Biocides susceptibility pattern and phenotypic detection of Efflux pump in *Staphylococcus aureus* isolates from two tertiary hospitals of Pakistan

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This study was conducted on wound isolates, collected both from in-patients and out-patients of two tertiary hospitals of Peshawar, Pakistan. A total of 70 *Staphylococcus aureus* isolates, including 35 methicillin resistant *Staphylococcus aureus* (MRSA) and 35 methicillin sensitive *Staphylococcus aureus* (MSSA) were tested using different biocides and antibiotics. The biocides used in this study were Cetrimide, Benzalkonium chloride, Chlorhexidine, Triclosan, Triclorcarban, PCMX, Cetylpyridinium chloride, Copper sulphate and Silver nitrate while antibiotics used in this study were Erythromycin, Clindamycin Neomycine, Minocycline, Ciproflaxacin, Ofloxacin, Fusidic acid, Vancomycin, Mupiracin, Rifampicin, Chloramphenenicol, Sulfamethoxazole/trimethoprim, Tetracyline and Penicillin G. Sensitivity of biocides was performed by minimum inhibitory concentrations (MICs) while the antibiotic susceptibility was carried out using Kirby Bauer disc diffusion method. The phenotypic detection of efflux pump was performed by agar dilution method using Ethidium bromide (EtBr). Most of the *S. aureus* isolates were found highly resistant to antibiotics, while they showed high susceptibility to biocides. Among the biocides tested, only 22.8% isolates showed resistance to Cetrimide. Triclosan was found the most effective biocide which showed MIC₉₀ ≤0.05 µg/ml for both MRSA and MSSA. Among the Cetrimide resistant isolates, three isolates of MRSA and one isolate of MSSA showed the presence of phenotypic efflux pump. Most of the isolates collected in Pakistan which exhibited high antibiotic resistance profile were sensitive to biocides. This indicates that biocides are infrequently used in Pakistani hospitals while antibiotics are overused. This study does not support the hypothesis of cross resistance between antibiotics and biocides.

**Key words:** Methicillin resistant *Staphylococcus aureus*, methicillin susceptible *S. aureus*, biocides.

**INTRODUCTION**

Antibiotics resistance is increasing amongst pathogenic bacteria, which is a serious problem worldwide (Waber and Rutala, 2006). Resistance pattern to antibiotics is also changing from time to time in these microorganisms. *Staphylococcus aureus* is the most abundant member of the indigenous flora of human skin (Tavares, 2000). It has been reported that 8% of healthy human adults are colonized with MRSA (Wertheim et al., 2005). Biocide is a term used for chemical agents (disinfectants and antiseptics) used to kill bacteria, virus and
moulds (Mcdonell and Russel, 1999). Triclosan is a bisphenol biocide which is used for the control of various gram negative and gram positive bacteria. It is bactericidal at lower concentrations, while bactericidal at higher concentrations (Suller and Russell, 2000). Its use began in the US in the 1970s in soaps; 76% of 395 soaps commercially available in the US contained it (Perencevich et al., 2001). Since then its use has risen dramatically (Adolfsson et al., 2000). Apart from its use in the soaps, it is also used in mouth washes, in preservation of food products and in many hospital used products (Boyce and Pittet, 2002). In 1998 its use was also recommended for the control of MRSA (Duckworth, 1998). Similarly in Europe, 350 tons of Triclosan is produced every year for use in commercial products, (Singer et al., 2002). TCC, 3,4,4-triclorocarbanilide (Triclorcarban) is the member of the most widely studied anilides group which are used as antiseptics. This is used in commercial soaps and deodorants. It has good activity against gram positive bacteria as compared to gram negative bacteria and fungi (Beaver et al., 1957). It is considered that it acts by adsorbing and destroying the semi-permeable membrane of cytoplasm which kills the cell (Hamilton, 1968).

Quaternary ammonium compounds (QACs) are another type of biocides which are used for the control of microorganisms both in the hospitals and in the community settings. These are the most important and beneficial antiseptics/disinfectants (Frier, 1971). Some of these compounds are known as cationic detergents, used as preoperative disinfectants on unbroken skin and also used for many other purposes. It is also used in cleaning hard surfaces and for neutralization of odours (Mcdonnell and Russel, 1999).

