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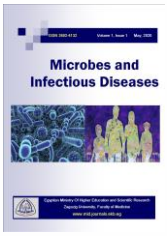

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Original article

Multidrug resistance and biofilm production among *Staphylococcus aureus* clinical isolates at a tertiary care hospital, Egypt

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ABSTRACT

Background: Treatment of persistent infections caused by biofilm producing *Staphylococcus aureus* is a concerning issue due to multidrug resistance (MDR). This work aimed to detect the correlation between production of biofilm and resistance to antimicrobials among *Staphylococcus aureus* isolates. **Methods:** Out of 300 clinical specimens, 128 *S. aureus* were isolated, and their antibiotics susceptibility testing were analyzed by disc diffusion method. Their ability to form a biofilm was investigated by micro-titer plate (MTP) method. Finally, their biofilm production genes were determined by polymerase chain reaction. **Results:** 70% of isolates were multidrug resistant and 85% were methicillin resistant *S. aureus* (MRSA), while the least resistance was detected for linezolid, azithromycin and vancomycin (2%, 7% and 9%, respectively). Biofilm was detected phenotypically in 84.8% of isolates while biofilm genes were detected in 94.5% of isolates where *ica A, ica B, ica C* and *ica D* were detected in 91%, 92%, 70% and 90%, respectively. Multidrug resistance was more significantly determined in biofilm forming isolates than biofilm negative ones (*p* value<0.001). **Conclusion:** Biofilm producing *S. aureus* isolates were prevalent among patients admitted at Surgical Intensive Care Unit. They harbored biofilm genes *like ica A, ica B, ica C* and *ica D* and displayed high MDR pattern.

Introduction

Staphylococcus aureus (*S. aureus*) is a primary leading cause of a wide range of human infections including hospital and community-acquired infections [1].

Several infections caused by *S. aureus* such as bacteremia, osteomyelitis, skin infections, pneumonia, meningitis and endocarditis. The virulence of the *Staphylococcus* species could be significantly increased by their ability to produce highly organized multicellular complexes called biofilms [2].

A chronic persistent bacterial infection is attributed to biofilm-producing organisms with enhancement of their resistance to antibiotics due to assisting in the transfer of the resistance genes such as insertion sequences, particularly in Gram-positive cocci [3].

Medical implants-associated infections are mainly attributed to biofilm-forming *S. aureus***.** Bacteria can get nutrients from their environment

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through biofilms channels. In addition, biofilm-producing bacteria are often resistant to antibiotics so, biofilm eradication is an unusually difficult task, especially in device associated infections that might require surgical intervention. Hence, impeding the biofilm formation on the medical devices before implantation could be the most effective preventive method of *S. aureus* infections [4, 5]. By inhibiting the initial bacterial adherence through antibacterial coatings surface using some materials such as quaternary ammonium silane or nanoparticles or by modification of device biomaterials via several methods, including matrix-assisted pulsed laser evaporation [6]

Biofilms production is a multi-step process including the initial reversible attachment of bacteria to a solid surface, then irreversible attachment followed by microcolonies formation due to bacterial multiplication then a mature biofilm is formed. Further, a mature biofilm dispersed to repeat another cycle of biofilm formation [7].

An intercellular adhesin (ica) operon encodes a polysaccharide intercellular adhesion (PIA) is necessary for biofilm establishment in *Staphylococci* [8]. The ica locus comprises four core genes, *namely ica A, ica D, ica B, and ica C*, as well as a regulatory gene, (*ica R)*. These genes encode the corresponding proteins ICAA, ICAD, ICAB, and ICAC [9].

A transmembrane protein with *N*-acetylglucosaminyl transferases enzymatic activity is encoded by *ica A* gene and it is responsible for the poly-*N* acetyl glucosamine polymer synthesis While the *ica D* gene encodes a product that is needed for the most favorable enzymatic activity of the *ica A* gene product [10, 11] on the other hand *ica B* role is not fully explained, but *ica C* acts as polysaccharides receptor. Accordingly, the ica *ADBC-carrying* strains are potential biofilm producers [9].

