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Original Article

Detection of *icaABCD* Genes in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* from Patients in Iran

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Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that causes several nosocomial or community-acquired infections. Adhesion to surfaces and subsequent biofilm formation are the major phases of a staphylococcal infection. The aim of this study was to detect the presence of *icaABCD* genes in clinical isolates of MRSA. **Methods:** A total of 110 clinical *Staphylococcus aureus* isolates were collected from two teaching hospitals in Shahrekord (Hajar and Kashani hospitals). The MRSA isolates were detected by an antibiotic susceptibility test. A microtiter tissue plate assay was used to detect the phenotypic biofilm formation. A polymerase chain reaction (PCR) was performed to detect the presence of *icaABCD* genes. **Results:** The microtiter plate assay results showed that attachment abilities were strong in 26 (23.6%) strains, moderate in 30 (27.2%) strains, and weak in 16 (14.54%) strains. The prevalence of the *icaA*, *icaB*, *icaC*, and *icaD* genes among the studied isolates were as follows: 42 isolates were *icaA* positive (38.18%), 34 *icaB* positive (30.9%), 46 *icaC* positive (41.8%), and 50 were *icaD* positive (45.4%). **Conclusion:** The high prevalence of *icaA/D* harboring *S. aureus* among the clinical isolates suggests that the risk of persistent infections in the hospital settings is considerably high. *J Med Microbiol Infec Dis, 2015, 3 (3-4): 67-70.*

Keywords: *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus, icaABCD.*

INTRODUCTION

The development of multidrug resistance in *Staphylococcus aureus* is a public health concern. In the hospital, infected and colonized patients contribute to the transmission and spread of *S. aureus* and hospital personnel, who serve as the reservoirs and facilitate further dissemination [1, 2]. The infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) are often difficult to treat due to the high resistance of the bacteria to multiple standard antibiotics conferred by both intrinsic and acquired mechanisms [3]. The biofilm produced by *S. aureus* is known as one of the main virulence factors of the bacteria and a primary agent of clinical infection. Biofilm production leads to infection and the failure of the indwelling medical devices, such as intravenous catheters, ear and central nervous system shunts, cosmetic surgical implants, and many other implanted devices [4]. In *S. aureus* biofilm formation, the synthesis of the polysaccharide intercellular adhesin (PIA) is the most important step that mediates the adhesion of bacterial cells to each other in the biofilm [1, 5]. The biofilm acts as a barrier to antimicrobial agents and the host immune system, and thus, assists sustained bacterial colonization [6]. The eradication of the biofilm in *S. aureus* is a challenging task due to its greater resistance to antibiotics, host defenses, and disinfectants [7, 8]. Researchers have demonstrated that the first stage of staphylococcal infections is the attachment to host tissue surfaces [9, 10]. Owing to the major role of biofilm-producing *S. aureus* in the infection of indwelling medical devices and their inherent resistance to therapeutic options, the early detection of such potential pathogens can be useful to decrease the morbidity rate and reduce healthcare service costs [11]. The *icaABCD* genes play a key role in the biofilm formation. Isolates of *S. aureus* and *Staphylococcus epidermidis* encode the major enzyme, *icaA*, which is essential for PIA synthesis. This enzyme might require an *icaD* gene product (called *IcaD*) for its activity [12]. The other genes within the ica operon are *icaB* (polysaccharide deacetylase), *icaC* (transporter of PIA), and icaR (the inhibitor gene). Most strains of *S. aureus* contain all four genes of the ica operon. However, several reports have detected some of these genes in different strains [12]. The aim of the present study was to determine the prevalence of biofilm-producing MRSA isolates in clinical specimens, compared to methicillin-susceptible *S. aureus* (MSSA) isolates.

MATERIAL AND METHODS

Bacterial isolates. One hundred and ten isolates of MRSA were chosen from a collection of *S. aureus* isolates collected from Jan 2015 to Oct 2015 from two hospitals in Shahrekord (Hajar and Kashani Hospitals).

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The isolates were identified as *S. aureus* by conventional microbiological assays, including procedures such as colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, DNase test and tube coagulase assay. Primary MRSA isolates were screened based on their resistance to cefoxitin (30 µg) discs (MAST, UK) by the disc diffusion method (MAST, UK), according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. The *S. aureus* ATCC 25923 strain, an MSSA, was used in this study as the control strain in antibacterial susceptibility testing. The confirmed isolates were stored at -70°C for subsequent studies.

