Original Research

DOI: doi.org/10.47363/AMR/2018(5)149

Determination of minimum inhibitory concentrations of common biocides to multidrug-resistant gram-negative bacteria

Rajendran Vijayakumar[1], Mohammad Saleh Al- Aboody[1], Meshal K. AlFonaisan[2], Wael Alturaiki[1], Suresh Mickymaray[1], Mariappan Premanathan[1], Suliman A. Alsagaby[1], Tim Sandle[3]

[1]Department of Medical Laboratories, Central Biosciences Research Laboratories, College of Science in Al Zulfi, Majmaah University, Kingdom of Saudi Arabia.
[2]Department of Medical Laboratories, College of Science in Al Zulfi, Majmaah University, Kingdom of Saudi Arabia.
[3]Head of Microbiology, Bio Products Laboratory, Elstree, Hertfordshire, United Kingdom.

Address for correspondence:
Rajendran Vijayakumar,
Department of Medical Laboratories, Central Biosciences Research Laboratories, College of Science in Al Zulfi, Majmaah University, Kingdom of Saudi Arabia.
vivegaviji@gmail.com

Received: August 30, 2016
Accepted: September 27, 2016
Published: October 12, 2016

ABSTRACT

Background: Until now, very few studies have investigated the susceptibility profile of biocides to nosocomial pathogens and none reported in the Saudi Arabia. Hence, the aim of this study was to detect the minimum inhibitory concentrations (MIC) of a range of multidrug resistant (MDR) bacteria: *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* against three common hospital disinfectants: chlorhexidine, benzalkonium chloride and cetrimide. **Methods:** The *in vitro* susceptibility tests of the three biocides were studied against 21 MDR *A. baumannii*, 11 MDR *P. aeruginosa* and 3 MDR *K. pneumoniae* strains, isolated from various clinical specimens in the Qassim region, Saudi Arabia. The susceptibility testing was performed by broth microdilution method following Clinical and Laboratory Standards Institute guidelines. **Results :** Among 35 isolates tested, there was no reduced susceptibility observed in *A. baumannii* and *K. pneumoniae*, however, two isolates of *P. aeruginosa* were showed reduced susceptibility (> 512 μg/mL) against benzalkonium chloride and cetrimide. **Conclusion :**Our observations imply that reduced susceptibility was observed with quaternary ammonium compounds against *P. aeruginosa* and no apparent relationship exists between specific disinfectants and their multidrug resistance character in *A. baumannii* and *K. pneumoniae*. Further studies are required to confirm these results in terms of biocides resistance.

KEY WORDS: Multidrug resistant *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae,* Biocides, Benzalkonium chloride, quaternary ammonium compounds, disinfectant, nosocomial pathogens

INTRODUCTION

Hospital-acquired infections (HAIs) are responsible for significant morbidity and mortality in today's healthcare environment. Recently the U.S. Centres for Disease Control and Prevention (CDC) reported that many of the most serious HAI cases are due to antibiotic-resistant bacteria. These cases include: Carbapenem-resistant Enterobacteriaceae (CRE); extended spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae; multidrug-resistant *Pseudomonas aeruginosa*; multidrug-resistant *Acinetobacter* species. These microorganisms have unique features that are advantageous for their persistence in harsh environmental conditions, including the presence of antimicrobial compounds. Since the environment serves as an important reservoir for infectious multidrug resistant organisms this is a matter of great concern and it presents a major task for healthcare organizations to eliminate [1]. Thus, CDC has advised healthcare professionals to continue to be proactive in the minimization of HAIs infections. This advice includes undertaking effective disinfection and controlled antibiotic use [2].

Biocides, including antiseptics and disinfectants, have long 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 from surfaces [3]. However, current procedures for infection control in hospitals have not always been successful in reducing the rise in infections by multi-drug-resistant pathogens (as global incidence rates suggest). This may not simply be related to the selecting inappropriate types of disinfectants or with inadequate procedures for their application. The potential emergence of bacterial resistance to biocides and the possible linkage between biocide and antibiotic resistance is now a major topic of discussion and concern. This resistance factor may lead to a failure with the disinfection of environmental surfaces and thus the spread of 'antibiotic- and disinfectant- resistant' nosocomial pathogens within the healthcare environment.

