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The Association of Biofilm Formation and Antibiotic Resistance in Staphylococcus Aureus Isolated from Otitis Media in Najaf Governorate

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Keywords: Staphylococcus aureus, Otitis media ,biofilm formation, antibiotic resistance, intracellular adhesion operon (A,B,C and D that encoding biofilm formation.

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Abstract

Background: antibiotic resistance and biofilm formation in Staphylococcus aureus Otitis media is an inflammation of the middle ear and the tympanic membrane that often ensues upper respiratory tract infection it is causes a spectrum of diseases If inadequately treated, it results in numerous severe problems. The aim of the study was to Assess biofilm formation using the microtiter plate method and establish presence. ica operon genes that encoded of biofilm production in some strains of Staphylococcus aureus and the correlation between biofilm formation and antibiotics resistance.

Patients and methods: Out of 130 otitis media isolates, we get 40 isolates of staphylococcus aureus and identified by using conventional technique then, confirming by Vitek 2compact system technique. then performed an antibiotic susceptibility test by using the automated identification usingVITEK2® compact system (BioMérieux, France), Following that, we used the microtiter plate method (MTP) to demonstrate the isolates' capacity to formation of biofilm. Following that, we carried out an identified to ica operons (A,B,C and D that encoding biofilm formation by using polymer chain reaction (PCR).

Results: after analysis of results all isolates resistance to beta lactam at varying resistance to another antibiotics and as a following: fusidic acid (80%), tobramycin (62.5%), tetracycline (45%), erythromycin (42.5%),gentamycin (42.5%), clindamycin (37.5%) and Rifampicin (35%) respectively .while all of isolates able to formation of biofilm and difference rates as a following (27.5%)as a strong isolates and (72 .5%) as a moderate isolates respectively, The molecular analysis of the ica operon found that ica A was detected in 6(15%) isolates, ica B in 3 (7.5%) isolates, and ica D in 19 isolates (47.5%). Furthermore, 6 isolates (15%) harbored and 2 (5%) isolates ica A and ica Band D, respectively. All isolate of S.aureus which taken from otitis media patients by physicians of Ear, Nose and Tonsillitis.

Conclusion: All isolates appearance high resistance to antibiotic that consider at serious health problem to individuals and populations, The findings indicated a correlation between treatment resistance and the existence of biofilm, It lowers the rate of healing or response to treatment and makes treatment more challenging and The low level of ica operon (A,B,C and D) in current study and non-correlation of biofilm formation but, entrance to detect typing of biofilm.

العلاقة بين تكوين الغشية الحيوية ومقاومة المضادات الحيوية في بكتريا المكورات العنقودية الذهبية المعزولة من التهاب الاذن الوسطى في محافظة النجف الاشرف

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الخلاصة

المقدمة: التهاب الاذن الوسطى هو التهاب يصيب الاذن الوسطى وغشاء الطبلة ، وغالبا ما يتبع عدوى الجهاز التنفسي العلوي. ويسبب مجموعه من الامراض والمشاكل الخطرة اذا لم يعالج بصورة كافية .هدفت الدراسة الى تقييم تكوين الأغشية الحيوية باستخدام طريقة صفيحة الميكرو تيتر وتحديد وجود جينات الاوبيرون ica المشفرة لا نتاج الاغشية الحيوية في بعض سلالات المكورات العنقودية الذهبية ، والعلاقة بين تكون الغشية الحيوية ومقاومة المضادات .

المرضى وطرق العمل: من بين 130 عينة مشخصه كالتهاب اذن وسطى ، حصلنا على 40 عينة من المكورات العنقودية الذهبية ، تم تشخيصها باستخدام الطرق التقليدية المتبعة وتم التأكد منها بواسطة جهاز الفايتك المصنع بواسطة شركة بايومريو الفرنسية .ثم اجرينا فحص الحساسية الدوائية باستخدام جهاز الفايتك ايضا بعد ذلك استخدمنا طريقة لوحة الميكروتيتر لا ثبات قدرة هذه العينات على تكوين الاغشية الحيوية ،واخيرا استخدمنا طريقة pcr المتسلسل لتحديد وجود ica operon A,B.C and D الذي يشفر تكوين الاغشية الحيوية في بعض العينات.

النتائج: بعد تحليل النتائج تبين ان كل العينات مقاومة للبيتا لاكتام وبدرجات متفاوته للمضادات الاخرى وكما يلي حامض الفيوسدك اسيد 80%،التوبرامايسين62.5%،التتراسايكلين 45%،الارثرومايسين والجنتاميسين بنسبة 42.5% ،الكلنداماسين 37.5% الريفامبيسين 35% على التوالي. فيما ظهرت جميع العينات القابلية على تكوين الاغشية الحيوية بدرجات متفاوته وكما يلي 27.5% كعزلات قوية و 72.5% كعزلات معتدلة على التوالي .في حين ان الدراسة الجزيئية لجينات ica operon بينت وجود ica A في6 عينات اي بنسبة 15% ،ica B في 3 عينات بنسبة 7.5% ، ica D في 19 عينة اي بنسبة 47.5% في حين لم تحدد اي عينة تحتوي على ica C عينة .علاوة على ذلك هنالك 6 عينة اي بنسبة 15% و 2عينة اي بنسبة 5% من فصيلة ica A D وica B D على التوالي .جميع عزلات المكورات العنقودية الذهبية ما خوذه من مرضى التهاب الاذن الوسطى من قبل اطباء الانف والاذن والحنجرة.

