Original Article

Study of biofilm formation in bacterial isolates from contact lens wearers

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Purpose: To detect biofilm forming capacity of bacterial isolates obtained from the conjunctiva, contact lens and accessories of contact lens wearers using phenotypic and genotypic methods. **Methods:** Bacterial strains were collected from the conjunctiva, contact lens and lens storage cases of contact lens wearers. The phenotypic detection of biofilm production was done using the tube method and congo red agar method. The biofilm-forming related genes, *icaA*, of Coagulase negative *Staphylococcus* (CONS) and *Staphylococcus aureus*, and *pslA*, of *P. aeruginosa*, were detected using PCR. **Results:** A total of 265 bacterial isolates which included *S. aureus*, CONS, *Pseudomonas*, Nil-fermenter Gram-negative bacilli (NFGNB), *Bacillus spp, Diphtheroids, Micrococci, Klebsiella pneumonia, Klebsiella oxytoca, E. coli, Proteus mirabilis, Proteus vulgaris, Citrobacter koseri, Citrobacter freundii, Enterobacter cloacae, Moraxella* were obtained. Of the 265 isolates, 53.5% were moderately positive, 33.2% strongly positive and 13.2% negative for biofilm production by tube method and 36.6% were moderately positive, 40% strongly positive and 23.3% negative for biofilm production by congo red agar method. Of the four *S. aureus* isolates, two (50%) showed the presence of *icaA* gene. Of the 23 CONS isolates, three (13%) showed the presence of *icaA* gene. All the *Pseudomonas* isolates were negative for presence *pslA* (1119 bp) gene though most of them were phenotypically positive for biofilm formation. **Conclusion:** Most of the bacterial isolates obtained from contact lens wearers had the potential to produce biofilms. Tube method and Congo red agar method exhibited significant statistical correlation (*P*-value = 0.006) and picked up a good number of biofilm-forming isolates, hence may be used for detection of biofilm production. The absence of biofilm-forming gene did not rule out the possibility for phenotypic biofilm production by bacteria.

Key words: Biofilm, congo red agar method, contact lens, lens storage case, polymerase chain reaction, tube method

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Contact lens (CL) use has been increasing for cosmetic or therapeutic purposes and is preferred because of their optical and cosmetic benefits over spectacles. Microbial contamination and eye infections are proved to be present in cases where there is a lack of compliance and poor hygiene towards lens care.[1,2] Microbial keratitis is a rare but feared complication of contact lens use as this may result in permanent loss of vision as a consequence of corneal scarring or perforation.[3] A biofilm has been defined as a "functional consortia of micro-organisms, organized at interfaces, within exopolymer matrices".[4] Biofilm protects microbes against antibiotics, phagocytes and bacteriophages and hence help in their survival.[5] Both Gram-positive and Gram-negative bacteria possess the ability to form biofilm such as *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Enterococcus faecalis, Klebsiella pneumonia, Proteus mirabilis* and *Pseudomonas aeruginosa*.[6] Bacterial biofilms are thought to play a major role in more than 80% of bacterial infection.[7,8] Biofilms were observed on CLs, IOLs, glaucoma tubes, stents, punctual plugs, corneal sutures, scleral buckle, or other ocular prostheses.[9,10] Extracellular polymeric substances (EPS) are secreted by bacteria that hold together heterogeneous mixtures of bacteria and therefore are an important component of biofilm production.[11] The main components of EPS in *S. aureus* and *S. epidermidis* are Polysaccharide intercellular adhesion (PIA) and capsular polysaccharide/adhesin (PS/A). Several studies have shown that the intercellular adhesion (ica) locus, particularly the icaA gene, encodes the production of both PS/A and PIA.[12,13] The *pslA* gene performs an essential function in biofilm formation of *P. aeruginosa*.[14,15] To our knowledge, biofilm formation ability of clinical strains of bacteria obtained from the conjunctiva, CL and its accessories have not been analyzed using both genotypic and phenotypic methods. Therefore, the aim of the present study was to: (a) Detect biofilm forming capacity of bacterial isolates obtained from conjunctiva, CL and accessories of CL wearers, (b) Comparison of biofilm detection by two methods: Tube method and Congo red agar (CRA) method, and (c) Corroboration of biofilm formation with detection of gene for biofilm formation by PCR (*Staphylococcus* and *Pseudomonas* isolates).

