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RESEARCH ARTICLE

Genetic diversity of *Staphylococcus aureus* isolated from ear infections in Iran: Emergence of CC8/ST239-SCC*mec* III as major genotype

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ABSTRACT

Increase in antibiotic resistance in *Staphylococcus aureus* isolated from ear infection is a serious public health problem. The objective of this investigation was to determine the antibacterial resistance profile and genetic variability of the *S. aureus* isolated from adult patients with otitis externa (OE) and otitis media (OM) infections, Tehran- Iran. The disk diffusion was employed to detect the susceptibility of 45 *S. aureus* strains. Biofilm production was evaluated by microtiter plate assay. Genetic diversity of the isolates was determined by staphylococcal cassette SCC*mec*, *spa*, and MLST techniques. Resistance to mupirocin and vancomycin were identified in 40 and 2.2% of isolates. Out of the 45 *S. aureus* isolates, 41 (91.2%) strains were considered as positive biofilm strains at different levels. According to our results, *S. aureus* isolated from OM (44.4%, 20/45) were including CC8/ST239-SCC*mec*III corresponded to *spa* types t860, t030, t037, t234, t421 (70%, 14/20) and CC/ST30-SCC*mec*IV corresponded to *spa* types t605 and t019 (30%, 6/20) while *S. aureus* isolated from OE (55.6%, 25/45) were including CC/ST30-SCC*mec*IV corresponded to *spa* types t605, t345 and t1130 (52%, 13/25), CC/ST22-SCC*mec*IV corresponded to *spa* type t790 (20%, 5/25), CC8/ST8-SCC*mec*IV corresponded to *spa* type t008 (16%, 4/25), and CC/ST45-SCC*mec*IV corresponded to *spa* types t004 and t038 (12%, 3/25). This study highlighted genetic variability and strong biofilm formation ability among our isolates revealing its crucial role in enhancing the resistance of this bacteria to drugs. Thus, it is necessary to continue the epidemiological analysis to improve the control of ear infections related to *S. aureus*.

KEYWORDS

Staphylococcus aureus, biofilm formation, otitis externa, otitis media, SCC*mec*, antibiotic resistance, multilocus sequence typing, *spa*

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INTRODUCTION

Ear infection have been brought to the fore the public health risk in recent times in both children and adults, remaining a major health challenge in many countries [1]. Ear infection can be acute or chronic and includes otitis externa (OE), which is an infection of the auricle and ear canal without rupture and otitis media (OM), which is characterized by middle ear infection. It is estimated that almost 350 million individuals have experienced at least an episode of ear infection during their lifetime and approximately half of them suffer from

significant hearing loss. This is a critical challenge especially in the developing countries which has a significant impact on the health status and overall well-being of individuals [2, 3]. Although many microorganisms such as *Serratia marcescens, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Proteus mirabilis* are frequent causes of ear infection, *Staphylococcus aureus* is known as the most common causative agents of ear infection [3]. The emergence and spread of multidrug resistant *S. aureus* strains portend a serious danger and is getting higher every year which have been raised a therapeutic problem. Several factors including especially mobile genetic elements, and biofilm formation, are known as the most common causative agents to mediate antimicrobial resistance in *S. aureus* strains [2, 4–7]. In Iran several researches have focused on the occurrence, phenotypic and genotypic tolerance of *S. aureus* retrieved from ear infections but little data has been published on the genetic variability and biofilm formation ability these isolates. Therefore, the present study was designed to evaluate the antimicrobial tolerance profiles and molecular traits, and biofilm pattern of *S. aureus* samples in OE and OM cases.

MATERIAL AND METHODS

S. aureus identification and ethics statement

A cross-sectional study was carried out in a hospital setting from December 2020 to November 2022 in two teaching hospitals of Shahid Beheshti University of Medical Sciences. Ear discharges by sterile swab and middle ear fluids by tympanocentesis were collected by otolaryngologists. Inclusion criteria included all samples that were related to ear infections cases, who had not taken any antibiotics for three weeks prior to the visit and had no history of hospitalization. Exclusion criteria included the people who had taken antibiotics for the past 3 weeks or been hospitalized. Ear purulent discharges after quick transfer to the laboratory were subsequently streaked over blood agar (HiMedia, Mumbai, India) and preliminarily recognized as *S. aureus* by bacteriological and biochemical techniques. Also, further identification was done by molecular diagnosis of *nuc*A gene in *S. aureus* isolates [5, 8].

