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Characterization of *Staphylococcus aureus* in Conjunctivitis: Efflux Pumps and Virulence Factors

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Abstract
Pink eye or conjunctivitis remains one of the most widespread eye infections where Staphylococcus aureus which is both virulent and antibiotic-resistant stands among several infecting pathogens as a Gram-positive bacterium. This study aimed to characterize Staphylococcus aureus in conjunctivitis, focusing on antimicrobial susceptibility Patterns, efflux pumps and virulence genes. The researchers obtained 150 sample collections from patients experiencing symptoms of conjunctivitis at From Al-Nasiriyah Teaching Hospital along with Al-Haboubi General Hospital in Ophthalmology Consultant at Dhi-Qar Governorate in Iraq throughout August to December 2024. These samples received culture on blood, chocolate, and MacConkey agar media. Of the isolates, 34 (40%) were typed as Staphylococcus aureus by the Vitek2 compact system. Antibiotic susceptibility testing revealed resistance in some of the isolates, and further assessment of efflux pump activity was conducted by the ethidium bromide cartwheel method. Of the 20 resistant isolates, 7 (20.6%) were found to have efflux pump activity. Molecular analysis by PCR identified the presence of three virulence genes sigB, katG, and clfA in all efflux-positive isolates. The genes perform multiple functions which include stress response together with oxidative defense and host adhesion mechanisms and specifically contribute to Staphylococcus aureus pathogenicity of conjunctivitis. The article highlights Staphylococcus aureus's survival and adaptation through antibiotic resistance and virulence factor mechanisms and producing difficulties in bacterial conjunctivitis control.

Keywords: Staphylococcus aureus, Conjunctivitis, Efflux Pumps, Virulence Factors.

Introduction

Pink eye represents an eye infection along with conjunctival inflammation which appears as pink or crimson buildup on the thin conjunctival membrane. The condition presents as subconjunctival hemorrhage through minor redness because of bacterial, viral or allergic causes [1],[2]. Conjunctivitis

is commonly known as "pink eye" because the white portion of the eye appears pink or red as a result of the blood vessels in it widening and filling with blood [3]. The causes of conjunctivitis vary depending on the etiologic agent; some are infectious and arise from bacterial, viral, or fungal infection of the eye, affecting one or both eyes; others are non-infectious and include allergic, chemical, or toxic conjunctivitis; these conditions spread quickly, are contagious, and affect people of all ages [4]. The common human pathogen Staphylococcus aureus causes different infections of the conjunctiva which range from acute invasive to chronic forms that prove difficult to treat [5]. Management of these infections is considerably more difficult owing to the organism's ability to develop resistance to antibiotics [6]. The capacity of Staphylococcus aureus to cause disease stems from a myriad of virulence factors that allow for adhesion to host tissues and immune evasion. In Staphylococcus aureus induced conjunctivitis, the impact of sigma factor B (SigB), catalase (KatG), and clumping factor A (ClfA) are the most important [7]. SigB is known to modulate expression of the different virulence genes in response to environmental stress. It further adds to biofilm development, which is a very important particular in chronic infections, and increases survival under harsh conditions such as oxidative stress found on the ocular surface [8]. The Hydrogen Peroxide-Detoxifying- Catalase (KatG) protects Staphylococcus aureus from the reactive oxygen species (ROS) that is formed by the immune response. KatG inactivates oxidative stress which helps the bacteria under the conjunctival epithelium survive and persist during infection [9]. While clumping factor A (ClfA) promotes bacterial attachment to host extracellular matrix components, such as fibrinogen. ClfA facilitates colonization of the conjunctiva and contributes to biofilm formation, enhancing resistance to mechanical clearance and antibiotic treatment [6]. Protein transporters called efflux pumps are essential for transporting various compounds and expelling their harmful effects from the cell. They are located in the cell membrane. Bacteria are able to develop antibiotic resistance because of these protective substances. These transporters carry a variety of molecules outside of cells, including toxic compounds. As well as colors like acriflavine, crystal violet, ethidium bromide, fatty acids, antiseptics, disinfectants, heavy metals, organic solvents, and antibiotics, these poisonous compounds can be hydrophilic, hydrophobic, or amphipathic. Antibiotics like novobiocin, tetracycline, macrolides, β-lactam, and chloramphenicol [10],[11]. Along with this line this study aimed to characterize Staphylococcus aureus in conjunctivitis, focusing on efflux pumps and virulence genes.

