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ORIGINAL ARTICLE

Molecular Study of Accessory-Gene-Regulator in *Staphylococcus aureus* Isolated from Sepsis in Pediatric Patients

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SUMMARY

Background: **Pediatric sepsis due to *Staphylococcus aureus* (*S. aureus*) is associated with high morbidity and mortality. Accessory-Gene-Regulator (agr) has a role in the pathogenesis of *S. aureus* through controlling and regulating the expression of virulence genes. Therefore, the aim of the present study was to investigate the prevalence of genotypes of the agr system in *S. aureus* isolated from children with sepsis and to assess their relationship to biofilm formation and antibiotic resistance.**

Methods: **The study was a retrograde cross-sectional study that included 131 children with health care associated sepsis due to *S. aureus*. The isolated *S. aureus* was investigated for their ability to form biofilm by microplate method, antibiotic susceptibility pattern by disc diffusion method, and molecular determination of agr genotypes by polymerase chain reaction (PCR).**

Results: **Methicillin resistant *S. aureus* (MRSA) was defined by resistance to cefoxitin antibiotic disc in 70 (53.4%) of the isolates and biofilm formation was positive in 67 (58%) of the isolates. Molecular study of the agr genes revealed that 54 (41.2%), 40 (30.5%), 27 (20.6%), and 10 (7.5%) of the studied isolates had agr I, agr II, agr III, and agr IV, respectively. In comparison between MRSA and methicillin sensitive *S. aureus* (MSSA), there was a significant increase in biofilm formation among MRSA (65.7%, p = 0.01) compared to MSSA (34.3%) and an increase in agr genotype I among MRSA (68.6%, p = 0.001) compared to agr I in MSSA (9.8%). There was a significant association with the presence of a central venous catheter (51.4%, p = 0.001) and urinary tract catheter (81.4%, p = 0.001) in children with MRSA compared to children with MSSA (21.3%, OR = 3.9, 95% CI = 1.8 - 8.5 and 36.1%, OR = 7.8, 95% CI 3.5 - 17.3, respectively).**

Conclusions: **There was an increase in the biofilm formation among *S. aureus* isolated from pediatric patients with sepsis with a significant increase in MRSA. The agr group I was the main agr gene among the isolated *S. aureus*. Moreover, agr I was the predominant gene in MRSA isolates and was significantly associated with biofilm formation.**

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KEYWORDS

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is associated with community and health care associated infections that range from skin infections up to invasive infections such as bacteremia and endocarditis. In children, there is a concern about the rising incidence of bacteremia in

hospitalized children due to *S. aureus* with increasing incidence of methicillin resistant *S. aureus* (MRSA) [1]. It is estimated that above 4% of all patients below 18 years that require hospitalization and around 8% of patients admitted to pediatric intensive care units in high-income countries have sepsis [2]. The mortality rate for children with sepsis ranges from 4% up to 50%. The mortality rates depend upon the severity of underlying diseases and the geographic location [3].

Virulence factors have a role in the pathogenesis of *S. aureus* [4]. The ability of *S. aureus* to form biofilm is one of these factors because it protects *S. aureus* from host immune reaction and antibiotic action [5].

The expression of virulence factors and antibiotic resistance is regulated by the Accessory-Gene-Regulator *(agr*) mediated quorum sensing (QS) system. The QS systems consist of two components, the synthase proteins which produce QS signals and the response regulators that bind QS signals and reprogram gene expression [6]. The function of the quorum sensing is to monitor the density of the bacterial cells through chemical signals that lead to the communication between bacterial cells and lead to regulation of the gene expression involved in virulence, competition, biofilm formation, pathogenicity, and antibiotic resistance [7]. Quorum-sensing gene regulation is population density-dependent and environment-dependent and presents through cell-to-cell communication [8]. The QS utilizes *luxS* in the context of bioluminescence regulation. The LuxS system uses the furanosyl borate diester molecule, an auto-inducer called AI-2, to perform its functions. The AI-2 molecule is involved in many phenotypes of bacteria like synthesis of the bacterial capsule, formation of the biofilm, and antibiotics sensitivity [8].

The QS has two components, one is the agr locus and the other is the LuxS system . The agr t acts through a secreted Auto-Inducing-Peptide (AIP) which regulates the cell density. The agr locus consists of two divergent transcriptional units, RNAII and RNAIII, which are driven by the P2 and P3 promoters [9]. There are four proteins agrA, agrB, agrC, and agrD encoded by P2 operon and P3 promoter encodes the effector molecule (RNAIII) [10].

