Study of Antibiotics Resistance and Biofilm Formation of *Staphylococcus aureus*

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Abstract

The aim of this study is to assess the biofilm-production rates in clinical *S. aureus* isolates and to correlate between multidrug resistance and their biofilm-forming capacity. One hundred ninety (190) clinical specimens were collected from different clinical sources (wounds, burns, urine, ear and blood) in Diyala province, during the period from September 15/ 2022 to December 15/ 2022. The isolates were diagnosed morphologically, microscopically, biochemically and confirmed by *16SrRNA* detection. All isolates were investigated for antibiotic sensitivity, using the disc diffusion method. The isolates under test showed a high resistance 100% to OX, while the rates of resistance ranged from C, F, AZM, VA, DA, TME, CN, RA, DOX, FEP, TEC 16%, 4%, 92%, 4%, 60%, 72%, 44%, 48%, 44%, 80%, and 42%, respectively. The ability of *S. aureus* isolates biofilm was investigated. All the isolates under study were coagulase producers and showed β-hemolysis. The results showed that all isolates were biofilm producer using micro-titer plate method.

Keywords: *Staphylococcus aureus*, virulence factors, biofilm, MDR.

دراسة مقاومة المضادات الحيوية وتكوين الأغشية الحيوية للمكورات العنقودية الذهبية

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الخلاصة

هدفت الدراسة الحالية الى تقييم معدلات انتاج الاغشية الحيوية لعزلات المكورات العنقودية الذهبية و العلاقة بين مقاومتها للمضادات المتعددة وقدرتها على انتاج الاغشية الحيوية. تم جمع مائة وتسعين (190) عينة من مصادر سريرية مختلفة (الجروح والحروق والبول والاذن والدم) في محافظة ديالى خلال الفترة من 15/ أيلول/ 2022 إلى 2022/12/15. تم تشخيص العزلات مظهرياً ومجهرياً وكيموحيويا وجينياً باستخدام الجين التشخيصي *16SrRNA* وتم اختبار قابليتها على تكوين الأغشية الحيوية. كما تم فحص حساسية جميع العزلات للمضادات الحيوية باستخدام طريقة انتشارالاقراص. حيث أظهرت العزلات تحت الاختبار مقاومة عالية 100% لـ OX بينما تراوحت معدلات المقاومة بين C, F, AZM, VA, DA, TME, CN, RA, DOX, FEP, TEC 16%, 92 %, 4%، 4%، 60%، 72%، 44%، 48%، 44%، 80%، و42% على التوالي. تم دراسة قدرة عزلات المكورات العنقودية الذهبية على إنتاج الأغشية الحيوية. بينت النتائج ان جميع العزلات قيد الدراسة كانت منتجة لإنزيم التخثر وانزيم الكتاليز وأظهرت انحلال الدم نوع بيتا. كما أظهرت النتائج أن جميع العزلات كانت منتجة للأغشية الحيوية بطريقة اطباق المعايرة الدقيقة.

الكلمات المفتاحية: *المكورات العنقودية الذهبية ، عوامل الضراوة ، الأغشية الحيوية ، المقاومة المتعددة .*

