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ORIGINAL ARTICLE

Adhesins Encoding Genes and Biofilm formation as Virulence Determinants in Methicillin Resistant *Staphylococcus aureus* Causing Hospital Acquired Infections

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Key words:
MRSA, adhesins, biofilm, virulence, SCCmec typing

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ABSTRACT

Background: *Methicillin resistant Staphylococcus aureus (MRSA) is a major infection-causing pathogen in health care settings. Adhesins, which are part of the class of molecules known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and the development of biofilms are crucial elements in the colonization and antibacterial resistance of this organism.* ***Objectives:*** *determines the prevalence of adhesins genetic determinants and biofilm formation among MRSA isolates in patients with hospital acquired infections (HAIs) at Mansoura University Hospitals (MUHs), as well as staphylococcal cassette chromosome mec (SCCmec) typing.* ***Methodology:*** *From patients with HAIs in MUH Departments, clinical samples were collected. Routine microbiological methods, cefoxitin sensitivity tests, and PCR for the mecA gene were used to identify MRSA isolates. The MRSA isolates' ability to form biofilms was evaluated using a microtitre plate assay. Multiplex PCR was used for SCCmec typing and screening for the genes ica D, eno, ebps, cna, fnbA, fnbB, fib, clfA and clfB. Eighty-five MRSA isolates were identified.* ***Results:*** *Type III of SCCmec MRSA was the most frequent (63.5 %). In 65 isolates (76.5 %), biofilm production was detected. Strong biofilm-forming isolates were the most prevalent; accounting for 49.4% of all MRSA isolates. The eno gene was the most common MSCRAMMs gene (95.3%), whereas clfB was the least (29.4%). The icaD gene was present in 90.6% of MRSA isolates and in all biofilm forming isolates. The icaD and the development of biofilms were significantly associated (P = 0.0001). None of the MSCRAMMs genes evaluated were significantly associated with biofilm development. The highest gene association was icaD with eno gene; 85.9%. The highest 3 MSCRAMMs genes co-existance was eno, ebps, and fnbA; 64.7%.* ***Conclusion:*** *The increased frequency of biofilm formers among infecting MRSA isolates revealed that biofilm formation may account for isolates persistence in hospital setting and hence transmission to patients. The prevalent existence of icaD and eno among biofilm formers indicates the possible molecular relationship that may link these genes and infection with biofilm forming MRSA which need further investigations.*

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a frequently encountered resistant pathogen. MRSA causes variety of infections ranging from minor skin infections to serious, potentially fatal types like bloodstream infections [1-2].

Prior to colonizing host tissue, *Staphylococcus aureus* must adhere to numerous cellular and extracellular matrix components. Additionally, host proteins may coat implanted biomaterials, allowing bacteria to adhere and colonize to their surfaces starting an infection [3].

S. aureus express a wide range of structural proteins that mediate this process and contribute to its pathogencity. These proteins include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [4]. Through MSCRAMMs, *S. aureus* can attach to the extracellular matrix of the host, such as laminin, fibrinogen, and collagen[3, 5]. The MSCRAMMs comprise, among others, clumping factor (CLF) A, B, collagen adhesin (Cna), fibronectin-binding protein (FnBP) A, B. These molecules enable the bacteria to adhere to and proliferate on injured tissues by binding to extracellular matrix proteins [5].

Furthermore, biofilm production defends microorganisms from opsonization, phagocytosis and antibacterial agents [6]. The intercellular adhesion *(ica)* genes-which control polysaccharide intercellular adhesion- are the most often investigated genes that control biofilm development in *S. aureus* [7]. There are currently different techniques for typing of *Staphylococcus aureus* isolates. Typically, these systems are used to investigate health care associated infection (HAIs) outbreaks [8].

The objective of the current study was to determine the prevalence of a group of MSCRAMMs and *icaD* genes among MRSA isolates causing HAIs in MUHs patients. Additionally, identification of biofilm forming capacity and staphylococcal cassette chromosome *mec* (SCC*mec*) types of these collected isolates.

METHODOLOGY

Clinical samples collection and bacterial identification

From April 2018 to October 2020, this study was carried out in Mansoura University Hospitals departments. During this period, clinical samples were collected from patients with suspected HAIs. Identification of *S. aureus* recovered from these samples was done by conventional microbiological techniques including; Gram stained film, haemolysis on blood agar. Biochemical reactions including catalase and coagulase assays were done for these isolates [9-10].

