**In vitro susceptibility of multidrug resistant *Pseudomonas aeruginosa* clinical isolates to common biocides**

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**ABSTRACT**

Biocides (antiseptics and disinfectants) play an essential role in infection control and the prevention of hospital acquired infections of pathogenic microorganisms. Now days, rates of antibiotic resistance in *Pseudomonas aeruginosa* are increasing worldwide and emerging of biocides resistant strains may lead to a failure in disinfection program. Until now, very few studies have investigated the susceptibility profile of nosocomial pathogens in particularly *P. aeruginosa* to antiseptics and disinfectant compounds and there are no reports available in the Kingdom of Saudi Arabia. Hence the aim of this study was to detect the minimum inhibitory concentrations (MIC) of a range of multidrug resistant (MDR) *P. aeruginosa* against three disinfectants common to the pharmaceutical and healthcare sectors: biguanide (chlorhexidine) and two quaternary ammonium compounds (benzalkonium chloride and cetrimide). The *in vitro* bactericidal activities of the three biocides were studied against 11 MDR *P. aeruginosa* organisms, isolated from various clinical specimens in the Qassim region, Kingdom of Saudi Arabia. The susceptibility testing performed by broth microdilution method following Clinical and Laboratory Standards Institute guidelines. Among 11 isolates tested, two (22%) were showed reduced susceptibility against benzalkonium chloride and cetrimide. Our observations imply an increased resistance observed against quaternary ammonium compounds among clinically isolated MDR *P. aeruginosa* isolates and further molecular studies are required to confirm these results in terms of resistance to the disinfectants.

**Keywords:** Multidrug resistant *Pseudomonas aeruginosa*; Biocides; Benzalkonium chloride; quaternary ammonium compounds; disinfectant.

**INTRODUCTION**

Rates of antibiotic resistance in *Pseudomonas aeruginosa* are increasing worldwide. The multidrug-resistant (MDR) phenotype in *P. aeruginosa* could be mediated by several mechanisms including multidrug efflux systems, enzyme production, outer membrane protein (porin) loss and target mutations. Rates of resistance to antimicrobials like imipenem, quinolones and third-generation cephalosporins have increased by 15, 9 and 20%, respectively. Similarly, a national surveillance study of intensive care unit (ICU) patients in the United States from 1993 to 2002, reported a significant increase in multidrug-resistance (MDR) *P. aeruginosa* isolates; defined as resistance to at least three out of four agents: imipenem, ceftazidime, ciprofloxacin and tobramycin (Obritsch et al., 2004). These MDR *P. aeruginosa* strains have been associated with healthcare associated infections and they can be spread within the hospital environment. For example, various studies reported that methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* have been isolated from hospital surfaces including stethoscopes, catheters, water supplies, and even disinfectant soap dispensers (Brooks et al., 2002; Guinto et al., 2002; Sandle, 2014). Hospital-acquired infections (HAIs) are responsible for significant morbidity and mortality in today's healthcare environment. Hospital disinfection policies have a main role to play in the control of HAIs (Fraise 2004).

Current procedures for infection control in hospitals have not been successful in controlling the rise in infections by multidrug-resistant pathogens. Biocides, including antiseptics and disinfectants, have been used extensively in hospitals and other healthcare settings for the sanitization of various medical devices and surfaces. In particular, disinfectants play an essential role in infection control and the prevention of nosocomial transmission of infectious microorganisms (Rutala, 1996). The increased usage of commonly used biocides products such as phenolics and quaternary ammonium compounds (QACs) in low concentrations has raised some concerns (Levy 2003; Daschner and Schuster 2004) about their overall efficacy, but also about the possible emergence of microbial resistance. Indeed,
there are now multiple laboratory reports discussing about the emergence of bacterial resistance to biocides, often as a result of exposure to a lower (sublethal) concentration (Walsh et al., 2003; Russell 2004; Thomas et al., 2005; Tumah, 2009).

The introduction of comprehensive disinfection protocols for the reduction of HAIs has been described in various publications (such as Vandini et al., 2014) and is in place in most developed healthcare systems. In addition, reduced susceptibility to biocides has been reported for various nosocomial pathogens (Russell, 1999a; Block and Furman, 2002; Kawamura-Sato et al., 2008). However, the available information about the relationship between disinfectant and antibiotics resistance profiles has so far been limited to a few bacterial species. The most predominant of these is P. aeruginosa. The bacterium is ubiquitous, flourishes in many environments such as soil, water, mammals and metabolically versatile. It can grow under both aerobic and anaerobic conditions. In terms of patient risks, the bacterium can cause infections of the airway, urinary tract, bloodstream, burns, and wounds (Fine et al., 1996). The organism also releases endotoxin when it undergoes cell lysis, which can trigger a pyrogenic response and cause endotoxic shock (Kurahashi, 1999).