Phenotypic analysis of biofilm production on CRA medium. The qualitative slime production was detected for MRSA isolates and assessed based on the color of *S. aureus* colonies developed on the Congo red agar (CRA) plate, as described previously [14]. The bacteria were grown in 10 ml tryptic soy broth (TSB) at 37°C for 24 h without shaking and then stored at room temperature for 48 h. The formation of reddish-black colonies on CRA plates indicates slime production. Non-slime-producing colonies were pinkish-red. ATCC 33591, the biofilm-producing strain of *S. aureus*, was used as the positive control, while the *S. epidermidis* ATCC 12228 strain was used as the negative control**.**

Polystyrene microtiter plate assay (MtP). Briefly, in the microtiter plate assay method, 20 µl of overnight cultures of the test pathogens (1%) were used to inoculate 24-well polystyrene MtPs, each well contained 1 mL of fresh TSB medium supplemented with 0.25% glucose. The plates were incubated for 24 h at 37°C, followed by washing with phosphate-buffered saline. Finally, ethanol was used to determine the biofilm-producing isolates. The adherence ability of tested isolates was classified into four categories based on the optical density (OD): strongly adherent (OD_{570}≥3.0), moderately adherent (OD_{570}≤1.5-2.0), weakly adherent (OD_{570}≤0.5-1.0), and non-adherent (OD_{570}-< OD_{570} of negative control). The *S. aureus* strain ATCC 33591 was used as the positive control and the *S. epidermidis* ATCC 12228 strain was used as the negative control.

Detection of Ica ABCD operon. PCR assays were used to detect the mecA, *icaA*, *icaD*, *icaB*, and *icaC* genes separately. The genes were amplified in an Eppendorf (Hamburg, Germany) thermocycler in a final reaction volume of 20 µl, containing 0.5 µl of Qiagen HotStarTaq master mix (Fermentas, Germany), PCR buffer with 1.5 µl $MgCl_2$, 1 µl of each dNTP, 1 µl of forward and reverse gene-specific primer (20 pMol, mecA, *icaABC* and *icaD*) (Table 1) [15]. Furthermore, 1 µl of DNA template was used per reaction. The products were then electrophoresed on agarose gel, and the presence or absence of any resulting bands was recorded.

Table 1. Target genes and their primers used in this study

Gene	Primers (5′→3)	Standard strain positive for the gene of interest	Product size (bp)	Reference
MECA	F TCCAGATTACAACTTCACCAGG R- CCACTTCATATCTTGTAACG	*S. aureus* ATCC 29247	162	[16]
ICAA	ICAA-F 5'-CCTAACTAACGAAAGGTAG-3' ICAA-R 5'-AAGATATAGCGATAAGTGC-3'	*S. epidermidis* ATCC 35556	188	[17]
ICAD	ICAD-F 5'-AAACGTAAGAGACGTGG-3' ICAD-R 5'-GGCAATATGATCAAGATAC-3'	*S. epidermidis* ATCC 35556	198	[17]

Table 2. Frequency of antibiotic resistance of MRSA strains

Antibiotics	Biofilm former N = 25		Non-biofilm former N = 1		Total N = 26	
	S	R	S	R	S	R
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Amikacin	11 (42.3%)	14 (53.8%)	0	1 (3.8%)	11 (42.3%)	16 (61.5%)
Ciprofloxacin	11 (42.3%)	14 (53.8%)	0	1 (3.8%)	11 (42.3%)	15 (57.6%)
Ceftriaxone	5 (19.2%)	20 (76.9%)	0	1 (3.8%)	5 (19.2%)	21 (80.7%)
Erythromycin	3 (11.5%)	22 (84.6%)	0	1 (3.8%)	3 (11.5%)	23 (88.4%)
Gentamicin	6 (23%)	19 (73%)	1 (3.8%)	0	7 (26.9%)	19 (73%)
Linezolid	25 (96.1%)	0	1 (3.8%)	0	26 (100%)	0
Mupirocin	20 (76.9%)	5 (19.2%)	1 (3.8%)	0	21 (80.7%)	5 (19.2%)
Oxacillin	0	25 (96.1%)	0	1 (3.8%)	0	26 (100%)
Rifampin	23 (88.4%)	2 (7.6%)	1 (3.8%)	0	24 (92.3%)	2 (7.6%)
Tetracycline	2 (7.6%)	23 (88.4%)	1 (3.8%)	0	3 (11.5%)	23 (88.4%)
Teicoplanin	25 (96.1%)	0	1 (3.8%)	0	26 (100%)	0
Tigecycline	25 (96.1%)	0	1 (3.8%)	0	26 (100%)	0
Tobramycin	10 (38.4%)	15 (57.6%)	1 (3.8%)	0	11 (42.3%)	15 (57.6%)
Vancomycin	25 (96.1%)	0	1 (3.8%)	0	26 (100%)	0

