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Association between accessory gene regulator alleles, *agr* functionality and biofilm formation in MRSA and MSSA isolated from clinical and nasal carrier specimens

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ABSTRACT

Background: *Staphylococcus aureus* has a huge armory of virulence factors which are under the control of the quorum-sensing accessory gene regulator (*agr*) system. *agr* dysfunctional strains usually have a higher ability to form biofilm. The aim of the work was to detect the association of *agr* groups, *agr* functionality and biofilm formation among methicillin resistant and methicillin sensitive *Staphylococcus aureus* (MRSA/MSSA) isolated from clinical and nasal carrier specimens. **Methods:** Antibiotic susceptibility testing (AST) was performed to 100 clinical samples and 50 nasal carriers. Isolates were then characterized by *agr* typing using multiplex PCR. *agr* activity was evaluated using *agr* CAMP assay. Biofilm formation was determined phenotypically by microtiter plate method and genotypically by amplifying *icaA* and *icaD* genes. **Results:** A high level of resistance to different classes of antibiotics was detected. Methicillin resistant *Staphylococcus aureus* was more prevalent among clinical samples than nasal samples. No vancomycin resistant *Staphylococcus aureus* was detected. The percentage of *agr* dysfunctional isolates and the ability to form biofilm were higher in clinical samples than in nasal swabs, and more prevalent in MRSA than in MSSA. *agr* I was the most predominant allele among all isolates. The percentage of biofilm formation was higher among non-functioning *agr* isolates. *icaD* gene was the most prevalent biofilm formation gene detected. **Conclusion:** The formation of the biofilm in MSSA depends on *ica* genes, while in MRSA a biofilm can be formed in absence of both genes. The *agrII* allele was statistically significant associated with strong biofilm formation ($p = 0.001$) and with *agr* dysfunction ($p = 0.030$).

Introduction

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen, that causes a broad range of human infections. Its pathogenicity is a complex process including a various array of virulence

factors which are expressed through different stages of infection via a web of virulence regulators [1].

Several reports suggested that the role of Accessory gene regulator (*agr*) in *S. aureus* virulence is sophisticated. Accessory gene regulator

dysfunction causes changes in the expression of genes and has global effects on bacterial phenotypes including pathogenicity [2].

agr locus; a quorum-sensing virulence regulator; plays an important role in perpetuating infection. Many staphylococcal infections are associated with communicating cell groups, known as biofilms. At high cell density, the *agr* locus leads to decreased production of cell-wall-associated factors, causing the dispersion of the biofilm, the spread of the infection and a simultaneous increase in exoproteins, including protease, hemolysin, and super-antigen production. Moreover, it leads to increased production of many murein hydrolases that are involved in autolysis. Therefore, the dysfunction of the *agr* locus can cause abundant biofilm formation and deficiency in autolysis even though the bacterial density is high. These changes can contribute to the persistence of the infection by hindering the host immune system [3]. There is increasing clinical evidence showing that alterations in *agr* in *S. aureus* are a key risk factor for poor clinical outcomes [4].

The aim of the work was to detect the association of *agr* groups, *agr* functionality and biofilm formation among methicillin resistant *S. aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) isolated from clinical specimens and nasal carriers.

Materials and Methods

Bacterial isolation and identification

In the present study, a total of 150 *S. aureus* isolates were included; 100 isolates from clinical samples, collected from patients attending the Microbiology department of the Medical Research Institute, Alexandria, Egypt and 50 isolates (33.3%) collected from nasal carriers by nasal swab.

Staphylococcus aureus isolates were fully identified phenotypically, and biochemically (catalase, haemolysis, oxidase, coagulase, DNase, and mannitol fermentation tests). The isolates were stored in glycerol- LB Broth (Merck, Germany) at -80 °C. The reference strain *S. aureus* ATCC 29213 was used as a quality control strain.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed to all antibiotics using the KirbyBauer disc-diffusion method except vancomycin which was determined using both vancomycin agar screening test and minimal inhibitory concentration (MIC) method, all

as recommended by the Clinical and Laboratory Standards Institute.

agr CAMP test and genotypic typing

agr CAMP test is used to determine the *agr* functionality via δ -haemolysin production. The test was done using *S. aureus* ATCC25923 [5]. *agr* typing was performed using conventional multiplex PCR [6].

Phenotypic and genotypic detection of biofilm formation

Biofilm formation assay was performed using microtiter plate method [7]. Each isolate was tested in triplicates. The negative control wells contained only broth. Detection of *icaA* and *icaD* genes: was done using conventional PCR [8].

