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High Prevalence of Antiseptic Resistance Encoding Genes and Reduced Phenotypic Antiseptic Susceptibility Among Antibiotic-Resistant *Pseudomonas aeruginosa* Isolates

Mohadeseh Radmehr[1], Majid Moghbeli[1], Hamed Ghasemzadeh-Moghaddam[2,*], Amir Azimian[2] and Alex van Belkum[3]

[1]Department of Microbiology, Damghan Branch, Islamic Azad University, Damghan, Iran
[2]Vector-Borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran
[3]Open Innovation & Partnerships, BaseClear, Leiden, Netherlands

[*]*Corresponding author*: Vector-Borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran. Email: hamedghupm@gmail.com

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Abstract

Background: Multi-drug resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* isolates are of clinical concern.

Objectives: To determine the distribution of antiseptic resistance genes and the associated level of phenotypic antiseptic resistance against quaternary ammonium compounds and biguanide compounds, we studied MDR and XDR *P. aeruginosa* isolates collected from different infections among patients from a single hospital.

Methods: *Pseudomonas aeruginosa* isolates were investigated in 2020 for *in vitro* susceptibility to benzethonium chloride (BTC), benzalkonium chloride (BKC), and chlorhexidine digluconate (CHG). The minimum inhibitory concentrations (MICs) against these antiseptic agents were determined using broth microdilution. Also, polymerase chain reaction (PCR)-mediated detection of *qac*E, *qac*EΔ1, and *bla*OXA-23 genes was used.

Results: Isolates were largely non-clonal according to their phenotypical and genotypical non-similarity (35 overall data-combination types detected). Most *P. aeruginosa* infections occurred in intensive care unit (ICU) patients (n = 43, 61.4%). Extensively drug-resistant and MDR phenotypes were detected in 20% and 12.6%, respectively. Among the 70 isolates retained, 53 (75.7%) harbored at least one resistance gene, comprising 11 (20.7%) isolates with solely the *qac*EΔ1 gene; seven (13.2%) isolates harbored the *qac*E gene. Both the *qac*E and *qac*EΔ1 genes were detected simultaneously in 35 (66%) isolates. The mean MICs for BTC (24.0 versus 10.56 μg/mL), BKC (46.1 versus 17.22 μg/mL), and CHG (107.7 versus 29.4 μg/mL) were statistically significantly higher among antiseptic resistance gene harboring isolates than in other isolates without such genes.

Conclusions: The significantly increased MICs against antiseptic agents among antibiotic-resistant *P. aeruginosa* isolates highlight the importance of monitoring such increases and implementing effective infection control.

Keywords: *Pseudomonas aeruginosa*, Antiseptic Resistance, Quaternary Ammonium Compound, Biguanide Compounds, *qac*E, *qac*EΔ1

1. Background

Pseudomonas aeruginosa is a saprophytic gram-negative bacterial species that can be isolated from soil, plants, and hospital environments (1). *Pseudomonas aeruginosa*-related infections cause death in patients who suffer from bacteremia (2), intensive care unit (ICU)-related septicemia (3), ventilator-related pneumonia, and even urinary tract infections (4, 5). Multiple drug-resistant (MDR) and even extensively drug-resistant (XDR) phenotypes among *P. aeruginosa* have been reported frequently and at elevated prevalence (4 - 60%) (6, 7). Reduced cytoplasmic membrane permeability, expression of efflux pump-related genes, the release of antibiotic-destroying enzymes such as beta-lactamases, and alginate production are the most common antibiotic resistance mechanisms among *P. aeruginosa* strains (8-10).

Efflux pumps are a group of proteins transferring energy packages, antibiotics, and other small molecules out of the bacterial cytoplasm or periplasmic space.

Continuous transfer of antibiotics out of bacteria will increase the minimum inhibitory concentrations (MIC) for such antibiotics and antiseptic agents. It helps the bacteria continue growing while the antibiotic or antiseptic target is still present in the bacterial cytoplasm (8-10). Also, *bla*OXA-23, *bla*OXA-24, and *bla*OXA-40 are genes responsible for resistance against different carbapenem antibiotics (11).

Utilizing disinfectants with quaternary ammonium compounds (QAC) and biguanide compounds included is one of the most applied prevention strategies against nosocomial infection (12). Reduced susceptibility against the active agents of the mentioned antiseptics has been reported before (13, 14). Antiseptic resistance may result from the expression of chromosomal genes and plasmid-located genes, including biocide resistance genes (BRGs) and *qac* (15). The *qac*E gene has been reported as one of the genes encoding resistance against QACs and chlorhexidine digluconate (CHG) among *Enterobacterales* and different *Pseudomonas* spp. (16).

