Journal of Infection and Public Health 11 (2018) 812–816

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Distribution of biocide resistant genes and biocides susceptibility in multidrug-resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* — A first report from the Kingdom of Saudi Arabia

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ARTICLE INFO

Article history:
Received 9 January 2018
Received in revised form 21 May 2018
Accepted 28 May 2018

Keywords:
Chlorhexidine
Quaternary ammonium compounds
Disinfectant
Biocide resistant genes
qacA
qacE
cepA

ABSTRACT

Purposes: The aim of this study was to determine the frequency of biocide resistant genes, *qacA*, *qacE* and *cepA* in multidrug resistant (MDR) bacteria: *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* and to correlate the presence or absence of resistant genes with biocides susceptibility.
Materials and methods: The study included 44 MDR *K. pneumoniae, P. aeruginosa* and *A. baumannii* microorganisms. The bacteria were screened for the presence of biocide resistant genes by the polymerase chain reaction (PCR) method. The test organisms were isolated from various clinical specimens in the Qassim region, Saudi Arabia. The *in vitro* susceptibility tests of the three biocides (benzalkonium chloride, cetrimide and chlorhexidine gluconate) were studied against the test isolates by broth microdilution method following Clinical and Laboratory Standards Institute guidelines.
Results: With the distribution of biocide resistant genes in *K. pneumoniae*, all 9 isolates (100%) possessed *cepA*; 4 (44.4%) and 1 (11.1%) isolate contained *qacA* and *qacE* genes respectively. Among 24 isolates of *A. baumannii* tested, *cepA*, *qacA* and *qacE* genes were found in 54.2%, 16.7% and 33.3% of isolates respectively. Among 11 *P. aeruginosa* isolates, 63.6% contained *cepA* gene, 18.2% contained *qacE* genes, and none of the isolates harboured *qacA* gene. There was no significant correlation between presence or absence of biocide resistant genes and high MIC values of the test isolates ($p \geq 0.2$).
Conclusion: Our observations imply that there was no significant correlation between presence or absence of biocide resistant genes and MICs observed in MDR *K. pneumoniae, P. aeruginosa* and *A. baumannii.* Further studies are required to find to confirm the trend of reduced susceptibility to biocides of problematic nosocomial pathogens.

Introduction

The number of hospital-acquired infections (HAI) has been growing exponentially worldwide since 1980, especially due to the emergence of wide spread of multidrug-resistant (MDR) bacteria, most notably non fermentative bacteria and Enterobacteriaceae resistant to various antibiotics [1,2]. These MDR isolates readily spread in hospital environments. Therefore, hospital disinfection policies have a major role to play in the control of HAIs [3]. Biocides, including antiseptics and disinfectants, have been used extensively in hospitals and other healthcare settings for the disinfection of various medical devices and to reduce environmental bioburden. In particular, disinfectants play an essential role in infection control and the prevention of nosocomial transmission of infectious pathogens [4]. However, microbial resistance to biocides has raised concern and current procedures for infection control in hospitals have not been successful in curbing the rise of HAI by MDR pathogens [5,6]. There are now several laboratory reports concerning the emergence of a possible bacterial resistance to biocides; these are often in relation to exposure to a lower (sublethal) con-

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https://doi.org/10.1016/j.jiph.2018.05.011

centration of the biocide [7,8]. In addition, reduced susceptibility to biocides has been described for various nosocomial pathogens, inferring a molecular resistance mechanism [9–12].

As with antibiotic resistance, reduced susceptibility to biocides may be an intrinsic property or it may arise either by chromosomal gene mutation or by the acquisition of genetic material (biocide resistant genes (BRGs) such as *qac* and *cepA* genes) in the form of plasmids or transposons [13]. The emergence of bacterial reduced susceptibility to biocides and the possible linkage between biocide and antibiotic resistance is a relatively new and important concern: the phenomenon may lead to a failure in disinfecting environmental surfaces and furthering the spread of 'antibiotic- and disinfectant-resistant' nosocomial pathogens [14,15].

