Biofilm Forming Bacteria Isolated From Human Eye Conjunctivitis and Keratitis Cases and their Ability to Adhere on Contact Lenses i*n vitro*

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Abstract

One hundred bacteria isolated from conjunctivitis and keratitis showed 14 different bacterial species as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were the most. All 100 isolates were tested for their ability to form biofilm, 65 isolates (65%) showed positive for congo red agar (CRA) test with the exception of *Enterobacter hormaechei, Staphylococcus haemolyticus* and *Bacillus pumilus* species, while 84 isolates (84%) showed positive for microtiter plate assay except *Bacillus pumilus* species. Nevertheless, *icaAD* genes were detected in 75 isolates (75%) within all species. Although 100 bacterial isolates showed different results toward biofilm production tests, CRA, MTP assay and *icaAD* genes, but there was no significant difference found among these tests. Appliedally, the ability to adhere on contact lenses (*In vitro*) appeared *Staphylococcus aureu*s has the highest adhesion 12(92.3%) followed by *P. aeruginosa* 3(75%) and *Staphylococcus epidermidis* 3(60%).

Keywords: *Biofilm, conjunctivitis, Enterobacter hormaechei, Staphylococcus haemolyticus, Bacillus pumilus*

Introduction

Biofilm is a community of microorganisms adhering to a surface and surrounded by a complex matrix of extra-polymeric substances[1]. It is a structure that gives single cell of organisms the ability to form temporary multicellular lifestyle in which group behavior facilitates survival in harsh environments[2]. It becomes like a community of microorganisms attached to the fix surface to be considered as a complex developmental process in nature[3]. The genes encoding the synthesis of PIA are organized in the intercellular adhesion (ica) operon, *icaA* is responsible of N-acetylglucosaminyl-transferase activity while *icaD* directs the correct folding of the membrane insertion of *icaA* and may act as a link between *icaA* and *icaC*[4]. Among the most common bacteria that are associated with biofilm production and causing ocular infections are *Pseudomonas* spp., *Staphylococci*, *Enterococci* and *Streptococci*[5, 6].The existence of biofilms in association with many eye infections has been recorded, especially in people wearing contact lenses and using contact lens storage case for a long time (Weissman and Mondino, 2002). Biofilm provides several advantages for pathogenic bacteria such as protection and resistance to antibiotics, antiseptics, avoiding host immune attacks, complement system and phagocytes[7].Because the increasing of eye bacterial infections associated with the using of contact lenses, the present study oriented to detect the adherence frequency of the bacteria isolated from eye infections and their ability to adhere on the contact lens materials (*In vitro*).

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Materials and Methods

Samples from eye infection cases were collected from conjunctivitis and keratitis, cultured and bacteria identified by *16S rRNA* sequencing as Mahdi *et al.* (2021) “under publishing”.

Congo Red Agar (CRA) Method

One hundred bacterial isolates were cultured by streaking technique on plates containing Congo red agar and incubated in 37ºC for 24 hrs. Plates were examined for black colonies as positive and colourless as negative [8].

Formation of biofilms in microtiter plates (MTP)

Two new sterile empty (96-well Microtiter) Plates were measured by micro ELISA auto reader wave length at 490 nm and the data were considered as a control [9]. 100 bacterial isolates were activated by inoculation (separately) in 20 ml tubes contain 10 ml brain heart infusion agar (LAB company, UK) with 2% sucrose and incubated at 37ºC for 18-24 hrs. Each well of Microtiter Plate was filled with 200 µl bacterial suspension of each isolate, except the first row of wells were filled with BHIB with 2% sucrose without bacterial culture as a blank, the Microtiter Plates were covered and incubated at 37ºC for 24 hrs. The contents of each well were then removed gently by inverting and tapping the plates, wells were washed four times with 200 µl phosphate buffer saline (LAB company, UK) to remove the free floating bacteria. Biofilms formed by adherent bacteria were fixed by adding 200 µl of 2% sodium acetate (Thomas baker company, India) for 30 min. at room temperature, the wells were stained by adding 200 µl of 0.5% crystal violet for 5 min., plates were washed with running tap water and left to dry at room temperature. The optical density (OD) of the post processed Microtiter Plates was measured using micro ELISA auto reader at wave length 490 nm [10]. The formation of biofilms were determined by calculating the differences between the readings of pre-culture and post-culture then the results recorded as OD Pre culture < OD Post-culture = positive and OD Pre culture ≥ OD Post-culture = negative.