Results

This study included 100 *S. aureus* clinical isolates (pus 51 (34%), wound 36 (24%), and blood 13 (8.7%)), in addition to 50 (33.3%) isolates from nasal carriers.

Susceptibility testing of the *S. aureus* isolates showed that the highest level of resistance was encountered to B-lactams as penicillin (98.7%), ampicillin (90%), cefoxitin (83.3%), and for fucidin (57.3%). Hundred twenty five (125) *S. aureus* isolates (83.3%) were identified as MRSA while 25 isolates (16.7%) were MSSA. Meanwhile the highest level of sensitivity was encountered for linezolid (100%), and vancomycin (100%).

The association between the sample type, and each of resistance to the methicillin, biofilm producers, *agr* functionality and *agr* alleles among the 150 *S. aureus* isolates is shown in **table (1)**. A statistically significant difference was found between clinical samples and nasal carriage regarding the resistance to methicillin ($p < 0.001$), biofilm production ($p < 0.001$) and *agr* functionality ($p < 0.001$).

On the other side, there was no association between different types of clinical samples and each of resistance to the methicillin, biofilm production, *agr* functionality and *agr* alleles shown in **table (2)**.

The correlation between biofilm production, *agr* functionality and *agr* alleles among MRSA and MSSA isolates is shown in **table (3)**. Among the 125 MRSA isolates 25(20%),32(25.6%),45(36%) and 23(18.4) were strong, moderate, weak and no- biofilm forming compared to 2(8%), 3(12%), 10(40%),10(40%) out of the MSSA strain. There has been a significant

difference in degree of biofilm formation between the MRSA and MSSA isolates, ($p=0.05$). Moreover, there was also a highly significant difference between the *agr* functionality in the MRSA and MSSA isolates ($p < 0.001$). Regarding the distribution of *agr* alleles, there has been a significant difference in the distribution of *agr* alleles among the MRSA & MSSA isolates ($p = 0.013$). Association of *agrIII* with MRSA was statistically significant as it was found among 24% of the 125 MRSA isolates compared to 0.0% in the MSSA isolates ($p = 0.006$).

Upon detection of biofilm genes *icaA* and *icaD* by PCR, it was found that 95 (63.3%) isolates were positive for *icaD* gene only, while 42 (28%) isolates were positive for *icaA* + *icaD* genes. On the other hand, none of the isolates were positive for the *icaA* gene only and 13 (8.6%) isolates were negative for both genes. There have been a high statistically significant association between degree of biofilm formation and detection of *icaA* and *icaD* gene by PCR ($p = 0.001$).

The correlation between degree of biofilm formation and detection of *icaA* and *icaD* genes in MRSA strains is shown in **table (4)** and in MSSA strains is shown in **table (5)**.

Furthermore, out of 106 *S. aureus* isolates with non-*agr* functioning, 98 isolates (92.5%) were biofilm producers. Meanwhile, out of 44 isolates with *agr*-functioning; 19 isolates (43.2%) were biofilm producers. There was a statistically significant association between *agr* functionality and biofilm formation ($p < 0.001$). The association between *agr* function and detection of *icaA* and *icaD* genes is shown in **table (6)**. There was a statistically significant association between *icaD* gene and *agr* dysfunction. ($p = 0.029$).

There was a highly significant association between *agrIV* and weak biofilm formation as 80% of *agrIV* isolates formed weak biofilm ($p = 0.001$), and also between *agrII* and strong biofilm formation as 66.7% of *agrII* type isolates were strong biofilm producers ($p < 0.001$) shown in **table (7)**.

Table 1. The association between the sample type, and each of resistance to the methicillin, biofilm producers, *agr* functionality and *agr* alleles

Specimen	Methicillin resistance		Biofilm		<i>agr</i> functionality		<i>agr</i> alleles No. (%)					Total
	MRSA No (%)	MSSA No (%)	Negative No (%)	Positive No (%)	Negative No (%)	Positive No (%)	I	II	III	IV	Non-typeable	
Clinical samples	99(99)	1(1)	12(12)	88(88)	84(84)	16(16)	47 ^a (47)	14 ^a (14)	26 ^a (26)	3 ^a (3)	10 ^a (10)	100(100)
Nasal carriage	26(52)	24(48)	21(42.0)	29(58)	22(44)	28(56)	34 ^b (68)	4 ^a (8)	2 ^a (4)	2 ^a (4)	6 ^a (12)	50(100)
Total	125(83.3)	25(16.7)	33(22.0)	117(78)	106(70.7)	44(29.3)	81(54.0)	18(12)	30(20)	5(3.3)	16(10.7)	150(100)
X ² p	53.016*(<0.001*)		17.483*(<0.001*)		25.729*(<0.001*)		9.347(0.053)					

χ^2 : Chi square test

p : p value for comparing between the studied categories

*: Statistically significant at $p \leq 0.05$

Frequency with Common letters is not significant (i.e. Frequency with Different letters is significant at $p \leq 0.05$).

