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Biofilm formation and molecular characterization of methicillin-resistant *Staphylococcus aureus* strains isolated from the patients, personnel, air and environment of ICUs

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ABSTRACT

The emergence of hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) as a main cause of nosocomial infections is a global health problem. The present study was conducted to reveal the prevalence of MRSA in intensive care units (ICUs) based on biofilm formation ability and genetic diversity in *agr* and *coa* genes. A total of 85 MRSA isolates were analyzed by molecular typing of *agr* and *coa* genes from different clinical sources in ICUs using Multiplex PCR and PCR-RFLP methods, respectively. Biofilm formation of all MRSA isolates was investigated using phenotypic and genomic methods. Among 85 isolated MRSA, majority of isolates were belonged to *agr* type I (71.8%) followed by *agr* types II (7.1%). PCR-RFLP *coa* typing revealed 5 different *coa* types and 13 RFLP patterns. Meanwhile, 51.8% of isolates were strong biofilm formative which were among hospitalized patients 54.5%, and medical staff 75%. Additionally, among 85 MRSA isolates, 60 (70.6%) and 53 (62.4%) isolates had *icaA* and *icaD* genes respectively. Moreover 39 (45.9%) of MRSA isolates had both genes, while 11 (13%) isolates had no studied genes. Based on our findings there is no correlation between biofilm formation and the presence of specific types of the *agr* and *coa* genes among MRSA clinical isolates. In conclusion, genetic diversity in *agr* and *coa* types indicated the presence of various clones of MRSA in our studied ICUs. High prevalence of MRSA strains with *agr* type I and strong ability of biofilm formation could be most considered as necessity of performance effective infection control policy.

1. Introduction

Staphylococcus aureus is one of the most important human pathogens responsible for clinical infections ranging from mild infections to life-threatening invasive diseases in both community and hospital settings worldwide (Goudarzi et al., 2016; Khashei et al., 2018). Hospital-associated infections caused by Methicillin-resistant *S. aureus* (HA-MRSA) with broad spectrum antibiotic resistance and widespread emergence in intensive care units (ICUs) is a major global health problem with high morbidity and mortality rates (Zinn et al., 2004; Nowrouzian et al., 2013; Goudarzi et al., 2017).

Pathogenesis of MRSA is related to expression of wide variety of virulence factors. The invasion ability of *S. aureus* strains is modulated by accessory gene regulatory (*agr*) as a major virulent factor. Also, Coagulase enzyme causing the clotting of plasma, that is encoded by the *coa* gene, is another main virulent factor and secreted by all *S. aureus* strains and is a main criterion to identify *S. aureus* in clinical infections (Mahmoudi et al., 2017). Furthermore, the other considerable virulence factor for MRSA, especially hospital-associated strains, is biofilm formation which has role in pathogenesis of intravenous catheter-related bacteremia, contaminated implemented medical devices, serious tissue damages and prolonged nosocomial infections (Cirkovic et al., 2015). Biofilm polysaccharide layers are involved in bacterial resistance to antibiotics and phagocytic macrophages. There is no sufficient literature about genetic background and biofilms formation in clinical MRSA strains. Synthesis of the polysaccharide intercellular adhesion (PIA) of *S. aureus* is encoded by the intercellular adhesion (*ica*) operon including *icaA*, *icaD*, *icaB* and *icaC* genes (Atshan et al., 2012; Zalipour et al., 2016).

Molecular typing is a proven important tool for studying the relatedness of MRSA strains, genetic diversity, clonal tracking and transmission modes in order to effective infection control. A great number of

Abbreviations: HA-MRSA, hospital-associated methicillin-resistant *S. aureus*; ICUs, intensive care units; PIA, polysaccharide intercellular adhesion; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; CLSI, Clinical and Laboratory Standards Institute; TSB, tryptic soy broth

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studies have been reported the MRSA genotyping using polymerase chain reaction (PCR). Among a large number of molecular typing methods, *agr* and *coa* typing are appropriate techniques in the identification and monitoring of the wide variety of MRSA clones and determination of the source of nosocomial infections in healthcare units. These methods can also evaluate the association between different molecular types and biofilm formation (Sakoulas et al., 2003; Salehzadeh et al., 2016; Shahin et al., 2018). According to the polymorphism of the *agr* genes, there are different *agr* types (I–IV) which HA-MRSA often belongs to *agr* type I (Sakoulas et al., 2003). PCR–restriction fragment length polymorphism (PCR-RFLP) analysis of the 3′ end region of *coa* gene has shown high variability in the number of 81 bp tandem sequence repeats in MRSA strains (Abdulghany and Khairy, 2014; Omar et al., 2014).

