ORIGINAL ARTICLE

A Study of Accessory Gene Regulator, Biofilm Formation, and Antibiotics Resistance of *Staphylococcus aureus* Isolated from Pediatric Patients with Healthcare-Associated Sepsis

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ABSTRACT

Background: *Among pediatric patients, Staphylococcus aureus (S. aureus) is a significant bacterium that causes healthcare-associated sepsis. Antibiotic resistance and biofilm development, a virulence factor in S. aureus, pose significant challenges to infection control. The accessory gene regulator (agr) is a regulatory gene believed to control multiple factors of virulence in Staphylococcus aureus.* ***Objective:*** *To investigate the ability of isolated S. aureus from pediatric patients with sepsis to produce biofilms and explore different types of agr systems and their interrelationships.* ***Methodology:*** *In this work, 115staph. aureus strains isolated from positive blood cultures from pediatric patients diagnosed with sepsis were tested for biofilm production, resistance to antibiotics and different types of agr genes by PCR.* ***Results:*** *Out of 115 of S. aureus strains, 91.3% of the isolates were capable of forming biofilm. We observed a significant biofilm formation capacity in 52 strains (49.5%), a moderate capacity in 31 strains (29.5%), and a weak capacity in (20.9%). PCR revealed that the agrI and agrIII genes were the most prevalent, with prevalence rates of 41.7% and 25.2 respectively, while the agrII gene was the least common at 9.6%, respectively. Also we reported a strong link between the placement of peripheral catheters and genotype Agr II. Also we reported a strong link between genotype agr II and the growth of robust biofilm.* ***Conclusion:*** *S. aureus forms a strong biofilm in pediatric patients with healthcare-associated sepsis which indicates virulence and difficulty in its eradication by antibiotics therapy. Agr II showed a positive association with the formation of strong biofilms.*

INTRODUCTION

Bloodstream infections leading to sepsis in healthcare settings are a worldwide problem resulting in a rise in morbidity, an increase in mortality, and an increase in hospital stays. *Staphylococcus aureus* (*S. aureus*) represents common pathogenic bacteria linked to such conditions, especially methicillin-resistant species (MRSA). MRSA-associated sepsis is increasing in the world in children up to 30% [1-3].

Risk factors related to *S. aureus* bloodstream infections in pediatric patients include prematurity, low birth weight, underlying conditions such as congenital heart diseases and pneumonia, and medical devices such as central venous catheters (CVC) and urinary catheters[4,5]. Early diagnosis and treatment of sepsis improves the prognosis [6-9].

The threat of bloodstream infection with *S. aureus* arises mainly from antibiotic resistance from mobile genetic elements and virulence factors [10]. The virulence factors of *S. aureus* include surface-related factors, hemolysin toxins, enzymes, and superantigenic toxins. The accessory gene regulator (*agr*) locus encodes two regulatory components, signal pathways that influence the expression of virulence proteins and induce antibiotic resistance. We categorize *agr* systems into four types, specifically I through IV [11]. This system activation causes *S. aureus* to emerge as a pathogenic organism capable of invasion.[12] The persistence of *S. aureus* being negative to the *agr* system may be attributed to additional mechanisms, including biofilm formation and the evasion of the immune system by bacteria lacking important antigenic determinants. [13,14].

S. aureus resistant to methicillin reflects this pathogen's resistance to beta-lactam antibiotics and denotes the limitation of options for antibiotic therapy. The *mecA* gene is linked to the emergence of methicillin-resistant *S. aureus*. The *mecA* gene encodes

Penicillin-binding protein 2a (PBP2a), which results in decreased binding affinity for beta-lactam antibiotics, including penicillinase-resistant penicillin. A mobile genetic element contains the *mecA* gene, also referred to as a staphylococcal cassette chromosome mec (*SCCmec*). The *mecA* gene complex's insertion sites for plasmids and transposons contribute to the emergence of antibiotic resistance [14].

Horizontal gene transfer facilitates the transfer of virulence and antibiotic resistance genetic elements to mobile genetic elements, making their transmission easier. Horizontal gene transfer is the main process by which virulence and antibiotic resistance arise simultaneously in bacteria during evolution and adaptation [15]. Furthermore, the process of biofilm development facilitates gene transmission and exchange [16]. The biofilm improves *S. aureus'* pathogenicity by impeding the immune system's bacterial elimination and resistance to antibiotic treatment.[14]

Monitoring virulence genetic components and antibiotic resistance patterns is crucial for effectively treating serious infections caused by *S. aureus*, such as bloodstream infections [15]. Limited literature exists on the correlation between biofilm development and *agr* genetic types in *S. aureus*, particularly in pediatric patients. Hence, the aims were to investigate the ability of biofilm development and the specific types of *agr* systems in isolated *S. aureus* from pediatric patients with sepsis, as well as their interrelationships.

