INTRODUCTION

The urinary tract is the most common site of nosocomial infection (30% - 40%) and is always associated with indwelling catheters. The risk of developing bacteriuria from a catheter increases by 3% - 6% per day and become 100% within 30 days [1]. Because of the high incidence rate, catheter associated urinary tract infection (CAUTI) is responsible for an enormous burden of morbidity, mortality and increased health care costs [2]. Hence several approaches have been adopted to limit the colonization of devices, such as, strict hygienic practices for health care personnel and environment. However, it should be noted that routine disinfection in hospital environment is an element of controversy [3].

Though most experts recommend treating CAUTI with 5-10 days of targeted antibiotic therapy [4, 5], the treatment usually promote the antimicrobial resistance in patients’ flora and transient bacteriuria [6, 7]. Likewise, many strategies were implemented, but these strategies achieved only short term suppression of bacteriuria [2, 8, 9, 10, 11] and merely delayed the onset of bacteriuria in chronically catheterized situations [12, 13]. Catheters with improved biomaterials and antimicrobial coatings have captured researcher’s attention [14, 15]. Impregnating urinary catheters with antimicrobial agents like silver ions and nitrofurazone found to be effective only in short term catheterization settings [2, 14] and leads to the emergence of antimicrobial resistance and adverse drug effects in patients [6, 16, 17]. Thus currently, no proven effective strategies exist for the prevention of CAUTI in subjects with long term catheterization [13].

Considering the fact that virulence factors (VFs) are central to the pathogenesis of infections [13, 15, 18], novel methods like hindering virulence factor production might assist in prevention of CAUTI [19].

Unlike antimicrobials, this strategy would not impart selective pressure to microbes, thus preventing development of resistance. Attenuation of pathogenic microbes would not alter the natural microbiome of host and work optimally to prevent infection than curing an established infection [19]. Inhibition of quorum sensing (QS), a mechanism which utilizes small molecules to regulate the production of VFs in microbes [20], recognized as a possible target for the prospected scheme. Modeling catheters which are either coated or irrigated with anti-QS compounds might attenuate the VFs in pathogens and can be used as an effective strategy to prevent device associated infections [19].Researchers have been focusing on developing chemical antagonists which can interfere with bacterial virulence by hampering the QS circuit [19, 21]. Pseudomonas aeruginosa PAO1 is one of the major candidates to study the effect of quorum sensing inhibition (QSI) as an anti-infective strategy [22] due to the expanded understanding about its pathogenic mechanisms [23, 24]. P. aeruginosa is the third most important organism associated with CAUTI in immuno compromised patients. [25]. Expression of diverse pathogenetic traits in P. aeruginosa is controlled by at least 2 QS systems, lasI/lasR [26] and rhlI/rhlR [27] which is arranged hierarchically. Hence QS interference has been an important anti-infective strategy against P. aeruginosa infections [19, 27].

The present study evaluated the anti-QS effect of sub-inhibitory concentration (SIC) of antibiotics upon the expression of VFs of isolates from CAUTI patients. The selected antibiotics were used against urinary tract infection (UTI) and their SIC levels were estimated against the test organisms. The effects of these antibiotics upon the VFs of the foresaid organisms at the SIC were also analyzed to compare the anti-QS effects. Our results offer new and diverse implications of antibiotics for the treatment of device associated infections.

MATERIALS AND METHODS

Chemicals

The antibiotics and other microbial media used in the study were purchased from Himedia laboratories PVT Ltd. (Bangalore, India). All the other chemicals, solvents and reagents used for the quantification of VFs were procured from Sisco Research Laboratories PVT Ltd. (Mumbai, India).