Antibiotic susceptibility testing

Susceptibility to ten antibiotic disks (Oxoid Ltd, Basingstoke, Hampshire, UK) including gentamicin, erythromycin, fusidic acid, nitrofurantoin, ciprofloxacin, rifampin, penicillin, clindamycin, tetracycline, and linezolid was verified by the Kirby-Bauer procedure. The findings were analyzed in accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI). All isolates of *S. aureus* underwent the cefoxitin (30 µg) disc diffusion test on Mueller Hinton agar plates for screening of methicillin-resistant *S. aureus* (MRSA) isolates. Each run of susceptibility test was done with reference strains of *S. aureus* ATCC 25923, ATCC 43300, and ATCC 29213 as control strains for quality assurance. The broth microdilution procedure was employed to confirm the resistance to vancomycin and mupirocin (low-level (LLMUPR) and high-level (HLMUPR) mupirocin resistance) (Sigma-Aldrich, St. Louis, Mo) following the CLSI criteria. D-zone examination was done for the identification of inducible clindamycin resistance phenotype (iMLS$_B$; inducible macrolide-lincosamide-streptogramin B). Multidrug resistant (MDR) strains of *S. aureus* were specified as resistant to 3≥ classes of antibacterial agents.

Microtiter plate (MtP) assay

In this method, 10^6 CFU mL^{-1} concentration of 24-h *S. aureus* culture in Trypticase Soy Broth (TSB, Merck, Darmstadt, Germany) including 1% glucose was prepared. Afterwards 200 µL of the 1:100 dilution of this suspension was added to individual wells of 96-well flat-bottomed microtiter polystyrene plates and incubated without shaking for 24 h at a temperature of 37 °C under static conditions. Then, the wells underwent a triple wash with phosphate buffer saline (PBS; pH 7.2) and were air-dried at ambient temperature. Subsequently, the fixation of attached bacterial cells were performed using 99% methanol. A total of 200 µL of 0.1% safranin solution was added to the wells for staining. After 15 min, safranin stain was removed and wells underwent multiple washes with phosphate buffer saline and dried at room temperature. The attached stain was dissolved through the addition of 1 mL of 95% ethanol per well. Finally, an ELISA reader was used to spectrophotometrically measure the optical density (OD) of the previously stained attached biofilm at 490 nm wavelength. The results were then interpreted as previously explained elsewhere [9]. *Staphylococcus epidermidis* ATCC 35984 strain was employed as a positive control strain for assessing biofilm formation.

Extraction of genomic DNA

The genomes of these bacteria were extracted using phenol-chloroform technique and minor modifications. In such a way that 3–5 pure colonies were suspended in 150 µL deionized water plus 30 µg mL^{-1} lysostaphin (Sigma-Aldrich, St. Louis, Mo). Qubit fluorometer and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, USA) were used to confirm the DNA content and purity [10].

Detection of *mecA* and toxin encoding determinants

Isolates were screened for the existence of genes encoding Panton-Valentine leukocidin, exfoliative toxin, and toxic shock syndrome toxin, including *pvl*, *eta*, *etb,* and *tst* by PCR assay and oligonucleotides sequences applied in Goudarzi et al.,'s study [10]. PCR was employed to detect the *vanA* and *mec*A-mediated resistance as described elsewhere [11].

Genotypic characterization

SCC*mec *typing. Staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by a multiplex polymerase chain reaction (MPCR) using method and pairs of primers

explained by Boye et al., [12]. The acquired banding patterns were then compared with that of the reference stains, as follows: ATCC 10442 (SCC*mec* type I), N315 (SCC*mec* type II), 85/2082 (SCC*mec* type III), MW2 (SCC*mec* type IVa), WIS (SCC*mec* type V), as reference strains.

spa *typing.* This technique was used to analyze the *S. aureus* protein A (*spa*) in concordance with method, specific primers and PCR conditions introduced by Goudarzi et al., [10]. A matching reaction mixture including water was used in each run as negative control instead of chromosomal DNA. The PCR products underwent purification through utilization of the QIAquick PCR Purification commercial kit. Subsequently, nucleotide sequence analysis was conducted via employment of an ABI Prism 377 automated sequencer (Applied Biosystems, Perkin-Elmer Co., Foster City, CA). The sequences were edited using Chromas software (Version 1.45, Australia). The collected data was then submitted to the Ridom SpaServer data repository (http://www.spaserver.ridom.de) to analyze the *spa* profiles.

