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جداسازی سویه‌های *استافیلوکوکوس اپیدرمیدیس* مقاوم به متی سیلین حاوی ژن *arcA* تولید آنتروتوکسین-های A، B، C، D، E، G و I از عفونت‌های بیمارستانی در اصفهان

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چکیده

استافیلوکوکوس اپیدرمیدیس انواع مختلفی از سموم پروتئین خارج سلولی از جمله انتروتوکسین‌های استافیلوکوکی را تولید می‌کند که به عنوان سوپر آنتی ژن عمل می‌کنند و می‌توانند شرایطی را برای حمله به سلول‌های میزبان فراهم کنند. هدف از مطالعه حاضر تعیین الگوی مقاومت آنتی بیوتیکی و تشخیص ژن‌های انتروتوکسین در سویه‌های *استافیلوکوکوس اپیدرمیدیس* مقاوم به متی سیلین حاوی ژن ACME-*arcA* در اصفهان بود. این بررسی بر روی ۱۵۰ ایزوله استافیلوکوکوس جدا شده از عفونت‌های مختلف بیمارستانی انجام شد. سویه های مقاوم به متی سیلین با روش PCR تایید شدند و الگوی ضد میکروبی ایزوله‌ها با روش انتشار دیسک تعیین شد. سرانجام، ژن‌های انتروتوکسین در سویه‌های مقاوم به متی سیلین حاوی ژن ACME-*arcA*با استفاده از PCR تشخیص داده شدند. از ۱۵۰ ایزوله استافیلوکوکوس مورد بررسی، ۱۰۰ ایزوله به‌عنوان *استافیلوکوکوس اپیدرمیدیس* شناسایی شدند. از ۱۰۰ ایزوله *استافیلوکوکوس اپیدرمیدیس* ۶۵ ایزوله (۶۵ درصد) ایزوله مقاوم به متی سیلین بودند .از این تعداد ، ۳۳ ایزوله (۷ /۵۰ درصد) هر دو ژن *mecA* و ACME-*arcA* را داشتند. الگوها ضد میکروبی ایزوله‌های مقاوم به متی سیلین در این مطالعه نشان داد، بیشترین میزان مقاومت را به اریترومایسین (۸۱/۵ درصد) و کلیندامایسین (۶۴/۶ درصد) نشان می‌دهند. در حالی‌که کمترین مقاومت را به ریفامپین (۷/۶ درصد) و لینزولاید (۱/۵ درصد) نشان دادند. علاوه بر این، فراوانی ژن‌های انتروتوکسین *sea, seb, sec,see, sed, seg, seh, sei* به‌ترتیب ۶۰/۶ درصد، ۶۳/۶ درصد، ۶۶/۶ درصد، ۱۵/۱ درصد، ۶ درصد، ۷۲/۷ درصد، ۰ درصد و ۷۵/۷ درصد گزارش شد. تنوع انواع ژن‌های انتروتوکسین و مقاومت آنتی بیوتیکی در سویه‌های *استافیلوکوکوس اپیدرمیدیس* مقاوم به متی سیلین از جمله دلایل نگرانی در مورد بهداشت عمومی است. بنابراین گزارش سریع و دقیق از وجود ژن‌های انتروتوکسین از عفونت‌های بیمارستان ضروری است.

INTRODUCTION

In the recent two decades, the importance of coagulase-negative staphylococci has raised. *Staphylococcus epidermidis* is the most important member of the coagulase-negative staphylococci (CoNS), and have been connected to significant hospital-associated infections (HAI) which today is resistant to methicillin (1). More than 70% of the hospital isolates are methicillin-resistant *Staphylococcus epidermidis* (MRSE), which makes it difficult, and costly to treat. MRSE is one of the most important bacteria that cause such complications as bacteremia, endocarditis, wound infection, urinary tract infections, pneumonia, and skin, and soft tissue infections, particularly in the intensive care unit (ICU). These bacteria are considered as the third cause of hospital infection (2, 3).