The increased usage of products containing low concentrations of commonly used biocides, such as phenolics and quaternary ammonium compounds (QACs), has raised some concerns about their overall efficacy [4,5]. In addition, there are also concerns about the possible emergence of microbial resistance. The

reduced susceptibility to biocides by some bacteria has been described for various nosocomial pathogens [6 – 9]. However, the available information about the linkage of resistance profiles to both disinfectants and antimicrobial agents has so far been limited to an assessment of the minimum inhibitory concentration (MIC) values of the biocides.

MICs are defined as the lowest concentration of an antimicrobial agent that will inhibit visible growth of microorganisms after a period of incubation. MICs are used by clinical laboratories mainly to confirm resistance; they are also employed as a research tool for determining the activity of new antimicrobial agent and their MIC breakpoints [10]. However there is a lack of guidelines and test methodologies for biocides that describe susceptibility or resistance profiles - with MICs break points - which are similar to the Clinical and Laboratory Standards Institute (CLSI) guidelines [11,12]. However, various researchers have analyzed the distribution of the MIC values of biocides against clinical isolates [8,13,14] and provided limited data relating to multidrug resistant organisms [15].

In the Kingdom of Saudi Arabia, as with many other regions of the world, MDR *P. aeruginosa* and *A. baumannii* are the serious nosocomial pathogen, despite their susceptibility towards common antiseptics and disinfectant being largely unknown. Therefore, healthcare personnel are in urgent need to know about MIC breakpoints of MDR nosocomial pathogens so that they can follow effective disinfection protocols to control such nosocomial pathogens.

This paper presents a study that provides methodology for making such an assessment. With the biocides selected, the aim was to select biocides in common use in many hospital settings. For this purpose, one biguanide (example – chlorhexidine) and two different QACs (benzalkonium chloride and cetrimide) were selected. Hence, this study aims to determine the minimum inhibitory concentration of biocides against MDR nosocomial pathogens. The pathogens selected were: *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Bacterial strains

The study included 35 multidrug resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* organisms. These bacteria were recovered from clinical sites from individual patients at the Hammadi hospital and Habib hospital of Qassim region, Kingdom of Saudi Arabia. All isolates were collected between January and June 2015. This study was approved by the ethics committee of hospitals and the deanship of scientific research, Majmaah University.

Susceptibility testing

In hospitals, MICs of antimicrobial compounds (namely imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, cefepime, ceftazidime, piperacillin/tazobactam, tigecycline, colistin) are performed by the standard antimicrobial susceptibility testing method following CLSI guidelines [16]. This is in order to classify a bacterium as susceptible, intermediate or resistant to an agent. All isolates included in this study were resistant to three or more antimicrobial classes of antibiotics. Microorganism identification and antimicrobial susceptibility testing of all drugs were performed again at central bioscience research laboratory - College of Science Zulfi, Majmaah University – using a VITEK 2– compact 15 (bioMérieux, France) for cross verification.

Testing the MIC of biocides

One biguanide of chlorhexidine gluconate 20% (Unilab Chemicals, India), two QACs of benzalkonium chloride 20% (Ubichem Fine Chemicals, U.K.) and 100% potency of 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 (Himedia, India), designed for the cultivation of fastidious and non-fastidious microorganisms, for further dilution preparations.

Bacterial inoculum preparation

Each bacterial inoculum was prepared by the colony suspension method with MHB and any 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 between 0.08 and 0.13 at 625nm spectrophotometrically (ThermoFisher scientific, USA), which is equal to 1 x 10^8 CFU/ml. The test methodologies were followed as per Wiegand *et al.*, [17].

