**INTRODUCTION**

*Staphylococcus (S.) aureus* is a major pathogen that colonizes biotic and abiotic surfaces and is emerging as one of the primary sources of nosocomial infections. Most of these hospital acquired infections are biofilm related. Attachment of planktonic cells to biotic or abiotic surfaces is the initial step in biofilm formation. Staphylococcal biofilm formation depends on the production of polysaccharide intracellular adhesions, which mediate the contact of bacterial cell to each other, resulting in the accumulation of multi-layered biofilm. The enzymes involved in polysaccharide intracellular adhesions were found to be encoded by the ica operon comprising the icaA, icaD, icaB and icaC genes. Several bacteria adopt biofilm mode of growth in response to antibiotics treatment. Fonseca *et al.* explained that sub-inhibitory concentrations of antibiotics although not able to kill bacteria can modify their physicochemical characters and the architecture of their outermost surface and may interfere with some systemic functions. During treatment with antibiotics there are periods of time when antimicrobials are below the MIC (minimum inhibitory concentration) and this may occur in an intermittent way at the site of infection. Moreover, the MIC for bacteria residing in biofilm matrix is considerably higher than planktonic bacteria, thus it is probable that antimicrobials are below the required MIC for bacteria adopted biofilm mode of growth. Majtan *et al.* described that sub-inhibitory concentration of Cefotaxime was found to induce biofilm formation as well as polysaccharide intracellular adhesions.

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production in Salmonella. Similarly, environmental factors may also affect the bacterial function i.e. use of disinfectants in hospital setup may induce biofilm mode of growth in nosocomial pathogens.9

The objective of this study was to determine the effect of physical and chemical stress factors e.g. antibiotics, NaCl, glucose, heat shock, cold shock and sonic waves on biofilm formation by icaA positive and negative strains of methicillin resistant Staphylococcus (S.) aureus.

**METHODOLOGY**

This experimental study was conducted during January 2010 through December 2010 at the Microbiological Analytical Centre, PCSIR Laboratories Complex, Karachi. Two test strains were used along with S. aureus ATCC#29213 as a control strain. One strain labelled as FA was isolated from food sample and the other strain labelled as CL was isolated from a clinical sample. For the identification of S. aureus, the growth was monitored on differential and selective media i.e. Mannitol Salt Agar (BioM), Baird Parker Agar (Oxoid) and DNase Agar (Merck). Staph latex kit (Promix Latex Agglutination System) was used for confirmation. Antibiotics Oxacillin, Ampicillin, Vancomycin, Ciprofloxacin, Erythromycin, Tetracycline, Linezolid, and Rifampicin were used for susceptibility testing by agar dilution method and the results were interpreted in accordance with the Clinical and Laboratory Standards Institute Guidelines.10

Biofilm formation was initially confirmed by Congo Red Agar method as described earlier.11 Briefly, BHI agar plates containing 50 g/L sucrose and 0.8 g/L Congo Red were prepared and streaked with subject strains and incubated aerobically for 24-48 hours at 37°C. Positive results were indicated by black colonies with dry crystalline appearance. Weak slime producers usually remained pink, though occasional darkening at the centre of colonies was observed. The experiment was performed in triplicate and repeated three times. Transmission electron microscopy was done to analyze the production of extracellular matrix material after exposure to Vancomycin. Biofilm positive tubes were divided into 4 mm sections and negatively stained with 2% uranyl acetate for 30 seconds and examined in a GOEL-JEM-1200 EX II transmission electron microscope.

