Major Article

Molecular analysis, biofilm formation, and susceptibility of methicillin-resistant *Staphylococcus aureus* strains causing community- and health care-associated infections in central venous catheters

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Abstract

Introduction: The behavior of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from central venous catheter-related infection was evaluated to determine its biofilm potential, antimicrobial resistance, and adhesion genes. **Methods:** A total of 1,156 central venous catheters (CVC) were evaluated to screen for pathogens. Antimicrobial sensitivity, biofilm formation potential, and molecular analysis of MRSA were examined following standard guidelines. **Results:** Of the 1,156 samples, 882 (76%) were colonized by bacteria or candida. Among the infected patients, 69% were male and 36% were female with median age of 32 years. *Staphylococcus aureus* infected 39% (344/882) of CVCs in patients. Of the 59% (208/344) of patients with MRSA, 57% had community acquired MRSA and 43% had hospital acquired MRSA. Linezolid and vancomycin killed 100% of MRSA; resistance levels to fusidic acid, doxycycline, clindamycin, azithromycin, amikacin, trimethoprim-sulfamethoxazole, gentamycin, tobramycin, and ofloxacin were 21%, 42%, 66%, 68%, 72%, 85%, 95%, 97%, and 98% respectively. Strong biofilm was produced by 23% of samples, moderate by 27%, and weak by 50% of MRSA. The presence of adhesion genes, *sdr*C and *sdr*D (90%), *eno* (87%), *fnb*A (80%), *clf*A and *sdr*E (67%), *fnb*B, *sdr*D (61%), and *cna* (51%), in most MRSA samples suggested that the adhesion genes are associated with biofilm synthesis. **Conclusions:** The superbug MRSA is a major cause of CVC-related infection. Antibiotic resistance to major classes of antibiotics and biofilm formation potential enhanced superbug MRSA virulence, leading to complicated infection. MRSA causes infection in hospitals, communities, and livestock.

Keywords: Community acquired MRSA. Hospital acquired MRSA. Central venous catheter. Antimicrobial resistance. Biofilm and adhesion genes.

INTRODUCTION

Drug resistance bacteria kill 700,000 people per year and this value is expected to reach 1 million in 2050[1]. Central venous catheters (CVCs) are indispensable in modern medicine practices, particularly in intensive care unit (ICU) patients. CVCs facilitate health management of critical patients who requires intermittent medication, fluids, and food[2]. CVC indwelling patients are at a high risk of mortality and morbidity along with other complications such as bloodstream infection and cardiac arrhythmia. Approximately 78% of critically ill patients require some type of CVC and 90% of catheter-related blood stream infections are -CVC related[3]. Two-thirds of these infections are caused by Gram-positive bacteria, predominantly Gram-positive cocci which are equally responsible for infections in ICU and non-ICU patients[4]. CVCs are colonized by microorganisms including *Staphylococcus aureus*, which is the most common cause of CVC infections[5-7]. *Staphylococcus aureus* is responsible for septic shock in 30% of CVC-associated septicemia cases[8]. Both types of MRSA, community acquired (CA-MRSA) and hospital acquired (HA-MRSA), infect hospitalized and non-hospitalized individuals[9,10]. Resistance to a large range of antibiotics complicates MRSA infection and increase the potential for biofilm formation on biotic and abiotic surfaces. Biofilms are complex heterogeneous structures with fluid-filled tunnels[11]. Interestingly, 60% of catheter-related infections are caused by biofilm-producing bacteria[12]. Microbial surface components recognizing adhesive matrix molecules of MRSA mediate attachment to host molecules and are potentially involved in biofilm formation[13,14]. Microbial surface components recognizing adhesive matrix molecules include fibronectin-binding proteins (*fnb*A and *fnb*B), clumping factors (*clf*A and *clf*B), collagen-binding protein (*c*na), fibrinogen-binding protein (*fib*), laminin-binding protein (*eno*), and three Sdr proteins (*sdr*C, *sdr*D, and *sdr*E)[13,15]. *Staphylococcus aureus* is found in 30% of healthy people, who are healthy carriers of infection[16].