Although, it is well known that Gram-negative bacteria are less susceptible to QACs than Gram-positive bacteria, and Pseudomonas spp. have generally high intrinsic resistance compared with other Gram-negative bacteria (Russell and Chopra 1996). P. aeruginosa possesses multifactorial mechanisms of responses (Schurek et al., 2012) as well as resistance to antimicrobials and disinfectants. The causes of development of bacterial resistance (both biocides and antibiotics), the benefit of biocide usage, and their possible role in development of multidrug-resistance, add further questions to the broad use of biocidal products (Bloomfield, 2002; Poole, 2002). The advantages and disadvantages of biocides usage in the hospitals environment needs to be strictly considered. Very few studies have discussed and analyzed MIC values of biocides with antibiotics. The MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after a period of incubation. Applying MIC testing to a number of microbial strains provides an estimate of the concentration that inhibits 90% (MIC90) of isolates and thus it can indicate shifts in the susceptibility of bacterial populations to a biocide (Sandle et al., 2014). With the biocides selected, the aim was to use biocides in common use in many hospital settings. For this purpose, one biguanide and two different QACs were selected. A biguanide is an organic compound, used as a medicinal product and as a disinfectant (examples include chlorhexidine, polyaminopropyl biguanide, polihexanide, and alexidine). Such broad spectrum disinfectants function by altering membrane permeability (Chawner and Gilbert, 1989). QACs are a large group of related compounds which primarily exhibit microcidal kill by denaturing proteins on the cell membrane (Denyer, 1995).

Hence this study aims to determine the minimum inhibitory concentration of biocides against multidrug resistant P. aeruginosa strains and analyse their susceptibility pattern.

**MATERIALS AND METHODS**

**Bacterial strains**

The study included eleven multidrug resistant P. aeruginosa isolates that were recovered from clinical sites of separate patients at the Hammadi hospital and Habib hospital of Qassim region, Kingdom of Saudi Arabia. All isolates were collected between January and June 2015.

**Susceptibility testing**

In hospitals, MICs of imipenem, meropenem and the remaining anti-pseudomonal antibiotics (amikacin, gentamicin, tobramycin, ciprofloxacin, cefepime, ceftazidime, piperacillin/tazobactam, ticigycline) were performed by standard antimicrobial susceptibility testing method following Clinical and Laboratory Standards Institute (CLSI,2014) guidelines (to classify the bacterium as susceptible, intermediate or resistant to the agent). Organism identification and antimicrobial susceptibility testing of all drugs were performed again with VITEK 2– compact 15 (bioMérieux, France) for cross verification.

**Testing the MIC of biocides**

One biguanide group of 20 % chlorhexidine gluconate (Unilab Chemicals, India), two QACs of 20% benzalkonium chloride (Ubichem Fine Chemicals, U.K.) and 100% cetrimide (Unilab Chemicals, India) were selected as biocidal agents, based on the commonality of use of such agents in global healthcare systems. To prepare, each biocide was dissolved in sterile distilled water following the protocol of CLSI and were prepared in stock solutions of 1000 µg/ ml, which subsequently were diluted in Mueller Hinton Broth (MHB) test medium, designed for the cultivation of fastidious and nonfastidious microorganisms, for further dilution preparations.

**Bacterial inoculum preparation**

Bacterial inoculum was prepared by colony suspension method with MHB and turbid solutions visually compared with the McFarland standard for turbidity vs. cell concentration, and verified by measuring the absorbance of the suspension spectrophotometrically. The absorbance should be in the range of 0.08 – 0.13 which is equal to 1 x 10^8 CFU/ml. The test methodologies were followed as per Wiegand et al., 2008.

**MIC plate preparation**

A sterile plastic, disposable microdilution plate with 96 wells was taken. Into the well in each column (from 1 –
10), 50µL from each of the tubes containing the corresponding concentration (2 x final concentration) of target disinfectant was dispensed from the stock. For example, with chlorhexidine, cetrimide or benzalkonium chloride: to column 1 the medium containing 1024 µg/mL (1024 mg/L) was dispensed; to column 2 the medium containing 512 µg/mL was dispensed; and so on to column 10 where the medium containing 2 µg/mL was dispensed. The final well concentrations reached were 256 to 1 µg/mL after the addition of inoculum (50 µL). For each test plate, two drug free controls were kept, one with the 100 µL medium alone (sterility control, column 12) and the other with 50 µL of medium plus 50 µL of inoculum suspension (growth control, column 11).

