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Biofilm Forming Multi Drug Resistant *Staphylococcus* spp. Among Patients with Conjunctivitis

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Abstract

Biofilm forming multidrug resistant *Staphylococcus* spp. are major reservoirs for transmission of ophthalmic infections. They were isolated from ocular patients suffering from conjunctivitis. In this study we analyzed biofilm forming ability, antibiotic resistance profile of the *Staphylococcus* spp. isolated from clinical ocular patients, and their phylogenetic relationship with other community MRSA. Sixty *Staphylococcus* spp. strains isolated from clinical subjects were evaluated for their ability to form biofilm and express biofilm encoding ica gene. Among them 93% were slime producers and 87% were slime positive. *Staphylococcus aureus* and *S. epidermidis* were dominant strains among the isolates obtained from ocular patients. The strains also exhibited a differential biofilm formation quantitatively. Antibiotic susceptibility of the strains tested with Penicillin G, Ciprofloxacin, Ofloxacin, Methicillin, Amikacin, and Gentamicin indicated that they were resistant to more than one antibiotic. The amplicon of ica gene of strong biofilm producing *S. aureus* strains, obtained by polymerase chain reaction, was sequenced and their close genetic relationship with community acquired MRSA was analyzed based on phylogenetic tree. Our results indicate that they are genetically close to other community acquired MRSA.

Key words: *Staphylococcus* spp., biofilm, conjunctivitis, *ica* gene

Introduction

The surface of the eye is rich in nutrients and, consequently, supports a diverse range of microorganisms that constitute the normal ocular flora, the growth of which is regulated and thus infection is prevented (Armstrong, 2000). The eye, though protected by number of natural defense mechanisms, suffers from number of infections caused by adapted microorganisms. Bacteria involved in ophthalmic infections principally infect the conjunctiva, cornea, and the uveal tract (Sankaridurg *et al.*, 1996). Conjunctivitis caused by bacteria occurs worldwide and affects people of all ages and both sexes. It has been cited as one of the most frequent causes of self-referral in the practice of comprehensive ophthalmology. According to the American Academy of Ophthalmology, conjunctivitis infrequently causes permanent visual loss or structural damage but the economic impact of the disease in terms of lost work time, although undocumented, is doubtless considerable (Schlech and Blondeau, 2005). Bacterial conjunctivitis is typically self-limiting though treatment with topical ophthalmic antibacterials can reduce symptoms, recovery time, contagious spread, possible reinfection, and risk of complications. In fact, reports of outbreaks of bacterial conjunctivitis underscore the benefit of controlling the spread of disease with immediate treatment (McDonald *et al.*, 2008). Prolonged use of older and previous-generation antibiotics only facilitate the development of resistant strains (Schlech and Blondeau, 2005). Chronic bacterial infections were reported to persist over a long duration, as many as six decades, and the causative agent was shown to be susceptible to antibiotics used in high and sustained therapeutic doses. Biofilm forming bacteria are one among those that cause serious infections. Costerton *et al.*, (1999) defined biofilm as a structural community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to inert or living surfaces. Once a biofilm has formed, the bacteria within them are protected from phagocytosis and antibiotics (Hoyle *et al.*, 1992). The various definitions

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of biofilm (Carpentier *et al.*, 1993; Costerton and Lappin Scott, 1999; Elder *et al.*, 1995) encompass three basic ingredients namely the microbes, slime exopolysaccharide, and the surface. The biofilm does not develop if any of these components is removed from the mix. The current concept is that biofilm bacteria can usually survive the sterilants and/or antibiotics in concentrations that are 1000–1500 times higher than the concentrations that kill floating (planktonic) cells of the same species (Costerton *et al.*, 1999). Hence, it is of utmost importance to understand the mode of infection, proliferation, and survival of pathogens towards the control of eye infections. Vasudevan *et al.*, (2003) concluded that adherent cells within a biofilm are significantly more resistant to antimicrobial agents compared with planktonic organisms.