Staphylococcus aureus harbors a variety of pathogenic tools, enzymes, and toxins that cause minor to life-threatening infections[17]. This bacterium is a causative agent of infections on biomedical device- and surgical tube-related infections, which greatly increase mortality, mortality, costs of treatment, and hospital stays. Conventional antibiotics are not effective against biofilm, worsening the situation[18]. Pathogenic tools, rapidly acquired resistance, and rapid mutation development are leading causes of methicillin-resistant *Staphylococcus aureus* (MRSA) as epidemic infectious agents[19,20].

The goal of this study was to isolate and identify the pathogens causing CVC-related infections and determine the antibiogram, biofilm potential, and adhesion genes in superbug MRSA.

METHODS

This study was conducted using 1,156 CVC samples collected from April 2012 to April 2016. The specimens studied were CVC tips, femoral, Jugular, and subclavian catheters. CVC specimens were processed as described by Maki et. al.[21] with slight modifications[21]. Briefly, catheter tips were cut into two pieces 5cm in length; one piece was directly rolled on culture plates and the other was incubated for 1h in a tube containing 1mL brain heart infusion [(BHI); Oxoid, Cheshire, UK]. Next, the samples were centrifuged and the pellet containing pathogens was inoculated on Sheep Blood Agar [(SBA); Oxoid], Chocolate [(CHO); Oxoid] agar, Sabouraud dextrose agar [(SDA); Oxoid], and MacConkey [(MAC; Oxoid] agar. All plates were examined after 24-h incubation at 37°C and further incubated for 48 and 72h if no growth was evident. The acceptable cut-off to declare CVC-related infection was 15 colony-forming units per milliliter (cfu/mL) after overnight incubation[22]. After preliminary identification of pathogens, *Staphylococcus aureus* was confirmed based on a high salt concentration, deoxyribonuclease (DNase) production, and mannitol fermentation. The same bacteria isolated from both techniques (direct and enrichment) were considered as pathogenic and further analyzed.

Antimicrobial sensitivity test

The Kirby-Bauer disk diffusion method was used for the antibiogram of *S. aureus* against eight classes of antibiotics following the performance guidelines and breakpoints recommended by Clinical and Laboratory Standards Institute (CLSI)[23]. *Staphylococcus aureus* (ATCC 29213) was used as a control strain for antibiogram. Based on cefoxitin (30µg) resistance (zone of inhibition ≤21nm), *S. aureus* was declared as MRSA and confirmed by amplification of *mec*[23]. The minimum inhibitory concentration of vancomycin was measured by the E-test (bioMérieux, Marcy-l'Étoile, France) following CLSI guidelines.

Slime production

Congo red agar was used to evaluate the slime production capability of MRSA isolated form CVC-related infections[24]. Red colonies were categorized as non-slime producers and black colonies as slime producers. The intensity of the black color was directly related to the slime production capability.

Quantitative biofilm formation on polystyrene

Biofilm formation was measured quantitatively by the crystal violet assay as described by O'Toole with some modifications[25]. Briefly, a fresh bacterial culture was diluted by 200-fold in BHI containing 1% glucose and then inoculated into a 96-well polystyrene plate. The plate was incubated aerobically at 37°C for 48h without agitation with a positive (*S. aureus* ATCC 35556) and negative (*Staphylococcus epidermidis* ATCC 12228) control strain. Plates were washed three times with phosphate-buffered saline, dried at room temperature, and stained with 0.1% (w/v) crystal violet (CV) for 10 min. After washing three times with phosphate-buffered saline, CV was solubilized by 95% ethanol for 10 min and the optical density (OD) was measured at 595nm. The biofilm formation index (BFI) was measured using the following equation[26]: BFI = (AB-CW)/G.

The OD of CV-stained attached bacteria was denoted as AB. CW represents the OD of the CV-stained negative control containing only broth medium. G denotes the OD of planktonic bacteria. Based on the ODs values, microorganisms were classified as weak ($0.1 > BFI \leq 0.5$), moderate ($0.5 > BFI \leq 1$), and strong ($BFI > 1$) biofilm producers. MRSA with an OD of less than 0.1 were classified as non-biofilm producers[27].

