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# Biofilm: The Haven for *Staphylococcus epidermidis* in Post-operative Endophthalmitis

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## Abstract

**Background:** *Staphylococcus epidermidis*, in spite of being a commensal of the eye, is regarded as one of the common etiological agents of bacterial endophthalmitis. The purpose of the study was to determine the pathogenic potential of this commensal organism in post-operative endophthalmitis.

**Methods:** A total of 63 *S epidermidids* isolates, comprising of 47 from vitreous samples of post-operative endophthalmitis cases and 16 controls from the healthy conjunctivae, were studied. Biofilm producing ability was determined by quantitative adherence test and "ica AB" Polymerase Chain Reaction (PCR) assay. Bacteria adhering onto the lens surfaces were enumerated by standard protocol. Scanning electron microscopy (SEM) was employed to visualize the biofilms on the lenses.

**Results:** Of the 47 isolates, 23 (48.9%) were adherent and 24 (51.0%) were nonadherent. PCR showed that 16 (34.0%) isolates possessed the *ica* locus whereas 31 (65.9%) did not possess this gene. All 16 (100%) *ica* positive organisms and only 7 (22.58%) of the 31 *ica* negative organisms adhered to artificial surfaces ($p<0.001$). SEM revealed that isolates carrying *ica* gene had formed intimate biofilms on intra ocular lenses. Clinical isolates showed significantly higher number of attaching bacteria onto the lens material as compared to the commensals.

**Conclusions:** This study documented that bacteria carrying *ica* gene were found to be adherent and biofilm producers. Biofilm could be incriminated as a virulence marker for *S epidermidis* causing post-operative endophthalmitis. PCR was a sensitive and rapid tool to detect biofilm forming ability.

**Keywords:** Endophthalmitis; *S epidermidis*; Biofilm; Adherence; Scanning electron microscopy; IOL

## Introduction

*Staphylococcus epidermidis* is regarded as one of the important causative agents of bacterial endophthalmitis following surgery [1]. However the pathogenicity of *S epidermidis* in post operative endophthalmitis (POE) is not known. In other words it is not clear as to how this organism, in spite of being a normal commensal of the eye, acquires such pathogenic potential. Recent evidences, both laboratory and clinical, support the role of *S epidermidis* in infections associated with indwelling medical devices [2] and its ability to form biofilms on such devices [3].

Biofilms are structured communities of microbial cells which are encased within a self-produced polymeric matrix and adherent to an inert or living surface. Once the organisms are embedded inside a biofilm they are well protected from the effects of antibiotics and the adversity of the host defenses [4,5]. The mechanism by which *S epidermidis* attaches to indwelling prosthetic materials and elaborates biofilm is being increasingly understood. One important element in this process is the *ica* operon, a gene cluster encoding the production of polysaccharide intercellular adhesin (PIA) that mediates intercellular adhesion giving rise to accumulation of multi-cellular sessile structures, known as biofilms [6].

Staphylococcal biofilms have widely been implicated in many implant based infections, including those associated with prosthetic heart valves, central venous catheters, orthopedic prostheses and urinary catheters [5,7]. However documentation of Staphylococcal biofilms in relation to IOLs is scanty. At the same time, it is well known that POE cases following implantation of devices such as intraocular lenses (IOL) are commonly caused by *S epidermidis*. Therefore, this study was aimed at determining if *S epidermidis* could form biofilms on IOLs and secondly, if the phenotypic as well as the molecular markers of *S epidermidis* virulence documented in other device related infections, also perpetuate for late onset POE.

## Materials and Methods

### Bacteria

A total of 63 *S epidermidis* isolates were studied. These comprised of 47 obtained from the vitreous samples in patients with late onset

POE and 16 commensal *S epidermidis* from healthy individuals' conjunctival sac, as controls.

## Case definitions of POE

Late onset POE was diagnosed when features of endophthalmitis such as increase in pain and redness, decreasing visual acuity, flare in the anterior chamber, corneal edema, hypopyon and poor glow in the fundus developed in a patient 4-6 wks following IOL implantation

## Collection and processing of samples

**Intraocular fluid:** 0.1-0.3 ml of vitreous fluid were collected using a 22 gauge needle and a tuberculin syringe under proper sterile conditions by approaching the anterior portion of vitreous cavity through pars plana region.

**Conjunctival swab:** In order to obtain commensal *S epidermidis*, conjunctival swabs from healthy individuals were collected by using sterile cotton tipped swabs pre-moistened with sterile normal saline by gently rolling the swab on the lower cul-de sac from lateral to medial side after retracting the lower eyelids properly. All the samples were processed and organisms were identified according to the standard protocol [8,9].

