Role of extra-cellular fatty acids in vancomycin induced biofilm formation by vancomycin resistant *Staphylococcus aureus*

Zulfiqar Ali Mirani[1] and Nusrat Jamil[2]
Microbiological Analytical Centre, PCSIR Laboratories Complex, Karachi, Pakistan
Department of Microbiology, University of Karachi, Karachi, Pakistan

Abstract: In the present study a vancomycin resistant *Staphylococcus aureus* (*S. aureus*) (VRSA) (Labeled as CP2) was isolated from the blood of a post-operative cardiac patient is described. It harbors a plasmid which carry *vanA* gene and exhibited low-level vancomycin resistance (MIC 16µg/ml), was sensitive to teicoplanin. It has been observed that sub-lethal dose of vancomycin induced biofilm formation by CP2 on nylon and silicon indwelling. The results divulge new insights into associations between vancomycin induced biofilms and extra-cellular fatty acids. Gas chromatography coupled with Mass spectrometry (GC-MS) revealed that biofilm matrix of CP2 contains a variety of saturated and un-saturated fatty acids, especially, diverse species of octadecanoic (C18:0) and octadecenoic acids (C18:1). A large difference in fatty acids composition was noticed in biofilms, isolated from hydrophobic and hydrophilic surfaces. CP2 produced thicker layer of biofilms on hydrophobic silicon and nylon surfaces which contains variety of saturated, un-saturated and cyclic fatty acids. Contrary to this on hydrophilic glass surfaces it produced thinner layer of biofilm which contains only straight chain saturated fatty acids. These fatty acid components seem to play a crucial role in cell-cell communication and in the establishment of biofilms, consequently, advantageous for pathogens to survive in hospital environment under enormous antibiotics pressure.

Keywords: *S. aureus*, vancomycin, oxacillin, resistance, biofilms

INTRODUCTION

S. aureus is one of the major causes of nosocomial infections. It causes variety of infections ranging from localized skin infections to life threatening infections including bacteremia and septicemia. Biomedical device-associated infections caused by *S. aureus* are of increasing importance in modern medicine. According to Edmiston *et al.* (2006) approximately 50% to 70% of all hospital-acquired infections are associated with an indwelling biomedical device. The most important factor in the pathogenesis of medical device-associated-staphylococcal-infections is the formation of adherent, multilayered biofilms (Mack *et al.,* 2006). Biofilms are composed of three layers. The first layer that mediates bacterial adhesion to the solid surface is composed of extra-cellular polymeric substances such as polysaccharides, glycoprotein, lipids, DNA and extra-cellular enzymes (Rode *et al*., 2007). The second layer is composed of bacteria, which produce extra-cellular metabolites to maintain the first layer. The third layer is the surface film on which free floating bacteria belonging to different genera, residing in same environment may stick and further strengthen it (Silverstein & Donatucci 2003). The large portion of surface film is composed of exo-polysaccharide matrix. The biofilm matrix provides protection to bacteria, because antibiotic diffusion is difficult in multi-layered films. Low oxygen tension leads to slow or reduced bacterial metabolism that make bacteria functionally resistant to antibiotics (Silverstein & Donatucci 2003). The establishment of biofilms is very organized and regulated by quorum sensing, diffusible signaling molecules, cell to cell signaling that controls population density-dependent gene expression (Estrela & Abraham 2010). It has been reported that various antibiotics are responsible for induction of biofilm mode of growth in resistant bacteria specifically amino-glycosides induced biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* (Hoffman *et al*., 2005). Sakoulas *et al.* (2005) demonstrated that adherence of VRSA to polystyrene is influenced by vancomycin therapy i.e. prolong vancomycin treatment resulting in increased biofilm formation. It has also been reported that staphylococcal lipase and sortase play an important role in biofilm formation (Xiong *et al*., 2009).The present study was designed to investigate the role of extracellular fatty acids in biofilm formation process of VRSA during growth under vancomycin stress environment.

**Corresponding author:* e-mail: mirani_mrsa@yahoo.com

METHODS

Identification of S. aureus

For isolation and identification of *S. aureus*, the growth was monitored on differential and selective media including Manitol salt agar (BioM), Staph-chromo agar (Merck), Staphylococcus 110 agar (BioM), Baird parker agar (Oxoid), DNase agar (Merck) and Blood agar.