Extraction of genomic DNA

Genomic deoxyribonucleic acid (DNA) was extracted using a conventional method[28]. Bacteria were grown overnight in BHI at 37°C in a shaking incubator and harvested by centrifugation. The pellet was incubated at 37°C for 1h in 10mM Tris-HCL and 34.5mg/mL lysozyme (Sigma, St. Louis, MO, USA). Lysis was conducted by incubation in lysis buffer containing 50mM Tris, 100mM ethylenediaminetetraacetic acid (EDTA), 1% SDS, 20µL proteinase k (Sigma), and lysostaphin (Sigma) at 55°C for 1h. DNA was eluted in DNase-free water after extraction and ethanol precipitation. The concentration was measured with a NanoDrop™ (Thermo Scientific, Waltham, MA, USA) and stored at 4°C until further investigation.

Prevalence of adhesion genes of MRSA

A total of 203 MRSA isolates were screened for 10 different adhesion genes (*clf*A, *clf*B, *eno*, *cna*, *fnb*A, *fnb*B, *fib*, *sdr*C, *sdr*D, *sdr*E), *mec*, and Panton-Valentine leucocidin. The following primers were used in this study: MecA-R (ATGCGCTATAGATTGAAAGGAT) and MecA-F (GTGAAGATATACCAAGTGATT) 310 base pairs (bp)[29]; Luk-PV2 (GCATCAAGTGTATTGGATAGCAAAAGC) and Luk-PV1 (ATCATTAGGTAAAATGTCTGGACATGATCCA) 433bp[29]; FnbA-R (TGTGCTTGACCATGCTCTTC) and FnbA-F (GATACAAACCCAGGTGGTGG) 191bp[30]; FnbB-R (CAAGTTCGATAGGAGTACTATGTTC) and FnbB-F (GTAACAGCTAATGGTCGAATTGATAC) 524bp; Cna-R (AATCAGTAATTGCACTTTGTCCACTG) and Cna-F (GTCAAGCAGTTATTAACACCAGAC) 423bp[31]; clfA-R (AGGCACTGAAAAACCATAATTCA) and clfA-F (TTACGAATCAGTTGACGAATGTG) 104bp[32]; clfB-R (CCGTCGGTTGAGGTGTTTCATTTG) and clfB-F (TGCAAGTGCAGATTCCGAAAAAAAC)

194bp[33]; eno-R (CAACAGCATCTTCAGTACCTTC) and eno-F (ACGTGCAGCAGCTGACT) 302bp[34]; fib-R (GCTCTTGTAAGACCATTTTCTTCAC) and fib-F (CTACAACTACAATTGCGTCAACAG) 404bp[34]; SdrC-R (ACGACTATTAAACCAAGAAC) and SdrC-F (TTCGCACTGTTTGTGTTTGCAC) 560bp[35]; SdrD-R (GGAAATAAAGTTGAAGTTTC) and SdrD-F (GTACTTGAAATAAGCGGTTG) 500bp[35]; SdrE-R (CAGTAAATGTGTCAAAAGA) and SdrE-F (ACTTTGTCATCAACTGTAAT) 767bp[35].

RESULTS

Among the 1,156 CVC samples, 882 (76%) were colonized with pathogens, while 274 (24%) CVC tips were negative for pathogens. The results of both inoculation techniques were compared for pathogen confirmation. Same microorganisms isolated from both techniques were considered as pathogens.