S. aureus isolates can be divided into four agr groups on the basis of the agrC gene which encodes the receptor of the AIP and agrD gene, which are responsible for encoding cyclic AIP [11].

There is a link between the agr system in *S. aureus* and antibiotic resistance and virulence factors [12]. This complex relationship needs full investigation for the establishment of effective control measures for infections with *S. aureus* in health care settings.

Therefore, the aim of the present study was to determine the frequency of agr system genotypes in *S. aureus* obtained from children with sepsis and to investigate their association with biofilm formation and antibiotic resistance.

MATERIALS AND METHODS

The study was a retrograde cross-sectional study that included children with health care associated sepsis. The children were recruited from intensive care units from Mansoura University Children Hospital from January 2019 until March 2021. The included children were below 18 years old and had signs of infection that started more than three days after admission with manifestations of fever or hypothermia, increase in respiratory and heart rates, and blood culture positive for *S. aureus*. The study was approved by the Mansoura ethical committee, and informed consent was obtained from the parent of each child.

The included children were subjected to full clinical history and clinical examination.

Microbiological laboratory investigation

Blood samples for microbiological culture were obtained under complete antiseptic procedure. Eight milliliters blood from each child was inoculated into two Pedi-Bact Alert aerobic bottles, and the bottles were incubated at the BacT/Alert system (bioMerieux, Inc., Durham, NC, USA). Positive blood culture bottles were further processed by subculture on blood agar media. *S. aureus* was identified according to standard microbiological techniques including Gram stain, catalase test, coagulase test, and specific growth on mannitol salt agar. Positive control strain *S. aureus* ATCC 25923 was used. Methicillin resistant *S. aureus* was identified as resistant to cefoxitin disc (30 µg) by disc diffusion method according to clinical standard laboratory guidelines [13].

Biofilm detection by microtiter plate method

The ability of the isolates to form biofilm was measured by microplate method. Briefly, isolates were inoculated on Muller-Hinton agar and incubated for 24 hours at 37°C. Suspension from the bacterial growth was performed by the use of Muller-Hinton broth and adjusted to 0.5 McFarland. Two hundred microliters of the prepared suspension were added to the wells of the microtiter plate (each isolate was done in triplicate). A negative control well contained only 200 µl of Muller-Hinton broth without isolate. *S. aureus* ATCC 35556 was used as positive control strain. After incubation at 37°C for 24 hours, the wells were washed with normal saline two times and stained with 1% crystal violet for 10 minutes. Subsequently, 250 µL of 95% ethanol solution were added to each well. The optical density (OD) was measured at 570 nm with a microplate reader (Stat Fax 2100). The calculation of OD was performed by subtracting the OD of negative control from the OD of each strain. The OD was considered to be high positive at ≥ 0.300, intermediate between 0.200 and 0.199, and negative at ≤ 0.100 [14].

Antibiotics sensitivity by discs diffusion method

Antibiotic sensitivity for *S. aureus* was performed by disc diffusion method as described by Clinical and Lab-

oratory Standards Institute [13]. The antibiotics discs used were amikacin (30 µg), amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), ciprofloxacin (10 µg), cefotaxime (30 µg), ceftazidime (10 µg), cefoxitin (10 µg), trimethoprim/sulfamethaxone (25 µg), erythromycin (15 µg), vancomycin (30 µg) (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA).

Molecular study of agr system by PCR
DNA Extraction
Pure overnight culture of *S. aureus* was used to prepare 1 mL suspension with phosphate buffer solution. This suspension was used to extract DNA using the commercial QIAamp blood and tissue kit (QIAGEN, Germany) according to the manufacturer's recommendations except for incubation with lysostaphin for 10 minutes instead of 30 minutes [15]. The extracted DNA was kept at -80℃ until the time of the amplification.

PCR for Agr system typing
Five microliters of the extracted DNA were used for the amplification. Each primer (1 µM) was mixed with the ready to use amplification mixture of Qiagen. The primers used were listed in Table 1. The amplification procedures were heating at 94℃ for 6 minutes, followed by denaturation at 95℃ for 45 seconds, primer annealing at 56℃ for 1 minute, and primer elongation at 72℃ for 70 seconds for 32 cycles and then a final extension at 72℃ for 8 minutes. Then electrophoresis was performed in a 1.5% agarose gel stained with ethidium bromide for 30 minutes [15].