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 μg/ mL 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 37^0 C with low humidity for 16 to 20 hours. Endpoint determination values were read visually with the aid of an inverted reading mirror. The MIC was 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 35 non-repetitive MDR clinical isolates, made up of 21 *A. baumannii* isolates; 11 *P. aeruginosa* isolates; and 3 *K. pneumoniae* isolates, were selected by analyzing susceptibility test results. These isolates were resistant to most antimicrobials, include cefepime, ceftazidime, amikacin, gentamycin, tobramycin, piperacillin/tazobactam and carbapenem groups (Table.1). The overall distribution of MICs of various biocides by the microbroth dilution technique is listed in Table.2. In *A. baumannii*, the MIC values of benzalkonium chloride showed 8 to 32 μg/mL and both cetrimide and chlorhexidine showed 32 to 128 μg/mL. MIC_{90} of benzalkonium chloride, cetrimide and chlorhexidine showed 16, 64, 32 μg/mL respectively.

In *P. aeruginosa*, the MIC values of benzalkonium chloride ranged from 32 to 512 μg/mL, whereas cetrimide showed 128 to >512 μg/mL. MIC_{50} and MIC_{90} 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 MIC_{90} was 16 μg/mL. Two isolates of *P. aeruginosa* among 11 tested, showed reduced susceptibility against both QACs. Here the MIC values were 512 μg/mL for benzalkonium chloride and >512 μg/mL for cetrimide; these were considerably higher than the remaining 9 isolates (mean 42.6 μg/mL).

With the 3 strains of *K. pneumoniae* tested, the MICs of benzalkonium chloride and chlorhexidine was 16 to 32 μg/mL and cetrimide showed 32 – 128 μg/mL. Based on the strain used, there was no observed reduced susceptibility of the biocides against the test cultures of *A. baumnaii* and *K. pneumoniae*.

The MICs outcomes of each biocides against all 35 clinical isolates were compared with the percentage of respective MICs for each organism (Table.3). Of the total 11 *P. aeruginosa* strains, 9 (81.8%) had an MIC of benzalkonium chloride of < 64 μg/mL, 2 (18.2%) of isolates had a higher MIC of >512 μg/mL; and 45% of isolates had an MIC of cetrimide of >512 μg/mL. The MICs of other compounds are shown in Table.3

Table 1. Antibiotic resistance patterns of MDR test isolates

Antibiotics tested	Number of resistant / Total number tested (%)		
	***A. baumannii* (21)**	***P. aeruginosa* (11)**	***K. pneumoniae* (3)**
Piperacillin/Tazobactem	19/19 (100)	6/11 (54.5)	3/3 (100)
Cefepime	NT	7/11 (63.6)	3/3 (100)
Ceftazidime	20/20 (100)	8/11 (72.7)	3/3 (100)
Ceftriaxone	15/15 (100)	NT	1/1 (100)
Aztreonam	NT	6/7 (85.7)	1/1 (100)
Imipenem	21/21 (100)	9/11 (81.8)	3/3 (100)
Meropenem	21/21 (100)	11/11 (100)	3/3 (100)
Amikacin	18/21 (85.7)	5/11 (45.4)	2/3 (66)
Gentamicin	18/21 (85.7)	5/11 (45.4)	1/3 (33)
Tobramycin	17/21 (80.9)	5/11 (45.4)	1/3 (33)
Ciprofloxacin	21/21 (100)	8/11 (72.7)	3/3 (100)
Levofloxacin	21/21 (100)	NT	1/1 (100)
Tigecycline	0/21 (0)	10/11 (90.9)	2/3 (66)
Colistin	5/21 (23.8)	0/11 (0)	1/3 (33)
Trimethoprim-sulfamathoxazole	NT	11/11 (100)	2/2 (100)

NT – Not tested

Table 2. Distributions of MIC of various biocides by microbroth dilution technique