Prevalence of MRSA among CVC-related infections

Of the 882 CVCs colonized with pathogens, 64% (564/882) were due to Gram-positive bacteria, 26% (230/882) by Gram-negative bacteria, and 10% (88/882) by *Candida*. Among Gram-positive bacteria, *S. aureus* (39%) and coagulase-negative *S. aureus* (16%) were dominant. Among Gram-negative bacteria, *Klebsiella pneumoniae* (10%) and *Pseudomonas aeruginosa* (7%) were the most common. Of *S. aureus*, 59% (203/344) were MRSA. Among the 203 patients infected by MRSA, 129 (64%) were male and 74 (36%) were female with a combined mean age of 32 ± 3 years. Of MRSA isolated from CVC, 57% (116/203) were CA-MRSA and 43% (87/203) were HA-MRSA (**Figure 1**), which were categorized based on CDC criteria (CA-MRSA harbors *pcv* and *SCCmec* IV/V)[36]. The prevalence of MRSA over 4 years is shown in **Table 1** with respect to age and gender. The age group 20-29 years was most commonly
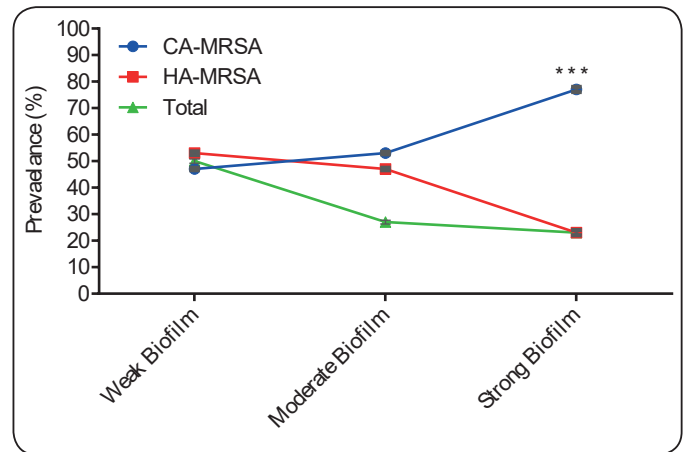

FIGURE 1: Biofilm formation potential between HA-MRSA and CA-MRSA. **CA-MRSA**: community acquired methicillin resistance *Staphylococcus aureus*; **HA-MRSA**: Hospital acquired methicillin resistance *Staphylococcus aureus*.

infected with MRSA, followed by the age groups of 40-49 and 30-39 years with infection rates of 63%, 30%, and 28%, respectively.

Antimicrobial sensitivity testing and biofilm production by MRSA

Antimicrobial sensitivity testing results are shown in **Figure 2** for CA-MRSA and HA-MRSA. Briefly, 72% (146/203) of MRSA samples were resistant to amikacin, 94% (192/203) to gentamicin, 97% (197/203) to tobramycin, 68% (138/203) to azithromycin, 42% (85/203) to doxycycline, 98% (199/203) to ciprofloxacin and ofloxacin, 85% (172/203) to trimethoprim-sulfamethoxazole (SXT), 66% (134/203) to clindamycin, and 21% (43/203) to fusidic acid. No isolates showed resistance to linezolid and vancomycin.

TABLE 1: Prevalence of MRSA in CVC infections.

Item	2013 n (%)	2014 n (%)	2015 n (%)	2016 n (%)	Total n (%)
Patients	41	53	44	65	203
male	24 (59.0)	33 (62.0)	26 (59.0)	46 (71.0)	129 (64.0)
female	17 (41.0)	20 (38.0)	18 (41.0)	19 (29.0)	74 (36.0)
Age					
<10	5 (12.0)	12 (23.0)	5 (11.0)	3 (5.0)	25 (12.0)
10–19	1 (2.0)	1 (2.0)	6 (14.0)	5 (8.0)	13 (6.0)
20–29	9 (22.	14 (26.0)	19 (43.0)	21 (32.0)	63 (31.0)
30–39	9 (22.0)	6 (11.0)	7 (16.0)	6 (9.0)	28 (14.0)
40–49	8 (20.0)	9 (17.0)	1 (2.0)	12 (18.0)	30 (15.0)
50–59	3 (7.0)	7 (13.0)	4 (10.0)	9 (14.0)	23 (11.0)
≥60	6 (15.0)	4 (8.0)	2 (4.0)	9 (14.0)	21 (10.0)
MRSA					
CA-MRSA	12 (29.0)	20 (38.0)	20 (45.0)	35 (54.0)	87 (43.0)
HA-MRSA	29 (71.0)	33 (62.0)	24 (54.0)	30 (46.0)	116 (57.0)