Statistical analysis
The data of the study was analyzed by IBM - SPSS22. The age was expressed as median, minimum, and maximum. Qualitative data was expressed as number and percentage. Comparison of qualitative data was performed by chi-squared test and p-value was considered significant if it was less than 0.05.

RESULTS

The study included 131 children diagnosed with sepsis. There were 70 (53.4%) males and 61 (46.4%) females. The median age was 4 months.
Sepsis was not associated with obvious source of infections in 67 (51.1%) of the studied children. However, it was associated with pneumonia in 58 (44.3%), with surgical site infections in 3 (2.3%), and with urinary tract infections in 3 (3.2%) of the investigated children. The most frequent manifestations of sepsis were fever (30.5%), oliguria (29%), and hypothermia (28.2%) (Table 2).
Methicillin resistant *S. aureus* was identified in 70 (53.4%**)** of the studied isolates. Sixty-seven (58%) of the investigated *S. aureus* isolates had the ability to form biofilm. PCR analysis of the agr system classified the *S. aureus* isolates into 54 (41.2%) isolates with agr group I, 40 (30.5%) isolates with agr group II, 27 (20.6%) isolates with agr group III, and 10 (7.5%) isolates with agr group IV, data not shown.
There was a significant increase in biofilm formation among MRSA compared to MSSA (65.7% vs. 34.4%, p = 0.01). Additionally, there was a significant increase of agr genotype I among MRSA when compared to MSSA (68.6% vs. 9.8%, p = 0.001) (Table 3).
Comparing the clinical data of children affected by MRSA with those affected by MSSA isolates showed a significant association between the presence of central venous catheter (51.4%, p = 0.001) and urinary tract catheter (81.4%, p = 0.001) in children with MRSA compared to children with MSSA isolates (21.3%, OR = 3.9, 95% CI = 1.8 - 8.5 and 36.1%, OR = 7.8, 95% CI 3.5 - 17.3, respectively). There was a significant increase in children with pneumonia associated with sepsis caused by MRSA isolates (55.7%) compared to children with MSSA isolates (31.1%, p = 0.01) (Table 4). Resistance to amoxicillin/clavulanic acid (77.1%, p = 0.001), ampicillin (65.7%, p = 0.001), cefotaxime (61.4%, p = 0.001), and ceftazidime (50%, p = 0.046) were significantly increased in MRSA compared to non-MRSA isolates (Table 5).
Agr group I was increased significantly among biofilm forming *S. aureus* compared to non-biofilm forming *S. aureus* (52.2% vs. 29.7%, p = 0.026) (Table 6).

DISCUSSION

Staphylococcus aureus is a pathogenic bacterium with high mortality rates in patients with sepsis. The pathogenic role of *S. aureus* is due to many virulence factors as well as its antibiotic resistance. A new therapeutic approach for *S. aureus* is the use of anti-virulence therapy that depends upon understanding the exact virulence factors [15].
In pediatric patients, methicillin resistant *S. aureus* (MRSA) is a major health problem with high mortality [16]. In the present study, the prevalence of MRSA, as identified by resistance to cefoxitin antibiotic disc, was 53.4%. A similar rate of MRSA was reported in a previous study among isolated *S. aureus* from patients with sepsis [17].
Biofilm formation of *S. aureus* is associated with an increase in resistance to antibiotics and decreases the effects of the host immune system to eradicate the organism. The production of biofilm by *S. aureus* is related to the synthesis of polysaccharide intracellular adhesin [18]. In the present study, biofilm formation among *S. aureus* isolates was 58% with a significant increase in biofilm formation among MRSA compared to MSSA. This rate of biofilm formation is similar to a previous report by Neopane et al. [19]. The rates of biofilm formation ranged from < 50% up to 100% in previous studies [20,21]. The difference in the rates of biofilm formation depends upon several factors such as the source of the isolates, environment of the study, avail-

Table 1. The genes and related primers used in this study.

Gene	Primers	Product size
agr I	5-ATG CAC ATG GTG CAC ATG C-3 5-GTC ACA AGT ACT ATA AGC TGC GAT-3	441
agr II	5-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3	575
agr III	5-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3	323
agr IV	5-CGA TAA TGC CG T AAT ACC CG-3	659

Table 2. Demographic and clinical data of the studied children.