It is remarkable, in the context, that there is a large volume of publications which describe antibiotic resistance mechanisms and yet there is only a comparatively small number of studies available worldwide addressing the mechanisms of biocides reduced susceptibility [14–16]. Moreover, very few studies have discussed and analyzed minimum inhibitory concentration (MIC) values of biocides with antibiotics together with their resistance mechanisms. [17–19].

In the Kingdom of Saudi Arabia (KSA), MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii* bacteria are the major nosocomial pathogens. Despite the ubiquitous nature of these pathogens, their susceptibility towards common hospital antiseptics and disinfectants is largely unknown. To date, the underlying genetic mechanisms responsible for mediating susceptibility to disinfectants in relation to these pathogens have not been investigated by researchers in KSA. Therefore, for healthcare personnel there is a pressing need to understand the frequency of biocide resistant isolates in the region and to understand the optimal disinfection protocols to effectively control these nosocomial pathogens. Prompted by the paucity of such information, a systemic study was undertaken using the nosocomial MDR isolates: the Gram-negative bacteria: *Klebsiella pneumoniae*, *P. aeruginosa* and *A. baumannii.* Hence, the study described in this paper was undertaken in order to determine the frequency of BRGs in the bacterial MDR pathogens and to determine the correlation of these genes and their MICs against common biocides.

Materials and methods

Bacterial strains

The study included 44 MDR *K. pneumoniae, P. aeruginosa* and *A. baumannii* organisms. These bacteria were recovered from clinical sites from individual patients at the Hammadi hospital and Habib hospital of Qassim region, KSA from January to June 2015. All isolates included in this study were resistant to three or more antimicrobial classes of antibiotics and tested as per the standard antimicrobial susceptibility testing method following Clinical and Laboratory Standards Institute (CLSI) guidelines [20]. Microorganism identification and antimicrobial susceptibility testing were performed at a central bioscience research laboratory – College of Science Zulfi, Majmaah University – using a VITEK 2– compact 15 (bioMérieux, France) for cross verification. This study was approved by the ethics committee of hospitals and the deanship of scientific research, Majmaah University.

Biocides susceptibility testing

One biguanide of chlorhexidine gluconate 20% (Unilab Chemicals, India), two quaternary ammonium compounds (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 a review of the commonality of use of such agents in the global healthcare system). To prepare, each biocide was dissolved in sterile distilled water following the CLSI protocol; from this stock solutions of 1000 μg/mL were prepared. These were subsequently diluted in Mueller Hinton Broth (MHB) test medium (Himedia, India), a medium 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. Any turbid solutions visually compared with the McFarland standard for turbidity *vs.* cell concentration and later verified by measuring the absorbance of the suspension on a spectrophotometer (ThermoFisher scientific, USA). For this analysis, the absorbance should be in a range between 0.08 and 0.13 at 625 nm spectrophotometrically, which is equal to 1×10^8 CFU/mL. The test methodologies were followed as per Wiegand et al. [21].

MIC plate preparation

MIC plates were prepared by the method described previously [18]. The final well concentrations reached were 512 μg/mL–1 μg/mL after the addition of inoculum (50 μL). After the addition of inoculum, the microdilution plates were incubated at 37 °C for 16–20 h. 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. For a standard assay, 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.

Extraction of total DNA and PCR assay

Total genomic DNA of all test isolates were extracted using DNA Extraction Kit (Invitrogen, USA). All isolates were subject to screening for the presence of *cepA*, *qacA*, *qacE* genes were detected by PCR with the primers and annealing temperatures described by Guo et al., for *cepA* and Abuzaid et al., for *qacA* and *qacE* genes [14,17]. *cepA* primer pairs were designed to amplify 1051 base pair (bp) F5′ CAACTCCTTCGCCTATCCCG3′; R5′TCAGGTCAGACCAAACGGCG 3′ with annealing temperature 66 °C for 45 s. The *qacA* and *qacE* primer pairs F5′ GCTGCATTTATGACAATGTTTG 3′, R5′ AATCCCACCTACTAAAGCAG3′, F5′ GCCCTACACAAATTGGGAGA3′, R5′ TTAGTGGGCACTTGCTTTGG3′ were customized to amplify 629 bp and 350 bp respectively, both PCR assays performed same annealing temperature of 55 °C for 30 s. The PCR amplification products were detected by 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized by gel documentation system (Gel Doc EZ imager, Biorad laboratories, USA).