P-ISSN: 2664-0562
E-ISSN:2664-0554

Introduction

The *Staphylococcus aureus* is a Gram-positive bacterium that is normally found as normal flora of the body. It can be found on skin as well as in the upper respiratory system [1]. Human pathogen *S. aureus* causes a wide variety of serious illnesses, including infective endocarditis and bloodstream, bone, skin, and soft tissue infections [2]. It can create a biofilm by adhering to various surfaces, which explains why it adheres to many medical instruments in a hospital setting [3]. A biofilm is a community of microorganisms adhered to a surface that provides a protection level of homeostasis and resilience in a changing environment. When compared to their planktonic cells, bacterial cells in biofilms are more vulnerable to environmental conditions and antimicrobials [4]. The production of biofilms by bacteria is a major aspect in their ability to survive in the host and is thought to be an important indication of their virulence, resulting in serious chronic infections [5]. Biofilm is crucial for *S. aureus* pathogenicity that antimicrobial treatment and innate host defense systems are ineffective against biofilm-associated *S. aureus* infections [6]. Furthermore, the development of biofilms by *S. aureus* and antimicrobial resistance are functionally related since the expression of the biofilm phenotype can be affected by the development of antimicrobial resistance. Additionally, many foodborne illnesses are linked to biofilms and are regarded as an urgent public health issue [7,8]. The development of MDR isolates in human illnesses significantly limits the capacity of healthcare providers to provide appropriate antibiotic treatment [9]. The assessment of the potential link between the multidrug-resistant (MDR) status and the biofilm-producing phenotype in bacteria has attracted a lot of attention [10]. The aim of this study is to evaluate the prevalence MDR phenotypes in *S. aureus* isolates collected from clinical sources as well as the relationship between MDR and biofilm-forming capacity.

Methods

Collection of *S. aureus* specimens

A total 190 clinical specimens from different sources were collected from two local hospitals in the Diyala province/Iraq. The samples were collected from burns, wounds, urine, ears, and blood at different ages patients for both sexes, and the patients' information, including age, sex, and source of isolation. The specimens were transferred directly on the appropriate previously prepared culture media. The specimens were numbered along the study as S_1, S_2, S_3, etc.

P-ISSN: 2664-0562
E-ISSN:2664-0554

Isolation and identification of *Staphylococcus aureus* isolates

The collected specimens were inoculated on Blood agar initially and on Mannitol salt agar to detect suspected *S. aureus* isolates and incubated at 37°C for 18-24 hrs. Other microbiological techniques were used for detection of *S. aureus*, such as gram stain, catalase and coagulase tests.

16SrRNA detection

The identification was confirmed by detection of *16SrRNA* gene. Briefly, total DNA was extracted from *S. aureus* grown on mannitol salt agar plates by using ABIOpure / USA extraction kit according to the manufacture instruction, and the primers were obtained from Macrogen/ Korea in lyophilized form, table (1).

Table (1): *Sequence and molecular size of PCR products of 16s rRNA.*

Gene	Primer Sequence	Size of product (bp)	Annealing temp. ºC	Ref.
16SrRNA – F	AACTCTGTTATTAGGGAAGAACA	756	55	[11]
16SrRNA – R	CCACCTTCCTCCGGTTTGTCACC			

The PCR program and reaction condition explained in Table (2).

Table (2): The conditions used for the amplification of *16s rRNA* gene.

Initial denaturation	Denaturation in each cycle	Annealing	Primers extension	Final extension
95 °C, 5 min	95°C, 30s	55°C,30s	72°C, 30s	72 °C,7 min
1 cycle	30 cycles			1 cycle

Antibiotic Susceptibility Test

Bauer-Kirby disc diffusion method was used to examine antibiotics susceptibility toward 12 commonly used antibiotics (Bioanalyse / Turkey), Table (3). Antimicrobial Susceptibility Test (AST) was performed according to the guidelines of clinical and laboratory standards institute (CLSI) (2022) on Muller Hinton agar plates (MHA).

Table (3): Antibiotics used against isolates in the current study.

Antibiotic families	**Antibiotics**	**Concentrations (µg)**
Macrolides	Azithromycin(AZM)	10
Aminoglycosides	Gentamicin (CN)	10
Glycopeptides	Vancomycin (VA)	30
	Teicoplanin (TEC)	30
Penicillins	Oxacillin (OX)	5
Amphenicol	Chloramphenicol (C)	30

P-ISSN: 2664-0562
E-ISSN:2664-0554

Rifamycins	Rifampin (RA)	5
Lincosamides	Clindamycin (DA)	10
Nitrofuran	Nitrofurantoin (F)	30
Tetracyclines	Doxycycline (DOX)	30
Cephalosporin	Ceftaroline (FEP)	30
Sulfonamides	Trimethoprime- sulfamethoxazdle (TME)	1.25/23.7

Biofilm formation assay

Microtiter plate method (MTP)

Quantification of biofilm formation *S. aureus* was performed using 96-well microtiter flat-bottomed polystyrene plate, as described by Samadi et al. [12]. The optical densities (ODs) of the plates were observed using ELISA reader at 630 nm. The results were measured according to their optical densities as follows: (1) strong biofilm producer ($4 \times ODc < OD$); (2) moderate producer ($2 \times ODc < OD \leq 4 \times ODc$); (3) weak biofilm producer ($ODc < OD \leq 2 \times ODc$); (4) non-biofilm producer ($OD \leq ODc$).