After the addition of inoculum, the microdilution plates were incubated at 37°C with low humidity for 16 to 20 hours. Endpoint determination values were read visually using inverted reading mirror. The MIC defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye. The MIC for the quality control organisms should be within one or two-fold dilutions of published values for routinely used antibiotics; however since the biocides had no normal values this was not performed for the quality control strains.

RESULTS
During the study period, 11 non-repetitive P. aeruginosa isolates were selected by analysing susceptibility test results. The isolates were resistant to most anti-pseudomonal antimicrobials which include ceftazime, amikacin, gentamycin, tobramycin, piperacillin/tazobactam and carbapenem groups (refer to Table 1). The MIC values of benzalkonium chloride showed 32 to 512 µg/mL, whereas cetrimide showed 128 to >512 µg/mL. Two isolates (PA6 and PA8) showed reduced susceptibility against both QACs and the MIC values are 512 µg/mL for benzalkonium chloride and >512 µg/mL for cetrimide. The 50th percentile of MIC of benzalkonium chloride and cetrimide were 32 and 128 µg/mL, respectively. The MIC value for the biguanide group (chlorhexidine gluconate) showed 8 to 64 µg/mL, and the 90th percentile of the MIC was 16 µg/mL (Table 2).

DISCUSSION
Biocides have a broad-spectrum activity that inactivates or kills microorganisms on living tissue and inanimate surfaces. Biocides are an important component of clinical and pharmaceutical industries and serve to prevent the contaminations (McDonnell and Russell, 1999).

P. aeruginosa is a true opportunistic pathogen that causes infections in a variety of clinical settings and can grow in many hospital environments. Today P. aeruginosa and Acinetobacter baumanii have developed resistance to many antimicrobial agents, with the highest frequency, in some cases expressing resistance to all clinically available antibiotics. Additionally these organisms have been reported to contaminate disinfectants in hospitals thereby reducing the ability of disinfection process (Davane et al., 2014). Therefore, susceptibility of MDR P. aeruginosa tested against biocides helpful for successful implementation of disinfection process in infection control program.

Chlorhexidine is the most widely used disinfectant in hospitals, (mainly as an antiseptic hand washing agents, oral products and preservative); QACs such as cetrimide and benzalkonium chloride are the widely used antiseptic agents in hospitals. Therefore high MICs of these biocides may not effectively control all hospital pathogens. At present, there are very few publications deploying a small number of isolates examining the relationship between biocides use and antibiotic resistance in the clinical sitting.

Benzalkonium chloride is one of the QACs examined. It is a membrane-active agent which primarily targets the cytoplasmic membrane of bacteria and the plasma membrane of yeast cells (McDonnell and Russell, 1999). In vitro efficacy of the present study showed that the MIC range of benzalkonium chloride was 32 to 512 µg/mL. Of the 11 test isolates, nine isolates (78%) showed MIC values that were between 32 and 64 µg/mL. The remaining two isolates (22%) PA6 and PA8 observed reduced susceptibility at 512 µg/mL.

There have been no major reports published on the assessment of the susceptibility profile to biocidal agents against MDR P. aeruginosa isolates. Hence, comparison of these data with others studies is very difficult. However, various researchers have analyzed the killing action of biocides on bacterial cell walls. Russell and Gould (1988) reported that MIC value of benzalkonium chloride against P. aeruginosa was 250 µg/mL, which is comparable with our present study findings; however high MIC values were observed in this study population. Similarly, in a paper by Lambert et al. (2001) a number of industrial and clinical isolated of P. aeruginosa were analyzed for their sensitivities towards several common biocides and antibiotics. The researchers found that MIC values of clinical isolated of P. aeruginosa to benzalkonium chloride were 78 to 625 µg/mL. The variations obtained by the available reports might be due to the nature of the isolates, quantity, cultural methods, and the test methods employed.