2. Objectives

The current study investigated the prevalence of antiseptic resistance genes and the resulting increase in MIC against QACs and CHG among *P. aeruginosa* isolates from different infections among patients in the Imam Hassan Hospital (IHH). The IHH is the biggest referral teaching and care hospital in North Khorasan province of Iran.

3. Methods

3.1. Study Samples

All *P. aeruginosa* isolates causing infections at different anatomical sites were collected by tracheal aspirate culture, blood culture, urine culture, and wound culture. Clinical specimens originated from hospitalized patients in various hospital wards (ICU, Cardiology, Emergency Department, Infectious Diseases Department, and Neurology) in the IHH during 2020. Bacteria were identified at the species level in the hospital laboratory, and this was confirmed in the microbiology laboratory at the Faculty of Medicine by Gram staining, oxidase testing, motility testing, and defining the ability of growth at 42°C according to Clinical and Laboratory Standard Institute (CLSI) guidelines (17). All isolates were stored at -30°C in trypticase soy broth (TSB) supplemented with 20% glycerol.

3.2. Antiseptic Susceptibility Testing

Bacterial susceptibilities to benzethonium chloride (BTC), benzalkonium chloride (BKC), and biguanide compounds such as CHG (Sigma-Aldrich, Steinheim, Germany) were determined using the Mueller-Hinton broth microdilution method (BMD) (18).

3.3. Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed on Mueller-Hinton agar (Merck, Germany) using the disk diffusion (Kirby-Bauer) technique, with zone size interpretation based on CLSI (2021) guidelines (17). The 11 antimicrobials agents used in characterizing the isolates of *P. aeruginosa* were: Carbapenems (doripenem, meropenem, imipenem), a tetracycline (tigecycline), aminoglycosides (amikacin, tobramycin), beta-lactamase/beta-lactamase inhibitor combinations (ampicillin + sulbactam, piperacillin + tazobactam), cephalosporins (cefepime, ceftazidime), and a fluoroquinolone (ciprofloxacin). Isolates were categorized as XDR when they revealed resistance to one or more antimicrobial agents in at least six categories or showed resistance to all except one or two antibiotics. Resistance to one or more agents in three or more categories was used for grouping the bacteria as MDR (19).

3.4. Detection of Genes

Chromosomal DNA was extracted using a commercial DNA extraction kit (Poyagene Azma, Iran) according to the manufacturer's instructions and kept at -30°C for further molecular investigations. All isolates were confirmed at the gene level as *P. aeruginosa* by detecting the *gyr*B gene using polymerase chain reaction (PCR) (20). All isolates were screened for antiseptic and antibiotic resistance genes such as *qac*E, *qac*EΔ1, and *bla*OXA-23 genes as described before (21, 22). The Supplementary File shows the primer sequences and related PCR protocols.

3.5. Typing of Bacterial Isolates

In order to verify that isolates were sufficiently heterogeneous and that past or ongoing outbreaks did not bias our study, we accumulated all data gathered above in a single figure. We defined the uniqueness of the strains by comparing phenotypic and PCR data (Figure 1). Typing the strains was performed using the combined similarities of isolates in their antibiotic resistance patterns and their resistance gene content. Similar *P. aeruginosa* isolates were placed under the same type and numbered accordingly.