A qualitative assessment of biofilm formation was determined as previously described by Christensen et al.12 Briefly, BHI broth (10 ml) supplemented with required concentration of antibiotic mentioned in Table II was inoculated with loop full of subject strains from overnight culture plates and incubated for 24 hours at 37°C. S. aureus (ATCC#29213) was also inoculated in a separate tube as a control. The tubes were decanted and washed with PBS (pH 7.0), dried out and stained with crystal violet (0.3%). Excess stain was removed and tubes were washed with de-ionized water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Initially, these two strains FA and CL along with S. aureus (ATCC#29213) as a control strain were grown without any treatment at 37°C for 24 hours in BHI broth and then exposed to sonication for 2 minutes, in an ice bath at a cycle of (15s burst, 15s rest) using a Virtis Virsonic 300 with a micro-tip at a depth setting 5. Similarly, overnight culture was exposed to heat shock at 50°C and cold shock at -20°C for 2 minutes in separate tubes, and then re-incubated at 37°C for 24 hours. Biofilm formation was observed and quantification was done. Biofilm formation was quantified by the addition of 2 x 200 µl of 95% ethanol as described by O'Toole et al.13 and absorbance was recorded with Spectrophotometer (Nicolet-Evolution 300) at a wave length of 569 nm. Two samples of each biofilm material were collected and each sample was analyzed three times. All the experiments were repeated at least three times. Results are presented as the mean and the standard deviation of the mean (STD) by Statistical Package for Social Sciences (SPSS) 17.0.

For molecular studies, genomic DNA was isolated by using the DNase kit (Qiagen), following the manufacturer’s instruction. PCR amplification of icaA and meca genes was performed with an MWG Thermal Cycler in a volume of 50 µl of Promega MASTER Mix. Primers described by Wang et al.14 and those described by Martin-Lopez et al.15 were used for amplification of meca and icaA genes, respectively.

icaA-S: 5’-AAACTTGGTGCGGTACAGG-3’
icaA-E: 5’-TCTGGCTTGACCATTGTTG-3’
Meca1 5’-GTA GAA ATG ACT GAA CGT CCG ATA A 3’,
Meca2 5’-CCA ATT CCA CAT TGT TTC GGT CTA A 3’.

**RESULTS**

The two subject strains labelled as FA and CL were identified as S. aureus on the basis of colonial characters i.e. black colonies with opaque zone on Baird Parker Agar (Oxoid) with egg yolk tellurite, Mannitol fermentation on Mannitol Salt Agar (Oxoid) and confirmed by positive reaction on Prolex Latex Agglutination System (Promega).

Antibiotic sensitivity pattern of FA and CL is depicted in Table I. Both of these strains exhibited multi-drug resistance and carried meca gene responsible for oxacillin resistance in MRSA. The strain FA showed typical characters of biofilm forming colonies with rough, dry and crystalline appearance on Congo-Red agar (Oxoid) and harbored icaA gene located on chromosome. Contrary to this CL did not show any change on Congo-Red agar surface or the presence of
icaA gene. Although FA strain showed biofilm formation without any exposure to stress environment, however, quantitative analysis showed that exposure to cell wall active antibiotics, 7% NaCl, heat shock and sonication increased the thickness of biofilm matrix materials (Table II, Figure 1).

Exposure to sonication and heat shock at 50°C also encouraged biofilm formation in CL and FA, the test strains. Contrary, 5% glucose and sub-inhibitory concentrations of Linezolid, Tetracycline, Erythromycin, and Rifampicin have no effect on biofilm formation process. The control strain i.e. S. aureus (ATCC#29213) also showed similar results after exposure to heat shock, cold shock and sonication. The addition of 16 µg/ml ciprofloxacin induced biofilm mode of growth in CL however, it did not show any effect on biofilm formation in strain FA. Table II illustrates the biofilm formation by CL, FA and control strain of S. aureus ATCC#29213.