METHODOLOGY

This retrospective cross-sectional study included 115 isolates of *S. aureus* taken from kids at Mansoura University Children's Hospital in Egypt who had bloodstream infections caused by being in the hospital between January 2022 and January 2024. The included children were diagnosed with bloodstream infections with clinical and laboratory findings according to the Centers for Disease Control (CDC) [18]. The culture of blood was positive for *S. aureus* bacterium. The culture of the blood samples was obtained from children admitted to the hospital with nosocomial sepsis from Mansoura University Children's Hospital, two hundred and fifty-bed hospital for tertiary care. The infection control program implemented the hospital-associated infection surveillance system to acquire the clinical data of pediatric patients.

The study specifically excluded children who had ac quired infections in the community or other healthcare settings. The Mansoura Faculty of Medicine's Ethical Council (R.24.02.2512) cleared the study, and each child's parents signed a consent.

Microbiological Methods

Five milliliters of blood samples were obtained from each child with clinical signs of bloodstream infections .Blood samples were collected under complete aseptic conditions and cultured into a bottle of an automated blood culture system, BacT/ALERT (Biomerieux, USA). We processed the culture of the blood showing growth signals to identify the organisms using conventional microbiological methods. The microbiological techniques included subculture of positive blood culture over blood agar plates *(*Oxoid Limited-Wade Road Basingstoke, Hampshire, RG24 8PW-United Kingdom), followed by identification by Gram-stained slides, colony morphology, coagulase, and catalase tests, sensitivity to furazolidone, the fermentation of mannitol, and the DNase test[17]. We used PCR to molecularly identify *S.* aureus by detecting its thermostable nuclease gene (nuc)[19].

Antibiotics Sensitivity Test by Disc Diffusion Method

The procedures from the Clinical and Laboratory St andards Institute (CLSI)
for detecting antibiotic sensitivity were used for antibiotics sensitivity. Cefoxitin, Ciprofloxacin, Clindamycin, Erythromycin, Gentamicin, Amikacin, Oxacillin, Rifampin, Tetracycline, Sulfamethoxazole, and Trimethoprim were the antibiotics used (Oxoid, Basingstoke, UK). The identification of MRSA relies on the observation of its resistance to Cefoxitin disc (30 μg)[20].

Biofilm formation

We performed a pure culture of *S. aureus* in tryptic soy broth, adjusted the suspension to a turbidity of 0.5 McFarland in 100 microliters, added this dilution to the wells of the flat bottom of the plate with 96 microtiter wells, and incubated for 24 hours at 37°C. After that, we washed the wells three times with sterile distilled water, stained them with crystal violet at room temperature for ten minutes, washed them again three times, allowed them to dry for twenty minutes, and then dissolved them with acetic acid at a concentration of 30%. The blank well with broth was the only blank well. *S. aureus* ATCC25923 was used as a positive control, and *Staphylococcus epidermidis* ATCC12228 was utilized as a negative control. The calculation of the optical density (OD) was considered negative if the OD was less than the OD of the negative control. Positive OD was reported as weak if OD <0.2, moderate if OD>0.2<0.4, and strong for biofilm producers if OD >0.4 [21].

Polymerase Chain Reaction

DNA extraction

We used the boiling and freezing method to extract DNA from bacterial growth. We performed the suspension by first putting the isolated *S. aureus* in sterile deionized water, boiling the suspension for ten minutes, and then directly placing it on ice for five minutes. We repeated the freezing-thawing cycles three times. After that, centrifugation was performed, and the supernatants were obtained then preserved at −20°C as DNA templates for PCR.