## Quantitative adherence test

Adherent properties of the organisms were determined by the quantitative slime test for adherence earlier standardized in our laboratory [4]. Briefly overnight bacterial culture in Trypticase Soy Broth (TSB) was diluted in 1:100 in fresh TSB and 1 ml of the diluted culture was added to separate quartz cuvettes and incubated overnight at 37°C. The cuvettes were then washed four times with phosphate buffer saline (PBS) of pH 7.2, fixed with Bouin's fluid and stained with Hucker's crystal violet. Excess stain was removed by gently washing with water and the cuvettes were then dried. The optical density (OD) of the stained biofilm was recorded at 570 nm. Organisms were considered adherent, if the OD was above the cut off value, which was calculated as 3X SD above the mean OD of 10 blank cuvettes stained similarly in that particular batch of the test.

## Scanning electron microscopy

Standard procedure was followed. *S epidermidis* overnight cultures were diluted 1:40 in fresh TSB and 200 ul of the each diluted culture was added to IOLs fixed at the bottom of 96 well polystyrene microtiter plates and incubated at 37°C for 48 hr, followed by three times washing with PBS. Fixation for scanning electron microscopy was done by fixative constituting 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer for 2 hours at 4°C followed by three times washing with PBS. A secondary fixation step was then performed with 1% osmium tetra oxide at room temperature for 1 hour followed by quick wash with 0.1M phosphate buffer. The samples were then dehydrated with the increasing ethanol concentrations such as 50%, 70%, 80%, and 95% for 15 min each and then two times wash with pure ethanol for 15 minutes. These were then subjected to critical point drying, finally mounted on aluminum stubs for sputter coating. Observations were made at 15kV with magnification of 1500 to 2500k using scanning electron microscope (LEO-435 VP, Co-operation Zeiss-Lieca)

## Enumeration of adherent bacteria on IOL

The method adopted was according to that described by Okajima et al. [10] with minor modifications. Briefly, bacterial suspensions were prepared in TSB to a concentration of $10^8$ organisms/ml. The sterile packing of each IOL was opened immediately before dipping in the bacterial suspensions to ensure minimal exposure to room air. The IOLs were placed separately under sterile conditions in 1 ml of *S epidermidis* suspension and incubated at 37°C for 20 minutes.

After incubation each IOL was removed from the suspension and placed in 5 ml of PBS (pH 7.2) and washed by vertexing for 60 seconds. This step was repeated with 5 ml of fresh PBS. Subsequently the IOL was placed in 1.5 ml microtube (Lock Fit: Treff, Degershem, Switzerland) containing 0.3 ml of 1 mm diameter sterile glass beads in 1 ml PBS. This regimen removed all adherent bacteria without affecting their viability. The disaggregated bacteria were then enumerated by the method of Miles and Mishra [11]. Briefly, the PBS containing the dislodged organisms was subjected to serial 10 fold dilutions ($10^{-1}$ to $10^{-6}$). A 100 µl quantity from every dilution was plated onto blood agar plate. The plates were incubated for 24 hours and the dilution yielding the highest number of discrete colonies was taken into consideration. Thus the number of organisms present in the original PBS containing the dislodged bacteria was calculated by multiplying with the appropriate power of 10. This was equal to the number of bacteria [expressed in terms of colony forming units (cfu) / ml] adherent per IOL.

Experiment was designed into four groups: Group I comprised of the 16 commensal isolates, group II comprised of the 24 non-adherent and *ica* negative organisms, group III of the 16 adherent and *ica* positive organisms, while group IV contained the 7 isolates which were adherent, but *ica* negative (vide infra, Table 1).

| ADHERENCE | *Ica*AB positive | *Ica*AB Negative |
|---|---|---|
| Adherent (23) | 16 | 7 |
| Non adherent (24) | 0 | 24 |
| X2 = 25.31; p<0.0001 | | |

**Table 1:** *ica* PCR positivity amongst adherent and nonadherent organism.

## Molecular characterization of the isolates

**DNA extraction:** Bacterial DNA was extracted by the recommended procedure developed earlier [12], with some modifications. An aliquot of 0.1 ml of an overnight culture in TSB was pelleted by centrifugation (5000 X g for 5 minutes). The culture pellet was then suspended in 300 µl of lysis buffer which consisted of (0.1 M Tris-Hcl {pH 8.0}, 100 mM EDTA, 30 µl of 10% Sodium Dodecyl Sulfate) along with 30 µl of 100 µg/ml lysostaphin and 30 µl of 100 µg/ml of proteinase K. The mixture was then incubated at 37°C for 1 hour and was finally treated with phenol-chloroform-isoamyl alcohol in the ratio of 25:24:1, and then with chloroform- isoamyl alcohol in the ratio, 24:1. DNA was precipitated by sodium acetate and isopropanol, followed by 70% ethanol wash. DNA pellet was finally suspended in distilled water.