Methods

The study was an observational study conducted in the Department of Microbiology and Ophthalmology of a tertiary

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care hospital attached to a medical college. Institution ethical clearance was obtained. Ethical committee approval was obtained. Informed written consent was obtained from those who volunteered to participate. A total of 40 CL wearers in the age group 18-35 years which consisted of undergraduate and postgraduate medical students were included in the study. All the 40 CL users were lens wearers for a period of more than 4-5 years.

The study subjects were silicone hydrogel soft contact lens users (SiHy). Frequency of change of CL wear is as follows:

- Monthly wear CLs: 37 participants
- Daily wear CLs: 1 participant
- Quarterly wear CLs: 2 participants.

The CLs storage case was used for duration of 4-6 months by the study population.

All the participants were examined by an ophthalmologist using a slitlamp. Individuals with ocular infections, co-existing ocular diseases, antibiotic use within 1 month and systemic diseases were excluded from the study.

Bacterial strains

Samples were collected from conjunctiva, CL and lens storage cases of both the eyes of CL wearers. Thus, a total of six samples each was collected from 40 CL wearers ($N = 240$). The samples were obtained by swabbing the lower conjunctival sacs, lens storage cases using sterile cotton swabs and CLs were collected (aseptically) from people just as they were to be discarded. All the samples were incubated for 24 hours at 37°C in Brain heart infusion broth and then sub-cultured onto blood agar, MacConkey agar and Sabouraud's dextrose agar (SDA). The blood agar and MacConkey agar was incubated at 37°C whereas SDA was incubated at 25°C. Organisms grown were identified using standard microbiological technique.[16] Of the 240 samples obtained, 6 samples had sterile growth and 27 of them exhibited polymicrobial growth; hence the total number of bacterial isolates obtained was 265.

Detection of biofilm production

The bacterial isolates obtained were subjected to two tests to detect biofilm production:

Tube method

The bacterial isolates (loopful of bacteria) obtained were inoculated into Trypticase soy broth supplemented with 1% glucose (TSBglu) and incubated for 24 hours at 37°C. Tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed, and tubes were washed with deionized water. Tubes were then dried in an inverted position and observed for biofilm formation.[17] Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Based on the intensity of the color formed, they were characterized as moderately positive and strongly positive [Fig. 1]. Lab-confirmed biofilm producer strain was used as a positive control.

CRA method

The bacterial strains obtained were inoculated into CRA and was incubated for 24-48 hours at 37°C.[17]

CRA was prepared as follows: BHI broth was supplemented with 5% sucrose and Congo red stain. Medium composed of BHI (37 g/L), Sucrose (50 g/L), Agar (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from other medium constituents and was added when the agar was cooled to 55°C. Isolates were considered as strongly positive when there was the presence of black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated a moderately positive biofilm producer. Colonies that remained pink were designated as non-biofilm producer [Fig. 2]. Lab-confirmed biofilm producer strain was used as a positive control.

Polymerase chain reaction (PCR)

Colony PCR method was used in order to detect genes related to biofilm formation in *Staphylococcus* species and *P. aeruginosa*. Bacterial cultures were lysed, DNA extracted, and gene-specific primers were used to amplify DNA fragments using PCR.[18] The DNA template was obtained using the crude method: Loop full of culture were picked up using sterile pipet tip, suspended in 50 µl nuclease free water, boiled at 95°C for 10 minutes and centrifuged at 14,000 rpm speed for 1 minute. The supernatant (2 µl) obtained was used as the template. The Primer Sequences and Product Length for *icaA* of *Staphylococcus* spp. and *pslA* of *P. aeruginosa* (common organisms implicated in biofilm formation):

PCR sequences product length

IcaA F: 5′-TCTCTTGCAGGAGCAATCAA-3′ 188 bp

R: 5′-TCAGGCACTAACATCCAGCA-3′

PslA F: 5′-CACTGGACGTCTACTCCGACGATAT-3′ 1119 bp

R: 5′-GTTTCTTGATCTTGTGCAGGGTGTC-3′

Reaction mix (20 µl)

2.0 µl of template suspension, 1.0 µl of 10 µM FP (Forward primer), 1.0 µl of 10 µM RP (Reverse primer), 6.0 µl of nuclease-free water, 10 µl of Master mix which consisted of: Taq DNA polymerase, dNTPs, Magnesium chloride and reaction buffers at optimal concentrations.

PCR reaction condition for gene 1 (icaA)

A thermal step program was used, including the following parameters: Incubation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 45 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 20 seconds (elongation) and 72°C for 10 minutes after conclusion of the 30 cycles. Amplification products were analyzed using 2% agarose gel electrophoresis.

