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Detection of Wzm gene in staphylococcus aureus ocular isolates to find out about biofilm formation and its link to antibiotic resistance

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***Abstract*---**Objectives:This study's purpose is to find out what detection of Wzm gene by PCR-based investigation in *staphylococcus aureus*isolates isolated from patients with eye infectionsto identify biofilm formation and thecorrelation with antibiotic resistance. Methods and Materials: 160 eye swabs specimenswere collected from iraq hospitals, including Ibn Al-Haitham Teaching Eye Hospital inBaghdad, Al-Imam Al-Sadiq Hospital andHilla Teaching Hospital in Babylon.Distributedaccording to types of ocular infections 119Conjunctivitis32Blepharitis (Eyelids Infection)7 Keratitis 2Dacryocystitis. These samples were collected between November 2021 and March 2022. S. aureus isolates were identified by inoculated on manitol salt agar, blood agar for primary screening of Staphylococcus spp. and then confirmed by biochemical tests and Vitek2 Compact System, and by specific primer for 16S rDNA gene of Staphylococcus spp.CLSI-2021were used to investigate bacterial antibiotic susceptibility testing, microtiter plates andWzm gene for biofilm production. Results:Among theculture-positive growth consist of 57(41.007%) bacterial isolates of *staphylococcus aureus*and all (57) isolates showedthe percentage of antibioticresistant (100%) to Cefoxitin, (96.49%)toPencillin G, (94.73%) to Oxacillin while the lowest resistant percentages were found with Nitrofurantoin (1.75%). PCR has been used to investigate Wzm gene for biofilm production instaphylococcus aureus and the results showed that 39(68.42%) has Wzm gene. Conclusion: staphylococcus aureus isolates producingbiofilm were found in high numbers in ocular isolates, the similarity of phenotypic and genotypic results ofbiofilm producing.All

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isolates were biofilm formerresistanttoCefoxitin andOxacillin (100%)which meansmethicillin resistance.

***Keywords*---**staphylococcus aureus, Wzm gene, ocular infections, methicillin resistance.

Introduction

Staphylococcus aureus is the most virulent and well-known member of the genus Staphylococci, inducing opportunistic infections and a broad range of life-threatening systemic illnesses(1). It is one of the most prevalent agents of eye infection.Eye infections have been linked to both hospital-associated (HA) and community-associated (CA) methicillin-resistant Staphylococcus aureus (MRSA). This bacterium is linked to numerous types of ocular infections, including conjunctivitis, keratitis, endophthalmitis, blepharitis, orbital cellulitis, and symptoms of dacryocystitis. Seventy percent of ocular and peri-orbital infections are caused by Staphylococcus aureus(2, 3).It is a bacterium residing in the mucous membrane and skin which has deadly pathogenic capabilities leading to different hospitalized and community infections(4, 5).

It is capable of producing several virulence factors that help to establish infection by facilitating tissue attachment, tissue invasion, and evasion from host immune response, such as enterotoxins, adhesions, hemolysis, invasions, superantigens, and surface factors that inhibit its phagocytic engulfment (6).Some virulence factors such as biofilm forming as well as The ability to acquire resistance to multiple antibiotics classes currently consider as major virulence factors and have an important role in increasing tissue damage and failure in antibiotic therapy, respectively(7).

Documentation supports the significance of biofilm generation in the pathophysiology of S. aureus and the evolution of MDR strains(8).In addition to other adhesion components, the ica operon-encoded polysaccharide intercellular adhesion (PIA) is necessary for biofilm formation in staphylococci(9). It is a phenomenon indicating aggregation bacteria, or this means that it is a group of these bacteriawhich were surrounded by a matrix of exopolysaccharides, they were it secretes exopolysaccharides to adherent microbial cells on a stationary surface (living or nonliving). The number of different pathsused in the synthesis of polysaccharides and export via the inner membrane is relatively limited.

The ATP-binding cassette (ABC) transporters a common type of active primary transporter with a wide variety of substances, from small ions to large macromolecule four structures of ABC transporters that mediate substance transport. Wzm-Wzt which one of them mediates O antigen transport for LPS synthesis, a polysaccharide is built on a lipid acceptor. Wzm gene mediates O-antigen transport for LPS synthesis also involved in biofilm production(10, 11).

