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ORIGINAL ARTICLE

Prevalence of resistance genes to biocides in antibiotic-resistant *Pseudomonas aeruginosa* clinical isolates

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Abstract

Background Biocides are frequently used as preservative, disinfectant and sterilizer against many microorganisms in hospitals, industry and home. However, the reduced susceptibility rate of *Pseudomonas aeruginosa* (*P. aeruginosa*) strains to biocides is increasing. The aim of this study was to evaluate the antimicrobial activity of four frequently used biocides against *P. aeruginosa* and to determine the prevalence of genes involved in biocide resistance.

Methods A total of 76 clinical isolates of *P. aeruginosa* strains were used in the present study. The minimum inhibitory concentrations (MICs) of four biocides, *i.e.* chlorhexidine digluconate, benzalkonium chloride, triclosan and formaldehyde, against *P. aeruginosa* strains were determined using agar dilution method. In addition, the prevalence of biocide resistance genes was determined using the polymerase chain reaction (PCR) method.

Results In the present study, the highest MIC_{90} and MIC_{95} (epidemiological cut-off) values were observed for benzalkonium chloride (1024 µg/mL), followed by formaldehyde (512 µg/mL), triclosan (512 µg/mL) and chlorhexidine digluconate (64 µg/mL). Furthermore, the prevalence of *qacEΔ1*, *qacE*, *qacG*, *fabV*, *cepA* and *fabI* genes were 73.7% (n = 56), 26.3% (n = 20), 11.8% (n = 9), 84.2% (n = 64), 81.5% (n = 62) and 0% (n = 0), respectively. A significant association was observed between the presence of biocide resistance genes and MICs ($p < 0.05$). Furthermore, there was no significant association between the presence of biocide resistance genes and antibiotic resistance ($p > 0.05$), except for levofloxacin and norfloxacin antibiotics and *qacE* and *qacG* genes ($p < 0.05$).

Conclusion Our results revealed that chlorhexidine digluconate is the most effective biocide against *P. aeruginosa* isolates in Ardabil hospitals. However, we recommend continuous monitoring of the antimicrobial activity of biocides and the prevalence of biocide-associated resistance genes for a better prevention of microorganism dissemination and infection control in hospitals.

Keywords *Pseudomonas aeruginosa* · Resistance · Triclosan · Benzalkonium chloride · Chlorhexidine digluconate · Formaldehyde

Abbreviations

MIC	Minimum inhibitory concentration
PCR	Polymerase chain reaction
P. aeruginosa	*Pseudomonas aeruginosa*
ICU	Intensive care unit
MDR	Multi-drug resistant

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CLSI	Clinical and Laboratory Standards Institute
ECOFF	Epidemiological cut-off value
ENR	Enoyl-acyl-carrier protein reductase