So, this work aimed to determine biofilm-forming *S. aureus* prevalence phenotypically and genotypically and to detect correlation between multidrug resistance and biofilm formation among these isolates

Subjects and Methods

This cross-sectional study was carried out in the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University from April 2023 to February 2024 where, the sample size was calculated using (open EPI_7). Assuming the frequency of MDR resistance was 95% versus 12% in biofilm-producers versus non-biofilm producers at 80% power and 95% CI, the estimated sample will be 90 MDR *S. aureus.* So, three hundred clinical specimens including pus, urine, endotracheal aspirates and blood were obtained from patients admitted at the Surgical Intensive Care Unit of Zagazig University hospitals. The infections caused by any organism other than *S. aureus* were excluded from this study.

Ethical approval and consent to participate: The Institution Review Board of the Faculty of Medicine, Zagazig University (ZU-IRB #10800-14-6-2023) approved this cross-sectional study. The Code of Ethics of the World Medical Association (Declaration of Helsinki) was followed in this study. Patients or their relatives gave written informed consent.

Under complete aseptic conditions the samples were collected and transported immediately for laboratory processing. Pus, urine and sputum samples were inoculated onto nutrient agar, blood agar and mannitol salt agar at 37°C for 24–48 h. while, blood samples were enriched on blood culture bottles for 48 h first, then they were subcultured on the same previous media under the same previous conditions. *Staphylococcus aureus* isolates were identified by standard microbiological techniques including colonial morphology, Gram stain, catalase, coagulase, and DNase tests [12]. MALDI TOF/MS using the VITEK MS system, (bioMérieux. Inc.Durham. USA) was used to confirm isolates' identification.

Antimicrobial susceptibility test

According to guidelines of CLSI, 2023, the following discs; cefoxitin (30µg), linezolid (30µg), clindamycin (2µg), levofloxacin (5µg), doxycycline (30µg), trimethoprim-sulfamethoxazole (2.5µg), azithromycin (15µg) and gentamicin (30µg), (Oxoid, UK) were used to detect antimicrobial susceptibility of *S. aureus* isolates on 0.5 McFarland standards isolates growth suspension inoculated on Muller Hinton agar plates by disc diffusion method. Vancomycin susceptibility was assessed by broth microdilution**.** *S. aureus ATCC 25923* was used as a control strain. *MRSA* was detected if isolates showed a cefoxitin inhibition zone ≤ 21mm [13].

Detection of biofilm formation by Micro-titer plate (MTP) method according to Stepanović et al. [14]:

Trypticase soy broth (TSB) containing glucose (1%) (Oxoid, UK) was used to inoculate *S. aureus* isolates then, growth suspension was adjusted to 1:100 dilution of 0.5 McFarland standards and added to a sterile 96-well flat-bottom micro-titer plate. After incubating aerobically at 37◦C for 24 hours, deionized water was used to wash the wells. Then, they were left to dry in air at 60°C for one hour. Subsequently, 100µl of crystal violet solution (0.1%) (Sigma Chemical Co., USA) was used to stain the adherent cells for 15 min. Washing of wells by water was done and then, ethyl alcohol was put on wells and left for 30 min. Using an ELISA plate reader (BioTek, USA), the optical density (OD) of each well was determined at 490nm. A negative control was prepared of sterile TSB and its OD was determined. The experiment was performed in triplicates. A cut-off value of OD (ODc) was estimated to equal negative control's average OD + (3 SD of negative control). Classification of biofilm production was reported according to **Table 1**.

PCR for biofilm genes

Using the QIAmp DNA Mini Kit (QIAGEN, Germany), DNA was extracted from isolates. PCR was performed using 50 µl PCR bead of Maxime PCR PreMix Beads (iNtron, Certified Company, Germany) where 5µl of DNA extract, 1pmol/µl of each primer and completed with sterile nuclease free water for each bead. Primer sequences and conditions were listed in **tables (**2 **&**3**)** according to **Diemond-Hernández et al.** [15]. The amplification products were analyzed by1.5% gel electrophoresis and compared with suitable DNA ladder.

Statistical analysis

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. Quantitative data were represented as the mean, standard deviation, and range. The Chi-square test ($\chi 2$) was used to compare proportions as appropriate. Reliability for categorical items was measured using Cohen's kappa coefficient. A *p*-value of < 0.05 was considered statistically significant at a 95% confidence interval.

Results

Isolation of *S. aureus*:

Our study revealed 128 *S. aureus* isolates out of 300 clinically different specimens collected from 300 patients with a mean age of 46 years (±18.3). The studied group was composed of 64.0% males and 36.0% females. The distribution of 128 isolates among the different specimens was: pus (48.7%), sputum (45%), urine (27.2%), and finally blood (20%) which was statistically significant (X^2 = 10.976, *p*= 0.0118) as shown in **table (4).**

Antibiotic susceptibility testing

As shown in **figure (1)**, 70% of isolates were MDR and showed high resistance to cefoxitin (85%) and levofloxacin (55%). Moreover, 50% were doxycycline resistant.