PCR for Identification of *S. aureus*

PCR for Identification of the thermostable nuclease gene (*nuc*) of *S. aureus* was listed in Table 1[19]. The amplification mixture was supplied from Thermofisher (Life Technologies Ltd3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF, UK). The used extracted DNA in the volume of 3 microliters was added to the mixture with o.4 µM of the used primer. Polymerase chain reaction program Incorporated denaturation at 94°C for 5 minutes; then thirty-five cycles including heating for 30 seconds at 94°C, annealing for 30 seconds at 61°C, and extension for 30 seconds at 72°C. Then, a final step of the extension for five minutes at 72°C was performed. ATCC 25923 *S. aureus* was used as the control strain. ATCC 25923 *S. aureus* was employed as the quality control strain**.**

PCR for Determination of the type of *Agr* Genes

The *agr* types I, II, III, and IV were typed by the polymerase chain reaction with the listed primers in Table 1 [22]. The thermofisher mixture (Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK) supplied the PCR mixture in a total volume of 25 microliters, adding 0.4 M of each primer and 3 microliters of extracted DNA. The thermocycler (Eppendorf, Germany) carried out the amplification steps through the following cycles: first, denaturation for 5 minutes at a temperature 94°C; then, thirty-five cycles of denaturation for one minute at a temperature 94°C; annealing for one minute at a temperature 55°C; and heating for one minute at a temperature 72°C for extension. The final step was to heat at 72°C for 5 minutes. Then electrophoresis was performed for the products of the amplification over 1.5% agarose gels for twenty minutes and visualized under ultraviolet light.

Table 1: Genes, the sequences of the primers used, and the product base pair (bp) size

Genes	Sequences of the primers	Bp
nuc1 *nuc2*	5′-GCGATTGATGGTGATACGGTT − 3′ 5′AGCCAAGCCTTGACGAACTAAAGC − 3′	279 bp
agrD gene-gene I	5′-ATGCACATGGTGCACATGC-3′ 5′-GTCACAAGTACTATAAGCTGCGAT-3′	440 bp
agrC gene- agr II	5′-ATGCACATGGTGCACATGC-3′ 5′GTATTACTAATTGAAAAGTGCCATAGC-3′	572 bp
agrD gene-agrIII	5′-ATGCACATGGTGCACATGC-3′ 5′-CTGTTGAAAAAGTCAACTAAAAGCTC-3′	406
agrC-agr Iv	5′-ATGCACATGGTGCACATGC-3′ 5′-CGATAATGCCGTAATAC CCG-3′	588

Statistical Analysis:

We conducted the analysis of the data using SPPS 24. We represented the qualitative data as numerical values and percentages, conducted a comparison using Chi-square analysis, and deemed the P value to be statistically significant if it was below 0.05. We represented the age statistics as the median, minimum, and maximum values.

RESULTS

This work included 115 pediatric patients diagnosed with healthcare-associated sepsis and *S. aureus* was isolated from blood cultures obtained from Intensive Care Units (ICUs).

Ages varied between 5.0 months and 8.4 years, with a median age of 3.9 years.

The cohort consisted of 59 (51.3%) males and 56 girl s (48.7%). The medical devices related to the condition were the central venous catheter (CVC) in 40 patients (34.8%), the urinary catheter (UTC) in 32.2%, and the peripheral venous catheter in 80 patients (69.9%). (Table 2)

S. aureus isolates had high resistance to oxacillin (55.6%), cefoxitin (53.9%), erythromycin (48.6%), gentamicin (47%), and amikacin (43.5%). Table 3 shows that the isolates showed less resistance to ciprofloxacin (17.4%) and rifampicin (22.6%).

Among the isolated *S. aureus*, 91.3% form biofilm. The capacity to form biofilm was strong in 52/105 (49.5%), moderate in 31/105 (29.5%), and weak in 20.9% (Table 4).

The methicillin resistance of *S. aureus* determined by resistance to cefoxitin revealed an insignificant association between MRSA and the formation of biofilm (P = 0.5) and an insignificant association between the strength of the formed biofilm and MRSA and methicillin-susceptible *S. aureus* (MSSA) (P = 0.67) (Table 5).

The PCR results revealed that the most common *agr* genes were *agrI* and *agrIII* (41.7% and 25.2%, respectively), and the less prevalent were *agr II* and IV (9.6% & 13.9, respectively). (Table 6)

There was a significant correlation between MRSA phenotype expressed as resistance to cefoxitin and *agrIII* genotype (P = 0.03), a significant association between peripheral catheter insertion and genotype *agr II* (P = 0.02), and a significant formation of strong biofilm and *agrII* genotype (P = 0.004). (Table 7)

Table 2: Age, sex and clinical data of the studied pediatric patients (n=115)

Age Minimum Maximum Median	 5.0 months 8.4 years 3.9 years
Sex Male (No.-%) Female (No.-%)	 59 51.3 56 48.7
Type of ICU Medical (No.-%) Surgical (No.-%)	 60 52.2 55 47.8
CVC (No.-%)	40 34.8
UTC (No.-%)	37 32.2
Peripheral venous catheter (No.-%)	80 69.6