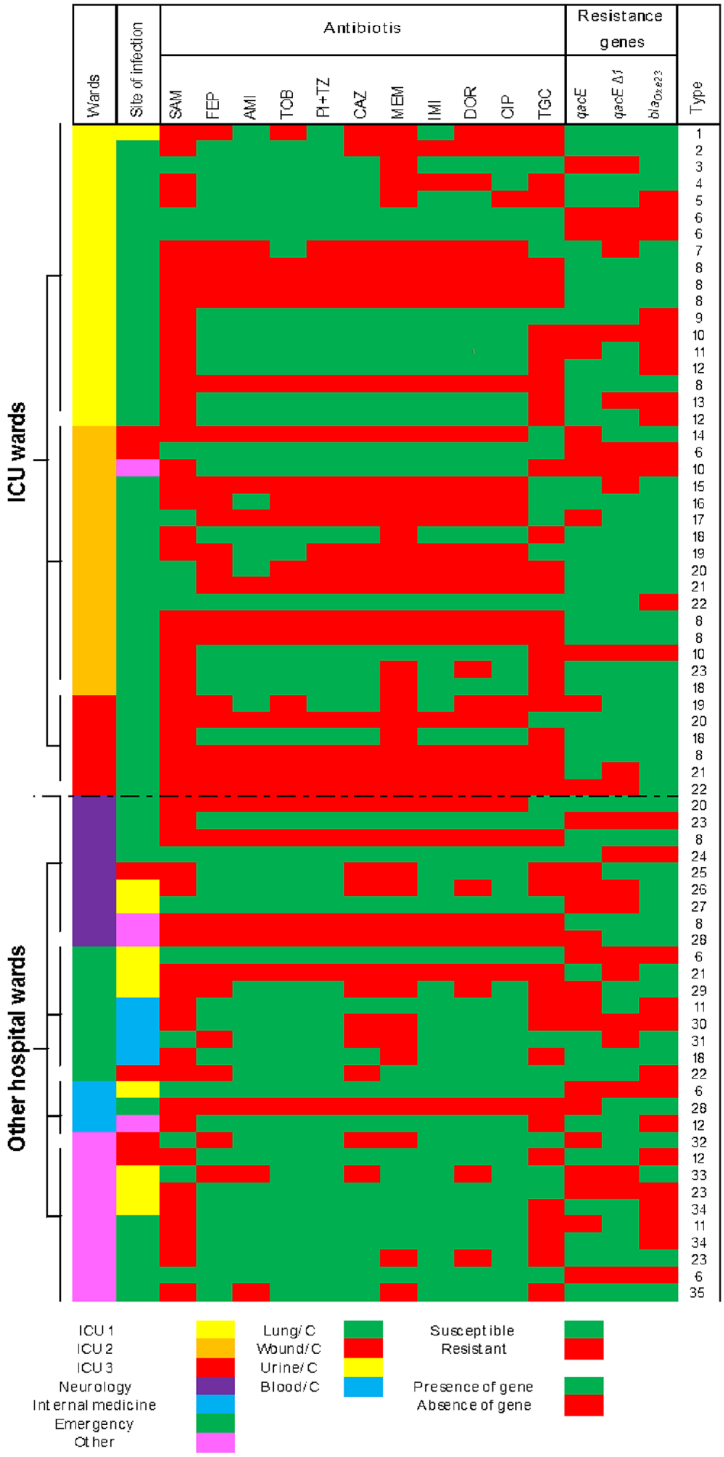

Figure 1. Accumulation of all strain-specific variables determined in the current study

3.6. Statistical Analysis

A one-way ANOVA test was performed to evaluate possibly significant differences using SPSS statistics 21. P-values < 0.05 were considered significant.

4. Results

From 78 documented *P. aeruginosa* infections detected in 2020, 70 *P. aeruginosa* isolates were still available and subjected to the current study. These *P. aeruginosa* isolates were obtained from different clinical samples such as tracheal aspirates (n = 46; 65.7%), wounds (n = 6; 8.5%), blood (n = 4; 5.7 %), and urine (n = 10; 14.2 %) (Appendix 2 in the Supplementary File). Among the infected patients, 58.5% were male (n = 41/70). The mean age of the infected patients was 63.3 (2 - 92) years, with 61.8 (2 - 92) years for males and 64.9 (42 - 87) years for females (Table 1).

The vast majority of *P. aeruginosa* infections occurred in the ICU (n = 43, 61.4%), comprising ICU I (n = 19, 27.1%), ICU II (n = 18, 25.7%), and ICU III (n = 6, 8.5%). An extensive diversity among ICU isolates (23 out of 43 strains, 53.4%) was spotted when an accumulation of all the strain-specific variables determined was used (Figure 1). Of note, when all isolates were considered, we documented 35 different types among all 70 isolates included. The main isolation site of *P. aeruginosa* isolates was lung infection (65.7%), followed by urine infection (14.2%), wound infections (8.5%), blood infection (5.7%), and other (5.7%) (Supplementary File).

4.1. Antibiotic Susceptibility Test

Among the 11 tested antibiotics, the highest resistance rate was detected against ampicillin + sulbactam (77.1%), followed by tigecycline (64.3%). The lowest resistance rate was for amikacin (32.9%) (Table 1). Carbapenem-resistant *P. aeruginosa* (CRPA) was detected in 48.5% of all cases.