Parameter	
Age, months	
Median	4.00
Minimum	1.00
Maximum	192.00
Gender	
Male, No. (%)	70 (53.4%)
Female, No. (%)	61 (46.4%)
Central venous catheter, No. (%)	43 (32.8%)
Urinary tract catheter, No. (%)	74 (56.6%)
Clinical diagnosis	
Primary sepsis, No. (%)	67 (51.1%)
Pneumonia, No. (%)	58 (44.3%)
Surgical site infections, No. (%)	3 (2.3%)
Urinary tract infections, No. (%)	3 (2.3%)
Fever, No. (%)	40 (30.5%)
Hypothermia, No. (%)	37 (28.2%)
Apnea, No. (%)	32 (24.4%)
Hypotension, No. (%)	33 (25.2%)
Oliguria, No. (%)	38 (29.0%)

Table 3. Comparison between MRSA isolates versus non-MRSA isolates with regard to biofilm formation and Agr types.

	MRSA isolates (n = 70)		Non-MRSA isolates (n = 61)		p-value
	No.	%	No.	%	
Biofilm formation	46	65.7	21	34.4	0.01
agr I	48	68.6	6	9.8	0.001
agr II	15	21.4	25	40.98	
agr III	6	8.6	21	34.4	
agr IV	1	1.4	9	14.8	

MRSA - Methicillin resistant *S. aureus*, agr - Accessory-Gene-Regulator.

Table 4. Comparison of demographic and clinical data between children affected by sepsis with MRSA isolates and children affected by sepsis with non-MRSA isolates.

	Children with sepsis by						
	MRSA (n = 70)		Non-MRSA (n = 61)		p-value	OR	95% CI
	No.	%	No.	%			
Gender							
Male	40	57.1	30	49.2	0.4	1.4	0.7 - 2.7
Female	30	42.9	31	50.8			
Central venous Catheter	36	51.4	13	21.3	0.001 *	3.9	1.8 - 8.5
Urinary tract Catheter	57	81.4	22	36.1	0.001 *	7.8	3.5 - 17.3
Sepsis associated Infections							
Primary sepsis	29	41.4	38	62.3			
Pneumonia	39	55.7	19	31.1			
Surgical site infections	2	2.9	1	1.6	0.01 *		
Urinary tract infections	0	0	3	4.9			
Fever	26	37.1	14	22.95	0.08	1.9	0.9 - 4.3
Hypotension	15	21.4	22	36.1	0.06	0.5	0.2 - 1.04
Apnea	18	25.7	14	22.95	0.7	1.2	0.52 - 2.59
Hypothermia	29	41.4	17	27.9	0.1	1.8	0.88 - 3.82
Oliguria	21	30	17	27.9	0.8	1.11	0.52 - 2.4

MRSA - Methicillin resistant *S. aureus*.

Table 5. Antibiotic resistance and biofilm formation in MRSA isolates compared to non-MRSA isolates.

Antibiotics	MRSA isolates (n = 70)		Non-MRSA isolates (n = 61)		p-value
	No.	%	No.	%	
Amoxicillin/clavulanic acid	54	77.1	11	18.02	0.001 *
Ampicillin	46	65.7	10	14.3	0.001 *
Amikacin	20	28.6	26	42.6	0.09
Ciprofloxacin	19	27.1	25	40.9	0.09
Cefotaxime	43	61.4	14	22.9	0.001 *
Ceftazidime	35	50	20	32.8	0.046 *
Trimethoprim/sulfamethoxazole	35	50	28	45.9	0.6
Eythromycin	56	80	48	78.7	0.8
Vancomycin	5	7.1	4	6.6	0.9
Biofilm formation	46	65.7	21	34.3	0.001 *

MRSA - Methicillin resistant *S. aureus*.
*** - p is significance.**

ability of the nutrients, and the method applied for the biofilm study [22].