The emergence of antibiotic-resistant MRSE strains in hospital environments might cause serious problems to hospitalized patients, especially in immunocompromised patients. This phenomenon ultimately leads to high mortality rates among patients in hospital environments (4-6). *Staphylococcus epidermidis* containing the ACME-*arcA* reserves are closely correlated with pathogens, and antimicrobial-resistant genes (7). ACME is a genetic region of the DNA integrated inside the *orfX*. This element adheres to the SCC*mec* element in MRSEs, and enhances the pathogenicity, and colonization of MRSEs in humans. However, the agent entry of this element into SCC*mec* has not yet been known (4, 8). ACME is in the downstream region of the staphylococcal cassette chromosome *mec* (SCC*mec*), and together make up the ACME-SCC*mec*. It seems the physical interactions between SCC*mec*, and ACME appear to increase antibiotic resistance, and pathogenicity in staphylococci (7, 8). Today, the infectious transmitted diseases in hospitals pose a major global problem, some of which are transmitted through contaminated foods in hospital (4, 8). Food poisoning occurs as a result of eating foods that contain heat-resistant enterotoxins, and are usually produced by staphylococci. These toxins are injected into the circulatory system, then affecting the incontinent vomiting center to produce such symptoms as nausea, vomiting, rarely diarrhea, and abdominal muscle pains (7-9).

Enterotoxins are transmitted horizontally through mobile genetic elements (MGEs) such as transposons, bacteriophages, conjugative plasmids, and pathogenicity islands (PAIs). They inflict serious dangers, particularly to the elderly, immunocompromised *individuals*, pregnant women, and infants. Staphylococcal enterotoxins in the group of superantigens (SAgs) have 23 different types. Depending on the type of enterotoxin, the amount of produced toxins ranges from 10 ng to 20 μg (7, 9, 11). Enterotoxins are similar in structure, and biological activity but differences in antigenic properties. More than 95% of the food poisoning enterotoxins produced by the *Staphylococcus epidermidis* are part of the A-E groups. In addition, enterotoxin to being the basic reason for

food poisoning, the main impression in pathological activities, such as osteomyelitis, sepsis, and respiratory distress syndrome (11,13, 28).

Staphylococcus enterotoxins can tolerate 100°C for half an hour. Their thermal resistance in food is higher than that in the laboratory cultures, so their biological activity remains unchanged after the heating stages of food preparation. Moreover, these toxins *gastric resistance* to acid, and activity remained even after food digestion.

Although pasteurization might destroy staphylococcal cells, the heat-resistant staphylococci enterotoxins remains biologically strenuous. Therefore, these toxins should be considered as important factors of food poisoning (10-13). All enterotoxins are mitogens of T cells that cause non-specific proliferation of T cells, and can provide conditions *to attack host cells* ; even 0.1mg of enterotoxins can stimulate T cells, and cause digestive disorders without damaging the intestinal tissue (10-14). The present study was designed, and implemented for the isolation ,and detection of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and s*ei* genes in *mecA*, and ACME-*arcA* positive strains isolated from hospital infections.

Materials and Methods

Strain isolation and identification

This cross-sectional study was done, during a period of 6 months in 2020. On a total of 150 staphylococcal isolates acquired from different nosocomial infections such as urine, blood, wound, catheter, throat in hospitals of Isfahan (Shariati, Gharazi, and Mehregan hospitals). Samples were incubated at 37 °C for 24 hours in blood agar (Merck, Germany). After colony growth, complementary tests based on using conventional bacteriological methods such as gram staining, culture on *mannitol salt agar* (MSA) (*Merck, Germany*), other biochemical tests such as coagulase, and novobiocin sensitivity assay were performed (15).

Detection of *mecA* genes by Polymerase Chain Reaction

The PCR method was used to detect MRSE isolates according to a described protocol in Hoveida et al (16). All DNA of strains was extracted, utilizing a DNA extraction kit (CinnaGen, Iran). The amplification reaction was performed in a final volume of 25 µl, containing 5 µl of genomic DNA, and 12.5 µL of Taq DNA polymerase, PCR master mix contained 2.5 µl 10x buffer, 0.6 µl $MgCl_2$, 0.4 µl dNTP, 0.5 µl of each primer (10 pmol/ µl), and 15.2 µl ddH_2O. A total of 35 cycles were performed with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15s, annealing at 55°C for 15s, and extension at 72°C for 20s, followed by a final extension at 72°C for 5 min. The *PCR products* were analyzed by electrophoresis on a 1.5% agarose gel, stained with *SYBR* Green. The primer sequence used in this reaction is shown in Table1.