Early identification and evolving effective control strategies against potentially pathogenic biofilm-forming *Staphylococci* can be one of the essential steps towards the prevention and management of the most problematic eye infection. The classic method most often used to phenotypically detect slime production among bacterial species is the Congo red agar (CRA) plate test (Freeman *et al.*, 1989). Newly developed molecular methods recently provided a direct evidence of the genetic basis of slime production complementary to the CRA test. They became available with the discovery that slime synthesis is controlled by the ica operon (Mack *et al.*, 1996; Gerke *et al.*, 1998). Polysaccharide intercellular adhesin (PIA), a main slime component consisting of linear β-1,6-linked glucosaminylglycans, is synthesized *in vitro* from UDP-*N*-acetyl glucosamine by the enzyme *N*-acetyl glucosaminyl transferase. This is encoded by the *icaA* gene. Slime production is considered to be a significant virulence factor for some strains of Staphylococci (Christensen *et al.*, 1982). The challenge in eradicating a chronic infection associated with slime formation is mainly due to the fact that the slime producing bacteria resist higher antibiotic concentrations than non slime producing strains (Gristina *et al.*, 1987). Hence, a precise approach is to use PCR technology for the detection of adhesion genes (Tristan *et al.*, 2003). Antibiotic resistance in bacteria is currently a major public health problem (Fishman, 2006). Recent reports indicate that resistance to earlier generation ocular antibiotics among clinical bacterial isolates is becoming more prevalent. Antibiotic resistance has been noted among ocular isolates, necessitating treatment with medications such as fortified vancomycin (Kim *et al.*, 2005). Multidrug-resistant MRSA is proliferating in serious ocular infections. Based on the rate of increase in the "The Surveillance Network" (TSN) database USA, it was predicted that MRSA cultures from serious ocular infections could be more common than methicillin-susceptible *S. aureus.* Hence large-scale national surveillance programs are required to monitor *in vitro* antimicrobial resistance trends in ocular isolates (Asbell *et al.*, 2008) towards efficient management of the problem.

The increasing number of reports concerning ocular bacterial resistance to currently used antibiotics warrants a detailed study on the possible factor conferring antibiotic resistance and the chances of acquiring same from other non clinical organisms possibly due to horizontal gene transfer (HGT). In this context we investigated biofilm forming ability and their associated role in antibiotic resistance profile of the *Staphylococcus* spp. isolated from clinical ocular patients and their phylogenetic relationship with other community MRSA.

Experimental

Material and Methods

Bacterial strains. Two hundred samples (from 88 males and 112 females) were collected from conjunctivitis infected patients (cataract, red eye, keratitis and contact lens infection) from patients undergoing treatment at the Dr. Agarwal Eye Hospital, Salem, Tamil Nadu, India during the period from June 2008 to June 2009. The patients were asked to look up and the inferior conjuctival sac was swabbed in a single swab for secretions. All the collected samples were processed within two hours. The swabs obtained were inoculated on to Nutrient Agar, MacConkey Agar, Blood agar and Mannitol Salt Agar plates and incubated at 37°C for 24–48 hours. Isolates obtained from plates were identified using conventional microbiological methods. Colonies showing Gram-positive cocci in clusters, which were catalase positive, oxidase negative and bacitracin resistant, were presumptively identified and labelled as *Staphylococcus* sp. Identification of staphylococcal isolates to the species level was carried out by detection of enzyme production (coagulase, phosphatase, ornithine, and urease), L-pyrolidonyl-β-naphthylamide hydrolysis, hemolytic properties on sheep blood agar, acid production from mannitol, mannose and trehalose and resistance to novobiocin by using a published protocol (Kloos and Bannerman, 1999).

Antibiotic sensitivity test. The antibiotic resistance/sensitivity profile of the conjunctivitis infection isolates against various antimicrobial agents commonly used to treat or prevent ocular infections were assessed *in vitro* by the disk diffusion method according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2006). The inoculum for each confirmatory isolate was prepared using a 0.5 McFarland standard, which was then swabbed onto a Mueller Hinton (HiMedia, Mumbai, India) agar plate supplemented with 2% NaCl. The commercial antibiotic disks

(HiMedia, Mumbai, India) were placed in Petri plates and seeded with 10^8 CFU/mL (0.5 McFarland) of bacterial isolates. Penicillin G (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Methicillin (5 µg), Amikacin (5 µg), Gentamicin (30 µg) antibiotic discs were placed in Petri dish maintaining equal distance (4 mm) with the help of forceps which was flame sterilized intermittently. Following incubation at 37°C for 18 h, the bacterial growth inhibition zone around the disks was analysed and compared with standard chart.

Slime producing ability of all the isolates were evaluated by two different methods.

