ORIGINAL ARTICLE

Detection of Biofilm Formation by Different Bacterial Isolates of Contact Lens

[1]Wageih S. El. Naghy*, [2]Sarah A. Hamam, [3]Tamer A. Wasfy, [1]Sara M. Samy
[1]Medical Microbiology & Immunology, Department, Faculty of Medicine, Tanta University, Egypt
[2]Clinical pathology Department, Faculty of Medicine, Tanta University, Egypt
[3]Ophthalmology Department, Faculty of Medicine, Tanta University, Egypt

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Corresponding Author:
Wageih S. El. Naghy, Assistant Prof. of Medical Microbiology & Immunology, Faculty of Medicine, Tanta University, Egypt.
Tel: 02-01067706363
wageh.elnaghi@med.tanta.edu.eg

ABSTRACT

Background: *Biofilms are groups of microorganisms that collect to each other and with different surfaces by adherence mechanisms. These are formed of cells and extracellular matrix manufactured by these cells. There may be a great problem in some situations e.g. on medical implants and resistance against antibiotics.* ***Objective:*** *The objective of this study is to determine biofilm forming power of bacteria isolated from the conjunctiva, contact lens and the lens storage case by both phenotypic and genotypic detection methods.* ***Methodology:*** *Samples were taken from (36) persons in the period from January 2020 to June 2020 at Ophthalmology Department, Tanta University Hospitals, all the samples were transported to the Medical Microbiology & Immunology Department, Tanta University where bacterial strains were isolated. The biofilm formation phenotypic detection was performed by both tube method and Congo red agar method. The biofilm-forming genes of coagulase negative Staphylococcus (CoNS) and Staphylococcus aureus (ica A) and that of P. aeruginosa (psl A), were detected by PCR.* ***Results:*** *The (216) samples (swabs & discarded lenses) gave rise to a total number of (247) bacterial isolates. By using tube method; (52.3%) were moderately positive, (31.5%) strongly positive and (16.2%) negative for biofilm formation while after using the Congo red agar method; (35.3%) were moderately positive, (38.4%) strongly positive and (26.3%) negative for biofilm formation. Regarding the Staphylococcus aureus isolates, two (50%) of these were containing (icaA) gene. Regarding the (21) CoNS isolates, three (14.3%) contained (icaA) gene. Although all of the Pseudomonas isolates didn't contain pslA (1119 bp) gene, these were positive for biofilm production by phenotypic methods.* ***Conclusion:*** *The majority of the isolates had the capacity to form biofilms. Both tube and Congo red agar methods showed clear significant correlation and detected a high number of biofilm-producing strains. The absence of genes responsible for biofilm formation did not exclude the phenotypic biofilm production by these bacteria which is a common state.*

INTRODUCTION

The use of contact lens [CL] is indicated for either cosmetic or optical correction purposes and is preferred over glasses. Contamination and infection of the eyes are common in persons who don't care about lens hygiene.[1,2] Bacterial keratitis may cause permanent blindness due to corneal scar or perforation.[3,4] Both Gram positive and Gram negative bacteria have the power to produce biofilm e.g. *Staphylococcus aureus, Staphylococcus epidermidis, E.coli, Klebsiella pneumonia*e and *Pseudomonas aeruginosa*.[5] Biofilms play a principal role in about 95% of bacterial infections.[6,7] Biofilm was found on contact lens, intraocular lens, stents and corneal sutures.[8,9] Bacteria secrete chemical substances like extracellular polymeric substances (EPS) that hold heterogeneous bacterial mixtures and act as an essential constituent of biofilms.[10] The essential 2 elements of EPS in *S. aureus* & *S. epidermidis* are polysaccharide intercellular adhesin (PIA) & capsular polysaccharide/adhesin (PS/A).[11] It was proved that *(icaA)* gene, encodes the production of both PS/A and PIA.[12,13] Also, in biofilm production of *Pseudomonas aeruginosa*, the (*pslA)* gene plays a similar function.[14,15] The aim of the present work is biofilm detection by the two known phenotypic methods and to confirm using PCR as a genotypic method.

METHODOLOGY

This study was conducted on thirty six contact lens users in the age from (17-30) years (most of them are medical students) at Ophthalmology Department, Tanta University Hospital, Egypt, in the period from January 2020 to June 2020. Written informed consents were

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obtained from the participants. They had contact lenses made of soft silicone hydrogel for a period from (4-5) years. Frequency of change of CL was variable: Every month; 33 persons, every day; one person and every 3 months; two persons. The (216) samples (two lower conjunctival swabs, two storage cases fluid swabs and two discarded contact lenses from each person) were transported to the Department of Medical Microbiology & Immunology, Tanta University. All the cases were clinically examined with a slit-lamp by an ophthalmologist.