Multilocus sequence typing (MLST)

All isolates were subjected to MLST assay. The experimental protocol adhered to the defined methodology outlined by Goudarzi et al. [8], including the use of primers, PCR reaction conditions, and detailed procedures. Determination of allele profiles and sequence types (STs) was conducted by comparing the obtained sequences of housekeeping genes (*tpi*A, *arc*C, *gm*K, *glp*F, *aro*E, *yqi*L, and *pta*) to the data bank at online MLST database website (https://pubmlst.org/).

RESULTS

In this research, out of 216 patients with ear infection, 45 *S. aureus* strains (20.8%) including 25 OE (55.6%) and 20 OM (44.4%) cases were isolated. Of all the retrieved samples, 18 (40%) were from male and 27 (60%) were from female participants with an average age of 31 years (range, 17–63 years). All the isolates were confirmed as MRSA. More than 77.8% of the patients had a record of taking antibiotics within the last 3 months. As presented in Table 1, the highest levels of drug resistance were indicated against penicillin (100%), tetracycline (91.1%), and erythromycin (86.7%) while resistance of less than 50% for antibiotics rifampin (44.4%), mupirocin (40%), nitrofurantoin (26.7%), fusidic acid (17.8%), and vancomycin (2.2%). All tested strains were susceptible to linezolid. Overall, four isolates (3.3%) belonging to OE cases tested had not indicated resistance to any of the examined antibiotics. In total, 40 isolates (88.9%) were confirmed as multidrug resistance (MDR) strains. In our study, nine resistance patterns were detected, wherein PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (22.2%; 10/45), PEN, GEN, ERY, TET, CLI, CIP, NIT, MUP (17.8%; 8/45), and PEN, TET, ERY, CIP (17.8%; 8/45) were the top three frequently identified profiles. Broth dilution for vancomycin indicated that 66.7% of the isolates were inhibited at MIC value 0.5 μg mL^{-1}, 31.1% at 1 μg mL^{-1}, and 2.2% at 32 μg mL^{-1}. One vancomycin-resistant *S. aureus* (VRSA) isolate carrying the *vanA* gene was isolated from OE case. In terms of clindamycin resistance, 28 (62.2%), and 11 (24.4%) isolates were confirmed as constitutive clindamycin resistance ($cMLS_B$) (18 OM and 10 OE cases) and inducible clindamycin resistance ($iMLS_B$) (10 OE and 1 OM cases) respectively. As shown in Table 1, 40% of isolates were confirmed as mupirocin resistant that high-level mupirocin-resistant (HLMUPR) and low-level mupirocin-resistant (LLMUPR) phenotypes were detected at a rate of 11.1% (all OM cases; 5/45) and 28.9% (7 OE and 6 OM cases; 13/45) respectively.

Table 1. Antimicrobial susceptibility pattern of *S. aureus* strains obtained from OE and OM cases

Antibiotic	OE cases n (%)	OM cases n (%)	Total n (%)
Penicillin	25 (55.6)	20 (44.4)	45 (100)
Gentamicin	11 (36.7)	19 (63.3)	30 (66.7)
Tetracycline	21 (51.2)	20 (48.8)	41 (91.1)
Erythromycin	20 (51.3)	19 (48.7)	39 (86.7)
Ciprofloxacin	16 (53.3)	14 (46.7)	30 (66.7)
Clindamycin	10 (35.7)	18 (64.3)	28 (62.2)
Rifampin	6 (30)	14 (70)	20 (44.4)
Fusidic acid	3 (37.5)	5 (62.5)	8 (17.8)
Nitrofurantoin	5 (41.7)	7 (58.3)	12 (26.7)
Mupirocin	7 (38.9)	11 (61.1)	18 (40)
Vancomycin	1 (100)	0 (0)	1 (2.2)