**Oligonucleotides:** The intercellular adhesin gene "*ica*" responsible for the phenotypic character such as biofilm, was amplified using the following primers [13]; forward: 5' TTA TCA ATG CCG CAG TTG TC, complimentary to nucleotides 1893 to 1919 and reverse 5': GTT

TAA CGC GAG TGC GCT AT which was complementary to nucleotides 2389 to 2408. The pair of primers amplified 516 bp sequence of the biofilm forming *ica* AB gene.

**PCR for *ica* AB gene:** The previously employed method [13] was followed, using the above set of primers, after standardization as per our own laboratory conditions. Each PCR reaction was carried out in 25 µl reaction volume. The reaction mixture consisted of 2.5 µl of 10x PCR buffer, 2 µl of 10 mM deoxynucleotidetriphosphate (dNTP) mix, 1 unit of Taq polymerase, 3 µl of DNA template, 10 pmol of both primers and sterile water to make up volume to 25 µl. DNA amplification was carried out in a Perkin Elmer thermo cycler with the following thermal cycling conditions: initial denaturation at 94°C for 2 minutes followed by 30 cycles each of amplification at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, ending with a final extension at 72°C for 5 minutes. PCR product was visualized by loading 10 µl volume of the amplified product on 2% agarose gel incorporating 0.5 µg/ml ethidium bromide in Tris borate buffer.

### Statistical analysis

Data were recorded in pre-designed proforma and managed in an excel spread sheet. Number of cells attached being non-normal variables, these were summarized as median (range: minimum to maximum). To compare differences in median values between the four groups, Kruskal Wallis test (nonparametric) followed by Wilcoxon Rank Sum test for pair-wise comparisons with p value adjustment for multiple comparisons, was used. Stata 12.0 statistical software was employed for data analysis. In this study, test adopted, was two sided.

## Results

The PCR assay was conducted for all 47 clinical isolates as mentioned in the methods for the amplification of the '*ica*AB' gene. As is evident from Figure 1, the '*ica*AB' amplified product was a 516 bp fragment. Out of the 5 strains shown, two i.e E5 and E8 (1*st* and the 3*rd* lanes respectively from the left) showed distinct 516 bp bands.

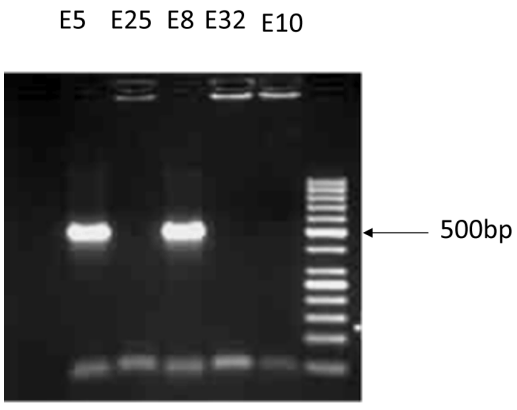


**Figure 1:** Gel picture of *ica*AB PCR. Amplified products run on lanes E 5 and E 8 show distinct 516 bp bands indicating PCR positivity, whereas lanes E 25, E 32 and E 10 show negative results. The lane on the right most shows 50 bp ladder molecular weight marker.

Of the 47 isolates, 16 (34.04%) were '*ica*AB' positive; whereas 31 (65.95%) were negative (Table 1). Whereas all 16 (100%) '*ica*' positive organisms showed adherence onto artificial surfaces, only 7 (22.58%) out of 31 '*ica*' negative organisms were able to do so. This difference was found to be statistically significant (Table 1, p<0.001).