Determination of vancomycin minimum inhibitory concentration

Mullher Hinton agar (MHA-oxoid) was used to measure

the vancomycin resistance level of CP2 following Clinical and Laboratory standard Institute (CLSI) guidelines (Hanaki *et al*., 2007) and MIC was re-confirmed by E-test (AB-Biodisk) according to manufacturer's instructions.

DNA extraction

For molecular studies, plasmid DNA was isolated by using the Promega Midi plasmid purification kit following the manufacturer's instruction.

Polymerase chain reaction (PCR)

PCR amplification of van genes was performed with an MWG thermal cycler in a volume of 50µl of Promega MASTER Mix. For amplification of the *vanA, vanB* and *vanC* genes, the primers described by Miele *et al.*, (1995) were used.

VANA-5´ATGAATAGAATAAAAGTTGCAATAC 3´
VANA1-5´CCCCTTTAACGCTAATACGAT 3´
VANB-5´-CCCGAATTTCAAATGATTGAAAA 3´
VANB1-5´- CGCCATCCTCCTGCAAAA 3´
VANC-5´-GCTGAAATATGAAGTAATGACCA 3´
VANC1-5´CGGCATGGTGTTGATTTCGTT 3´

Biofilm assay

A qualitative assessment of biofilms formation of VRSA strain on different biomedical indwelling was determined by tube method. Variable biomedical tube devices made up of glass capillaries, silicon folly catheters (10ML 5cc-Medline) and Bard Nylon Catheter Plug (MED Worldwide), used in this experiment. Two inch pieces each of these tubing were submerged in MH broth supplemented with 14µg/ml vancomycin containing 4 hours young culture of VRSA and incubated at 37˚C for 48 hours. After 48 hours these tube pieces were washed with phosphate buffer saline (pH 7.0) and dipped in acetic acid to fix the biofilms, stained with 3% crystal violet for 15 minutes and finally washed with distilled water for 24 hours. Following controls were run in parallel:

1) CP2 without antibiotics
2) Sensitive control of *S. aureus* (ATCC# 29213) with and without antibiotics
3) Sterile MH broth

Quantification of biofilms

Biofilm formation was quantified by the addition of 2×200µl of 95% ethanol as described by O'Toole *et al.*, (1998) and absorbance was recorded with spectro-photometer (Nicollet Evolution 300 BB) at 563 nm wave length.

Electron microscopy

Scanning electron microcopy was done to analyze the production of extra-cellular matrix material after exposure to vancomycin. Biofilm positive tubes was divided into 4mm sections and washed with distilled water to remove debris negatively stained with 2% uranyl acetate for 30 seconds. These 4mm tube sections showed the presence of biofilms material on lumen were directly examined in a GOEL-JEM-1200 EX II electron microscope.

Isolation of matrix material

Biofilms were formed on sections of Silicon, Nylon and Glass tubes and extracted as described by Al-Fattani & Douglas (2006). The tubing were incubated for 1 hour in deionized distilled water and then washed with ethanol to remove non adherent cells. Tubing with adherent biofilm matrix were transferred to new tube containing 10ml of distilled water and sonicated (Virtis Virsonic 300 with a micro-tip at setting 5) for 5 minutes in an ultrasonic water bath (45s burst, 45s rest) then vortexed for 1 minute to disrupt the biofilms. Cell suspension was then collected and centrifuged. The supernatant was concentrated in freeze drier to one-tenth of original volume.

Extraction of phospholipids

The phospholipids were extracted from biofilm matrix material as described by Rahman *et al.* (2000). The membranes were suspended in the glass tubes by the addition of solvent (2:1 chloroform and methanol respectively) in a 17:2 (v/w) ratio of solvent to pellet and stirred constantly using micro-stir bars for 30min at room temperature. The suspension was transferred to pre-washed glass tubes and the debris was collected by 15min centrifugation at 10,000rpm (Sigma 3K30) at 4°C. The supernatant was collected and the pellet was again extracted with solvent as before. The two supernatants were combined and 2vol upper phase reagent (chloroform/methanol/water containing 0·75% potassium chloride; 3:48:47, by vol.) was added. The mixture was briefly vortexed to form a single phase. The phases were separated by low speed 1min centrifugation in a clinical centrifuge. The upper phase and the precipitate at the interface were discarded. The lower phase was extracted with the upper phase reagent at least three more times or until there was no precipitate at the interface. After the final extraction, the lower organic phase was transferred to a glass vial and the solvent evaporated under methane gas. The dried phospholipids were stored at -70°C under a nitrogen atmosphere.