METHODOLOGY

Ethical approval

The research received approval from the College of Medicine at the University of Kufa in Iraq as IRB 5628/2024 which meets the requirements of the International Declaration of Helsinki for Human Research Protection. All specimens were collected with the consent of adult patients or parents of children with conjunctivitis.

Sample Collection

150 samples were collected from patients infected with conjunctivitis of different ages (from birth -7 years) in Dhi Qar Governorate "Nasiriyah" for the period from August to December 2024 in the Ophthalmology Consultant at Nasiriyah Teaching Hospital and Al-Haboubi General Hospital. The specialist doctor performed an examination before the swab collection process where purulent conjunctival eye material was obtained using sterile swabs for inoculation on blood agar along with chocolate agar and MacConkey agar plates which received incubation at 37 °C for 24 hours.

Identification of bacterial isolates

The Vitek2 compact system, manufactured by BioMerieux, is used for phenotypic identification of bacterial isolates based on cultural and microscopic characteristics. This system, which relies on biochemical reactions between bacterial isolates and media, has shown high accuracy and specificity (95%-99%).

Antimicrobial Susceptibility Testing

It was carried out according to [12].

Detection of Active Efflux Pump

The ethidium bromide cartwheel method was carried out according to [13].

Selection of Staphylococcus aureus isolates

For all the Staphylococcus aureus isolates, an antimicrobial susceptibility test was done. For some of the isolates that were antibiotic-resistant, an efflux pumps test was done. Finally, for the isolates that made efflux pumps, a gene detection test was done.

Extraction of Bacterial DNA

The gDNA was extracted according to the "Presto mini gDNA Bacteria (Geneaid) Kit Presto protocol", according to the manufacturer's instruction [15].

PCR amplification

All Staphylococcus aureus isolates were screened for the presence of SigB, KatG and Clfa. The PCR technique was performed by a Thermo cycler device (Professional TRIO/Biometra/Germany). It detected the presence of genes in Staphylococcus aureus using specific primers for these genes (Macrogen company /Korea) as in Table (1). PCR (Polymerase chain reaction) technique was applied to all isolates of Staphylococcus aureus, the PCR reaction contents (20 µl) included 8tµl GoTaq Green master mix PCR (2X) (Promega, USA), 1 µl for each forward and reverse primer (Macrogen/ Korea), 0.5µl of MgCl2 (25mM), 2µl of DNA template and 7.5µl of ddH2O. PCR program is shown in Table (2) for genes. Also, electrophoresis was applied using agarose gel (2%) to reveal PCR amplicon using staining with ethidium bromide stain.

Table 1: Primers sequences of genes

Genes	5`-3` sequence	Annealing temp(C^0)	Product size(bp)	References
sigBf	TTCCATTGAAGCTGATAAAGATGGT	55	68	(Ishii *et al*., 2014)
sigBr	GGTCATCTTGTTGCCCCATAA			
clfAf	GGCACGCAATCTGCAATTAA	55	61	(Ishii *et al*., 2014)
clfar	CATGAATCCCCCAATGGAAA			
katGf	CAACTGATGGATACGGCTATGA	55	104	(Liao *et al*., 2021)
katGr	TTCTTTAGCGTCCTCTGATTGT			
16Sf	CAACGCGAAGAACCTTACCAA	55	136	(Ishii *et al.,* 2014)
16Sr	GCGGGACTTAACCCAACATCT			

Table 2: PCR programs for genes

Genes	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
SigB	94°C(5min) 1cycle	95°C(30sec) 35 cycle	55°C(30sec) 35 cycle	72°C(30sec) 35 cycle	72°C(5min) 1 cycle
Clfa	94°C(5min) 1cycle	95°C(30sec) 35 cycle	55°C(30sec) 35 cycle	72°C(30sec) 35 cycle	72°C(5min) 1 cycle
KatG	94°C(5min) 1cycle	95°C(30sec) 35 cycle	55°C(30sec) 35 cycle	72°C(30sec) 35 cycle	72°C(5min) 1 cycle
16RNA	94°C(5min) 1cycle	95°C(30sec) 35 cycle	55°C(30sec) 35 cycle	72°C(30sec) 35 cycle	72°C(5min) 1 cycle

RESULTS

85 specimens 56.67% showed positive growth of bacterial culture on different media, with heavy growth of bacteria appearing after 24 hours of incubation at a temperature of 37 °C, while 65 specimens 43.33% of conjunctivitis showed negative growth of bacterial culture on different media even after 48 hours of incubation at 37°C. Based on bacterial growth on different culture media, this study showed that, among the bacterial isolates, 74 (87.06%) of them were Gram-positive isolates. These bacterial isolates are Staphylococcus aureus, which was the most frequent isolate, accounting for 34 (40%).