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of MRSE strains were done according to disk diffusion procedure (Kirby–Bauer). For this purpose, antibiotics including erythromycin (15 µg), clindamycin (2 µg), cefoxitin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), rifampicin (5 µg), linezolid (30 µg) (*Mast, UK*) have been used (14), After 24h of incubation, the diameter of zone of inhibition of bacterial growth was measured, and results *compared* to the reference tables prepared by CLSI (15).

Screening of ACME-*arcA* gene

Identification of the ACME-*arcA* gene in the MRSE isolates was done using a thermal cycle, and specific primers for this gene. The primer sequence, and PCR condition are shown in Table1 (7). Briefly, all DNA of strains was extracted, utilizing a DNA extraction kit (CinnaGen, Iran). The amplification reaction was performed in a final volume was adjusted to 25 µl, containing 1 µl of genomic DNA, and 12.5 µL of Taq DNA polymerase, PCR master mix (CinnaGen, Iran), 1 µl of each primer, and 15.2 µl ddH_2O. A total of 35 cycles were performed with an initial denaturation at 96°C for 5 min, 35 cycles of denaturation at 94°C for 45min, annealing at 45.5°C for 60 min, and extension at 72°C for 1min, followed by a final extension at 72°C for 10 min. The *amplification products* were analyzed by electrophoresis on a 1% agarose gel, stained with *SYBR* Green (7).

Detection of enterotoxin genes

To detection of enterotoxin genes, DNA of each isolate was extracted, utilizing a DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instructions.

The DNA concentration, and purity was confirmed using Nanodrop a 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR specific primers, and conditions utilized to sequence genomic DNA was shown in Table 1.

Statistical Analysis

All analyses were carry outed using SPSS Statistics version 20 (SPSS, Chicago, USA), using chi-square test, and kappa coefficient and values < 0.05 were considered statistically significant.

RESULTS

Out of 150 nosocomial infections, 100 *Staphylococcus epidermidis* strains were isolated. Among the 100 of *Staphylococcus epidermidis* recovered from nosocomial infections, 65 isolates (65%) were found resistant to methicillin (MRSE) (Figure1(A)), and of these, 33 isolates (50.76%) had both *mecA*, and ACME-*arcA* genes (Figure1(B)). The antimicrobial susceptibility patterns of MRSE isolates in this study showed that the MRSE isolates exhibited the highest rates of resistance to erythromycin (81.5%), clindamycin (64.6%), cefoxitin (63.07%), and tetracycline (61.5%), while they showed the lowest resistance to rifampicin (7.6%), and linezolid (1.5%) (Table 2). The antimicrobial resistance patterns of various types of hospital infections against commonly utilized

antibacterial agents is shown in Table 3. Also, the frequencies of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and s*ei* genes were 60.6% (20 strains), 63.6% (21 strains), 66.6% (22 strains), 15.1 % (5 strains), 6% (2 strains), 72.7% (24 strains), 0% (0 strains), and 75.7% (25 strains) respectively, in the isolates containing the *mecA*, and ACME-*arcA* genes. Figure 1 shows the patterns according to the primers utilization in the PCR test. The *sei* gene isolated from urine infection that showed the highest ratio between enterotoxin genes (Figure 2).

Table 1: Primer sequence used in the PCR reaction

Gene	Amplicon size	Sequence	Annealing temperature	Reference
mec	310 bp	F:TGGCTATCGTGTCACAATCG R:CTGGAACTTGTTGAGCAGAG	55°C	(16)
arcA	486 bp	F: GAG CCA GAA GTA CGC R: CAC GTA ACT TGC TAG AAC GA	45.5°C	(7)
sea	120bp	F: CCTTTGGAAACGGTTAAAACG R: TCTGAACCTTCCCATCAAAAAC	55°C	(16)
seb	164bp	F: GTATGGTGGTGTAACTGAGC R:CCAAATAGTGACGAGTTAGG	50°C	(32)
sec	257 bp	F: GCAGGTACTCTATAAGTGCC R:GACATAAAAGCTAGGAATTT	50°C	(33)
sed	278bp	F: CCAATAATAGGAGAAAATAAAAG R: ATTGGTATTTTTTTTCGTTC	50°C	(32)
see	170bp	F:CAAAGAAATGCTTTAAGCAATCTTAGGCCAC R: CTTACCGCCAAAGCTG	55°C	(31)
seg	287 bp	F: TGCTCAACCCCGATCCTAAATTAGACGA R: CCTCTTCCTTCAACAGGTGGAGACG	55°C	(17)
seh	376 bp	F:CAATCACATCATATGCGAAAGCAG R:CATCTACCCAAACATTAGCACC	50°C	(31)
sei	454 bp	F: TGGAGGGGCCACTTTATCAGGA R: TCCATATTCTTTGCCTTTACCAGTG	50°C	(17)