- **Inclusion criteria:** Persons (17-30 years) wearing CLs for cosmetic or optical cause without any type of ocular infections.
- **Exclusion criteria:** Persons with eye infections or diseases, antibiotic intake in the last month or systemic disease.

Bacterial isolates

Sterile cotton swabs were taken from the two lower conjunctival sacs of both eyes , the two storage case fluids and the two contact lenses of each person aseptically collected just at moment of discard, so, six samples were taken from each user from the (36) CL users (N = 6x36 = 216). All the samples were inoculated in Brain heart infusion (BHI) broth & incubated for 24 hrs at 37°C and then subcultured onto blood agar, MacConkey agar & Sabouraud's dextrose agar (SDA) **[Oxoid]**. The blood and MacConkey agars were incubated at 37°C whereas (SDA) was incubated at a lower temperature 25°C. Identification of organisms were done using standard microbiological methods.[16]

Detection of biofilm formation:

Two phenotypic tests were used for detection of the biofilm formation:

***The tube method*:**

It is a qualitative assay for detection of biofilm producing microorganisms. A loopful of bacteria are inoculated in polystyrene test tube which contained trypticase soya broth (TSB) with (1%) glucose and incubated for 24 h at 37°C. The broth was carefully decanted and the tubes were washed using phosphate-buffered saline "PBS" (pH=7.3) then dried. The sessile isolates of which biofilms formed on the walls of polystyrene test tube are stained with crystal violet (0.1%). The free excessive crystal violet was washed using deionized water. Then the planktonic cells are discharged by rinsing twice with (PBS). Then, crystal violet-stained polystyrene test tube is rinsed twice with PBS to discharge stain. Tubes were put in an inverted position, then dried and observed for biofilm production. After air drying, the occurrence of visible film lining the walls, and the bottom of the tube indicates biofilm production.[17] Depending on the color degree formed on the lining wall of the tubes (observational), these were categorized as moderately + ve and strongly +ve. Laboratory ensured biofilm producing strains were utilized as a +ve control strains (Fig.1).

Fig. 1: The tube method showing bacterial biofilm formation; strongly positive (a), moderately positive (b) and negative result (c)

Congo red agar (CRA) method:

It is also a qualitative method for detection of biofilm producing microorganism. The obtained bacterial strains were carefully inoculated into Congo red agar plates (CRA) then incubated for 24-48 hours at 37°C.[17] CRA preparation was as follows: BHI broth (37 g/L), agar (10 g/L), (5%) sucrose and Congo red stain (0.8 g/L). Congo red is an aqueous solution with high concentration that autoclaved at 121°C for 20 minutes alone and added when the agar got cooled down to 55°C. Isolates were recorded as strongly +ve when black colonies with a dry crystalline consistency appeared. Dark colonies with no dry crystalline morphology were interpreted as moderately +ve biofilm producers. Colonies that did not change from pink were recorded as non-biofilm producers (Figure 2). Laboratory ensured biofilm producing strains were utilized as a + ve control strains.

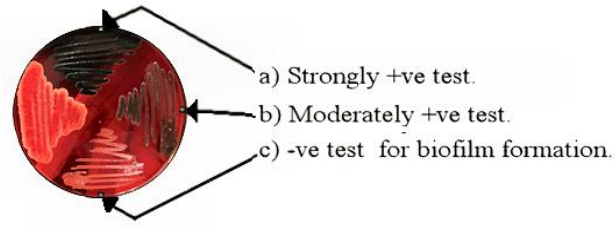

Fig. 2: The Congo red agar test

Polymerase chain reaction (PCR)

PCR was used to detect the genes responsible for biofilm formation in *Staphylococci* (4 *Staphylococcus aureus* and 21 coagulase negative *Staphylococcal isolates*) and 12 *Pseudomonas aeruginosa* isolates. Bacterial colonies were lysed, then DNA was extracted, & gene-specific primers were utilized to amplify DNA fragments.[19] To get the DNA template: A loopful of colonies were taken using sterile pipette tip, then suspended in (50) µl nuclease-free water, heated at (95°C) for ten minutes and then centrifuged at (14,000) rpm. The (2 µl) supernatant was utilized as the template. The Primer sequences and product length for [*icaA*] of *Staphylococci* and [*pslA*] of *Pseudomonas aeruginosa* as these bacteria are common organisms producing biofilm:

IcaA F: 5′-TCTCTTGCAGGAGCAATCAA-3′ (188) bp.
R: 5′-TCAGGCACTAACATCCAGCA-3′
PslA F: 5′-CACTGGACGTCTACTCCGACGATAT-3′ (1119) bp.
R: 5′-GTTTCTTGATCTTGTGCAGGGTGTC-3′

Reaction mix (Qiagen; Germany):

This mix consists of (2.0) µl template suspension, (1.0) µl of (10) µM FP (Forward primer), (1.0) µl of (10) µM RP (Reverse primer), (6.0) µl nuclease-free water, (10) µl Master mix which contained: Taq DNA polymerase, dNTPs, Magnesium chloride and reaction buffers at suitable concentrations. Thus, the total volume is (20) µl.

PCR for gene 1 (icaA of Staphylococci) & gene 2 (pslA of Pseudomonas aeruginosa):

A thermal cycler program was utilized & included: Incubation at (95°C) for (5) minutes, followed by (30) cycles of denaturation at (95°C) for (45) seconds, (55°C) of annealing for (30) seconds, (72°C) of elongation for (1 minute 20 seconds) for gene **2** while only (20 seconds) for elongation of gene **1** and (72°C) for (10) minutes after conclusion of the (30) cycles. Amplification products were detected by using (2%) agarose gel electrophoresis for gene (1) (188 bp) & (1%) agarose gel electrophoresis for gene (2) (1119 bp), then stained with ethidium bromide and U.V. photographed .

Statistical analysis: The results were in the form of percentages, analyzed in suitable statistical tests & shown as tables and graphs.

RESULTS

The bacterial isolates obtained from the lower conjunctival sac, contact lens and lens storage cases were as follow: *Staphylococcus aureus*, CoNS, *Pseudomonas*, Non-fermenter Gram-negative bacilli, *Bacillus*, *Diphtheroids*, *Micrococci*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterococci*, *E. coli*, *Proteus spp.*, *Citrobacter spp.*, *Enterobacter cloacae* & *Moraxella*. Five from (216) samples were completely sterile & (25) gave polymicrobial results. The total number of the isolated bacterial strains was (247) divided into (2) categories; Gram-positive strains (186) & Gram-negative strains (61) (Table 1).

Table 1: Detection of biofilm production of bacterial strains isolated from contact lens.

Organism	Frequency	Tube Method Positive	Congo red Method Positive
Staphylococcus aureus	4	2	2
CoNS	21	20	19
Pseudomonas	12	12	11
Non-fermenter Gram negative bacilli	9	8	8
Bacillus	53	44	34
Diphtheroids	33	21	20
Micrococcus	70	58	53
Enterococci	5	4	4
K. pneumonia	4	4	4
K. oxytoca	3	3	3
E. coli	4	4	4
Proteus mirabilis	1	0	0
Proteus vulgaris	3	3	3
Citrobacter koseri	11	10	6
Citrobacter freundii	2	2	1
Enterobacter cloacae	2	2	2
Moraxella spp.	10	10	8
Total	**247**	**207**	**182**

Detection of biofilm formation:

The whole bacterial strains (N = 247) were analysed by both tube method and CRA method to determine phenotypic formation of biofilm. By the tube method, (52.3%) showed moderately positive, (31.5%) strongly positive and (16.2%) were negative. By the CRA method, (35.3%) showed moderately positive, (38.4%) strongly positive and (26.3%) were negative. There is a significant statistical correlation between these two methods & the consistency between them was (74.5%) (P-value = 0.006) (Table 2).

Table 2: Tube method and Congo red agar (CRA) method results analysis

	Tube Method	
	Positive	**Negative**
Congo red agar method		
▪ Positive	157	25
▪ Negative	50	15

Microbial isolates and biofilm formation:

Regarding (53) *Bacillus* species grown, 44 (83.0%) were biofilm + ve in tube method & 34 (64.1%) in CRA method. Of the (33) *Diphtheroids* isolates, 21 (63.6 %) of them were biofilm + ve in tube method & 20 (60.6%) in CRA. Among (70) *Micrococci* isolates, 58 (82.8%) of them were biofilm + ve in tube method & 53 (75.7%) in CRA method. (95.2%) of CoNS isolates were biofilm producers. Also, for the (4) *Staphylococcus aureus* grown, 2 (50.0%) were biofilm +ve in both methods, the (12) *Pseudomonas* strains were (12) (100%) biofilm + ve in tube method & (11) (91.6%) in CRA method. In other bacterial strains, most of them were biofilm producers (table 1).