Eight antibiotic resistance patterns were detected (A-H). Five contained XDR and MDR phenotypes (n = 23 isolates, 32.8%) (Table 1). Non-MDR patterns (F-H) were the dominant resistance phenotypes detected among 25 (35.7%) isolates. Also, *P. aeruginosa* showing pattern A (XDR) expressed resistance against 11 antibiotics (Table 1). The MDR isolates were spotted among pattern B isolates (10 antibiotics) (N = 3, 4.3%), pattern C (four antibiotics) (N = 2, 2.8%), pattern D (three antibiotics) (N = 2, 2.8%), and pattern E (four antibiotics) (N = 2, 2.8%) (Table 1). Twenty-two isolates showed a unique resistance pattern comprising 15 ICU-collected isolates and seven isolates of other wards. The majority of *P. aeruginosa* infections were observed among ICU patients (n = 46/70, 65.7%), comprising 29 (63%) male and 17 (36.9%) female patients. Moreover, MDR and XDR phenotypes were detected in 29/46 (63%).

4.2. Antiseptic Resistance Gene Distribution

Among 70 isolates, 53 isolates harbored at least one antiseptic resistance gene (75.7%), comprising 11 (20.7%) isolates with the *qac*E $\Delta 1$ gene alone and seven (13.2%) isolates with the *qac*E gene alone. Simultaneous occurrence of *qac*E and *qac*EΔ1 genes were spotted in 35 (66%) isolates. As stated above, grouping the isolates according to their antibiotic resistance phenotypes and resistance genes pattern similarity defined 35 overall combination types among the 70 (50%) strains we studied (Figure 1). The highest prevalence of antiseptic resistance genes (*qac*E and *qac*EΔ1) was detected in antibiotic resistance patterns B, D, and E (100%), followed by pattern A (64.2%). The most frequent single occurrence of *qac*E and *qac*EΔ1 (50%) genes was in MDR pattern C, followed by XDR pattern A (14.28%). The highest co-occurrence of resistance genes was observed in pattern D (50%) (Table 1).

4.3. Antibiotic Resistance Gene Distribution

The *bla*OXA-23 gene was spotted in 59 (84.3%) isolates. Most *bla*OXA-23 gene-positive isolates had at least one antiseptic resistance gene (n = 44/59, 74.5%) (Table 2). The highest occurrence of the *bla*OXA-23 gene was among patterns A, B, D, and E (100%), following pattern C (50%) (Table 1).

4.4. Minimum Inhibitory Concentrations Against Antiseptics

The MICs ranged from 1.9 to 62.5 μg/mL for BTC, 1.9 to 125 μg/mL for BKC, and 31.2 to 250 μg/mL for CHG. The mean MICs for BTC (24.0 versus 10.56 μg/mL, P = 0.001), BKC (46.1 versus 17.22 μg/mL, P = 0.001), and CHG (107.7 versus 29.4 μg/mL, P = 0.001) among antiseptic resistance gene-harboring isolates were statistically significantly higher than those for strains that did not possess resistance genes (Table 2). There was a statistically significant difference in the mean MICs between isolates harboring *qac*E, *qac*EΔ1, and *qac*E + *qac*EΔ1 for BTC, BKC, and CHG and those having no gene (P = 0.001) (Table 2).

5. Discussion

The current study illustrated the high prevalence of antiseptic resistance genes (*qac*E and *qac*EΔ1) and a significant relationship between their presence and increased phenotypic resistance against BTC, BKC, and CHG among infectious *P. aeruginosa*. The reported rate of MDR and XDR isolates (68.5%) was slightly higher than the average rate for Iran (58%) (23). The accumulation of all the strain-specific variables determined illustrated an extensive diversity among all isolates (35 out of 70 strains, 50%), highlighting that data interpretation had

Table 1. Antibiotic Resistance Patterns, Antiseptic, and Antibiotic Resistance Genes Distribution Among *Pseudomonas aeruginosa* Isolates [a, b]