الاستنتاج: اظهرت جميع العزلات مقاومة شديده للمضادات الحيوية مما يشير الى مشكلة صحية كبيره للفرد والمجتمع، واشارت الدراسة الى وجود علاقة بين مقاومة المضادات الحيوية ووجود الاغشية الحيوية، حيث انها تقلل من معدل الشفاء والاستجابة للعلاج وتجعل العلاج اكثر تحديا، كما ان انخفاض مستوى ica operon في الدراسة الحالية وعدم ارتباط تكوين الاغشية الحيوية ، يعد مخلا لتحديد نوع الاغشية الحيوية

1. Introduction

Otitis media is an inflammation of the middle ear and tympanic membrane that frequently follows an upper respiratory tract infection. It encompasses a spectrum of conditions, including acute otitis media, otitis media with effusion, and chronic suppurative otitis media. persistent suppurative otitis media is defined by recurrent ear discharge through a perforated tympanic membrane resulting from persistent inflammation of the middle ear and mastoid cells. It is the principal cause of preventable hearing damage in developing nations.(Sonbol *et al.*, 2022);(Khairkar *et al*., 2023). Otitis media represent a significant chronic illness in countries with low or middle incomes, manifesting 2 to 8 times more commonly in compare with developed nations(Bhatia *et al.,* 2024). This disorder poses a substantial public health concern due to its high prevalence and the huge financial burden it imposes on patients, communities, and healthcare systems. The predominates etiology of otitis media are microbial infection of the tympanomastoid cavity that including Bacterial, viral and fungal infection. Bacterial infection is the most predominant and according to studies *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus* spp., and *Klebsiella* spp. are the most commonly isolated aerobic pathogens (Abdulkareem and Qahtan, 2023); (Khairkar *et al*., 2023). The rising resistance of Staphylococcus aureus to beta-lactam antibiotics and other antimicrobial agents poses a significant health threat to individuals and populations(Liu et al., 2016);(Gonzalez Rivero, 2023). Staphylococcus aureus possesses the capability to produce several pathogenic components, including derived enzymes, toxins, adhesion proteins, and biofilms. Biofilm production transpires in two primary stages: first, microorganisms adhere to a substrate's surface, and subsequently, cells aggregate via intercellular adhesion. Strains of S. aureus involved in biofilm-related infections may have distinct genetic origins, leading to the production of various virulence factors during the infection process(Mahmood *et al*., 2022) .Biofilm development appears to be linked to various regulatory variables, including polysaccharide. Biofilm development appears to be linked to various regulatory variables, including polysaccharide intercellular adhesion (PIA), which is synthesized and regulated by the intercellular adhesion (ica) ADCB operon(Mahmood *et al.*, 2022).Its gene products facilitate the synthesis and transport of extracellular polysaccharide adhesions that assist in the adhesion of bacterial cells to one another, to host cells, and to surfaces. Treatment for otitis media is sometimes predicated solely on clinical diagnosis, which can precipitate antibiotic resistance and elevate the risk of avoidable consequences, including hearing loss and meningitis. Currently, there is a paucity of data regarding the prevalence and molecular epidemiology of staphylococcal ear infections in hospitals in Najaf. **Aim of the study:** This study sought to Isolation and identification of staphylococcus aureus from ear infections(chronic otitis media), assessing the antibiotic susceptibility profile by vitek-2 system, evaluate the biofilm production ability of staphylococcus aureus isolates, Investigating the presence of

adhesion genes (*ica A, B, C, D*)that detect the types of biofilm and investigation of correlation between antibiotic resistance and biofilm formation .The purpose of this aims to provide data base of otitis media in Najaf governorate.

2. Materials, Patients and Methods

2.1. Patients' Constant and Enrollment

Between October 2023 and March 2024, a total of 130 ear discharge swabs were collected for this study. The specimens were obtained from patients with chronic otitis media attending the Ear, Nose, and Throat (ENT) and clinical outpatient departments at Al-Sader Teaching Hospital and Al-Manathera General Hospital. All Participants were suffering from chronic middle ear infections. Data on age, sex, duration of discharge , antibiotics or steroids treatment, recent surgery, and presence of chronic or immune-related conditions, (cancer ,autoimmune disease), were collected from each study participant prior to sample collection .

2.2. Inclusion Criteria

Patients of any sex or age presented with signs and symptoms of middle ear discharge (unilateral or bilateral) persisting for more three-month. patients with chronic otitis media must not receive systemic or topical antibiotics during the past week were excluded. chronic otitis media patients who agreed or for whom a legal guardian had agreed to participate in the study.

2.3. Samples Collection

From October 2023 to March 2024, a total of 130 clinical specimens were collected during this study from the Ear, Nose, throat and the Clinical outpatient department from Al Sader Teaching ENT Hospital and Menathraa. General Hospital were the resources of that current study. Were aged from 3 to 60 years. A total of 120 clinical specimens were collected during this study From Patients who suffered from middle ear infection. Swab was taken from infection area and then transported by transport media to the College of Medicine, Jaber Ibn Hayan for the laboratory examination. Data about type of infection, sex and age of patients were recorded on the swab. Specimens were collected from patients who did not receive antibiotic for one week before sample. Samples were collected in sterile wide mouthed containers and then streaked by loop on plates of blood agar and mannitol salt agar.

2.4. Biochemical and Standard Assay Methods

The following techniques were used to investigate each different diagnosis in current study:

cultivation of the specimens, standard assays were performed, including Gram staining and various biochemical tests such as mannitol fermentation on mannitol agar, beta hemolysis on blood agar, coagulase, and catalase tests, to identify Staphylococcus aureus(De la Maza et al., 2020);(Tille, 2021).

A conformation test for Staphylococcus bacteria was subsequently performed utilizing the automatic identification provided by the VITEK2® compact system (BioMérieux, France), recognized as a precise approach for determining the type and strain (Weinstein and Lewis, 2020).

2.5. Antibiotic Sensitivity Test of S.Aureus By VITEK2 Compact System

The automated Vitek® 2 systems are employed for the identification of antibiotic susceptibility testing (AST). The Vitek® 2 systems determined the antibiotic susceptibility profile for all S. aureus isolates in accordance with the Clinical and Laboratory Standards Institute guidelines. The Vitek® 2 compact system's antibiotic sensitivity cards were utilized to ascertain minimum inhibitory concentration (MIC) values. The cards utilized in this trial are designated AST-GP 580 and contain 15 antibiotics. The antibiotics were offered in various concentrations. The turbidity level was calibrated to (0.5–0.63) McFarland and quantified using a Densi Chek plus turbidity meter. Following 6–12 hours of incubation, findings were interpreted and subsequently processed using the Vitek® 2 Compact System card (Weinstein and Lewis, 2020).