Oxacillin susceptibility assay:

The Clinical Laboratory Standards Institute (CLSI) recommended disc diffusion cefoxitin (30 µg) (Mast Diagnostics, Merseyside-UK) sensitivity assay was used to test for oxacillin resistance [11]. Oxacillin resistant *S. aureus* were tested for *mec*A gene using primers and protocol previously described [12] DNA extraction was carried out by QIAamp DNA extraction kits (Qiagen, Hilden, Germany) in accordance with the manufacture instructions.

Staphylococcal cassette chromosome *mec* typing

For SCC*mec* typing, multiplex PCR was used according to the protocol described before [13]. The protocol involved a cycle of initial denaturation lasting 5 minutes at 94°C, followed by 35 cycles of denaturation lasting 30 seconds at 94°C, annealing at 52°C for 30 seconds, extension lasting 45 seconds at 72°C, then a final extension lasting 7 minutes at 72°C. Agarose gel electrophoresis was used for visualization of all PCR products [13-15]. MRSA isolates were typed according to SCC*mec* into type I (415 bp), II (937 bp), III (518 bp) and IV (937 bp and 415 bp).

Assay of biofilm forming capacity of the MRSA isolates

In 96 well microtiter polystyrene plates, the biofilm formation assessment was carried out in accordance with the protocol described before by Stepanovi et al[16]. The tested MRSA strains were cultured in 2 mL of tryptic soya broth (TSB) with 0.25 percent glucose, and incubated overnight at 37°C. Then, 0.5 McFarland turbidity suspension was prepared. For each strain, 200 µL was placed in triplicate on a flat-bottomed microtiter plate with a lid. After that, the cultures were kept at 37°C for 24 hours. Each microtiter plate well was gently aspirated after incubation to remove nonadherent bacteria, and the wells were then washed twice with 300 L of phosphate-buffered saline (PBS, pH 7.4). After three gentle rinses with the addition of 300 µL of sterile water to remove any remaining non-attached cells, the microtitre plates were placed inverted at room temperature for air drying. Crystal violet 0.1% was used to stain the adhering biofilm mass for 10 to 15 minutes. After being washed with sterile distilled water, the wells were left to air dry. Finally, 300 µL of 95% ethanol were poured into each well. The uninoculated media was considered as a negative control. The cut-off OD (ODc) =average OD of negative control+ (3×SD of negative control). The MRSA isolates were classified into (16): non biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤2 times ODc), moderate biofilm producers (2timesODc < OD ≤4 times ODc) and strong biofilm producers (OD> 4 times ODc).

Adhesin of MSCRAMM gene determinants and *icaD* gene:

The PCR was used to test for the presence of the MSCRAMM and *icaD* genes. The genes of collagen-binding protein (*cna*), laminin-binding protein (*eno*), fibronectin-binding protein (*fnbA, fnbB*), elastin-binding protein (*ebps*), intercellular adhesion D (*icaD*), clumping factor (*clfA, clfB)* and fibrinogen-binding protein (*fib*) were included.

Multiplex PCR was performed using primers illustrated in (Table 1) and the reactions described before. Briefly, initial denaturation (5 minutes at 94 °C) was done, then 25 cycles of amplification (1 minute at 94 °C for denaturation, 1 minute at 55 °C for annealing, and 1 minute at 72 °C for extension) were carried out. The reaction was terminated with a 10 min extension step at 72 °C. The products of PCR reaction were visualized using agrose gel electrophoresis [17-18].

Table 1: Primers sequence and amplicone size (bp) of adhesine genes investigated in this study.

Gene	Primer sequences	Amplicon size (bp)
icaD	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	198
cna	GTC AAG CAG TTA TTA ACA CCA GAC AAT CAG TAA TTG CAC TTT GTC CAC TG	423
eno	ACG TGC AGC AGC TGACT CAA CAG CAT TCT TCA GTA CCTTC	302
ebps	CAT CCA GAA CCA ATC GAA GAC CTT AAC AGT TAC ATC ATC ATG TTT ATC TTT G	186
fnbA	GTGAAGTTTTAGAAGGTGGAAAGATTAG GCTCTTGTAAGACCATTTTTCTTCAC	643
fnbB	GTAACAGCTAATGGTCGAATTGATACT CAAGTTCGATAGGAGTACTATGTTC	524
fib	CTACAACTACAATTGCCGTCAACAG GCTCTTGTAAGACCATTTTCTTCAC	404
clfA	ATTGGCGTGGCTTCAGTGCT CGTTTCTTCCGTAGTTGCATTTG	292
clfB	ACATCAGTAATAGTAGGGGGCAAC TTCGCACTGTTTGTGTTTGCAC	205

cna: collagen binding protein, *eno*: encoding laminin binding protein, *ebps* :elastin binding protein, *fnb*A: fibronectin binding proteins A, *fnbB:* fibronectin binding proteins B, *fib*: fibrinogen binding protein, *clfA: clumping factor A, clfB: clumping factor B.*