PCR-based confirmation of bacterial biofilm formation from CLs wearers:

From the four *Staphylococcus aureus* isolates, two isolates (50 %) gave bands for *(icaA*) (188 bp) gene so, two isolates of *Staphylococcus aureus* were positive for the presence of the (*icaA*) gene. Among (21) CONS isolates, three (14.3 %) gave bands for (*icaA*) gene so, 3 CoNS isolates were positive for the presence of the (*icaA*) gene (Figure 3). The isolates which gave bands for *(icaA*) gene were phenotypically (+ve) for biofilm production by both the phenotypic methods. While the isolates that were phenotypically (-ve) for biofilm production did not give any bands for *(icaA*) gene and hence contained no (*icaA*) genes at all. All isolates for *Pseudomonas* were (-ve) for (*pslA*) (1119 bp) gene & gave no bands in U.V. photograph, although majority of them were phenotypically (+ve) for biofilm production but no (*pslA*) genes were detected in the 12 isolates.

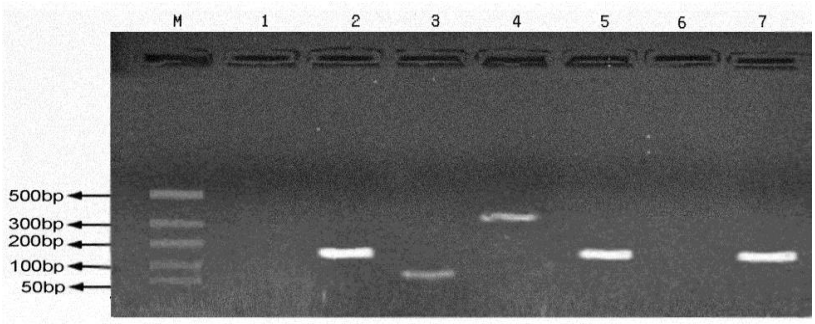

Fig. 3: U.V. photograph for Ethidium bromide stained agarose gel electrophoresis showing marker lane (M); (100) bp DNA stepladder with Lanes (2,5 & 7) demonstrating bands for Staph. aureus & CoNS icaA PCR amplicons (188 bp).

DISCUSSION

Biofilms are really groups of microorganisms that collect to each other and with different surfaces by adherence mechanisms. They are formed of cells and extracellular matrix manufactured by these cells. A biofilm make organisms resistant to anti-microbial agents through failure of antibiotics to penetrate the polysaccharide matrix. It was found that the antibiotic concentration required to kill biofilm forming bacteria is higher than that required to inhibit planktonic cells. Biofilm protects microoganisms from external environment, host immunity and antibiotic therapy. They are the survival strategy that helps bacteria to bypass bad conditions.[20,33,35,36] Our aim is to determine the biofilm forming power of these bacteria by both phenotypic and genotypic detection methods.

In our work, the total number of the isolated bacterial strains was (247); divided into different Gram-positive strains (186) & Gram-negative strains (61). By using tube method; (52.3%) were moderately positive, (31.5%) strongly positive and (16.2%) negative for biofilm formation while after using the Congo red agar method; (35.3%) were moderately positive, (38.4%)

strongly positive and (26.3%) negative for biofilm formation. The total biofilm production was (83.8 %) by tube method and (73.7 %) by CRA method which is higher compared to the study conducted by Hassan et al.,[21] where the percentage of biofilm formation was (63.6%). This difference may be due to that they worked on a fewer number & different types of strains.

In agreement with our work, Raksha et al.[18] detected a similar percentages as (86.7%) by tube method and (76.7%) by CRA method which is also higher compared to the study of Hassan et al.[21]. This concordance in the percentages may be due to that the study time & number of samples are somewhat similar.

In concordance with our results, Raksha et al.[18] found among the (265) isolates assessed, (53.5%) were moderately positive, (33.2%) strongly positive and (13.2%) negative by tube method. While when using CRA method, (36.6%) were moderately positive, (40%) were strongly positive and (23.3%) were negative which is closely related to our results.

On contrary, Hassan et al.[21] found among (110) isolates, strong biofilm producers were (21), moderate (33) and weak producers (56) which is lesser compared to our results. Also, Mathur et al.[17] by tube Method, they detected 18 (11.8%) isolates as strong biofilm producers and 45 (29.6%) were moderate producers as they worked on a fewer number & different types of strains. Our results were higher compared to Mathur et al.[17], Hou et al.[22] and Hassan et al.[21] where biofilm formers percentages using CRA were 3.4%, 34.38% and 3.6% respectively.

