitivity of *S. aureus* isolates to the tested antibiotics is shown in Table III. A percentage of 68 were resistant to antibiotic cefoxitin and was considered as MRSA strains. The highest resistance related to antibiotics of penicillin (98%), ampicillin (90%), amoxicillin and trimethoprim (86%) and the least resistance related to antibiotics of vancomycin (sensitive 100%) and ciprofloxacin (sensitive 56%). Total resistance rate of antibiotic in strains separated from urine and wound samples was more than other strains. Resistance to vancomycin was not observed in any strain.

Table II. Distribution of *S. aureus* according to the type of samples and gender

	Female		Male		Total	
Type of samples	%	*N*	%	*N*	%	*N*
Blood	6	15	8	20	14	35
Trauma	12.8	32	4	10	16.8	42
Skin	11.2	28	6	15	17.2	43
Urine	50	60	28	70	52	130

Table III. Antibiotic susceptibility patterns for *S. aureus*

	Resistant		Intermediate		Sensitive	
Antibiotics	%	*N*	%	*N*	%	*N*
Cefoxitin	68	34	0	0	32	16
Vancomycin	0	0	0	0	100	50
Ciprofloxacin	42	21	2	1	56	28
Penicillin	98	49	0	0	2	1
Erythromycin	56	28	18	9	26	13
Trimethoprim	86	43	6	3	8	4
Amikacin	42	21	6	3	52	26
Ampicillin	90	45	0	0	10	5
Gentamicin	40	20	6	3	54	27
Amoxicillin	86	43	0	0	14	7
Chloramphenicol	8	4	14	7	78	39
Clindamycin	46	23	12	6	42	21

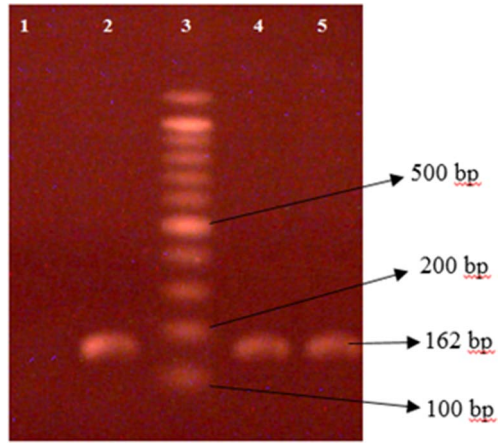

Figure 1. 1% agarose gel electrophoresis analysis of PCR amplification products of *mec*A gene of 162 bp extracted from *S. aureus*. Lane 1: negative control (no DNA template); lanes 2, 4: positive isolates; lane 3: DNA molecular size marker (100 bp ladder); lane 5: positive control (*mec*A-positive strain ATCC 33591)

Amplification of mec*A gene*

The results showed that the distribution of *mec*A gene existed in staphylococcus in 60% of samples (30 samples). It should be noted that the rate of distribution of *mec*A gene in samples of *S. aureus* separated from wound samples was more than other samples; from 30 samples, 15 samples belonged to wound samples ($p < 0.032$) (Figure 1).

Amplification of pvl *gene*

For considering existence of *pvl* gene in strains of separated *S. aureus*, specific primers of this gene were used and we expected existence of the band 575 bp that its image was observed in electrophorese gel. *pvl* gene was positive in 20% of samples (10 samples) (Figure 2). It is necessary to mention that there was not meaningful relationship between existence of *pvl* gene, type of sample ($p < 0.046$), and *mec*A gene among strains ($p < 0.015$).

Amplification of coa *and* spa *genes*

For considering existence of *coa* gene and *spa* gene in strains of separated *S. aureus*, specific primers of these genes were used and we expected existence of 575 bp band for both genes. The result showed that each has two patterns of

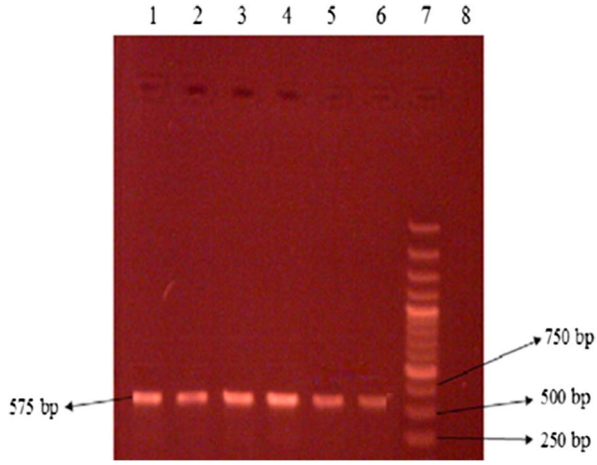

Figure 2. 1% agarose gel electrophoresis analysis of PCR of *pvl* gene. Lanes 1–5: positive isolates; lane 6: positive control (*pvl*-positive strain ATCC 49775); lane 7: DNA molecular size marker (100 bp ladder); lane 8: negative control

amplification and size of product PCR of genes was varied. PCR products of *coa* gene were 891 bp in some isolates, 810 and 405 bp in others. PCR products of *spa* gene were 1,200 bp in some isolates, 1,296 and 240 bp in others (Figures 3 and 4).