Dendogram analysis

The genetic relationship between all bacterial isolates under study was found using a Dendogram by converting the results that appeared into a characterization table. When the result is positive, the number 1 is placed, and when the result is negative, the number is 0, then this data was entered into Past software using the Dic option in order to obtain a Dendogram.

Results and Discussion

Among 190 clinical samples (wounds, burns, urine, blood and ear) during the study period, 50 (26%) *S. aureus* isolates were identified distributed as shown in (Table 4), using morphological, microscopic, biochemical and genetic methods. According to source of the samples, the prevalence of *S. aureus* was higher in urine samples and lower in blood samples, while on isolate was obtained from ear samples.

Table (4): Percentage of *S. aureus* isolation according to sample source.

Samples source	No. of samples	No. of isolates (%)
Wound	60	15 (8%)
Burn	50	14 (7%)
Urine	40	18 (9%)
Blood	20	3 (2%)
Ear	20	0 (0%)
Total	190	50 (26%)

P-ISSN: 2664-0562
E-ISSN:2664-0554

S. aureus were detected by conventional techniques through their ability to yield β hemolysis on blood agar, fermentation of mannitol salt, and production of catalase, and coagulase (Table 5), Figure (1) shows the *16SrRNA* gene product for diagnosis conformation.

Table (5): Biochemical tests results.

Test	Result
Catalase	+
Oxidase	–
Coagulase	+
Mannitol fermentation	+
Hemolysis	β- hemolysis
Gram stain	G+ve

(+): Positive; (-): Negative

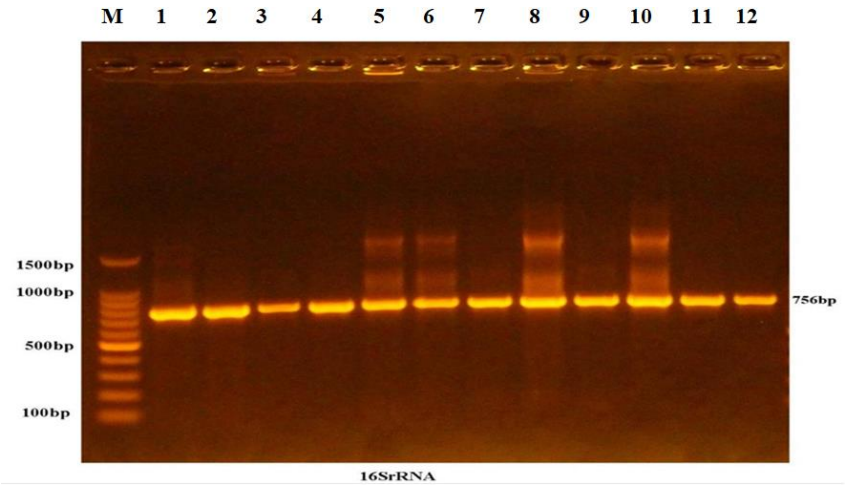

Fig. (1): *16SrRNA* gene amplicon on agarose gel using the PCR technique for *S. aureus*, at a concentration of 2%. (Marker) M represents 100-1500 bp, while numbers from 1 to 22 indicate the number of isolates.

Isolates that can grow on mannitol salt agar (MSA) are considered to be from *Staphylococci* genus because it is a selective and differential medium. The high concentration of sodium chloride (7.5%) makes it toxic to most bacteria, except *Staphylococci*, which can survive mostly in this medium due to their tolerance to salty environments such as human skin. If the species can ferment the mannitol sugar and create acid, which reduces the pH of the medium, changing the red color of the phenol reagent in the medium from red to yellow, which makes it a differential medium [13].