In another interesting study reported by Lambert, the MIC of 111 P. aeruginosa clinical isolates against eight antimicrobial biocides (which includes benzalkonium chloride, chlorhexidine and triclosan) and several clinically relevant antibiotics were run. It was found that many biocides compounds had a significantly lower mean MIC in the year of 2000 relative to 1989 (Lambert, 2004). This infers a change in resistance profile over time.
With the third biocide investigated - cetrimide - this is another type of QAC disinfectant. The MIC of this compound showed the range 128 – 512 µg/mL. This data showed a strong resemblance to that of previous study by Russel et al. (1999b) where it was reported the MIC range of cetrimide against *P. aeruginosa* was between 64 and 128 µg/mL. Although the same two isolates (PA6 and PA8), as with benzalkonium chloride, showed reduced susceptibility >512 µg/mL against cetrimide, these two organisms showed resistance to imipenem, merapenem and cefepime, ceftazidime and piperacillin/tazobactam. However other organisms, that have the multidrug resistance character, not have such reduced susceptibility against biocides. This reaffirms our premise that biocide response depends on the environmental stress factor.

In an examination of clinical *P. aeruginosa*, Joynson et al. (2002) conducted an *in vitro* study to explain the gaining of resistance to QACs by *P. aeruginosa*. They reported that an isolate of *P. aeruginosa*, trained by subculture to be resistant to two aminoglycosides, had an increased resistance to benzalkonium chloride; however, the same organism trained by subculture to be resistant to benzalkonium chloride was more sensitive to the same two antibiotics. Usually the term resistance is not used for biocides susceptibility; nevertheless, as per literature reference, Russell’s statement about resistance to biocides suggests a four- to eight-fold increase in the minimum inhibitory concentration above an average value can occur (Russell, 1998a). This is in keeping with the study described here, which showed that two isolates were resistant to both QACs.

With the present study, *in vitro* efficacy of chlorhexidine gluconate against MDR *P. aeruginosa* was between 8 to 64 µg/mL. Here the 50th and 90th percentile were 16 µg/mL. The results of present study findings are comparable with study by McDonnell and Russell, where the MIC of chlorhexidine against *P. aeruginosa* was 5 to 60 µg/mL (McDonnell and Russell, 1999).

Regarding antibiotic susceptibility panel all the isolates were resistant to common anti-pseudomonal drugs such as β-Lactams, cephalosporins (cefepime, ceftazidime), carbapenems (imipenem, meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (aminoglycosides) and tobramycin. Alexander et al., (1991) found that high antibiotic resistant organisms are generally more resistant to disinfectants. An analysis of the present study findings showed 22% of MDR isolates indicated reduced susceptibility to QACs and there was no correlation observed between reduced susceptibility to disinfectants and multidrug resistance to anti-pseudomonal drug groups. This is very similar to a finding by Martro et al. (2003) who found no evident correlation between the multidrug resistance and biocides resistance for the nine *Acinetobacter* spp. that caused a continued ICU outbreak in Spain. This indicates a study with a large series of data with more an-

### Table 1: Biocides MICs and antimicrobials resistance profiles of MDR *P. aeruginosa*

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>Benzalkonium chloride</th>
<th>Cetrimide</th>
<th>Chlorhexidine gluconate</th>
<th>Resistance to other anti-pseudomonal antimicrobials</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1</td>
<td>32</td>
<td>128</td>
<td>8</td>
<td>CEF, CFZ, PT, AK, GEN, TOB, CIP, TIG, IMI, MER</td>
</tr>
<tr>
<td>PA2</td>
<td>64</td>
<td>256</td>
<td>16</td>
<td>CEF, CFZ, PT, CIP, TIG, IMI, MER</td>
</tr>
<tr>
<td>PA3</td>
<td>32</td>
<td>128</td>
<td>8</td>
<td>PT, CIP, TIG, GEN, IMI, MER</td>
</tr>
<tr>
<td>PA4</td>
<td>32</td>
<td>256</td>
<td>16</td>
<td>CEF, CFZ, PT, GEN, CIP, TIG, IMI, IMI, MER</td>
</tr>
<tr>
<td>PA5</td>
<td>32</td>
<td>128</td>
<td>16</td>
<td>TIG, AK, GEN, IMI, MER</td>
</tr>
<tr>
<td>PA6</td>
<td>512</td>
<td>&gt;512</td>
<td>8</td>
<td>CEF, CFZ, PT, AK, GEN, TOB, CIP, TIG, IMI, IMI, MER</td>
</tr>
<tr>
<td>PA7</td>
<td>32</td>
<td>128</td>
<td>64</td>
<td>AK, GEN, TOB, CIP, TIG, IMI, MER</td>
</tr>
<tr>
<td>PA8</td>
<td>512</td>
<td>&gt;512</td>
<td>16</td>
<td>CEF, CFZ, PT, TIG, IMI, MER</td>
</tr>
<tr>
<td>PA9</td>
<td>64</td>
<td>128</td>
<td>8</td>
<td>CEF, CFZ, PT, AK, GEN, TOB, CIP, TIG, IMI, IMI, MER</td>
</tr>
<tr>
<td>PA10</td>
<td>64</td>
<td>256</td>
<td>16</td>
<td>CEF, CFZ, PT, AK, TOB, CIP, TIG, IMI, IMI, MER</td>
</tr>
<tr>
<td>PA11</td>
<td>32</td>
<td>128</td>
<td>16</td>
<td>CEF, CFZ, PT, TIG, IMI, IMI, MER</td>
</tr>
</tbody>
</table>