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacillus, which is frequently isolated from hospital environments, especially from medical equipments used in intensive care units (ICUs) [1–3]. *P. aeruginosa* is an opportunistic human pathogen that is associated with various community-acquired and nosocomial infections such as ventilator-associated pulmonary infections, skin and soft-tissue infections, catheter-related urinary tract infections, eye and ear infections, bloodstream infections, endocarditis and surgical site/transplantation infections [1–3]. Therefore, it seems that hygiene control of surfaces and medical equipments in the hospital settings in terms of *P. aeruginosa* contamination is important because nosocomial infections are considered as a growing global threat in terms of economical and public health [3]. The most common antiseptic and disinfectant biocides used in clinical settings are chlorhexidine digluconate (a biguanide, disrupting the cell membrane), benzalkonium chloride (a quaternary ammonium compound, disrupting the cell membrane), triclosan (a bisphenol, blocking of lipid biosynthesis) and formaldehyde (an aldehyde, alkylating agent) [4, 5]. However, several studies have reported bacterial resistance to various biocides due to the presence of resistance genes [6]. On the other hand, the risk of *P. aeruginosa* infections in ICU-hospitalized patients is high (up to 30%) despite applying hygiene programs [5]. Such a high prevalence can be attributed to the reduced susceptibility of *P. aeruginosa* strains against a variety of antiseptics and disinfectants over time [7]. Another problem is the emergence of multi-drug resistant (MDR) *P. aeruginosa* strains, which can lead to treatment failure [3]. Interestingly, *P. aeruginosa* strains have shown cross-resistance to biocides and antibiotics with probably similar mechanisms, thereby making bacterial elimination from hospital environments difficult [5, 8]. Therefore, obtaining information on the prevalence and mechanisms of bacterial resistance to antimicrobial agents and the selection of suitable biocides and antibiotics can be helpful for controlling hospital-acquired infections caused by *P. aeruginosa*. Several mechanisms of resistance to antibiotics and tolerance to biocides have been identified in *P. aeruginosa* strains including: 1) simple growth requirements and ability of biofilm formation, 2) enzymatic degradation, 3) target site modification, 4) outer membrane impermeability, and 5) presence of efflux pumps. These properties are involved in bacterial persistence in medical environments and development of nosocomial infections [2, 9–14]. Among these resistance mechanisms to biocides, efflux pumps encoded by *qacEΔ1*, *qacE*, *qacG* and *cepA* genes play an important role in *P. aeruginosa* resistance to benzalkonium chloride and chlorhexidine [14]. On the other hand, *fabV* gene confers high-level triclosan resistance. Various compounds are used in hospitals and non-hospital environments in Ardabil city but there is no exact data on the rate of biocide effectiveness against local isolates of *P. aeruginosa*. Moreover, the mechanisms of biocide resistance in *P. aeruginosa* clinical isolates of Ardabil are still unclear. Therefore, the aim of the current study was to assess the distribution of biocide resistance genes, namely *qacEΔ1*, *qacE*, *qacG*, *fabV*, *cepA* and *fabI*, and to determine the minimum inhibitory concentration (MIC) and the epidemiological cut-off (ECOFF) values of various biocides against antibiotic-resistant *P. aeruginosa* strains isolated from clinical samples in Ardabil.

Methods

Data on *P. aeruginosa* isolates

A total of 76 confirmed clinical *P. aeruginosa* isolates were obtained from various specimens in Ardabil hospitals and then used to assess the distribution of biocide resistance genes as well as determination of the MIC and the ECOFF of four biocides. Drug resistance characteristics of *P. aeruginosa* isolates was evaluated using the disk diffusion method based on the Clinical and Laboratory Standards Institute (CLSI, 2018) guideline [15]. Data on the prevalence of class I integron, harboring resistance genes to biocides and antibiotics, was used in this study in order to evaluate the association with resistance to biocides. It is noteworthy, the prevalence of antibiotic resistance along with class I integrin rate were previously determined by authors [16, 17].

Preparation of biocide solutions

Biocides used in this study were chlorhexidine digluconate (20%) (Sigma-Aldrich, USA), benzalkonium chloride (>95%) (Sigma-Aldrich, USA), triclosan (98%) (Bio Basic, Canada) and formaldehyde (37%) (Thermo Fisher Scientific, USA). Stock solutions of antimicrobial agents were prepared in distilled water or water-alcohol for water-insoluble antimicrobial agents. All antibacterial solutions were sterilized using sterile syringe filters (0.22 µm) before use.

Determination of the MICs of biocides

The MICs of antiseptics and disinfectants including chlorhexidine digluconate, benzalkonium chloride, triclosan and formaldehyde were determined by agar dilution technique

and according to the CLSI guideline [15]. The CLSI and similar organizations have not been defined a standard protocol describing bacterial resistance or susceptibility against non-therapeutic antimicrobials in susceptibility tests. Therefore, estimation of the ECOFF value for each biocide was done based on the MIC distributions *in vitro*. For this purpose, at first a range of biocide concentrations (0.125-1024 µg/mL) was prepared in Mueller-Hinton agar medium and then a 0.5 McFarland standard concentration of *P. aeruginosa* isolates (1.5×10^8 CFU/mL) was prepared in normal saline. Finally, diluted bacterial inoculum (1:10) (1.5×10^7 CFU/mL) was poured onto medium containing different biocides as a spot (10^4 CFU per spot). The plates were incubated at 37 ˚C overnight and then checked in terms of bacterial growth. In the current study, the ECOFF value for the antibacterial susceptibility testing against four biocides was determined equal to MIC_{95} (95% rule). Therefore, *P. aeruginosa* strains with higher MIC value compared with ECOFF value were considered as non-wild-type isolates, *i.e.* organisms with detectable resistance and reduced susceptibility for each biocide.