Biocides	MDR Organisms (Numbers tested)	Number of isolates with MICs (µg/mL) indicated								Mean MICs	MIC_{50}	MIC_{90}
		8	16	32	64	128	256	512	>512			
Benzalkonium chloride (QACs)	*A. baumannii* (21)	17	3	1	0	0	0	0	0	10.3	8	16
	P. aeruginosa (11)	0	0	6	3	0	0	2*	0	128	32	512
	K. pneumoniae (3)	0	2	1	0	0	0	0	0	21.3	NA	NA
Cetrimide (QACs)	*A. baumannii* (21)	0	0	16	4	1	0	0	0	42.7	32	64
	P. aeruginosa (11)	0	0	0	0	6	0	3	2*	325.8	128	>512
	K. pneumoniae (3)	0	0	1	1	1	0	0	0	74.7	NA	NA
Chlorhexidine gluconate (Biguanide)	*A. baumannii* (21)	0	0	21	0	0	0	0	0	32	32	32
	P. aeruginosa (11)	4	6	0	1	0	0	0	0	17.4	16	16
	K. pneumoniae (3)	0	1	2	0	0	0	0	0	26.7	NA	NA

*same isolates showed reduced susceptibilities to QACs; NA – Not applicable

Table 3. Grouping of biocides MICs (µg/mL) of MDR clinical pathogens

MDR Organisms (Numbers tested)	Biocides	Numbers (%) of isolates with MICs (µg/mL) range indicated			
		8 – 16	32 – 64	128 – 256	≥512
A. baumannii (21)	Benzalkonium chloride	20 (95.2)	1 (4.8)	0	0
	Cetrimide	0	20 (95.2)	1 (4.8)	0
	Chlorhexidine gluconate	0	21 (100)	0	0
P. aeruginosa (11)	Benzalkonium chloride	0	9 (81.8)	0	2 (18.2)
	Cetrimide	0	0	6 (54.5)	5 (45.5)
	Chlorhexidine gluconate	10 (90.9)	1 (9.1)	0	0
K. pneumoniae (3)	Benzalkonium chloride	2 (66.7)	1 (33.3)	0	0
	Cetrimide	0	2 (66.7)	1 (33.3)	0
	Chlorhexidine gluconate	1 (33.3)	2 (66.7)	0	0

DISCUSSION

Biocides are an integral component of clinical and pharmaceutical industries and serve to prevent the dissemination of nosocomial pathogens in the hospital environment and control the growth of environmental isolates in industrial cleanrooms. Biocides have a broad-spectrum activity that inactivates or kills microorganisms on living tissue and inanimate surfaces in either specific or non-specific ways [18].

Today both *A. baumannii* and *P. aeruginosa* have developed resistance to multiple antimicrobial agents, with some strains expressing resistance to all antimicrobial compounds. Additionally, these organisms have been reported to contaminate disinfectants in hospitals or other such environments, thereby compromising the ability of the disinfectant to reduce or eliminate bacterial contamination [19]. These strains are very common environmental bacteria growing in water, soil, compost and drainage and they are critical nosocomial pathogens due to the recent increase in their isolation from various clinical specimens. The U.S. CDC has warned healthcare professionals to pay particular attention to avoiding the spread of MDR *Pseudomonas*, *Acinetobacter* and carbapenem-resistant / ESBL producing Enterobacteriaceae [2]. Several studies focus on antimicrobial resistance issues relating to *A. baumannii* and *Pseudomonas*, *K. pneumoniae* [8,15,20]. However, there have been few studies that have analyzed the *in-vitro* susceptibility and development of resistance to biocidal compounds.

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less characterized. In theory, such resistance is less likely. This is because disinfectants are more powerful biocidal agents than antibiotics and are applied in high concentrations, normally against a low population of microorganisms. Moreover, these organisms, due to the absence of optimal environmental growth conditions and due to limited nutrients, are usually

not growing actively. Hence, the selective pressure for the development of resistance is less profound. However, international pharmacopoeial guidelines suggest that the most frequently isolated environmental isolates from cleanroom environments should be periodically tested against their susceptibility to biocides [21].