Benzalkonium Chloride is a QAC. This compound is mainly active against gram positive organisms and is used to disinfect surfaces in healthcare environments. It is also used as a preservative for contact lenses and as a veterinary disinfectant (Fraise, 2002). Cetrimide is a halogenated QAC. It has antiseptic as well as detergent properties with a wide spectrum activity against bacteria and fungi (Gathwala et al., 2006). Chloroxylenol (4-chloro-3, 5-dimethylphenol; p-chloro-m-xylene) (PCMX) is a bactericidal halophenol which is used in the preparation of antiseptics and disinfectants. Some bacteria like Pseudomonas spp. and moulds are resistant to it. Although it has been used worldwide for many years, but its mode of action is not well understood. However, it is thought that it affects membrane of the microbes (Mcdonell and Russell, 1999).

Chlorhexidine is a bactericidal which is used worldwide as antiseptic in different concentrations in topical solutions since 1954. It is present in hand washes, body washes, and mouth wash solutions, as preservative and in many hospitals used antiseptic solutions. It acts by binding to negatively charged cell wall of bacteria thereby disturbing the osmotic equilibrium leading to death of the cell (Milstone et al, 2008; Dayner, 1995).

Metals in the form of ions alone or in combinations with other chemicals have been used as disinfectants for the decontamination of fluids, living tissues and solid surfaces for more than a century (Borkow and Gabby, 2005). In the 18th century, in the western world, copper was used extensively in hospitals for the treatment of various mental and lungs disorders. Being bacteriostatic, copper has many potential uses in the field of medicine being used as an antibacterial, antiviral and antifungal (Borkow and Gabby, 2005).

Silver has been used since 335 BC for the control of microbial growth and it is still used as coating in water tanks to prevent growth of bacteria (White, 2002). Due to its biocidal characteristics, silver is used in a variety of products like wound dressings, and clinical devices (Thomas, 2003). Silver ions are nowadays, used for the control of bacterial growth in many medical procedures like dental work, catheterization, etc. It is also used in nonmedical objects like the lining in toilet seats, refrigerators, dishwashers, and laundry machines (Jung et al., 2008).

The activities of biocides are different in different types of organisms. Even in the same species, it may be different. Favero et al. (1991) prepared a guide in which they grouped the biocides on the basis of its appropriate application. But it could not explain the exact interaction of different organisms and biocides (Marilad, 2002).

Researchers are now investigating resistance in bacteria to commonly used biocides. In UK and Australia, MSSA and MRSA isolates were found resistant to disinfectants (Mcdonell and Russel, 1999). However, resistance to biocides in bacteria is not very common as compared to antibiotics. This variation may be associated with different modes of action of biocides to kill microorganisms (Russell, 2002). That is why resistance in bacteria to sanitizers is not well documented (Nuñez and Moreton, 2007). Some reports indicate that bacteria use similar tools in the development of resistance process against biocides like alteration of target sites, and efflux pump which have been documented for antimicrobial agents. However, level of resistance to biocides, whenever studied is low (Fairese, 2002). There are many studies which have described antibiotic and biocides resistance in bacteria which may be innate, or acquired (Murtough, 2001).
incubated at 37°C for 18 to 24 h aerobically and antibiotic susceptibility was performed according to guidelines of British Society for Antimicrobial and Chemotherapy (BSAC version 10.2 (2011)). The different antibiotics used in this study are listed in Table 1. Biocides including benzalkonium chloride, cetrimide, triclosan and silver nitrate (AgNO₃) were obtained from Sigma-Aldrich (UK). Copper sulphate (CuSO₄) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Tryptone soya agar (TSA) Oxoid Basingstoke, UK) and Lady Reading hospital. All the isolates were tested against 21 different antibiotics, including benzalkonium chloride, cetrimide (PCMX) and silver nitrate (AgNO₃) were obtained from Sigma-Aldrich (UK). Copper sulphate (CuSO₄) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Ethidium bromide was purchased from Sigma-Aldrich (UK). ATCC 9144 Oxford S. aureus, was used as a control strain. Biocides used in this study, their diluents and concentrations are listed in Table 2.

**Determination of Minimum inhibitory concentration (MICs) for Biocides**

Iso-sensitest agar was used for the determination of MICs of biocides in the agar incorporation method, using BSAC guidelines. A multipoint inoculator (Denley Industries, Sussex, UK) was used to dispense approximately 1 µL of each bacterial suspension equivalent to 0.5 McFarland solution to the plate. Different concentrations were used for different biocides. The ranges of concentrations are given in Table 2. The concentrations of AgNO₃ and CuSO₄ were used in 0.05 to 1.0 mM and 0.1 to 10.0 mM respectively, while µg/ml concentrations were used for the rest of biocides.

**Ethidium bromide (EtBr) agar screening method**

The isolates expressing MICs on the extreme of upper and lower sides for quaternary ammonium compounds (QACs) were tested using a method described by Martin et al. (2006). All the isolates were streaked out on the TSA plates containing the EtBr with a concentration range from 0.5 to 30.0 µL. All the inoculated plates were incubated for 18 to 24 h at 37°C in air. After incubation, all the plates were viewed under the UV light for the florescence.