Previous Iranian studies indicated that the increased prevalence of MRSA isolates containing different toxin and antibiotic resistance genes is a serious threat for the hospitalized patients in the ICUs, which the isolates from the patients and environment were usually indistinguishable (Mirzaii et al., 2015; Rashidi Nezhad et al., 2017; Goudarzi et al., 2019). In order to clarify our hospitals situation this study was aimed to evaluate the prevalence and molecular characteristics of MRSA isolates based on *agr* and *coa* genes and relatedness of biofilm formation ability of different genotypes in hospitalized patients, medical staff, air and environment, to control the nosocomial infection in ICUs.

2. Materials and methods

2.1. Sample collection and identification of S. aureus

This cross-sectional study was implemented over the period from July 2017 to May 2018 in two educative and remedial hospitals (Poursina and Velayat) of Rasht city in the north of Iran and approved by the Research Ethics Committee Guilan University of Medical Sciences (IR.GUMS.REC.1396.293). Signed informed consent forms were obtained from all participants prior to inclusion in the study. The demographic information for each participant including age, gender, ward and duration of hospitalization, antibiotic usage, and underlying disease were recorded. Among 147 *S. aureus* isolates, a total of 85 non-duplicated MRSA strains were collected using Anderson pump containing nutrient agar plate for air or sterile cotton swabs for the environment and also nares, hands and wounds of hospitalized patients with over 96 h of admission and nares and hands of medical staff in ICUs. Swabs immediately were inoculated into nutrient agar and then were incubated at least 24 h at 37 °C. *S. aureus* isolates were identified on the basis of positive catalase, slide and tube coagulase and DNase tests, and also ability to Mannitol fermentation on Mannitol salt agar (QUELAB-UK).

2.2. Phenotypic screening of MRSA

Among *S. aureus* clinical isolates, MRSA strains were screened using cefoxitin disc (30 μg) on Muller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI 2017) guideline (Wayne, 2017). MRSA isolates were stored at −80 °C in brain-heart infusion broth (BHI) (QUELAB-Canada) containing 20% glycerol for future investigations.

2.3. Phenotypic biofilm formation

Biofilm assay was performed according to Iara Rossi Goncalves in 96-well Microtiter plates (Nunc) with some modifications (Rossi Goncalves et al., 2017). Briefly, after overnight incubation of bacterial suspension in tryptic soy broth (TSB) supplemented with 2% glucose, the bacterial suspension was adjusted to 0.5 McFarland standard tube. 200 μl of this suspension was diluted 1:100 in TSB containing 2% glucose and inoculated into each well. The plates were incubated at 37 °C for 24 h. Then, supernatants were removed and each well was washed twice with distilled water. After drying in room temperature, each well was stained with crystal violet 1% and incubated for 15 min. After removing the stain, the glacial acetic acid 30% was added to each well. The eluted dye was removed from each well and transferred to a new well, and then the absorbance was read in an ELISA plate reader (BIOTEC ELX 808-USA) at 570 nm. All assays were performed in triplicate. The results were obtained based on TSB as a negative control. Based on the optical density of the samples (ODi) the samples were classified as strong ($0.5 < OD570\ nm$), moderate ($0.24 < OD570\ nm < 0.5$), weak ($0.12 < OD570\ nm < 0.24$), or non-producer of biofilm ($OD570\ nm < 0.12$).

2.4. Extraction of genomic DNA

High pure DNA template preparation kit (Roche, Germany) was used for genomic DNA extraction of MRSA strains on the basis of manufacturer instruction. Concentration of DNA was measured using Nano drop.

2.5. Amplification of nuc A, fem A and mec A genes by PCR

PCR amplification for *nucA*, *femA*, and *mecA* genes for MRSA additional confirmation was performed using specific primers (Table 1) (Oliveira and de Lencastre, 2002; Shopsin et al., 2003). PCR reactions were done in a final volume of 20 μ containing 10 μl master mix (2× PCRBIO Taq Mix Red - PCR BIOSYSTEMS), 1 μl of extracted DNA and sterile Double distilled water to reach to 20 μl. Amplification was carried out as follows: pre-incubation (94 °C for 2 min), followed by 30 cycles of denaturation (94 °C for 30 s), annealing (47 °C for 20 s), extension (72 °C for 40 s) and a final extension (72 °C for 10 min). PCR products were electrophoresed in 1.5% agarose gel (Roche, Germany) containing Sybrsafe. DNA ladder (Roche Co, Germany) was used to detect the molecular weights of observed bands under UV lamp.