Among the tested isolates, 42.2% (19/45) were toxigenic. The *pvl* gene (37.8%) was recovered most, followed by *tst* gene (20%). The prevalence of biofilm formation in different degrees were including strong (40%; 18/45), moderate (35.6%; 16/45), weak (15.6%; 7/45). Four isolates did not have any ability in biofilm formation (8.8%). Among *S. aureus* recovered from 20 OM cases, 10 isolates indicated strong (50%), 7 isolates (35%) moderate and 3 isolates (15%) weak ability in biofilm formation. Out of 25 *S. aureus* isolated from OE cases, 8 (32%) had strong, 9 (36%) moderate, 4 (16%) weak biofilm production, and the remaining 4 (16%) did not show the ability to produce biofilms. According to the multiplex PCR test for SCC*mec* typing of 45 tested isolates, type IV had highest prevalence representing 68.9% (31/45), followed by types III (31.1%; 14/45). *spa* typing was used to study all 45 isolated recovered from OE and OM cases. This technique was able to classify all 45 isolates in this investigation. *spa* typing indicated twelve particular *spa* types, namely t019 (26.7%; 12/45), t030 (13.3%; 6/45), t790 (11.1%; 5/45), t008 (8.9%; 4/45), t345 and t421 (6.7%; each 3/60), t037, t234, t605, t1130, and t004 (4.4%; each 2/45), t860, and t038 (2.2%; each 1/45). Table 2 depicts the molecular types distribution of 45 *S. aureus* strains in OE and OM samples. The isolates were resolved into 5 clonal complexes (CCs) including CC30 (42.2%; 19/45 [13 OE and 6 OM]), CC8 (40%; 18/45 [4 OE and 14 OM]), CC22 (11.1%, [OE]), and CC45 (6.7%, [OE]). All five HLMUPR strains were recovered from OM cases and belonged to ST239-SCC*mec*III/t030 (60%; $n = 3$), ST239-SCC*mec*III/t037 (20%; $n = 1$), and ST30-SCC*mec*IV/t019

Table 2. Characteristics of the 45 *S. aureus* strains obtained from OM and OE cases

Ear infection (n; %)	MRSA clone (*n*; %)	Toxins (*n*; % indicated when not 100%)	biofilm formation degree (*n*; % indicated when not 100%)	Resistance pattern (*n*; % indicated when not 100%)
OM (20; 44.4)	CC8/ST239-SCC*mec*III/ t030 (6; 30)	*tst* (5; 83.3)	Strong	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (3; 50)
				PEN, GEN, TET, ERY, CLI, RIF (2; 33.3)
				PEN, GEN, TET, ERY, CLI, CIP, RIF, NIT, FUS (1; 16.7)
	CC8/ST239-SCC*mec*III/ t421 (3; 15)	–	Moderate (2; 66.7), Weak (1; 33.3)	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (2; 66.7)
				PEN, GEN, TET, ERY, CLI, RIF (1; 33.3)
	CC8/ST239-SCC*mec*III/ t037 (2; 10)	*tst*	Strong	PEN, GEN, TET, ERY, CLI, CIP, NIT, MUP (1; 50)
				PEN, GEN, TET, ERY, CLI, CIP, RIF, NIT, FUS (1; 50)
	CC8/ST239-SCC*mec*III/ t234 (2; 10)	–	Moderate	PEN, GEN, TET, ERY, FUS (1; 50)
				PEN, GEN, TET, ERY, CLI, CIP, RIF, NIT, FUS (1; 50)
	CC8/ST239-SCC*mec*III/ t860 (1; 5)	–	Moderate	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP
	CC/ST30-SCC*mec*IV/t019 (4; 20)	*pvl*	Strong (2; 50), Moderate (1; 25), Weak (1; 25)	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (1; 25)
				PEN, GEN, TET, ERY, CLI, CIP, NIT, MUP (3; 75)
	CC/ST30-SCC*mec*IV/t605 (2; 10)	*tst*	Moderate (1; 50), Weak (1; 50)	PEN, TET, FUS (1; 50)
				PEN, GEN, TET, ERY, CLI, RIF (1; 50)
OE (25; 55.6)	CC/ST30-SCC*mec*IV/t019 (8; 32)	*pvl*	Strong (2; 25), Moderate (3; 37.5), Weak (3; 37.5)	PEN, TET, ERY, CIP (6; 75)
				PEN, GEN, TET, ERY, CLI, RIF (1; 12.5)
				PEN, GEN, TET, ERY, FUS (1; 12.5)
	CC/ST30-SCC*mec*IV/t345 (3; 12)	*tst*	Weak (1; 33.3), No biofilm (2; 66.7)	PEN, TET, FUS (1; 33.3)
				PEN (2; 66.7)
	CC/ST30-SCC*mec*IV/ t1130 (2; 8)	*tst*	No biofilm	PEN
	CC/ST8-SCC*mec*IV/t008 (4; 16)	*pvl* (2; 50)	Strong	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (2; 50)
				TET, ERY, VAN (1; 25)
				PEN, GEN, TET, ERY, CLI, CIP, NIT, MUP (1; 25)
	CC/ST22-SCC*mec*IV/t790 (5; 20)	*pvl* (3; 60)	Strong (2; 40), Moderate (3; 60),	PEN, GEN, TET, ERY, CLI, CIP, RIF, MUP (1; 20)
				PEN, GEN, TET, ERY, CLI, CIP, NIT, MUP (2; 40)
				PEN, TET, ERY, CIP (2; 40)
	CC/ST45-SCC*mec*IV/t004 (2; 8)	–	Moderate	PEN, GEN, TET, ERY, CLI, CIP, NIT, MUP (1; 50)
				PEN, GEN, TET, ERY, CLI, RIF (1; 50)
	CC/ST45-SCC*mec*IV/t038 (1; 4)	–	Moderate	PEN, GEN, TET, ERY, CLI, CIP, RIF, NIT, FUS