Tube adherence (Christensen *et al.*, 1982). A loopful of the isolate from agar plate was inoculated into a glass tube containing 5 ml of trypticase soya broth (TSB) and incubated at 37°C for 48 h. Each tube was decanted, stained with 0.25% safranin, and then gently rotated to ensure uniform staining and the contents were gently decanted. The tubes were then placed upside-down to drain. The color of the inner surfaces of the tubes was observed. An adherent film on the surface of the glass tube was taken as an evidence of slime formation. The absence of a film or the mere presence of a ring at the liquid-air interface was interrupted as a negative result (–). Based on slime production, the positive results were recorded as strong (+++), moderate (++), week (+). Each test was interpreted by two different observers.

Congo red agar method (Freeman *et al.*, 1989). Slime production by the isolates was determined by CRA method as described by Freeman *et al.*, (1989). The media used contained brain heart infusion broth 37 g/l, sucrose 0.8 g/l, agar-agar 10 g/l, and Congo red stain 0.8 g/l. The Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar was cooled to 55°C. Plates of the medium were inoculated and incubated aerobically for 24 h at 37°C. All the chemicals and reagents were procured from HiMedia, Mumbai, India. The appearance of reddish black colonies with a rough, dry, and crystalline consistency was considered to be indicative of slime production. Non slime isolates produced pinkish red, smooth colonies with a darkening at the centre.

Biofilm assay (Cucarella *et al.*, 2001). The isolates were individually grown overnight in TSB at 37°C, and diluted 1:40 in the same broth incorporated with 0.25% glucose. Sterile 96 well 'U' bottom polystyrene tissue culture plates (Tarsons, Mumbai, India) were inoculated with 200 µl of the bacterial suspension and incubated overnight (24 hours) at 37°C without agitation and visualized by staining with 1% crystal violet for 15 minutes after washing thrice with 200 µl of sterile phosphate buffer saline (pH 7.4) and drying. After rinsing three times with distilled water and subsequent drying, the formed biofilm was quantified in duplicate by a micro plate reader (model 680, Bio-Rad, Hercules, CA) at 570 nm. Uninoculated wells containing TSB with glucose served as blanks. *S. epidermidis* ATCC 35983, a known slime producer was used as positive control for slime production. Blank corrected absorbance values of strains were used for reporting biofilm production. Strains producing a blank corrected mean absorbance value of >0.1 were considered biofilm producers. Each strain was tested for biofilm production in duplicate and the assay was repeated three times.

Isolation of genomic DNA. Bacterial isolates grown overnight in a 50 ml LB broth, at 37°C in a rotary shaker (200 rpm) were used for genomic DNA isolation employing standard protocols outlined by Sambrook *et al.*, (1989). Two ml of overnight bacterial culture was transferred to an Eppendorff tube and spun at 6000 rpm for 4 minutes. The supernatant was discarded and drained well on to tissue paper. The bacterial pellet was re-suspended in 400 µl of TE buffer. Vortex rapidly then, 600 µl of 1M NaCl was added and allowed to heat at 65°C for 10 minutes. After 10 minutes, cooling it to room temperature 1000 µl of saturated phenol solution was added, mixed well centrifuged at 10,000 rpm for 10 minutes. (In the case of very dense aqueous phase, it was diluted with sterile distilled water). The aqueous phase was collected and mixed with 2 volume of absolute ice cold ethanol. Later it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, the pellet was allowed to air dry completely. The pellet was washed with 70% ethanol and allowed to dry at room temperature. After drying, the pellet was stored in 20–80 µl of TE at –20°C. The extracted DNA was visualized by electrophoresis in 0.7% agarose gel and viewed under the Gel Documentation/Imaging Systems (Alpha Innotech, San Leandro, CA, USA) at 262 nm.

Detection and sequencing of *ica*A gene. The nucleotide sequences of the *ica*A gene available in the GenBank Sequence Database of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov) were utilized for designing primer sequences using genetyx version7 software. The primers used were: Forward: AAGTCATACACTTGCTGGCG and reverse: CTGTCTGGGCTTCACCATGT.

DNA from strong biofilm producing isolates was screened for *ica*A gene by the polymerase chain reaction (PCR) in a DNA thermal cycler (Eppendorf, Hamburg, Germany) (Vasudevan *et al.*, 2003). The reaction was carried out in a 25 µl volume containing 3 mmol of the primers, together with 25 ng of DNA, 1 U Takara ExTaq HS and buffer (containing 20 mmol MgCl2), 200 mmol each of dATP, dCTP, dGTP and dTTP. Thirty cycles of amplification, each consisting of denaturation at 92°C for 40 seconds, annealing at 49°C for 45 seconds and elongation at 72°C for 1 min,

along with a final extension at 72°C for 7 min was carried out. The presence and size of the amplicons were confirmed by electrophoresis on 1.5% agarose gel. Further nucleotide sequence of the amplicons were determined by sequencing which was carried at Ocimum Biosolution, MCV Chennai, India using the instrument ABI 3130, Applied Biosystems, CA, USA. The obtained sequences were submitted to NCBI (Accession numbers GQ 214387 and GQ214388).