MRSA: methicillin resistance *Staphylococcus aureus;* **CVC:** central venous catheters; **CA-MRSA**: community acquired methicillin resistance *Staphylococcus aureus*; **HA-MRSA**: hospital acquired methicillin resistance *Staphylococcus aureus*.
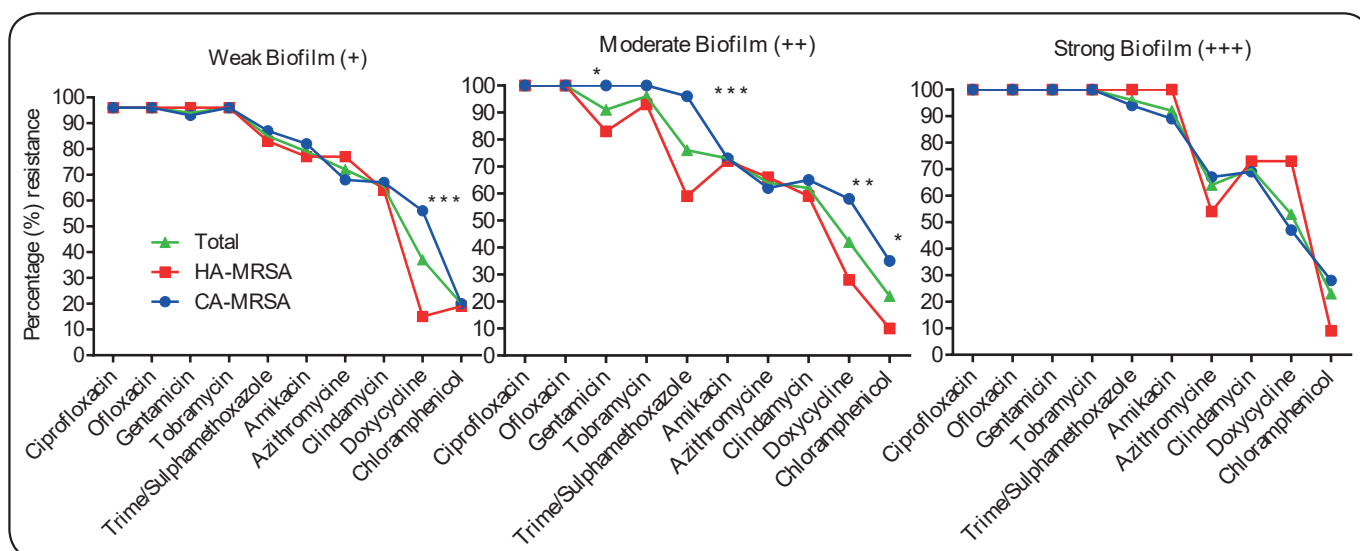
FIGURE 2: Prevalence of resistance between HA-MRSA and CA-MRSA. **HA-MRSA**: Hospital acquired methicillin resistance *Staphylococcus aureus*; **CA-MRSA**: community acquired methicillin resistance *Staphylococcus aureus*.

All MRSA isolated from CVC were biofilm producers. Half of MRSA (50%) were weak biofilm producers followed by moderate (27%) and strong biofilm (23%) producers.

Prevalence of adhesion genes of MRSA

Ten adhesion genes were amplified using specific terminal sequences. The amplification results showed that *clf*B was present in all isolates of MRSA, followed by *sdr*C and *sdr*D (90%), *eno* (87%), *fnb*A (80%), *clf*A and *sdr*E (67%), *fnb*B and *sdr*D (61%), and *cna* (51%). For moderate and weak biofilm producers, no significant (p > 0.05) differences were found among different types of MRSA (CA-MRSA, HA-MRSA) and the presence of adhesion genes (**Table 2**). However, for strong biofilm, there was a significant (p < 0.05) difference between the types of MRSA (CA-MRSA and HA-MRSA) because of the presence of adhesion genes.