PEN, Penicillin; CLI, Clindamycin; NIT, Nitrofurantoin; ERY, Erythromycin; TET, Tetracycline; CIP, Ciprofloxacin; MUP, Mupirocin; GEN, Gentamicin; VAN, Vancomycin; RIF, Rifampicin; FUS, Fusidic acid.

(20%; $n = 1$). LLMUPR strains belonged to ST8-SCC*mec*IV/t008 (23.1%; 3/13), ST22-SCC*mec*IV/t790 (23.1%; 3/13), ST30-SCC*mec*IV/t019 (23.1%; 3/13), ST239-SCC*mec*III/t421 (15.5%; 2/13), ST45-SCC*mec*IV/t004 (7.6%; 1/13), and ST239-SCC*mec*III/t860 (7.6%; 1/13). VRSA strain belonged to ST8-SCC*mec*IV/t008 which harbored *vanA* determinant. All CC8 and CC30 strains belonged to OM cases with the exception of CC8/ST239-SCC*mec*III/t234 and CC30/ST30-SCC*mec*IV/t605 exhibited $cMLS_B$ phenotype. Isolates with $iMLS_B$ phenotype belonged to CC30/ST30-SCC*mec*IV/t019 (7 isolates), CC8/ST8-SCC*mec*IV/t008 (1 isolates), CC22/ST22-SCC*mec*IV/t790 (1 isolate), and CC8/ST239-SCC*mec*III/t234 (1 isolate). According to our results, eight *S. aureus* strains (17.8%) were resistance to fusidic acid which belonged to lineages CC8/ST239-SCC*mec*III/t030, CC8/ST239-SCC*mec*III/t037, CC/ST30-SCC*mec*IV/t605, CC/ST30-SCC*mec*IV/t019, CC/ST30-SCC*mec*IV/t345, CC/ST45-SCC*mec*IV/t038 (each 1 isolate), CC8/ST239-SCC*mec*-III/t234 (2 isolates). More than a third of the MRSA strains isolated from ear infections were positive for strong biofilm production. The proportion of strong biofilm positivity was higher in OM (50%; 10/20) than in OE (32%; 8/25). Strong biofilm producers belonged to CC8/ST239-SCC*mec*III/t030 (33.4%; 6/18), CC8/ST239-SCC*mec*III/t037 (11.1%; 2/18), CC/ST30-SCC*mec*IV/t019 (11.1%; 2/18), CC/ST30-SCC*mec*-IV/t019 (11.1%; 2/18), CC/ST22-SCC*mec*IV/t790 (11.1%; 2/18), and CC/ST8-SCC*mec*IV/t008 (22.2%; 4/18) clonal lineages. All the isolates without ability to form biofilm belonged to CC/ST30-SCC*mec*IV/t345 (2 isolates) CC/ST30-SCC*mec*-IV/t1130 (2 isolates) clonal lineages obtained from OE cases.