Table 2. The association between the type of clinical samples and each of methicillin resistance, biofilm formation, *agr* functionality and *agr* alleles.

Specimen	Methicillin resistance		Biofilm		<i>agr</i> functionality		<i>agr</i> alleles No. (%)					Total
	MRSA No (%)	MSSA No (%)	Negative No (%)	Positive No (%)	Negative No (%)	Positive No (%)	I	II	III	IV	(Non-typeable)	
Pus swab	51(100)	0(0)	7(13.7)	44(86.3)	39(76.5)	12(23.5)	22(43.1)	6(11.8)	16(31.4)	3(5.9)	4(7.8)	51(100)
Wound swab	35(97.2)	1(2.8)	3(8.3)	33(91.7)	33(91.7)	3(8.3)	18(50)	6(16.7)	8(22.2)	0(0)	4(11.1)	36(100)
Blood	13(100)	0(0)	2(15.4)	11(84.6)	12(92.3)	1(7.7)	7(53.8)	2(14.4)	2(14.4)	0(0)	2(18.2)	13(100)
Total	99(99)	1(1)	12(12)	88(88)	84(84)	16(16)	47(47)	14(14)	26(26)	3(3)	10(10)	100(100)
χ^2 , <i>p</i>	2.123 (MC <i>p</i> = 0.497)		0.927 (MC <i>p</i> = 0.693)		4.393(0.111)		5.072 (MC <i>p</i> = 0.768)					

 χ^2 : Chi square test

MC: Monte Carlo

p: *p* value for comparing between the studied categories*: Statistically significant at $p \leq 0.05$ **Table 3.** The correlation between biofilm production, *agr* functionality and *agr* alleles among MRSA and MSSA isolates.

Specimen	Biofilm		<i>agr</i> functionality		<i>agr</i> Alleles No. (%)				
	Negative No (%)	Positive No (%)	Negative No (%)	Positive No (%)	I	II	III	IV	Non-typeable
MRSA (n=125)	23(18)	102(82)	95(76)	30(24)	64(51.1)	15(12)	30(24)	3(2.4)	13(10.4)
MSSA (n= 25)	10(40)	15(60)	11(44)	14(56)	17(68)	3(12)	0(0)	2 (8)	3(12)
Total (n=150)	33(22)	117(78)	106(70.7)	44(29.3)	81(54.0)	18(12)	30(20)	5(3.3)	16(10.7)
X2, <i>p</i>	7.827 (=0.05*)		10.292 (<0.001*)		11.316(0.013*)				

 χ^2 : Chi square test*p*: *p* value for comparing between the studied categories*: Statistically significant at $p \leq 0.05$ **Table 4.** The association between degree of biofilm formation and detection of *icaA* and *icaD* gene in MRSA strains.

	Degree of biofilm formation				Total	χ^2	<i>p</i>
	Strong No (%)	Moderate No (%)	Weak No (%)	Non biofilm No (%)			
<i>icaA</i> gene only	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	-	-
<i>icaD</i> gene only	15 (16.9)	22 (24.7)	32 (36.0)	20 (22.5)	89 (71.2)	4.408	0.221
<i>icaA</i> & <i>icaD</i> genes	8 (28.6)	8 (28.6)	12 (42.9)	0 (0.0)	28 (22.4)	8.560*	0.036*
Negative for both <i>icaA</i> and <i>icaD</i>	2 (25.0)	2 (25.0)	1 (12.5)	3 (37.5)	8 (6.4)	3.327	MC <i>p</i> = 0.355
Total	25 (20.0)	32 (25.6)	45 (36.0)	23 (18.4)	125(100)		

 χ^2 : Chi square test

MC: Monte Carlo

p: *p* value for comparing between the studied categories*: Statistically significant at $p \leq 0.05$

Table 5. The association between degree of biofilm formation and detection of *icaA* and *icaD* gene in MSSA strains.