Materials and Methods

Specimen's collection

One hundred and sixty eye swabs specimens were collected from patients witha bacterial eye infections from different parts of the eye, including(conjunctiva (119) samples, cornea (7) samples, eyelids (32) samples, Dacryocystitis (2) sample)for both sexes with ages ranging from (4-65) years who attended to Al-Imam Al-Sadiq Hospital,Hilla Teaching Hospital / Babylon, and Ibn Al-Haitham Teaching Eye Hospital/ Baghdad, from November 2021 to March 2022, and 20 swabs specimens were collection from healthy persons as control specimens, by using sterile swabs,using the expertise of specialists in the field of ophthalmology, taking into account that the patients did not use antibiotics, the samples were transferred to the laboratory and then inoculated on the differential and diagnostic media.

Laboratory Diagnosis

After collecting the swabs and transporting them to the laboratory. Specimens are inoculated onto blood agar and cultured for 24 hours at 37 °C before being evaluated for shape, size, color, and pigments. As a result, the samples were transferred to mannitol salt agar and incubated at 37°C for 24 hrs, a procedure that is both selective and differential. All colonies from primary cultures were purified by subculture on brain heart infusion agar (BHI) then reinoculated onto(MSA) and incubated at 37 °C for 24 h. Then one pure isolated colony was transferred to Nutrient agar medium for preservation and further biochemical tests Confirm identification of isolates (12).

Extraction DNA whole (total) genomic

The Staphylococcus aureus whole genomic DNA was extracted by using Geneaid bacterial DNA extraction (Korea kit), and DNA extraction according to manufacturer's protocol.

Polymerase Chain Reaction (PCR) Technique

In recent years, various PCR-based molecular approaches have been developed as an alternate method for precise identification. PCR is a very effective method to amplify a specific DNA.

PCR Amplification

57 samples of bacterial isolates were analyzed by polymerase chain reaction. DNA, primers, and PCR premix were thawed at room temperature, then briefly vortexed and centrifuged to bring the contents of the tubes to the bottom. The PCR mixture was made with a volume of 25 l containing the extracted DNA, primers, and PCR premix mentioned in the table (1), as well as Nuclease-Free Water and PCR Master Mix.

Table (1): Contents of the Reaction Mixture for amplification of genes in this study

No.	PCR mix		Volume
1.	Master mix		12µL
2.	Template DNA		5µL
3.	Primer	Forward Primer	1.5µL
		Reverse Primer	1.5µL
4.	nuclease free water		5µL
5.	Total volume		25µL

Primers used in this study

The primer pair for 16S rDNA gene were purchased from (Realgene/China) While for Wzm gene from (Macrogen/Korea) (Table 2) which used in this study were dissolved using sterile ddH2O. Stock solution (100 pmol/µl) was prepared by adding ddH2O to the vial containing lyophilized primer as recommended by provider and stored in deep freezer until used in Polymerase Chain Reaction (PCR) Technique, while working stock of 10 pmol/µl was made by mixing 10 µL from each primer and add 90µL ddH2O.

Table (2): The primers used in the current study

Gene Name	Primer	Sequence 5`.....3`	Amplicon (bp)	Reference
16S rRNA	Stap16SF	CCTATAAGACTGGGATAACTTCGGG	791	(13)
	Stap16SR	CTTTGAGTTTCAACCTTGCGGTCG		
Wzm	Wzm-F	TGCCAGTTCGGCCACTAAC	148	(14)
	Wzm-R	GACAACAATAACCGGGATGG		

PCR program

the PCR program done by using DNA template extracted from all isolates of S. aureus for detection the genes. The optimum conditions and temperature for the polymerase chain reaction was used for each gene shown in table (3).

Table (3): Thermal Cycler Programs Used in This Study

Gene	Temperature (°C)Time					No. of cycles
	Ini. Dent.	Cycling condition			Final Ext.	
		Denat.	Anneal.	Ext.		
Staph16S rDNA	95 °C/ 2 min.	95 °C/ 30 sec.	56 °C/ 30 sec.	72 °C/ 70 sec.	72°C/5 min.	30
Wzm	95°C/ 2 min.	95°C/ 30 sec.	58°C/40 sec.	72°C /30sec.	72°C/5 min.	35

Electrophoresis with Agarose Gel

In (1.5%) agarose gel 5µL of PCR products and promega DNA ladder (100-10,000bp) carefully loaded in the wells and electric current was matched (72 volts for 80 minutes). Gel was observed under a UV trans-illuminator to detect DNA band (PCR Products) and compare with ladder (100-10,000 bp).