PCR-RFLP of coa *and* spa *genes*

For being certain about different genetic content of PCR bands, RFLP technique was used. Regarding research in computer (*in silico*) on *coa* and *spa*

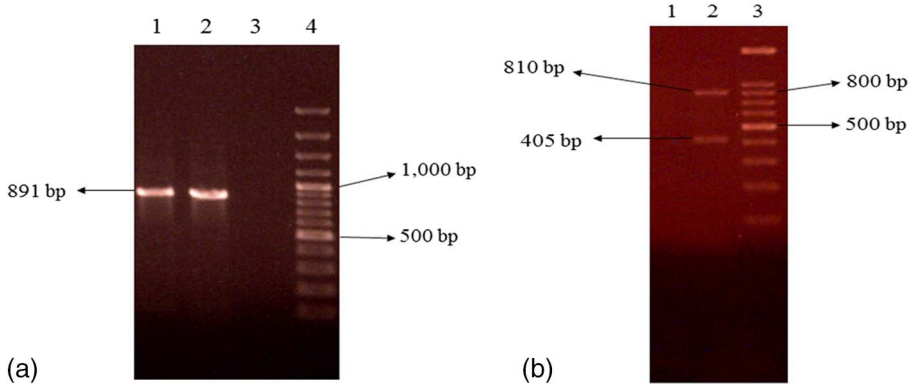

Figure 3. Representative 2% agarose gel electrophoresis of *coa* gene PCR products where 3 (b) and 4 (a) are DNA molecular size markers (100 bp ladder). (a) Isolates 1 and 2 showing single band, (b) isolate 2 showing two bands

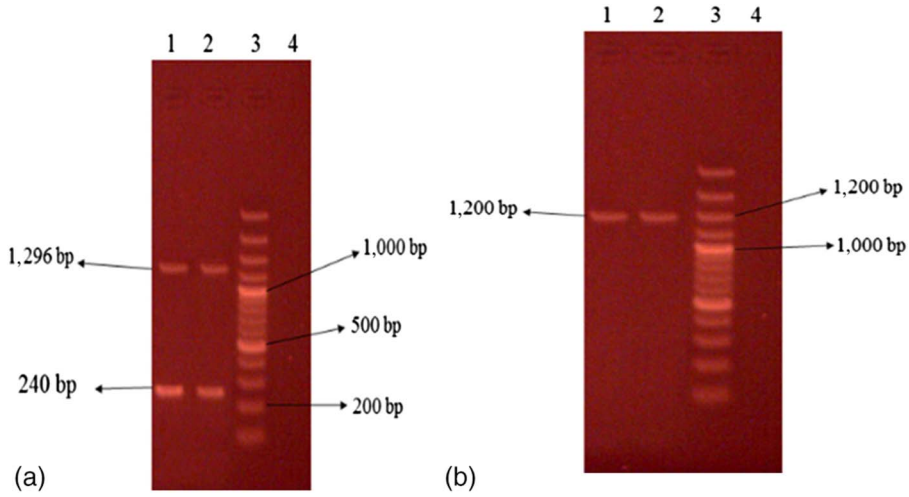

Figure 4. Representative 2% agarose gel electrophoresis of *spa* gene PCR products where lane 3 is DNA molecular size marker (100 bp ladder). (a) Isolates 1 and 2 showing two bands, (b) isolates 1 and 2 showing single band

genes in database GenBank, enzymes *Hae*III and *Bsp*1431 were used that has proper restriction sites on *coa* and *spa* genes (Figures 5 and 6).