Amplification of *icaAD* gene

IcaA and *icaD* genes were amplified separately by PCR for 100 bacterial isolates according to Hussein (2013). The sequence of primers *icaA* Forward: 5-TCTCTTGCAGGAGCAATCAA-3, Reverse: 5-TCAGGCCACTAACATCCAGCA-3, and *icaD* Forward: 5-ATGGTCAAGCCCAGACAGAG-3, Reverse: 5-CGTGTTTTCAACATTTAATGCAA-3. 25µl of PCR reagent mixture contains 12.5 µl of Go Taq Green master mix (Bioneer , Korea) , 5 µl of DNA template, 1 µl from each primers (Macrogen, Korea) and 5.5 µl of Nuclease Free water (Bioneer , Korea). The Verity thermo cycler (Applied Biosystem , USA) was used with conditions for amplifying one cycle at 94°C for 5min. followed by 50 cycles at 94 °C for 30 sec. 55.5° C for 30 sec. and 72°C for 30 sec. Final extension at 72°C for 1 min . The bands were detected on agarose gel electrophoresis and photographed under UV transilluminator (Wisd, Korea).

Adhesion of bacteria on contact lenses (*In vitro*)

Twenty five sterile cosmetic soft contact lenses (Bella brand) were used for studying the practically adherence ability of some isolates as a modified techniques from Abd Al Wahid and Abd Al-Abbas (9). 25 test tubes (20 ml) containing 10 ml of BHIB with 2% sucrose prepared and autoclaved at 121°C for 15 min. a single new contact lens was inoculated in each tube. 22 isolates (13 isolates of *S. aureus ,* 5 isolates of *S. epidermidis* and 4 isolates of *P. aeruginosa*) were cultured in the 22 tubes and the 3 tubes left without culturing as blank. After incubation all tubes at 37ºC for 24 hrs. each contact lens from all tubes was washed five times with sterile PBS to remove any free-floating bacteria, then washed by solution of 2% sodium acetate to fix the adherent bacteria, each contact lens was re-inoculated in test tube containing 10 ml BHIB and incubated at 37ºC for 24 hrs. all contact lenses were cultured on petri dishes containing BHIA and incubated at 37ºC for 24 hrs. The bacterial growth observing around the contact lens in each petri dish referring to the ability of adherence.

Results

Congo red agar (CRA) test

Sixty five isolates (65%) showed positive results to CRA test (Figure 1 and Table 1). *S. aureus* 25(75.76%) , *S. epidermidis* 16(57.14%), *P. aeruginosa* 6(85.71%), *E. faecalis* 4(57.14%), *B. subtilis* and *S. pyogenes* 3(100%) for both, *S. hominis* and *P. mirabilis* 2(66.67%) for both , *S. lugdunensis* 2(100%) , *B. amyloliquefaciens* and *E. cloacae* 1(100%) for both, with high significant difference (P≤0.01).

Microtiter plate (MTP) assay

Eighty four (84%) of isolates showed positive results to Microtiter plate (MTP) assay (Table 1) as violet wells (Figure 2). *S. aureus* 28(84.85%) , *S. epidermidis* 22(78.57%), *P. aeruginosa* and *E. faecalis* 6(85.71%) for both, *B. subtilis* 5(83.33%), *E. hormaechei* 4(100%), *S. pyogenes* and *S. hominis* 3(100%) for both, *P. mirabilis* 2(66.67%) , *S. lugdunensis* 2(100%) , *B. amyloliquefaciens*, *S. haemolyticus* and *E. cloacae* 1(100%) for each, with high significant difference (P≤0.01).

Adherence gene *icaAD*

Figure (3) showing the bands of *icaA* gene on agarose gel with size 188 bp. While, Figure (4) showing the bands of *icaD* gene with size 198 bp. *IcaAD* genes showed the presence of *icaA* and/or *icaD* genes in 75(75%) of the total bacterial isolates. *S. aureus* 26(78.79%) , *S. epidermidis* 19(67.86%), *P. aeruginosa* 5(71.43%), *E. faecalis* 4(57.14%), *B. subtilis* 5(83.33%), *E. hormaechei* 3(75%), *S. pyogenes* and *S. hominis* 3(100%) for both , *P. mirabilis* 1(33.33%) , *S. lugdunensis* 2(100%) , *B. amyloliquefaciens*, *S. haemolyticus*, *E. cloacae* and *B. pumilus* 1(100%) for each, with high significant difference (P≤0.01) as (Table 1). However, there was no significant differences between CRA, MTP and *icaAD* genes. Although 100 bacterial isolates showed different results toward biofilm production tests, CRA (65%), MTP assay (84%) and *icaAD* gene (75%), but, there was no significant difference found among these tests, even within bacterial species.