	Degree of biofilm formation				Total	□□	p
	Strong No (%)	Moderate No (%)	Weak No (%)	Non biofilm No (%)			
<i>icaA</i> gene only	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	-	-
<i>icaD</i> gene only	0 (0.0)	0 (0.0)	1 (16.7)	5 (83.3)	6 (24)	4.918	^{MC} p=0.131
<i>icaA</i> & <i>icaD</i> genes	2 (14.3)	3 (21.4)	9 (64.3)	0 (0.0)	14 (56.0)	21.526*	^{MC} p<0.001*
Negative for both <i>icaA</i> and <i>icaD</i>	0 (0.0)	0 (0.0)	0 (0.0)	5 (100)	5 (20)	7.508*	^{MC} p=0.024*
Total	2 (8)	3(12.0)	10(40.0)	10(40.0)	25(100)		

χ²: Chi square test

MC: Monte Carlo

p: p value for comparing between the studied categories

*: Statistically significant at p ≤ 0.05

Table 6. The association between *agr* function and detection of *icaA* and *icaD* genes.

	<i>agr</i> -Functioning No (%)	<i>agr</i> Non functioning No (%)	Total	χ ²	p
<i>icaA</i> gene only	0	0	0	7.086*	0.029*
<i>icaD</i> gene only	27(28.4%)	68(71.6%)	95(100%)		
<i>icaA</i> + <i>icaD</i>	9(21.4%)	33(78.6%)	42(100%)		
Negative for both <i>icaA</i> and <i>icaD</i>	8(61.5%)	5(38.5%)	13(100%)		
Total	44	106	150		

χ²: Chi square test

p: p value for comparing between the studied categories

*: Statistically significant at p ≤ 0.05

Table 7. The association between the degree of biofilm formation and *agr* alleles.

Biofilm degree	<i>agr</i> alleles				Total	□□	^{MC} p
	<i>agrI</i>	<i>agrII</i>	<i>agrIII</i>	<i>agrIV</i>			
Weak	31 ^{ab} (38.3%)	0 ^c (0%)	10 ^b (33.3%)	4 ^a (80.0%)	45	16.744*	0.001*
Moderate	21 ^a (25.9%)	4 ^a (22.2%)	9 ^a (30%)	0 ^a (0.0%)	34	1.675	0.676
Strong	10 ^a (12.3%)	12 ^b (66.7%)	3 ^a (10 %)	0 ^a (0%)	25	24.053*	<0.001*
non biofilm	19 ^a (23.5%)	2 ^a (11.1%)	8 ^a (26.7%)	1 ^a (20.0%)	30	1.696	0.658
Total	81	18	30	5			

χ²: Chi square test

MC: Monte Carlo

p: p value for comparing between the studied categories

*: Statistically significant at p ≤ 0.05

Means with Common letters are not significant (i.e., Means with Different letters are significant)

Discussion

The accessory gene regulator quorum sensing circuit controls *S. aureus* pathogenesis. It is a cell-to-cell communication system which

harmonizes bacterial behavior and organizes expression of virulence factors. Several reports suggested that the role of *agr* in human infection is complex, compounded by the isolation of *agr* dysfunctional strains from clinical samples [9-12].

In this study, (83.3%) of the *S. aureus* isolates were MRSA and (16.7%) were MSSA. Statistically significant difference ($p = 0.001$) was found between percentage of MRSA isolates in clinical samples (99 %) and nasal carrier samples (52 %). A high percentage of MRSA isolates in clinical samples was also reported by **El-Sherbini et al.** 69.2% (173/250) [13].

The results also revealed that percent of *agr* dysfunctionality was higher in clinical samples (84%) compared to nasal carrier (44%) isolates ($p < 0.001$), and also in MRSA (76%) compared to MSSA (44%) isolates ($p < 0.001$). The result of the present work agreed with the observation of **Yang et al.** [14] who reported that methicillin resistance might lead to *agr* dysfunction while its actual role whether it's a result or a cause of drug resistance remains unclear.

Statistically significant differences in biofilm formation between the clinical samples (88%) and nasal carrier (58%) isolates ($p < 0.001$) and also between MRSA (82%) and MSSA (60%) isolates ($p=0.05$) were detected. Similarly, a study conducted in Poland stated that MRSA strains had significantly higher ability to form biofilm than MSSA strains ($p = 0.0002$) [15].

icaA and *icaD* genes have been described to play a crucial role in biofilm production in *Staphylococcus* isolates. *icaA* alone has almost no enzymatic activity, but concurrent expression with *icaD* initiates the enzymatic activity and production of biofilm oligomers [16]. Our results agreed with this statement as all biofilm producers were either positive for *icaD* only; 95/150 (63.3%) or positive for both *icaA* + *icaD*; 42/150 (28%) while none of the isolates were positive for the *icaA* only.