**RESULTS**

**Antibiotic susceptibility pattern of S. aureus**

Seventy (70) isolates of *S. aureus* were tested against 21 different antibiotics. Among these isolates, 35 were MRSA and 35 were MSSA. All these *S. aureus* isolates were susceptible to vancomycin and resistant to penicillin. Most of the MRSA isolates were resistant to more than four of antibiotics tested. Among the aminoglycosides, gentamicin showed high level of resistance in MRSA (88.6%) and very low level resistance in MSSA isolates (2.9%). In MRSA, 88.6% isolates and in MSSA 54.3% isolates were resistant to neomycin. High level of resistance was recorded for CIP (94.3%) and OFX (86.0%) in MRSA. In MSSA, OFX showed high level of resistance (82.9%) and CIP showed low level of resistance (5.7%). In MRSA, 74% isolates were resistant to E while only 8.5% isolates were resistant in MSSA to E.
Table 3. Resistance pattern recorded for MRSA and MSSA (n=70).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistance MRSA (%)</th>
<th>MSSA Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIP</td>
<td>94.3</td>
<td>5.7</td>
</tr>
<tr>
<td>OFX</td>
<td>94.3</td>
<td>82.9</td>
</tr>
<tr>
<td>N</td>
<td>88.6</td>
<td>54.3</td>
</tr>
<tr>
<td>CN</td>
<td>88.6</td>
<td>2.9</td>
</tr>
<tr>
<td>E</td>
<td>74.3</td>
<td>8.5</td>
</tr>
<tr>
<td>FD</td>
<td>71.4</td>
<td>77.1</td>
</tr>
<tr>
<td>TET</td>
<td>68.6</td>
<td>51.4</td>
</tr>
<tr>
<td>MH</td>
<td>65.7</td>
<td>40</td>
</tr>
<tr>
<td>SXT</td>
<td>54.3</td>
<td>14.28</td>
</tr>
<tr>
<td>RD</td>
<td>45.7</td>
<td>17.14</td>
</tr>
<tr>
<td>DA</td>
<td>42.9</td>
<td>11.4</td>
</tr>
<tr>
<td>LZD</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>MUP</td>
<td>8.6</td>
<td>11.4</td>
</tr>
<tr>
<td>VA</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Using tetracycline, 68.6% of MRSA and 51.4% of MSSA isolates were resistant to it. Resistance level to MH was 65% in MRSA isolates and 40% in MSSA isolates. In MSSA, 77.1% isolates and in MRSA 71.4% isolates showed resistance to Fusidic acid. Similarly, for MUP, the resistance level observed was 11.4% of MSSA isolates and only 8.6% of MRSA isolates. In 45.5% isolates of MRSA, resistance was recorded for RD while in MSSA, 17.1% isolates showed resistance to it. Very low level of resistance was recorded in isolates both from MRSA and MSSA for Linezolid (2.9%) (Table 3).

Among the biocides, the QACs showed the highest MIC value that is cetrimide 15.0 µg/mL and cetlypyridinium chloride and benzalkonium chloride 3.0 µg/mL. MRSA showed higher MIC90 for cetrimide (15.0 µg/mL), while the MIC90 value for the MSSA was 6.0 µg/mL. Overall, 22.9% S. aureus isolates showed resistance against cetrimide. Triclorcarban showed MIC90 value of 0.5 µg/mL for MRSA which was higher than the MIC50 value of 0.25 µg/mL recorded for MSSA. Triclosan showed MIC ≤0.05 µg/mL and inhibited growth of all the 70 S. aureus isolates while PCMX showed MIC ≥100 µg/mL, to inhibit the growth of all isolates. Triclosan was the most effective biocide while PCMX was the least effective biocide recorded in this study. In metals, silver nitrate had MIC50 value of 0.4 µg/mL while copper sulphate had MIC50 value of 2.0 µg/mL. Silver nitrate was found more effective than the copper sulphate. The isolates which showed resistant to cetrimide were investigated for phenotypic detection of efflux pump by using EtBr agar screening method (Figure 1). Among the biocides, no resistance was found except for cetrimide. Table 4 shows the MIC50 and MIC90 values for MRSA and MSSA for all biocides used in this study.