DISCUSSION

Although, there has been no report of the precise occurrence of *S. aureus* involved in ear infections in Iran [3], previous studies have reported the growing incidence of *S. aureus* in ear infections all over the world. However, OE incidence in Iran ranges from 12.4 to 63.46%. In present study, the prevalence of *S. aureus* isolates in patients with ear infection was reported at 20.8%. Various occurrence rates have been recorded in USA (25.6%) [7], China (16.2%) [13], and Italy (11.3%) [14]. Ear infection related to *S. aureus* is becoming difficult to treat as a result of antimicrobial resistance. Therefore, it is necessary to depict resistance pattern and its distribution among clonal lineages. In this study, fusidic acid resistance was detected in 17.8% of the examined isolates. Hajikhani et al., in a meta-analysis study reported low rate of fusidic acid resistance in *S. aureus* isolates (0.5%) [15]. This percentage is inferior than that recorded in other countries such as Greece (57%) and Ireland (19.9%) [16] and higher than studies done in Kuwait (9.3%) and Germany (10.3%) [15, 16]. In a similar study carried out by Udo et al. also showed increased trends of resistance to fusidic acid from 22% in 1994 to 92% in 2004 [17]. Similarly, our findings compare with previous researches in Iran highlights increasing rate in the prevalence of clinical *S. aureus* strains resistant to fusidic acid [15]. Several researches indicated that fusidic acid resistance rates vary greatly across geographic locations which might be associated with unrestricted administration of this antibiotic, lack of proper alternatives, diverse attitudes towards antimicrobial protocols and the dissemination of fusidic acid resistant clonal lineages.

VRSA is a problem for both the hospital and the community environments and could limit treatment options to control staphylococcal infections leading to high level of mortality. This study demonstrated 1 isolate VRSA (2.2%) which carried *vanA* gene. The prevalence of VRSA strains varied among the various geographic area, such that Shariati et al.'s study in 2020 displayed various prevalence rate of resistance to vancomycin in Asia (1.3%), Europe (1.1%), America (3.6%), and Africa (2.5%). They also indicated that about two thirds of VRSA strains were reported from India and Iran [18]. However, increasing resistance of *S. aureus* to vancomycin may be because of their easy availability, scarcity of appropriate alternatives to vancomycin, the lack of guidelines for antibiotic use and diverse attitudes towards antimicrobial protocols. Therefore, antibiotic stewardship committees should adopt an appropriate policy for the benefit of antibiotic use and the antibiotics like vancomycin are only used after their susceptibility testing. However, increasing resistance of *S. aureus* to vancomycin show the need to develop new antibiotics and alternative antimicrobial strategies.

Mupirocin is the choice of drug for managing the proliferation of *S. aureus* isolates within populations and healthcare facilities, as well as mitigating the incidence of major infections. Resistance to mupirocin among MRSA strains is substantially growing and is now recognized as a worldwide problem. The prevalence rate of mupirocin resistant MRSA varied among the various countries, 27.8% in South Africa, 12.1% in Canada, 31.3% in USA and 45.5% in Turkey, and 39.6% in Iran [19]. In this report, the data revealed an occurrence rate of HLMUPR in 11.1% of MRSA isolates. The prevalence of HLMUPR-MRSA observed in this investigation exceeded the overall rate documented by Dadashi et al. [19]. The reason behind the elevated prevalence of mupirocin resistance remains poorly understood, but it seems to be connected to an inadequate governance of antibiotic administration strategies, improper policies and extensive use of antibiotics, which likely increase the chance of genetic variations and acquisition of mupirocin resistance genes.

In the present study, approximately one quarter of isolates were phenotypically iMLSB positive. However, this reported rate is higher than earlier study with a prevalence of 10.9% in Iran. In this study, Goudarzi et al. indicated a three-fold increase in the trend for the prevalence of iMLSB phenotype from 7.5% in 2013 to 21.7% in 2018 [8]. iMLSB phenotype among *S. aureus* has been reported by several authors in Jordan (76.7%) [20], Nepal (21%) [21], and Brazil (7.9%) [22]. This variation might be influenced by the excessive usage of macrolides, regional locations of study population, infection prevention protocols in healthcare facilities, and the prior history of antibiotic usage in patients.