Biofilm formation assay

The quantitative measurementsformation of the biofilm test by using tissue culture plate method (TCP) were done according to the method described by (15). . And results classified to absorbance into 4 categories: non, weak, moderate, and strong according to(16) this classification is demonstrated in the table (4).

Table (4): classification of S. aureus as biofilm formation

"OD" ≤ "ODc"	non-biofilm
"ODc< OD ≤ 2 x ODc"	Weakly biofilm producer
"2 x ODc< OD≤ 4 x ODc"	Moderate biofilm producer
"4 x ODc< OD"	Strong biofilm producer

*"OD" Optical density reader average of Isolate, "ODc" Optical density reader average of control.

Antibiotic test for sensitivity

The antibiotic sensitivity of S. aureus isolates to different antimicrobials determined according to Kirby-Bauer disk diffusion method on Mueller Hinton agar (MHA)(17) .Different antibiotic tablets were used in different concentrations such as Cefoxitin 30, Clindamycin 2, Ciprofloxacin 5, Chloramphenicol 30, Doxycycline 30, Erythromycin 15, Gatifloxacin 5, Gentamicin 10, Levofloxacin 5, Linezolid 30, Nitrofurantoin 300, Oxacillin 1, Ofloxacin 5, Pencillin G 10, Rifampin 5, Trimethoprim 10, Vancomycin 30. According to the Clinical and Laboratory Standards Institute (CLSI 2021) for sensitivity or resistance of the organism to each antibiotic.

Results and Discussion

Fifty-sevenS. aureus isolates obtained from a total of one hundred and sixtyeye swabs were collected from patients (males and females)with ocular bacterial infections,41 isolates obtained from Conjunctivitis,12 obtained from Blepharitis,4 obtained from Keratitis, after clinical diagnosis by the consultant physician during the period fromNovember 2021to March 2022.The results of identification by culturing are as follows [blood agar (colony with β-hemolysis)(18), mannitol salt agar (yellow colony)(19)] and gram stain(gram positive cocci) and biochemical tests(Catalase test,Oxidase test,coagulase test(20))confirmed viaamplification of specific primer for 16S rDNA gene of Staphylococcus spp.all fifty-seven isolates give +ve result for this gene (Fig. 1).

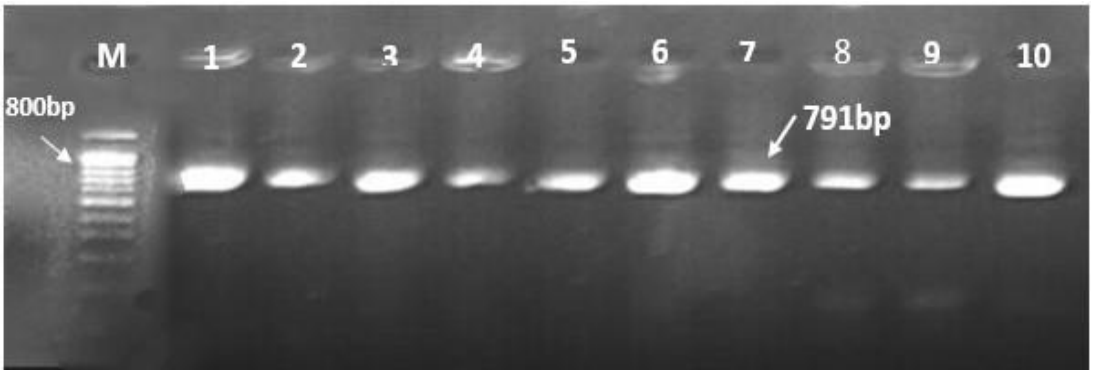

Fig. 1. 1.5%Agarose gel electrophoresis at 72 volts for 80minutes in TBE of PCR products (791 bp) after amplification of 16S rDNA gene of Staphylococcus Spp. M lane represent 100 bp DNA Marker while the rest lanes represents positive samples for gene.

A total of (160) specimens were distributed into three groups according to age, 4-18 years (31) patients, 19 – 40 years (79) patients, >40years (50) patients as shown in Table (5) and the percentage of female was 28.75%, while the percentages ofmale in this study 71.25%. The results showed that the highest rate of infection was in the age group (19 – 40 years) in the rate 49.375%,while less percentage with age (4__18 years) in the rate of 19.375%, and the infection occur in male was high than female in all age group.