Detection of biocide resistance genes

Extraction of total genomic DNA was done using a simple boiling method according to previous instructions [18]. DNA quantity and quality was controlled using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, USA) and then validated using sequencing of amplified genes. Extracted DNA was stored at -20 ˚C until use for detection of biocide resistance genes. The presence of *qacEΔ1*, *qacE*, *qacG*, *fabV*, *cepA* and *fabI* genes was detected by specific primers in polymerase chain reaction (PCR). According to the thermal cycling condition for amplification and the oligonucleotide primer sequences presented in Table 1, PCR was performed in a volume of 25 µL containing 20 µL of master mix (Ampliqon, Denmark), 1 µL of each primers (10 µmol/L) and 3 µL of extracted DNA. PCR products were detected by electrophoresis on 1% agarose gel in a TBE 0.5x buffer and then confirmed by sequencing technique. In addition, a *fabI* gene positive *Acinetobacter baumannii* clinical isolate was used as positive control. Also, in this study, identified positive isolates for *qacEΔ1*, *qacE*, *qacG*, *fabV* and *cepA* genes were used for quality control.

Data analysis

All data on the MICs of biocides against *P. aeruginosa* strains, the presence of resistance genes to biocides and *P. aeruginosa* resistance to various antibiotics were collected and their correlation analyzed by the SPSS software version 16. The Chi-square test was used to analyses and a p value of <0.05 was considered statistically significant.

Results

In the present study, among 76 *P. aeruginosa* clinical isolates, the prevalence of *qacEΔ1*, *qacE*, *qacG*, *fabV*, *cepA* and *fabI* genes were 73.7% (n=56), 26.3% (n=20), 11.8% (n=9), 84.2% (n=64), 81.5% (n=62) and 0% (n=0), respectively. As shown in Table 2, the MIC range for various biocides was as follows: benzalkonium chloride

Table 1 Used primers and PCR programs in the present study

Gene	Primer sequence	Thermal cycling condition					Amplicon size (bp)	Reference
		Initial denaturation (°C/time)	Denaturation (°C/time)	Annealing (°C/time)	Extension (°C/time)	Cycle		
qacEΔ1	F: AATCCATCCCTGTCGGTGTT	94	94	53	72	30	190	[19]
	R: CGCAGCGACTTCCACGATGGGGAT	4 min	1 min	50 s	1 min			
qacE	F: TTAGGATGGAGACGAAATTTTCA	94	94	59	72	30	240	[This study]
	R: CGCTTAACACCTAGTATTATTACCGT	4 min	1 min	1 min	1 min			
fabI	F: ATGCTGAAAATTGTTTTGAGTGAGA	94	94	59	72	30	830	[This study]
	R: TTCATCATCCTTCATAGATTGGCTC	4 min	1 min	1 min	1 min			
qacG	F: TTGAATAATTGGTTATTTCTGGCT	94	94	59	72	30	333	[This study]
	R: TTAGTGAACACTTGCCTTAGATAG	4 min	1 min	1 min	1 min			
cepA	F: GCTCGCTGATGTCGGTAGG	94	94	59	72	30	481	[This study]
	R: CTGCTGGCAGTGCACTATTC	4 min	1 min	1 min	1 min			
fabV	F: TCGACCTGGTGGTCTACAGC	94	94	59	72	30	530	[This study]
	R: GACCTGCTCGATGCAACC	4 min	1 min	1 min	1 min			

Table 2 Minimal inhibitory concentration of biocides against clinical isolates of *P. aeruginosa*