Based on the evidence, *S. aureus* possesses the capability to generate biofilm, leading to the development of persistent and long-lasting infections, as well as therapeutic inefficacy. Our results showed that more that 90% of MRSA strains isolates from ear infections (OM and OE cases) displayed biofilm formation in different degrees. Similar to our findings, several reports of Nepal (69.8%) [6], China (72.2%) [4], and Hungary (38%) [23]. In concordance with the other reports, our study confirmed that the prevalence of MDR among strong biofilm-producers was greater than in non-biofilm producers. In fact, simultaneously resistance to multiple antibiotics and strong ability in biofilm formation will increase the chance of survival of the isolates and will complicate the treatment of ear infections.

In the present research, the 45 isolates belonged to twelve particular *spa* types and mostly were clustered into the 4 main CCs (CC30 42.2%, CC8 40%, CC22 11.1%, CC45 6.7%). The majority of CCs detected in the present survey have also been declared reports from other countries in the region as common CCs in *S. aureus* recovered from ear infections [7, 13]. In this study, a great proportion of the isolates belonged to CC/ST30-SCC*mec*IV corresponded to five *spa* types, including t019, t605, t345, and t1130. According to our data CC30 isolates were identified in both OM and OE cases. CC/ST30-SCC*mec*IV/t019 carrying *pvl* genes (26.7%), pandemic Southwest Pacific-(SWP) clone or USA1100, was the most prevalent type. SWP lineage was former described in Iran (21.5%) (MDR), Kuwait (2.8%) (BMC), UAE (14.6%), and some of European countries [24, 25]. Based on the evidence, the presence of PVL could enhance the dissemination of the SWP clone in both hospital settings and communities. Simultaneous carriage of PVL along with other toxin genes could allow the SWP clone to cause serious diseases [24, 25]. Present data warrants further extensive studies to characterize SWP clone with their concomitant antibiotic resistance profile. Our further study on the CC/ST30-SCC*mec*IV isolates showed PVL-negative, but *tst1*-positive strain of ST30-MRSA-IV corresponded to *spa* type t605, t345, and t1130. Similarly, CC/ST30-SCC*mec*IV strains *tst*-positive and *pvl*-negative were reported sporadically in Australia and Ireland [24]. Boswihi et al. in Kuwait also displayed CC/ST30-SCC*mec*IV/t605 as the new detected genotypes in MRSA strains isolated from hospitalized individuals [25]. The above results as well as our results are in contrast to the study of Goudarzi et al., who reported the existence of PVL-positive CC/ST30 SCC*mec*IV/t605 in 1.6% of tested isolates [10]. A previous study conducted in 2018 by Wurster and colleagues showed CC30 as the second predominant *S. aureus* clone isolated from ear infections (19.4%) [7]. Our data exhibited that the majority of CC/ST30 tested isolates had ability to form biofilms at different intensities confirming the earlier finding of Chamon et al., which indicated high rate of biofilm formability among ST30 strains [26]. Similar to our work a low prevalence of CC/ST30-SCC*mec*IV/t345 was also reported in Hashemizadeh 's study [27], and Aggarwal's study [28].

The result of this study revealed a relatively high frequency of CC8/ST239-SCC*mec*III resembled Vienna/Hungarian/Brazilian clone, corresponded to five *spa* type, including t860, t030, t037, t234, and t421. The presence of CC8/ST239-SCC*mec*III/t030 and CC8/ST239-SCC*mec*III/t037 strains were also reported earlier in Middle East, Europe, South and North America, and Asia [24, 25, 29]. This study found that CC8/ST239-SCC*mec*III/t030, CC8/ST239-SCC*mec*III/t037, CC8/ST239-SCC*mec*III/t234 were resistance to fusidic acid. Goudarzi et al., over a period of five years on 126 inducible clindamycin resistant *S. aureus* strains reported the presence of FA-resistant *S. aureus* in two t030-MRSA III strains [8]. Yu et al., in a similar study on 392 *S. aureus* isolates during a three years period reported a 7.1% prevalence fusidic acid resistant t030-MRSA III strains [30]. In another research carried out by Chen et al. in Taiwan, two *spa* types t037-MRSA III and t002-MRSA II were reported as the most eminent identified genotypes in fusidic acid resistant MRSA strains isolated from hospitalized individuals, accounting for 62 and 29% respectively [31]. A study in Kuwait on 4726 MRSA isolates obtained in 2016–2017, aimed to investigate diversity in the clonal composition of MRSA isolates circulating within hospitals located in Kuwait. The results showed the presence of CC8/ST239-MRSAIII with nine *spa* types, t1247, t1339, t15224, t6258, t713, t16187, t421, t860, t945 [29], which was almost in line with the findings of our study indicating the CC8/ST239-SCC*mec*III/t421 and CC8/ST239-SCC*mec*III/t860 in 3 and 1 of the MRSA strains. These results brought forward a possibility that common genotypes of fusidic acid resistant *S. aureus* strains may be diverse from country to country and even city to city. Hence, these lineages should be monitored to avoid an ongoing outbreak of aforementioned clones.

