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Biofilm formation and pan antibiotic resistance in isolates from contact lens cleaning solution used by CLARE patient

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ABSTRACT

Characterization of antibiotic resistance, biofilm formation ability and resistance to oxidative stress and halostress in isolates from contact lens cleaning solution (CLCS) and contact lens used by CLARE (Contact lenses associated red eye) patient. Trypticase soy peptone media was used for the microbiological isolation and maintenance. Antibiotic resistance was determined by using Kirby Bauer disk diffusion assay as per CLSI nomenclature. Biofilm formation assay was carried out by recommended techniques. In the present study, Enterobacter aerogenes CIN3, Burkholderia cepecia A2P1 and Proteus vulgaris B2P1 strains were isolated from CLCS used by CLARE patient. All three isolates were resistant to ampicillin, chloramphenicol, co-trimoxazole, colistin sulphate, streptomycin, sulfatriad, tetracycline and sensitive to ciprofloxacin and imipenem. B. cepecia and P. vulgaris also showed resistance to levofloxacin, norfloxacin and moxifloxacin. The multiple drug resistant isolates were characterized for their ability to form biofilms and produce exopolymeric substances implicated in the etiology of CLARE. In addition, biofilm formation of B. cepecia and P. vulgaris was induced by following exposure to oxidative (25 and 50 mM hydrogen peroxide) and salinity (5.0 and 9.5% NaCl) stress at antimicrobial concentrations typically found in CLCS. Only E. aerogenes growth reduced following treatment with oxidative and salinity stress. The importance of biofilm forming isolates in the pathogenesis of ocular infections mediated through usage of abiotic prosthetic devices such as contact lenses and contact lens cleaning solutions is emphasized.

INTRODUCTION

Microbial contamination of contact lenses can often lead to infection and inflammation of the ocular tissue [1]. Contact lenses induced acute red eye (CLARE) is characterized by pain, red eye, tearing, photophobia, corneal infiltration and blurred vision upon waking [2]. One of the major predisposing factor of contact lens induced corneal pathology includes poor contact lenses hygiene. In this respect, the role of contaminated contact lens cleaning solutions in introducing infections of environmental saprophytic micro-organisms has been previously reported [3,4,5]. Contaminated contact lens cleaning solutions have been previously reported to show contaminants particularly of *Pseudomonas spp., Staphylococcus spp., Serratia spp., Fusarium spp., Acanthamoeba Spp*. leading to contact lenses associated microbial keratitis [3-6]. Additionally, lipopolysacharide rich gram negative bacteria are powerful activators of pro-inflammatory innate immune response [8].

Contact lenses cleaned with contaminated cleaning or storage solutions permits adherence of micro-organisms onto the contact lenses. Extended contact lens wear ensures prolonged contact of the infectious agents with the corneal tissue [3, 9]. Micro-organisms isolated from contact lenses are typically in the biofilm mode of growth [10].

Biofilms are surface adhering structured community of micro-organisms encapsulated in an exopolymeric substance which protects it from environmental stress [11]. The exopolymeric substance also renders the biofilm dwellers with increased resistance to antimicrobial agents. The exopolysaccharide production in the biofilm mode of growth triggers inflammatory reaction in the ocular milieu.

The role of biofilm formers and their contribution to antibiotic resistance and innate immune stress in the environment has not been well studied particularly in context with CLARE cases. In this report, a microbiological analysis of the contact lens and cleaning solution ReNu Multiplus used by a patient suffering from sterile CLARE due to extended contact lens wear is presented. Our data report the effect of halo and oxidative stress on the isolates in the planktonic as well as biofilm mode of growth.

MATERIALS AND METHODS

2.1 Sampling and characterization

Sampling was performed from the contact lens cleaning solutions (CLCS) of Bausch & Laumb ReNu Multiplus of a patient suffering from contact lens associated red eye (CLARE). The condition of the patient improved after removal of the contact lens. Isolates were grown in trypticase soy broth (TSB) (Hi Media, India) at 37°C. Microbiological and biochemical characterization was used for the identification of the isolates [12].

2.2 Antibiotic susceptibility

Antibiotic susceptibility tests for each isolates were performed by disk diffusion method (Hi Media, India) as per CLSI nomenclature [10]. The antibiotic tested include Ampicillin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Colistin sulphate (10 µg), Co-Trixamoxazole (25 µg), Gentamicin (10 µg), Imipenem (10 µg), Moxifloxacin (5 µg), Norfloxacin (10 µg), Streptomycin (10 µg), Sulphatriad (200 µg), Tetracycline (30 µg), Vancomycin (30 µg). Standard American Type Culture Collection (ATCC) bacteria *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 15442 were used for quality control.

