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Virulence Profile of an Emerging Coagulase Negative *Staphylococcus auricularis* NC Clinical Isolate

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Coagulase negative *Staphylococci* are emerging pathogenic bacteria, associated particularly with abiotic medical prosthetic surfaces. A multiple drug resistant biofilm forming strain of *Staphylococcus auricularis* NC isolate, an infrequently associated coagulase negative *Staphylococci* from intraocular lenses of post operative endophthalmitis case was isolated and its virulence characteristics studied. Multiple drug resistant, biofilm former and coagulase positive *Staphylococcus aureus* ATCC 33592 was used as a reference strain. Herein we report the biofilm formation ability and resistance to oxidative stress in the biofilm growth of *Staphylococcus auricularis*. This emerging pathogenic isolate showed resistant to over 16 different antibiotics of all classes including novobiocin. As a strong biofilm former it also showed resistance to oxidative stress (25mM H_2O_2, 50mM H_2O_2, 500 μM sodium nitroprusside as nitric oxide donor) but not to high osmotic stress (9.5% NaCl). This study is of basic interest to the field of emerging infectious agents, particularly infrequently isolated coagulase negative *Staphylococci auricularis* in medical microbiology.

Keywords: *Staphylococcus auricularis*, *S. aureus*, Endophthalmitis, Biofilm former.

Infectious endophthalmitis is a rare but devastating complication of post operative cataract surgery[1]. Coagulase negative Gram positive cocci are significant cause of endophthalmitis, particularly, *Staphylococcus spp.* where the source of the pathogen may be from exogenous sources such as operating conditions, equipment etc [2, 3] or from the patient's own ocular micro flora[4]. According to one study in 43% of the cases following cataract surgery microbial flora can be isolated from patient's anterior chamber[5]. Organisms may be carried into the eye as surface fluid refluxes through the wound during surgery[6]. Interestingly, incidence of endophthalmitis is very low compared to these statistics. Common ophthalmic practitioners use preoperative antibiotic therapy to decrease the microbial load of eyelid and cul-de-sac micro flora and hence prevent postoperative complications due to contamination[5-6]. Despite such practices, the incidence rates are found to be 0.01% in the Western world and incidences may be higher in India[3]. Thus the virulence profiles and antimicrobial resistance pattern of isolates from cases of endophthalmitis are important means of developing future prophylactic treatments. The emerging role of coagulase negative *Staphylococci* in prosthetic device associated infections is on the rise. *Staphylococcus aureus*

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followed by *S. epidermis* are the most well studied *Staphylococcal* pathogens. However in recent years with the advent of genomic profiling the role of diverse *Staphylococci* in microbial infections is being recognized. Some of the less well studied infectious coagulase negative Staphylococci include *S. auricularis*, *S. lugdunensis*, *S. sciuri*, *S caprae* amongst others [1, 2]. Understanding the virulence mechanisms of such infrequent infectious isolates will provide us with a better understanding of prophylactic and therapeutic measures to be undertaken in several chronic and persistent infections.

In the present study, we report the virulence profile of coagulase negative *Staphylococcus auricularis* isolate from a case of post operative endophthalmitis.

MATERIAL AND METHODS

Sampling and characterization

Sample obtained from the extracted intraocular of patient and intravitreal fluid of patient suffering from endophthalmitis was grown in Tryptone Soypeptone Broth (TSB) (Hi Media, India) at 37°C. Microbiological and biochemical characterization was used for the identification of the isolate[7]. *Staphylococcus aureus* ATCC 33592 was used as a reference strain and also maintained on Tryptone Soypeptone Broth (TSB) (Hi Media, India) at 37°C.

Antibiotic susceptibility

Antibiotic susceptibility tests for each isolates were performed by disk diffusion method (Hi Media) as per CLSI nomenclature[8]. Plates were spread with cultures and antibiotic using antibiotic disks, and incubated at 37° C. The inhibition zones were measured after 18-24 h. Isolates were characterized as resistant, moderate and susceptible following the standard antibiotic disk diffusion sensitivity method. The antibiotic tested include Amoxicillin (25mcg), Ampicillin (10 mcg), Azithromycin (15 mcg), Cephalothin (30 mcg), Clindamycin (2 mcg), Co-Trimoxazole (25 mcg), Ciprofloxacin(5mcg), Erythromycin (15 mcg), Gentamicin (10 mcg), Imipenem (10mcg), Linezolid (30 mcg), Moxifloxacin (5 mcg), Novobiocin (5 mcg), Ofloxacin (5mcg), Oxacillin (1 mcg), Penicillin- G (10 units), Streptomycin (10 mcg), Tetracycline (30 mcg), Tiecoplanin (10 mcg), Vancomycin (30 mcg). Multiple antibiotic resistance (MAR) index for the isolates was calculated as a/b where 'a' is the number of antibiotics to which the isolate is resistant and 'b' is the number of antibiotics to which the isolate is subjected[9]. *Staphylococcus aureus* ATCC 25932 was used as quality control organism for *S. aureus* and CoNS standards for *S. auricularis* NC [8].