Antibiotic Resistance Patterns

Antibiotic susceptibility testing was performed for all Staphylococcus aureus isolates. 20 isolates resistant to some antibiotics were selected for efflux pump testing using the ethidium bromide - agar cartwheel method (EBC). 7 isolates that showed efflux pumps were subjected to gene detection testing (PCR), with 5 isolates showing virulence genes. High (MRSA) Rate, the data presented show that a striking 76%, approximately 26 out of 34 sampled

isolates, exhibited resistance to Cefoxitin, confirming the alarming presence of Methicillin-resistant Staphylococcus aureus (MRSA) in the samples. Vancomycin remains effective in this case, all isolates were either sensitive or intermediate, with no resistance, meaning 27 (sensitive) and 7 (intermediate), suggesting that vancomycin is still effective. Erythromycin shows high resistance, reduced activity of erythromycin against Staphylococcus aureus was demonstrated by the fact that half of the isolates (14) were resistant, and an additional 14 were intermediate. Doxycycline appeared to outperform tetracycline with regard to resistance, as only 1 isolate was resistant compared to 5 for tetracycline, but both antibiotics demonstrated moderate to good activity against the targeted bacteria. Ciprofloxacin and Ofloxacin, these two antibiotics were among targets with higher potency, as more than 30 of the isolates were sensitive to Ciprofloxacin and Ofloxacin, with only one of the isolates being resistant to Ciprofloxacin, and no resistance to Ofloxacin. SXT (Trimethoprim-Sulfamethoxazole), also retained considerable activity, as it proved to be effective against all of the samples except three of the isolated copper samples as showed in (Table 3).

Table 3: Antibiotic sensevitiy of Staphyloccocus aureus isolates

Antibiotics	Resistance		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Cefoxitin (MRSA marker)	26	7.64	_	_	8	2.35
Ciprofloxacin	1	0.29	3	0.88	30	8.82
Doxycycline	1	0.29	7	2.05	26	7.64
Erythromycin	14	4.11	14	4.11	6	1.76
Ofloxacin	0	0.00	1	0.29	33	9.70
Tetracycline	5	1.47	10	2.94	19	5.58
Trimethoprim-sulphamethoxazole	3	0.88	_	_	31	9.11
Vancomycin	0	0.00	7	2.05	27	7.94
Susceptibility %	**14.68**		**12.32**		**52.91**	

Detection of Efflux pump

Twenty bacterial samples of Staphylococcus aureus underwent the ethidium bromide - agar cartwheel method (EBC) detector for observing efflux pump activity. The results showed that 7 (20.6%) Staphylococcus aureus isolates were positive for the phenotypic detection as illustrated Table (4). The identification of inhibitory concentrations was established through identification of the lowest dose that eliminated UV light activation (UV) among the isolates as showed in (Figure 1).

Table 4: Results of phenotypic detection of efflux pumps of Staphylococcus aureus isolates using different concentrations of ethidium bromide dye in A tryptic soya agar.

EtBr (mg/L) and degree of fluorescence produced				
Isolate No.	**1(mg/L)**	**2(mg/L)**	**3(mg/L)**	**Efflux activity**
Staphylococcus aureus 1	-	-	-	**Negative**
Staphylococcus aureus 2	-	-	-	**Negative**
Staphylococcus aureus 3	+	+	+	**Positive**
Staphylococcus aureus 4	-	-	-	**Negative**
Staphylococcus aureus 5	+	+	+	**Positive**
Staphylococcus aureus 6	-	-	-	**Negative**
Staphylococcus aureus 7	-	-	-	**Negative**
Staphylococcus aureus 8	-	-	-	**Negative**
Staphylococcus aureus 9	-	-	-	**Negative**
Staphylococcus aureus 10	-	-	-	**Negative**
Staphylococcus aureus 1	-	-	-	**Negative**
Staphylococcus aureus 11	-	+	+	**Intermediate**
Staphylococcus aureus 12	+	+	+	**Positive**
Staphylococcus aureus 13	+	+	+	**Positive**
Staphylococcus aureus 14	+	+	+	**Positive**
Staphylococcus aureus 15	+	+	+	**Positive**
Staphylococcus aureus 16	-	-	-	**Negative**
Staphylococcus aureus 17	-	-	-	**Negative**
Staphylococcus aureus 18	-	-	-	**Negative**
Staphylococcus aureus 19	-	-	-	**Negative**
Staphylococcus aureus 20	-	-	-	**Negative**