Fatty acids Analysis

Extracted phospholipids were methylated to prepare fatty acids methyl esters by standard IUPAC method 2.301 described by Dielenbacher (1997). After drying under methane gas about 100mg phospholipids were taken in round bottom flask; 20 ml of chromatographic grade methanol was added followed by the addition of 0.15ml dry 1N methanolic potassium hydroxide solution, refluxed for 30min at 750ºC. Cooled at room temperature and placed in separating funnel followed by addition of 10ml hexane. The pressure of funnel was released by

Table 1: Biofilm formation by isolate CP2 in the presence of vancomycin on different biomedical in-dwelling devices

	Tubing material and biofilm optical density(OD^{563})		
Combinations	Nylon	Silicon	Glass
In the presence of vancomycin	0.645	0.721	0.090
Control 2. *S. aureus* (ATCC#29213) with vancomycin 4 mg/L	ND	ND	ND
Control 4. *S. aureus* (ATCC#29213) without antibiotic	ND	ND	ND
Control 5. Sterile BHI broth	0.077	0.071	0.007

ND- Not Detected

Table 2: Saturated and unsaturated fatty acids composition (mean percentage FAMEs) of Biofilm Matrix Produced by CP2 in Presence of 14µg/ml Vancomycin on Silicon and Nylon and Glass Tubing

S. No.	Compound	(Percentage) Mean		
		Nylon	Silicon	Glass
1.	Undecanoic acid methyl ester	1.34	1.29	1.50
2.	Dodecanoic acid,methyl ester	0.39	0.40	ND
3.	Tridecanoic acid methyl ester	3.21	7.73	1.58
4.	Tridecanoic acid,12methyl-,methyl ester	2.08	1.39	0.72
5.	Tetradecanoic acid methyl ester	6.45	4.86	6.81
6.	Pentadecanoic acid,14-methyl, methyl ester	8.17	8.74	10.57
7.	Hexadecanoic acid methyl ester	1.16	1.97	1.72
8.	Octadecanoic acid methyl ester	15.04	26.38	16.71
9.	Oxiraneudecanoic acid,3-pentyl-,methyl ester	9.79	ND	ND
10.	Cyclopentanetridecanoic acid,methyl ester	12.37	9.79	ND
11.	Octadecanoic acid,9,10-Dihydroxy-,methyl ester	6.35	ND	ND
12.	Nanodecanoic acid methyl ester	4.22	1.79	ND
13.	Cyclopropanebutanoic acid2-	ND	4.83	ND
14.	9,12,15-Octadecanoic acid	3.73	ND	ND
15.	Octadecanoic acid,2-(hexadecyloxy)methyl ester	9.87	10.99	ND
16.	9-Octadecanoic acid,2-(Octadecyloxy) methyl ester	6.61	6.49	ND
17.	9-octadecane,3-	3.98	3.82	ND
18.	Octadecane,1,1	4.58	7.82	ND

ND= Not Detected

shaking, the NaCl and water was added into mixture. Finally organic layer was separated by adding small amount of anhydrous sodium thiosulphate in the vial and analyzed by GC-MS technique.

GC-MS conditions or temperature parameters

The GC-MS analysis for FAMEs was performed on Agilent 6890 N gas chromatographic instrument coupled with an Agilent MS-5975 inert XL mass selective detector and an Agilent auto-sampler 7683-B injector (Agilent technologies, Little Fall, NY, USA). A capillary column HP-5MS (5% Phenyl methylsiloxane) with dimension of 60m x 0.25mm i.d × 0.25µm film thickness (Agilent technologies, Palo, Alto, CA, USA) was used for the separation of fatty acids methyl esters. The initial temperature was 150ºC which was maintained for 2 min raised till 240ºC at the rate of 3ºC/min and kept at 230ºC for 10 min. the split ratio was 1:50, and helium was used as a carrier gas with the flow rate of 0.8ml/min. the injector and detector were 240 and 270ºC, respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70eV the scan range of 50-800m/z. Library search identification of compounds from mass spectra were optimized and tested by matching test spectra against reference spectra in the NIST mass spectral database.

