Investigation of *fnbA* and *fnbB* Genes coding Adhesin specific fibronectin-binding proteins in *Staphylococcus aureus*

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Received: September 20, 2018 Accepted: November 01, 2018

ABSTRACT: *The colonization and infection in intra vascular catheters is mainly due to the presence of different virulence determinants including toxins, tissue degrading enzymes and immune evasion factors. Several virulence genes are implicated in biofilm formation, like icaA and icaD, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase. The staphylococcal surface adhesins named as "microbial surface components recognizing adhesive matrix molecules" enable bacteria to bind to the fibronectin, fibrinogen, and collagen of the host. In staphylococci, binding to proteins such as fibrinogen, elastin, and fibronectin is mediated by adhesins, which are named FNBPA and FNBPB and are under the control of fnbA and fnbB genes. Fibronectin binding proteins were investigated in both methicillin susceptible and resistant S. aureus strains isolated from patients with staphylococcal infections such as osteomyelitis and skin and soft tissue infections. Presence of the fnbA gene was reported as approximately 100% and for the fnbB gene this proportion was reported to be between 0% and 98% in clinical isolates. The aim of the present study was to investigate the genes that regulate the production of the fibronectin binding proteins in the Staphylococcus sp using polymerase chain reaction.*

Key Words: *Staphylococcus aureus, biofilm formation, fibronectin, fnbA and fnbB genes*

INTRODUCTION

Intravascular catheters are the most common assist devices utilized every year. These catheters and its types are used for the administration of fluids, medications, parenteral nutrition, and blood products; to monitor hemodynamic status; and to provide hemodialysis (Donlan, 2001). Due to these multiple functions, after exposure to body fluids the device becomes a suitable environment for the support of biofilm production and subsequent infection. Microbial adhesion to medical device surface is considered the base of the pathogenic mechanism of prevalent *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Donlan (2001) reported biofilm producers are highly associated with the increased risk of central line-associated bloodstream infections (CLABSI) which results in significant morbidity, mortality, and costs for health care delivery. World Health Organization (WHO) proposed several methods and guidelines for the prevention of CLABSI.

The colonization and infection is mainly due to the presence of different virulence determinants including toxins, tissue degrading enzymes and immune evasion factors. Several virulence genes are implicated in biofilm formation, like *icaA* and *icaD*, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase (Cos and Tote, 2010). The staphylococcal surface adhesins named as "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) enable bacteria to bind to the fibronectin, fibrinogen, and collagen of the host. In staphylococci, binding to proteins such as fibrinogen, elastin, and fibronectin is mediated by adhesins, which are named FNBPA and FNBPB and are under the control of *fnbA* and *fnbB* genes. Fibronectin binding proteins were investigated in both methicillin susceptible and resistant *S. aureus* strains isolated from patients with staphylococcal infections such as osteomyelitis and skin and soft tissue infections. Presence of the *fnbA* gene was reported as approximately 100% and for the *fnbB* gene this proportion was reported to be between 0% and 98% in clinical isolates (Arciola et al., 2005).

For the first time, in the present research, different combinations of antibacterial compounds showing synergism to fabricate the intravascular catheter materials by surface coating were studied. To determine the sustained release of drugs from the fabricated materials (Matl *et al*., 2008); appropriate drug-carriers (beta-cyclodextrin and DL-Lactic acid) were blended with antibacterial agents. Among the drug combinations, one is a fluoroquinolone compound and the other is nitroimidazole compound which has good antimicrobial activity against aerobic and facultative anaerobic microorganisms. The combination

therapy of these two groups of drugs managed to accept the factors determined by Boeckh *et al*., (1990), Gorman and Jones (2002) and Saginur *et al*., (2006). The character of synergism mainly depends on the mode of action of a drug. Both of these compounds act on the DNA of bacteria, targeting the inhibition of DNA synthesis and replication. Since the mode of action of fluoroquinolone and nitroimidazole are same these two drugs proves the synergism. One major application of antimicrobial coating in medical products is to help maintain sterile environments. Antimicrobial treatment for biomedical products are necessary to avoid cross infection by pathogenic microorganisms; to control the infestation by microbes; to arrest metabolism in microbes in order to reduce the formation of odour; to safeguard the product from staining, discoloration and quality and over all to increase the standard of hygiene in hospitals and healthcare centers

Thus in the present study the tissue reactions of coated catheters with drugs and carriers and investigation on the virulence genes, *fnbA* and *fnbB* that regulate the production of the fibronectin binding proteins in *Staphylococcus sp* using polymerase chain reaction was investigated.