2.6. Accessory gene regulatory (agr) typing

Identification of different *agr* types was performed for all 85 MRSA isolates using Multiplex PCR (Shopsin et al., 2003). A common forward primer (Pan) and specific reverse primers for each *agr* groups (*agr* I, *agr* II, *agr* III, *agr* IV) were used for amplification of 440, 572, 406 and 588 bp fragments for *agr* I, *agr* II, *agr* III and *agr* IV, respectively. The primer sequences are listed in Table 1.

DNAs were amplified in a proflex (ABI, USA) under the following conditions: initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, then extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel. PCR products were visualized under ultraviolet illumination.

2.7. Coagulase (coa) gene typing

Polymorphism in the 3′ end region of *coa* gene was evaluated by PCR-RFLP (Hookey et al., 1998). Amplification of the 3′ end of *coa* gene was carried out using PCR. DNA was amplified in a proflex (ABI, USA) under the following conditions: initial denaturation for 2 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55.7 °C for 30 s, and extension at 72 °C for 50 s. A final extension was performed for 5 min at 72 °C.

Restriction analysis of PCR products was performed using the restriction enzyme *Alu*I (Fermentas, USA) according to manufacturer's instruction. Briefly, 10 μl of the PCR product diluted in 18 μl of nuclease-free distilled water was digested with 10 U (1 μl of AluI enzyme and 2 μl Buffer 10× tango) and then incubated at 37 °C for 16 h

Table 1
Primers sequences used for amplification of studied genes.

Gene fragment		Primer sequences (5′ → 3′)	Product size (bp)	Ref
femA	Forward	AAA AAA GCA CAT AAC AAG CG	132	(Oliveira and de Lencastre, 2002)
	Reverse	GAT AAA GAA GAA ACC AGC AG		
nucA	Forward	GCG ATT GAT GGT GAT ACG GTT	280	(Shopsin et al., 2003)
	Reverse	AGC CAA GCC TTG ACG AAC TAA AGC		
mecA	Forward	ACT GCT ATC CAC CCT CAA AC	163	(Oliveira and de Lencastre, 2002)
	Reverse	CTG GTG AAG TTG TAA TCT GG		
Pan-*agr*	Forward	ATG CAC ATG GTG CAC ATG C	–	(Shopsin et al., 2003)
agr I	Reverse	GTC ACA AGT ACT ATA AGC TGC GAT	441	
agr II	Reverse	TAT TAC TAA TTG AAA AGT GGC CAT AGC	573	
agr III	Reverse	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323	
agr IV	Reverse	CGA TAA TGC CGT AAT ACC CG	659	
ica A	Forward	TCT CTT GCA GGA GCA ATC AA	188	(Arciola et al., 2001)
	Reverse	TCA GGC ACT AAC ATC CAG CA		
ica D	Forward	ATG GTC AAG CCC AGA CAG AG	198	
	Reverse	CGT GTT TTC AAC ATT TAA TGC AA		
coa	Forward	ATA GAG ATG CTG GTA CAG G	603	(Hookey et al., 1998)
	Reverse	GCT TCC GAT TGT TCG ATG C	660	
			875	
			603 & 660	
			603 & 875	

followed by 20 min incubation in the water bath at 65 °C. The restricted fragments were then analyzed using a 1.5% gel electrophoresis.

2.8. Amplification of icaA and icaD genes

Amplification of *icaA* and *icaD* genes was performed for all 85 MRSA isolates using traditional PCR (Arciola et al., 2001). Oligonucleotide primers are listed in Table 1. PCR reaction containing 1 μl of each forward and reverse primers, 10 μl master mix (2× PCRBIO Taq Mix Red - PCR BIOSYSTEMS), 1 μl of extracted DNA and sterile double distilled water to reach to final volume of 20 μl. A thermal step program for both *ica A* and *ica D* was used, including the following parameters: initial denaturation in 95 °C for 2 min followed by 38 and 35 cycles including 95 °C for 30 s, 56.2° and 55.5 °C for 30 s, and 72 °C for 20 and 30 s for *icaA* and *ica D* respectively, followed by a final extension at 72 °C for 5 min. PCR products were then analyzed using 1.5% gel electrophoresis stained with sybrsafe (Sinaclon).

2.9. Statistical analysis

The SPSS software version 22 was used for statistical analysis in this study. Chi-square or Fisher's exact tests were used to determination of significance.