Biocides	MIC (µg/mL)										ECOFF* (µg/mL)
	4	16	32	64	128	256	512	1024	MIC_{50}	MIC_{90}	
Formaldehyde	–	–	2 (2.6%)	47 (61.9%)	15 (19.7%)	–	12 (15.8%)	–	64	512	512
Benzalkonium chloride	–	–	–	–	-	12 (15.8%)	53 (69.7%)	11 (14.5%)	512	1024	1024
Triclosan	–	–	11 (14.5%)	3 (3.9%)	5 (6.6%)	–	57 (75%)	–	512	512	512
Chlorhexidine digluconate	4 (5.3%)	10 (13.2%)	22 (28.9%)	40 (52.6%)	–	–	–	–	64	64	64

*ECOFF-epidemiological cut-off value

256-1024 µg/mL, formaldehyde 32-512 µg/mL, triclosan 32-512 µg/mL and chlorhexidine digluconate 4-64 µg/mL. In total, the highest MIC_{90} was observed for benzalkonium chloride (MIC_{90}=1024 µg/mL), followed by formaldehyde (MIC_{90}=512 µg/mL), triclosan (MIC_{90}=512 µg/mL) and chlorhexidine digluconate (MIC_{90}=64 µg/mL), Therefore, it seems that chlorhexidine digluconate and benzalkonium chloride had the highest and the lowest effects, respectively, in terms of growth inhibition of *P. aeruginosa* isolates in this study. As shown in Table 3, a significant association was observed between the presence of biocide resistance genes and MICs ($p<0.05$). Additionally, biocide resistance gene profiles revealed that isolates simultaneously harboring *qacEΔ1, cepA* and *fabV* genes are more prevalent (n=35, 46%), while 8 isolates (10.5%) did not harbor any biocide resistance genes (Table 4). Moreover, strains containing biocide resistance genes had higher MIC_{50} and MIC_{90} values compared with isolates without these genes. In this study, ECOFF value for the reduced susceptibility to benzalkonium chloride, triclosan, formaldehyde and chlorhexidine digluconate were determined as 1024, 512, 512 and 64 µg/mL, respectively.

Isolates with and without biocide resistance genes were compared in terms of resistance rate to the following antibiotics: piperacillin, piperacillin-tazobactam, ticarcillin-clavulanate, ceftazidime, cefepime, aztreonam, doripenem, imipenem, meropenem, gentamicin, tobramycin, amikacin, netilmicin, ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin and ofloxacin. There was no significant association between the presence of biocide resistance genes and antibiotic resistance ($p>0.05$), except for levofloxacin and norfloxacin antibiotics and *qacE* and *qacG* genes ($p<0.05$).

Table 3 Association between biocide resistance genes and MIC

Gene	Biocide (MIC)							
	Formaldehyde		Benzalkonium chloride		Triclosan		Chlorhexidine	
	≤128	256≤	≤256	512≤	≤128	256≤	≤16	32≤
qacEΔ1+	46	10	3	53	5	51	6	50
qacEΔ1-	18	2	9	11	13	7	8	12
p value	0.019		0.00		0.00		0.02	
qacE+	15	6	2	19	2	19	2	19
qacE-	49	6	10	45	16	39	12	43
p value	0.24		0.00		0.426		0.491	
qacG+	7	2	0	9	0	9	0	9
qacG-	57	10	12	55	18	49	14	53
p value	0.88		0.00		0.53		0.32	
cepA+	50	12	7	55	8	54	9	53
cepA-	14	0	5	9	10	4	5	9
p value	0.00		0.03		0.00		0.00	
fabV+	52	12	7	57	8	56	10	54
fabV-	12	0	5	7	10	2	4	8
p value	0.00		0.01		0.00		0.00	