Phylogenetic tree construction. The evolutionary relationships among the isolates were determined by phylogenetic analysis. The sequences were aligned first using CLUSTAL W that calculate a crude similarity measure between all pairs of sequences by using a fast and approximate alignment algorithm described by Wilbur and Lipman (1983) and then determined the order of sequences to be aligned in the final multiple alignment. The resulting distances were used to calculate a phylogenetic guide tree which uses pairwise sequence distance calculation to perform multiple sequence alignment The guide tree was calculated with the MEGA 4 method (Saitou *et al.*, 1987; Tamura *et al.*, 2007).

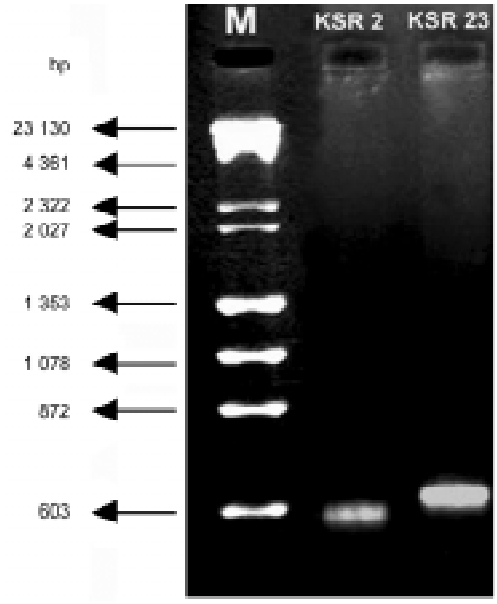

Fig. 1. PCR amplified product of ica A gene sequences of biofilm forming conjunctivitis Staphylococcus aureus isolates.

Results

Conjuctival specimens were examined from 200 patients and 108 bacterial isolates were obtained from them. All the isolates were presumptively identified as *Staphylococcus* spp., *Pseudomonas* spp., *Escherichia coli* and *Proteus* spp. Further it was observed that *Staphylococcus aureus* (36 isolates) and *S. epidermidis* (24 isolates) were dominant among the isolates obtained from ocular patients.

The biofilm forming ability of *S. aureus* and *S. epidermidis*, the dominant group among the isolates, was determined using tube adherence test, congo red plate method and confirmed using microtitre plate assay. It was observed that 38 out of 60 isolates were weakly positive (+1), 16 moderately positive (+2), two strongly positive (+3) and four isolates were biofilm negative. Further, the biofilm formed was quantified by microtitre plate adherence assay and their results indicated that isolates KSR 2, and KSR23 belonging to *S. aureus* were more prone to biofilm formation recording 0.564–1.157 OD, an indicative of higher activity.

The antibiogram study revealed that all the isolates of *S. aureus* and *S. epidermidis* were resistant to methicillin (Table I). Out of 60 *Staphylococcus* isolates 11 were penicillin resistant, 3 ciprofloxacin resistant, 49 ofloxacin resistant, 13 amikacin resistant and 13 gentamicin resistant. In total, 13 strains were resistant to three or more antibiotics. The antibiotic susceptibility data showed diminished activity of number of antibiotics on this conjunctivitis causing isolates which

Table I
Shows the antibiotic susceptibility and biofilm forming ability of the ophthalmic isolates

Strain No	Name of the isolate	No of isolates	Antibiogram of the isolates																		Biofilm Forming Ability Score					
			Penicillin (10 µg)			Ciprofloxacin (5 µg)			Ofloxacin (5 µg)			Methycillin (5 µg)			Amikacin (5 µg)			Gentamycin (30 µg)			Congo Red			Christenson		
			R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	+++	++	+	+++	++	+
1	*Staphylococcus aureus*	36	7	2	27	2	26	8	33	3	–	35	1	–	3	3	30	7	5	24	1	10	22	1	9	22
2	*S. epidermis*	24	4	3	17	1	18	5	16	5	3	22	2		10	11	3	6	3	15	1	6	16	1	9	12
3	*Pseudomonas* sp.	17	2	3	7	3	9	6	1	3	13	3	8	7	2	6	9	3	9	6	2	9	6	2	7	6
4	*Proteus* sp.	8	5	2	1	1	3	6	–	2	6	2	2	4	–	2	6	1	2	5	–	1	7	–	0	7
5	*Escherichia coli*	15	4	3	8	1	6	8	1	4	10	2	4	9	1	4	10	2	3	10		6	8	–	5	8
6	Others	8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	2	5	–	0	5