Calculation and standard analysis

Peak identification of the fatty acid in analyzed membrane samples was covered out by the comparisons with retention time and mass-spectra of known standards. Standard methyl esters of octadecanoic acid (C18:0) and octadecenoic acid (C18:1) were used for the confirmation of GC-Ms library results. All samples were used in duplicate, analyzed three times and reported as n=2×3.

RESULTS

The subject strain of present study CP2 was identified as *S. aureus* on the basis of colonial characters as black colonies with opaque zone on Baird Parker Agar with egg yolk tellurite, mannitol fermentation on Mannitol Salt Agar and confirmed by positive reaction on Prolex Latex Agglutination System. It exhibited low-level of

vancomycin resistance (MIC 16µg/ml) and found to carry *vanA* gene on plasmid. This strain exhibited high level of oxacillin resistance (MIC>1000µg/ml) and was sensitive to teicoplanin (MIC 3µg/ml).

Supplementary data

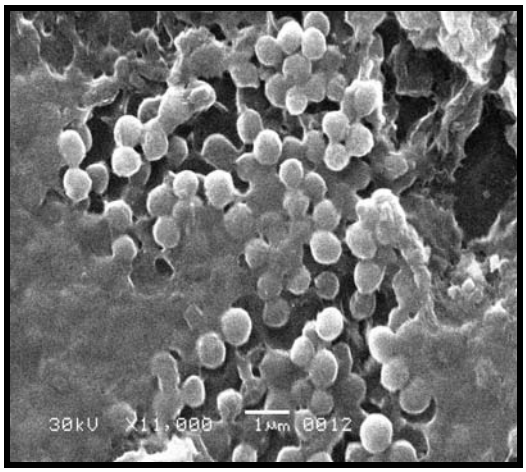
Table 1 shows the comparative analysis of biofilm on different biomedical tubing by CP2 grown in the presence of vancomycin. Crystal violet stained biofilm matrix deposited on lumen and surfaces of tubing were quantified by solubilizing the dye (crystal violet) in ethanol and determined the absorbance at 563nm. The highest optical density values for biofilm matrix were achieved after addition of sub-lethal concentration of vancomycin in growth medium.

Biofilm formation

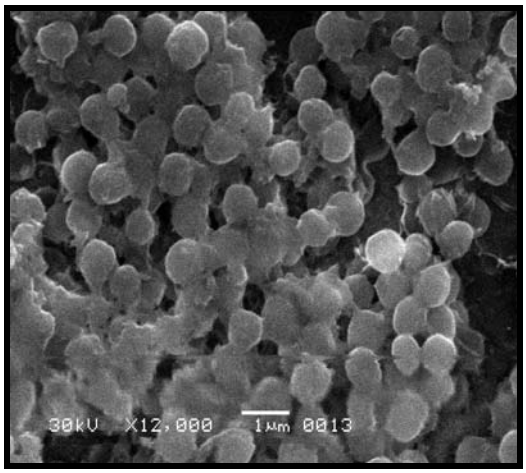
Under vancomycin (14µg/ml) stress environment CP2 was found to produce biofilm (figs. 1A & B) and prefer the lumen of silicon and nylon tubing for this. These tubing materials are hydrophobic in nature and lumen may comparatively have low oxygen tension then outer surfaces. Conversely, in the absence of vancomycin CP2 and sensitive control showed very weak biofilm formation on hydrophilic glass surfaces exclusively (Table 1, Figure 1C). Maximum biofilm formation was achieved after 48h of incubation at 37°C (table 1 and figs. 1A & B). Electron micrographs of the matrix material recovered from lumen of nylon and silicon tubing after 48 hours of incubation supported this finding (fig. 1).