PCR reaction condition for gene 2 (pslA of P. aeruginosa)

A thermal step program was used, including the following parameters: Incubation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 45 seconds (denaturation), 55 C for 30 seconds (annealing), 72°C for 1 minute 20 seconds (elongation) and 72°C for 10 minutes after conclusion of the 30 cycles. Amplification products were analyzed using 1% agarose gel electrophoresis.

Data analysis

The data obtained were in the form of percentages and were analyzed using appropriate statistical tests and represented using tables and bar graphs.

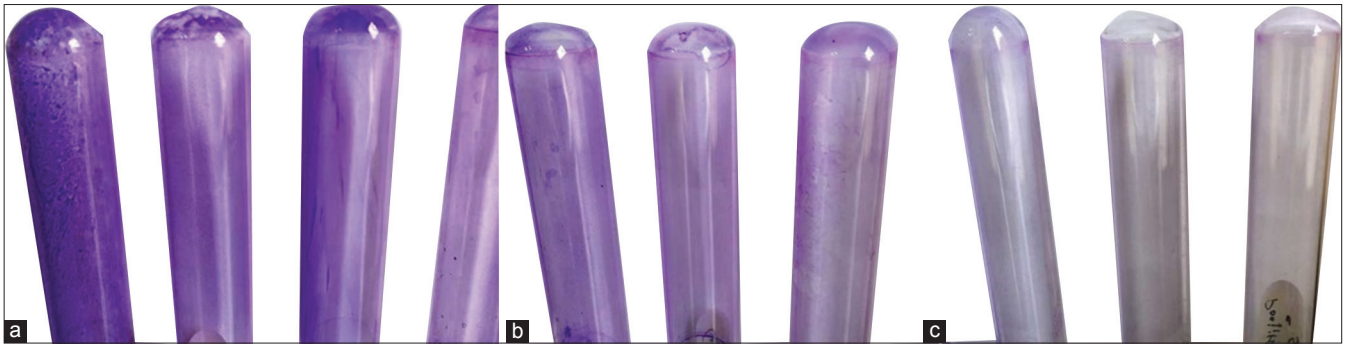

Figure 1: Bacterial biofilm detection by the tube method, showing strongly positive (a), moderately positive (b) and negative (c) results

Figure 2: Biofilm detection by Congo red agar method, showing strongly positive (a), moderately positive (b) and negative (c) results

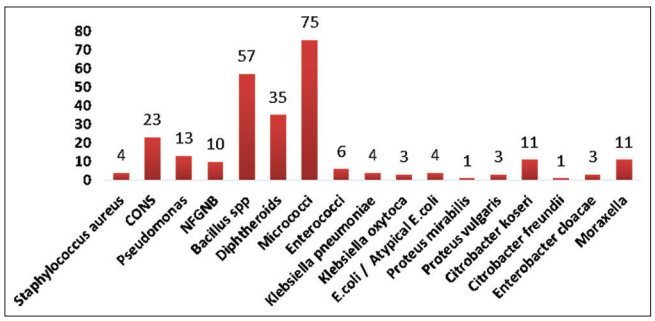

Figure 3: Frequency of bacterial isolates obtained from the conjunctiva, Contact lens (soft Contact lenses –monthly disposable) and lens storage cases of contact lens wearers

Results

Bacterial strains

The bacterial isolates obtained from the conjunctiva, CL (soft CLs-monthly disposable) and lens storage cases of contact lens wearers were: *S. aureus*, Coagulase negative *Staphylococcus* (CONS), *Pseudomonas*, Nil-fermenter Gram-negative bacilli (NFGNB), *Bacillus spp, Diphtheroids, Micrococci, Klebsiella pneumonia, Klebsiella oxytoca, E. coli, Proteus mirabilis, Proteus vulgaris, Citrobacter koseri, Citrobacter freundii, Enterobacter cloacae* and *Moraxella*. The distribution of the bacterial isolates obtained is depicted in Fig. 3.

Table 1: Analysis of results obtained in tube method and congo red agar method

	Tube method	
	Positive	Negative
Congo red agar method		
Positive	188	19
Negative	45	13

Detection of biofilm formation

Total bacterial isolates (n = 265) were assessed by both tube method and CRA method to look for phenotypic production of biofilm. Of the 265 isolates, 53.5% were moderately positive, 33.2% strongly positive and 13.2% negative by the tube method. Of the 265 isolates, 36.6% were moderately positive, 40% strongly positive and 23.3% negative by the CRA method. The consistency between the CRA method and the tube method was 75.8% (P-value = 0.006). The results of tube method and CRA are depicted in Table 1.