CEF – Cefepime; CFZ – Ceftazidime, PT – Piperacillin/tazobactam, AK - Amikacin; GEN – Gentamycin; TOB - Tobramycin, CIP - Ciprofloxacin, TIG-Tigecycline, IMI – Imipenem, MER – Meropenem

### Table 2: Biocides MICs (µg/mL) of MDR *P. aeruginosa*

<table>
<thead>
<tr>
<th>Descriptions</th>
<th>Benzalkonium chloride</th>
<th>Cetrimide</th>
<th>Chlorhexidine gluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>32 to 512</td>
<td>128 to &gt;512</td>
<td>8 to 64</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>32</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>512</td>
<td>&gt;512</td>
<td>16</td>
</tr>
</tbody>
</table>
tibiotics and biocides is needed to confirm or challenge this conclusion.

Various authors suggest a positive linkage between bacterial resistance and the use of biocides. For example, Russell et al. (1998b) revealed that chlorhexidine gluconate resistance in Pseudomonas stutzeri correlated with resistance to polymyxin B, gentamicin, erythromycin and ampicillin. Similar observations were found for other nosocomial pathogens, such as MRSA and P. aeruginosa (Fraise, 2002; Köljalg et al., 2002).

Moreover, P. aeruginosa possesses a natural resistance to many antimicrobials because of the bacterium’s outer membrane barrier; the presence of multidrug efflux transporters and endogenous antimicrobial inactivation. Efflux proteins in P. aeruginosa have been widely studied and shown to be associated with some antibiotics and biocides (Russell, 2002).

Apart from microbiological in-vitro susceptibility testing, Morita et al. (2003) found that a multidrug efflux pump plays an important role in conferring multidrug resistance to wild-type P. aeruginosa in hospitals, an environment where disinfectants, including chlorhexidine, are used frequently. Morita and colleagues state that biocides induce efflux pumps, which causes the bacterium surviving in toxic environments and emerging resistance to clinically relevant antibiotics. Additionally, various researchers have reported that laboratory studies have shown that bacteria can become less susceptible to a biocide that cross-resistance may occur to other biocides, as well as to antibiotics (Russell et al., 1998b; Tattawasart et al., 2000; Andrews, 2001). Controlling of these microorganisms is very important, because they are associated with biofilm formation in niche environments and they create endotoxin on undergoing cell lysis, such as after sterilization. However, in clinical and industrial settings, the biocides are normally used at concentrations far in excess of the observed MIC values, which control these MDR organisms.

Overall, the susceptibility pattern of biocides is comparable with antibiotics. Two strains showed resistance (decreased susceptibility to benzalkonium chloride and cetrimide); and there was no significant correlations observed antibiotic-biocides resistant profiles. Based on the analyses in this study it is hard to support a hypothesis that increased biocide resistance is a cause of increased antibiotic resistance in P. aeruginosa.

CONCLUSION

Among 11 MDR P. aeruginosa isolates, 22% isolates showed reduced susceptibility against QACs and there was no significant reduction observed against the biguanide chlorhexidine tested. The present findings conclude that no apparent correlation exists between specific disinfectants and antimicrobial drugs. However, our observations indicate notable reduced susceptibility observed against QACs among MDR P. aeruginosa clinical isolates. In addition, the findings of this study highlight the importance of MDR P. aeruginosa having biocide resistant mechanisms. These were investigated by in-vitro susceptibility testing. This resistance may be the result of phenotypic adaptation and survival in an environment, where growth conditions are limiting and the bacterial cells are subject to stress. Further studies are needed, with a control group of environmental isolates, together with detail of their resistance mechanism shown by molecular level studies and efflux protein analysis.

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REFERENCE


Schurek K. N., Breidenstein E. B. M, Hancock R. E. W. *Pseudomonas aeruginosa*: a persistent pathogen in...


Wiegand, I., Hilpert, K., Hancock, R.E. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75.