Globally there is a paucity of susceptibility breakpoint studies to multidrug resistant clinical isolates in worldwide and there are no reports available from Saudi Arabia. Hence, the present study analyzed the *in-vitro* susceptibilities of nosocomial MDR Gram-negative isolates to biocides isolated in hospitals of the central region of Saudi Arabia. The susceptibility of MDR nosocomial pathogens tested against biocides presented here is helpful for the successful implementation of disinfection process in the infection control program. However, there are some limitations that exist in this study. First, there was no reference breakpoint available for MIC of biocides and hence quality control strains were not included in this study. Secondly, while high concentrations of biocides are used as disinfectants, with antiseptic preparations the minimum inhibitory doses differ for each type of organisms and this study only set out to determine MIC value of biocides and analyze the reduced susceptibility by comparing the mean MIC value of the strains. Thirdly, the study looked at only three disinfectants.

QACs such as benzalkonium chloride and cetrimide are the widely used antiseptic agents in hospitals. Benzalkonium chloride is a membrane-active agent, which primarily targets the cytoplasmic (inner) membrane of bacteria and the plasma membrane of yeasts [18]. *In vitro* efficacy of the present study showed that the MIC range of benzalkonium chloride against A. *baumannii* was 8 to 32 µg/mL, *P. aeruginosa* was 32 to 512 µg/mL and *K. pneumoniae* was 16 to 32 µg/mL. The finding relating to apparent resistance with two *P. aeruginosa* isolates is in keeping with other published research. For example, Kawamura-Sato *et al.*, conducted a large series of studies with 283 strains of *A.baumannii* and reported that several *Acinetobacter* clinical isolates have developed augmented resistant to multiple antibiotics and disinfectants. The researchers reported that the MIC_{90} of benzalkonium chloride was 50 µg/mL [8]. This is slightly higher than our study findings, which showed the mean MIC_{90} was 16 µg/mL. This is probably because of the testing methods varied between the studies.

In considering test method variations further, our study findings are very similar to Wand *et al.*, reports, MICs of wild-type colistin susceptible A.*baumannii* showed 4 – 8 µg/mL; Wand and colleagues carried out the microbroth dilution technique which is a similar technique used in our study [20]. However, Kawamura-Sato *et al.* analyzed MIC by agar well dilution method, which differs from the present study methodologies [8]. This may be a reason why higher MIC values were recorded compared with our study findings. This variant reaffirms the lack of testing methodologies for determination of biocides MIC breakpoints.

Of the 11 *P. aeruginosa*, nine isolates (81.8%) showed MIC values that were between 32 and 64 µg/mL. The remaining two (and different) isolates (18.2%) observed reduced susceptibility at >512 µg/mL. Russell and Gould reported that the 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 [22]. Similarly, in a paper by Lambert *et al.* found that MIC values of clinical isolates of *P. aeruginosa* to benzalkonium chloride were 78 to 625 µg/mL [11]. 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) had a significantly lower mean MIC in the year of 2000 relative to 1989 [23]. This infers a change in resistance profile over time. Furthermore, Abuzaid and his associates reported MIC range of benzalkonium chloride against clinical *K.pneumoniae* isolates was 16 to 64 µg/mL [24], which is very similar to our study results.

With the second biocide investigated cetrimide, this is a second type of QAC disinfectant. The MIC of this compound showed 32 to 128 µg/mL against both *A. baumannii* and *K. pneumoniae* group of strains and the range for the *P. aeruginosa* isolates was 128 to 512 µg/mL. Regarding the cetrimide MICs of *P. aeruginosa*, data showed a strong resemblance to that of the previous study by Russel *et al*, where it was reported the MIC range of cetrimide against *P. aeruginosa* was between 64 and 128 µg/mL [25].