The significance of the frequency of BRGs and the range of MIC values among the various tested organisms were compared using Student's *t* test; here a *p* value less than 0.2 was considered as statistically significant.

Results

During the study period 44 non-repetitive MDR clinical isolates, made up of 24 *A. baumannii* isolates; 11 *P. aeruginosa* isolates; and 9 *K. pneumoniae* isolates, were selected by analyzing susceptibility test results. These isolates were resistant to most antimicrobials including cefepime, ceftazidime, amikacin,

Table. 1
Distribution of three different biocide resistant genes and biocides susceptibility values of each *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*.

Name of the isolate (n)	Organisms identification number	Biocide resistant genes			MIC value (μg/mL)		
		qacA	*qacE*	*cepA*	BKC	CET	CHG
K. pneumoniae (9)	KP 60	–	+	+	16	128	32
	KP 134	–	–	+	32	32	16
	KP 249	–	–	+	16	64	32
	KP 250	–	–	+	8	64	32
	KP 301	–	–	+	32	128	64
	KP 302	+	–	+	16	32	32
	KP 303	+	–	+	16	64	64
	KP 304	+	–	+	8	32	32
	KP 339	+	–	+	16	64	32
P. aeruginosa (11)	PA 3	–	–	+	32	128	8
	PA 9	–	–	–	64	>128	16
	PA 42	–	–	–	32	128	8
	PA 56	–	–	+	32	>128	16
	PA 62	–	–	+	32	128	16
	PA 68	–	–	–	512	>512	8
	PA 71	–	–	–	32	128	64
	PA 125	–	–	+	512	>512	16
	PA 131	–	+	+	64	128	8
	PA 142	–	+	+	64	>128	16
	PA 143	–	–	+	32	128	16
A. baumannii (24)	AB 4	–	–	–	8	32	32
	AB 8	–	–	–	16	64	32
	AB 19	–	–	–	8	64	32
	AB 22	–	–	–	16	32	32
	AB 30	–	+	–	8	32	32
	AB 32	–	–	+	8	32	32
	AB 43	–	+	+	8	64	32
	AB 44	–	–	+	8	32	32
	AB 50	+	–	–	8	32	32
	AB 57	–	+	+	8	32	32
	AB 59	+	–	+	8	32	32
	AB 61	–	+	+	16	128	32
	AB 74	–	+	–	8	32	32
	AB 80	–	+	+	8	32	32
	AB 83	–	–	+	8	32	32
	AB 126	–	–	+	8	64	32
	AB 130	–	+	–	8	32	32
	AB 132	–	–	–	16	64	32
	AB 133	–	+	+	8	32	32
	AB 135	–	–	+	32	32	32
	AB 146	+	–	–	8	32	32
	AB 161	–	–	+	16	64	32
	AB 162	+	–	–	8	32	32
	AB 169	–	–	+	16	64	16

+ Present, – absent; BKC — Benzalkonium chloride; CET — Cetrimide; CHG — Chlorhexidine gluconate.

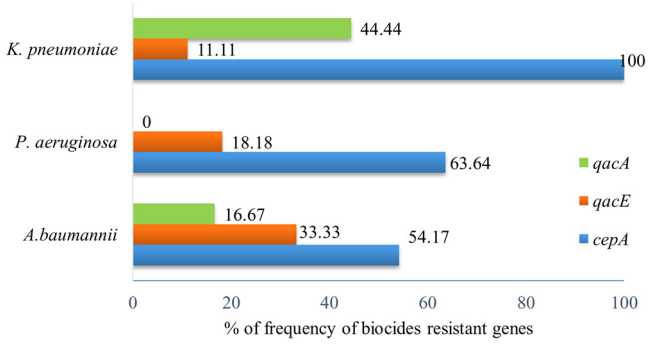

Fig. 1. Distribution of biocide resistant genes in MDR *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*.

gentamycin, tobramycin, piperacillin/tazobactam and carbapenem groups. The distribution of BRGs and the MIC outcomes of each biocide against all 44 clinical isolates were compared and listed in Table 1 and Fig. 1.