Table 4 Gene profile and minimal inhibitory concentration of biocides

Gene	Isolate (n)	Chlorhexidine digluconate		Triclosan		Benzalkonium chloride		Formaldehyde	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{90}	MIC_{50}
qacEΔ1	2	32	32	0.5	64	256	512	128	64
qacE	1	32	32	512	512	512	512	64	64
qacEΔ1 + *cepA*	1	64	64	512	512	512	512	64	64
qacEΔ1 + *fabV*	2	32	64	512	512	512	512	128	128
cepA + *fabV*	5	32	64	128	512	256	512	64	64
qacE + *cepA* + *fabV*	6	32	64	512	512	512	1024	512	128
qacEΔ1 + *cepA* + *fabV*	35	64	64	512	512	512	512	512	64
qacEΔ1 + *qacE* + *cepA* + *fabV*	7	64	64	512	512	512	1024	512	64
qacEΔ1 + *qacG* + *cepA* + *fabV*	2	64	64	512	512	512	1024	64	64
qacEΔ1 + *qacE* + *qacG* + *cepA* + *fabV*	7	64	64	512	512	1024	1024	512	128
No gene	8	4	64	0.5	64	256	512	64	64

The prevalence of integron I positive *P. aeruginosa* strains harboring *qacEΔ1* gene was 32 out of 76 (42.1%). No significant association was observed between the presence of class I integron and biocide resistance genes (*qacEΔ1, qacE, cepA* and *fabV)*, except for *qacG* gene ($p = 0.00$).

Discussion

Biocides are basic compounds to control microorganism dissemination and ensuing infections and are frequently used as preservative, disinfectant and sterilizer against various microorganisms, in particular *P. aeruginosa* [20, 21]. One of the most useful biocides against microbes, especially Gram-positive bacteria, is triclosan, which is widely used in toothpastes, soaps and other daily products [20]. Triclosan is an anionic and lipophilic compound, which its anti-bacterial function stems from inhibition of enoyl-acyl-carrier protein reductase (ENR), an enzyme involved in fatty acid synthesis [22–24]. However, *P. aeruginosa* strains are inherently resistant to this biocide (MIC>2000 µg/mL) [24]. ENR enzymes show diversity among different bacteria in terms of sequence and structure, and contain four isozymes including FabI (triclosan- sensitive ENR), FabL, FabV and FabK (triclosan-resistant ENRs) [22–24]. The FabV isozyme is involved in swimming motility, energy metabolism, protein secretion and adherence, and is responsible for *P. aeruginosa* resistance to triclosan biocide [24]. Genes encoding FabI and FabV enzymes are found in most bacterial chromosomes such as *P. aeruginosa* [22–24]. Bacterial resistance to triclosan is associated with mutation in the active site of *fabI* gene and the presence of *fabV* gene [20, 24]. In the current study, the prevalence *fabI* resistance gene among *P. aeruginosa* isolates was 0%. Unlike *fabI* gene, the frequency of *fabV* gene was high in the present study (84.2%). Zhu *et al.* showed that deletion of *fabV* gene confers extremely high susceptibility to triclosan (>2,000 folds) in *P. aeruginosa* isolates [22]. Similar result was reported by Huang *et al.* [24]. In this study, the MIC_{50} and MIC_{90} values of triclosan for *fabV* resistance gene- harboring *P. aeruginosa* strains was higher than *fabV* gene-negative strains (Table 4).

Chlorhexidine digluconate, an antiseptic, disinfectant and preservative, is a bactericidal biocide, which has higher antibacterial activity against Gram-positive compared with Gram-negative bacteria [25]. This biocide is used in oral health antiseptics, hand washes and other hygienic solutions. The antibacterial mechanism of chlorhexidine digluconate is *via* the bacterial cell membrane [20]. However, *P. aeruginosa* is intrinsically resistant to this biocide due to the presence of an outer membrane [25]. Adaptive resistance to chlorhexidine biocide is mediated by a membrane protein encoded by *Acinetobacter* chlorhexidine efflux gene (*aceI*). The AceI protein identified in *Acinetobacter baumannii* is involved in chlorhexidine efflux *via* an energy-dependent mechanism [26]. However, genes encoding this protein were not identified in *P. aeruginosa* strains in the current study (data not shown). The antiseptic resistance gene *cepA*, an efflux pump gene, is associated with chlorhexidine resistance in Gram-negative bacteria causing high chlorhexidine MICs [27, 28]. In our study, 62 (81.5%) *cepA*-positive strains were found, which is higher than those reported by Mendes *et al.* (44.5%) and Vijayakumar *et al.* (63.6%) [27, 28]. According to MIC results, chlorhexidine digluconate is more effective than other biocides against *P. aeruginosa* isolates (MIC range=4–64 µg/mL) (Table 2). In this study, the presence of *cepA* gene had variable effects on the MIC_{50} and MIC_{90} values of chlorhexidine (Table 4).