2.3. Biofilm assay

Static biofilm formation assay was used as per O'Toole et al [14] with some modifications. Isolates were grown in 1.5 ml polypropylene tubes as well as in 96 well polystyrene micro titer plates containing 500 µl of TSB and 96 well micro titer plates containing 200 µl of TSB for 24h at 37 °C. Cultures were removed and planktonic growth measured spectrophotometrically at A_{630}. Static surface with biofilms were washed with sterile saline. Adherent bacteria were stained with 1% w/v crystal violet for 20 min. Tubes and wells were washed, stained adherent bacteria were detached using 200 µl of dimethyl sulfoxide and solubilized biofilms measured using ELISA microreader (Rayto, USA) at A630. Results are mean of 3 experiments done in triplicates.

2.4. Hydrophobicity assay

Microbial hydrophobicity assay was performed as described [15]. Briefly, bacteria were grown in TSB, washed and resuspended in sterile saline. Initial absorbance was measured spectrophotometrically at A_{630} (Spectronics, Merck). 2 ml of culture was mixed with same quantity of xylene using a vortex. Phases were allowed to separate for 30 min at room temperature. Absorbance of the aqueous phase was measured as before. Hydrophobicity index was calculated as:

$$[A_{\text{initial}} - A_{\text{aqueous phase}} / A_{\text{initial}}] \times 100$$

2.5 Motility assay

Motility was determined by swimming plate, swarming plate and twitch plate assay as described previously [16]. Briefly, cultures were inoculated on 0.3% w/v agar for swimming motility and diameter of migration zone was measured in mm after incubation at 37°C for 24h. Swarming motility was measured by stabbing on overnight dried TSA containing 0.5% w/v agar. Twitching motility was performed by stabbing on TSA with 1% w/v agar. The zone of migration at the interface of agar and plate was measured.

2.5 Exopolysaccharide production

Measurement of exopolysaccharide was done by phenol sulfuric method [17] as well as Congo red (CR) binding assay [18]. Briefly, Congo red binding assay was determined by culturing the strain on TSA plate containing 0.003% CR. For determination of Congo red binding activity, strains were incubated for three days. Cultures were

then centrifuged and resuspended the precipitate in PBS and set $O.D_{630}$ of 1.0. Further cells were incubated in the presence of 50 µg/ml CR and centrifuged, absorbance of residual dye in the supernatant was measured at 490nm.

2.6 Acyl Homoserine Lactone (AHL) and Polyphosphate Kinase (PPK) assay
In AHL assay quantification of lactones was done by spectrophotometer as described previously [19]. Briefly, 50 µl of a 1:1 mixture of hydroxyl amine (2M): NaOH (3.5M) was aliquoted and mixed with 40 µl sample. Subsequently same amount of 1:1 mixture of ferric chloride (10% in 4M HCl): 95% ethanol added. A dark brown color indicating presence of lactones, Mixture was measured at 520 nm. PPK was quantified using toludine metachromatic shift as described earlier [20].

2.7 Effect of Halostress and Oxidative Stress on biofilm formation
Static biofilm assay was performed as described previously NaCl (0.5, 5 and 9.5%) or H_2O_2 (10, 25 and 50 mM) was added to cultures in 96 well micro titer plates in triplicates to determine the effect on planktonic growth and biofilm formation.

2.8 Statistical Analysis
Statistical analysis was done using student's t test. All experiments were repeated at least thrice in triplicates. $p \leq 0.05$ was considered as biologically significant.

RESULTS

3.1 Microbial Characterization

Gram negative facultative aerobes were isolated from the used contact lens cleaning solutions Baucsh & Laumb ReNu Multiplus obtained from patient suffering from CLARE (Table 1). Microbiological and biochemical identification was used to characterize the isolates as *Enterobacter aerogenes* C1N3, *Burkholderia cepecia* A2P1 *and Proteus mirabilis* B2P1. The isolates are not part of the ocular microflora and are indicative of environmental contamination through soil or contaminated water in the contact lens cleaning solution.