DISCUSSION

There is very limited data available in international literature regarding the use of biocides in hospitals of Pakistan; therefore, it is impossible to compare this data with findings of other researchers from Pakistan. As expected, most of the MRSA isolates were resistant to more than four antibiotics tested as compared to MSSA. In the present study, three QACs showed higher MICs against the tested S. aureus isolates as compared to other biocides used. These included cetrimide, benzalkonium chloride, and cetlypyridinium chloride.

Low level of resistance was found in isolates tested for cetrimide as compared to other QACs tested. Cetrime showed MIC90 values of 4.0 and 5.0 µM/mL for MRSA and MSSA respectively. McDonnell and Russell (1999) in their studies reported similar results for cetrimide. In the present study, 22.9% MSSA and MRSA had MIC90 of 6.0 and 15.0 µg/mL respectively which are similar to the findings of Al-Masaudi et al. (1991) who have reported resistance in MRSA to cetrimide with MIC90 of 15 µg/mL. This resistance in Pakistani isolates towards QACs could be because of the fact that these compounds have been used for more than hundred years in the world including Pakistan as disinfectants (Russell, 2002).

Cetylypyridinium chloride and benzalkonium chloride (BKC) showed MIC of 2.0 µM/mL for 50% isolates both of MSSA and MRSA. The MIC values observed for BKC in this study lies in the susceptible range as reported by Zmantar et al. (2011). They found 4 and 8 µg/mL for 54% staphylococci isolate which were considered resistant while those with MIC value ≤20 µg/mL was reported in 28% of isolates and were considered as sensitive. Using the criterion of Zmantar et al. (2011) all the isolates tested in the present study are susceptible to BKC. However, another study reported by Sekiguchi et al. (2004) classified S. aureus from a hospital in Tokyo resistant to BKC with MIC value 6.25 µg/mL. Al-Masaudi et al. (1988) reported that both MRSA and MSSA strains tested were susceptible to chlorhexidine. Results of the present study are in agreement with their findings in the case of chlorhexidine susceptibility. However, another study has pointed out that chlorhexidine MICs vary from strain to strain and are independent of the antibiotic resistance pattern (McDonnell et al., 1997).
Triclosan was found highly effective against all the *S. aureus* isolates tested in this study. MIC$_{50}$ and MIC$_{90}$ for triclosan were ≤ 0.05 µg/mL for all *S. aureus* isolates used in the study. Doori et al. (2003) reported in their study MIC$_{50}$ of 0.03 mg/L and MIC$_{90}$ 0.06 mg/L for the clinical isolates of MRSA. Although their MIC$_{90}$ value is slightly greater than our findings, they labelled their isolates as sensitive to triclosan (Doori et al., 2003). The MIC values observed in this study were different from the MIC values for *S. aureus* isolates tested by Schmid and
Kapaln (2004). Another study which is also in disagreement to the findings of this study was reported by Aiello (2004). Both of those studies were conducted in the US where 76% commercial soaps contain triclosan and more than 140 home care and personal care products contain this compound (Perencevich et al., 2001; Sutton et al., 2008). Another reason for high MIC values of triclosan for those *S. aureus* in the US could be that a survey has reported that 74.5% of US population contain triclosan in their urine (Calafat et al., 2008). These studies support our results why isolates from Pakistan have exhibited low MIC values for triclosan. Another study by Cole et al. (2011) found that the *S. aureus* isolates which were sensitive to the tested antibiotics had higher MIC for triclosan which reject the claim of cross resistance of biocides and antibiotics (Cole et al., 2011). In the present study, it was recorded that the antibiotic resistant isolates were sensitive to triclosan which supports the hypothesis of Cole et al. (2011). In Pakistan, very limited and infrequent use of antiseptic hand washes and gels could be a reason that most of the isolates from Pakistan are sensitive to the tested biocides (Baqi et al., 2009).

Bamber and Neal (1999) reported that out of 16 MRSA isolates having low-level resistance to mupirocin, no one expressed high level MICs to triclosan and also rejects the hypothesis of cross resistances between antibiotic and biocides. In the present study, no resistance was found to triclosan even not in antibiotic resistant isolates. Similarly, Cole et al. (2003) found no relationship between the use of triclosan and other biocides and antibiotic resistance in homes isolates where biocidal products were or were not being used. In another study, Cole et al. (2011) found that *S. aureus* had triclorcarban (TCC;) MIC ranges from 0.0029 to 0.1875µg/L among TCC user group and 0.0469 to 0.1875µg/L among non users which is less than our findings. In this study, TCC MIC range was found both for MRSA and MSSA 0.25 to 0.5 mg/L.