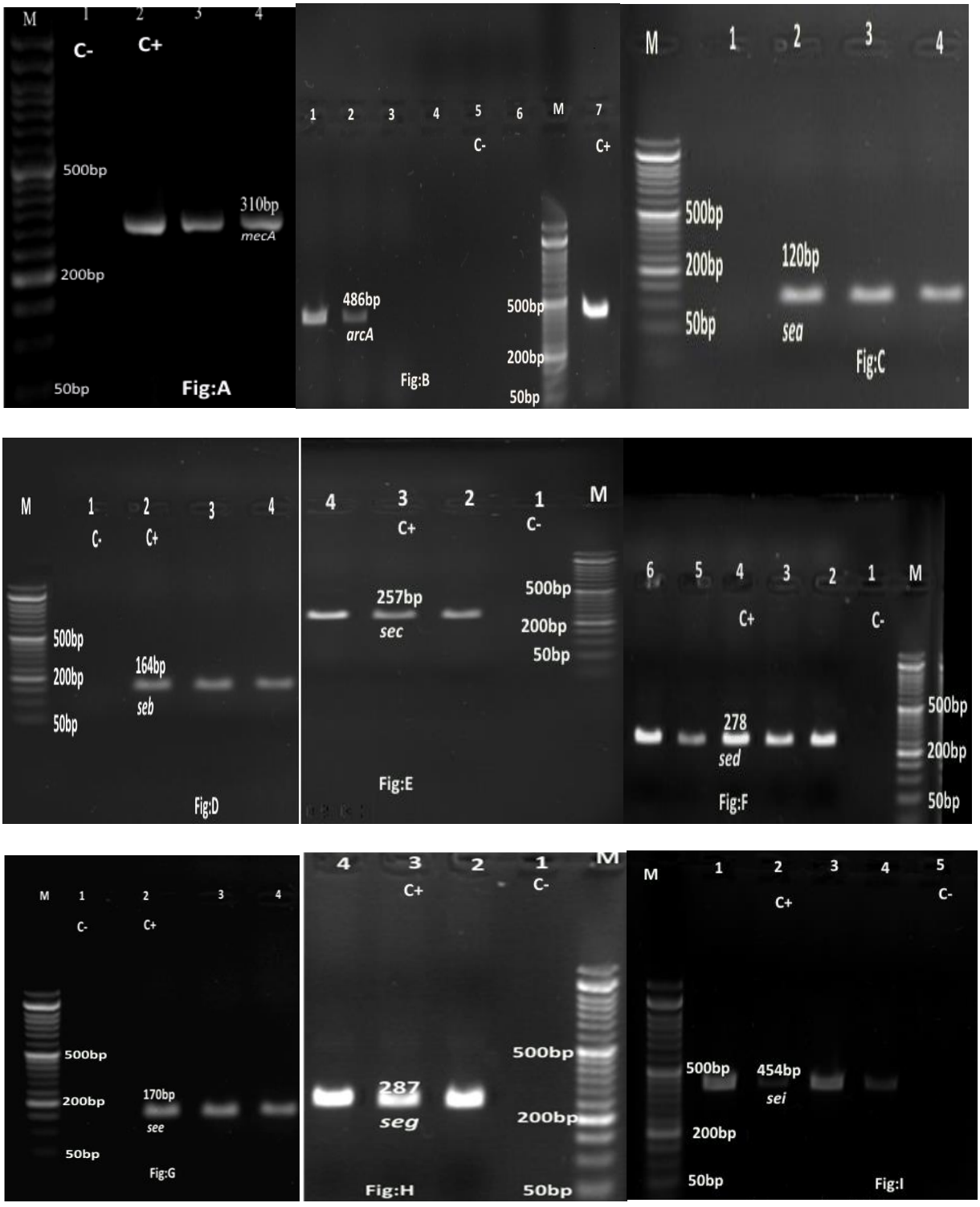


Figure (1).Electrophoresis gel images of *mecA* (A), *arcA* (B), *sea* (C), *seb* (D), *sec* (E), *sed* (F), *see* (G), *seg* (H), and *sei* (I) genes. In each image, C+: positive control, C-: negative control, and M: marker ladder