Adhesion of bacteria on contact lenses (*In vitro*)

Out of 22 different bacterial isolates, only 18(81.8%) isolates were win to adhere and grow on BHIA by culturing the contact lenses, the highest adhesion was 12(92.3%) of *S. aureus* with significant difference (P≤0.05) than 3(75%) of *P. aeruginosa* and 3(60%) of *S. epidermidis* (Table 2). Only four isolates (*S. aureus* 1 , *S. epidermidis* 2 , *P. aeruginosa* 1) were failed to grow. Interestingly, no growth was shown in any control contact lenses (Figure 5)

Figure 1. Congo Red Agar (CRA) test of two *S. aureus* isolates.

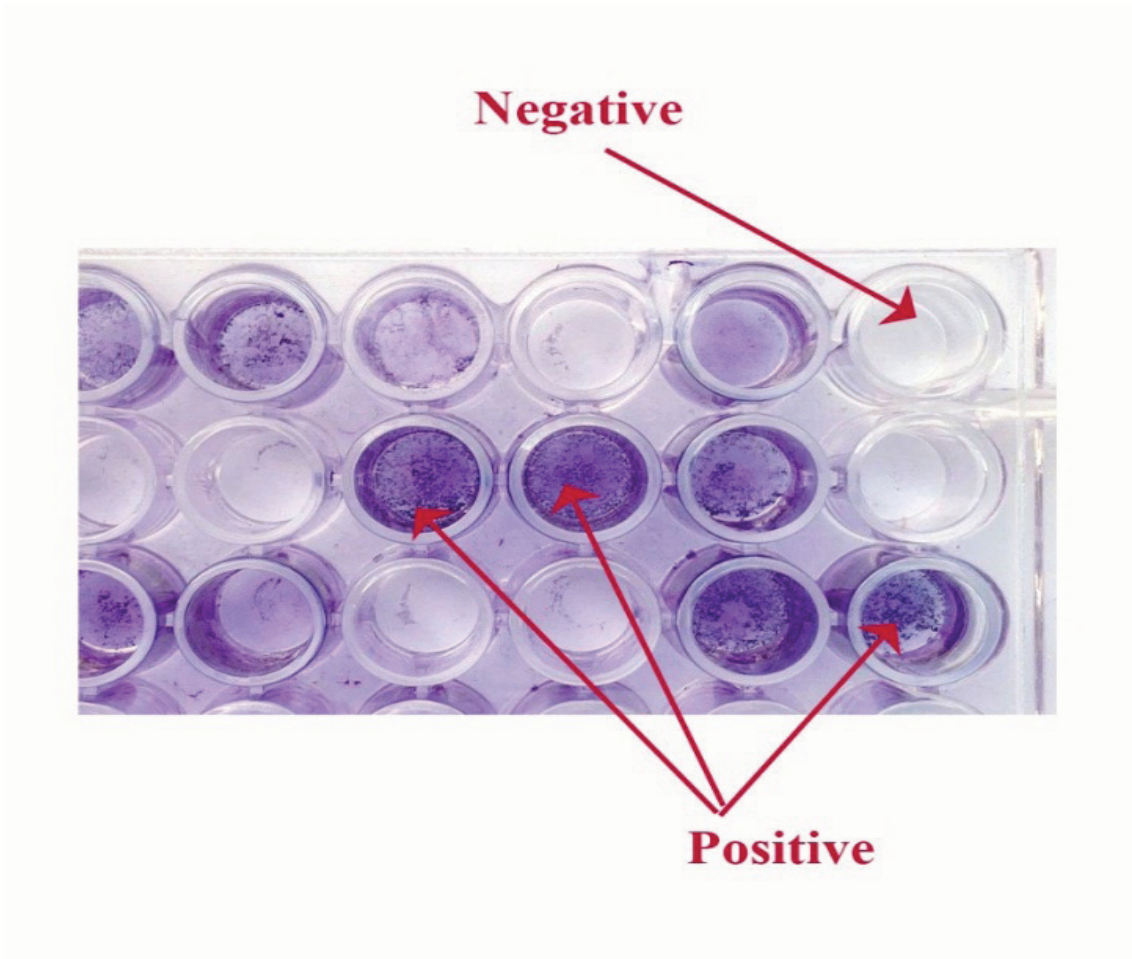

Figure 2. Microtiter plate (MTP) assay. Positive: Violet dye showed the adherence of bacteria in the wells. Negative: colourless, no adhesion.