Usually, the term resistance is not used for biocide susceptibility; nevertheless, as per literature, Russell's statement about resistance to biocides suggests a four- to eightfold increase in the minimum inhibitory concentration, above an average value, can occur [26]. This is in keeping with the study described here, which showed that two isolates of *P. aeruginosa* were resistant to both QACs (that is the mean value of benzalkonium chloride of 9 strains was 42.6 µg/mL). Two isolates showed reduced susceptibility >512 µg/mL against benzalkonium chloride and cetrimide, these two organisms showed resistance to imipenem, meropenem and cefepime, ceftazidime and piperacillin/tazobactam. However, other organisms, that have the multidrug resistance character, did not have such reduced susceptibility against biocides. This reaffirms our premise that biocide response depends on the type of environmental stress factor.

In an examination of clinical *P. aeruginosa*, Joynson *et al.* (2002) conducted an *in vitro* study to explain the acquisition of resistance to QACs by *P. aeruginosa*. They reported that an isolate of *P. aeruginosa*, reproduced by subculture to become 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 [27].

Chlorhexidine is the most widely used antiseptic agent in hospitals and pharmaceutical industries, applied in various applications such as surface cleaning, hand washing and skin preparation before invasive procedures. With the present study findings, the MIC_{90} of chlorhexidine gluconate to *A.baumannii* was 32 μg/mL, which is very similar to large series of a study conducted by Kawamura-Sato *et al.*, (2010) [8]. With *P. aeruginosa*, the results of this study are comparable with a study by McDonnell and Russell, where the MICs of chlorhexidine against *P. aeruginosa* was 5 – 60 μg/mL [18].Guo and his team members analyzed the susceptibility of carbapenem-resistant *K. pneumoniae* to disinfectants and investigated possible correlations between drug resistance genes and increased resistance to disinfectant [14]. They found that the range of chlorhexidine MIC was 8 to 64 μg/mL, and 70% (19 of 27) of the tested isolates showed 32μg/mL. Another study report from the UK showed MIC_{50} and MIC_{90} were 32 and 64 μg/mL against 64 clinical *K. pneumoniae* isolates [24]. This is comparable with this our study which found that all three isolates tested showed MICs of between 16 and 32μg/mL.

Concerning the antibiotic susceptibility panel all tested isolates were resistant to common drugs such as β-Lactams, cephalosporins (e.g. ceftazidime, cefepime), carbapenems (e.g. imipenem, meropenem) fluoroquinolones (e.g. ciprofloxacin), aminoglycosides (e.g. amikacin, gentamycin, and tobramycin). Alexander et al., (1991) found that highly antibiotic-resistant organisms are generally more disinfectant-resistant [28]. Although not correlated, this research found high antibiotic resistance can leads to some biocide-resistant organisms. The analysis of our study findings showed 18.2% and 45.5% of MDR *P. aeruginosa* isolates indicated reduced susceptibility to QACs of benzalkonium chloride and cetrimide, respectively. An another important findings observed in this study was that there was no correlation observed between reduced susceptibility to all biocides and multidrug resistance to drug groups of *A. baumannii* and *K. pneumoniae*. This is very similar to a finding by Martro *et al.* (2003) who found no evident correlation between the resistance profiles of antimicrobial agents and biocides for the nine *Acinetobacter* spp. that caused a sustained ICU outbreak in Spain [29]. However, the above statement contrast with the findings from Naparstek *et al.* These researchers analyzed MICs of chlorhexidine across a large series of 126 isolates of extremely drug resistant *K. pneumonia*, using the agar dilution method. The researchers reported that 90% of isolates had a high MIC and reduced susceptibility compared with a control strain [15]. This indicates a study with a larger data set with more antibiotics and biocides is needed to confirm or challenge this conclusion and there is pressing need for uniform test methodologies to prove or disprove this statement.