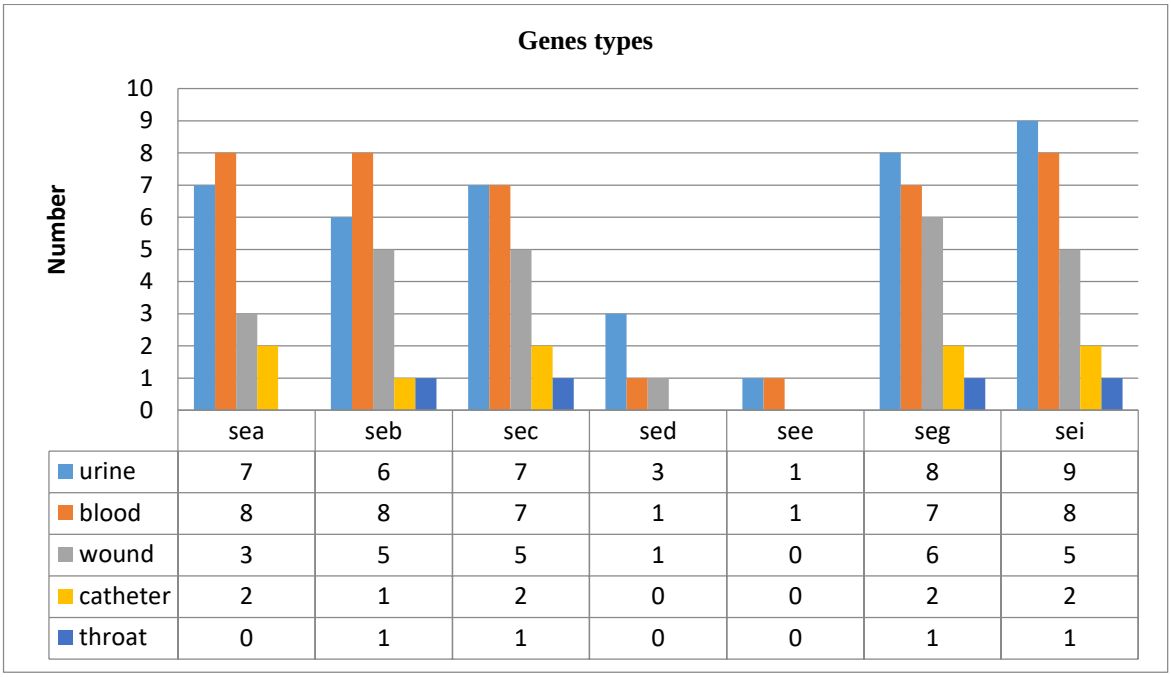
Table 2: Antibiotic susceptibility patterns of MRSE isolates

Antibiotic	No. of strains	Resistance (%)	No. of strains	semi resistance (%)	No. of strains	Sensitivity (%)
erythromycin	53	81.5	7	10.7	5	7.6
clindamycin	42	64.6	21	32.3	3	4.6
cefoxitin	41	63.07	20	30.7	4	6.1
tetracyclin	40	61.5	19	29.2	6	9.2
gentamicin	39	60	21	32.3	5	7.6
cotrimoxazole	38	58.4	20	30.7	7	10.7
ciprofloxacin	30	46.1	23	35.3	12	18.4
rifampicin	5	7.6	-	-	60	92.3
linezolid	1	1.5	4	6.1	60	92.3

Table 3. Antimicrobial resistance patterns of various types of hospital infections against commonly utilized antibiotics

Antimicrobial Agents	Types of Infections					
	Urine (28)	**Blood (18)**	**Wound (12)**	**Catheter (5)**	**Throat (2)**	**Total (65)**
erythromycin	24 (85.7%)	17 (94.4%)	8 (66.6%)	3 (60%)	1 (50%)	53 (81.5%)
clindamycin	20 (71.4%)	11 (61.1%)	8 (66.6%)	3 (60%)	-	42 (64.6%)
cefoxitin	13 (46.4%)	14 (77.7%)	11 (91.6%)	2 (40%)	1 (50%)	41 (63.07%)
tetracyclin	21 (75%)	8 (44.4%)	9 (75%)	2 (40%)	-	40 (61.5%)