3. Results

3.1. Prevalence of MRSA

Among 147 identified *S. aureus*, 85 MRSA strains were isolated from air, environment, nares and hands of hospitalized patients and medical staff (carriers) in ICUs [General: 46 (54.1%), Burn: 12 (14.1%) and Trauma: 27 (31.8%)]. Accordingly, the frequency of MRSA isolated from hospitalized patients, medical staff, hospital environment, and air samples were 66 (77.6%), 8 (9.4%), 9 (10.6%), and 2 (2.4%), respectively. Of 74 MRSA isolated from hospitalized patients and medical staff, 46 (62.2%) and 28 (37.8%) of strains obtained from men and females samples, respectively.

3.2. Distribution of agr types

The distribution of different *agr* types in various clinical sources is shown in Fig. 1. MRSA strains with *agr* type I were predominant (71.8%) among all isolates. Furthermore, 13 (15.3%) MRSA strains were un-typable and *agr* types I was circulated commonly among all clinical sources.

3.3. Distribution of the coa genotype

Amplification of the 3′ end region of the *coa* gene yielded 5 different classes, ranging from 603 bp to 875 bp, among MRSA isolates. The 660 bp amplicon or class 2 (34.1%) were predominant among all MRSA isolates. *AluI* digestion of the PCR products revealed 13 distinct RFLP patterns (pattern 1-pattern 13). The distribution of the *coa* types and RFLP patterns of MRSA clinical isolates are shown in Tables 2 and 3, respectively. RFLP pattern 1 (25.9%) was the most predominated among hospitalized patient and RFLP patterns 1, 3, 5, 9 and 11 were distributed among all MRSA clinical sources. Furthermore, 10 (11.8%) of MRSA strains were un-typable.

3.4. Biofilm formation assays

According to spectrophotometer criteria and microtiter plate assays, the ability of biofilm formation in different MRSA isolates in ICUs is shown in Fig. 2.

3.5. Presence of icaA and icaD genes

The frequency of *icaA* and *icaD* genes among 85 MRSA were 60 (70.6%) and 53 (62.4%) respectively and 39 (45.9%) isolates had both genes, while 11 (13%) isolates had no studied genes.

4. Discussion

The emergence and widespread prevalence of MRSA as a major human pathogen in ICUs with broad-spectrum antibiotic resistance and wide range of life-threatening invasive diseases, is becoming a global problem of public health (Goudarzi et al., 2016; Eshaghi et al., 2017). Molecular typing of bacteria is a proven important tool to study of epidemiological aspects of infectious diseases. Genomic diversity survey using amplification of *agr* and *coa* genes of *S. aureus* as main virulence factors has been proposed as a simple and accurate technique for typing especially MRSA strains for determination of the infection source and more effective infection control (Godwin et al., 2001; Mirzaii et al., 2015; Mohajeri et al., 2016). For this main aim, in the current study, a total of 85 MRSA clinical isolates from hospitalized patients, medical staff, environment and air in ICUs were studied for

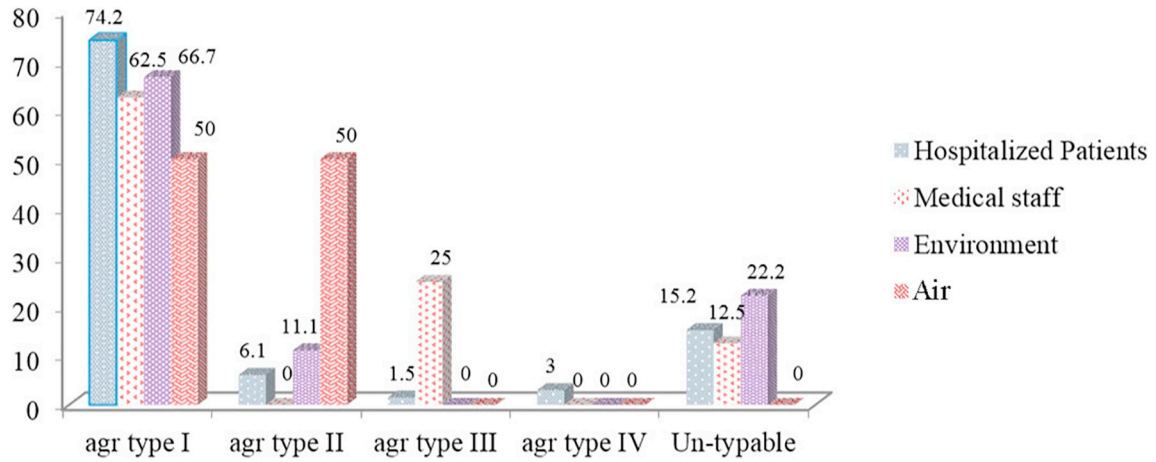

Fig. 1. Distribution percentage of *agr* types in all MRSA isolates in ICUs.