Fatty acids analysis

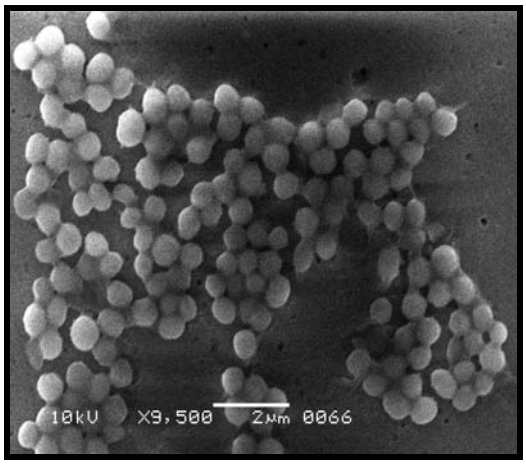
By GC-MS sixteen fatty acids were recovered and identified with two unknown compounds from biofilm matrices produced by CP2 in presence and absence of vancomycin on silicon, nylon and glass tube (Table 2).The sensitive control strain without exposure to any treatment also showed biofilm formation only on glass surface which contained saturated fatty acids (Table 2). All analyzed sample contain the higher amount of tetradecanoic acid, pentadecanoic acid, octadecanoic acid, oxiraneudecanoic acid, cyclopentanetridecanoic acid, nanodecanoic acid and other fatty acids were also present in significant amount. Biofilms exhibited a greater proportion of hydroxy fatty acids and cyclopropane fatty acids but lower proportion saturated fatty acids. Simultaneously, the shear-removable portion of the biofilm showed an increase in the proportion of *trans*-monounsaturated fatty acids and cyclopropane fatty acids. Octadecenoic acid with the unsaturated group was dominant among all of the analyzed fatty acids; its highest amount was at 26.38% in the biofilm isolated from silicon tube, while in biofilms materials isolated from glass and nylon it was at 16.71% and 15.04% respectively. Second highest fatty acid was cyclopentanetridecanoic at 12.37 and 9.79% in the biofilms produced by CP2 in the presence of 14µg/ml vancomycin on nylon and silicon tubing respectively (Table 2), and was not recovered from the glass surface lowest biofilm formation by CP2 was noticed in the absence of vancomycin (table 1).



(A)



(B)



(C)

Figs. 1A, 1B, 1C: Electron Micrographs of CP2; Cells Embedded in extra-cellular biofilm matrix martial produced after exposure to vancomycin fig. 1C control without exposure to vancomycin.