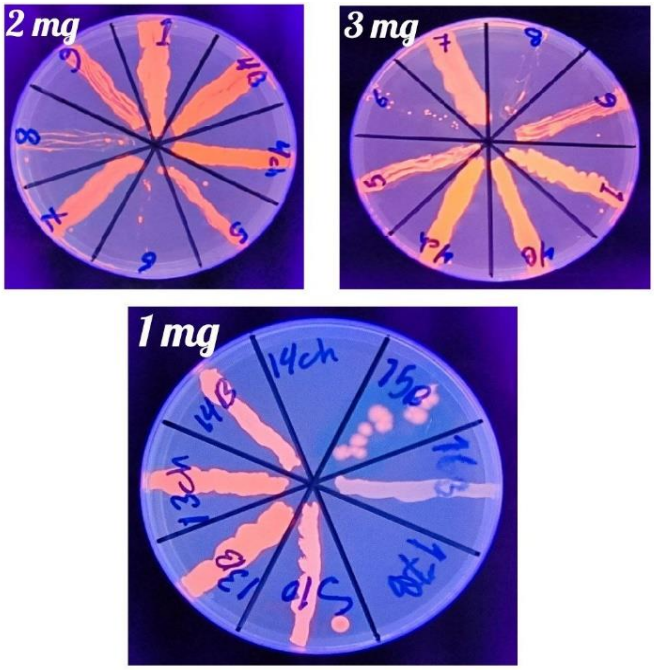

Figure 1: Phenotypic detection of effluent pumps using Cart-Wheel method.Fluorescent and non fluorescent bacterial isolates of Staphyloccoccus aureus at different concentrations of ethidium bromide dye under ultraviolet light.

Virulence factor genes detection

The results showed that all isolates of Staphylococcus aureus have SigB genes at (100%), Clfa genes (100%), and KatG genes are found in all isolates (100%) as shown in Figures (2, 3, 4 and 5).

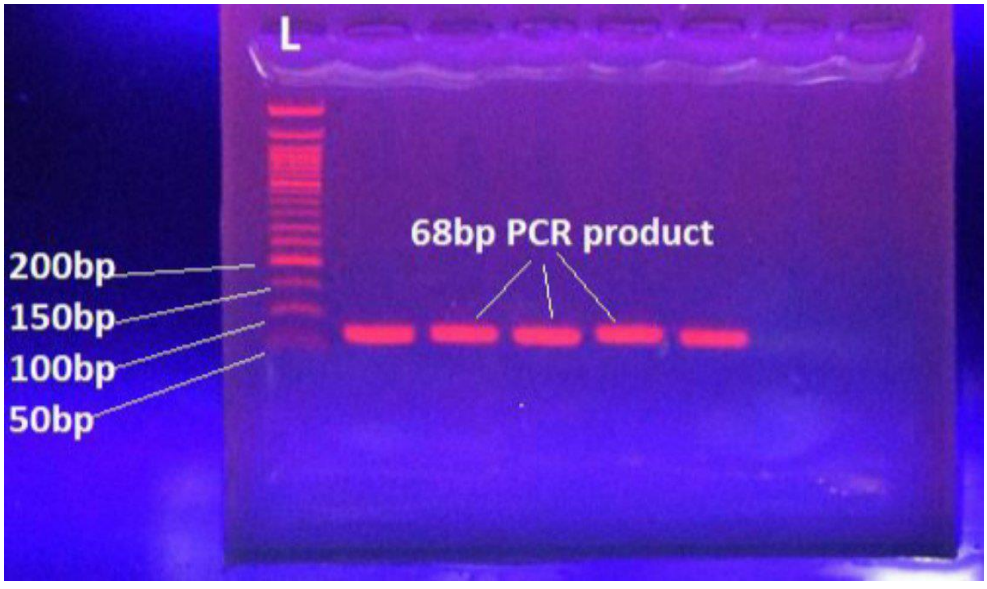

Figure 2: The amplification PCR product of SigB gene (68bp) for Staphylococcus aureus isolates was electrophoresed on a 2% agarose gel. M: 50bp ladder marker.