Bacterial strains used in this study

Two clinical isolate of Pseudomonas sp. (PA-1 and PA-2) were collected from CAUTI patients and used along with a reference strain.
**Effect of antibiotics upon pyocyanin production**

In order to analyze the pyocyanin production, the cells were cultivated in the presence and the absence of the test compounds (Table 2, 3 and 4). A significant decrease in pyocyanin production by PAO1 was observed to the level of 80% by CXM (20 µg/mL) which was on par with AZM (2 µg/mL). NAL (1µg/mL) reduced 74% pyocyanin production in PAO1. CXM (36 µg/mL) produced 65% reduction in pyocyanin production by PA-1, while AZM (3 µg/mL) reduced it to a factor of 76.6%. In PA-2, CXM (32 µg/mL) inhibited the pyocyanin production by a factor of 76.36% while with the positive control (2 µg/mL) the reduction was 81%.

The other antibiotics in the investigation reduced the pyocyanin production but the reduction rate was not comparable with that of the positive control.

**Effect of antibiotics upon pyoverdine production**

The effect of different antibiotics on pyoverdine production was studied through quantifying the pyoverdine biomass through microtitre plate (MTP) assay [32]. The pyoverdine biomass was quantified by measuring the absorbance at 600 nm using a UV-visible spectrophotometer (ELx-880, Biotek).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>P. aeruginosa PAO1 (µg/mL)</th>
<th>Clinical Isolates (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA-1</td>
<td>PA-2</td>
</tr>
<tr>
<td>CXM</td>
<td>102</td>
<td>112</td>
</tr>
<tr>
<td>AMX</td>
<td>46</td>
<td>82</td>
</tr>
<tr>
<td>COT</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>NAL</td>
<td>34</td>
<td>52</td>
</tr>
<tr>
<td>AMP</td>
<td>30</td>
<td>58</td>
</tr>
<tr>
<td>AZM</td>
<td>82</td>
<td>108</td>
</tr>
</tbody>
</table>

Table 1: MIC of antibiotics against the test organisms

After incubation OD_600 was determined at SIC level of the antibiotics. The mixture was incubated for an hour in dark and the absorbance was measured at 490nm using a UV-visible spectrophotometer (ELx-880, Biotek) [29].

**Statistical analysis**

All experiments were performed independently (n=6) and the data was represented as mean values ± Standard Deviation (SD). The % inhibition of virulence factors by the antibiotics was measured using the formula: % inhibition = 100 - (a-b)/b where, ‘a’ is the respective virulence factor expression with antibiotics and 'b' is that of growth control. The difference between the control and the test were analyzed using one way ANOVA with a p value of 0.05 being significant using GraphPad Prism 5.

**RESULTS**

**Determination of MIC of the antibiotics**

Five antibiotics, which are conventionally used against UTI, were selected for the study. Azithromycin (AZM) was used as the positive control. MIC of all the antibiotics was assessed for all the test organisms (PAO1, PA-1 and PA-2) and represented in Table 1. Concentrations below the MIC level considered as SIC and all the further assays were carried out in the SIC of these selected antibiotics.

The effect of antibiotic SIC levels upon biofilm formation was studied through quantifying the biofilm biomass through microtitre plate (MTP) assay [32]. The biofilm biomass was quantified by measuring the absorbance at OD_590 using a UV-visible spectrophotometer (ELx-880, Biotek).

**Effect upon Extracellular polymeric substances (EPS) formation**

Test organisms were allowed to form biofilms over glass slides immersed in appropriate media (2X2 cm) with or without the antibiotics by incubating at 37°C and allowed to grow till late stationary phase. After the incubation the EPS was quantified by total carbohydrate assay [29]. Brieﬂy, 1% of the overnight culture of the test organisms were inoculated and incubated at 37°C to reach late stationary phase. After incubation OD_590 was analyzed using a UV-visible spectrophotometer (ELx-880, Biotek) and the viable cells were counted through spread plate method on a nutrient agar plate. All measurements were performed with 1/10 dilutions to verify the results [31].

Effect upon E. coli growth

The other antibiotics in the investigation reduced the pyocyanin production but the reduction rate was not comparable with that of the positive control.
81.60% in PA-1 while in PA-2; it was 82.45% at 32 µg/mL. Among the other antibiotics, NAL (14 µg/mL) inhibited 79.0% of the pyoverdine production by PA-1 where as in PA-2 COT (12 µg/mL) reduced 76.0%. The culture supernatants retained a pH of 7.0, regardless of the amount of the solvent or diluents added along with the test compound (data not shown).