Table 2
Distribution of *coa* genotypes among all MRSA clinical isolates.

PCR amplicon class	Restriction fragments (~bp)	No. (%)
Class 1 (603 bp)	300-230-110	28 (32.9)
Class 2 (660 bp)	260-400 or 250-300-110	29 (34.1)
Class 3 (875 bp)	400-200-250	11 (12.9)
Class 4 (6602–875 bp)	100-250-340-470	14 (16.5)
Class5 (603–660 bp)	130-220-310	3 (3.5)

investigation of relatedness between ability of biofilm formation and genotype variety of the *agr* and *coa* genes.

In agreement with our results, some studies were reported a high prevalence of MRSA strains in other regions of Iran (61.9%) (Havaei et al., 2014; Moshtagheian et al., 2018). Similar results were reported from Nigeria (O'Malley et al., 2015), and Taiwan (Wang et al., 2012).

It is well confirmed that the expression of most virulence factors including biofilm formation of *S. aureus* is modulated by the *agr* locus (Ando et al., 2004). In the present study, *agr* typing for MRSA isolates showed that the majority types were belonged to *agr* group I (71.8%) followed by *agr* group II (7.1%). According to *agr* types, this could be explained that the most isolation sources were harboring type I and this *agr* type was more virulent among MRSA strain. Our results are consistent with other studies, in which they found *agr* group I as the predominant type in MRSA strains (Machuca et al., 2013; Bibalan et al., 2014; Havaei et al., 2014; Moshtagheian et al., 2018).

In contrast, Goudarzi et al. (2017) and Moshtagheian et al. (2018) identified *agr* type III as a predominant *agr* group among studied isolates. But we should notice that *agr* type III belongs to community-acquired MRSA (CA-MRSA) (Paul et al., 2014). However, these differences can be due to several factors, including differences in geographical regions, infection control policies, nature of infections and sample size.

PCR-RFLP of the other virulence gene, the *coa* gene, has been widely used as an effective discriminatory tool for the genotyping of *S. aureus* especially MRSA strains. *coa* gene amplification is a simple, specific and reproducible method for epidemiological studies (Ishino et al., 2007; Javid et al., 2018). In the present study, five different size of amplicons of the *coa* gene (*coa* genotypes) were obtained from MRSA strains. Our results about genotypes diversity were in line with other previous studies in different regions of Iran (Mohajeri et al., 2016), and some other countries (Janwithayanuchit et al., 2006; Talebi-Satlou et al., 2012), but in contrast with Abdolghany and da Silva studies who reported 15 and 27 *coa* types respectively (Rodrigues da Silva and da Silva, 2005; Abdulghany and Khairy, 2014). Our results showed significant heterogeneity in 3′ end of the *coa* gene due to several point mutations among MRSA strains isolated from clinical samples.

Recent studies have shown that prolonged nosocomial infections by MRSA strains can be related to biofilms formation and high antimicrobial resistance (Cha et al., 2013). In this study, we investigated biofilm formation of MRSA clinical isolates based on genotypic and phenotypic characteristics using amplification of *icaA* and *icaD* genes and spectrophotometric micro plate method respectively. According to our results, 51.8%, 29.4% and 17.7% of the isolates were strong, intermediate and weak-positive biofilm formatives, respectively. Strong biofilm formatives of MRSA isolates only were found among hospitalized patients (54.5%) and medical staff samples (75%).

In the present study, 78.6% of MRSA isolates that were harboring both *icaA* and *icaD* genes were strong biofilm formative. This finding demonstrated that expression of *icaA* and *icaD* genes simultaneously is more effective to stimulate the PIA biosynthesis similar to other studies (Arciola et al., 2001). Interestingly, 11 (13%) MRSA isolates that were negative for the above mentioned genes were positive for biofilm formation. This indicated that additional genes in *ica* operons or other determinants may be involved in biofilm formation (Ghasemian et al., 2016).