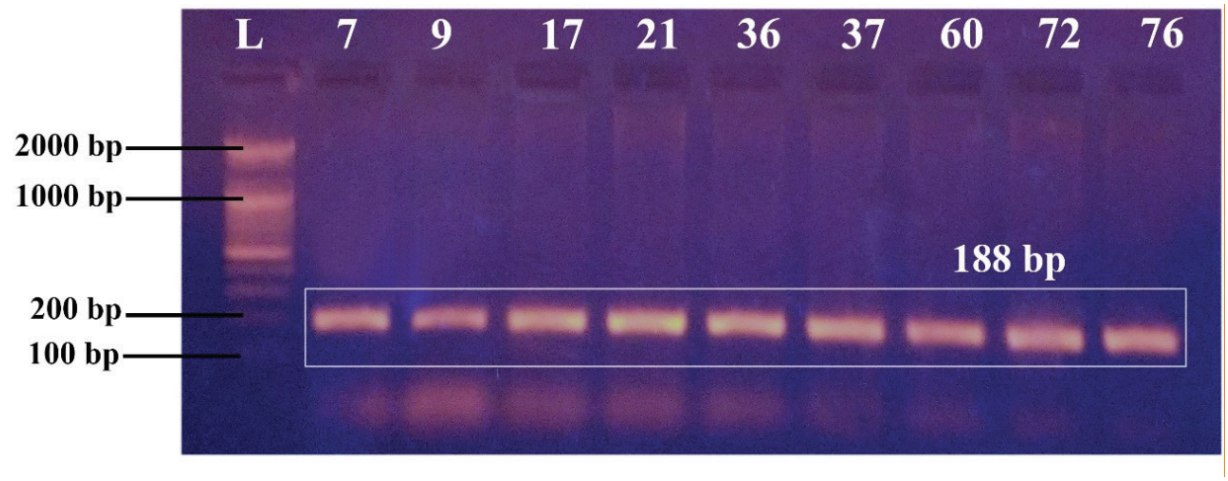

Figure 3. Agarose gel electrophoresis showing amplified ica A gene (188 bp). Lane L: 100 bp Marker, Lane 7, 9, 17, 21, 36, 37, 60, 72 and 76 were the No. of bacterial