As mentioned in the methods, all 16 *ica* positive adherent; 24 *ica* negative non adherent, 7 *ica* negative adherent and 16 commensal bacteria were tested for their abilities to attach onto the IOL surfaces. Table 2 presents the statistical comparison of the enumerated (cfu/ml) figures with respect to the attached bacterial cells. As is evident from the data analysis, all clinical isolates, irrespective of whether those were *ica* positive or negative, had the capability to attach to the lens surfaces in significantly higher numbers as compared to the commensals (I vs. II, p<0.001; I vs. III, p<0.001; I vs. IV, p<0.001). Similarly, larger number of adherent organisms, irrespective of their *ica* positivity were found to have attached to the lens material as against the *ica* negative non adherent bacteria, and this difference, too, was statistically significant (Table 2; II vs II, p<0.001; III vs.IV, p<0.001). No significant correlation, however, could be found between the adherent *ica* negative and adherent *ica* positive groups (III vs. IV, p>0.05).

| Groups | No. of organisms (n) | Quantification (cfu/ml) of bacteria attached to the IOL surface | |
|---|---|---|---|
| | | Median | Range (Minimum to Maximum) |
| Commensals (I) | 16 | $0.925\times10^5$ | $(0.004 \text{ to } 4.0)\times10^6$ |
| Non adherent & *ica* negative (II) | 24 | $6.25\times10^6$ | $(0.025 \text{ to } 80)\times10^6$ |
| Adherent & *ica* positive (III) | 16 | $5.25\times10^9$ | $(0.5 \text{ to } 60)\times10^9$ |
| Adherent & *ica* negative (IV) | 7 | $3.0\times10^9$ | $(1.5 \text{ to } 40)\times10^9$ |
| One way ANOVA using Kruskal Wallis test : $\chi^2=49.368$, p<0.0001 | | | |
| Post Hoc analysis with adjustment in p values for multiple comparisons | | | |
| Group I vs Group II : p<0.001 | | | |
| Group I vs Group III : p<0.001 | | | |
| Group I vs Group IV : p<0.001 | | | |
| Group II vs Group III : p<0.001 | | | |
| Group II vs Group IV : p<0.001 | | | |
| Group III vs Group IV : p<0.05 (not significant) | | | |

**Table 2:** Statistical comparison of the enumeration of IOL Adherent Bacteria of different\ phenotypic and molecular characters and the commensals.

Scanning electron microscopy picture showed multi layered viscid biofilm formation covering the entire IOL surfaces by all the strains, which were adherent and *ica* positive, in contrast to only scattered patchy and localized attachment of organisms that were nonadherent and *ica* negative (Figure 2).

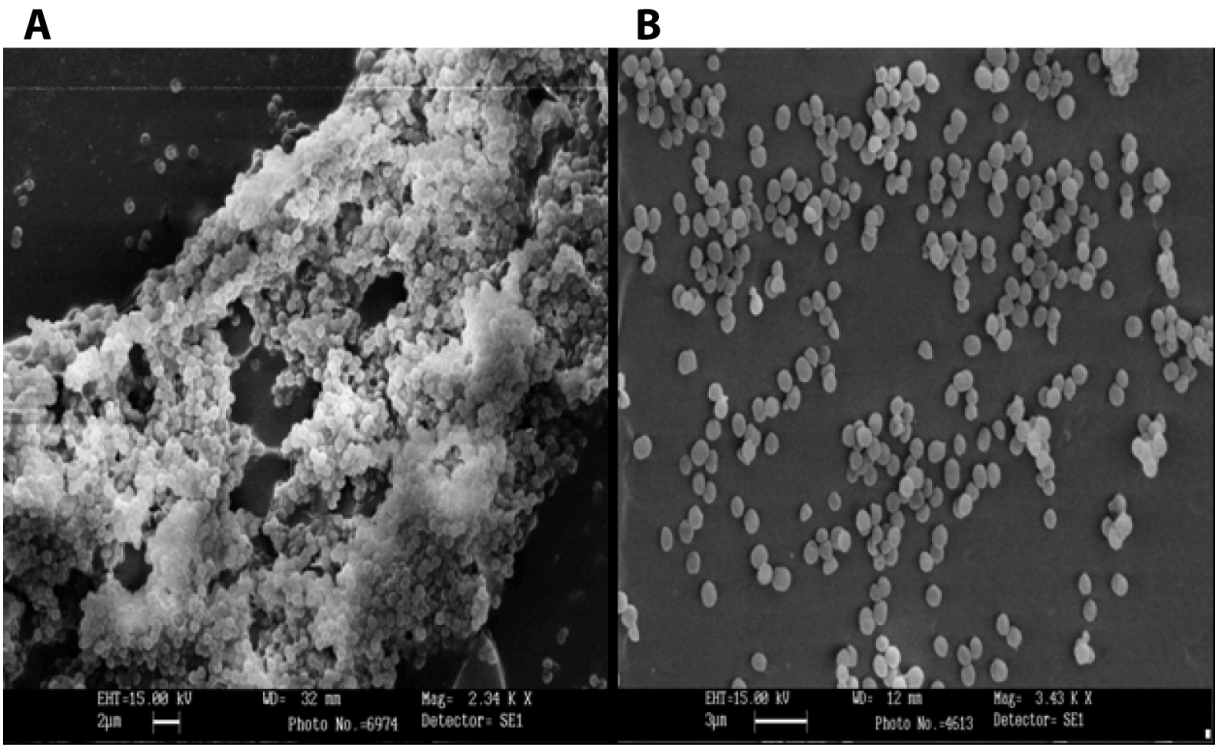


**Figure 2:** Scanning electron microscopic picture of PMMA intra-ocular lens showing multi layered biofilm formation (panel A) and discrete non-biofilm producing cells (panelB).