Table 3: Antibiotic resistance of *S. aureus*

Antibiotics	**No. %**
Amikacin	50 43.5
Rifampicin	26 22.6
Tetracycline	26 22.6
Ciprofloxacin	20 17.4
Cefoxitin	62 53.9
Erythromycin	56 48.6
Sulfamethoxazole	39 33.9
Gentamicin	54 47
Clindamicin	33 28.7
Oxacillin	64 55.6

Table 4: Biofilm formation of isolated *S. aureus*

	No.	**%**
Positive Biofilm formation	105	91.3
Strength of Biofilm Strong Moderate Weak	 52 31 22	 49.5 29.5 20.95

Table 5: Association between biofilm formation and methicillin resistance phenotype of *S. aureus*

	MRSA (n=62) No. %	**MSSA (n=53) No. %**	**P**
Biofilm formation	58 93.5	47 88.7	P=0.5
Strong biofilm Moderate biofilm Weak biofilm	27 43.5 19 30.6 12 19.4	25 47.2 12 22.6 10 18.9	P=0.67

Table 6: PCR results of detection of *agr* genes.

Gene	**No.**	**%**
Agri	48	41.7
AgrII	11	9.6
Agri	29	25.2
Agr IV	16	13.9

Table 7: Association between *Agr* genes, biofilm formation, cefoxitin resistance, and different risk factors of sepsis

	***AgrI* (n=48) No. %**	***AgrII* (n=11) No. %**	***AgrIII* (n=29) No. %**	***AgrIV* (n=16) No. %**
Biofilm formation P	45 93.8 0.52	11 100 0.59	25 86.2 0.27	14 87.5 0.81
Cefoxitin resistance P	23 47.9 0.31	6 54.5 0.9	20 68.9 0.03**	6 37.5 0.54
CVC P	20 41.7 0.24	3 27.3 0.75	7 24.1 0.18	6 37.5 0.37
Urinary catheter P	19 39.6 0.16	3 27.3 1.0	10 34.5 0.76	2 12.5 0.14
Peripheral catheter P	34 70.8 0.84	11 100 0.02**	19 73.1 0.64	11 68.7 0.31
Strong biofilm Moderate biofilm Weak biofilm P	24 50 8 16.7 13 27.1 0.07	10 90.9 0 0 1 9.1 0.004**	11 37.9 10 34.5 4 13.8 0.4	6 37.5 5 31.3 3 18.8 0.9

Chi-square P** significant

DISCUSSION

The accurate and rapid diagnosis of sepsis is the lifesaving in sepsis. The management includes the use of antibiotics appropriately, fluid resuscitation, and vasoactive medications. The insertion of treatment protocols, adjusted resource sepsis bundles, and well-performed technologies will lead to the reduction of sepsis mortality [23], especially in pediatric patients.

In this study, the associated medical devices were central venous catheter urinary catheter, and peripheral venous catheter. Venous catheters, either central or peripheral, are among the main medical devices used and are essential in the protocol of the treatment of ICU patients. The common complication of these catheters is their association with bloodstream infections[24]. Moreover, the use of a catheter in the urinary system is a known risk factor for sepsis, and it should be used if clinical indication is reported[25]. To decrease the bloodstream infection associated with these medical devices, preventive measures should be assessed using adequate preventive bundles.

The antibiotics resistance was reported in 2019 by the CDC, and MRSA was defined as serious health threats that require prompt action. It was reported as a responsible etiology for around 323,700 infections in patients admitted to the hospitals, with approximately 10,600 deaths in 2017 [26].

The isolated *S. aureus* had high resistance to oxacillin (55.6%), cefoxitin (53.9%), erythromycin (48.6%), gentamicin (47%), and amikacin (43.5%). The less resistance was toward ciprofloxacin (17.4%), and rifampicin (22.6%),

A prior study revealed that S. *aureus* isolated from clinical samples was highly resistant to oxacillin and erythromycin with lower resistance to amikacin and ciprofloxacin[27]. In a recent study from Alexandria, Egypt, *S. aureus* had high rates of gentamicin resistance (90%) [28]. The pattern of resistance to antibiotics varied according to the type of screened patients, geographical location, and antibiotic prescription policy.