Statistical analysis:

Data were analyzed using the SPSS application, version 20.0. Number and percentage-based qualitative data were used for descriptive analysis. Monte Carlo correction and the chi-square tests were used to compare categorical data. P value less than 0.05 was considered significant.

RESULTS

Distribution of MRSA among clinical samples and SCC*mec* typing

During the study period a total of 85 MRSA isolates were collected from patients admitted to MUHs presented manifestations of HAIs. The included patients were 44 females and 41 males representing 51.8% and 48.2%, respectively. The patients' age was ranged from 25 to 61 years (mean ± SD: 46.4±9.5).

The collected MRSA were from blood, endotracheal tube aspirate, urine and wound samples (swabs and aspirate) (Figure 1).

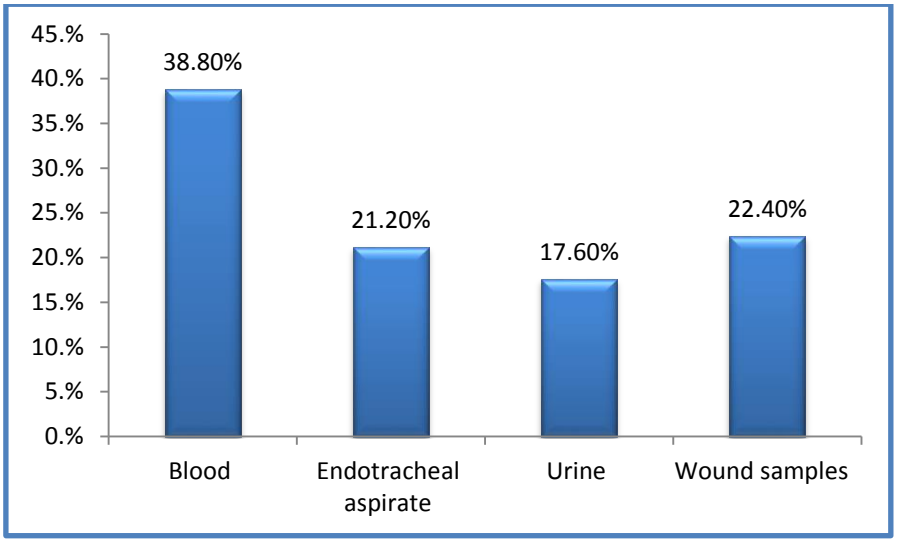

Fig. 1: Distribution of MRSA isolates among clinical samples

Regarding SCC*mec* typing, type III is the highest prevalent type representing 63.5% (54 isolates). No isolates of type V was detected, (Table 2).

Biofilm formation ability of the collected MRSA samples

Biofilm formation capacity of the collected isolates was evaluated by the microtiter plate assay. Sixty-five isolates (76.5%) of total MRSA were biofilm formers. Regarding the biofilm degree, the isolates were classified as strong, moderate and weak biofilm formers representing (42, 49.4%), (19, 22.4%) and (4, 4.7%) of the total isolates, respectively table (3).The highest prevalence of biofilm formers were from blood isolates (25 isolates representing 75.8% of total bloodstream MRSA isolates). There was no significance difference in the distribution of biofilm forming MRSA isolates among clinical samples (Table 3).

Prevalence and distribution of MSCRAMMs adhesins and *icaD* genes among collected MRSA isolates

The *eno* gene had the highest frequency among MSCRAMM adhesin genes (95.3%), followed by the *ebps* (82.4%). While, *clfB* had the lowest prevalence (34.1%). The intercellular adhesion gene (*icaD*) was reported in 90.6%, (Table 2).