Moreover, various authors have suggested a positive linkage between bacterial resistance and the use of biocides. For example, Russell *et al.* revealed that chlorhexidine gluconate resistance in *Pseudomonas stutzeri* correlated with resistance to polymyxin B, gentamicin, erythromycin, and ampicillin [30]. Similar observations have been found for other nosocomial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* [31,32]. Additionally, various researchers have reported that laboratory studies have shown that bacteria can become less susceptible to a biocide and that cross-resistance may occur to other biocides, as well as to antibiotics [15,30,33,34]. Our study findings very similar to the available reports, however, based on the analyses performed here it is very difficult to support a hypothesis that increased biocide resistance is a cause of increased antibiotic resistance in *P. aeruginosa*.

CONCLUSION

Overall, we show here, by an MIC analysis of MDR clinical isolates, the reduced susceptibility or high MIC value (> 512 μg/mL) with MDR *P. aeruginosa*. There were no significant variations of MICs observed in other groups of MDR *A. baumannii* and *K. pneumoniae*. The present findings conclude that the likelihood of a relationship existing between specific disinfectants and the multidrug resistance character of the selected bacteria examined, is low. In addition, the findings of this study highlight the global lack of biocide MIC reference limits and consequently there is a need for a large series study from hospitals to confirm the trend of reduced susceptibility of problematic nosocomial pathogens.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Waseem Barakat and Dr. Mazen Krawi for providing bacterial strains for this study. This research work was funded by the Deanship of Scientific Research at Majmaah University, Kingdom of Saudi Arabia (project number BHSRC/10, 1434-35).