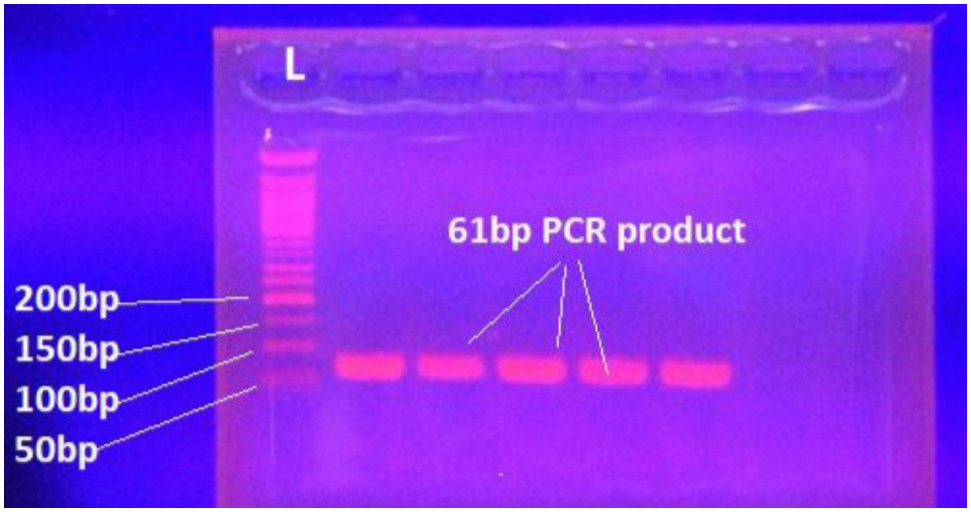

Figure 3: The amplification PCR product of Clfa gene (61bp) for Staphylococcus aureus isolates was electrophoresed on a 2% agarose gel. M: 50bp ladder marker.

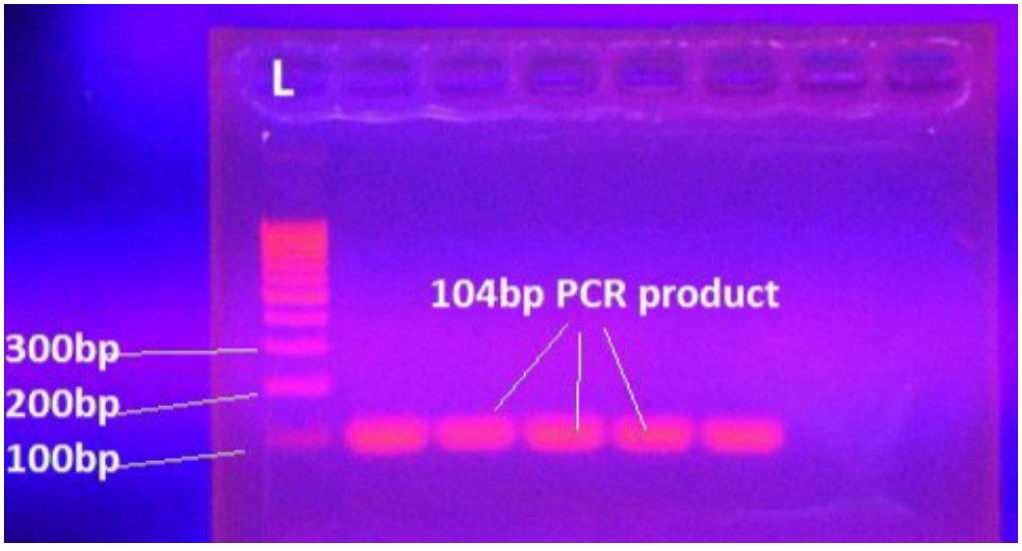

Figure 4: The amplification PCR product of KatG gene (104bp) for Staphylococcus aureus isolates was electrophoresed on a 2% agarose gel. M: 100bp ladder marker.