Biofilm assay

Static biofilm formation assay was used as per Toole et al with some modifications[10]. Isolates were grown in 1.5 ml polypropylene tubes as well as 96 well polystyrene microtiter plates containing 500 µl of TSB and 96 well microtiter plates containing 200 µl of TSB for 24h at 37°C. Cultures were removed and planktonic growth measured spectrophotometrically at A630. 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 at A630. Results are mean of 3 experiments done in triplicates.

Hydrophobicity assay

Microbial hydrophobicity assay was performed as described by [11]. Briefly, bacteria were grown in TSB, washed and resuspended in sterile saline. Initial absorbance was measured spectrophotometrically (A630). Two 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 initial – A aqueous phase/ A initial] x100

Exopolysacharride production

Exopolysacharride measurement was performed by phenol sulfuric method[12] as well as Congo red binding assay[13]. 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 incubate for three days and then centrifuge collected precipitate were resuspended in PBS and set $O.D_{630}$ of 1.0. Cells were incubated in the presence of 50µg/ml CR then centrifuged and supernatant were collected. Absorbance of residual dye in the supernatant was measured at 490nm.

Colony Spreading

Isolate was spot inoculated on overnight dried soft agar plates (0.3 %w/v). The spread of the colony was measured in mm after 24 hrs of incubation.

Effect of Halo stress on biofilm formation

Static biofilm assay was performed as described previously. NaCl (0.5%, 5% and 9.5%) was added to cultures in 96 well microtiter plates in triplicates to determine the effect on planktonic growth and biofilm formation.

Effect of oxidative stress using hydrogen peroxide and nitric oxide

Log phase cultures were treated with 25mM and 50 mM H_2O_2 or 500 µM sodium nitroprusside as nitric oxide donor in a static biofilm assay as described previously. Growth was measured of planktonic as well biofilm formation as described previously.

Statistical Analysis

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

RESULTS AND DISCUSSION

Culture Characteristics

In this study, Gram positive cocci was isolated from the excised intraocular lens and intra vitreal fluid of a 60 year old male patient suffering from post operative endophthalmitis following cataract surgery. Microbiological and biochemical characterization confirmed that the isolate was oxacillin and novobiocin resistant, ß hemolytic, coagulase negative *Staphylococcus auricularis NC*. The isolate was catalase +ve, oxidase -ve, urease -ve, citrate -ve, and gelatin -ve, utilized mannitol, lactose, sucrose, arabinose, trehalose, maltose and glucose with acid production. For all further studies, the isolate was maintained on Tryptone Soy media and incubated at 37°C for 24

Table 1. Antibiogram and zone of inhibition (mm) of *Staphylococcus auricularis NC* and MRSA *Staphylococcus aureus*

Antibiotic	Concentration	Range (CLSI, 2008)	*S. auricularis* NC	*S. aureus* ATCC 33592
Ampicillin	10 mcg	27-35	R(8)	R(0)
Amoxicillin	25 mcg	28-36	R(8)	R(0)
Azithromycin	15 mcg	21-26	R(15)	R(0)
Cephalothine	30mcg	29-37	R(0)	R(0)
Clindamycin	2 mcg	24-30	S(30)	R(15)
Co-trimoxazole	25 mcg	24-32	R(17)	R(0)
Ciprofloxacin	5 mcg	22-30	R(0)	R(0)
Erythromycin	15 mcg	22-30	R(9)	R(0)
Gentamicin	10 mcg	19-27	R(15)	R(11)
Imipenem	10 mcg	17-51	I(33)	I(27)
Linezolid	30mcg	25-32	I(30)	R(24)
Moxifloxacin	5 mcg	28-35	R(0)	R(20)
Novobiocin	5 mcg	22-31	R(13)	R(21)
Ofloxacin	5mcg	24-28	R(18)	R(20)
Oxacillin	1 mcg	18-24	R(0)	R(0)
Penicillin	10 unit	26-37	R(9)	R(0)
Streptomycin	10 mcg	14-22	R(0)	R(0)
Teicoplanin	10 mcg	15-21	I(17)	R(15)
Tetracycline	30 mcg	24-30	R(18)	R(13)
Vancomycin	30 mcg	17-21	R(16)	R(15)
MAR(index)			0.8	0.95