Phenotypic detection of *S. aureus* biofilm

Biofilm production was evaluated phenotypically with the following results: non-producer (14.2%), weak producer (29.6%), moderate producer (23.4%) and strong producer (32.8%) as shown in **table (5)**.

Genotypic detection of *S. aureus* biofilm

Out of 121 (94.5%) isolates carrying biofilm-producing genes, 116 isolates (91%) were positive for the *Ica A* gene while *Ica B* was detected in 118 isolates (92%) and *Ica D* was found in 115 isolates (90%) while 90 isolates (70%) harbored *Ica C* as represented in **table (6)** and **figure (2)**.

Relation between biofilm formation and MDR in *S. aureus* isolates

In studying the relation between biofilm formation and MDR: All strong biofilm producers (100%) were MDR while 6 MDR (33.3%) were non biofilm-producer, and this was statistically significant ($p < 0.001$**) **(Table 7)**.

Agreement between phenotypic and molecular methods of biofilm detection in *S. aureus* isolates

Concerning biofilm determination methods among *S. aureus* isolates, a Kappa test was used to evaluate the agreement between phenotypic and molecular characteristics of biofilm formation. There was a statically significant agreement (Kappa = 0.522, $P < 0.001$) where all phenotypic biofilm producers were genotypically positive **(Table 8).**

Table 1. Biofilm categories according to OD readings.

Biofilm categories	Readings
No biofilm production	OD ≤ ODc
Weak production	ODc< OD ≤ 2ODc
Moderate production	2ODc < OD ≤ 4ODc
Strong production	4ODc < OD

Table 2. Primer sequences of biofilm genes.

Gene	Primer sequence	Bp
Ica A F	5' GAC CTC GAA GTC AAT AGA GGT 3′	814
Ica A R	5' CCC AGT ATA ACG TTG GAT ACC 3′	
Ica B F	5' ATG GCT TAA AGC ACA CGA CGC 3′	526
Ica B R	5' TAT CGG CAT CTG GTG TGA CAG 3′	
Ica C F	5' ATA AAC TTG AAT TAG TGT ATT 3′	989
Ica C R	5' ATA TAT AAA ACT CTC TTA ACA 3′	
Ica D F	5' AGG CAA TAT CCA ACG GTA A 3′	325
Ica D R	5' GTC ACG ACC TTT CTT ATA TT 3′	

Table 3. PCR conditions.

Gene	Initial denaturation 1 (cycle)	Denaturation (30 cycles)	Annealing (30 cycles)	Extension (30 cycles)	Final Extension (1cycle)
Ica A	5 min at 94°C	1 min at 95°C	1 min at 60°C	1.5 min at 72°C	2.5 min at 72°C
Ica B			1 min at 59°C		
Ica C			1 min at 45°C		
Ica D			1 min at 59°C		

Table 4. The distribution of *S. aureus* among the different clinical specimens.

Specimen	**No**	**isolates**		**X^2 test**	***p* value**
		No	**%**		
Pus	156	76	48.7%	10.976	0.0118*
Urine	44	12	27.2%		
Sputum	80	36	45%		
Blood	20	4	20%		
Total	300	128	42.7%		

X^2= Chi-Square test * Significant

Table 5. Biofilm production by phenotypic methods.

Phenotypic	**No.**	**(%)**
Non producer	18	14.2
Weak	38	29.6
Moderate	30	23.4
Strong	42	32.8
Total	128	100.0

Table 6. Distribution of biofilm genes among isolates.

Genes	**No.**	**(%)**
Biofilm genes positive	**121**	**94.5**
Ica A	116	91.0
Ica B	118	92.0
Ica C	90	70.0
Ica D	115	90.0
Biofilm genes negative	**7**	**5.5**
Total	**128**	**100.0**

Table 7. Biofilm formation in MDR and non- MDR *S. aureus* isolates.