A major biocide resistance mechanism in Gram-negative bacteria including *P. aeruginosa* is the action of efflux pumps such as the small multidrug resistance family (SMR)

[14, 21]. Biocide resistance genes *qacEΔ1*, *qacE* and *qacG* encode multidrug efflux pumps, which confer resistance to quaternary ammonium compounds like benzalkonium chloride [14, 21]. In our study, the *qacEΔ1* gene was observed in 73.7% of clinical isolates of *P. aeruginosa*, while in studies conducted by Subedi *et al*., Roma˜o *et al*., Kücken *et al*., Helal *et al*. and Mahzounieh *et al*. the *qacEΔ1* gene was detected in 46.1%, 48%, 10%, 48% and 91.5% of the isolates, respectively [14, 21, 29–31]. According to the reports of Subedi *et al*., Kücken *et al*., Helal *et al*. and Mahzounieh *et al*., 100%, 2.7%, 13.5% and 50% of *P. aeruginosa* strains, respectively, had the *qacE* gene [14, 29–31], while we detected this gene in 26.3% of isolates. The frequency of *qacG* gene in the present study was 11.8%, which is higher compared to the frequency reported by Subedi *et al*. (0%) [14]. The MIC_{50} and MIC_{90} values of benzalkonium chloride were significantly high for *qacEΔ1*-, *qacE*- and *qacG*-positive *P. aeruginosa* strains compared with the negative strains (Table 4).

Class I integron carries *qacEΔ1* and antibiotic resistance genes in clinical isolates of *P. aeruginosa* [14]. Therefore, *P. aeruginosa* strains harboring class I integron are resistant to benzalkonium chloride and various antibiotics [29]. Comparison of our current and previous study [17] showed that the frequency of integron I-positive *P. aeruginosa* strains harboring *qacEΔ1* gene was 32 out of 76 (42.1%). No significant association was observed between the presence of class I integron and biocide resistance genes (*qacEΔ1, qacE, cepA* and *fabV*), except for *qacG* gene ($p = 0.00$).

Formaldehyde is an organic electrophilic biocide, which its mechanism of action involves cross-linking of macromolecules (proteins, RNA and DNA) [20, 32]. Our results indicated that the MIC_{50} and MIC_{90} values of formaldehyde were high for biocide resistance genes-positive *P. aeruginosa* strains compared with the negative strains (Table 4).

A study by Chuanchuen *et al*. showed a cross-resistance between biocide and antibiotic resistance. They demonstrated a link between *P. aeruginosa* exposure to triclosan biocide and efflux-mediated resistance to ciprofloxacin [13]. In the present study, there was no significant association between biocide resistance genes and antibiotic resistance, except for levofloxacin and norfloxacin antibiotics and *qacE* and *qacG* genes. However, more studies are needed to substantiate the existence of biocide-antibiotic cross-resistance.

Conclusion

Our results revealed that the frequency of resistance genes to benzalkonium chloride, chlorhexidine digluconate, triclosan and formaldehyde was high in clinical isolates of *P. aeruginosa*. Furthermore, *P. aeruginosa* isolates harboring resistance genes had higher MIC values compared with those lacking these genes. On the other hand, chlorhexidine digluconate was the most effective biocide against *P. aeruginosa* isolates in Ardabil hospitals. We recommend continuous monitoring of the antimicrobial activity of biocides and biocide-associated resistance genes in order to prevent microorganism dissemination and infection control in hospitals.

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Authors' contributions MN, SS and SAB collected the data. FK and HV analyzed the data and led the writing of the manuscript. SH, MA and AS revised the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This research was approved by the Research Ethics Committee of Ardabil University of Medical Sciences.

Consent to participate Informed written consent was given to subjects from whom the samples were obtained for this study.

Consent for publication Not applicable.

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