Table 3. Colonial morphology of MRSA strains on CRA after 72 h

	Red	Almost black	Black	Very black
MRSA strain	1 (3.8%)	7 (26%)	12 (46.1%)	6 (23%)

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Table 4. Results of biofilm production for MRSA isolates using the microtiter plate method

	Non-biofilm producer 1 (ODs ≤ 0.08) N (%)	Weak biofilm producer (0.08 < ODs ≤ 0.16) N (%)	Moderate biofilm producer 3 (0.16 < ODs ≤ 0.32) N (%)	Strong biofilm producer4 (0.32 < ODs) N (%)
MRSA strain	1 (3.8%)	7 (26%)	10 (38%)	8 (30.7%)

RESULTS

MRSA distribution. *S. aureus* was isolated from 110 out of 200 samples (55%) collected from two hospitals in Shahrekord. Using phenotypic (disk diffusion method) and genotypic (PCR for detection of genes) methods, 26 (23.6%) isolates were confirmed to be MRSA.

Antibiotic susceptibility. We found that a total of 26 isolates from 110 collected specimens were resistant to MRSA. These isolates were evaluated for their biofilm-production ability in a CRA medium culture. The most common resistance observed in the isolates (Table 2) was against erythromycin (88.4%). The least common resistance was against gentamicin (73%). The phenotypic method showed that 30.7% of isolates were highly capable of biofilm production, while 38% were intermediate biofilm producers and 26% of isolates were low biofilm producers. The prevalence of the 12 genes involved in biofilm production was as follows: *icaA* positive: 42 (38.18%), *icaB* positive: 34 (30.9%), *icaC* positive: 46 (41.8%), and *icaD*: 50 (45.4%).

The formation of rough, black colonies on CRA plates was indicative of slime production in contrast to the smooth red colonies of the non-slime producing strains. Among the 26 MRSA clinical isolates tested, 12 were found to produce typical black colonies within 72 h (Table 3). The individual strains of *S. aureus* varied in their biofilm-forming ability on tissue culture plates (Table 4).

All MRSA strains in this study were susceptible to linezolid, teicoplanin, tigecycline, and vancomycin.

DISCUSSION

S. aureus is the most recurrent cause of nosocomial infections and infections on indwelling medical devices, which characteristically involve biofilms. There are only a limited number of studies on the expression profiles of genes involved in biofilm production. The *icaABCD* and other related genes are known as key genes in biofilm formation [18]. The *icaA* and *icaD* genes are regarded as necessary factors for intercellular adhesion [17]. *S. aureus* is the most common pathogen isolated from intensive care unit infections. This result is consistent with the results of other studies [16, 17]. The prevalence rate of MRSA at a burn center differs in various regions. For example, this rate varies from a low of 33% in the United States [19] to a high of 98% in Korea [20]. In this study, 23.6% of the analyzed *S. aureus* isolates were MRSA. All MRSA strains in this study were susceptible to fusidic acid, linezolid, teicoplanin, tigecycline, and vancomycin. The agr locus in *S. aureus* isolates works as a global regulator of virulence genes, including secreted virulence components and surface proteins. This finding agrees with other studies that have reported previously that MRSA is almost always susceptible to these antibiotics [21]. Detection of the ica operon is used to measure the ability of biofilm formation of the bacteria *in vitro* [5, 7, 16]. As CRA has an excellent sensitivity for screening the biofilm-formation ability of *S. aureus* [22], it was used for the phenotypic detection of biofilm formation in our recovered MRSA isolates. The results of CRA assays in our *in vitro* study revealed that the majority (30.7%) of MRSA isolates produce biofilm. Using the microtiter plate method, all MRSA strains studied were found to produce biofilm except for one isolate; this rate was much lower (70%) in MSSA isolates. In our study, wound and skin specimens were three principal sources of biofilm producing isolates with frequencies of 82%, 81.3%, and 56.5%, respectively. In conclusion, a high prevalence of the biofilm-producing isolates involved in hospitals may indicate a potential outbreak of MRSA in the hospitals. Additionally, most of the previous studies have conducted surveys on the *icaAD* genes. A study in 2012 reported that the *icaAD* genes were present in 32% of blood and catheter isolates [22]. In the study by Szweda in 2012, 36 out of 46 Staphylococcal isolates harbored *icaA* and *icaD* genes; however, Grinholc did not detect *icaD*. Handke *et al.* showed that all strains contained *icaA* [23]. Thus, genetic methods for the detection of the genes involved in biofilm formation are appropriate for predicting the biofilm production phenotype *in vitro***.**

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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