MATERIALS AND METHODS

The entire research work was carried out from June 2018 to November 2018. All the lab work was done in Microbiology Laboratory, Sree Narayana Guru College, Coimbatore, Tamil Nadu, India.

Preparation of antibacterial drug and coating IVCs by slurry-dipping technique (Boccaccini *et al*., 2003)

Drug releasing intravascular catheters were made using a standard slurry-dipping technique. The technique started with the preparation of stable slurry with specific amount of drugs (levofloxacin-ornidazole and moxifloxacin-ornidazole) in the molten polyethylene glycol (PEG). Appropriate slurry temperature (37°C) was determined by an optimization process based on a trial-and-error approach to achieve optimum coating thickness, uniformity and stability of composite coating as well as adequate infiltration of drug particles into coating structure. PEG (2g) with a predefined molecular weight was mixed with drug powder (0.5g) in a glass vial. The mixture was heated at the range of 60–70°C in a water bath to obtain homogeneous slurry. The resulting slurry was homogenized in a magnetic stirrer for 5 to 10 min. Each piece of catheter (length - 6mm) was dip-coated twice with intermittent drying (suspension coating method). The dip-coating procedure was carried out in sterile glass beakers on a shaker (120 rpm) for 30min, with a drying period of about 15 minutes between the two coating procedures, followed by drying at room temperature. All coating steps were carried out under strict aseptic conditions.

All samples were coated by a thickness of about 3–10% of catheters outer diameters. After coating procedure, the catheter samples were stored at 4°C for upto 15min. In order to increase drug loading and prevent excessive increase in catheter thickness, the coating process was repeated four times for each sample. Subsequently, in order to slow down the release rate of drug from PEG coating and mitigate the friction effect between catheter surface and mucosa, second coating layer was formed on the catheter surface. Carrier (Betacyclodextrin/ DL-lactic acid) was dissolved in DMSO to acquire a 10 w/w% solution. PEG-coated samples were submerged into Carrier (Betacyclodextrin/ DL-lactic acid) solution three times for 1 min each. Thereafter, these samples were stored at 0°C or in a deep freezer to implement one freeze–thaw cycle and physically crosslink the samples. The coated catheters were left to dry on a clean bench for 1 week at room temperature to remove residual DMSO.

Analyzing the tissue reactions of coated catheters using IEC-6 animal cell lines (Yang *et al*., 2004)

The intestinal epithelial cell lines IEC-6 [American Type Culture Collection (ATCC) - 57090] was used to investigate the tissue cytotoxic reactions of the coated materials by both direct and indirect contact method. For direct contact method, the test sample was placed on sub-confluent monolayer of IEC-6 cells and incubated. Following incubation, the cell culture was examined microscopically for cellular response around the samples. For indirect method, IEC-6 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 5% FCS, insulin 5µg/ml (Sigma) and 2 mmol/*l* glutamine. They were grown to confluence in 96-well plates for MTT assay. The cells in suspension containing approximately 1×10^6 were added to each well of a 96-well culture plate and were incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. IEC-6 cells were treated with treated and untreated catheter. Control cultures were treated with DMSO. The cultures were again incubated as above. After 36 h, 20 µl of MTT solutions were added to each well and the cultures were further incubated for 4 h and then 200 µl of DMSO was added. The formed crystals were dissolved gently by pipetting two to three times slowly. The absorbance at 655 nm was measured using plate reader. Percent inhibition was recorded as per the standards.

Analysis of virulence genes of *Staphylococcus sp*

The test bacteria *Staphylococcus sp* were grown upto logarithmic phase under sterile conditions. The genetic materials of the organisms were extracted separately as per standard DNA extraction method. Following were the steps involved in DNA extraction and electrophoresis.

a) Culturing the test bacteria for DNA extraction

About 50 ml of LB broth was prepared and the selected test organism was inoculated. Culture tubes were incubated in the appropriate condition (37°C for 12 hours). Dispensed 5 ml of the culture separately and centrifuged at 8,000 rpm for 15 minutes. The pellets were washed in normal saline twice and then suspended in 2ml normal saline buffer.