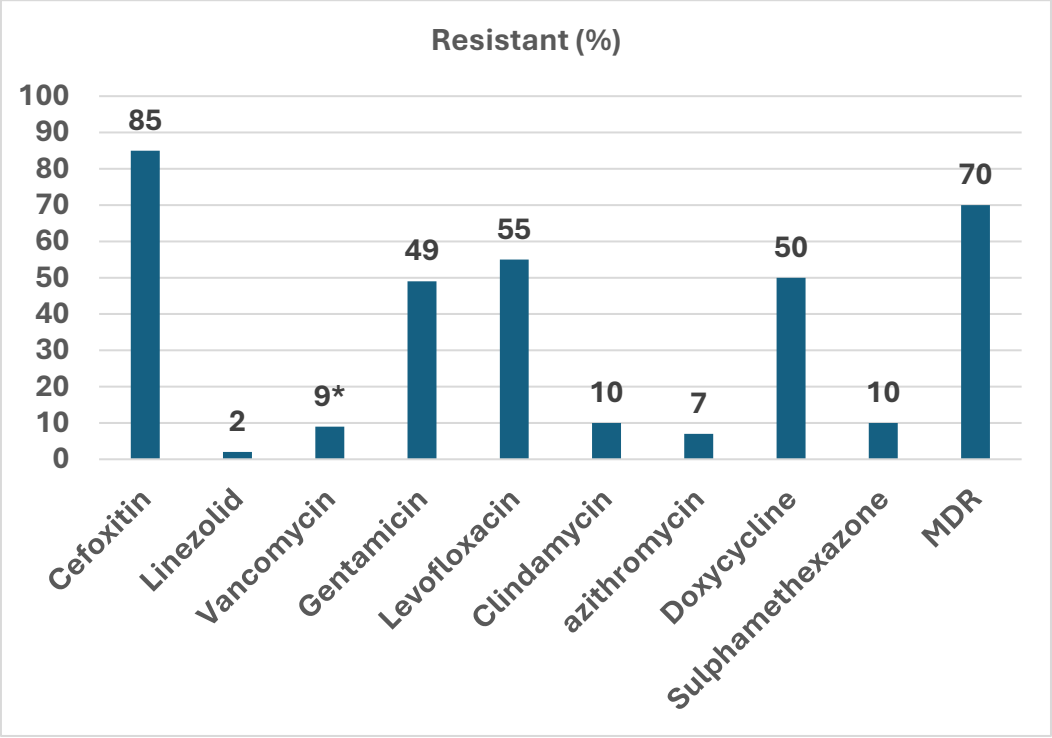
Biofilm formation	MDR				X^2	*p* value
	No (No.=38)		Yes (No.=90)			
	No.	(%)	No.	(%)		
Non producer(n=18)	12	66.7	6	33.3	63.234	<0.001**
Weak(n=38)	25	65.8	13	34.2		
Moderate(n=30)	1	3.0	29	97.0		
Strong(n=42)	0	0.0	42	100.0		

X^2= Chi-Square test, ** Highly significant, MDR, multidrug resistant

Table 8. Agreement between phenotypic and molecular methods of biofilm detection among *S. aureus* isolates.

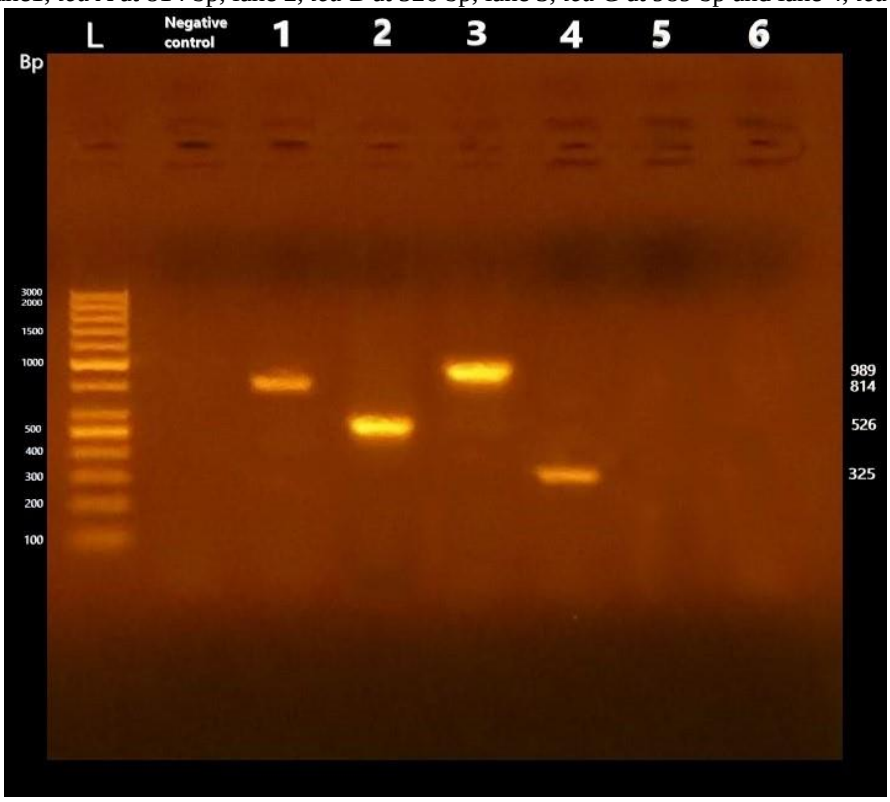
Variables		Genotypic				Kappa test	*p* value
		Negative (No.=7)		Positive (No.=121)			
		No.	(%)	No.	(%)		
Phenotypic	Non produced (n=18)	7	38.9	11	61.1	0.522	<0.001**
	Biofilm producer (n=110)	0	0.0	110	100.0		