2.6. Biofilm Formation Assay

The microtiter plate (MTP) technique was employed to assess the biofilm formation capability of each isolate(Zhao *et al.*, 2023)The isolate was inoculated in the broth medium and cultured for approximately 18 hours at 37°C in a static condition prior to being diluted 1 in 100 with fresh 3% glucose brain heart infusion broth medium. 0.2 ml aliquots of the diluted culture were deposited into many wells of a sterile polystyrene 96-well flat-bottom culture plate, with broth utilized as a control for sterility and non-specific medium binding. The tissue culture plate was incubated for eighteen to twenty-four hours at 37°C. Following incubation, the contents of each well were carefully extracted by tapping the plate. The wells were rinsed four times with 0.2 cc of phosphate-buffered saline (pH 7.2) to remove free-floating planktonic bacteria. Sodium acetate (2%) was employed to fix the biofilm created by adhering "sessile" bacteria on the plate, whereas crystal violet (0.1% w/v) was utilized for staining. Following the application of deionized water to eliminate residual discoloration, the plates were allowed to dry. Bacterial adhesion was consistently demonstrated using crystal violet staining, resulting in biofilm formation on both sides of the wells. The optical density (OD) of stained adhesion bacteria at 570 nm was assessed using a micro-ELISA auto reader. Classification of Bacterial Biofilm Formation by Microtiter Plate (MTP) (Sultan and Nabiel, 2019) Table1.

Table1: Distribution Results of Biofilm in Current Study

Formation of biofilm	Acceptance	Mean OD Values
Non/Weak	**Non/Weak**	**< 0.120**
Moderate	**Moderately**	**0.120-0.240**
Elevated	**Strong**	**> 0.240**

2.7. DNA Extraction of S.Aureus

DNA is extracted with S.aureus colonies by the boiling method in current study. Bacteria was grown for 18-24 hours ,then centrifuged for 2 minutes and washing by 0.85% sodium chloride solution (0.85%Nacl

)then, centrifuged again.the pellet is re suspended in 1ml of Tris-EDTA Buffer (TE) and boiled for 15 minutes (Araújo et al., 2004);(Zou et al., 2019).

Cells lysis is heated to 85 °C for 20 minutes then freezing for 10 minutes and centrifuged at 1000 rpm for 10 minutes. the supernatant and represent extraction of DNA.

2.8. Molecular Detection of Ica ABCD Operon

Biofilm-associated genes including ica operon (A, B, C and D) were examined via standard PCR reaction. List of the used primers are summarized in Table2. PCR reaction was carried out in 25µl of Premix (Promega -USA). The mixture comprised of 12.5µl all master mix, 2µL of each primer, 3.5µl nuclease free water, 5 µl template DNA. The thermal profile included initial denaturation at 95°C for 5 min, followed by 34 cycles of 94°C/ 30 s of denaturation, 54/30sec of annealing (different for each gene) and 72/1min and final extension of 72/5minute. PCR products were electrophoresed in 2% gel agarose in 1X TBE buffer and then melted in microwave until the solution becomes clear. Once the a agarose was cooled to 50-55°C, 5µl of simply safe dye (10 mg/ml) was added to 100 ml of melting a agarose gel to get final concentration 0.5µg/ml (Yala et al., 2011)The agarose was poured into the gel tray with sealed ends, the comb was positioned correctly, and then it was allowed to dry. The samples were placed in a distinct well of the gel, with a marker in another well. Electrodes were properly attached, and the run was executed in accordance with the gel percentage and dimensions. The duration for agarose gel electrophoresis is 45 minutes for genomic DNA and 1 hour and 30 minutes for PCR.

Table2: List of Oligonucleotide Primers Synthesized by Microgen, Korea

Primers	Primer direction	primer Sequence (5-3)	Base pair size	reference
Ica A	IcaA F	TGATCCAAAACTTGGTGCAG	306 bp	(Setiabudy *et al.,* 2023)
	IcaA R	TCTGGAACCAACATCCAACA		
Ica B	IcaB F	TGCAGATGACGATCCACCTA	378 bp	
	IcaB R	TTTTCTTCCCCAACATGACC		
Ica C	IcaC F	CGGCATGATATTGCGTGAAT	566 bp	
	IcaC R	TGAAAACTGAAAAGCTGACTGA		
Ica D	IcaD F	ATGGTCAAGCCCAGACAGAG	335 bp	
	IcaD R	CGAAAATGCCCATAGTTTCAA		

2.9. Statistical Analysis

The current data have thus been analyzed on Statistical Package for the Social Sciences (SPSS) version 21. The differences between the categories.have been ascertained applying Chi-square test with a considered cut-off 5% and 1% (significant level at $P \leq 0.05$ and ≤ 0.0001 respectively)(Berghoff et al., 2016).

3. Results

3.1 Collection and Identifications

The results of this study indicate that gram-positive bacteria (G+ve) are more prevalent than gram-negative bacteria,(G-ve).these results comparable with studies occurring in Duhok Governorate of Iraq in 2021 and Kirkuk Governorate of Iraq in 2023 (Agha and Al-Delaimi, 2021);(Al-Jubouri and Dahham, 2023).the results is 57%as gram positive bacteria (G+ve), 43% as a gram negative bacteria (G-ve).and (65.5%)as a gram positive bacteria (G+ve), 34.5% as gram negative bacteria (G-ve) respectively. In the other hand, they were distribution of difference type of bacteria in these study can be Staphylococcus aureus had the highest number, as (40) bacterial isolates were obtained, representing 33.3%, P.aerogenosa (26)21%,, Streptococcus pneumonia(12)10 %, , Escherichia coli(11)9%,, Enterococcus fecalis(9)7.5% , Proteus(8)6.6%,, Klebsiella Spp (6)5%, , S. epidermis and S. lantus (3)2.5%,, respectively. Fig.1.