3.2 Antibiotic Resistance

All the isolates show resistance to ampicillin, chloramphenicol, co-trimoxazole, colistin sulphate, streptomycin, sulphatriad and tetracycline. The isolates were analyzed for their antibiotic resistance to third and fourth generation antibiotics such as norfloxacin, moxifloxacin, vancomycin and imipenem. *B. cepecia* A2P1, and *P. mirabilis* B2P1 showed resistance to norfloxacin and moxifloxacin as determined by the disk diffusion assay as per CLSI guidelines [13](Table 1). Interestingly, isolates *B. cepecia* A2P1 and *P. mirabilis* B2P1 were found to be resistant to fourth generation fluoroquinolones: moxifloxacin but sensitive to ciprofloxacin. Hence, the three isolates from the contaminated contact lens solution, *E. aerogenes, B. cepecia* and *P. mirabilis* were pan drug resistant biofilm formers.

3.3 Biofilm characterization

The isolates were further characterized for their ability to produce biofilms and exopolysaccharide which have been implicated in the etiology of CLARE in extended contact lens wearers. Fig.1 shows the ability of the three isolates to form biofilm by the static biofilm assay, exopolysaccharide production as measured by phenol sulfuric acid assay and Congo red binding assay. Congo red binding assay also verifies the ability of all the isolates to bind to exopolysaccharides and secrete exopolymeric matrix upon binding to solid substrates [15]. Hydrophobicity measurements by MATH assay show that all the organisms were hydrophilic and their percentage hydrophobicity was below 50% (Fig.1E). All the isolates were capable of showing swimming, swarming and twitching motility (Fig.1F).

3.4 Resistance to halostress and oxidative stress

The ability of the isolates to withstand halo and oxidative stress was checked by exposing 24 hour biofilm formed cells to increasing concentrations of NaCl (0.5, 5 and 9.5%) and hydrogen peroxide (10, 25 and 50 mM). Table 2 reports the ability of the isolates to withstand halostress (9.5%) as well as treatment with hydrogen peroxide (25 and 50 mM) which are the concentrations typically present in contact lens cleaning solutions. No effect was seen on treatment with 5% NaCl and 10 mM hydrogen peroxide. The ocular tissue uses salinity as well as oxidative stress as part of its innate immune response to prevent growth of opportunistic pathogens as well as contamination from the open environment. Hence, growth of both planktonic and biofilm formation of *E. aerogenes* C1N3 was inhibited by

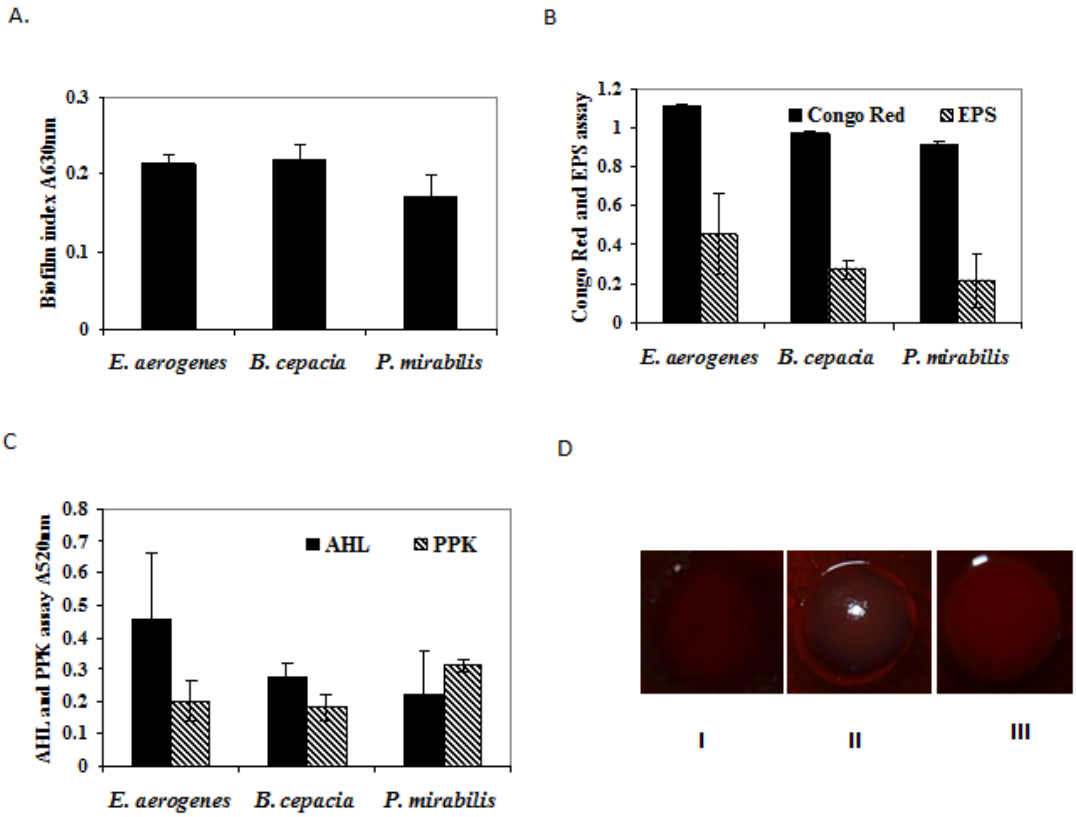
NaCl and hydrogen peroxide. While there was no effect on planktonic growth of *B. cepecia* A2P1, biofilm was found to be induced at higher concentrations of hydrogen peroxide. Only planktonic growth was decreased at high salt concentration of *P. mirabilis* B2P1 while no effect was seen in biofilm formation. There was also no effect of oxidative stress on *P. mirabilis* B2P1.