DISCUSSION

Pathogenic bacteria have tendency to protect themselves from toxic effects of antibacterial agents by adopting biofilm mode of growth (Stoodley *et al*., 2004). *S. aureus* is one of the major pathogen causing problems in clinical as well as out side clinical (community) environments (Levy 2000). The multi-drug resistant strains of *S. aureus* are also known to form biofilms on biomedical implants and are difficult to treat with routine antibacterial agents (O'Toole *et al*., 2000). In present study a *vanA* type strain CP2 exhibited low-level vancomycin resistance (MIC16µg/ml) was isolated from a post operative cardiac patient has been described. It has been reported by the previous workers including (Gu *et al*., 2009) that most of the vanA type VRSA strains exhibit high level of vancomycin and teicoplanin resistance (Gu *et al*., 2009). The CP2 seems to be a different strain of vanA family with vanA genotype and vanB phenotype. Gu *et al.*, (2009) described that vanA type strain of Enterococci shows high-level of resistance to both vancomycin and teicoplanin, whereas vanB type strains are characterized by various level vancomycin resistance and sensitive to teicoplanin. The mechanism of vancomycin resistance in *S. aureus* involves a complex reorganization of cell wall metabolism, leading to a grossly thickened cell wall with prolonged doubling times or slow growth rate and production of extracellular matrix materials (Walsh & Howe 2002; Sieradzki & Tomasz 1997). The extra-cellular matrix material is considered as an initial step in bacterial biofilm formation (Costerton *et al*., 2005). In present study, it has been observed that vancomycin induce biofilm formation in CP2 (subject strain of this study) and which prefers lumen of hydrophobic silicon and nylon tubing for biofilm formation. Interestingly, highest biofilm formation was achieved when cells entered in stationary phase i.e. after 48 hours of incubation at 37°C. One long-standing explanation for reduced susceptibility in the biofilm state is the possibility that some of the bacteria in a biofilm grow slowly or enter a stationary-phase state in which they are protected (Anderl *et al*., 2003). Moreover, change in the cell envelop that from starvation reflect the need for protection and insulation from stressful environment. Starved cells are covered with more hydrophobic molecules that favor adhesion and biofilm formation. During stationary phase membrane may also become less fluid and less permeable as fatty acids composition changes from un-saturated to cyclopropyle derivatives (Velkov 1999). During current study silicon and nylon tubing of hydrophobic nature was used and highest biofilm matrix was recovered from lumen of these tubing after addition of vancomycin to the growth media. The electron micrographs reveled that cells adhered on the lumen of silicon and nylon tubing were embedded in extracellular matrix material (fig. 1). These matrix materials provide shelter against the toxic effect of vancomycin, helpful in the formation of multi-cellular aggregates and adhesion to solid surfaces. Interestingly, this biofilm matrix material contained verity of fatty acids i.e. saturated, unsaturated and cyclic with C18:0 and C18:1 as major component. These fatty acids are seemed to be responsible for bacterial adhesion followed by biofilm formation on hydrophobic surfaces under vancomycin stress environment. Interestingly, majority of these fatty acids were long chain i.e., C19 to C51 (table 2). de Carvalho *et al.* (2009) described that cells with high percentage of long chain fatty acids exhibit more hydrophobic characters and are responsible for strong biofilm formation. Incorporation of fatty acids also results in high negative surface charges, according to the extended theory of stability by Derjaguin, Landau, Verwey, and Overbeek (DLVO) "the adhesion capacity is inversely correlated with the (negative) surface charge of the cell in which electrostatic repulsion, van der Waals attraction, and acid–base (hydrophobic) interactions are major contributors" (Boks *et al*., 2008). This is due to the fact that most natural surfaces are negatively charged, and adhesion will take place only when the resulting electrostatic repulsion is overcome by attraction (e.g., van der Waals forces or hydrophobic interactions) between the bacteria and the surface (Jucker *et al*., 1996). Interestingly, octadecanoic acid and its unsaturated and cyclic forms (table 2) i.e. octadecenoic acid, 2-(hexadecyloxy) and 9-octadecenoic acid, 2-(octadecyloxy) were found to be major components of biofilm matrix of CP2. Consistent to our observation, it has been reported that octadecenoic acid is modifiable by a bacterial enzyme named fatty acid-modifying enzyme (FAME), ensuring bacterial survival in tissues (Cerca *et al*., 2005; Stenz *et al*., 2008). Stenz *et al.*, (2008) has demonstrated that 9-octadecenoic acid help *S. aureus* in biofilm formation on polystyrene surfaces. In a series of experiments they observed that *cis* 9-octadecenoic acid (oleic acid) reduces the *S. aureus* adhesion on abiotic surface but after primary attachment it stimulates biofilm formation, particularly, in anaerobic conditions. Similarly, in current study it has been observed that CP2 produces biofilms on lumen of biomedical indwelling where oxygen concentration could be comparatively low. Moreover, cis 9-octadecenoic acid may have a major role in the formation of cellular aggregates, which could result from an ionic interaction of the positively charged polysaccharides intracellular adhisins with the negatively charged *cis* 9-octadecenoic acid (Gotz 2002) and isolates them from direct contact with the growth inhibitor. Moreover, it has also been documented that un-saturated fatty acids and straight chain saturated fatty acids i.e. decanoic acid, tridecanoic acid and pentadecanoic acid act as extracellular signals for bacterial and fungal cell-cell communication (Liaw *et al*., 2004) and in biofilms bacteria grow as organized communities and they interact through a very complex communication system (Stoodley *et al*., 2002). Possibly, these fatty acids work as extracellular signals for bacterial

communication during biofilm mode of growth. Although the role of these fatty acids has not been defined in biofilms earlier, we are for the first time proposing that CP2 released such type of fatty acids and a direct relationship was found between the vancomycin resistance, hydrophobic surfaces and biofilm formation. Possibly, vancomycin up-regulates the phospholipids synthesizing enzymes which may not be utilized in proper architecture of cell wall and exported out of cell and facilitates cellular adhesion or biofilm formation to survive under vancomycin stress environment. However, these aspects of the medical device related-infection may need to be evaluated independently to ascertain the contribution of fatty acids in the virulence of VRSA/VISA causing device-related infections.

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