Figure 1. Antibiotic resistance pattern by disc diffusion method.



*Vancomycin susceptibility was detected by the broth microdilution method.

Figure 2. Gel electrophoresis of *Ica ADBC* genes in *S. aureus* isolates. L, Ladder 100- 3000 bp, negative control, then lane1, *ica A* at 814 bp, lane 2, *ica B* at 526 bp, lane 3, *ica C* at 989 bp and lane 4, *ica D*, at 325 bp.



Discussion

Bacterial biofilms pose a serious problem for public health. Staphylococcal biofilm-associated lethal infections eradication is a challenging and costly issue **[**16, 17].

In our study, 128 *S. aureus* were recovered from a total of 300 specimens with an isolation rate (42.7%). Most isolates were recovered from men (64.0%) over 46 years old and this was matched with **Omidi et al.** [18]. They recovered most of their isolates from men over 50 years old. However, other previous studies demonstrated no significant association between the ages and gender of the patients and isolation of biofilm forming bacteria [19- 21].

Isolates were mainly from pus followed by sputum which was of a statistical significance (*p* value 0.0118), and this was following **Sapkota et al.** who reported that most of their isolates were from pus and wound swab samples indicating their role in pyogenic soft tissue and wound infections [22]. Additionally, a previous similar study agreed with our results [23]. Furthermore, in partial agreement to our results, **Abdelraheem et al.** [24] found biofilm forming *S. aureus* was more significantly isolated from wound samples than respiratory samples.

Concerning the results of antibiotic susceptibility, more than two-thirds of isolates (70%) were MDR and showed high levels of resistance to cefoxitin (85%) and levofloxacin (55%). Moreover, (50%) were doxycycline resistant. This agreed with **Gitau et al. [**25] who detected MRSA in 91.97% of their isolated *S. aureus* which was slightly higher than **Hasan et al.** [26], **Saeed et al.** [27] and **Abdelraheem et al.** [24] who identified MRSA in 75%, 76% and 79.4% of their *S. aureus* isolates, respectively. However, **Dilnessa** [28] found that only 12.8% were MRSA. In addition, **Shahmoradi M et al.** [5] reported a higher level of resistance where their *S. aureus* isolates were highly resistant to penicillin (100%), AMC (100%), streptomycin (100%), nalidixic acid (84%) and methicillin (36%) moreover, 49 (98%) of the clinical isolates were MDR. The increased prevalence of MDR *S. aureus* suggested the necessity for strict infection control measures implementation together with adherence to antibiotic policy in our hospital.

In this study, biofilm-producing *S. aureus* represented 94.5% (121/128) of our isolates. This result was near to results observed in a previous

study which showed that 96 % of the isolates were biofilm producer [29] while **Abdelraheem et al.** [24] **and Karki et al.** [30] determined biofilm in 81.6% and 86.3% respectively of their isolates. Conversely, **Nasr et al.** reported that only 46% of their *S. aureus* isolates were biofilm producers [31]. The variance in biofilm production might be influenced by several factors such as differences in the virulent ability of bacteria to form biofilm and the number of adherent bacterial cells, specimen type, country of origin and the genetic background of the *S. aureus* isolate. Moreover, biofilm formation is affected by environmental factors like growth medium, surface type (rough or smooth) and the charge and porosity of the surface [24].