Note: R – Resistant; I – Intermediate; S – Sensitive; +++ Strong; ++ Moderate; + Weak; * Not Determined

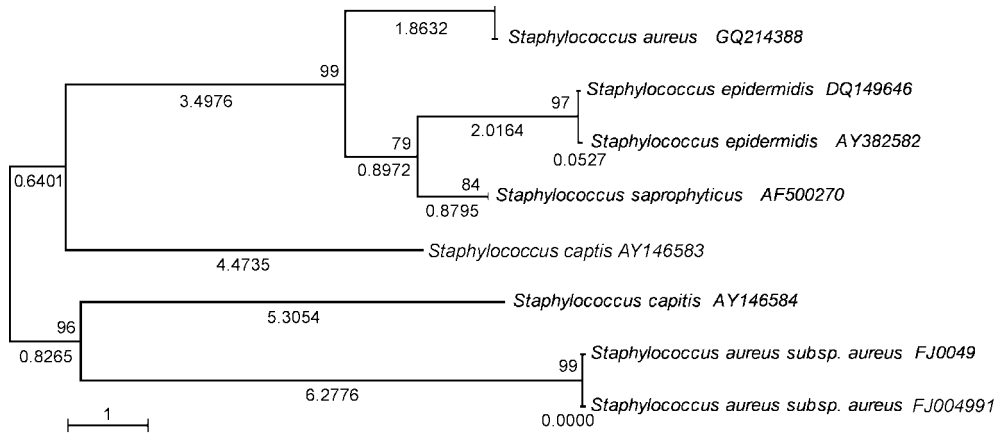

Fig. 2. Phylogenetic relationship of ica gene sequences among biofilm forming conjunctivitis *Staphylococcus aureus* isolated from ocular patients in Tamilnadu, India with reference sequences obtained through BLAST analysis.

increases with their biofilm forming ability. Though the other isolates *Pseudomonas* spp., *Proteus* spp., *E. coli etc.*, showed moderate antibiotic resistance; it acquires less significance because of their low biofilm forming ability.

The gene responsible for biofilm formation inter cellular adhesion (*ica*) was detected in 20 isolates of *S. aureus* and 16 of *S. epidermidis* by PCR amplification. Amplicon with 630 bp and 580 bp for *S. aureus* was obtained (Fig. 1). Automated sequencing of these amplicons provided partial sequences which were submitted to GenBank and were assigned the name and accession (GQ 214387 and GQ214388) (www.ncbi.nih.gov.). The phylogram presented in figure 2 indicates close relatedness of isolates obtained from the ocular patients with the sequences available in the genome database. The optimal tree with the sum of branch length equal to 26.69712461 as shown (Fig. 2) was obtained. Sequence analysis comparison of the *ica*A gene from these species revealed very high sequence similarity, suggesting the possibility of horizontal gene transfer of biofilm encoding genes.

Discussion

Coagulase negative Staphylococci (CoNS) commonly isolated mixed with more typical ocular flora lead to major infections including keratitis, conjunctivitis and endophthalmitis. CoNS, were considered as harmless skin commensal flora and dismissed as culture contaminants. But in recent years, they are increasingly being recognized as important human pathogens. The American Society of Cataract and Refractive Surgery survey for the year 2004 revealed that sixty-one percent of cases reported were due to *Staphylococci* (Donnenfeld *et al.*, 2005). *S. aureus* is the most common pathogen recovered from conjunctivitis (Knauf *et al.*, 1996) and its role in the pathogenesis of chronic allergic conjunctivitis due to colonisation has been suggested (Tuft *et al.*, 1992). *Staphylococcus epidermidis*, an opportunist microorganism, is now recognised as a real "new" pathogen, in particular as etiologic agent of infections associated bacterial colonies on the surface. *Staphylococcus aureus* was shown to undergo physiological changes in the early stages of biofilm formation (Williams *et al.*, 1999). In the present study both *S. aureus* and *S. epidermis* were isolated as dominant species from the conjunctivitis patients corroborating with the earlier reports.