Microbial isolates and biofilm formation

Among 57 *Bacillus* sp obtained, 44 (77.1%) of them were biofilm positive in tube method and in CRA method. Of the 35 *Diphtheroids* isolates obtained, 22 (62.8%) of them were biofilm positive in tube method and 21 (60%) in CRA. Among 75 *Micrococci* isolates obtained, 63 (84%) of them were biofilm positive in tube method and 58 (77.3%) in CRA method. 86.9% of CONS isolates were biofilm producers. In other bacterial organisms, most of them were biofilm producers. The results are depicted in Table 2.

PCR-based confirmation of bacterial biofilm formation from CLs wearers

Of the four *S. aureus* isolates, two (50%) showed the presence of *icaA* gene. Among 23 CONS isolates, three (13%) showed the presence of *icaA* gene [Fig. 4]. The isolates which showed the presence of *icaA* gene were phenotypically positive for biofilm formation by both the methods (tube method and CRA method). The isolates which were phenotypically negative for biofilm formation did not show the presence of *icaA* gene. All

Table 2: Detection of biofilm production of bacterial isolated obtained from contact lens wearers

Organism	Frequency	Tube method Positive	Congo red method Positive
Staphylococcus aureus	4	4	4
CONS	24	22	20
Pseudomonas	13	13	12
NFGNB	10	9	11
Bacillus sp	57	44	36
Diphtheroids	35	22	21
Micrococci	75	63	58
Enterococci	6	5	4
Klebsiella pneumonia	5	7	7
Klebsiella oxytoca	3	3	3
E. coli/Atypical *E. coli*	4	4	4
Proteus mirabilis	1	0	0
Proteus vulgaris	3	3	3
Citrobacter koseri	11	10	5
Citrobacter freundii	1	1	1
Enterobacter cloacae	3	3	3
Moraxella	11	10	8
Total	265		

the *Pseudomonas* isolates were negative for presence *pslA* (1119 bp) gene though most of them were phenotypically positive for biofilm formation.

Discussion

Biofilms are the survival strategy of bacteria which help them survive harsh environmental conditions.[5] They have the ability to bear a high concentration of antimicrobial agents.[19] To best of our knowledge, there are few large studies analyzing the biofilm-forming capacity of clinically obtained bacterial isolates (conjunctiva, CLs and its accessories). Various gram-positive (200) and gram-negative bacterial (65) isolates were obtained from the conjunctiva, CLs and its accessories of CLs wearers and subjected to detection of biofilm production using tube method and CRA. The percentage of biofilm producers in our study was 86.7% by tube method and 76.7% by CRA method which is higher compared to studies conducted by Mathur *et al*.,[17] Afreenish Hassan *et al*.[20] and Juárez-Verdayes *et al*.[21] where the percentage of biofilm formers is 41.4%, 63.6% and 66% respectively. Of the 265 isolates assessed, 53.5% were moderately positive, 33.2% strongly positive and 13.2% negative by tube method. As compared with results of a study conducted by Mathur *et al*.,[17] where tube Method picked up 18 (11.8%) isolates as strong biofilm producers and 45 (29.6%) were moderate biofilm producers, the present study showed a higher proportion of biofilm producers. In a study conducted by Afreenish Hassan *et al*.[20] – Among 110 isolates, strong biofilm producers were 21, moderate were 33 and weak or non-biofilm producers were 56 which again is lesser compared to results obtained in the present study.

CRA enables for the direct analysis of the colonies. Of the 265 isolates, 36.6% were moderately positive, 40% strongly positive and 23.3% negative which is higher compared to other studies conducted by Mathur *et al*.[17] Hou *et al*.[22] and Afreenish Hassan *et al*.[20] where the percentage of biofilm formers using CRA was 3.4%, 34.38% and 3.6% respectively. Tube method picked up higher positive isolates when compared with CRA method. Based on our experience with the tube method and CRA, the interpretation of results was easier with CRA as tube method was subjective in nature. Consistency between the tube method and CRA method in our study was 75.8%. Though there are differences between the results of the tube method and CRA method, they show significant statistical correlation *P* value = 0.006). Therefore, we suggest that the phenotypic methods (tube method and CRA method) can be used as a convenient way to detect biofilm-related infections. The unique attempt of the study was to detect biofilm production by commensals. Among the commensals, 77.1% of *Bacillus* isolates, 62.8% of *Diphtheroids,* isolates and 84% of micrococci isolates were biofilm producers which show that they are also indeed potential biofilm producers. In all, 86.9% of CONS isolates were positive for biofilm formation which is similar to studies conducted by Catalanotti *et al*.[23] where 74.1% of *S. epidermidis* strains were biofilm positive. All the *Pseudomonas* isolates (13/13 by tube method and 12/13 by CRA method) obtained were phenotypically positive for biofilm formation. Oncel *et al*.[24] reported that 60% (6/10) of *P. aeruginosa* isolates from chronic rhinosinusitis produced bacterial biofilms. Coban *et al*.[25] also reported 33.3% (20/60) of *P. aeruginosa* samples tested for the biofilm-formation ability of isolates in patients with cystic fibrosis were biofilm-positive.