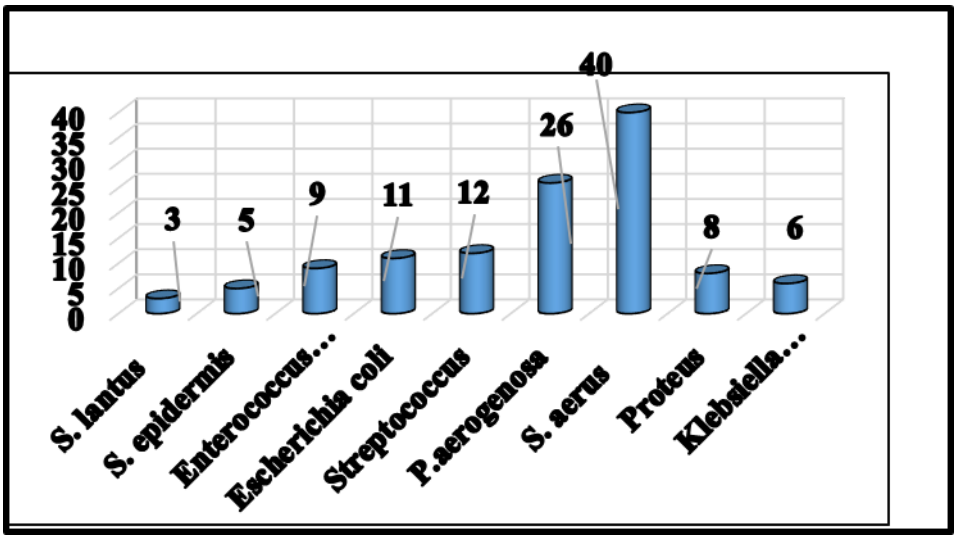

Figure1: Distribution of Bacterial Isolates Identified in The Study Samples. The figure shows the frequency of different bacterial species isolated, including *Staphylococcus aureus* (n = 40), *Pseudomonas aeruginosa* (n = 26), *Streptococcus* spp. (n = 12), *Escherichia coli* (n = 11), *Enterococcus* spp. (n = 9), *Proteus* spp. (n = 8), *Klebsiella* spp. (n = 6), *Staphylococcus epidermidis* (n = 5), and *Staphylococcus haemolyticus* (n = 3).

These study appearance S.aureus as high rate of a causative agent compare with another type of causative agents in similar to studies occurring in Kirkuk Governorate of Iraq in 2023 and in tanta of Egypt in 2022 Agha & Al-Delaimi, 2021);(Sonbol *et al.*, 2022). The colonies of *Staphylococcus aureus* grown on blood agar appeared as opaque, white to creamy formations, measuring approximately 1–2 mm in diameter. Hemolysis activity was confirmed through complete lysis of red blood cells, indicating β-hemolysis. Isolates

capable of fermenting mannitol were preliminarily identified as *Staphylococcus aureus*. These isolates strains formed large, yellow -colored colonies encircled by wide yellow zones on the medium, which changed from pink to yellow, indicating mannitol fermentation. Microscopic examination confirmed that the *Staphylococcus aureus* isolates were gram-positive, cocci-shaped organisms typically arranged in grape-like clusters, Identification of the isolates was confirmed using the coagulase test. Isolates that produced a positive coagulase result—evidenced by their ability to clot human plasma—were classified as *Staphylococcus aureus*. This is due to the fact that coagulase is a distinctive enzyme produced solely by *S. aureus*, For further confirmation of identification, the VITEK® 2 system was employed. This automated system delivers precise results based on colorimetric readings derived from a range of biochemical tests. It is especially effective in distinguishing among closely related bacterial species due to its high level of specificity. Analysis using the VITEK® 2 system with GP-ID and 47 biochemical tests. The isolates of 40 isolates of *Staphylococcus aureus* were identified after 6 hours as *S. aureus* with a probability rang most of result that reveals confirmation test may be probability at 100% to conventional biochemical test also, these study similar to studies which , using VITEK® 2 system as codification identification(Agha and Al-Delaimi, 2021);(Al-Jubouri and Dahham, 2023, Vinshia et al., 2024). the reasons of high rate of S.aureus may be return to commensalism nature and nosocomial , multi-drug resistance of antibiotics (MDR), ability to form biofilms and a variety of genetic (1). In the current study, the majority of S. aureus isolates were isolated from otitis media 40/120 (33.3%). after data analysis of results in the current study, conducted the rate of male more effect to chronic otitis media in compare with female, the rate of these study male23(57.5%) and female 17 (42.5%). This may have been stated by differences between the sexes in anatomy, life - style, behavior, socio-economic status and genetic differences between male and female (Afolabi *et al*., 2012);(Mohanna and Bahannan, 2016).

3.2 Antibiotics Susceptibility Test

In current study appearance all Staph. aureus strains were resistant to beta lactam antibiotics indicating that they were methicillin resistant Staphylococcus aureus (MRSA). The high rate of resistance in the current study records in the following: benzyl penicillin and oxacillin, with both exhibiting (100%) resistance. This was followed by fusidic acid (80%), tobramycin (62.5%), tetracycline (45%), erythromycin (42.5%), gentamycin (42.5%), clindamycin (37.5%) and Rifampicin (35%) respectively .in the other hand, records high rate of sensitive in the S.aureus as a following: Linezolid (100%), Tigecyclin (97.5%),Trimethobrin & sulfamethaxzol (92%), ,and Levofloxacin, Moxifloxacin (87.5%) and (77.5%) respectively ,figure (4-11) illustrate distribution of antibiotic susceptibility test in current study. The results indicate that the majority of bacterial isolates exhibited resistance to most drugs. Infections caused by *S. aureus* have grown increasingly challenging due to the rise of multidrug-resistant forms, particularly MRSA, which poses a significant issue for healthcare providers globally (Mohanna and Bahannan, 2016); (Bassetti *et al*., 2022);(Dawaiwala *et al*., 2023).Table3and Fig.2, illustrate distribution of antibiotics susceptibility test.