[a)] Multiple antibiotic resistance (MAR) index for the isolates was calculated as a/b where 'a' is the number of antibiotics to which the isolate is resistant and 'b' is the number of antibiotics to which the isolate is subjected[9]. R: Resistant, I: Intermediate, S: Sensitive

h to obtain log phase cultures. *Staphylococcus aureus* and *Staphylococcus epidermis* have been previously implicated in post operative endophthalmitis[14]. The incidence of endophthalmitis due to *Staphylococcus auricularis* has been rare[1]. The CoNS has been isolated under rare conditions typically as a blood contaminant in septicemia patients[2]. Since, most virulence profile characterizations studies are typically of *Staphylococcus aureus*, a comparative study of virulence profiles between endophthalmitis causing *Staphylococcus auricularis* and reference *Staphylococcus aureus* was performed.

Antibiogram and biofilm formation ability

The isolates were characterized for its ability to form biofilms and antibiotic susceptibility. Antibiotic resistance in the isolate was determined by disk diffusion method using antibiotic disks (Hi Media) as per the CLSI nomenclatures [9]. The isolate was found to be resistant to several antibiotic classes; aminoglycoside [Gentamicin (10 mcg), Streptomycin (10 mcg)], ß lactam (Amoxicillin (25mcg), Ampicillin (10mcg), Oxacillin (1 mcg), Penicillin- G (10 units)], fluoroquinolones [Ciprofloxacin (5mcg), Ofloxacin (5mcg); Moxifloxacin (5 mcg), macrolides [Azithromycin (15 mcg), Erythromycin (15 mcg)], Cephalosporins [Cephalothine (30 mcg)], Co-Trimoxazole (25 mcg), aminocoumarin antibiotics Novobiocin (5 mcg), Tetracycline (30 mcg). The isolate was found to be sensitive to Clindamycin (2 mcg) and showed intermediate resistance to Imipenem (10 mcg), Linezolid (30 mcg), Teicoplanin (10 mcg) and Vancomycin (30 mcg). (Table 1). Multiple antibiotic resistant indexes calculated for the *S. auricularis* strain was 0.8 while for *S. aureus* was 0.95. Fluoroquinolones are considered the drugs of choice for endophthalmitis due to increased penetrability, and persistence in the eye film as well as low toxicity and broad spectrum nature. The isolate shows increased resistance to fluoroquinolones, Ofloxacin, Gatifloxacin and Moxifloxacin and routinely used antibiotics in cataract operations.

Isolate MRSA *Staphylococcus aureus* ATCC 33592 (standard biofilm former) was used as standard reference strain for further biofilm experiments. Biofilm assay was done as per static biofilm formation protocol[10]. *S. auricularis* NC was isolated from surface of the intraocular lens and formed strong biofilm on polyethylene as well polystyrene surfaces (Table 2). Biofilm characteristics such as surface hydrophobicity, exopolymeric substances production, colony spreading ability and biofilm formation assay were performed for the isolate. *S. auricularis* NC is highly hydrophilic (11.94±5.25%) while *S. aureus* was found to be moderately hydrophilic (39.4±5.2%) according to MATH assay as per

Table 2. Comparison of Biofilm formation ability of *S auricularis* NC and *S aureus* ATCC 33592

Biofilm characteristics	*S. auricularis NC*	*S. aureus* ATCC 33592
Biofilm formation assay [a)]		
Polyethylene	1.305±0.54	1.576±0.275
Polystyrene	0.296±0.091	0.188±0.011
Hydrophobicity [b)]	11.94±5.25%	39.4±5.2%
Exopolysaccharide [e)] production $_{(A490)}$	0.5±0.177	0.37 ± 0.2
Congo Red Binding Assay $_{(A490)}$ [d)]	0.392±0.005	0.54 ± 0.8
Colony spreading (mm) [e)]	35.33±1.24	1±0

a) Biofilm formation on substrate[10].
b) Hydrophobicity is defined as percentage of cells partitioning into xylene phase in a microbial adhesion to hydrocarbon assay[11]
c) Carbohydrate content of EPS produced by bacteria in biofilm determined by the phenol sulfuric acid method[12].
d) Congo red binding assay measured amount of Congo red binding to exopolymeric matrix[13].
e) Colony spreading was performed by spot inoculating on dried 0.3%w/v TSA plates and measuring zone of colony spreading in mm after 24 hrs of incubation.