With the 9 strains of *K. pneumoniae* tested, all isolates (100%) having *cepA* and 4 (44.4%) and 1 (11.1%) isolates containing *qacA* and *qacE* genes respectively. Among 24 isolates of *A. baumannii* tested *cepA*, *qacA* and *qacE* genes were found in 54.2%, 16.7% and 33.3% isolates respectively. Among 11 test isolates of *P. aeruginosa*, 63.6% contained *cepA* gene and 18.2% were contained *qacE* genes, none of the isolates harbour *qacA* gene.

Regarding the MIC values of test isolates against three biocides ranged from 8 to >512 μg/mL and there was no observed reduced susceptibility of the biocides against the test cultures of *A. baumnaii* and *K. pneumoniae*. However, two isolates of *P. aeruginosa* (PA 68, PA125) showed reduced susceptibility against both QACs (≥512 μg/mL). Moreover, there was no significant correlation between presence or absence of BRGs and high MIC values of test isolates ($p > 0.2$). The overall distribution of MIC values of each test group of isolates against various biocides and the presence of BRGs are mentioned in Table 2.

Discussion

Globally there is paucity of studies mapping out the distribution of BRGs and susceptibility breakpoint data relating to MDR clinical strains [22,23]. Specifically there are no reports available relating to KSA. Thus, the aim of this present study was to evaluate the

Table 2
The overall distribution of MICs and biocide resistant genes in *K. pneumoniae, P. aeruginosa* and *A. baumannii.*

Organism (n)	Frequency of biocide resistant genes (number)		*MIC* range (μg/mL)			*p* Value*
	qacA or *qacE*	*cepA*	Benzalkonium chloride	Cetrimide	Chlorhexidine gluconate	
K. pneumoniae (9)	Positive (5)	Positive (9)	8–32	32–128	16–64	0.8248
	Negative (4)	Negative (0)	8–32	32–128	16–64	
P. aeruginosa (11)	Positive (2)	Positive (7)	32–512	128 to >512	8–16	0.5895
	Negative (9)	Negative (4)	32–512	128 to >512	8–64	
A.baumannii (24)	Positive (12)	Positive (13)	8–32	32–128	16–32	0.5421
	Negative (12)	Negative (11)	8–16	32–64	16–32	

* The result is not significant at $p < 0.2$.

frequency of BRGs in MDR *Acinetobacter*, *Pseudomonas*, *Klebsiella* isolates and make a correlation with susceptibility breakpoints to common biocides.

Genes conferring reduced susceptibility to quaternary ammonium compounds are called *qac* genes. So far, a range of various *qac* genes have been described and these genes are widely spread among clinical and environmental bacteria [24–26], as seen among two genes (*qacA* and *qacE*) which were analyzed in this study. The frequency of *qacA* and *qacE* observed in *K. pneumoniae* are coincidental with recent studies performed by Guo et al., where the researchers found 41% *qacA* and 11.1% *qacE* in carbapenem resistant *K. pneumoniae* [14]. Another test isolate, *A. baumannii* was found to be carrying 16.7% of *qacA* genes and 33.3% of *qacE* genes. This is comparable with recent study conducted by Babaei et al., which while reporting no positive outcomes for *qacA*, found 73% of tested isolates were carrying *qacE* genes [26]. This is a higher rate than in the study presented here. However, another study from Iran reported that 40% of *A. baumannii* clinical isolates were carrying *qacE* genes, which is very similar to the present study [27]. However, many reports have shown the frequency of *qacA* gene to be predominantly in Gram-positive bacteria and less so in Gram-negative bacteria [24–26]. This reaffirms the findings of our study where we found that none in *P. aeruginosa* and only a low percentage in *A. baumannii*.

In relation to the next gene, *cepA, this* is associated with chlorhexidine resistance in *K. pneumoniae* and possibly in other related Gram-negative bacteria [9,22]. Therefore, we attempted to find out the distribution of *cepA* genes among the test population and it was detected 100% in *K. pneumoniae,* 63.6% in *P. aeruginosa* and 54.2% in *A. baumannii*. This is similar to the findings of Abuzaid et al., conducted in the United Kingdom, where it was reported that 87.5% of *K. pneumoniae* clinical isolates harbored *cepA* genes [17]. It is noteworthy that Azadpour et al. reported that the presence of *cepA* gene in clinical isolates of *K. pneumoniae* in Iran was 22.4% [28]. However, there are no reports are available to compare the frequency of *cepA* genes in *Acinetobacter* and *Pseudomonas* [17,22]. Hence, we could not compare this with other findings.