Table3: Antibiotic Resistance Patterns of Staphylococcus Aureus Isolates (P < 0.001)

Type of antibiotic	Resistance	sensitive
Benzyl pencillin	100%	0%
Oxacillin		
Gentamycin	42 .5%	57.5%
Tobramycin	62.5 %	37.5 %
Levofloxacin	12.5%	87.5%
Moxifloxacin	22.5%	77.5%
Erythromycin	42.5 %	57.5%
Clindamycin	37.5%	62.5%
Teicoplanin	0%	100%
Linzolide	0%	100%
Tigecyclin	2.5 %	97.5%
Tetracyclyne	45%	55%
Fucidic acid	80%	20%
Rifampicin	35%	65%
Trimethoprine&Sulfamethaxzol	8%	92%

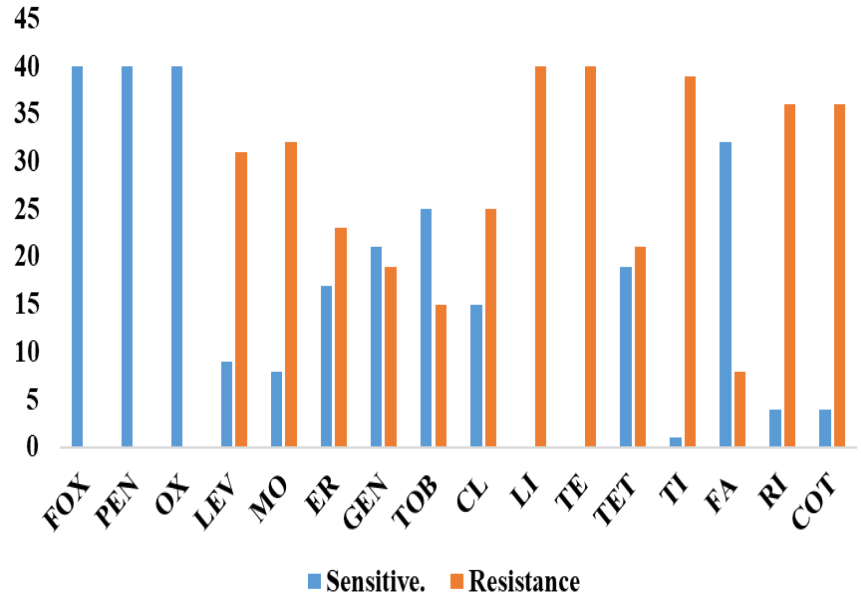

Figure2: Antibiotic Resistance and Susceptibility Patterns of *Staphylococcus Aureus* Isolates. The figure illustrates the percentage distribution of *Staphylococcus aureus* isolates showing resistance and susceptibility to the tested antibiotics. Resistance rates were generally higher for several commonly used antibiotics, whereas lower resistance and higher susceptibility were observed for others. Statistical analysis revealed a highly significant difference in resistance patterns among the tested antibiotics ($p < 0.001$). FOX: Cefoxitin; PEN: Benzylpenicillin; OX: Oxacillin; LEV: Levofloxacin; MXF: Moxifloxacin; ERY: Erythromycin; GEN: Gentamicin; TOB: Tobramycin; CLI: Clindamycin; TET: Tetracycline; TIG: Tigecycline; LZD: Linezolid; TEC: Teicoplanin; FA: Fusidic acid; RIF: Rifampicin; COT: Trimethoprim–sulfamethoxazole.

All isolated of S.aureus in current study were resistance to all beta lactam antibiotics benzyl penicillin and oxacillin these result similar to studies conducted in Iraq (Raheema *et al.,* 2021);(SA et al., 2023).the reason for this result The resistance is due to The emergence of extended-spectrum beta-lactamase (ESBL) enzymes produced by Staphylococcus aureus. These may be chromosomal or plasmid in origin, functioning to hydrolyse the beta-lactam ring or by mutations in carboxy/transpeptidase or penicillin-binding proteins (PBPs), which are involved in the last stages of peptidoglycan production, leading to diminished efficacy(Farhan et al., 2020);(Raheema and Qaddoori, 2020) (Bassetti *et al*., 2022. In the current study appears resistance to Aminoglycoside Tobramycin and Gentamycin were about 62.5 % and 42.5%,respectively.Theis study compatible to another studies occurring in Iraq (Al-Jubouri and Dahham, 2023) appearance high resistance to Tobramycin is 93 %and result of Gentamycin was conducted (Heyar *et al.,* 2017).resistance at rate of 40% the reasons resistance to Aminoglycoside returned to Staphylococcus aureus produced to B-lactamase enzymes or the cause may be due to the occurrence of a mutation(Al-Jubouri and Dahham, 2023); (Heyar *et al.,* 2017) .The resistance rate of fluoroquinolones which include Moxifloxacin and Levofloxacin in current study at 22.5 and 12.5 respectively, This study compatible to studies in Iraq but, difference in results occurring (may be resistance at 0%to Moxifloxacin and (Mohammed *et a*l., 2022).the result may be 31.6%to Levofloxacin. Resistance to fluoroquinolones in *S. aureus* outcomes from mutational alterations in the gyrA and gyrB (topoisomerase II) and parC (grlA) 2024 (topoisomerase IV) genes. and enhances the formation of chromosomally encoded efflux pumps(Yahya et al., 2024);(Hajhamed *et al*., 2023).The resistance rate to erythromycin in current study was 42.5%.in compare with another results of studies conducted in Iraq (Al Ani and Al Meani, 2018).that reported a resistance rate at 53%. Resistance of Staphylococcus aureus to erythromycin frequently results in inducible clindamycin resistance, culminating in treatment failure. This intricate phenomenon encompasses many pathways within the Erm gene family, comprising around 40 types. These genes can induce resistance to a broad spectrum of antibiotics, including macrolides, lincosamides, and streptogramins, by mechanisms such as efflux pumps, enzymatic modifications, or changes in the ribosome binding site (resulting from mutations or methylation in the 23S rRNA unit) (Mahfouz *et al.,* 2023). The resistance rate of Clindamycin in current study at 37.5% in compare with another study occurring in Iraq (Al Ani and Al Meani, 2018). resistance rate at 47%. The resistance rate appear at high level against Fucdic Acid in current study at 80% and compare similarity with other study occurring in Iraq (34)resistance rate at 80%. Strains of Staphylococcus aureus have exhibited increased resistance to fusidic acid in recent years. This resistance results from either plasmid-mediated reduced permeability of bacterial cell wall or membrane, or from spontaneous mutations in the gene producing EF-G (fusA), hindering fusidic acid's ability to bind to EF-G and eliminate the bacterium. (Hajhamed *et al*., 2023) . The resistance rate of Tetracycline in current study at 45% several studies occurring in Iraq high resistance