In the present study, tube method showed more +ve isolates when compared with the (CRA) method. The results were easier to be interpreted with the CRA method as tube method was mainly observational. Consistency between the two methods in our work was (74.5%). Although there are some differences between the results of both methods, there was a significant statistical correlation (*P* value = 0.006).

In coordination with our statistical results, Raksha et al.[18] found that the consistency between the two methods was (75.8%) that was very close to ours & there was a significant statistical correlation.

In the present study, it was found that biofilm production by commensal bacteria was (83.0%) of *Bacillus* isolates, (63.6%) of *Diphtheroids,* isolates and (82.8%) of *Micrococci* isolates which indicate that they are potential biofilm formers. In agreement with our results, Raksha et al.[18] found that among the commensals, (77.1%) of *Bacillus* isolates, (62.8%) of *Diphtheroids,* isolates and (84%) of *Micrococci* isolates were biofilm producers which are relatively very near to our percentages.

In our study, (95.2 %) of CoNS isolates were biofilm producers. Similarly, Raksha et al.[18] reported that (86.9%) of CoNS isolates were biofilm positive supporting the studies conducted by Catalanotti et al.[23] where (74.1%) of *Staphylococcus epidermidis* strains were biofilm producers. [24]

In our work, *Pseudomonas aeruginosa* strains were (12), (100%) biofilm + ve in tube method and (11) (91.6%) in CRA method.

In agreement with our results, Raksha et al., [18] all the *Pseudomonas* isolates (13/13 by tube method & 12/13 by CRA method) obtained were +ve for biofilm producing.

On the contrary, Oncel et al.[25] stated that (60%) (6/10) of *P.aeruginosa* isolates from chronic rhinosinusitis produced biofilm. Also, Coban et al.[26] found only (33.3%) (20/60) of *P. aeruginosa* samples were +ve for biofilm-formation in cystic fibrosis. These differences may refer to that they conducted their work on a fewer limited number of cases and different diseases.

The four *Staphylococcus aureus* isolates, 2 (50.0 %) were biofilm + ve by both methods. The other bacterial isolates, most of them were biofilm producers**.** These results come in agreement with that of Raksha et al.[18]

In the present study, of the four *Staphylococcus aureus* isolates; two (50%) of these were containing (*icaA*) gene by PCR and of the (21) CoNS isolates; three (14.3%) contained (*icaA*) gene. The biofilm formation in *Staphylococcus* species are encoded by *icaA* gene that is formed of four areas namely *ica* A, D, B and C.[24,27]

In agreement with our results, Raksha et al.[18] *Stahylococcus aureus* and CoNS strains were assessed for the presence of *icaA* gene (188 bp) using PCR. Of (23) CoNS isolates three (13%) and two out of four (50%) *Staphylococcus aureus* isolates showed the presence of *icaA* gene.

Our results are different from the study of Hou et al.[22] in which (40.63%) of *Staph. epidermidis* and (11.11%) of *Staph. aureus* strains were proved to carry *icaA* gene. These results suggest that the production of biofilms needs a multiple factors like *icaC, icaD* [28] and the *icaA* gene seems to be an important factor determining biofilm formation. The genotypically +ve isolates for *icaA* gene were also phenotypically +ve for biofilm formation by both the phenotypic methods which is in agreement with the study conducted by Suzuki et al.,[29] The isolates which were phenotypically -ve for biofilm production did not show the presence of *icaA* gene.

In the present study, although all of the *Pseudomonas* isolates didn't contain *pslA* (1119 bp) gene[27,34], these were +ve for biofilm production by phenotypic methods that agrees with results of Raksha et al.[18]

Our result is completely different from the study of Hou et al.[22] in which (31.03%) of *Pseudomonas* strains contained *pslA* gene. Reports by Colvin et al., [15] proposed an important role of the *pslA* gene in the initiation of *P. aeruginosa* biofilm formation,[30] So, further studies are needed to genotypically detect the

biofilm formation by *Pseudomonas* as biofilm formation process is a net result of interaction of many genes.[31,32]

CONCLUSIONS

The majority of bacteria isolated had the capacity to form biofilms. Both tube and Congo red agar methods showed significant correlation and detected a good number of biofilm-producing strains but the Congo red agar method is more preferable as the tube method is mainly observational. The absence of genes responsible for biofilm formation did not exclude the phenotypic biofilm production by these bacteria which is a common state.

Recommendations:

The present study recommended further genetic studies of *icaA* and *pslA* independent biofilm formation mechanisms and regular surveillance of biofilm formation by bacterial strains and their antibiograms to prevent probable infections. It is also recommended to take care with the contact lens hygiene and to adjust the frequency of their change to avoid this contamination and infection.

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Conflicts of interest:

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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