Resistance Phenotype	Pattern	Values	Resistant Antibiotic	No.	Sensitive	No.	Having at Least A Gene	*qac*E	*qac*EΔ1	*qac*E + *qac*EΔ1	*bla*OXA-23
XDR	A	14 (20)	SAM, FEP, AMI, TOB, PI + TZ, CAZ, MEM, IMI, DOR, CIP, TGC	11	-	-	9 (64.2)	2 (14.28)	2 (14.28)	9 (64.2)	14 (100)
MDR	B	3 (4.2)	SAM, FEP, AMI, TOB, PI + TZ, CAZ, MEM, IMI, DOR, CIP	10	TGC	1	1 (33.3)	0	1 (33.3)	2 (66.6)	3 (100)
	C	2 (2.8)	SAM, CAZ, MEM, TGC	4	FEP, AMI, TOB, PI + TZ, IMI, DOR, CIP	7	1 (50)	0	1 (50)	0	1 (50)
	D	2 (2.8)	SAM, MEM, TGC	3	FEP, AMI, TOB, PI + TZ, IMI, DOR, CIP, CAZ	8	2 (100)	0	0	2 (100)	2 (100)
	E	2 (2.8)	SAM, DOR, MEM, TGC	4	FEP, AMI, TOB, PI + TZ, IMI, CAZ, CIP	7	2 (100)	0	0	2 (100)	2 (100)
Non-MDR	F	13(18.5)	SAM, TGC	2	FEP, AMI, TOB, PI + TZ, CAZ, MEM, IMI, DOR, CIP	9	4 (30.7)	1 (7.6)	3 (23)	6 (46.1)	0
	G	3 (4.2)	SAM	1	FEP, AMI, TOB, PI + TZ, CAZ, MEM, IMI, DOR, CIP, TGC	10	1 (33.3)	0	0	1 (33.3)	0
	H	9 (12.8)	0	0	SAM, FEP, AMI, TOB, PI + TZ, CAZ, MEM, IMI, DOR, CIP, TGC	11	2 (22.2)	1 (11.1)	0	1 (11.1)	1

[a] DOR, doripenem: 10 mg; MEM, meropenem: 10 mg; TGC, tigecycline: 15 mg; IMI, imipenem: 10 mg; CAZ, ceftazidime: 30 mg; SAM, ampicillin + sulbactam: 20 mg; FEP, cefepime: 30 mg; AMI, amikacin: 30 mg; TOB, tobramycin: 10 mg; CIP, ciprofloxacin; PI + TZ, piperacillin + tazobactam.
[b] Values are expressed as No. (%) unless otherwise indicated.

Table 2. Distribution of Antiseptic Resistance Genes and Minimum Inhibitory Concentrations Among *A. baumannii* Isolates [a]

PCR Result for Antiseptic Resistance Genes	Values	BTC	P [b]	BKC	P [b]	CHG	P [b]	Resistance Gene, Distribution Pattern	BTC	BKC	CHG	*bla*OXA-23 [c]
Positive	53 (75.7)	24.0 (1.9 - 62.5)	0.001 [d]	46.1 (1.9 - 125)	0.001 [d]	107.7 (31.2 - 250)	0.001 [d]	*qac*E, 7 (13.2)	15.90 (1.9 - 31.2)	28.1 (1.9 - 62.5)	53.3 (31.2 - 62.5)	4 (6.7)
								*qac*EΔ1, 11 (20.7)	21.4 (1.9 - 62.5)	27.8 (1.9 - 62.5)	56.6 (31.2 - 62.5)	9 (15.4)
								*qac*EΔ1 + *qac*E, 35 (66.0)	26.5 (7.8 - 62.5)	55.4 (7.8 - 125)	134.7 (31.2-250)	31 (52.5)
Negative	17 (24.3)	10.56 (7.8-15.6)		17.22 (3.9 - 62.5)		29.4 (15.6 - 31.2)			10.56 (7.8 - 15.6)	17.22 (3.9 - 62.5)	29.4 (15.6 - 31.2)	15 (25.4)

Abbreviations: BTC, benzethonium chloride; BKC, benzalkonium chloride; CHG, chlorhexidine digluconate.
[a] Values are expressed as mean (μg/mL) range or No. (%).
[b] One-way ANOVA test.
[c] *bla*OXA-23, antibiotic resistance gene.
[d] Significant.

no biases by clonality among isolates; even the isolates from ICU departments showed significant diversity (23 out of 43 strains, 53.4%). Multi-drug resistant and XDR phenotype frequencies were different in China (MDR 18.5%, XDR 3.5%) (24), Pakistan (MDR 36.3%, XDR 18.1%) (25), Iraq (MDR 50%, XDR 45%) (26), Thailand (46.4%) (27), and Nigeria (MDR 61%, XDR 5%) (28). Comprehensive monitoring of antibiotic resistance among *P. aeruginosa* isolates in 30 European Union countries revealed an MDR rate of zero to 49.4% when among a group of 27 European countries, the reported average rate was under 25% (29).