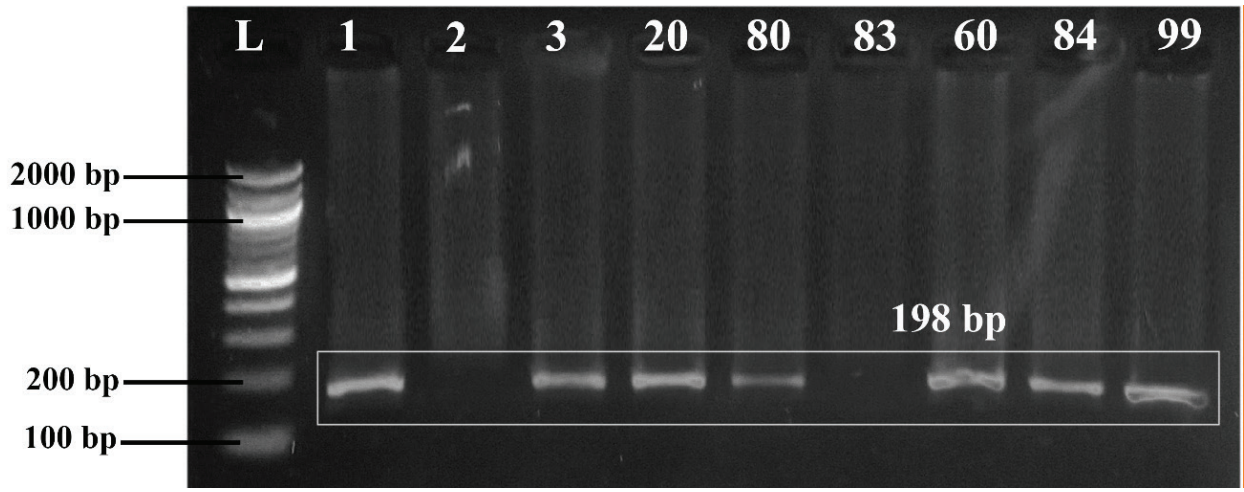

Figure 4. Agarose gel electrophoresis showing amplified icaD gene (188 bp). Lane L: 100 bp Marker, Lane 1, 2, 3, 20, 80, 83, 60, 84 and 99 were the No. of bacterial isolates containing icaD gene bands .

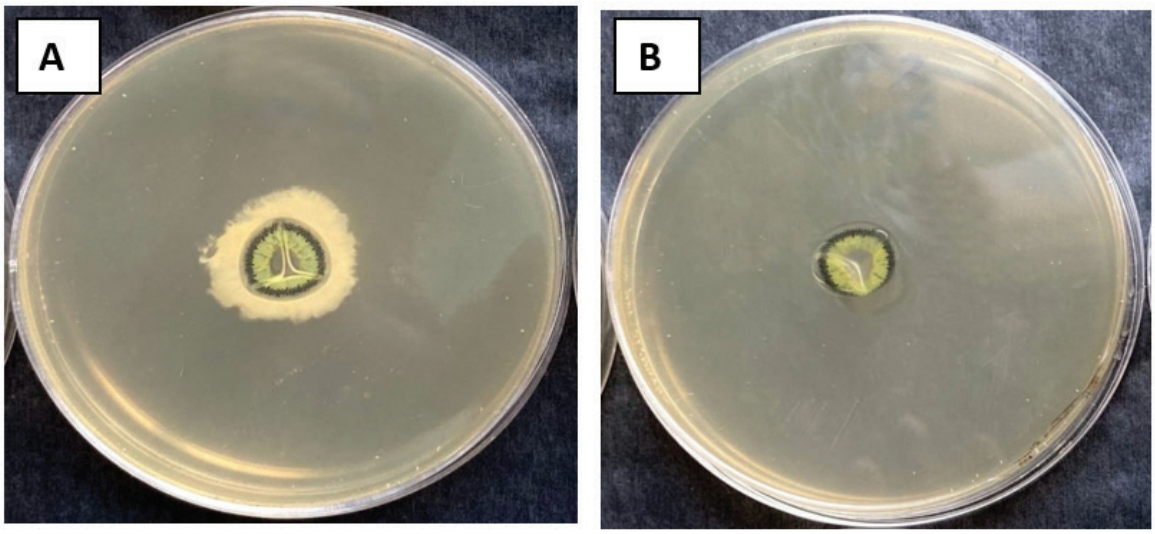

Figure 5. A- Growth of bacteria around contact lens after washing with PBS and re- cultured on BHIA. B. No growth of bacteria around contact lens after washing with PBS and re-culture on BHIA.

Table 1. Frequency of bacterial species isolated form human eye infections against different tests.

No.	Bacterial species	n	Congo red agar n(%)	Microtiter plate (MTP) assay n(%)	IcaAD gene n(%)	n	Adhesion on contact lens n(%)
1	Staphylococcus aureus	33	25(75.76%)	28(84.85%)	26(78.79%)	13	12(92.3%)
2	Staphylococcus epidermidis	28	16(57.14%)	22(78.57%)	19(67.86%)	5	3(60%)
3	Pseudomonas aeruginosa	7	6(85.71%)	6(85.71%)	5(71.43%)	4	3(75%)
4	Enterococcus faecalis	7	4(57.14)	6(85.71%)	4(57.14)	0	0

Cont... **Table 1. Frequency of bacterial species isolated form human eye infections against different tests.**

5	Bacillus subtilis	6	3(50%)	5(83.33%)	5(83.33%)	0	0
6	Enterobacter hormaechei	4	0	4(100%)	3(75%)	0	0
7	Streptococcus pyogenes	3	3(100%)	3(100%)	3(100%)	0	0
8	Staphylococcus hominis	3	2(66.67%)	3(100%)	3(100%)	0	0
9	Proteus mirabilis	3	2(66.67%)	2(66.67%)	1(33.33%)	0	0
10	Staphylococcus lugdunensis	2	2(100%)	2(100%)	2(100%)	0	0
11	Bacillus amyloliquefaciens	1	1(100%)	1(100%)	1(100%)	0	0
12	Staphylococcus haemolyticus	1	0	1(100%)	1(100%)	0	0
13	Enterobacter cloacae	1	1(100%)	1(100%)	1(100%)	0	0
14	Bacillus pumilus	1	0	0	1(100%)	0	0
	Total	100	65(65%)	84(84%)	75(75%)	22	18