Furthermore, **McCarthy et al.** [17] stated that the presence of *ica* genes are essential for biofilm formation by MSSA but not MRSA, this support our findings as, all (100%) MSSA negative for *icaA* and *icaD* genes were non-biofilm forming whereas 5 (62.5%) out of 8 MRSA negative for both genes were biofilm producers. Therefore, MRSA may not use the traditional route to form biofilm and may use polysaccharide intercellular adhesion (PIA)- independent pathways as reported by **Shivaee et al.** [18].

Regarding the inability of biofilm formation in some *Staphylococcal* strains, 25 isolates were non-biofilm forming despite of their

positivity to *icaD* gene. This can be elucidated by point mutations in the gene or a negative regulation either translational or post-translational, which affects the production of the proteins associated with the biofilm [16].

In the current study, a statistically significant association was found between *agr* dysfunction and biofilm formation ($p < 0.001$). as (92.5%) of *S. aureus* isolates with dysfunctional *agr*, were biofilm producers. Meanwhile, (43.2%) of *agr* functioning isolates were biofilm producers. There was also a statistically significant association between *icaD* gene and *agr* dysfunction ($p = 0.029$) as 71.6% of the *icaD* gene positive isolates and 78.6% of the *icaA* + *icaD* genes positive isolates were *agr* non-functioning. On the other hand, 61.5% (8/13) isolates negative for both *icaA* and *icaD* were *agr* functioning.

In contrast to these results, **Yang et al.** [14] reported no significant difference between *agr* dysfunctional and *agr* functional isolates regarding the biofilm formation ability ($p = 0.4972$); however, they found that 9/10 *agr* dysfunctional isolates could effectuate strong biofilm formation and multidrug resistance.

One of the purposes behind using bacterial *agr* typing is to characterize the *S. aureus* isolates and to find the relationship between *agr* alleles and the types of infection. Therefore, in this study, *agr* typing of the 150 clinical and nasal isolates was carried out. It was found that *agrI* was the most prevalent *agr* type 81(54%), followed by *agrIII* 30(20%), *agrII* 18(12%), and *agrIV* 5(3.3%) while 16(10.7%) isolates were non-typeable. Similarly, **Javdan et al.** [19], **Xu et al.** [20] and **Nasirian et al.** [21] reported *agrI* as the most prevalent *agr* type. Different results were reported by a study conducted in Iran [3]. They revealed that *agrIII* was detected in 55 isolates (44.7%) and *agr I* in 25 isolates (20.3%). Both *agr II* and *IV* were not detected, while 43 (35%) were non-typable.

Concerning the relation between *agr* typing and type of infection, the results of the present work revealed that the *agrI* was the prevalent among all types of clinical specimens (43.1% - 53.8%). However, the differences in the distribution of *agr* types among the different types of clinical specimens was not statistically significant ($p = 0.768$).; *agrI* was higher in blood samples (53.8%). *agrII* was relatively higher in wound swabs (16.7%), *agrIII* was higher in pus (31.4%), and

agrIV was found only among 5.9% of pus specimens. In addition, no statistically significant difference was found regarding the relation between the source of specimens whether from clinical cases or from nasal carriers and *agr* types ($p=0.053$). The present results are also consistent with previous reports [22,23]. More or less similar data was reported by **Peerayeh et al.** [24] who found that *agr* group I was prevalent in all clinical and healthy specimens, but it was higher in urine samples (70.8%) than others.

In the present work the relation of *agr* types with both biofilm formation and methicillin resistant *Staphylococci* was observed, there was a highly significant relation between *agrII* and strong biofilm formation ($p < 0.005$), and between *agrIV* and weak biofilm formation. In accordance with these results, **Cafiso et al.** [25] found that *agrII* (MRSA and MSSA) showed strong ability to produce biofilm, while *agrI* and *IV* showed a medium or weak biofilm. Moreover, the association of *agrIII* with MRSA was statistically significant as it was found among 24% of the 125 MRSA isolates compared to 0.0% in the MSSA isolates ($p=0.006$). **Hasani et al.** [26] as well observed a significant relationship between *agr* types and methicillin resistance as 94% of MRSA isolates belonged to *agrI* ($p < 0.05$). This difference in the results may be due to differences of geographical location and source of isolation and suggest that *agr* allele varies for each region and identifying predominant types are useful [27].

Conflict of interest :None.

Financial disclosure: None to declare.

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