**DISCUSSION**

It has been reported that most of S. aureus and S. epidermidis strains carry ica cluster, responsible for biofilm formation.15-17 Loss of the ica locus results in an inability to produce polysaccharide intercellular adhesin and to develop biofilms.16 The expression of the ica operon and biofilm formation seems to be highly variable among Staphylococci.17 Thus, biofilm expression is influenced by environmental signals and can be induced in response to external stress and sub-inhibitory concentrations of certain antibiotics.3,17,18 The present study is focused on the effect of stress application on biofilm formation by icaA positive and negative strains of MRSA. One strain of MRSA labelled as FA was isolated from food sample (rice) and the other strain labeled as CL was a clinical strain, isolated from the pus of a diabetic foot patient. Both of these strains were multi-drug resistant MRSA (Table I). The FA was found to produce biofilm while CL did not show any signs of biofilm formation or adhesive material on Congo-Red Agar plate or on silicon tubing. Moreover, FA was found to carry icaA gene which was not detected in CL.

**Table I: Minimum inhibitory concentrations (MICs) of different antibiotics against the test strains.**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>FA (µg/ml)</th>
<th>CL (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Linzolid</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>16</td>
<td>08</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16</td>
<td>32</td>
</tr>
</tbody>
</table>

FA-icaA Positive MRSA Strain isolated from food sample.
CL-icaA Negative MRSA Strain isolated from clinical sample.

**Table II: Biofilm quantification in S. aureus strains after exposure to stress conditions.**

<table>
<thead>
<tr>
<th>Stress agents</th>
<th>FA (µg/ml)</th>
<th>CL (µg/ml)</th>
<th>Control (µg/ml)</th>
<th>OD 569 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>2 min</td>
<td>2 min</td>
<td>2 min</td>
<td>0.93</td>
</tr>
<tr>
<td>Heat shock (50°C)</td>
<td>2 min</td>
<td>2 min</td>
<td>2 min</td>
<td>0.82</td>
</tr>
<tr>
<td>Cold shock (-20°C)</td>
<td>2 min</td>
<td>2 min</td>
<td>2 min</td>
<td>0.06</td>
</tr>
<tr>
<td>NaCl</td>
<td>7%</td>
<td>7%</td>
<td>7%</td>
<td>0.43</td>
</tr>
<tr>
<td>Glucose</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>0.05</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>32 µg/ml</td>
<td>16 µg/ml</td>
<td>ND*</td>
<td>0.56</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2 µg/ml</td>
<td>2 µg/ml</td>
<td>ND</td>
<td>0.54</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>132 µg/ml</td>
<td>132 µg/ml</td>
<td>ND</td>
<td>0.84</td>
</tr>
<tr>
<td>Linzolid</td>
<td>2 µg/ml</td>
<td>2 µg/ml</td>
<td>ND</td>
<td>0.26</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16 µg/ml</td>
<td>16 µg/ml</td>
<td>ND</td>
<td>0.22</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>32 µg/ml</td>
<td>16 µg/ml</td>
<td>ND</td>
<td>0.26</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>8 µg/ml</td>
<td>4 µg/ml</td>
<td>ND</td>
<td>0.29</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>8 µg/ml</td>
<td>16 µg/ml</td>
<td>ND</td>
<td>0.21</td>
</tr>
</tbody>
</table>

FA-icaA Positive MRSA Strain isolated from food sample; CL-icaA Negative MRSA Strain isolated from clinical sample; Control- S. aureus ATCC#29213; ND= Not determined; STD= Standard deviation.

This table shows the comparative analysis of biofilm by strains of S. aureus e.g. FA, CL and ATCC#29213 (control) on silicon tubing under different conditions. Crystal violet stained biofilm matrix deposited on surfaces of tubing were quantified by solubilizing the dye (crystal violet) in 1 ml ethanol and determining the absorbance at 569 nm using Spectro Photometer. The strong biofilm formation was observed by isolate FA after exposure to sonication (OD 0.93). The conditions which showed OD below 0.4 at A 569 nm were considered as biofilm negative.
It was noticed that icaA gene is not always required for biofilm formation, as strain CL did not carry icaA gene but showed biofilm formation while growing in the presence of Vancomycin, Oxacillin, Ampicillin, Ciprofloxacin, and after treatment with sonication and heat shock. Similar treatments increased the quantity of biofilm matrix material by FA strain which was found to carry icaA gene. This is in agreement with Cho et al.17, who have described that polysaccharide intercellular adhesion production of biofilm-negative isolates could be stimulated in response to environmental stress and sub-inhibitory concentrations of antibiotics. Probably, induction of biofilm formation occurs in hospitals environment where disinfectants and antibiotics are being used frequently. It has also been noticed that anti-cell wall active antibiotics i.e. Vancomycin, Oxacillin and Ampicillin seems to be more effective agents for the induction of biofilm mode of growth in MRSA, whereas antibiotics active against protein synthesis, like Tetracycline and Erythromycin, have not shown any effect on biofilm formation process in either icaA positive (FA) or icaA negative (CL) strains. Contrary to this, Rachid et al.3 reported that Penicillin and Vancomycin have no effect on icaA gene expression whereas sub-inhibitory concentration of Tetracycline enhances icaA expression 9 to 11 fold.