gentamicin	17 (60.7%)	9 (50%)	10 (83.3%)	3 (60%)	-	39 (60%)
cotrimoxazole	11 (39.2%)	13 (72.2%)	9 (75%)	4 (80%)	1 (50%)	38 (58.4%)
ciprofloxacin	14 (50%)	8 (44.4%)	5 (41.6%)	3 (60%)	-	30 (46.1%)
rifampicin	1 (3.5%)	3 (16.6%)	1 (8.3%)	-	-	5 (7.6%)
linezolid	-	1 (5.5%)	-	-	-	1 (1.5%)



	sea	seb	sec	sed	see	seg	sei
urine	7	6	7	3	1	8	9
blood	8	8	7	1	1	7	8
wound	3	5	5	1	0	6	5
catheter	2	1	2	0	0	2	2
throat	0	1	1	0	0	1	1

Figure(2). Distribution of enterotoxin genes between methicillin-resistant *Staphylococcus epidermidis* strains gathered from different hospital infections

DISCUSSION

It has been more than two decades since *coagulase-negative staphylococci* have emerged as opportunistic pathogens. The use of embedded medical devices (e.g. various types of prostheses, and catheters) over the past decades has made methicillin-resistant *Staphylococcus epidermidis* an important pathogen in the hospital. The global studies showed that 60–85% of clinical

Staphylococcus epidermidis isolates are resistant to methicillin (18,19, 20).

In our study, we found that 65 *Staphylococcus epidermidis* strains out of 100 strains (65%) were MRSE. According to the outcomes of the studies systematic review, and meta-analysis by Razavi et al in 2018, MRSE had a higher diffusion level in Isfahan, Tabriz, Sanandaj, and Arak than Tehran, because of in Tehran better MRSE infection controlled (19). Also, the obtained results by Razavi et al. indicating that the level of MRSE infections was higher in the western departments of Iran than the other departments (19, 20). In the study of Xu et al. in the community of Shanghai area of China in 2018, the prevalence of *Staphylococcus epidermidis*, and MRSE in healthcare personnel were reported to be 61.3%, and 30% respectively (21). According to Shamansouri et al. (2016) in Isfahan, the frequency of *mecA* in clinical isolates were reported to be 56.41% (7). Clearly, the abundance of MRSE isolates in these three studies was consistent with our findings. In this study, the methicillin-resistant *Staphylococcus epidermidis* isolates exhibited the highest resistance to erythromycin (81.5%), clindamycin (64.6%), cefoxitin (63.07%), and tetracycline (61.5%), while they showed the lowest resistance to rifampicin (7.6%), and linezolid (1.5%). Here, the highest resistance to erythromycin is consistent with those reported for the hospitals in Tehran, Tabriz, and Isfahan (2, 22, 23).

According to the results of the current, and similar previous studies across Iran, we can conclude that the antibiotics linezolid and, daptomycin can be effectively used in the therapy of infections due to MRSE (5). In our study, we found that from 65 *Staphylococcus epidermidis* isolates, 33 isolates (50.76%) had ACME-*arcA* genes. In the study of Diep et al. the spread of ACME-*arcA* was presented 60% (25). In a survey conducted by Shamansouri et al. (2016) in Isfahan, ACME-*arcA* was reported in 14 (20.58%) of MRSE isolates, and they reported that the ACME-*arcA* gene was able to promote the increase, maintenance, and diffusion of strains with multiple-drug resistance (7).

In the study of Xu et al. in the community of Shanghai area of China in 2018, the prevalence of ACME-*arcA* was reported in 67.7% of MRSE isolates, and 74.1 of *Staphylococcus epidermidis* strains, and 83.3% of Methicillin-sensitive *Staphylococcus epidermidis* (MSSE) isolates (21). The result of this study was almost close to ours. Granslo et al. (2010) observed that 40% of the *Staphylococcus epidermidis* isolates extracted from contaminated blood cultures contained the ACME gene (26). *Miragaia* et al. (2009) found that 51% of the *Staphylococcus epidermidis* in the world areas were the ACME positive (27, 7). the results of this study are in agreement with our research.