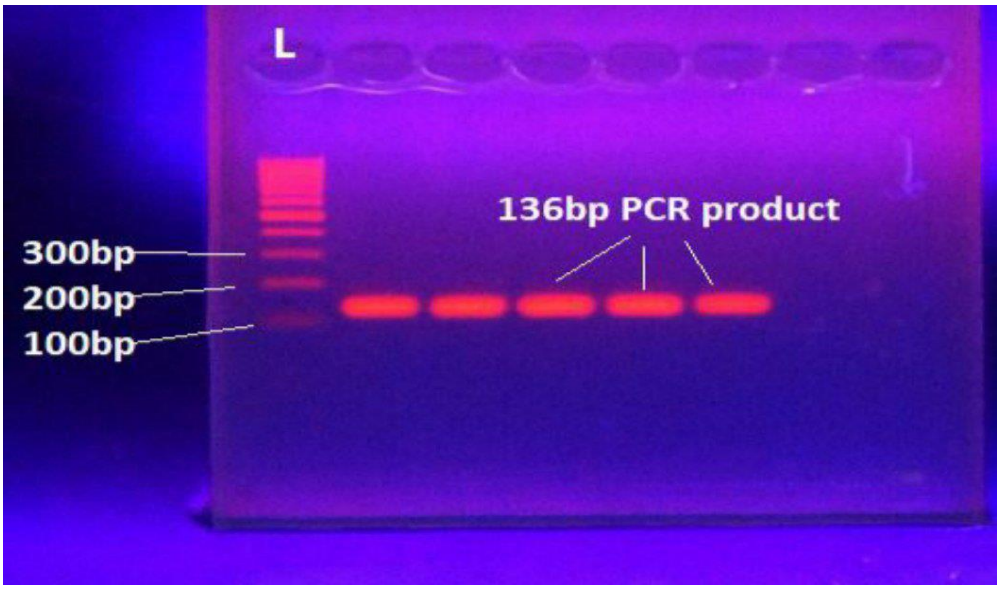

Figure 5: The amplification PCR product of 16RNA gene (136bp) for Staphylococcus aureus isolates was electrophoresed on a 2% agarose gel. M: 100bp ladder marker.

Discussion

The most often identified Gram-positive bacteria from the normal ocular flora of human beings is *Staphylococcus aureus* [16,17]. This study showed a high incidence of *Staphyloccocus aureus* bacteria in the occurrence of conjunctivitis. The research conducted in Guangdong PR China found *Staphylococcus aureus* existed in 37.5% of patients with bacterial conjunctivitis while the developed molecular biology method detects *Staphylococcus aureus* infection in eyes with enhanced speed and sensitivity compared to bacterial culture testing methods [18]. While [19] A 227 *Staphylococcus aureus* isolates were collected in 2013, 2014, and 2018–2019 from three hospitals in Hainan/China province for investigation of their antimicrobial resistance, virulence gene profiles, and molecular characteristics. showed susceptible to VAN LZD, in agreement most previous studies China. One Iraqi study, that diagnosed *Staphylococcus aureus* in hospitals by PCR using 16SrRNA found that 100% were diagnosed as *Staphylococcus aureus* [20]. Another study reported in which *Staphylococcus aureus* showed the highest prevalence (25%) compared to *Streptococcus pneumonia* (7.95%), with ($\boldsymbol{p} < .\mathbf{0002}$) significant differences [21]. Researchers from 2017 derived results about *Staphylococcus aureus* being the dominant pathogen for conjunctivitis from their examination of Iraq patients [22]. PCR was a reliable technique and sensitive enough for the detection of *Staphylococcus aureus* virulence gene such as *SigB* gene, *Clfa* gene and *KatG* gene. Stress response genes such as *sigB*, *katG*, and *clfA* relating to oxidative defense and host adhesion are associated with *Staphylococcus aureus* virulency as well as eye infections. *Staphylococcus aureus* global regulator, Alternative sigma factor B, *sigB* is crucial for

stress resistance because it allows the pathogen to deal with oxidative stress and immune response within the eye**.** For increased colonization in conjunctivitis, *sigB* augments cap region encoding adhesin (fibronectin binding proteins) expression while down-regulating toxins, thus increasing virulence [23]. The presence of *sigB* has been correlated with the ability to withstand extreme conditions, highlighting the value of this protein in ocular infections, which corroborates findings from this study [24]. *Staphylococcus aureus* ability to withstand neutrophil destruction in the tear film and conjunctival epithelium is largely attributed to *katG*, which encodes catalase-peroxidase [25]. Adhesion to conjunctival epithelial cells, a pivotal moment for infection, is promoted by *clfA* [26]. In *Staphylococcus aureus*-mediated bacterial conjunctivitis, genes *sigB*, *katG*, and *clfA* are critical for adhesion, oxidative defense, and stress adaption. Their interactions with pathogenicity, pose new possibilities for treatment strategies [24].

Conclusion

Staphylococcus aureus, which is a type of Gram positive bacterium, is the most common cause of bacterial conjunctivitis. The study showed that some of the isolated bacterial strains demonstrated considerable resistance to several antibiotics. This is likely due to inefficient or inappropriate antibiotic usage and the development of bacterial resistance strategies which rely on ineffective, though biologically rich, efflux pumps and virulence factor gene systems, leading to strong antibiotics defiance and bacterial survival in the eye.

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Conflict of interest statement: None to declare

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