The present study revealed a relatively low frequency of CC/ST8-SCC*mec*IV/t008. CC8-MRSA IV/t008 carrying *pvl* encoding genes that resembled the USA300 was also reported earlier in Australia, China, Iran, Japan, Kuwait, Spain, Switzerland, and UAE [24, 25, 29]. As already reported in previous studies, multi-drug resistance especially resistance to vancomycin and mupirocin among USA300 strains is increasing globally [24]. Havaei et al.in their recent study investigated 171 isolates of *S. aureus* isolated from two cities of Iran. In their research, it was found that 2.9% of tested isolates were vancomycin-intermediate *S. aureus* (VISA) and out of which 2% belonged to CC/ST8-*spa* t008 [32].

In present research the emergence of CC22 (11.1%) corresponded to *spa* type t790, as the fourth prevailing type in *S. aureus* recovered from OE infections with a notable carriage of *pvl* (60%). This lineage as a well-known epidemic MRSA clone is also common in UK, Kuwait, Iran, KSA, and other European countries such as Ireland, and Germany [24, 29]. In contradiction to the observations of earlier researchers reporting *spa* type t790 as the most common type among *S. aureus* isolates, our data displayed low prevalence of this *spa* type in *S. aureus* related to OE (11.1%). Although TST and PVL-positive ST22-MRSA-IV strains have been reported by several researchers, our results indicated that 60% of CC/ST22-SCC*mec*IV/t790 isolates harbored *pvl* encoding genes. CC/ST22 strains carrying PVL have also been reported in studies of Kuwait [29], and Ireland. Shore

et al., in a similar study done in Ireland between 2002 and 2011 reported a low percentage of CC22/t005 and CC22/t1869 strains [33]. Boswihi et al., in their study over an eighteen-year period, investigated clonal diversity of MRSA strains in Kuwait health care settings. In their research, it was found that CC/ST22-MRSA-IV corresponded to *spa* type t790 was present in 9.2% of tested isolates [25]. The current study revealed that the rate of resistance to mupirocin was 60% that all exhibited LLMUPR phenotype. Similar finding was shown by Goudarzi et al. which reported HLMUPR-MRSA strains among CC15 (40%), CC22 (23.3%), CC8 (36.7%) [5]. However, the emergence and dissemination of CC/ST22-SCC*mec*IV/t790 along with MDR pattern may be a significant risk to public health due to rising rates of treatment failure and illness severity.

USA600, or CC/ST45-SCC*mec*IV, is described to be a prevalent clone with worldwide dissemination capacity and high survival rates which posed a great threat to health care settings and community health. In this study, the frequency of CC/ST45-SCC*mec*IV corresponded to *spa* types t004 and t038 was 6.7%. Interestingly, Liang and coworkers reported USA600/ST45 as the third prevalent type with a notable prevalence of 18.8% in *S. aureus* strains in China [34]. However, the prevalence of CC/ST45-SCC*mec*IV in our study was lower than similar study with a prevalence of 26.1% in Poland [35], and higher than a reported rate of Iran (4%) [8]. The lower incidence of USA600 observed in this study, as compared to other nations, could potentially be ascribed to variances in geographic factors, type of samples, pattern of distribution of this clone in hospital and community.

This study highlights the occurrence of biofilm production in MDR *S. aureus* related to ear infection which significantly limit the availability of effective antimicrobial treatments. These findings confirmed the dissemination of specific lineages in *S. aureus* strains causing OM and OE infections. High prevalence of CC8 and CC30, as the most prevalent types in OM and OE cases respectively highlights the need to continue epidemiological investigations aimed at delineating the molecular epidemiological map of these isolates in Iran.

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