Discussion

Distribution of resistance against different antibiotics among strains of *S. aureus* has created many problems in different parts of the world. Unfortunately, there is little information on the resistance rate of *S. aureus* strains against different antibiotics in some cities of Iran. In this study, 68% of *S. aureus* strains were isolated from patients who are resistant to cefoxitin (MRSA) and this rate is more than that of studies conducted by some researchers. These results showed that the frequency of MRSA strains in the subjects is more related to infectology and surgical departments. Potential danger of the distribution of MRSA strains in a special care section has been paid attention due to the occurrence of more problems in the hospitals, various medical manipulation, and extensive consumption of antibiotics. Study of Zamani et al. [18], on 70 samples of *S. aureus*, showed that 50% were MRSA and considering antibiotic-resistance pattern, they showed resistance to tetracycline (74.2%), co-trimoxazole (68.5%), erythromycin (68.5%), and ceftazidime (51.4%). Studies by Moradi et al. [19], on 104 samples of *S. aureus*, showed that the highest rate of sensitivity was toward vancomycin (96.2%), chloramphenicol (88.2%), rifampin (81.7%), and the lowest rate of resistance was toward cefoxitin (40.4%). In a study by Lepsanovic et al. [20],

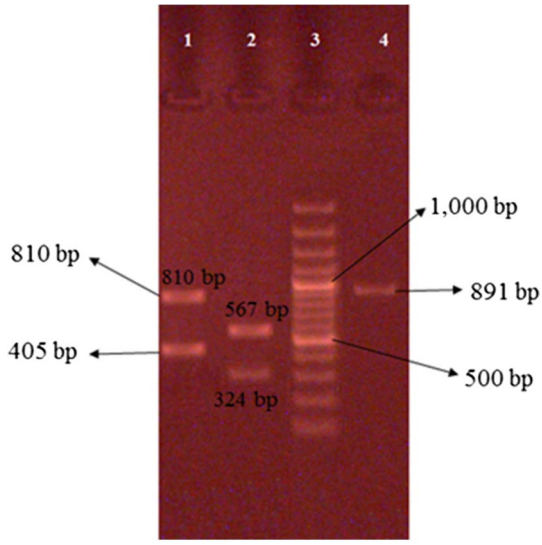

Figure 5. Restriction analysis of *coa* gene with *Hae*III restriction enzyme. Column 3 is DNA molecular size marker (100 bp ladder), isolates 1–4 showing 1 band of *coa* gene that remained uncut, and isolate 2 showing two bands of *coa* gene PCR product

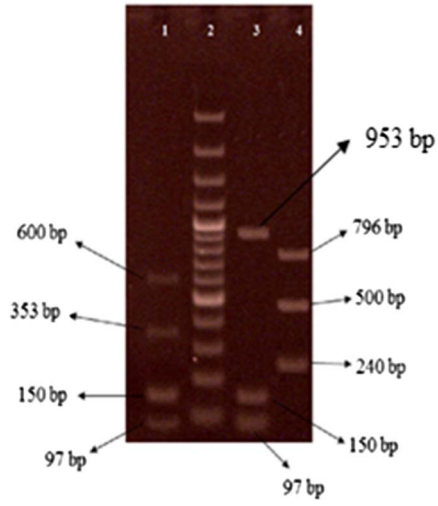

Figure 6. Restriction analysis of *spa* gene with *Bsp*1431 restriction enzyme. Column 2 is DNA molecular size marker (100 bp ladder). Isolate 1 showing four bands of *spa* gene, isolate 3 showing three bands of *spa* gene, and isolate 4 showing three bands of *spa* gene PCR product

all MRSA strains from hospitalized patients were resistant to one or more non-β-lactam antibiotics while 52% were multiresistant. In isolates from healthy people, 16% were sensitive to all non-β-lactam antibiotics and 40% were