Majtan et al.8 reported that Cefotaxime, a third-generation Cephalosporin antibiotic, induced biofilm formation in Salmonella enterica. However, inhibition of biofilm was observed in strains with the highest biofilm forming capacity when grown in the presence of sub-inhibitory concentration of Ciprofloxacin (a second-generation Fluoroquinolone).8 According to Wang et al.14 Fluoroquinolones do not influence biofilm formation process in Staphylococci. In the present study, Ciprofloxacin induced biofilm formation in CL, an icaA negative MRSA.

Jin et al.20 also described that glucose can induce the biofilm formation by inducing icaA gene. Contrary to this, decrease in biofilm density after exposure to 5% glucose by FA, icaA positive strain was observed. However, 7% NaCl was found to induce biofilm mode of growth in icaA negative strains of MRSA and enhance biofilm matrix material production in icaA positive strain, which is in agreement with Knobloch et al.21 and Rachid et al.3 who also reported that high salt levels stimulate biofilm formation in S. aureus and S. epidermidis. It is also reported that glucose and NaCl can induce biofilm formation by inducing the icaADBC operon in S. epidermidis.22 In the present study, glucose was found to decrease biofilm formation process in icaA positive strain while on icaA negative strain it has no effect on this process, suggesting that MRSA strain might respond in a different way than S. epidermidis. Moreover, exposure to sonic waves for 2 minutes and heat shock also augment biofilm formation in icaA positive and induce biofilm mode of growth in icaA negative strain. It has been reported that exposure to sonication left most of the cells in a viable state with partial damage to cell wall and bacterial cell surfaces play a crucial role in their adhesion to surfaces.23 Similarly, heat shock also causes holes in the membrane which results in the loss of sensitive enzymes with shrinkage and precipitation and leakage of intracellular materials of heated bacterial cells.24,25 Exposure to heat and sonic waves possibly damages the Staphylococcal envelope resulting in release of some cellular materials that mediate bacterial adhesion to surfaces followed by biofilm formation. It has also been reported that high temperature induces over-expression of stress sigma factor RpoS (δ).

These are general stress sigma factors induced when bacteria enter into stationary phase of growth and in response to multiple stress conditions. They are also involved in the control of motility, biofilm formation and virulence.26

It has also been noticed that anti-cell wall agents may play a crucial role in biofilm formation and bacteria with partially damaged cell wall could be more prone to adapt biofilm mode of growth. Accordingly, Page et al. described that the state of cell wall deficiency encourages adherence to glass and plastic thus biofilm formation in S. aureus, providing a protection to cells against chemical and physical agents.27,28 These findings suggest that the presence of the ica locus alone is not sufficient or necessary factor for biofilm formation, some other factors may also exist in bacterial system which may regulate biofilm formation process under stress environment.

CONCLUSION

There is a role of anti-cell wall factors i.e. sonication, heat shock, NaCl and antibiotics in the induction of biofilm mode of growth in MRSA and Methicillin sensitive S. aureus. The factors which partially damage bacterial cell wall, equally, induce biofilm formation in icaA positive or negative S. aureus.

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