P-ISSN: 2664-0562
E-ISSN:2664-0554

Biochemical tests of the growing isolates showed differential and diagnostic results. All isolates were subjected to a catalase test and gave a positive result. All isolates had the ability to break down hydrogen peroxide (H_2O_2 3%) and convert it to water and oxygen gas. The positive result appeared in the form of gas bubbles, which is a differential test between the types of Staphylococcus spp. that are positive for it and the types of Streptococcus spp. and Micrococci that are negative for this test.

Catalase production is considered one of the factors related to virulence in staphylococci, as it allows them to better resist killing by hydrogen peroxide H_2O_2 [14]. All of the isolates also gave positive results for the plasma coagulase test, which is a differential test between *S. aureus*, which are all positive, and *S. epidermidis*, which is negative for this test [15].

Antibiogram profile

The results for antibiotic susceptibility test are illustrated in (Figure 2). The test showed a clear variation in the response of the bacterial isolates toward antibiotics used.

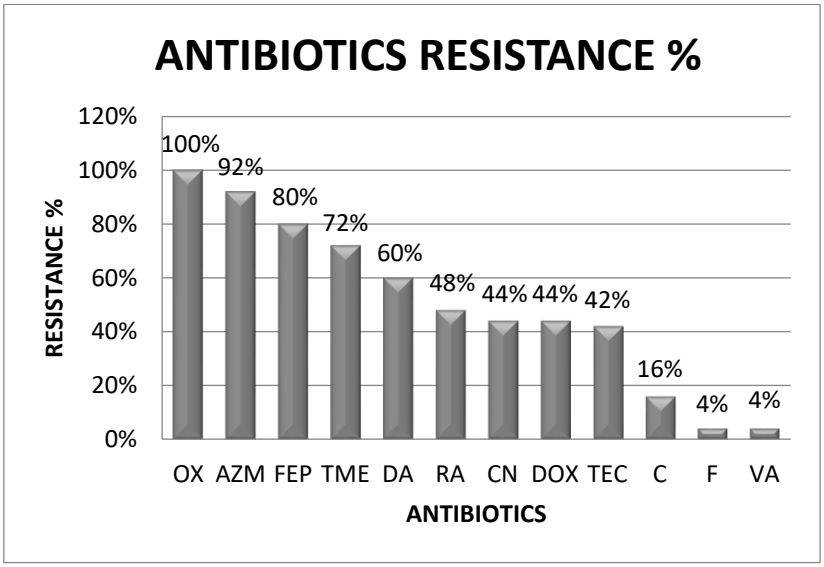

Fig. (2): Antibiotic resistance pattern of *S. aureus*.

The isolates showed 100% resistance to Oxacillin, this result disagree with Abd Abdullah et al. [16] that presented 73.17% resistance toward OX. The resistance of *S. aureus* to this antibiotic due to resistance genes to penicillin antibiotics, which are either chromosomal or plasmid in origin, or it may be the result of mutations in the genes for proteins binding to penicillin (PBPS), which is more common in gram-positive bacteria, specifically in the 30S ribosomal unit, thus leading to the antigen losing its affinity for binding to the target protein and reducing the permeability of bacterial cell to the antibiotic [17]. The resistance to Azithromycin reached 92%. results disagree with Al-Khfaji et al. [18] that was 64.51% resistance to AZM.

P-ISSN: 2664-0562
E-ISSN:2664-0554

The resistance of bacteria to the Azithromycin may be due to the ability of the isolates to produce the enzyme RNA methylase, which is encoded by the genes *ermA* and *ermB*, which are called methylase genes, or may be the target location of the antibiotic has changed, or due to the widespread and indiscriminate use of this group of antibiotics [19].