Statistical analysis

All tests were performed in duplicate with a confidence level of 95% and significance level of <0.05. Statistical Package for the Social Sciences (SPSS) software (SPSS, Inc., Chicago, IL, USA) was used for data analysis with the Chi-square test.

DISCUSSION

Antimicrobial resistance is a major threat to people and worsens when lifesaving devices become contaminated. CVC is essential for critically ill patients but leads to life-threatening consequences when inserted in the central venous system[37] and become colonized by multidrug-resistant superbugs such as MRSA.

This study evaluated CVC-related infections on different types of catheters and hospital units and directly involving CVC rather than blood culture. This study revealed that age, gender, and sex were not significant predictors of CVC-related

infections. Interestingly, Gram-negative bacteria outnumbered *S. aureus* in causing CVC infection.

This study revealed that 76% of CVCs were infected by bacteria (63% by Gram-positive) or candida; a similar study conducted in Italy showed that 73% of all CVCs from ICU patients were infected and 54% were due to Gram-positive bacteria[38].

Staphylococcus aureus is the major pathogen causing CVC infections (39%), followed by CONS (16%) and *K. pneumoniae* (10%). These results agree with those of previous studies[39,40]. Most studies demonstrated that Gram-negative bacteria are the major cause of catheter-related blood stream infection, but these studies used blood samples to detect infection. A surveillance study in Australia revealed that *Enterococci species* is a major pathogen (26%) of central line catheter-related infection[41]. The prevalence of MRSA (59%; 203/344) among CVC-related infections agreed with the results of previous similar studies[42,43]. In India, *P. aeruginosa* was found to be the most common cause (42%) of CVC infection, which contradicted our study because the previous study was conducted in only cancer patients[44].

Our study revealed that 57% of MRSA infections were due to HA-MRSA, which disagreed with the results of a single-center study conducted in France in 2012 which showed that 34% of CVC-related infections were caused by HA-MRSA[45]. CA-MRSA is also emerging as a notorious pathogen in CVC-related infections, particularly in children[45].

Biofilm producers are more resistant to antibiotics than their counterparts[46]. All isolates were sensitive to vancomycin and linezolid, which agrees with previous studies[47]. Among MRSA cases, 85% were resistant to SXT, which agrees with the literature[47]. Among macrolides, erythromycin resistance was observed in 96% of cases and ciprofloxacin resistance in 98% of MRSA isolates, which agrees with studies conducted in Japan[48,49]. The same studies showed different resistance to SXT

TABLE 2: Prevalence of adhesion genes in MRSA isolated from CVC related infection.

Adhesion genes	clfA	clfB	eno	cna	fnbA	fnbB	fib	sdrC	sdrD	sdrE
Biofilm weak (+)										
Total	69% (70/101)	100% (101/100)	83% (84/101)	60% (61/101)	83% (84/101)	66% (67/101)	91% (92/101)	91% (92/101)	66% (67/101)	68% (69/101)
CA-MRSA	43% (30/70)	47% (47/101)	45% (38/84)	66% (40/61)	45% (38/84)	42% (28/67)	47% (43/92)	46% (42/92)	42% (28/67)	45% (31/69)
HA-MRSA	57% (40/70)	54% (54/101)	54% (46/84)	34% (21/61)	55% (46/84)	58% (39/67)	49% (53/92)	54% (50/92)	58% (39/67)	55% (38/69)
Biofilm moderate (++)										
Total	71% (39/55)	100% (55/55)	89% (48/55)	46% (25/55)	76% (42/55)	51% (28/55)	89% (49/55)	87% (48/88)	51% (28/55)	64% (35/55)
CA-MRSA	46% (18/39)	53% (29/55)	53% (26/49)	84% (21/25)	55% (23/42)	57% (16/28)	53% (29/49)	52% (25/48)	57% (16/28)	54% (19/35)
HA-MRSA	54% (21/39)	47% (26/55)	47% (23/49)	16% (4/25)	45% (19/42)	43% (12/28)	47% (23/49)	48% (23/48)	43% (12/28)	46% (16/35)
Biofilm strong (+++)										
Total	57% (27/47)	100% (47/47)	94% (44/47)	36% (17/47)	79% (37/47)	60% (28/47)	90% (42/47)	92% (43/47)	60% (28/47)	68% (32/47)
CA-MRSA*	22% (6/27)	23% (11/47)	23% (10/44)	65% (11/17)	24% (9/37)	25% (7/28)	24% (10/42)	23% (10/43)	25% (7/28)	25% (8/32)
HA-MRSA*	78% (21/27)	77% (36/47)	77% (34/47)	35% (6/17)	76% (28/47)	75% (21/28)	76% (32/42)	77% (33/43)	75% (21/28)	75% (24/32)