Biofilm production was evaluated phenotypically among 128 *S. aureus* isolates which were graded as follows: non-producer (14.2%), weak producer (29.6%), moderate producer (23.4%), and strong producer (32.8%) and agreed to some degree with **Abdelraheem et al.** [24] who found non-biofilm producers (18.4%), weak (47.5%), moderate (28.4%) and strong (5.6%) biofilm producers. In addition, **Omidi et al.** [18] investigated phenotypic detection of biofilm production showed that 93.1% of *S. aureus* isolates were biofilm producers, from which, 111, 6, and 19 isolates were identified as strong, moderate, and weak biofilm producers respectively. A similar study by **Neopane et al.** [32] investigated biofilm production by *S.aureus* isolated from wounds and showed 69.8% of isolates were biofilm producers where 6.97% were strong, 27.9% were moderate, 34.88% were weak and 30.2% were non-biofilm producers.

A molecular study of biofilm genes found that out of 121(94.5%) biofilm-producing genes isolates, 116 isolates (91%) were positive for *Ica A* gene while *Ica B* was detected in 118 isolates (92%) and *Ica D* was detected in 115 isolates (90%) while *Ica C* was detected in 90 isolates (70%). This agreed with **Abdelraheem et al.** [24] who observed the prevalence of *ica A, ica B and ica D among S. aureus* isolates were 91.4%, 92.9% and 90% respectively. Also, **Khlaf et al.** [33] reported that the prevalence of *ica A, ica B* and *ica D* genes were 95.8%, 91.6% and 95.8%, respectively Meanwhile **Torlak et al.** [34] and **Tekeli et al.** [35] demonstrated that *ica* genes were harbored by all *S. aureus* isolates.

In studying the relationship between biofilm formation and MDR: All strong biofilm producers (100%) were MDR while 6 MDR (33.3%) were non biofilm producers and this agreed with **Moghadam et al. [**36], **Neopane et al.** [32] and **Ibrahim et al.** [37] who observed that biofilm-forming *S. aureus* had a higher antimicrobial resistance pattern than biofilm non-producers.

Finally, comparing the phenotypic and genotypic characteristics related to biofilm formation among *S. aureus* isolates. The phenotypic biofilm producers and genotypic positive results agreed with a (100%), this indicates that all phenotypic biofilm-producing isolates possessed the genotypic markers for biofilm production.

However, 38.9% of isolates were non-producers of biofilm neither phenotypically nor genotypically. On the other hand, the majority of 61.1% were genotypically positive only. In accordance with our results, **Torlak et al.** [34] reported that the agreement between phenotypic and genotypic methods was 91% and that 9 % of their isolates had the biofilm genes but were unable to form biofilm. This discrepancy suggested that some isolates had genetic potential for biofilm formation but could not express it phenotypically under the conditions tested. This might be due to environmental conditions, regulatory mechanisms, or experimental conditions that affect the expression of biofilm-related genes. And for more accurate results both phenotypic and molecular methods should be used to detect biofilm production among *S. aureus* isolates.

The moderate Kappa value indicates that there is only a moderate level of agreement between genotypic testing and the actual biofilm production observed suggesting that while phenotypic testing is a good indicator, relying solely on phenotypic testing may lead to incomplete or inaccurate conclusions about the biofilm production capabilities of *S. aureus* isolates as biofilm genes expression might be affected by environmental factors. Therefore, using both phenotypic and molecular methods provides a more comprehensive and accurate assessment, ensuring that the biofilm production potential of the isolates is correctly identified.

Conclusion

The study highlights the significant role of biofilm production in the clinical outcomes of *Staphylococcus aureus* infections as biofilm formation was strongly associated with MDR, particularly among moderate and strong biofilm-producers that lead to worse outcomes with

prolonging infection, high cost and mortality. The high prevalence of biofilm-related genes ensures the genetic predisposition of the studied isolates to form biofilms. This has important implications for treatment strategies, as biofilm-associated infections are difficult to treat due to their resistance to antibiotics. Effective management of such infections requires targeted therapies that eradicate both the biofilm and the MDR characteristics of the isolates.

Author contributions

HME contributed to the study conception and design. HME, ASLA, EEO contributed to methodology, analysis, and interpretation of data. HME, ASLA wrote the manuscript draft, provided review and editing. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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