Ophthalmologists believe that excessive and inadequate systemic use of antibiotics is one of the most important factors causing antibiotic resistance and that resistance among ocular isolates is a reflection of the practice pattern of this community. A study conducted between 1996 and 2001 showed that number of conjuctivitis causing isolates susceptible to methicillin decreases and the number of MRSA isolates increased from 8.5% in 1990 to 27.9% in 2001. It also revealed a 160% increase in ciprofloxacin resistance among keratitis and conjuctivitis *S. aureus* isolates (Marangon *et al.*, 2004). Goldstein *et al.* (1999) reported that resistance of *Staphylococcus aureus* to ciprofloxacin and ofloxacin increased 7-fold from 1993 to 1997. Hwang (2004) showed that several microorganism causing ophthalmic infections had developed resistance to ciprofloxacin and its sister fluoroquinolones, ofloxacin and levofloxacin, more quickly than imagined, and resistance levels are increasing each year.

Both *S. aureus* and *S. epidermidis* the well recognised etiologic agents of ophthalmic infections exploits the production of a polysaccharide biofilm for wrapping up and armouring their colonies on the surface as one pathogenic mechanism. Studies conducted using animal models have shown that biofilm-producing *S. epidermidis* strains are more virulent in causing

infections than biofilm negative strain (Deighthon *et al.*, 1996; Gelosia *et al.*, 2001). Though a number of tests are available to detect slime production by Staphylococci, all those methods including TM and CRA are often subject to severe analytical limitations and hence unable to detect bacterial adherence accurately (Mathur *et al.*, 2006). It is reported that *S. epidermidis* enters the eye during and after intraocular surgery and causes postoperative suppurative endophthalmitis. However, the factors contributing to the virulence of *S. epidermidis* are not well understood. It has been suggested that the ability to form biofilms on polymer surfaces greatly contributes to the virulence of *S. epidermidis*. This ability depends on the production of polysaccharide intercellular adhesion (PIA) molecules, encoded by the intercellular adhesion (*ica*) locus including the *ica*A gene, *ica*B gene, *ica*C gene, and *ica*D gene However, the prevalence of biofilm-forming strains of *S. epidermidis* in the conjunctival microflora has not yet been determined (Takashi Suzuki *et al.*, 2005). Results obtained in the present study and reports made in earlier studies prove that *S. aureus* and *S. epidermidis* are common pathogens causing eye infections. Their high prevalence and antibiotic resistance may be due to their biofilm forming nature. The PCR amplification of the *ica*A gene demonstrates the inherent biofilm producing nature of the isolates.

The evolutionary relationship of the strains *Staphylococcus aureu*s KSR2 and *S. aureus* KSR23 obtained from ocular patients in the present study was analyzed by comparison with sequence data available with National Center for Biotechnology Information (NCBI). Several studies have shown that the presence of genes encoding intra cellular adhesion (*ica*) is associated with the formation of slime and biofilm in *S. aureus* and *S. epidermidis* (Ammendolia *et al.*, 1999; Arciola *et al.*, 2001; Cramton *et al.*, 1999; Vasudevan *et al.*, 2003). In the community, hospital strains of methicillin resistant staphylococci may contribute to the emergence of methicillin resistant staphylococci *de novo*, through horizontal acquisition of the methicillin resistant encoding gene (Salmenlinna *et al.*, 2002). It has been reported that Methicillin Resistant (MR) staphylococci in haemodialysis units in hospitals, which were resistant to multiple antibiotics, probably contributed to the overall increase in the incidence of staphylococcal infections rather than simply replacing the more susceptible strains. The majority of the MR *Staphylococcus epidermidis* isolates from patients belonged to one main clone. The clonal relatedness of isolates strongly suggests that CoNS infections were probably contracted from a common source in the haemodialysis unit and that this clone was transferred by patient-to-patient transmission leading to infections (Liakopoulos *et al.*, 2008). In the present study, the antibiotic resistant *S. aureus* strains recovered from clinically significant conjunctivitis infection, as well as among colonizing isolates from community, were found to carry the ica operon and produce biofilms. Hence, it is presumed that there was a possible horizontal transfer of genes encoding biofilm among conjunctivitis isolates. Of course, the biofilm forming ability, their antibiotic resistance, the presence of *ica* gene and their close relationship with other community associated counterparts and probable HGT warrants an in depth study to draw final conclusions. Efficient detection of biofilm forming ability and expression of the encoding *ica*A gene, as well as the effective suppression of PIA or PS/A synthesis would facilitate development of feasible strategies for the treatment of conjunctivitis and prevention of the further transmission of their infection.

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