Isolates showed 80% resistance to Ceftaroline which was contrary to the results of Lee et al. (2018) [20], where the resistance was 44%. For Trimethoprime-sulphametoxazol the resistance was 72%, as these results disagree with Bokharaei et al. [21] result that was 0% resistance.

The resistant to Clindamycin reached 60%, This result agrees with the findings of Khodabandeh et al. [22], where the resistance was 68.8%. The resistance of *S. aureus* to Rifampin reached 48% which was almost near Parastan et al. [23] result which presented 32.21% resistance. The results showed that 44% of the isolates were resistant to both Gentamicin and Doxycycline. Aminoglycosides, represented by Gentamicin, have an effective effect against clinically important staphylococci through their binding to the 30S ribosomal subunit and their interference with protein synthesis,

which leads to a lethal effect on bacteria [24]. The widespread use of aminoglycosides has led to the development of aminoglycoside-modifying AME enzymes, the most common mechanism of acquired resistance to these antibiotics in *S. aureus*, in addition to other resistance mechanisms such as target modification, efflux pumps, and targeted mutations [25]. Vancomycin and Teicoplanin showed 4% and 42% resistant rate respectively. Finally, chloramphenicol and nitofurination showed 16 % and 4% respectively.

Multi-drug resistance (MDR)

The results of the current study showed that 48 (96%) of the isolates was multiple resistance ranged from 3 to 9 antibiotics, as shown in Table (6).

Table (6): MDR of *S. aureus* isolates.

No. of antibiotics that resist	No. of resistant isolates %		Isolates numbers
3	6	12%	S_2, S_{10}, S_{21}, S_{37}, S_{38}, S_{39}
4	7	14%	S_1, S_{14}, S_{19}, S_7, S_{22}, S_{36}, S_{46}
5	7	14%	S_{16}, S_{23}, S_{24}, S_{45}, S_{50}, S_{40}, S_{49}
6	6	12%	S_{17}, S_{25}, S_{43}, S_{13}, S_{44}, S_{31}
7	3	6%	S_{11}, S_5, S_{41}
8	14	28%	S_4, S_8, S_9, S_{15}, S_{33}, S_6, S_{12}, S_{18}, S_{42}, S_{47}, S_{26}, S_{27}, S_{34}, S_{35}
9	5	10%	S_{48}, S_{28}, S_{29}, S_{30}, S_{32}
Total	**48**	**96%**	

P-ISSN: 2664-0562
E-ISSN:2664-0554

Dendogram

Dendogram results of *S. aureus* AST in the current study, showed the presence of two main groups 1 and 2, with a similarity rate of 30%, Figure (2). The first group included one isolate (S20), that similar to the rest of the isolates in its resistance to the OX, while sensitive to other antibiotics. The second group included 49 isolates, which we

re divided into A that included 19 isolates, while group B included 30 isolates, with 50% similarity.

Group A was divided into two other subgroups, A1 and A2, with 60% similarity. The first group, A1, included three clones, and single isolates included S7, S49, S46, S23, S2, S14, S50, and S1. The clones were similar in their resistance to antibodies OX, AZM, and FEP, and their sensitivity to antibodies TEC, C, F, and RA, while varied in sensitivity to DA, TMP, CN, DOX, and VA. The second group, A2, included two isolates, S10 and S3, that similar in their resistance to OX and TMP and their sensitivity to FEP, CN, VA, CN, and C. F, TEC, RA, and DOX, and they varied in their resistance to AZM.

Group B was subdivided into two other subgroups, B1 and B2, with 70% similarity. Group B1 included five clones and 11 single isolates. The clones were similar in their resistance to OX, AZM, DA, FEP, and TEC and its sensitivity to antibodies C, F, and VA, while varied in sensitivity to TMP, CN, RA, and DOX. Group B2 included one clone, that similar in their resistance to OX, AZM, DA, TMP, RA, and CN, and their sensitivity to antibiotics C, F, and VA, while varied in their sensitivity to FEP and DOX.