multiresistant. Study by Haghgoo et al. [21], on 13 samples of *S. aureus*, isolated from the cultivation of blood of patients kept in Shahid Madani Hospital in Tabriz, Iran, showed that the highest resistance was toward methicillin and ceftriaxone (31%). In a study by Karasartova et al. [22], 43 phages isolated from 48 MRSA were investigated for carrying toxin genes including the *sak, eta, lukf-pv, sea, selp, sek, seg, seq chp*, and *scn* virulence genes using PCR and Southern blot. The results indicate that prophages encode a significant proportion of MRSA virulence factors. Distribution of strains of cefoxitin-resistant *S. aureus* in this study is more than that in the mentioned studies. *pvl* is a virulence factor that is carried by a bacteriophage and it can be transferred to other *Staphylococcus* strains. Positive strains of *pvl* have high virulence and are responsible for severe infections like infection of bone joint and necrosis pneumonia. Therefore, rapid distinguishing and on time control of the *pvl*-positive strains is necessary. This is the first study to determine the frequency of *pvl* gene in clinical samples from Rasht, Iran. In this study, it was shown that from 50 isolates of *S. aureus*, 20% (10 samples) carried this gene and were more frequent in samples of urine and also it was shown that no significant relationship existed between *pvl* and *mec*A genes among the strains. Molla-abbaszadeh et al. [23], considered the distribution of *pvl* gene in strains of *S. aureus* and from 100 strains, 18 (18%) were reported to be positive, regarding the existence of *pvl* gene and from these subjects, 94.4% MRSA and 56.6% were methicillin-sensitive *Staphylococcus aureus* (MSSA). Khosravi et al. [17] reported distribution of *pvl* gene in MRSA strains was 7.2% and in MSSA strains, it was 33.3% [17]. Recently, more studies have been conducted on positive *pvl* strains of MRSA, whereas positive *pvl* infections of MSSA play important role in the distribution of positive *pvl* strains. About 60% of the total positive strains for *pvl* in England in the past 5 years were sensitive to methicillin [24]. A study of Cupane et al. [25] showed that that 75% of strains of *S. aureus* had *pvl* gene and most of them (60.7%) were MRSA. In a study by Osman et al. [26], among 210 *S. aureus* samples, *pvl* gene was observed in 58%. Brown et al. [8] considered 1,055 *S. aureus* regarding existence of *pvl* gene, 377 strains (35.7%) had this gene, which is most resistant to methicillin. In a study of Lima et al. [27], on strains of MRSA isolated from patients with cystic fibrosis showed that almost half of the strains carried *pvl* gene. In this study, distribution of *pvl* gene in MRSA strains was more that 60% of the samples of positive *pvl* (six samples) in strains MRSA and 40% (4 samples) in MSSA strains. The distribution of *pvl* gene in the study of Molla-abbaszadeh et al. [23] and Khosravi et al. [17] was more than that of the study of Cupane et al. [25] and Osman et al. [26], and in the study of Lima et al. [27], it was less, and it seems that the distribution of positive *pvl* gene was more in MRSA strains in all the mentioned studies. *Coa* gene exists in all *Staphylococcus* strains and shows capability of typing for this gene; therefore,

it is a main gene for distinguishing *S. aureus*. In this study, three types of *coa* genes were observed among the strains that there was band of 405 bp in most strains. Existence of more than one band shows more than one allele in *coa* gene, which shows that the strain produces different variants of this protein. Polymorphism existing in *coa* gene resulted from deletion and insertion at the end of 3′ of *coa* gene and gene sizes changes in this way. Himabindu et al. [28] showed that PCR product of *coa* gene has three band patterns and most isolates had 812 bp, whereas in this study, band of 405 bp existed in most strains. According to studies of Lawrence et al. [29], typing of *coa* gene creates common band of 402 bp, which is similar to the present studies in which 405 bp band occurred in most strains. In this study, the PCR product of some strains was not cut by *Hae*III enzyme and it showed that they do not have cutting site for *Hae*III. Study of Lawrence et al. [29] demonstrated that band of 402 bp by *Hae*III enzyme was changed to bands 176, 146, and 81 bp. In case that in our studies, band of 405 bp have not been cut by *Hae*III enzyme. In a study by Karahan et al. [30], in Turkey, it was shown that from 200 strains of *S. aureus*, 161 (80.5%) had polymorphism in *coa* gene. About 83.9% of these strains had a band with size of 500–1,400 bp and 16.1% had two pieces. Size of *spa* gene based on its X region is polymorphic and has repetitive sequence of 24 bp; this number and sequence in different strains are different. Size of *spa* gene in this study was created in the 3′ end with different sizes, which were observed to be 1,200, 1,296, and 240 bp in gels. Inexistence of *spa* gene was observed in three strains. Study by Shakeri et al. [10] showed that *spa* gene was not present in 5% of the strains. Schmitz et al. [31] showed that five types of *spa* gene were reported among strains of *S. aureus*. They reported that the number of repetitive sequences of X region of *spa* gene is related to the epidemiological compatibility. Strains having shorter length cannot attach to the epithelium of nose; therefore, exit through breath, caught, and sneezed out through the nose. It should be noted that this is the first study conducted on molecular typing of clinical isolates of *S. aureus* based on *coa* and *spa* genes samples, from hospitals of Rasht, Iran; this shows difference of genetic patterns. In some cases, some similarities are observed in different sections, which probably show a kind of bacterial transfer between the staff and hospitals.

Conclusions

The results of this study showed increasing resistance of clinical samples of *S. aureus* to different antibiotics. The resistance rate of *S. aureus* to cefoxitin was high and on the other hand, its resistance toward other antibiotics, such as β-lactam, aminoglycosides, macrolides, and quinolone, is high. The *pvl* gene shows

relatively high frequency in this region as compared with other places in the world. Since infection with the bacteria, *S. aureus* is very prevalent and toxin-producing bacteria create problems at the society level, so it is necessary to use proper diagnostic method, especially molecular methods to identify virulence factors.

Conflict of Interest

No conflict of interest associated with this work.

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