In the current study, there was a significant increase in biofilm formation among MRSA compared to MSSA. This finding is contrary to a previous study by Ghasemian et al. [23] but is in line with a previous study by Mahmoudi et al. [24]. This discrepancy suggests a difference in the genetic pathway of biofilm formation between MRSA and MSSA. This is supported by Pozzi et al. [25] who reported that biofilm formation in MSSA

Table 6. Distribution of Agr system among biofilm forming versus non-biofilm forming *S. aureus* isolates.

agr group	Biofilm formation				p-value
	Positive		Negative		
	No.	%	No.	%	
agr I	35	52.2	19	29.7	0.026 *
agr II	20	29.9	20	31.3	
agr III	9	13.4	18	28.1	
agr IV	3	4.5	7	10.9	
Total	67	100	64	100	

agr - Accessory-Gene-Regulator.
*** - p is significance.**

mainly occurs via PIA synthesis while in MRSA it is more related to the *fnbB* adhesion. Biofilm production in MSSA depends upon on polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine (PIA/PNAG) with its expression on hydrophilic surfaces and an Atl/PNAG-dependent biofilm on hydrophobic surfaces, while methicillin-resistant isolates express an Atl/FnBP-mediated biofilm phenotype. The autolytic activity is Atl-dependent, and extracellular DNA releases are responsible for the early stages of biofilm formation in the MRSA, while FnBPs encourage the intercellular accumulation and biofilm formation [25].

The presence of urinary catheter and central venous catheter were significantly more prevalent among children with MRSA than those with MSSA. This finding was in line with previous bacteremia studies [26,27]. Among different virulence determinants in *S. aureus* is the quorum-sensing system agr that controls toxins that lyse leukocytes and control other immune evasion factors [28]. For that reason and in the light of frequent antibiotic resistance in *S. aureus*, recently, quorum-quenching approaches that target agr functionality are often proposed as potential alternatives to antibiotic-based treatment of *S. aureus* infection [29]. Thus, as is the case for most bacteria causing bloodstream infections, the role of virulence and quorum-sensing in *S. aureus* sepsis remains incompletely understood [30]. In the current study, the frequency of agr group I was 41.2% which is lower than a previous study from China that reported a frequency of 84.4% for agr I in Chinese children [31,32]. There is a known difference in the clonal types of *S. aureus* isolates in different regions that might provide an explanation. In the present study, agr I was the predominant type in MRSA. This finding is similar to previous studies [33,34]. By contrast, a previous study from France determined equal distribution of the four genotypes of agr among clinical isolates [35]. The difference in the origin of the clinical isolates and the geographical distribution of different genotypes of *S. aureus* may explain the difference in the results [36].

There is evidence about the role of quorum sensing in biofilm formation by *S. aureus* and in severity of infections [15]. However, there is a lack of literature about the role of agr mediated quorum-sensing associated with biofilm. In the present study, there was a significant association of agr I with biofilm formation in *S. aureus*, a finding that needs further investigation and *in vitro* studies in animal models to verify the therapeutic value of quorum-sensing blockers for systemic *S. aureus* and MRSA infection [15].

There was a significant increase in resistance to amoxicillin/clavulanic acid, ampicillin, cefotaxime, and ceftazidime in MRSA compared to MSSA isolates. MRSA has resistance to penicillin like antibiotics and to other classes of antibiotics. Vancomycin has been used in treatment of MRSA species but there is a noticeable emergence of reduced sensitivity to vancomycin [37] as well as to daptomycin [38] and linezolid resistance [39].

CONCLUSION

The present study highlighted the prevalence of MRSA among clinical isolates from pediatric patients with sepsis and showed the relationship between agr genotypes and each of the biofilm formation and antibiotic resistance virulence factors of sepsis with *S. aureus* in pediatrics. The majority of *S. aureus* isolates, particularly MRSA, were biofilm forming. The agr I was the predominant genotype among the studied isolates of *S. aureus* and it was significantly associated with MRSA and biofilm formation.

Declaration of Interest:
There are no any conflicts of interests for any of the authors.

References:

1. Anubha J, Shampa A, Vineeta G. A rising and alarming threat of *Staphylococcus aureus* Bacteremia in under 5 children. Europ Resp J. 2019;54(63):992-8. https://erj.ersjournals.com/content/54/suppl_63/PA992

2. Weiss SL, Fitzgerald JC, Pappachan J, et al. Global epidemiology of pediatric severe sepsis: the sepsis prevalence, outcomes, and therapies study. Am J Respir Crit Care Med 2015 May 15; 191(10):1147-57. (PMID: 25734408)

3. Ruth A, McCracken CE, Fortenberry JD, Hall M, Simon HK, Hebbar KB. Pediatric severe sepsis: current trends and outcomes from the Pediatric Health Information Systems database. Pediatr Crit Care Med 2014 Nov;15(9):828-38. (PMID: 25226500)