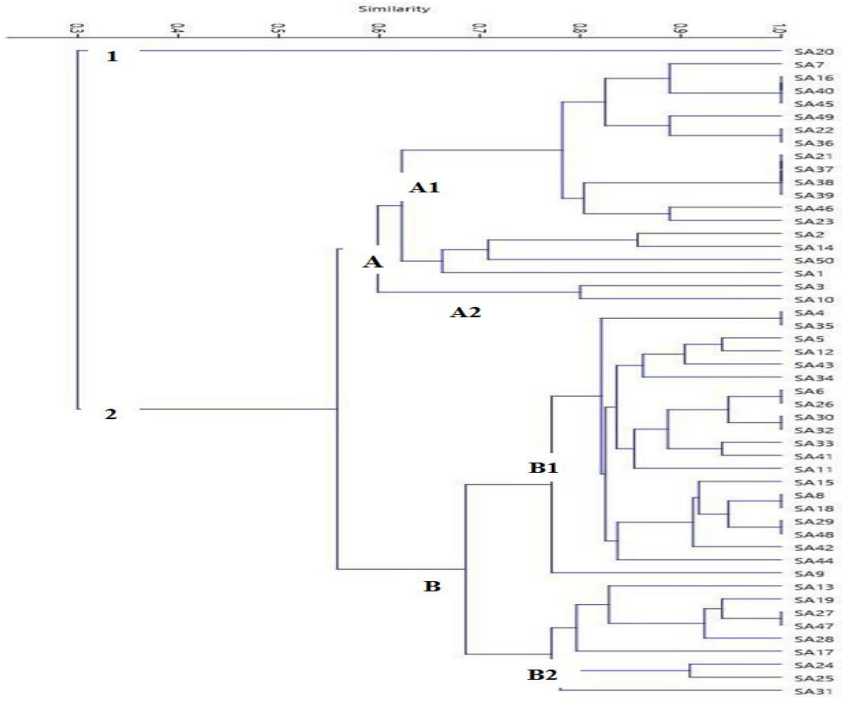

Fig. (3): Dendogram for AST results to *Staphylococcus aureus* bacteria.

P-ISSN: 2664-0562
E-ISSN:2664-0554

Detection of Biofilm Formation

The results showed that 100% of all isolates were biofilm producers in different degrees compared to the negative control, Table (7). This result is in agreements with the study conducted by Hatem et al. [26] which found that all of *S. aureus* isolates were capable to produce a biofilm.

Table (7): Biofilm formation on MTP.

Biofilm	Wound	burns	Urine	Blood	Total
Strong	3 (6%)	7 (14%)	6 (12%)	2 (4%)	18 (36%)
Moderate	7 (14%)	7 (14%)	7 (14%)	1 (2%)	22 (44%)
Weak	5 (10%)	0 (0%)	5 (10%)	0 (0%)	10 (20%)
Total	15 (30%)	14 (28%)	18 (36%)	3 (6%)	50 (100%)

Among our biofilm producers isolates 36% and 22% were strong and moderate biofilm producers respectively, this agree with Parastan et al. [23] study that found 39.91% and 20.67% isolates were strong, and moderate respectively, The reason for the variance in the results between studies may be due to the differences in the type of medium used or at incubation period difference, as if the incubation period is increased, the cellular density of the biofilms increases, or the reason may be the difference in the size of the sample taken [27].

Biofilm formation by the bacterial isolates under study is one of the most widespread virulence factors, because it indicates the presence of bacteria in contaminated and pathogenic environments [28]. The biofilm works to provide protection against host immune defenses, and thus works to concentrate nutrients and protect them from antibiotics and phagocytic cells [29].

Conclusion

Antibiotic resistance was shown to be highest against Oxacillin and lowest against Vancomycin, respectively. Results of this study suggest that Oxacillin should not be utilized as a first-line treatment for *S. aureus* infections. In addition, the percentage of biofilm-producing isolates that were multi drug-resistant (MDR) was much greater than non-biofilm forming.

P-ISSN: 2664-0562
E-ISSN:2664-0554

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P-ISSN: 2664-0562

E-ISSN:2664-0554