rate occurring ((Mahmood et al., 2022), (Al-Jubouri and Dahham, 2023) ,(Al-Hasnawy *et al*., 2019) .(74% ;74.4% and 93%), respectively. The resistance rate of Trimethoprine & Sulfamethaxzol at 8% that difference result rate in compare with another studies occurring in Iraq (Yahya *et al.*, 2024); (Al-Jubouri and Dahham, 2023) the result resistance rate at 40% and 70%, respectively. While other study occurring (Al-Khafaji, 2018).in Iraq appear result of resistance rate at 0%. The resistance rate of Rifampicin in current study at 35% these results incompatible with other study occurring in Iraq (Fadhil and Mohammed, 2022)at resistance rate at 20%. All Staphylococcus aureus isolates exhibited a high sensitivity of 97.5% to linezolid and tigecycline. These results correspond with the studies conducted in Iraq(Mohammed *et al*., 2022). the sensitive rate at 100% also another study occurring in Iran (Kananizadeh *et al.,* 2019) appear result as well as sensitive rate at 100%.The sensitive rate of teicoplanin at 100% in current study.in compare with another result of study occurring in Iraq (Rasheed and Hussein, 2020).may be similarity, Linezolid, tigecycline, and teicoplanin were regarded as the most potent antibiotics against MRSA strains. according to several studies designed to treatment of MRSA (Al-Khafaji, 2018); (Agha and Al-Delaimi, 2021).

3.3. Biofilm Formation

All isolates of *S. aurus* in the current study able to formation of biofilm in microtiter plate (MTP) method that consider the most method to demonstrate biofilm. with variations in intensity classified strong as 27.5% and moderate as 72.5% Table4 illustrates rate of biofilm distribution in current study.

Table4: Biofilm-Forming Ability of Staphylococcus Aureus Isolates Assessed by The Microtiter Plate Method

Biofilm formation degree	**Frequency *S. aureus* isolates**	***Mean* + SD -**	**P- value**	**95% confidence interval**	
Strongly biofilm formation	11 (27.5%)	0.22+0.16 -	0.00001	lower	upper
				0.11	0.19
Moderately biofilm formation	29 (72 .5%)	0.07 + 0.03 -		0.09	0.22

Biofilms play a pivotal role in the pathogenesis of middle ear infections. Compared to their planktonic counterparts, biofilm-associated bacteria exhibit distinct morphological and physiological traits, enhanced resistance to environmental stressors, greater tolerance to antimicrobial treatment, and evasion of host immune defenses (Fadhil and Mohammed, 2022)These colonizing bacteria synergistically form a cooperative microenvironment that facilitates the persistence and spread of infection, contributing to its chronicity (Rather et al., 2021);(Zafer et al., 2024). Methicillin-resistant *Staphylococcus aureus* (MRSA) has adapted to increase biofilm production in response to selective pressures encountered in both clinical and community settings. Among biofilm-associated infections, staphylococcal species—particularly MRSA—are recognized as the most prevalent, accounting for over 80% of all documented MRSA infections in humans (Moulic *et al*., 2024). The investigation into biofilm production among MRSA isolates yielded results consistent with

previous studies, indicating that MRSA strains possess a strong propensity for biofilm formation. This capability is linked to the synthesis of a glycocalyx—a viscous extracellular layer composed of polysaccharides, polypeptides, or both—that encapsulates the bacterial cell. When loosely attached to the cell wall, the glycocalyx manifests as an irregular structure referred to as a slime layer. Alternatively, when more firmly associated, it resembles a capsule that closely adheres to the bacterial cell wall (Moulic *et al.,* 2024).The other studies occurring in Iraq (Idrees *et al.,* 2021). the record of result all S.aureus otitis media able to produce biofilm formation at 100% while distribution 70% isolates strong,15% moderate and weak at 15%. Also, another study to Methicillin resistance *Staphylococcous aureus* isolated from otitis media(Alwan *et al.*, 2021) recorded all isolates able to produce of biofilm at 18.92% isolates, moderate in 18.46%, weak in 20.00%, These findings correspond with the results presented (Araújo *et al.,* 2004)who noted analogous patterns, with robust and moderate biofilm producers constituting 13.4% and 62% of their respective samples.

3.4. Detection of Ica Operon

Detection of intracellular adhesion (ica locus) classes A, B, C and D in current study. The amplification of Ica operon revealed the presence of ica A, ica B, and ica D, while ica C was not detected. Specifically, ica A was detected in 6(15%) isolates, ica B in 3 (7.5%) isolates, and icaD in 19 isolates (47.5%). Furthermore, 6 isolates (15%) harbored both ica A and ica D. The current results are illustrated in Fig.3 and Table5.