b) Extraction of DNA from test bacteria

About 2ml of test bacterial culture was centrifuged at 10,000 rpm for 15 minutes to pelletize the cell mass. The supernatant was removed and the cell pellet was incubated on ice till next use. To the obtained pellet 500µl of solution-A (Annexure-1) was added and gently mixed to make a uniform cell suspension. The cell suspension was incubated on ice for 30 minutes. To the mixture 100µl of freshly prepared solution-B (Annexure-2) was added and vortexed well. The suspension was incubated in ice for 5 minutes (cells get lysed and the solution becomes clear). About 750µl of solution-C (Annexure-3) was added and vortexed for 2 min. The suspension was kept in ice for 60 minutes (chromosomal DNA and cell material will precipitate into whole viscous clump). The tubes were centrifuged at 6000 rpm for 10 minute at 4°C. Transferred the supernatant to fresh eppendorf tube and centrifuged at 10,000 rpm for 10 minute at 4°C. The supernatant was discarded and to the pellet, 50 µl of ice cold ethanol was added along the sides of the tube. The solvent used was removed immediately without disturbing the pellet. The tubes were blot dried using blotting paper by inverting the tube over it. The pellets of DNA were stored by adding 100µl of 1X TAE buffer and placed in a deep freezer. The DNA precipitate thus obtained was resuspended in bi-distilled water and used as a template for Polymerase Chain Reaction (PCR) method.

c) PCR method for the amplification of the fnbA and fnbB sequences

Two pairs of primers were designed for the amplification of the *fnbA and fnbB* genes with previously published sequences. The sequences of *fnbA and fnbB* were taken from the GenBank sequence database of the National Center for Biotechnology Information. Primers specific for *fnbA and fnbB* were picked on the gene sequences by the Primer program.

Staphylococcus sp isolates are screened for the presence of *fnbA and fnbB* virulence genes using the designed primers (Table-1). All amplifications were performed in 25µL reactions, each consisting of 12.5µL of 2X master mix, 0.5µL of each oligonucleotide, and an appropriate volume of nuclease free water. PCR products were separated by electrophoresis in 1–2% agarose gels containing 5µg/mL ethidium bromide run at 100V for 45min and visualised under a UV transilluminator. PCR reactions were performed using the method described by Arciola et al. (2001). The reaction mixture consisted of a reaction buffer (50mM KCl, 10mM Tris-HCl [pH 9.0], 2,5mM $MgCl_2$) in a total volume of 25µl containing 5µl of template DNA (150ng), 0,2mM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 1U Taq DNA polymerase and the above-mentioned primers (0.5µM each for *fnbA and fnbB*). DNA amplification was carried out in a thermocycler with the following thermal cycling profile: initial denaturation at 94ºC for 5min, followed by 50 cycles of amplification (denaturation at 94ºC for 30s, annealing at 59ºC for 30s and extension at 72ºC for 30s) ending with a final extension at 72ºC for 1min. After the first 30 cycles, a further 1U of Taq DNA polymerase was added. Eight microliters of PCR products were analyzed by electrophoresis in 2% agarose gel during 50min at 80V. The bands were stained with ethidium bromide (0.5 µg/ml) and observed under UV light.

$$\text{Bacterial reduction (\%)} = A - B/A \times 100$$

Where, A = number of adhered organisms (in CFU) obtained from the uncoated materials B = number of adhered organisms (in CFU) obtained from the coated materials.

RESULTS AND DISCUSSION

Analyzing the tissue reactions of coated catheters using IEC-6 animal cell lines

When the IEC-6 cells were cultured with the uncoated DMSO samples, IEC-6 cell proliferation was found slightly increased after 2 days of culture. Similarly, the increase in the IEC-6 cell numbers were found for the coated materials with drug and carrier mixture samples [levofloxacin-ornidazole + betacyclodextrin (D1C1); and moxifloxacin-ornidazole + DL-lactic (D2C2)]. In Table-2 and 3, the effect of drug and carrier mixtures (D1C1 and D2C2) was presented. The obtained value showed that the effect of mixtures on IEC-6 cell proliferation was dose-dependent with a maximum at 0.4% of the D1C1 containing supernatant and at 0.6% of the D2C2 containing supernatant. The number of cells proliferated indicated that

the coated catheter materials with the synergistic drugs and carriers (levofloxacin-ornidazole + betacyclodextrin; and moxifloxacin-ornidazole + DL-lactic acid) are highly biocompatible to the tissues.