Table 1: Antibiogram of isolates from contact lenses cleaning solution

Isolates	CFU/ml	Antibiogram[a]
Enterobacter aerogenes C1N3	$1x10^5$	AMP^R CIP ^{S} C^R Cl^R COT^R GEN^R IMP^S MO^S NX^S S^R SF^R TE^R VA^S
Burkholderia cepecia A2P1	$2x10^5$	AMP^R CIP ^{S} C^R Cl^R COT^R GEN^s IMP^S MO^R NX^R S^R SF^R TE^R VA^S
Proteus mirabilis B2P2	$2x10^5$	AMP^R CIP ^{S} C^R Cl^R COT^R GEN^R IMP^S MO^R NX^R S^R SF^R TE^R VA^S

[a]*Abbreviations:*

AMP: Ampicillin (10 mcg), CIP: Ciprofloxacin (5 mcg), C: Chloramphenicol (30 mcg), Cl: Colistin sulphate (10 mcg), COT: Co-Trimoxazole (25 mcg), GEN: Gentamicin (10 mcg), IMP: Imipenem (10 mcg), MO: Moxifloxacin (5 mcg), NX: Norfloxacin (10 mcg), S: Streptomycin (10 mcg), SF: Sulphatriad (200 mcg), TE: Tetracycline (30 mcg), VA: Vancomycin (30 mcg).



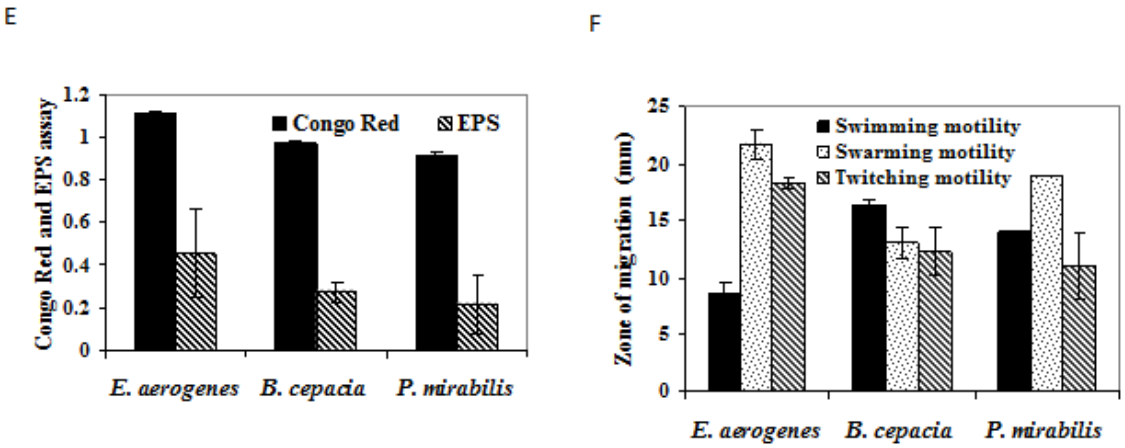


Figure 1 (A) Biofilm index of *Enterobacter aerogenes*, *Burkholderia cepacia* and *Proteus mirabilis*. (B) Absorbance of EPS and Congo red binding assay of *Enterobacter aerogenes*, *Burkholderia cepacia* and *Proteus mirabilis*. (C) Absorbance of AHL and PPK assay. (D) Congo red agar binding assay (I) *Enterobacter aerogenes*, (II) *Burkholderia cepacia* and (III) Proteus *mirabilis*. (E) Percentage Hydrophobicity of *Enterobacter aerogenes*, *Burkholderia cepacia* and *Proteus mirabilis*. (F) Swimming, swarming and twitching motility of *Enterobacter aerogenes*, *Burkholderia cepacia* and *Proteus mirabilis*.