Table 2: Characters of collected MRSA isolates

Character	(NO, %)
SCC*mec* types	
I	7 (8.2)
II	21 (24.7)
III	54 (63.5)
IV	3 (3.5)
Prevalence of MSCRAMMs adhesins and intracelur adhesin D *(icaD)* genes	
eno	81 (95.3)
ica D	77 (90.6)
ebps	70 (82.4)
cna	33 (38.8)
fnbA	69 (81.2)
fnbB	44 (51.8)
fib	53 (62.4)
clfA	46 (54.1)
clfB	25 (29.4)
Degree of Biofilm formation (total=65)	
Strong	42 (49.4)
Moderate	19 (22.4)
Weak	4 (4.7)

The *icaD* was present in all clinical samples. The highest *icaD* gene prevalence was in urine sample (14/15). No statistically significant difference in *icaD* distribution among clinical samples. Regarding MSCRAMMs gens, the *eno* gene represents the highest prevalence of studied virulence genes in all clinical samples. Regarding blood samples, *fnbA* follow the *eno* gene (30/33). The e*bp*s gene follow *eno* gene in the prevalence among ETA, urine and wound samples. However, no statistically significant difference was detected in distribution of studied virulence genes among different clinical samples. Additionally, no statistically significant association between difference MRSA SCC*mec* types and type of clinical samples SCC*mec* types (Table 3).

Table 3: Distribution of adhesions genes, biofilm formation and SCC*mec* types among MRSA isolates from different clinical samples

Clinical sample (total no)	MSCRAMMs adhesins genes No (%)									Biofilm** No (%)	SCCmec type** No (%)			
	icaD	*cna*	*eno*	*ebps*	*fnbA*	*fnbB*	*fib*	*clfA*	*clfB*		I	II	III	IV
Blood (33)	30 (90.9)	12 (36.4)	32 (97)	26 (78.8)	30 (90.9)	21 (63.6)	19 (57.6)	15 (45.5)	11 (33.3)	25 (75.8)	3 (9.1)	8 (24.4)	20 (60.6)	2 (6.1)
Respiratory tract samples (18)	16 (88.9)	4 (22.2)	16 (88.9)	15 (83.3)	12 (66.7)	8 (44.4)	14 (77.8)	10 (55.6)	4 (22.2)	12 (66.7)	3 (16.7)	4 (22.2)	11 (61.1)	-
Urine (15)	14 (93.9)	5 (33.3)	15 (100)	14 (93.3)	12 (80)	6 (40)	10 (66.7)	10 (66.7)	4 (26.7)	12 (80)	-	3 (20)	11 (61.1)	1 (6.7)
Wound aspirate (19)	17 (89.5)	12 (63.2)	18 (94.7)	16 (84.2)	15 (78.9)	9(47.3)	10 (52.6)	11 (57.9)	6 (31.6)	16 (84.2)	1 (5.3)	6 (31.6)	12 (63.2)	-
P value	0.97	0.78	0.45	0.49	0.2	0.36	0.36	0.55	0.44	0.63	0.78			

The association between biofilm formation and investigated genes in MRSA isolates

The genes that had the highest prevalence among biofilm producing isolates were *icaD* (65/65), *ebps* and *eno* (63/65, each). There is a statistically significant association between biofilm production and the presence of *icaD* (P=0.0001) (Figure 2).