*= P≤0.01

Discussion

The ability of 100 isolates to produce biofilm were examined by three methods: Congo red agar (CRA), Microtiter plate assay (MTP) and *icaAD* genes detection. Biofilm producers give black colour colonies and non-biofilm producers give pink colour colonies on CRA. This is because interaction between CR and polysaccharide to form black colored complex [11]. CRA showed 65(65%) isolates positive (Table 2). The study focused on *S. aureus , S. epidermidis* and *P. aeruginosa* because of their highest frequencies in isolation and their infectious importance comparing with other species which may show high frequency with low number as in many studies [12-14]. Microtiter plate (96-well plate) assay is a method used for detection of biofilm formation which allows the observation of bacterial adherence to an abiotic surface [15]. The advantages of this method for its simplicity, use of routine lab materials, ability to be adjusted to small or large numbers of samples, capability to test variety samples in a single assay and it is useful for detecting the early steps of biofilm formation such as initial surface attachment by spectrophotometric measurements [16]. Because of the difference in the thickness, all wells measured by micro ELISA auto reader wave length at 490 nm and the data were considered as a control [9]. In the present study, 84 (84%) of isolates showed positive results to Microtiter plate (MTP) as violet wells with high significant differences (p≤0.01). Nevertheless, the addition of sucrose was to enhance the biofilm production by bacteria (Eftikhar and Speert , 2009). However, de Castro Melo, Ferreira (17) reported that the congo red method has higher specificity while MTP method has higher sensitivity.

Multiple studies have stated PCR as an essential tool for ica gene identification, it is a simple, efficient, fast technique and requirs only minimal DNA amounts [18, 19]. Despite *icaA* or *icaD* can be used separately, most studies were used *icaAD* genes together [20]. In the presnt study, the *icaAD* was detected in all bacterial species with different percentages. Despite biofilm production depends on the presence of either or both *icaA* and *icaD* genes, the lack of *icaC* gene may explain why biofilm not produced by some isolates who have *icaA* and *icaD* [21]. Although 100 bacterial isolates showed different results toward biofilm production tests, CRA (65%), MTP assay (84%) and *icaAD* gene (75%), but there was no significant difference found among these tests even within bacterial species, this result agreed with many studies suggested that there was no differences in results between phenotypic methods (congo red agar and microtiter plate) and *icaAD* PCR for detecting the biofilm producing bacteria [22, 23]. Biofilm plays important roles in ocular infections by attaching to each other and to ocular surfaces by which the bacteria can cause infection and avoiding killing effects by antibodies and antibiotics (bispo 2015, zegans 2002)

Twenty five sterile cosmetic contact lenses (Bella brand) were used to study the ability of some studied bacteria to adhere and to show the role of contact lens for transmitting the pathogenic bacteria to cause keratitis. The isolates were chosen randomly from three species (*S. aureus* , *S. epidermidis* and *P. aeruginosa*) the focusing on these three species was of their highest frequency in isolation and their medical importance in clinical ifections. There are several factors affecting on the bacterial adhesion to contact lenses such as bacterial species or strains, chemicals substances of the contact lens (silicon or hydrogel) and physical properties "hydrophobic or hydrophilic" [24]. In the present study, the adhesion of *S. aureus* was higher than *P. aeruginosa* and *S. epidermidis,* this is disagreed with many studies that reporting *P. aeruginosa* has the highest adhesion on contact lens [25, 26], this is may be due to the isolation frequency of *S. aureus* in the present study was higher than *P. aeruginosa*.

Conclusion

S. aureus , S. epidermidis and *P. aeruginosa* were the most species in eye infections comparing to other bacterial species. All isolated species showed the ability to produce biofilm with no significant differences among them. The contact leses materials showed to be good surfaces for some bacterial species to adhere on contact lenses directly.

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Conflict of Interest: we declare that there is conflict of interest

Ethical Approval: the research approved by scientific and ethical committee at our department

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