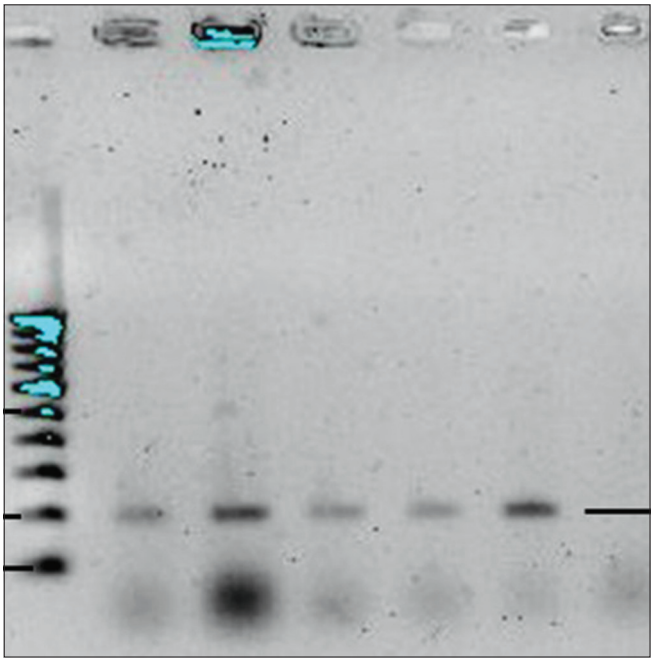

Figure 4: Lane 1 – 100 bp DNA ladder; Lane 2-7 – Staph *icaA* PCR amplicon

Major synthetic pathway of biofilm formation in *Staphylococcus* species are encoded by *icaA* operon which is comprised four genes namely *ica* A, D, B and C.[26,27] Hence, *ica* A was considered as the representative gene for the *icaA* operon in our study. *S. aureus* and CONS where assessed for

the presence of *icaA* gene (188 bp) using PCR. Of 23 CONS isolates three (13%) and two out of four (50%) *S. aureus* isolates showed the presence of *icaA* gene. Our results differ from study conducted by Hou *et al*.[22] where 40.63% of *Staphylococcus epidermidis* and 11.11% *S. aureus* strains carried *icaA* gene. These results indicate that the formation of biofilms requires a complicated network of factors such as icaC,[28] icaD[28] and the *icaA* gene is likely to be one of many factors that regulate biofilm formation. The isolates which were genotypically positive for *icaA* gene were also phenotypically positive for biofilm formation by both the methods (tube method and CRA method) which is similar to study conducted by Takashi *et al*.[29] The isolates which were phenotypically negative for biofilm formation did not show the presence of *icaA* gene.

All the *Pseudomonas* isolates were negative for presence *pslA* (1119 bp) gene but most of them were phenotypically positive for biofilm formation. The finding in our study is different from the study conducted by Hou *et al*.[22] where 31.03% of *Pseudomonas* strains carried *pslA* gene. Previous reports by Overhage *et al*.[14] and Colvin *et al*.[15] suggested an essential role for the psl gene cluster in the initial step of *P. aeruginosa* biofilm formation, so we focused on the functional assessment of the pslA gene. P. aeruginosa produces at least three polysaccharides (alginate, Pel and Psl) which determine the stability of the biofilm structure.[30,31] Among the 60 two-components systems found in the genome of P. aeruginosa,[32] the GacS/GacA system acts as a super-regulator of the QS system and is involved in the production of multiple virulence factors as well as in biofilm formation.[33] Hence, further studies are required to genotypically detect biofilm production by *Pseudomonas* spp as the process of biofilm formation is determined by the interaction of many gene clusters, some of which have not yet been identified.

Conclusion

In conclusion, most of the bacterial isolates obtained from CLs were potential enough to produce biofilms. Tube method and CRA exhibited significant statistical correlation and picked up a good number of biofilm-forming isolates, hence may be used for detection of biofilm production. The absence of biofilm-forming gene does not rule out the possibility of phenotypic biofilm production by bacteria.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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