4. Yang X, Dong F, Qian S, et al. Accessory gene regulator (agr) dysfunction was unusual in *Staphylococcus aureus* isolated from Chinese children. BMC Microbiol 2019 May 14;19(1):95. (PMID: 31088356)

5. Chung PY, Toh YS. Anti-biofilm agents: recent breakthrough against multi-drug resistant *Staphylococcus aureus*. Pathog Dis 2014 Apr;70(3):231-9. (PMID: 24453168)

6. Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. Annu Rev Genet 2009;43:197-222. (PMID: 19686078)

7. Paul D, Gopal J, Kumar M, Manikandan M. Nature to the natural rescue: Silencing microbial chats. Chem Biol Interact. 2018 Jan 25;280:86-98. (PMID: 29247642)

8. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 2005;21:319-46. (PMID: 16212498)

9. Novick RP, Projan SJ, Kornblum J, et al. The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Gen Genet 1995 Aug 30;248(4):446-58. (PMID: 7565609)

10. Bibalan MH, Shakeri F, Javid N, Ghaemi A, Ghaemi EA. Accessory Gene Regulator Types of *Staphylococcus aureus* Isolated in Gorgan, North of Iran. J Clin Diagn Res 2014 Apr;8(4):DC07-9. (PMID: 24959440)

11. Shopsin B, Mathema B, Alcabes P, et al. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J Clin Microbiol 2003 Jan;41(1): 456-9. (PMID: 12517893)

12. Derakhshan S, Navidinia M, Haghi F. Antibiotic susceptibility of human-associated *Staphylococcus aureus* and its relation to agr typing, virulence genes, and biofilm formation. BMC Infect Dis 2021 Jul 1;21(1):627. (PMID: 34210263)

13. Clinical and Laboratory Standards Institute (CLSI). 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI, Wayne, Philadelphia, PA. https://clsi.org/media/1928/m07ed11_sample.pdf

14. Jaffe RI, Lane JD, Albury SV, Niemeyer DM. Rapid extraction from and direct identification in clinical samples of methicillin-resistant staphylococci using the PCR. J Clin Microbiol 2000 Sep;38(9):3407-12. (PMID: 10970392)

15. Cheung GYC, Bae JS, Liu R, Hunt RL, Zheng Y, Otto M. Bacterial virulence plays a crucial role in MRSA sepsis. PLoS Pathog 2021 Feb 25;17(2):e1009369. (PMID: 33630954)

16. Turner NA, Sharma-Kuinkel BK, Maskarinec SA, et al. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. Nat Rev Microbiol 2019 Apr;17(4):203-18. (PMID: 30737488)

17. Foster C, Bagdure D, Custer J, et al. Outcomes of Pediatric Patients With Sepsis Related to *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* Infections Requiring Extracorporeal Life Support: An ELSO Database Study. Front Pediatr 2021 Oct 8;9:706638. (PMID: 34692605)

18. Piechota M, Kot B, Frankowska-Maciejewska A, Grużewska A, Woźniak-Kosek A. Biofilm Formation by Methicillin-Resistant and Methicillin-Sensitive *Staphylococcus aureus* Strains from Hospitalized Patients in Poland. Biomed Res Int 2018 Dec 27; 2018:4657396. (PMID: 30687745)

19. Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y. *In vitro* biofilm formation by *Staphylococcus aureus* isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. Int J Gen Med 2018 Jan 18;11:25-32. (PMID: 29403304)

20. Cha JO, Yoo JI, Yoo JS, et al. Investigation of Biofilm Formation and its Association with the Molecular and Clinical Characteristics of Methicillin-resistant *Staphylococcus aureus*. Osong Public Health Res Perspect 2013 Oct;4(5):225-32. (PMID: 24298437)

21. Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y. *In vitro* biofilm formation by *Staphylococcus aureus* isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. Int J Gen Med 2018 Jan 18;11:25-32. (PMID: 29403304)

22. Kokare CR, Chakraborty S, Khopade AN, Mahadik KR. Biofilm: importance and applications. Indian J Biotechnol 2009;8:159-68. https://www.semanticscholar.org/paper/Biofilm%3A-Importance-and-applications-Kokare-Chakraborty/6563f644541bee822a62c80db8f7c449dba1b462