Our findings clearly demonstrate that there is a significant association between sampling site including hands and nares with biofilm formation. This could be important in the aspect of bacterial spreading to air and medical devices such as ventilators. Also, there was no significant association between *agr* types and *coa* genotypes and the ability of biofilm formation in MRSA isolates. Moreover, there was no

Table 3
RFLP patterns of *coa* in MRSA clinical isolates with different amplicon sizes.

MRSA clinical samples	No. (%)												
	Pattern 1	Pattern 2	Patter 3	Pattern 4	Pattern 5	Pattern 6	Pattern 7	Pattern 8	Pattern 9	Pattern 10	Pattern 11	Pattern 12	Pattern 13
Hospitalized patients	16 (24.2)	8 (12.1)	4 (6.1)	6 (9.1)	2 (3)	5 (7.6)	3 (4.5)	3 (4.5)	1 (1.5)	3 (4.5)	2 (3)	2 (3)	2 (3)
Medical staff	1 (12.5)	1 (12.5)	0	0	0	0	2 (25)	2 (25)	1 (12.5)	0	0	0	0
Environment	4 (44.4)	0	2 (22.2)	0	1 (11.1)	1 (11.1)	0	0	1 (11.1)	0	0	0	0
Air	1 (50)	0	0	0	1 (50)	0	0	0	0	0	0	0	0

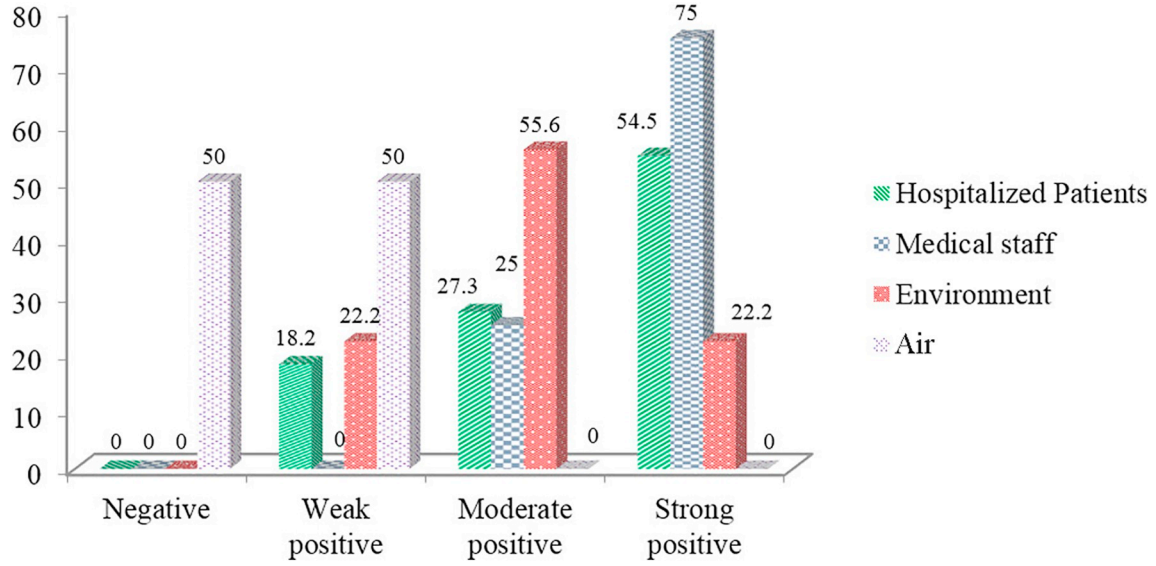

Fig. 2. Percentage of biofilm formation ability in different MRSA isolates in ICUs.

significant association between *agr* types and *coa* genotypes and the presence of *icaA* and *icaD* genes. Among studied ICUs, general ICU is assumed as the main source of spreading the MRSA isolates among clinical and environmental samples.

The main limitations of the present study which must be acknowledged were the lack of antibiotic resistance assay, patients' follow up, and limitation of results to few hospitals.

In conclusion, genetic diversity in *agr* and *coa* types indicated the presence of various clones of MRSA strains in our studied ICUs. High prevalence of MRSA strains with *agr* type I and strong ability of biofilm formation highlight the necessity of performance effective infection control policy. These findings support the need for future studies focused on genotyping methods to reveal the distribution of circulating MRSA clones and detect the emergence of new MRSA clones in our ICUs.

CRediT authorship contribution statement

Fatemeh Tahmasbi: Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing, Funding acquisition. **Raheleh Sheikhi:** Methodology, Writing - review & editing, Supervision. **Ali Ashraf:** Methodology, Investigation, Validation. **Ali Mojtahedi:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

None declared.

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