**Effect of antibiotics upon biofilm formation**

Amoxicillin (5 µg/mL) significantly inhibited 68.38% of PAO1’s biofilm production which was in par with that of AZM’s (2 µg/mL) 74.70%. Among the other antibiotics, COT (3 µg/mL), NAL (1 µg/mL) and CXM (20 µg/mL) inhibited the biofilm formation by the factor of 40.17%, 40.16% and 38.46% respectively. CXM (36 µg/mL), AMX (14 µg/mL) and NAL (14 µg/mL) dislodged the biofilm formation by 68.50%, 63.74% and 65.57% respectively in PA-1 which was in par with that of 77.25% of AZM (3 µg/mL). AMX at 12 µg/mL and CXM at 32 µg/mL effectively dislodged the biofilm biomass by 51.72% and 48.76% respectively. AZM (2 µg/mL) reduced the biofilm formation by 73.3% in PA-2. The biofilm formation by the test organisms under the influence of the test compounds are represented in Table 2, 3 and 4.

**Effect of antibiotics upon EPS formation**

Gefuroxime at 20 µg/mL, 36 µg/mL and 32 µg/mL inhibited EPS production by 71.67%, 69.92% and 65.13% correspondingly in PAO1, PA-1 and PA-2. But AZM (2 µg/mL) inhibited the EPS production in PAO1 by 83.37%. Among the clinical isolates; PA-1 and PA-2, AZM at 3 µg/mL and 2 µg/mL inhibited the EPS production by 79.29% and 80.98% respectively. The effect of various antibiotics at SIC levels were represented in Table 2, 3 and 4.

**Cell density analysis**

During every assay the cell density was analyzed and compared with the growth control and expressed as percentage growth/mL of culture. The obtained data are given in Table 2, 3 and 4.

Table 2: Effects of antibiotics upon virulence factors produced by P. aeruginosa PAO1

<table>
<thead>
<tr>
<th>Antibiotics (µg/mL)</th>
<th>Pyocyanin * formation</th>
<th>Pyoverdine * formation</th>
<th>Biofilm * formation</th>
<th>EPS * production</th>
<th>Cell density * (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth ctrl</td>
<td>1.17 ± 0.01</td>
<td>2.73 ± 0.08</td>
<td>1.21 ± 0.005</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>CXM (20)</td>
<td>1.17 ± 0.01</td>
<td>2.73 ± 0.08</td>
<td>1.21 ± 0.005</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>NAL (1)</td>
<td>1.17 ± 0.01</td>
<td>2.73 ± 0.08</td>
<td>1.21 ± 0.005</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>AMP (4)</td>
<td>0.90 ± 0.01</td>
<td>1.90 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td>98.75 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>AZM (2)</td>
<td>1.17 ± 0.01</td>
<td>2.73 ± 0.08</td>
<td>1.21 ± 0.005</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

* Pyocyanin production was expressed as OD 420 after the incubation with 0.2N HCl. Pyoverdine production was expressed as relative florescent units at 465nm (excitation λ=405nm) per mL of the culture. Biofilm production was expressed as OD 540 after incubating with crystal violet. EPS production was expressed as OD 540 after 5% Phenol-H2SO4 (1:5 v/v) treatment. Cell density was expressed as % growth per mL of the culture in comparison with the growth control. ns- non significant; * significant at p<0.05; **significant at p<0.01 and ***significant at p<0.001.