MRSA: methicillin resistance *Staphylococcus aureus;* **CVC:** central venous catheters; **clfA:** clumping factors A; **clfB:** clumping factors B; **eno:** laminin-binding protein; **can:** collagen-binding protein; **fnbA:** fibronectin binding protein A; **fnbB:** fibronectin binding protein B; **fib:** fibrinogen-binding protein; **sdrC:** serine aspartate peptide C ;**sdrD:** serine aspartate peptide D; **sdrE:** serine aspartate peptide E; **CA-MRSA:** community acquired methicillin resistance *Staphylococcus aureus*; **HA-MRSA:** Hospital acquired methicillin resistance *Staphylococcus aureus.* *Strong significant *P* < 0.01;

(2%) and clindamycin (90%) because of the use of antibiotics in different settings[50,51]. Variable resistance to fusidic acid was reported previously, and some reports showed the same resistance found in this study[52]. Similar fusidic acid resistance results were reported by Decousser[53]. Our study revealed a 42% resistance rate to doxycycline, while previous studies reported higher resistance[54,55]. For weak biofilm producers, there was a significant ($p < 0.05$) relationship between the type of MRSA (CA-MRSA and HA-MRSA) and doxycycline resistance. For moderate biofilm producers, a significant relationship ($p < 0.05$) was found for gentamycin, fusidic acid, doxycycline, and trimethoprim-sulfamethoxazole. For strong biofilm producers, both were equally resistance to antibiotics ($p > 0.05$). MRSA is well-known to thrive under antibiotic treatment. The variable resistance pattern to MRSA explains why antibiotics usage differs according to local guidelines in different locations. First-line drugs are more resistant because their use is common than other antibiotics. An imbalance has been detected among antibiotic usage, the discovery of new antibiotics, and emergence of resistance, which leads to serious consequences of infectious diseases[56,57].

No significant difference was evident between weak and moderate biofilm producing MRSA via adhesion genes ($p > 0.05$). Interestingly, a significant difference was observed between the strong biofilm producers CA-MRSA and HA-MRSA because of the adhesion genes evaluated in this study ($p < 0.01$). Twenty-five isolates contained all of the adhesion genes, suggesting that adhesion genes were exclusively involved in biofilm formation[58]. No gene or set of genes can function as a sole indicator of biofilm formation potential; this outcome supports previous findings[59]. A study conducted in Morocco revealed adhesion genes prevalence rates of 96% *fnbA*, 60% *eno*, 43% *clfA*, 43% *clfB*, 11% *cna*, 6% *fib*, and 2% *fnbB* in MRSA isolated from clinical specimens. These results contradicted our findings because these studies used different specimens to isolate the pathogens[58,60]. The results of the prevalence of adhesion genes among MRSA agrees with those of previous studies[61,62]. The presence of adhesion genes in most MRSA isolated from CVC-related infection was complementary to biofilm formation and posed resistance to antibiotics.

This study revealed a significant difference among the strength of biofilm potential, type of MRSA, and antibiotic resistance. The strong biofilm producers CA-MRSA and HA-MRSA are equally resistant to antibiotics. Adhesion genes are indispensable for biofilm formation. The presence of adhesion genes is independent of biofilm strength.

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