In the current study, among 70 *S. aureus* isolates, efflux pump was detected in three MRSA isolates and in one MSSA isolate by using a phenotypic method as described by Marteins et al. (2006). In the current study, it was found that MRSA isolates had the MIC values for EtBr >30.0, 30.0 and 25.0µg/mL which is similar to a study conducted by Patal et al. (2010). They reported that the MIC value ≥25.0 µg/mL for EtBr indicates the presence of efflux pump in such isolates. MSSA isolate which was phenotypically positive for efflux pump had MIC of 15 µg/mL for EtBR which was in contrast to the findings reported by Patal (2010). Some of the other MSSA isolates also had 15 µg/ml MIC but were phenotypically negative for efflux pump. Therefore, the possibility of some other type(s) of efflux pump could not be excluded in such isolates. Further molecular studies are required to confirm this possibility. Three MRSA isolates were also resistant to EtBr, cetrimide, gentamicin, and neomycin. Cookson and Philips (1988) presented a hypothesis that resistance to all these antimicrobials and biocides are transfer collectively. Therefore, these findings support the idea presented by Cookson and Philips (1988) because these isolates were also resistant to SXT. However, Chang et al. (2007) reported that four different types of qac (qacA, qacB, qacC and qacD) genes carried on plasmids are related to the presence of resistance to this particular antibiotic and biocides. So the possibility of qac genes in these three isolates could not be excluded. It has also been reported that in *S. aureus* including MRSA, qac genes are responsible for multidrug efflux along with antimicrobial resistance genes, are associated with high values of MICs to a number of biocides like ethidium bromide, acriflavine, QACs such as cetrimide, BAK, and chlorhexidine (Tennent et al., 1989; Littlejohn et al., 1992; Reverdy et al., 1993).

In the last ten years, resistance to antiseptics and disinfectants have been reported in Australia and in United Kingdom in MRSA isolates (Mcdonell and Russell 1999). Mansouri et al. (2006) reported that cetrimide is useful and can be used as disinfectants against gram negatives and gram positives organisms. Their findings are in contrast with the finding of this study. In the present study, 22.9% of *S. aureus* isolates were found resistant to cetrimide showing high resistance among all the biocides tested in this study.

In the present study, two metal salts were tested in which silver nitrate was found more effective having MICs of 0.4 µg/mL while copper sulphate had MICs of 2.0 µg/mL. The metallic form of silver is nonreactive but becomes biologically active in the presence of liquids. The ionic form of silver has an antimicrobial activity and can be used as biocide. Its efficacy is lowered by the organic compounds (Dune, 2004). In such cases concentration >50 ppm are required (Hall, 1987). The silver inside the living cells bind to the proteins like DNA and RNA and inhibits the replication of microbial cell. The silver in ionic form has a biocidal and antimicrobial activity against many pathogens and also used in many clinical disorders like influenza, skin disorders including wounds, cuts and warts (Lansdown, 1987). In this study, it was found that silver nitrate was more effective against clinical isolates of *S. aureus*. Its ionic form is used in manufacturing of wound dressings (Kalson, 1999). Copper sulphate was less effective as compared to silver nitrate in this study. Silver nitrate is used on surfaces and
active against the microbes in its metallic form (Wood et al., 2007).

All the above mentioned studies were reported from developed countries where the biocides use in the hospitals and community settings is very common. In contrast to developed countries, many hospitals in Pakistan lack proper use of biocides and antibiotics. Many studies in this regard have been conducted in the US and Europe, while limited data is available from developing countries like Pakistan. Results of a survey conducted in 2007 in two tertiary hospitals of Karachi Pakistan supports this fact that why isolates from Pakistan are more sensitive to most of the biocides used in this study (Anwar et al., 2009). They have reported lack of facilities and resources such as sinks, water, soap, antiseptic lotions and gels in these hospitals. The use of antiseptics, disinfectants and sanitizing chemicals should be encouraged in our hospitals in order to minimize the rate of infection, morbidity, and morality. All these practices are considered the first line defence against the infections and will also reduce the prescription of antibiotic (Ioannou et al., 2007).

Conclusion

This study suggests that there is no consistent pattern in the relationship between biocide and antibiotic resistance pattern. This study discounts the claim of cross resistance of biocides and antibiotics in bacteria. But resistance to aminoglycoside, cetrimide and EtBr can suggest a cross resistance pattern. Efflux pump was detected in three MRSA isolates and in one MSSA isolate by using a phenotypic method. MSSA isolate which was phenotypically positive for efflux pump had MIC value of 15 µg/mL for EtBr. Some of the other MSSA isolates also had 15 µg/ml MIC but were phenotypically negative for efflux pump. Therefore, the possibility of some other type(s) of efflux pump could not be excluded in such isolates. Further molecular studies are required to confirm this possibility.

REFERENCES