Table 3: Effects of antibiotics upon virulence factors produced by clinical isolate PA-1

<table>
<thead>
<tr>
<th>Antibiotics (µg/mL)</th>
<th>Pyocyanin * production</th>
<th>Pyoverdine * production</th>
<th>Biofilm * formation</th>
<th>EPS * production</th>
<th>Cell density * (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth ctrl</td>
<td>476.70 ± 16.06</td>
<td>2.73 ± 0.08</td>
<td>1.21 ± 0.005</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>CXM (36)</td>
<td>87.67 ± 4.36</td>
<td>0.86 ± 0.07</td>
<td>0.36 ± 0.028</td>
<td>99.32 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>AMX (14)</td>
<td>273.87 ± 8.78</td>
<td>0.99 ± 0.045</td>
<td>1.10 ± 0.045</td>
<td>99.45 ± 2.01</td>
<td></td>
</tr>
<tr>
<td>COT (10)</td>
<td>117.23 ± 9.01</td>
<td>1.52 ± 0.040</td>
<td>0.55 ± 0.036</td>
<td>99.83 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>NAL (14)</td>
<td>95.53 ± 1.69</td>
<td>0.94 ± 0.031</td>
<td>0.57 ± 0.018</td>
<td>97.96 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>AMP (10)</td>
<td>420.23 ± 1.23</td>
<td>1.93 ± 0.046</td>
<td>0.91 ± 0.009</td>
<td>99.23 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>AZM (3)</td>
<td>34.80 ± 5.96</td>
<td>0.621 ± 0.023</td>
<td>0.25 ± 0.032</td>
<td>98.79 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

* Pyocyanin production was expressed as OD 420 after the incubation with the 0.2N HCl. Pyoverdine production was expressed as relative florescent units at 465nm (excitation λ=405nm) per mL of the culture. Biofilm production was expressed as OD 540 after incubating with crystal violet. EPS production was expressed as OD 540 after the 5% Phenol-H2SO4 (1:5 v/v) treatment. Cell density was expressed as % growth per mL of the culture in comparison with the growth control. ns- non significant; * significant at p<0.05; **significant at p<0.01 and ***significant at p<0.001.

Table 4: Effects of antibiotics upon virulence factors produced by clinical isolate PA-2

<table>
<thead>
<tr>
<th>Antibiotics (µg/mL)</th>
<th>Pyocyanin * production</th>
<th>Pyoverdine * production</th>
<th>Biofilm * formation</th>
<th>EPS * production</th>
<th>Cell density * (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth ctrl</td>
<td>427.37 ± 8.63</td>
<td>2.03 ± 0.042</td>
<td>1.26 ± 0.027</td>
<td>98.97 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>CXM (32)</td>
<td>75.3 ± 3.44</td>
<td>0.10 ± 0.044</td>
<td>0.44 ± 0.037</td>
<td>99.34 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>AMX (12)</td>
<td>125.67 ± 1.75</td>
<td>0.98 ± 0.072</td>
<td>0.76 ± 0.033</td>
<td>99.13 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>COT (12)</td>
<td>94.53 ± 1.75</td>
<td>0.64 ± 0.037</td>
<td>0.87 ± 0.019</td>
<td>99.67 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>NAL (10)</td>
<td>109.17 ± 3.12</td>
<td>0.99 ± 0.042</td>
<td>0.53 ± 0.029</td>
<td>98.56 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>AMP (8)</td>
<td>328.80 ± 2.96</td>
<td>1.53 ± 0.022</td>
<td>0.79 ± 0.029</td>
<td>98.76 ± 1.02</td>
<td></td>
</tr>
<tr>
<td>AZM (2)</td>
<td>24.40 ± 2.10</td>
<td>0.54 ± 0.022</td>
<td>0.24 ± 0.032</td>
<td>99.12 ± 0.79</td>
<td></td>
</tr>
</tbody>
</table>

* Pyocyanin production was expressed as OD 420 after the incubation with 0.2N HCl. Pyoverdine production was expressed as relative florescent units at 465nm (excitation λ=405nm) per mL of the culture. Biofilm production was expressed as OD 540 after incubating with crystal violet. EPS production was expressed as OD 540 after the 5% Phenol-H2SO4 (1:5 v/v) treatment. Cell density was expressed as % growth per mL of the culture in comparison with the growth control. ns- non significant; * significant at p<0.05; **significant at p<0.01 and ***significant at p<0.001.