23. Ghasemian A, Najar Peerayeh S, Bakhshi B, Mirzaee M. Comparison of Biofilm Formation between Methicillin-Resistant and Methicillin-Susceptible Isolates of *Staphylococcus aureus*. Iran Biomed J 2016 Jul;20(3):175-81. (PMID: 26948126)

24. Mahmoudi H, Pourhajibagher M, Chiniforush N, Soltanian AR, Alikhani MY, Bahador A. Biofilm formation and antibiotic resistance in meticillin-resistant and meticillin-sensitive *Staphylococcus aureus* isolated from burns. J Wound Care 2019 Feb 2;28(2): 66-73. (PMID: 30767648)

25. Pozzi C, Waters EM, Rudkin JK, et al. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. PLoS Pathog 2012; 8(4):e1002626. (PMID: 22496652)

26. Ishikawa K, Soejima S, Masuda F, Saitoh S. Implementation of dCas9-mediated CRISPRi in the fission yeast Schizosaccharomyces pombe. G3 (Bethesda) 2021 Apr 15;11(4):jkab051. (PMID: 33617628)

27. Arias-Ortiz PM, Calderón LD, Castillo JS, et al. [Risk factors for methicillin-resistant *Staphylococcus aureus* bacteremia: A multicenter matched case-control study]. Biomedica 2016 Dec 1;36(4): 612-9. (PMID: 27992988)

28. Le KY, Otto M. Quorum-sensing regulation in staphylococci-an overview. Front Microbiol 2015 Oct 27;6:1174. (PMID: 26579084)

29. Dickey SW, Cheung GYC, Otto M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. Nat Rev Drug Discov 2017 Jul;16(7):457-71. (PMID: 28337021)

30. Painter KL, Krishna A, Wigneshweraraj S, Edwards AM. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? Trends Microbiol 2014 Dec;22(12):676-85. (PMID: 25300477)

31. Wu D, Li X, Yang Y, et al. Superantigen gene profiles and presence of exfoliative toxin genes in community-acquired meticillin-resistant *Staphylococcus aureus* isolated from Chinese children. J Med Microbiol 2011 Jan;60(Pt 1):35-45. (PMID: 20829395)

32. Gu FF, Chen Y, Dong DP, et al. Molecular Epidemiology of *Staphylococcus aureus* among Patients with Skin and Soft Tissue Infections in Two Chinese Hospitals. Chin Med J (Engl) 2016 Oct 5;129(19):2319-24. (PMID: 27647191)

33. Xu Y, Qian SY, Yao KH, et al. Clinical and molecular characteristics of *Staphylococcus aureus* isolated from Chinese children: association among the agr groups and genotypes, virulence genes and disease types. World J Pediatr 2021 Apr;17(2):180-8. (PMID: 33660136)

34. van Leeuwen W, van Nieuwenhuizen W, Gijzen C, Verbrugh H, van Belkum A. Population studies of methicillin-resistant and -sensitive *Staphylococcus aureus* strains reveal a lack of variability in the agrD gene, encoding a staphylococcal autoinducer peptide. J Bacteriol 2000 Oct;182(20):5721-9. (PMID: 11004170)

35. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 2002 Feb; 70(2):631-41. (PMID: 11796592)

36. Tahmasebi H, Dehbashi S, Arabestani MR. Association between the accessory gene regulator (agr) locus and the presence of superantigen genes in clinical isolates of methicillin-resistant *Staphylococcus aureus*. BMC Res Notes 2019 Mar 12;12(1):130. (PMID: 30871616)

37. Ghahremani M, Jazani NH, Sharifi Y. Emergence of vancomycin-intermediate and -resistant *Staphylococcus aureus* among methicillin-resistant *S. aureus* isolated from clinical specimens in the northwest of Iran. J Glob Antimicrob Resist 2018 Sep;14:4-9. (PMID: 29454049)

38. Roch M, Gagetti P, Davis J, et al. Daptomycin Resistance in Clinical MRSA Strains Is Associated with a High Biological Fitness Cost. Front Microbiol 2017 Dec 5;8:2303. (PMID: 29259579)

39. de Dios Caballero J, Pastor MD, Vindel A, et al. Emergence of cfr-Mediated Linezolid Resistance in a Methicillin-Resistant *Staphylococcus aureus* Epidemic Clone Isolated from Patients with Cystic Fibrosis. Antimicrob Agents Chemother 2015 Dec 14;60(3):1878-82. (PMID: 26666940)