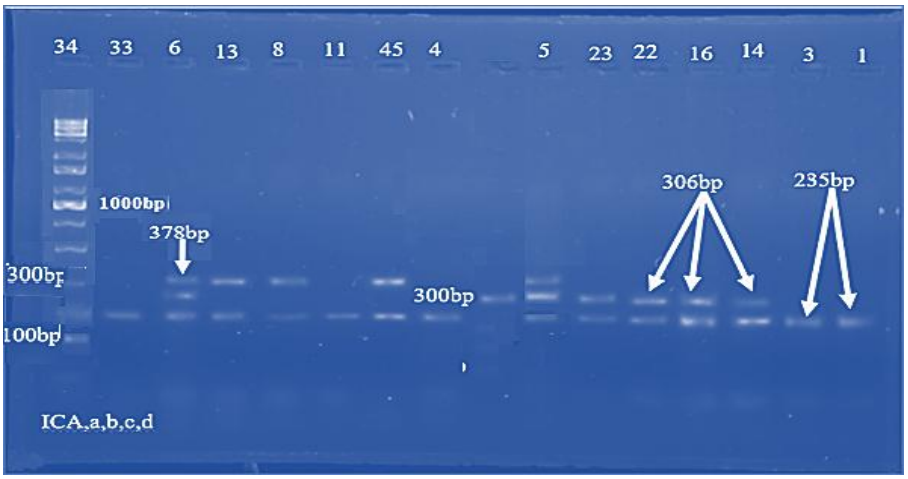

Figure3: Gel Electrophoresis of Polymerase Chain Reaction Result for Ica Gene Operon In MRSA isolates with an amplicon size of 378, 306, 235 and 300 bp. Line M: DNA marker (50-1500 bp); Lines (1-12): methicillin-resistant S. aureus isolated middle ear infections. Migrated in (1.5% agarose, TBE buffer (1x), and current 200 A with 80 volts for 50 minutes).

Table5: Distribution of *ica* Gene operon Combinations Among *Staphylococcus Aureus* Isolate

Pattern	No. of isolated	Frequency %
Ica A	6	15%
Ica B	3	7.5 %
Ica D	19	59.4%
Ica A D	6	15 %
Ica B D	2	5%

These findings are relatively consistent with a study conducted in Iran (46), which reported detection of icaA in 2 isolates (6.3%) and icaD in 19 isolates (59.4%), with no detection of icaB or icaC. Biofilm formation in *Staphylococcus aureus* represents a pivotal process in the establishment and persistence of chronic infections. Consequently, a comprehensive understanding of the mechanisms underlying biofilm development is essential for effective control and therapeutic intervention(Ghaioumy *et al.*, 2021) ;(Lister and Horswill, 2014). The ability of S. aureus to aggregate and form biofilms is primarily facilitated by the secretion of a mucoid extracellular polymeric matrix known as polysaccharide intercellular adhesion (PIA). This matrix is encoded by the ica operon, which comprises the genes icaA, icaD, icaB, and icaC (Mahmood *et al*., 2022)Among these, ica A and ica B have been extensively studied for their role in the synthesis of PIA, which consists predominantly of N-acetylglucosamine—an essential component of the exopolysaccharide matrix within biofilms. The icaD gene enhances the mucoid characteristics of the PIA, contributing to capsule formation and structural integrity of the biofilm (Mahmood *et al*., 2022).The present study identified a higher prevalence of icaD compared to other ica operon genes. This finding supports the notion that icaD plays a significant role in promoting capsular polysaccharide features, which are recognized as virulence factors in S. aureus pathogenesis. Additionally, the co-expression of icaA and icaD is crucial for the efficient production of N-acetylglucosamine, further enhancing the biofilm's extracellular matrix and virulence potential. The relatively low prevalence of *ica* operon genes observed in the present study suggests that *ica* gene expression may be influenced by environmental factors, as noted (Mahmood *et al*., 2022).Several *Staphylococcus aureus* strains are capable of forming biofilms independently of polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG). In such strains, secreted proteins and extracellular DNA can compensate for the absence of PIA/PNAG during biofilm formation (Cue *et al*., 2012);(Guzmán Soto, 2021). Notably, certain strains exhibit the ability to switch between PIA-dependent and PIA/PNAG-independent biofilm mechanisms(Guzmán Soto, 2021);(Hennig *et al*., 2007). Additionally, some researchers have proposed that wall teichoic acid (WTA) may contribute to biofilm development and reduce reliance on PIA synthesis (Patel and Rawat, 2023). Despite the overall low expression rates, *icaA* and *icaD* were detected more frequently than other *ica* operon genes. This may be attributed to the functional synergy of the transmembrane proteins IcaA and IcaD, which form an N-acetylglucosaminyltransferase complex capable of synthesizing short PNAG oligomers (less than 20 residues) that contribute to biofilm structure and capsular

polysaccharide characteristics. The co-expression of *icaA* is essential for optimal *icaD* function and the subsequent production of N-acetylglucosamine, a key component in biofilm matrix formation.

3.5 Investigating the Biofilm Production in MR*SA* Isolates and Their Association with Antibiotic Susceptibility

The results of antibiotic resistance and biofilm formation of isolates presented in Tables6 indicate that all antibiotics employed in this study exhibited resistance. demonstrates that all antibiotics resistance examined in this study.