**DISCUSSION**

Catheter associated urinary tract infection is one of the most common nosocomial infections [2, 4, 5, 17], and its pathogenesis is related to the susceptibility of inert catheter material to microbial colonization and biofilm formation. Survival advantages conferred by biofilm community include resistance against being swept away by shear forces, phagocytosis and anti-microbial agents. Due to these aspects it is difficult to completely eradicate the biofilm associated nosocomial infections, which causes unwanted clinical and economic outcomes [33, 34]. Currently no proven strategies are available for the prevention of CAUTI in chronically catheterized conditions [14].
Attenuation of the pathogens' virulence has been gaining interest as a new anti-infective approach. Depletion in pathogenicity will make the microbes incompatible to adapt and proliferate in host milieu hence readily cleared by the immune system [19]. For instance SIC levels of AZM found to be effective against cystic fibrosis (CF) by reducing the virulence factor expression through interfering with QS mechanism in P. aeruginosa and used as an effective method to prevent the complications [35]. Since P. aeruginosa is one of the most causative agents for CAUTI, and majority of the VFs was found to be QS controlled, attenuation of pathogenicity by the means of QSI has been found to be a potent plan of action against infections [25].

In the present study we have investigated the inhibitory effect of SIC levels of selected antibiotics upon the QS controlled virulence factor expression in test organisms that cause CAUTI. So that these antibiotics can be used as an improved biomaterial coating and for as lock solution to prevent device associated infections.

The SIC of the selected antibiotics were assessed from the obtained MIC against the test organisms (Table 1). The test organisms used were: laboratory reference strain P. aeruginosa PAO1 and 2 clinical isolates of Pseudomonas sp. from CAUTI patients (PA-1 and PA-2). Choice of SIC as assay concentrations was also supported by the finding that anti-QS compounds permit unabated cell proliferation hence minimize the risk of emergence of resistance [36]. Among the clinical isolates, PA-2 exhibited similar MIC pattern with PAO1, while PA-1 displayed an elevated level in MIC than PAO1. Similar to the MIC profile, PA-2 had comparable variable virulence factor production with that of PAO1, while PA-2 evinced a raised virulence factor production (Table 2, 3 and 4).

Comparing the VFs produced by P. aeruginosa strains from UTI and CF patients, concluded that significant variation was observed in the expression pattern depending on the site of infection [37]. Features like enzymes, siderophores, toxins, EPS production and biofilm mode of growth help to evade antibiotic shock and host defense mechanisms. All these pathogenic aspects are directly regulated by the QS circuitry, which bring about the reduction in biofilm biomass by all the test organisms after the treatment [38]. In our study we have also observed the inhibitory effect of SIC levels of selected antibiotics upon the QS controlled virulence factor expression in test organisms that cause CAUTI. So that these antibiotics can be used as an improved biomaterial coating and for as lock solution to prevent device associated infections.

It has been well documented that interfering with the QS mechanism does not inhibit growth. All the assays performed in the study were at sub-MIC level, which had no effects on the growth of test organisms. The cell density analysis (Table 2, 3 and 4) at the tested concentrations allowed unabated cell growth and it can be concluded that in all the assays the reduction in the expression of VFs was not influenced by the reduction in the cell population [39].

Skindersoe et al., (2008) analyzed the anti-QS property of 12 antibiotics against P. aeruginosa PAO1 and found 3 antibiotics, Azithromycin (AZM), cefazidime (CFT) and ciprofloxacin (CIP) with potent activity at the QS regulated levels. In our study we have investigated the inhibitory effect of SIC levels of selected antibiotics upon the QS controlled virulence factor expression in test organisms that cause CAUTI. So that these antibiotics can be used as an improved biomaterial coating and for as lock solution to prevent device associated infections.