Investigating the presence of genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* using standard polymerase chain reaction method

The prevalence of virulence genes *fnbA* and *fnbB* that regulate the production of the fibronectin binding proteins in the *S. aureus* strains using polymerase chain reaction was selected as the primary objective of this study. In the selected strain, both *fnbA* and *fnbB* genes were detected (Table-4: Fig-1). The ability of *Staphylococcus sp* to adhere to plasma and extracellular matrix (ECM) proteins deposited on biomaterials is a significant factor in the pathogenesis of device-associated infections. Several specific adhesions are expressed on the surface of *Staphylococcus sp*, which interact with a number of host proteins, such as fibronectin, fibrinogen, collagen, vitronectin and laminin, and have been designated. In the present study, the potential of *Staphylococcus sp* to form biofilm was detected through amplification of Adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* genes by PCR. This study showed high prevalence of *fnbA* and *fnbB* gene in *Staphylococcus sp*. Thus, the presence of fnb locus in most of the *Staphylococcus sp* isolates confirms its role as virulence factor in biofilm formation.

CONCLUSION

The colonization and infection in intra vascular catheters is mainly due to the presence of different virulence determinants including toxins, tissue degrading enzymes and immune evasion factors. *Staphylococcus sp* is considered to be major biofilm forming organism in catheter. The tissue reactions of coated catheters with drugs and carriers and the genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* using standard polymerase chain reaction method were evaluated. The proliferation of cells indicates that the coated catheter materials with the synergistic drugs and carriers (levofloxacin-ornidazole and betacyclodextrin; and moxifloxacin-ornidazole and DL-lactic acid) are highly biocompatible to the tissues. Prevalence of *fnbA* and *fnbB* gene in *Staphylococcus sp* confirmed its ability to act as a virulence factor in biofilm formation. Thus results of present study signify the potentiality of drug carriers against biofilm producers in intra vascular stents which may leads to efficient stenosis.

Table – 1: Forward and reverse primers of selected virulence genes of *Staphylococcus sp*

S. No	Target genes	Encodes for	Primer type	Nucleotide sequence
1	*fnbA*	Adhesin specific fibronectin-binding proteins	Forward Reverse	5’ – CCACCTGGGTTTGTATCTTCTTC – 3’ 5’ – GATTACCACACAGCTATAGATGGTG – 3’
2	*fnbB*	Adhesin specific fibronectin-binding proteins	Forward Reverse	5’ – CGTGACCATTTTCAGTTCCTAAACC – 3’ 5’ – GATACAAACCCAGGTGGTGG – 3’

d) Agarose gel electrophoresis study

Table-2: Dosage effects of drug and carrier mixtures (D1C1) used for antibacterial catheter surface coating on IEC-6 cell proliferation

S. No.	Samples and concentrations used	IEC-6 cell numbers ($X10^5$)
1	Uncoated (control)	12.4 ± 1.2
2	0.2%*	14.4 ± 1.4
3	0.4%*	18.6 ± 2.8
4	0.6%*	16.8 ± 1.2

*(D1C1) Drug and carrier mixture concentrations

Table-3: Dosage effects of drug and carrier mixtures (D2C2) used for antibacterial catheter surface coating on IEC-6 cell proliferation

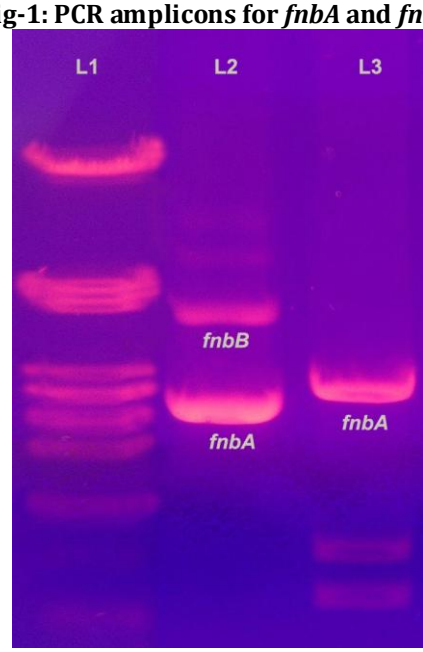
S. No.	Samples and concentrations used	IEC-6 cell numbers ($X10^5$)
1	Uncoated (control)	12.4 ± 1.2
2	0.2%*	15.6 ± 2.2
3	0.4%*	16.1 ± 1.2
4	0.6%*	19.2 ± 2.4

*(D2C2) Drug and carrier mixture concentration

Table-4: Genes with Amplicon size

S.No.	Gene Name	T(°C)	Amplicon Size
1.	*fnbA*	50° C	244bp
2.	*fnbB*	50° C	276bp

Fig-1: PCR amplicons for *fnbA* and *fnbB*



Lane1: DNA ladder
Lane2: PCR amplicons of both *fnbA* and *fnbB*
Lane3: PCR amplicons of *fnbA* from standard biofilm producing *Staphylococcus* strain

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