## Discussion

*S epidermidis* is one of the leading causes of bacterial endophthalmitis following IOL implantation [14]. However the exact link between the IOL implant and *S epidermidis* pathogenicity remains unclear. Bacterial biofilms, especially staphylococcal biofilms in association with IOLs were the subjects of discussion during the recent times [15-17].

We attempted to see if clinical isolates of *S epidermidis* could form biofilms on PMMA lenses. Our SEM results showed multilayered biofilm formation on the lense surfaces (Figure 2). Similar observations were noted by Okajima et al. [10] who demonstrated that *S epidermidis* formed biofilms intensely on acrylic lenses and such biofilm formation could be recognized as early as 24 hours post-incubation. Our study reflected exactly similar findings.

Unlike others [10,18], who noted biofilms on IOLs of various compositions, such as PMMA, silicone or MPC (methacryloyloxyethyl phospharyl choline); we conducted the study using PMMA lenses alone. While Kodjikian et al. [18] emphasized only on the SEM study of *S epidermidis* biofilm on silicone IOL with PMMA haptic, ours was on a different perspective altogether, because we had opted not only to detect biofilm formation, but also to enumerate the bacterial population attaching on PMMA lenses using organisms with different phenotypic and molecular characteristics (Table 2).

On comparing the quantification of bacteria attaching on the IOL material, it was found that organisms that were categorized as *ica* positive (by the PCR assay) and phenotypically adherent (by our quantitative *in vitro* adherence test), attached in much larger numbers as compared to either the commensal organisms or organisms which were *ica* negative and nonadherent. This was an interesting and clinically relevant observation because *ica* gene cluster encoding polysaccharide intercellular adhesin (PIA), as previously emphasized, could mediate intercellular adhesion of bacteria resulting in multilayered biofilm formation [19]. Exactly similar picture was presented by others that *ica* gene and biofilm forming ability were present amongst 85% of the strains of *S epidermidis* obtained from blood and CSF as against those obtained from the skin and mucosal surfaces of healthy volunteers [20,21].

Based upon the above findings of ours, it could be derived that bacteria carrying *ica* operon and having the phenotypic adherence property could very well colonize on the IOL surface. These organisms having the potential to adhere, as shown by our data (100% of the *ica* positive bacteria being adherent, Table 1) could accelerate cell to cell aggregation, further consolidating the adhesion process and facilitating the formation of rigid sessile multi-layered cell communities over the IOL surface [22].

In our study, it was interesting to note that organisms which were even *ica* negative and non-adherent showed higher rate of attachment as compared to the commensals (Table 2). This could denote that clinical isolates, even if were non-adherent and *ica* negative, probably possessed other virulence properties as opposed to the mere commensals. Over and above, comparison of the colony counts on IOL material between Gr III (adherent and ica positive organisms) and Gr IV (adherent but *ica* negative organisms) showed no statistically significant difference. Thus it could also be possible that there could be the existence of yet uncharacterized *ica*-independent biofilm

mechanism as stated by others [2], who proposed that different staphylococcal surface components and even teichoic acid could well recognize the host derived conditioning film formed on the surface of the implant following its insertion into the body cavity.

Besides the afore-mentioned observations, the present study depicted another interesting finding. All the adherent organisms (as determined by the quantitative adherence test) carried the *ica* AB' gene. Earlier studies [19,20] documented that quantitative biofilm production was significantly higher in strains of *S epidermidis* isolated from bacteremic patients and patients with other deep seated infections.

To our knowledge, the present study is the first of its kind that puts forth a comparative data between *in vitro* quantitative adherence and the *ica* positivity. Therefore our results substantiate further the previously proposed view on the mechanism of *S epidermidis* adherence onto various indwelling devices in general and intra-ocular devices in particular, and addresses the means to get away with the solution of the yet unresolved riddle of biofilm in IOL implant related infections, which may help in clinical judgments to decide on the overall management of patients with IOL induced POE.

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