DISCUSSION

Multiple drug resistant and biofilm forming bacteria were isolated from contact lens cleaning solution and characterized for their resistance to halo and oxidative stress, both antimicrobial components in CLCS. *E. aerogenes* has been implicated as nosocomial opportunistic pathogenic bacteria which can also be frequently isolated from soil. *E. aerogenes* has also been isolated from contact lens associated microbial keratitis [21]. This is however the first report of *E. aerogenes* contamination of the CLCS. *B. cepecia* has a wide distribution, typically isolated from soil, water and rhizosphere as plant pathogens. However, due to their ability to survive in well hydrated environments, they are also acquiring status of nosocomial pathogens in health care settings. *B. cepecia*, formerly *P. cepecia* has been isolated from contact lens and contact lens cleaning solutions from keratitis patients [22]. *P. mirabilis* has also been reported to be the most frequently contaminating bacteria in 8% of the ophthalmic solutions used in a long term care facility [23]. Typically microbial contamination of contact lens, lens cases predominantly contamination of gram positive coagulase negative *Staphylococci* are correlated with microbial keratitis [24]. Herein, in the case of sterile contact lens associated red eye, the gram negative bacteria were contributing to the inflammatory response. The role of gram negative bacteria *Aeromonas hydrophila, Haemophilus influenza, P. aeruginosa, P. putida* and *Serratia marcescens* in CLARE has been previously reported [21, 25-27].

Multiple antibiotic resistance particularly to third and fourth generation antibiotics in micro-organisms isolated from CLCS is a cause for concern [4, 7]. Introduction of MDR environmental strains through contact lenses in ocular tissue provide environment for the emerging pathogenic bacteria. The ability to form biofilms further compounds their resistance to antimicrobial treatments. Further genetic detailing of the isolates may provide information regarding determinants that may be present on mobile elements which could cause horizontal spread of drug resistance.

The role of gram negative bacteria in CLARE is associated with the production of endotoxins which can incite a proinflammatory milieu in the ocular tissue [28]. However, the role of biofilm formers on contact lens or contact lens cleaning solutions, which may not be invasive, in the generation of ocular pathology has not been previously appreciated. Hence, biofilm confers the ability to resist up to thousand fold higher concentrations of antimicrobial compounds and innate immune mediator [3]. The isolates were characterized for biofilm formation using several assays. The role of twitching and swarming motility in biofilm formation has been described earlier [29]. PPK is reported to be responsible not only biofilm formation but also production of many virulence factors in *P. aeruginosa* [29]. AHL molecules which are part of quorum sensing play a very important role in biofilm formation [30, 31]. Herein we report microbial contaminants with the biofilm formation ability capable of releasing exopolymeric matrix in the lens cleaning solution. The lipopolysachharide contamination in the contact lens cleaning solution may

itself be sufficient to cause inflammation characteristic of CLARE. Previously, CLARE has been associated with the presence of bacterial exopolysacharide, a massive content of the biofilm [10, 11, 26].

P. aeruginosa and *S. aureus* biofilms susceptibility to hydrogen peroxide and a polyquaternium-preserved care solution, and the resistance of *Serratia marcescens* biofilm to a polyquaternium-preserved care solution but not to hydrogen peroxide disinfection have been reported [32]. In this study, the planktonic forms were always susceptible to the biocides but variable results were found for the biofilm growth. This is again indicative of the increase resistance provided by biofilm mode to various environmental stressors.

CONCLUSION

Emergence of pathogenic abilities in typically saprophytic micro-organisms is an increasing trend in infectious biology, particularly since the introduction of abiotic prosthetic devices. Awareness amongst contact lens wearers regarding hygienic practices will help to decrease incidences of red eye. Contact lens solution formulations can also be developed to degrade any bacterial endotoxins that may remain in the solutions which are essentially antimicrobial in nature.

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