Table (5) Distribution of the specimens according to Age/ years

Age/ years	Number of patients	Percentage %	P value
4__18	31	19.375%	0.000
19__40	79	49.375%	
>40	50	31.25%	
Total	160	100%	

In this study observed conjunctivitis was the dominant type of clinical manifestation of ophthalmic patients (74.37%) followed by, blepharitis (20%),keratitis (4.37%),dacryocystitis (1.25%) as shown in Table (6).

Table (6) Distribution of the specimens according to Type of Infection

Type of Infection	Number of patients	Percentage %	P value
Conjunctivitis	119	74.375%	0.000
Blepharitis	32	20%	
Keratitis	7	4.375%	
Dacryocystitis	2	1.25%	
Total	160	100%	

In this study 18 types of antibiotics were selected to test the susceptibility of the isolated bacteria used according to the (CLSI 2021) which are: [Cefoxitin (CX), Clindamycin (CD), Ciprofloxacin (CIP), Chloramphenicol (C), Doxycycline (DXT), Erythromycin (E), Gatifloxacin (GAT), Gentamicin (CN), levofloxacin (LEV),

Linezolid (LZN), Nitrofurantoin (F), Norfloxacin (NX), Oxacillin (OX), Ofloxacin(OFX), Pencillin G (P), Rifampin (RD), Trimethoprim (TMP)and Vancomycin (VA)]shown in Table (7)with theirpercentage of susceptibility pattern, which showed that the percentage of antibiotic resistant (100%) to Cefoxitin, (96.49%) to Pencillin G, (94.73%) to Oxacillin while the lowest resistance rates were found with Nitrofurantoin (1.75%).

Table (7): Percentage of antibiotic susceptibility pattern

NO.	Antibiotics	Percentage of antibiotic susceptibility pattern			P value
		S	IN	R	
1.	CX	0%	0%	100%	0.000
2.	CD	89.47%	0%	10.52%	0.000
3.	CIP	84.21%	3.5%	12.28%	0.000
4.	C	91.22%	0%	8.77%	0.000
5.	DXT	68.42%	19.29%	12.28%	0.000
6.	E	29.82%	0%	70.17%	0.000
7.	GAT	91.22%	0%	8.77%	0.000
8.	CN	63.15%	8.7%	28.07%	0.000
9.	LEV	84.21%	0%	15.78%	0.000
10.	LZN	96.49%	0%	3.50%	0.000
11.	F	98.24%	0%	1.75%	0.000
12.	NX	84.21%	0%	15.78%	0.000
13.	OX	5.26%	0%	94.73%	0.000
14.	OFX	75.43%	3.5%	21.05%	0.000
15.	P	3.5%	0%	96.49%	0.000
16.	RD	91.22%	0%	8.77%	0.000
17.	TMP	59.64%	0%	40.35%	0.000
18.	VA	96.49%	0%	3.50%	0.000
P value		0.000			

Figure (2)Showthe resultsof biofilm formation test by using tissue culture plate methodwhich are: non 17(30%), weak 12(21%), moderate 22(39%), and strong 6(10%).