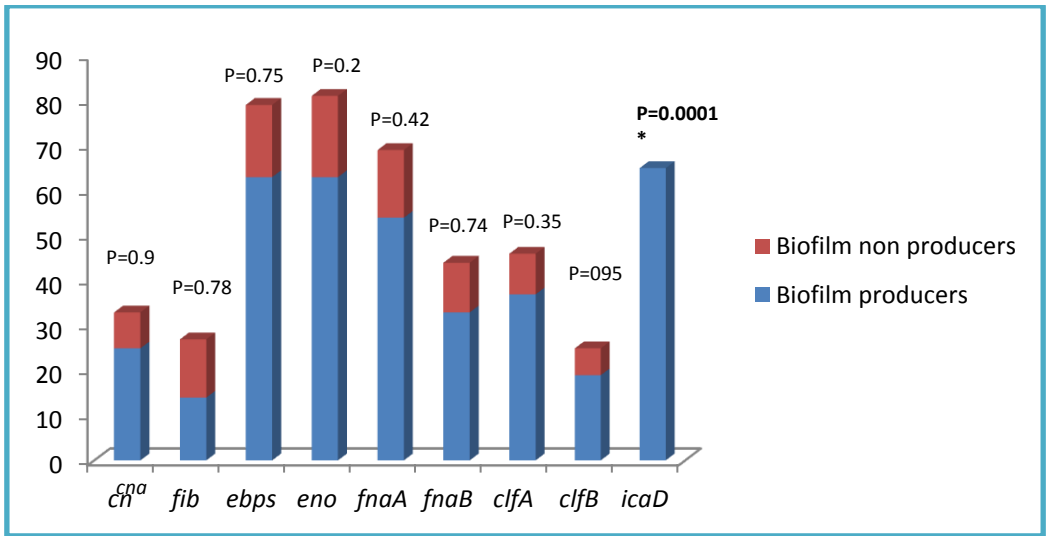

Fig. 2: Distribution of studied genes among biofilm producers and non producers MRSA isolates
*****Significant association between *icaD* gene and biofilm formation

Strong biofilm producers represented 54.5% of *icaD* positive isolates. About 16% (15.6) of *icaD* positive isolates fail to form biofilm. Concerning MSCRAMMs genes, the highest prevalence of strong and moderate biofilm formers were among *eno* positive MRSA isolates (40/42 and 19/19, respectively) (Table 4).

Table 4: Association of MSCRAMMs adhesins with different biofilm degree

Biofilm degree	MSCRAMMs adhesins genes No (%)								
	ica **65 (84.4)**	***cna*** **30(90.9)**	***eno*** **61(75.3)**	***ebps*** **54(77.1)**	***fnbA*** **54(78.3)**	***fnbB*** **33(75)**	***Fib*** **40(75.5)**	***clfA*** **37(80)**	***clfB*** **18(72)**
Strong	42 (100)	19 (45.2)	40(95.2)	35 (83.3)	36 (85.7)	21 (50)	27 (64.3)	24 (57.1)	12 (28.6)
Moderate	19 (100)	11 (57.9)	19 (100)	17 (89.5)	14(73.7)	10 (52.6)	11(57.9)	11 (57.9)	6 (30.6)
Weak	4 (100)	-	2 (50)	2 (50)	4 (100)	2 (50)	2 (50)	2 (50)	-
P value	-	0.3	0.2	0.06	0.2	0.9	0.7	0.9	1

Regarding the coexistence of studied genes, the most common association of *icaD* was with *eno* gene (73isolates, 85.9%) and then with *ebps* (64 isolates, 75.3%). Fibrinonectin binding proteins A and B were coexisted in 38 isolates (44.7%). While, clumping factor A and B genes were present together in 17 MRSA isolate (20%). The highest 3 MSCRAMMs genes coexistance was *eno, ebps,*and *fnbA* (55 isolates, 64.7%) (Table 5).

Table 5: Association of MSCRAMMs and *icaD* genes in MRSA isolates

Genes association	No (%)
eno, icaD	73 (85.9)
eno, ebps	68 (80)
ebps, icaD	64 (75.3)
eno, ebps, icaD	62 (72.9)
eno, ebps, fnbA	55 (64.7)
eno, ebps, fnbA, cna	26(30.6)
fnbA, fnbB	38 (44.7)
icaD ,eno, ebps, clfa	35 (41.2)
eno, ebps, fnbA, fnbB	31 (36.5)
icaD, eno, fib	43 (50.6)
clfA, clfB	17 (20)
icaD, clfA, clfB	16 (18.8)
fnbA, fnbB, clfA, clfB	9 (10.6)
eno, ebps, clfA, clfB	16 (18.8)
fib, fnbA, fnbB, clfA, clfB	2 (2.4)

DISCUSSION

Methicillin resistant *S. aureus* represents a major health care problem has been spread rapidly and seriously in many areas of the world. [1]

Typing of MRSA helps to understand the epidemiology of infections [8].

Using SCC*mec* for MRSA typing, our study revealed that type III was the most frequent type representing 63.5% (54 isolates) then, type II 24.7% (21 isolates). SCC*mec* type V wasn't detected. According to previous reports, SCC*mec* types are distributed differently in various parts of the world, variably in hospital and community setting [19]. The most prevalent hospital acquired MRSA strains worldwide are of SCC*mec* types I to III. Matching with this finding, the majority of hospital isolates in the current study were SCC*mec* type III. Similar to this, it had been reported that SCC*mec* type III MRSA isolates are more abundant in isolates from many Asian countries like Saudi Arabia and India. While SCC*mec* type II strains are more common in other countries like Japan and Korea [20].