REFERENCES

1. Fraise, AP. Decontamination of the environment and medical equipment in hospitals. In Fraise AP, Lambert PA, Maillard JY, (Eds.). Principles and practice of disinfection, preservation and sterilization, 4th ed. *Oxford: Blackwell Sci.* 2004, pp563–585.
2. The Centers for Disease Control and Prevention's, Superbugs threaten hospital patients, http://www.cdc.gov/media/releases/2016/p0303-superbugs.html [Accessed 25.05.16]
3. Rutala WA. APIC guidelines for selection and use of disinfectants. *Am J Infect Control* 1996; 24: 313 – 42.
4. Levy SB. Antibacterial household products: cause for concern. *Emerg Infect Dis* 2001; 7:512–15.
5. Daschner F, Schuster A. Disinfection and the prevention of infectious disease: no adverse effects? *Am J Infect Control* 2004; 32: 224–225.
6. Block C, Furman M. Association between intensity of chlorhexidine use and micro-organisms of reduced susceptibility in a hospital environment. *J Hosp Infect* 2002;51: 201–206.
7. Russell AD. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *J Hosp Infect* 2004; 57:97–104.
8. Kawamura-Sato K, Wachino J, Kondo T, Ito H, Arakawa Y. Reduction of disinfectant bactericidal activities in clinically isolated Acinetobacter species in the presence of organic material. *J Antimicrob Chemother* 2008; 61: 568–576.
9. Vijayakumar R, Al-Aboody, MS, AlFonaisan MK, Sandle T. In vitro susceptibility of multidrug resistant *Pseudomonas aeruginosa* clinical isolates to common biocides, *Int J Res Pharm Sci* 2016; 7 (1): 110-116
10. Andrews JM, Andrews JM. Determination of minimum inhibitory concentrations. *The J Antimicrob Chemother* 2001; 48 Suppl 1, 5–16.
11. Lambert RJW, Joynson JA, Forbes B. The relationships and susceptibilities of some industrial, laboratory and clinical isolates of *Pseudomonas aeruginosa* to some antibiotics and biocides. *J Appl Microbiol* 2001; 91, 972–984.
12. Sandle T, Vijayakumar R, Saleh Al Aboody M, Saravanakumar S. In vitro fungicidal activity of biocides against pharmaceutical environmental fungal isolates, *J Appl Microbiol* 2014; 117 (5): 1267 – 1273
13. Russell AD. Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *J Appl Microbiol* 2002; 92 (Suppl):121–135.
14. Guo W, Shan K, Xu B, Li J. Determining the resistance of carbapenem-resistant *Klebsiella pneumoniae* to common disinfectants and elucidating the underlying resistance mechanisms. *Pathog Glob Health* 2015;109 (4): 184 – 192.
15. Naparstek L, Carmeli Y, Chmelnitsky I, Banin E, Navon-Venezia S. Reduced susceptibility to chlorhexidine among extremely-drug-resistant strains of Klebsiella pneumoniae. *J Hosp Infect* 2012; 81(1), 15–19.
16. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement.* CLSI document M100-S24, 2014; Vol. 34 No. 1
17. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc.* 2008; 3 (2):163-175.
18. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev*. 1999; 12(1):147-179.
19. Davane M, Suryawanshi N, Pichare A, Nagoba B. *Pseudomonas aeruginosa* from hospital environment, *J Microbiol Infect Dis* 2014; 4 (1): 42-43
20. Wand ME, Bock LJ, Bonney LC, Sutton JM. Retention of virulence following adaptation to colistin in Acinetobacter baumannii reflects the mechanism of resistance. *J Antimicrob Chemother* 2015;70 (8): 2209 – 2216.
21. United States Pharmacopoeial Convention, Inc. United States Pharmacopeia 32-National Formulary 23. Rockville, MD: US Pharmacopoeial Convention, Inc. Chapter < 1072> disinfectants and antiseptics, 2010.
22. Russell AD, Gould GW. Resistance of Enterobacteriaceae to preservatives and disinfectants. *J Appl Bacteriol Symp* 1988; 65:167S–195S.
23. Lambert RJW. Comparative analysis of antibiotic and antimicrobial biocide susceptibility data in clinical isolates of methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant Staphylococcus aureus and *Pseudomonas aeruginosa* between 1989 and 2000. *J Appl Microbiol* 2004;97, 699–711.
24. Abuzaid A, Hamouda A, Amyes SGB. *Klebsiella pneumoniae* susceptibility to biocides and its association with cepA, qacΔE and qacE efflux pump genes and antibiotic resistance. *J Hosp Infect* 2012; 81(2), 87–91.
25. Russell AD. Antifungal activity of biocides. In Principles and Practice of Disinfection, Preservation and Sterilization, 3rd edn (Eds. Russell, A.D., Hugo, W.B. & Ayliffe, G.A.J.) *Oxford: Blackwell Science*.,1999; pp.149-167.
26. Russell AD. Mechanisms of bacterial resistance to antibiotics and biocides. *Prog Med Chem* 1998;35,133–197.
27. Joynson JA, Forbes B, Lambert RJW. Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. *J Appl Microbiol* 2002;93, 96–107.
28. Alexander DM, Jeawon RH, Persad S. Disinfection resistance in antibiotic resistant organisms. *SA J Sci* 1991; 87,614–617.
29. Martro´ E, Herna´ndez A, Ariza J, Dominquez MA, Matas L, Argerich MJ, Martin MJ, Ausina V. Assessment of *Acinetobacter baumannii* susceptibility to antiseptics and disinfectants. *J Hosp Infect* 2003; 55: 39–46.
30. Russell AD, Tattawasart U, Maillard JY, Furr JR. Possible link between bacterial resistance and use of antibiotics and biocides. *Antimicrob Agents Chemother* 1998;42: 2151.
31. Fraise AP. Susceptibility of antibiotic-resistant cocci to biocides. *Symp Ser Soc Appl Microbiol* 2002;(31):158S-162S.
32. Kõljalg S, Naaber P, Mikelssar M. Antibiotic resistance as an indicator of bacterial chlorhexidine susceptibility. *J Hosp Infect* 2002; 51:106-113.
33. Tattawasart U, Maillard JY, Furr JR, Russell AD. Outer membrane changes in *Pseudomonas stutzeri* resistant to chlorhexidine and cetylpyridinium chloride. *Int J Antimicrob Agents* 2000;16: 233–238.
34. Andrews JM. The development of the BSAC standardization method of disc diffusion testing. *J Antimicrob Chemother* 2001;48: 29–42

Source of Support: Nil, Confl ict of Interest: None declared