The prevalence of CRPA isolates was reported higher in Tehran (55.8%) (30) and the Southwest of Iran (52.2%) (31), while the rate of resistance was lower in other parts of Iran, including Yazd (37%) (32) and Golestan provinces (28.1%) (33) than in the present study (48.5%). The CRPA phenotype was detected differently among Asian *P. aeruginosa* isolates (10.2% to 72.7%) (34-36). The reported rate of CRPA in Egypt was from 42.5% to 100%, depending on hospital localization (37). The rate of CRPA among isolates was significantly lower in European countries (17.2%) and the U.S. (12%) than in the present study (38). Resistance mechanisms in *P. aeruginosa* are either intrinsic or acquired. Mutations in efflux pumps have been observed in carbapenem-resistant isolates, causing strains to display an MDR phenotype (32). In combination with gene mutations and the acquisition of genetic elements such as *qac*EΔ1 and *qac*E genes, efflux pumps play a critical role in this process (39). The rate of *qac*EΔ1-positive *P. aeruginosa* isolates in Iran (73.7% to 92.5%) (Table 3) was relatively higher than in our current study (20.7%). The situation differed for the *qac*E gene, which was reported

differently from prior Iranian studies (1.1% to 26.3%) (Table 3).

Table 3 . Prevalence of Genes Among *Pseudomonas aeruginosa* Isolates in Iran and Other Countries

Place of Study	Genes (Rate in %)		References
	*qac*E	*qac*EΔ1	
Iran			
North Khorasan	13.2	20.7	Current study
Ardabil	26.3	73.7	(40)
Hamadan	1.1	36.9	(41)
Qazvin	17.5	92.5	(42)
Tehran and Esfahan	59	91.5	(43)
Other countries			
Australia and India	100	46.2 %	(44)
Brazil	-	48	(45)
Egypt	13.4	47.2	(46)
Egypt	33	78	(44)
Germany	2.7	10	(22)
Saudi Arabia	18.2	-	(39)
Iraq	-	97.1	(47)
Bangladesh	34.72	93.05	(39)

The documented rate of the *qac*EΔ1 gene was lower in Germany (10%) (22) when its reported rate was higher in other countries (Table 3) (39, 44-49). The *qac*E gene frequency was lower among *P. aeruginosa* isolates from Germany (2.7%) (22), but several other studies reported a higher rate of *qac*E genes (33% - 100%) (39, 44, 46, 48, 49) (Table 3). The coexistence of antiseptic resistance genes (*qac*EΔ1 and *qac*E) and carbapenem resistance gene (*bla*OXA-23) among *P. aeruginosa* isolates here can be explained by the location of genes on the same plasmid (48).

The significant increases in MIC against BTC, BKC, and CHG among *P. aeruginosa* isolates that harbor *qac*E and *qac*EΔ1 genes were already reported in Iran (50) and Egypt (48). However, in studies conducted in Saudi Arabia (39) and Brazil (45), no relation between the presence of *qac*E and *qac*EΔ1 genes and increased MIC against biocides was recognized. Bacterial distribution and spread reduce susceptibility among them, affecting biological, socio-economic, and physical aspects (39). The recommended working concentrations for BKC, BTC, and CHG in commercial disinfectants (2000, 1000, and 5000 μg/mL, respectively) (40) are higher than the highest measured MIC in the current study. Still, increased MICs for antiseptic agents active against *P. aeruginosa* indicate the importance of targeted screening for such *P. aeruginosa* isolates in hospitals.

5.1. Conclusions

The results of our study highlighted the importance of close monitoring of *P. aeruginosa* isolates causing infection in hospitals for antiseptic resistance development. This will ultimately help prevent the spread of such organisms.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Footnotes

Authors' Contribution: M. R.: Investigation and laboratory work; M. M.: Project administration; H. G. M.: Conceptualization, project administration, writing, and original draft preparation; A. A.: Investigation and resources; A. V. B.: Writing, review, editing, and scientific advice.

Conflict of Interests: Hamed Ghasemzadeh-Moghaddam and Amir Azimian are North Khorasan University of Medical Sciences academic members; Alex van Belkum is the BaseClear, Sylviusweg 74, 2333 BE Leiden, the Netherlands staff.

Data Reproducibility: The data presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: Sample collection was performed as set by the Islamic Azad University Ethics Committee, Damghan, Iran (project number: IR.IAU.DAMGHAN.REC.1401.001). Link: ethics.research.ac.ir/IR.IAU.DAMGHAN.REC.1401.001.

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