Regarding the *in vitro* efficacy of the data presented in our findings were agreement with various reports by Wand et al. [29] Lambert et al. [19] and Abuzaid et al. [17]. Russell and Gould reported that the MIC value of benzalkonium chloride against *P. aeruginosa* was 250 μg/mL, which is comparable with our study findings; however high MIC values were observed in this study population [30]. Regarding the cetrimide MICs of *P. aeruginosa,* data showed a strong resemblance to that of the study by Russel et al. [31].

With our 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. [12]. With *P. aeruginosa* and *K. pneumoniae* the results of this study are comparable with research by McDonnell and Russell, where the MICs of chlorhexidine against *P. aeruginosa* was found to be between 5–60 μg/mL [9]; moreover, Guo et al. found similar ranges [14]. Overall, comparing the available reports this showed variations of MIC values described as one or two dilutions in the present study and there was no reduced susceptibility observed in *A. baumannii* and *K. pneumoniae*; however, two *P. aeruginosa* isolates showed reduced susceptibility against benzalkonium chloride and cetrimide. Moreover, the variations of MIC values and reduced susceptibility will not affect the disinfection program. This is because, in practice, biocides are typically used at concentrations well in excess of MIC values (more than 100–200 times). At the same time, MICs provide a useful reference point for biocides when used as preservatives, whereby the prevention of microbial multiplication and reduction of viability to official levels are more appropriate than inactivation. Thus, reduced susceptibility or high MIC values of biocides does not infer resistance. Hence, the present study investigated the correlation of these MIC values with frequency of BRGs. Here, by comparing reduced susceptibility of two strains of *P. aeruginosa* with presence of BRGs, only one isolate harbors *cepA* gene, and other isolate contained none. Indeed, the present study results showed that there is no significant correlation between reduced susceptibility and harboring genes with other tested isolates. The present study results indicated that reduced susceptibility to the biocides and multidrug resistant was independent of the presence and absence of *cepA*, *qacA* and *qacE* genes. There are similar findings observed by Azadpour et al. [28], where the researchers reported that no significant association of reduced susceptibility to biocides with the presence of *qacEΔ1* and *cepA* genes in *K. pneumoniae*; and in a separate review by Naparstek et al. [15], which did not find a correlation between chlorhexidine susceptibility and *cepA* gene expression [16]. A further study conducted using 122 isolates of MDR *A. baumannii* showed that no significant difference was observed in the MIC of biocides and the presence or absence *qacE* gene [26]. Interestingly another study by Lie et al. reported that carbapenem resistant *A. baumannii* strains harboring *qacE* displayed a higher MIC (64 μg/mL) for benzalkonium chloride at the same time *qacEΔ1* genes have no significant impact on reduced susceptibility [32].

Consequently, the distribution and transmission of bacterial reduced susceptibility to biocides is likely to be affected and constrained by biological, physical and socio-economic factors and these vary among different countries, regions and communities. The reason for the difference may be due to variations with the study population, BRGs, and MIC analysis of different group of biocides. According to this observation, a large-scale geographical specific study is required for each specific nosocomial pathogen in order to confirm the reduced susceptibility and presence of BRGs.

Conclusion

Overall, we show here, that a high frequency of *cepA* genes was observed in *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* compared with *qacA* and *qacE* genes. None of the *P. aeruginosa* isolates harbored *qacA* genes. There were no significant correlation between presence or absence of BRGs and MICs observed in MDR *K. pneumoniae, P. aeruginosa* and *A. baumannii*. As far as we know, the present study is the first report showing distribution of BRGs and their

biocide MIC values among MDR *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* from the KSA. Consequently, there is a need for a large-scale study, using isolates from hospitals, to confirm the trend of reduced susceptibility to biocides of 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, Saudi Arabia.

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