Table6: Relationship Between S. Aureus Isolates' Biofilm Development and Antibiotic Resistance

Biofilms	**Strong**		**Moderate**	
Antibiotics	**Resistance**	**Sensitive**	**Resistance**	**Sensitive**
Benzylpenicillin	11(27.5%)	0(0%)	29(72.5%)	0(0%)
Oxacillin	11(27.5%)	0(0%)	29(72.5%)	0(0%)
Gentamicin	8 (20%)	2 (5%)	13(32.5%)	17 (42.5%)
Tobramycin	10(25%)	1(2.5%)	15(37.5%)	14(35%)
Levofloxacin	3(7.5%)	7(17.5)	6(15%)	25(62.5%)
Moxifloxacin	4(10%)	7(17.5%)	5(12.5%)	24(60%)
Erythromycin	7(17.5%)	4(10%)	10 (25%)	19(47.5%)
Clindamycin	7(17.5	2(5%)	8 (20%)	23(57.5%)
Linezolid	0(0%)	11(27.5%)	2(5%)	27(67%)
Teicoplanine	0(0%)	11(27.5%)	2(5%)	27(67%)
Tetracycline	9(22.5%)	1(2.5%)	10(25%)	20(50%)
Tigecycline	1(2.5%)	10(25%)	0(0%)	29(72.5%)
Fusidic Acid	10(25%)	0(0%)	22(55%)	8(20%)
Rifampicin	8(20%)	3(7.5%)	6(15%)	23(46%)
Trimethoprim/Sulfamethoxazole	4(10%)	7(17.5%)	0(0%)	29(72.5%)

The current investigation demonstrated that biofilm-producing isolates were linked to an increased prevalence of antimicrobial resistance and exhibited a significant percentage of benzylpenicillin and oxacillin resistance. They exhibited resistance to the majority of antibiotics, with the exception of Teicoplanine and linezolid. The results showed that MRSA isolates had a significant capacity for biofilm formation. The predominant rate of isolates exhibiting biofilm development was with no XDR or PDR isolates identified, only MDR isolates were present. These results correlate with study conducted in Karbala of Iraq(Al-Khfaji et al., 2023) .which determined that a greater prevalence of antimicrobial resistance is exhibited among biofilm producers. Out of the 31 isolates 35.4% and 51.6% were XDR and MDR, respectively. The investigation's results correspond with the results of(Elmanama *et al*., 2020) . conducted in Nepal, which determined that biofilm producers exhibit a greater rate of antibiotic resistance compared to non-producers. Biofilm-producing MRSA had a much greater prevalence (90.5%) compared to biofilm-non-producer MRSA (9.5%). Significantly, 89.2% of biofilm-producing S. aureus exhibited multidrug resistance. The results of the current investigation contradict those reported (56). in Diyala, which indicated 41 (82%) cases of

multidrug resistance (MDR), 7 (14%) cases of extensive drug resistance (XDR), and 2 (4%) cases of pan-drug resistance (PDR). Biofilm formation was assessed using the microtiter plate method, revealing robust formation at 14%, moderate at 42%, and weak at 44%. Antibiotic resistance has emerged as a significant issue due to the widespread and indiscriminate application of these drugs in treatment, especially among Staphylococcus aureus bacteria. This may result from the formation of biofilms, which enhance their pathogenic capacity(Jasim and Alzubaidy, 2022) . The ability of bacterial strains to produce biofilms is closely linked to their persistence and pathogenicity. Moreover, persistent bacterial infections, such as Chronic otitis media (COM) , are associated with biofilm formation. The capacity to produce biofilm is intricately associated with clinical strains of S. aureus, genetic lineages, multidrug resistance profiles, and extremely virulent strain. (Ioannidis *et al.,* 2023).The biofilm's protective characteristics allow the bacteria within it naturally susceptible to numerous antibiotics. Antibiotic resistance in bacterial strains within biofilms can grow by as much as 1000-fold. The resistance of bacterial biofilms to antibiotics is multifaceted, encompassing many mechanisms such as the impediment of drug penetration, a reduced bacterial growth rate, and the existence of antibiotic degradation pathways. Moreover, biofilm development provides a medium for horizontal gene transfer among bacteria, facilitating the dissemination of antibiotic resistance genes and other virulence factors. Recent scientific findings indicate that bacteria do not consistently exist as independent cells in nature; rather, they typically form a cohesive community within a resilient matrix comprised of polysaccharides, extracellular DNA, proteins, lipids, and various other constituents. Biofilms consist of bacterial aggregates encased in a self-generated extracellular matrix composed of exopolysaccharides (EPSs), proteins, and other micro molecules, including DNA. They can develop on both biotic and abiotic surfaces. Moreover, biofilm safeguards the encapsulated bacterial cells from host immune cells and antibiotics, hence promoting the prolonged life of infections. The capacity of S. aureus to generate biofilm. The primary virulence characteristic is its presence on biotic and abiotic surfaces. The biofilm formation of Staphylococcus aureus poses a significant human and animal health issue. nevertheless, the matrix may enable the accumulation of antibiotic-degrading enzymes, such as β-lactamases. Bacteria exhibiting elevated levels of chromosomal β-lactamase within biofilms would encounter diminished amounts of β-lactam antibiotics owing to the enzyme's accumulation in the polysaccharide matrix. The extracellular beta-lactamase would deactivate the antibiotic upon penetration, therefore safeguarding the deeper cells (Božić, 2024).

4. Conclusion

In the present study, *S.aureus* is the predominant and most Gram positive Bacterium in mid ear infection. All of the *Staphylococcus aureus* samples had a significant rate of resistance to other antibiotics and 100% resistance to beta-lactams. To varied degrees of resistance to another antibiotics. All of the *Staphylococcus*

aureus samples were capable of forming biofilms in varied degrees of formation. All of the of the *Staphylococcus aureus* samples that developed biofilms exhibited great resistance, there is a strong correlation between the antibiotic resistance and biofilm formations. The ICA operon was not present in each sample, suggesting that the samples in this investigation were capable of forming two different kinds of biofilms: one dependent biofilm and the other independent biofilm formation.

5. Ethical Approval

The study protocol received the approval from the Research Ethics Committee at the Faculty of the Medicine Jaber ibn Hayan and was conducted in accordance with the Hel-AND guidelines. Each participant of the study provided informed consent through a questionnaire that they completed. Consent was obtained verbally for this research

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