Results are mean of triplicate and represent one of three similar experiments.

protocol of [11], strong exopolysacharride producer as determined by spectrophotometry using phenol sulfuric acid method[12] and has the ability to bind congo red[13] previous reports have shown that congo red binds to the extracellular components of microbial biofilms[15]. The ability of coagulase negative *Staphylococcus* and their ability to adhere to polymeric surfaces of medical devices have been widely reported[2]. The role of exopolysacharride formation in biofilm is essential for microbial adherence, nutrient absorption, and protection from environmental stress such as oxidative stress, dehydration, nutrient limitation and antimicrobial surfaces [15]. *Staphylococci* are non flagellated non motile organisms. However, the role of quorum sensing dependant surface migration or colony spreading in biofilm formation in pathogenic *Staphylococci* has been reported[16]. Both *S. auricularis* and *S. aureus* showed significant colony spreading. The biofilm forming abilities of the two isolates were found to be comparable and as such may be contributing to the pathogenecity of *S. auricularis* NC in bacterial endophthalmitis.

Effect of oxidative stress on biofilm formation

Bacteria have developed several mechanisms to evade oxidative stress, an essential arm of the innate immune response[17]. The ability of the isolate to resist oxidative stress was determined by treating cells in microtiter plates with 25 mM and 50mM hydrogen peroxide or 500 µM sodium nitro prusside (SNP) as nitric oxide donor. Planktonic growth was measured spectrophotometrically at A_{630nm} and biofilm formation was performed as described earlier[10]. Table 3 shows that the both the isolates were resistant to treatment with hydrogen peroxide or sodium nitroprusside as nitric oxide donor particularly in the planktonic as well as biofilm mode of growth. In fact biofilm formation was induced in *S. aureus* at 50 mM hydrogen peroxide treatment. The increased resistance to oxidative stress can also be one of the mechanisms of increased antibiotic resistance. Several antibiotics have shown to show antibacterial activity by generating the release of reactive oxidative species[18, 19]. Alternatively, within biofilm, oxidative stress may also cause increased rates of mutability resulting in selection of resistant cells[20]. High osmotic stress treatment reduced biofilm formation for both isolates however it was found to be biologically significant only for *S. aureus* biofilm. No effect of 5% NaCl was observed on the biofilm forming ability (data not shown). Thus, it is likely that induction of osmotic stress resistance phenotype is not well correlated with biofilm formation, oxidative stress and antibiotic resistance. Significant differences were also not found in the means between the two isolates implicating the highly resistant nature of the *S. auricularis* NC strain in comparison with the reference pathogenic *S. aureus* ATCC 33529 strain.

Table 3. Effect of oxidative stress and halo stress on planktonic cells and biofilm formation

	Planktonic	Biofilm
S. auricularis NC		
Untreated Control	0.286±0.058	1.305±0.538
500 µM Sodium Nitro Prusside (NO donor)	0.174±0.029*	1.164±0.378
25 mM H_2O_2	0.3±0.07	1.31±0.384
50 mM H_2O_2	0.299±0.04	1.12±0.117
9.5% NaCl	0.381±0.08*	0.844±0.077
S. aureus ATCC 33592		
Untreated Control	0.0775±0.005	1.576±0.275
500 µM Sodium Nitro Prusside (NO donor)	0.085±0.013	1.197±0.39
25 mM H_2O_2	0.083±0.011	1.704±0.44
50 mM H_2O_2	0.084±0.04	2.07±0.44*
9.5% NaCl	0.096±0.008	0.724±0.031*

Data represents spectrophotometric reading at A_{630}nm for planktonic cells and standard biofilm assay[10] after 24hrs of incubation at 37°C. Results are mean of triplicate.
*Statistical significance was determined by student's t test at p= 0.05

CONCLUSION

The study reports biofilm forming pan drug resistant including novobiocin resistant coagulase negative *Staphylococcus auricularis* as the cause of post operative endophthalmitis. The isolate was found to be sensitive to Clindamycin and showed intermediate resistance to Imipenem, Linezolid, Teicoplanin and Vancomycin. The isolate showed biofilm formation, resistance to oxidative stress comparable to the clinical pan drug resistant *S. aureus* ATCC 33592.

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