Among 115 isolated S. *aureus*, 91.3% of them form biofilm. The capacity to form biofilm was strong among 52/105 strains (49.5%), moderate in 31/105 strains (29.5%) and weak in (20.9%).

In a prior study conducted on isolated *S. aureus* from infections in the urinary tract, all 55 isolates were biofilm producers, which included 55% moderate biofilm producers and 35% powerful biofilm producers[29]. In another study carried out on isolated *S. aureus* from bloodstream infections associated with CVC , all isolates had the capacity of biofilm formation and biofilm strongly was produced by 23% of *S.aureus*, moderately by 27% of the separates, and weak by 50% of *S.aureus* [30].

There was an insignificant association between MRSA and the formation of biofilm. The finding may reflect the difference in the background of genetic elements of the biofilm formation as stated previously by *Pozzi* et al.[31] that the formation of biofilm in MSSA is mainly through PIA synthesis while in MRSA, it is more related to the *fnbB* adhesion.

The PCR study revealed that the most prevalent *agr* genes were *agrI* and *agr III* followed by *agr II* and *agr IV*. Similar results were reported previously [32,33] Another study revealed *agr* I, and II as the most frequently detected types isolated from patients in burn units [34]. These discrepancies might be attributed to the variations in the patient's age and the types of the samples obtained from the patients and the differences in the geographic regions. The variations in the infection control policies over different times are modifying in a healthcare setting with different types of isolates recovered during various periods of sample collection [34].

There correlation was significant between the MRSA detected by phenotype test expressed as resistance to cefoxitin and the *agr III* genotype (P=0.03). Contrary to this result, an earlier study revealed an association of *S. aureus* with genotype *agr* II in healthcare-associated infections [35]. Another study reported that *agr* I type was the prevalent *agr* gene among MRSA isolates [36]. These differences could be attributed to the association between different lineages of isolated *S. aureus* and different *agr* types in different clinical settings [37]. Our findings reported that the association was significant between peripheral catheter insertion and genotype *agr* II and this may indicate that a specific lineage of S. *aureus* is present and associated with risk factors for the pathogenesis of sepsis in our patients.

The locus of *agr* was hypothesized to down-regulate the biofilm formation and this is according to the situation by which the biofilm develops. However, there is involvement of various mechanisms regulating in the formation of biofilm and its thickness of *Staphylococcus aureus*[38].

In this present study, there is a significant formation of strong biofilm and *agrII* genotype (P=0.004). A similar result was reported previously [38]. There are different genes associated with biofilm formation in *S. aureus* at different stages of growth including *icaR*, *icaA*, *hld*, *agrDCA*, and *hla* genes, and the regulators *sarA* and *rsbU*, the study indicated that the *icaR* gene, a putative transcriptional repressor engaged in the regulation in the environment, was not transcribed in *agr*II leading to formation of robust biofilm, demonstrating an early *icaA* activity of the transcription capable to elucidate the production of strong biofilm by strains that had *agr* II [39,40].

CONCLUSION

This study highlights the capacity of the isolated *S. aureus* from children with healthcare-associated sepsis to form a strong biofilm which indicates virulence and difficulty in its eradication by antibiotics therapy. Also, there was resistance to antibiotics significantly detected among isolated *S. aureus*. The main genetic types of *agr* system were I and III. The *agr II* was associated with strong biofilm formation.

Declaration

Ethics approval and consent to participate. We conducted every procedure following the ethical guidelines specified in the Proclamation of Helsinki, and subsequent revisions, The study received approval of the ethics from the Ethical committee of Mansoura, Faculty of Medicin (R.24.02.2512) and a written informed signed consent was collected from the parents of each pediatric case.

Availability of data and materials

The SPPS sheet obtained during the current work is found in the fighshare repository at https://doi.org/10.6084/m9.figshare.25304209.v1

Competing interests:

There is no conflict of interest for any of the authors.

Self-Funded

Authors' contributions

Within the laboratory investigation, MAR provided th e preliminary preparation of the article's data analysis a nd the subsequent revision of the draft. Evidence-based medicine (ABM) was used in the data of the patients collection and subsequent draft writing of the publication. AGE provided information on the laboratory investigation, including the article's initial writing, the study's data analysis, and the subsequent revision of the publication draft. The RAR collaborated on the laboratory investigation, the initial article compilation, and the study data analysis. MESZ participated in the laboratory study and contributed to the article's initial writing. MEA participated in the laboratory study and contributed to the initial draft of the publication. All authors have thoroughly reviewed and given their approval to the final paper.

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