Biofilm is a feature that is closely correlated with bacterial persistence and virulence. It allows bacteria to colonize host tissues, escape from host immunological mechanisms, and allows protection from antibacterial agents facilitating the emergence of antibacterial resistance [6, 21]. In our study, sixty-five isolates (76.5%) of MRSA isolates were biofilm formers. Strong, moderate, and weak biofilm producers represented 49.4%, 22.4%, and 4.7 %, respectively of the isolates. This result agrees with previous reports of Cha et al. (68.3% of MRSA isolates were biofilm producers) [22]. Matching with result of Serray et al. major sector of MRSA isolates in our study were strong biofilm producers[23]. However, moderate biofilm forming capacity was the highest among biofilm producers in other studies like Ali and Seiffein [24]. These variable results can be attributed to the different detection methods and different biofilm categorization limits. Polysaccharidic intercellular adhesin produced by proteins encoded by *icaD* gene cluster, is necessary for the development of biofilm in *Staphylococcus aureus* [7]. Our study confirms this finding; the prevalence of *icaD* among MRSA isolates was 90.6%, all biofilm forming isolates were positive for *icaD* gene. Significant association was found between biofilm formation and the presence of *icaD* gene. However, no significant difference in *icaD* distribution between different degrees of biofilm producers, this is in line with finding of Serray et al [23]. About 16% of *icaD* positive isolates failed to produce biofilm. This finding like result before that of Conlon et al. This can be explained by the suppression of the *ica* operon by the action of repressor molecule [25].

A significant virulence factor in staphylococcal infections is thought to be the collagen adhesin encoded by *cna* gene. The *cna* gene product can bind to collagen which is an important component of human connective tissue [26]. *The cna* gene in our study was reported in 38.8% of MRSA isolates. This finding is matching with that of Kot et al., (30.8%) [27]. However, different result was obtained by Kurlenda et al. (4%) [28]. Furthermore, in our study most of *cna* positive isolates were mostly from wound and blood stream infection which may be linked to tissues rich in collagen and increased device insertion, respectively.

The *eno* and *ebps* genes code for adhesins to laminin and elastin. Enolase; a protein that may bind laminin is produced by the *eno* gene. Laminin makes up a significant portion of the vasculature's basal membrane. Therefore adhesion to laminin could promote tissue invasion and the spread of staphylococcal cells through blood to different parts of the host[29]. Elastin is widely found in the skin, lungs, and blood vessels. In particular, the bacterial colonization of damaged elastin-rich regions is facilitated by the elastin-binding protein of *S. aureus* [30].

In our study, the frequencies of *eno* and *ebps* genes were 95.3% and 82.4%, respectively. Similar to this result, high prevalence of *eno* gene was found in hospital acquired MRSA isolates [31], and in all isolates Kasela et al., [30] and Kot et al. [27]

The most crucial proteins for *S. aureus*'s binding to fibrinogen and fibrin are the products of clumping factors (*clfA*, *clfB*) (32), fibronectin binding proteins (*fnbA*, *fnbB*) [33] and fibrinogen binding protein (*fib*) genes. Given that fibronectin is a widely dispersed host protein that can be present in cellular matrix in fibrillar form as well as soluble form in the blood [34]. The *clfA* and *clfB* genes were detected in 54.1% and 29.4% of the isolates under study, respectively. Our result matches with that of Gowrishankar et al.[35] and slightly higher than that of Serray et al. [23] Additionally, 81.2% and 51.8% of MRSA isolates in our study were positive for *fnbA* and *fnbB* gene, respectively. This finding matches with that of Mirzaeea et al. [36]. However, Arciola et al.[37]; reported 98.4% and 99.5% for *fnbA* and *fnbB*, respectively. Regarding *fib* gene, 62.4% of MRSA isolates were positive for *fib* gene similar to other report before [38]. These different results can be explained by the variation in genetic background and isolation source which reflected in MSCRAMMs gene predominance among MRSA isolates.

CONCLUSION

Biofilm formation and MSCRAMMs adhesins are prevalent virulence determinants in MRSA causing HAIs. The high prevalence of biofilm formers among infecting isolates revealed that biofilm formation may be responsible for the persistence of MRSA isolates in the hospital environment and hence transmission to patients. The prevalent MSCRAMMs adhesins are responsible for more adhesion and colonization of damaged tissue. The highest prevalence of *icaD* and *eno* genes indicates the possible molecular relationship that may link these genes and infection with biofilm forming MRSA which need further investigations.

This study was approved by the ethical committee in the Faculty of Medicine, Mansoura University. Informed consent was received from each participant in the study.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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