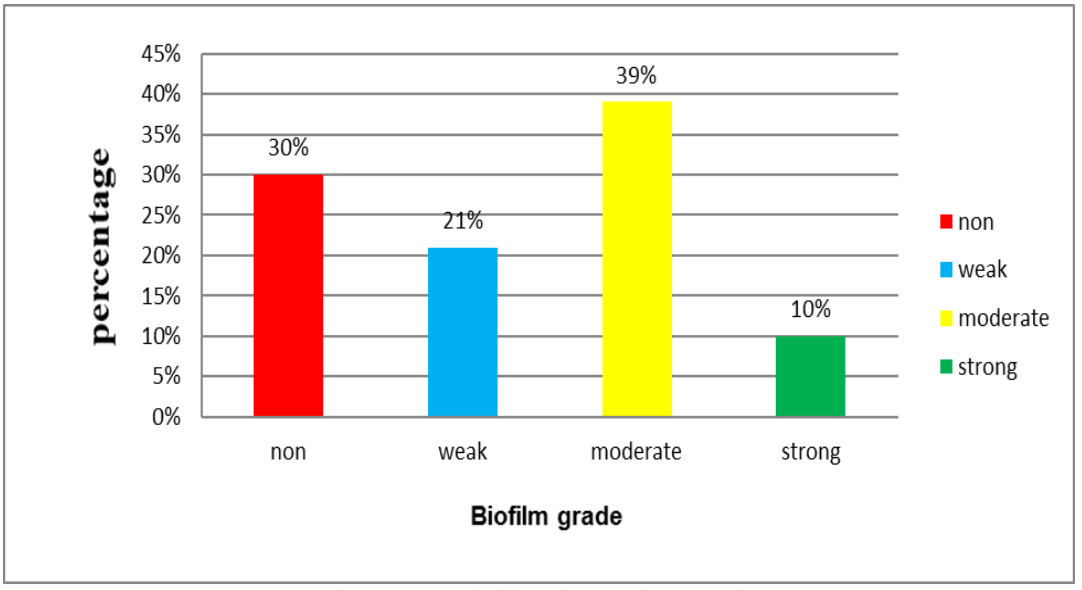

Figure (2) Biofilm formation grade

According to PCR results out of 57 bacterial isolate, presence of the wzm gene was noticed in 39(68.4%) isolate Figure (3).

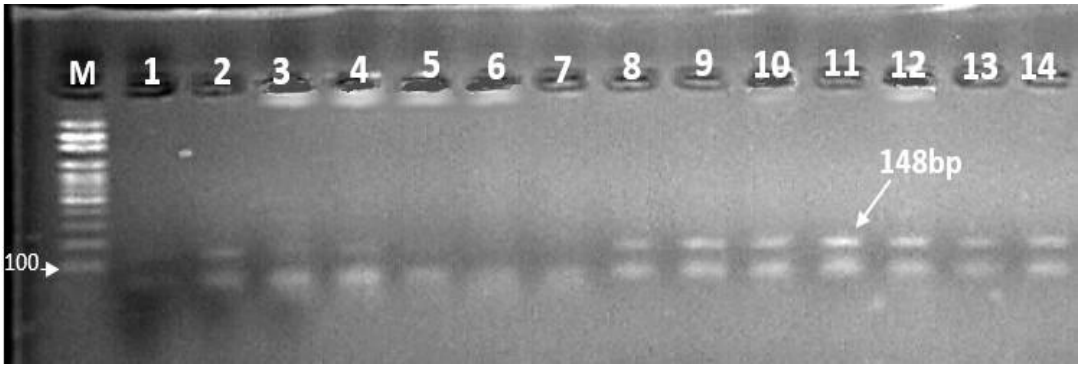

Figure (3) 1.5%Agarose gel electrophoresis at 72 volts for 80minutes in TBE of PCR products (148 bp) after amplification of PCR to wzm amplicon (148 bp), M lane represent 100 bp DNA Marker while the rest lanes (1-14) represented bacterial isolate.

Table (8) Showed the Correlation between biofilm formation and antibiotic resistance.

Table (8) Correlation between biofilm grade and antibiotic resistance

Antibiotic	Biofilm grade								*P.* value
	Weak		Moderate		Strong		none		
	No	%	No	%	No	%	No	%	
CX	6	50	12	54	2	33	17	100	0.000
CD	0	0	3	14	0	0	3	18	0.000

CIP	4	33	1	5	2	33	0	0	0.000
C	0	0	2	9	1	17	2	12	0.000
DXT	0	0	2	9	1	17	2	12	0.000
E	9	75	16	73	3	50	12	71	0.000
GAT	0	0	3	14	0	0	0	0	0.000
CN	5	41	6	27	3	50	2	12	0.000
LEV	3	25	2	9	2	33	2	12	0.000
LZN	0	0	1	5	0	0	1	6	0.000
F	0	0	1	5	0	0	0	0	0.000
NX	4	33	2	9	2	33	1	6	0.000
OX	12	100	22	100	6	100	14	82	0.000
OFX	4	33	5	23	2	33	0	0	0.000
P	12	100	21	95	6	100	16	94	0.000
RD	1	8	3	14	0	0	1	6	0.000
TMP	8	66	7	32	3	50	5	29	0.000
VA	0	0	1	5	1	17	0	0	0.000

Conclusion

The current study demonstrated the effectiveness of using genetic determinants for biofilm formationby the Wzm gene among isolates of Staphylococcus aureus, and these isolates had a high degree of biofilm-forming ability and played a role in the pathogenicity of these isolates by improving antibiotic resistance and bacterial infections are associated with bacterial biofilms. This may lead to a synergistic effect of virulence factorsand confirmed that there is an increased prevalence of MRSA strains among Staphylococcus aureuswas isolated from patients with ophthalmic infections in our study was 100%, Our isolates exhibited a high rate of resistance (100%) to Cefoxitin, (96.49%) to Pencillin G, (94.73%) to Oxacillin. There is a significant relationship between biofilm-forming and antibiotic resistance patterns.

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