Food poisoning is a foodborne illness. *Staphylococcus epidermidis* in nosocomial infection produce toxins called enterotoxins that are a cause of food poisoning agents. Previous studies have shown patients carriers enterotoxin can be a source of infection in the community,

especially in hospitals. PCR analysis conducted, in the present study, revealed that the frequencies of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and s*ei* genes were 60.6% (20 strains), 63.6% (21 strains), 66.6% (22 strains), 15.1 % (5 strains), 6 % (2 strains), 72.7% (24 strains), 0% (0 strains), and 75.7% (25 strains) respectively, in the isolates.

According to Pereira et al. (2017) *Staphylococcus epidermidis* had a high prevalence of superantigenic genes in coagulase-negative staphylococci that cause severe infections. In this study, the frequency of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *she*, and *sei* genes were 69.8%, 64.3%, 69.5%, 71.4%, 50%, 69.7%, 61.5%, and 72% respectively (28). The result of this study was almost close.

The *sea* gene is carried by a prophage that facilitates its diffusion among staphylococcal strains, and its product is enterotoxin A, as one of the major food poisoning toxins (28). Many studies have shown that these genes are predominant in CoNS (29).

Vasconcelos et al. (2011) showed that the most common enterotoxin genes were *seg*, and *sei* staphylococci, with a frequency of 62.7%, and 66.7%, respectively (30). These genes exist in the egc cluster, which also includes genes encoding other staphylococcal enterotoxins, and these genes have shown to be highly correlated with each other. The result of this study was similar to ours.Differences between the results of different studies may be attributed to differences in the method used, and in the samples studied, including the number, environment, and geographical source of the samples (29). Pinheiro et al. (2015) detected enterotoxin genes in 95.3% of the blood cultures infected with *Staphylococcus epidermidis* isolates, and that had at least one enterotoxin gene, the most common of which was the classical enterotoxin genes such as *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and *sei* with frequencies equal to 59%, 29%, 30%, 3%, 3%, 73%, 13%, and 74% (31). The results of the *sea*, *seg*, *see*, and *sei* genes in this study were similar to ours.

Conclusion

Tracing of antibiotic resistance, and enterotoxin genes among ACME-*arcA* encoding MRSE strains indicated that clinical samples might represent a potential health hazard. The general objective of controlling hospital infections is to reduce these infections as much as possible. Not only these infections cause secondary complications but they also increase the possibility of food poisoning, which in turn leads to high mortality rates, prolonged hospitalization, and high-cost treatment. Therefore, studies about tracing of such isolates in the nosocomial infections are very recommended.

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Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Isolation of Methicillin-Resistant *Staphylococcus epidermidis* Strains Containing the *arcA* Gene Producing Enterotoxins A, B, C, D, E, G, and I from Hospital Infections in Isfahan, Iran.

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ABSTRACT

Staphylococcus epidermidis produces various types of extracellular protein toxins including staphylococcal enterotoxins that act as superantigens, and can provide conditions to attack host cells. The goal of the current study was to determine antibiotic resistance patterns, and detection of prevalent enterotoxin genes in MRSE strains containing the ACME-*arcA* gene in Isfahan. This survey was done, on a total of 150 staphylococcal isolates acquired from different nosocomial infections. MRSE strains isolated by the PCR procedure. The antimicrobial patterns of MRSE isolates were determined by the disk diffusion procedure. Finally, enterotoxin genes in MRSE strains containing the ACME-*arcA* gene were detected by using the PCR system. Out of 150 clinical infections, 100 *Staphylococcus epidermidis* strains were isolated. Among 100 *Staphylococcus epidermidis* strains, 65 (65%) isolates were MRSE. Of these, 33 isolates (50.7%) had both *mecA*, and ACME-*arcA* genes. The antimicrobial patterns of MRSE isolates in this study showed that the MRSE isolates exhibited the highest rates of resistance to erythromycin (81.5%), and clindamycin (64.6%), while they showed the lowest resistance to rifampicin (7.6%), and linezolid (1.5%). Moreover, the frequency of enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and s*ei* was reported 60.6%, 63.6%, 66.6%, 15.1%, 6%, 72.7%, 0%, and 75.7% respectively, in the isolates containing the *mecA*, and ACME-*arcA* genes. The variety of enterotoxin genes types and resistance index among ACME-*arcA* encoding MRSE are causes for a public health concern; so rapid and accurate reporting of the presence of enterotoxin genes from hospital infections is essential.

Keywords: Antibiotic Resistance pattern, Enterotoxins, Methicillin-Resistant.