Reduction in pyocyanin production has been largely used as an analysis in anti-QS research. Since the pyocyanin production is directly regulated by the QS circuitry (rhl), the QS reduction pattern can be directly correlated with the reduction in the transcription levels [29, 30, 32]. In the present study, all the tested antibiotics at distinct concentrations reduced the pyocyanin production by the test organisms (Table 2, 3 and 4). Among the antibiotics, CXM reduced the test organisms’ QS regulated pyocyanin production in a similar pattern with that of positive control. Other antibiotics also inhibited pyocyanin production; however the reduction rates were not proportionate with the positive control. Since the antibiotics used in the study were structurally different and enormous, the possibility of a high affinity competitive receptor site binding was of lesser feasibility. This hypothesis was supported by the in silico docking study results of antibiotics with the QS receptor sites, by Skindersoe et al., (2008) [40]. Elevated spatial penalties due to the improper fitting have been resulted in reduced affinity scores in docking studies. So it’s been nearly confirmed that the underlaying mechanism for the anti-QS activity of the tested antibiotics will be other than a direct interaction with the QS receptor protein [40]. We speculate that the adaptive response of microbes towards stress conditions could be the mechanism for the observed anti-QS property of the tested antibiotics. Reduction in membrane permeability has been found to be a prime response adopted by microbes in response to stress conditions, which bring about decreased influx concentration of QS signal molecules, resulting in reduced expression levels of QS regulated genes [41, 42].

It has been reported that antibiotics display pleiotropic effects in both clinical and environmental conditions, which are the practical demonstrations of the hormesis effect [41]. This concept was examined in our study by comparing the anti-QS property of the antibiotics with 2 clinical isolates from CAUTI patients (PA-1 and PA-2). The test compounds exhibited diverse inhibition pattern in all the tested QS regulated traits of clinical isolates, contrast to the laboratory reference strain. In other study, conducted by Wagner et al., (2005) compared the effectiveness of AZM against QS controlled expression of VFs by reference strains and clinical isolates from CF patients. CF clinical isolates exhibited a variable expression pattern in comparison to the reference strains. This variation could be due to the slow growth and limited exo product expression rate of clinical isolates in comparison to the reference strains [41]. But we speculate that, unlike in reference strains, clinical strains were capable to overcome the antibiotic stress by producing diverse exo-enzymes which could cleave antibiotics to small molecules. These small molecules could specifically bind to the QS receptors like conventional QSIs.

A significant variation in the effective dosage of the tested antibiotics was visible among all the test organisms. Even same antibiotics showed significant QS activity against different test organisms at various concentrations. Similarly among the clinical isolates, alike antibiotics exhibited varied effects at diverse concentrations. This variation in the effective concentration can be due to the change in
the transcription levels of the various characters. The contrast in the observations could be due to the slow growth rate and the limited virulence factor production rate of clinical isolates in the in vitro conditions. The lack of these responses might be due to the different intracellular concentrations of the test compounds with corresponding hermetic effects as well.

All the antibiotics tested in the study exhibit diverse mechanisms of antimicrobial action and are structurally very different. Thus, it is interesting that all the antibiotics exhibited reduction in QS regulated characters at varied degrees and at diverse concentrations indicating that the observed QS action has less correlation with their anti-microbial mechanism of action. A detailed reverse transcription PCR and DNA-microarray analysis will provide a clear cut idea about the mechanism as well as the cluster of genes which are affected by the hermetic action of the test compounds [40, 41]. Since the present study compares the effect of anti-QS activity of the antibiotics upon QS regulated behaviors of both PA01 and CAUTI isolates, further detailed study in this aspect warranted to design a QS dependent therapy against nosocomial infections like CAUTI.

CONCLUSION

To the best of our knowledge, this is the first report demonstrating the anti-QS property of CXM against PA01 and 2 other Pseudomonas sp. clinical isolates from CAUTI patients. QS Inhibitory property of the test compound indicates its propitious anti-infective drug against CAUTI. Since the chances of resistance development is unlikely, since at SIC levels the test compounds pose no or little selective pressure. A further comprehensive study using CXM coated or wash solutions against chronically catheterized conditions will envisage the actual clinical impact of the observations that made in the study.

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REFERENCES