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QacE and QacEΔ1 Genes and Their Correlation to Antibiotics and Biocides Resistance *Pseudomonas aeruginosa*

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Abstract

One of the serious and growing challenges for infection control programs worldwide is hospital acquired infections. *Pseudomonas aeruginosa* is considered the most common cause of hospital acquired Gram-negative infections. Intensive exposure of hospital pathogens to biocides may result in the emergence of resistance not only to the biocides, but possibly to antibiotics as well. Thus, the current study was done to investigate the prevalence of *qacEΔ1* and *qacE* genes and their correlation to antibiotics and biocides resistance in *Pseudomonas aeruginosa* isolated in Egypt. The antimicrobial activity of six biocides against drug sensitive and multi-drug resistant *Pseudomonas aeruginosa* was evaluated. Phenol and formalin displayed a higher antimicrobial activity compared to other biocides tested. Minimum inhibitory concentration (MIC) values of chlorine releasing agents were higher than the dilution prescribed by their manufactures. The *qacEΔ1* gene was identified in 57.8% of multidrug resistant isolates and 21.4% in susceptible strains, While, *qacE* gene was only detected among multi-drug resistant *Pseudomonas aeruginosa*. This study reported a correlation between *qacEΔ1* and multidrug resistance *Pseudomonas aeruginosa*, while there was no correlation between the presence of qac genes and increased MIC values to biocides. The effectiveness of biocides is very important to control microbial population and prevent the transmission of infections in hospitals. In conclusion we highlight the need for health care facilities to assess the antimicrobial effectiveness of biocides periodically.

Keywords: Biocides, MIC, qacE, qacEΔ1and *P. aeruginosa*.

Introduction

Biocides, including disinfectants and antiseptics, are used for a variety of topical and hard surface applications in health care facilities [1]. Biocides have an important role in preventing and controlling hospital-acquired infections. However, failures in the antimicrobial activities of biocides have been reported [2, 3]. Generally, the susceptibility of Gram-negative bacteria is lower than gram-positive bacteria. This outcome is likely to be intrinsic rather than plasmid mediated, due to the protective barrier of the outer membrane [4].

Due to its ability to survive in unfavorable environmental conditions, biofilm formation and its high resistance to antibiotics, antiseptics, and disinfectants, *Pseudomonas aeruginosa* remains to be an important bacterium in nosocomial infections [5, 6, 7, 8]. It can infect multiple organ systems and can cause serious illness, especially among the immunocompromised [9]. One of the mechanisms of resistance to biocide is the expression of efflux systems. *P. aeruginosa* harbors multidrug transporter efflux systems, involving QacE and QacEΔ1, as do other Gram-negative bacteria [10,11]. The *qacEΔ1* gene is a defective form of the *qacE* gene and is included in the 3′ conserved segment of class I integrin as described by Paulsen *et al*., [12, 13].

The possibility of *P. aeruginosa* developing resistance to biocide formulations, as they do with antibiotics, underscores the need to continuously evaluate the antimicrobial activities of various biocide formulations against this pathogen. In Egypt, investigations regarding the determinants of resistance and susceptibility to biocides at their currently prescribed concentrations are very confined. Resistance to antimicrobial agents is usually tested as minimum inhibitory concentration (MIC), which is a very useful investigation tool [2]. The purpose of this work was to study the susceptibility of multidrug resistant (MDR) *P. aeruginosa*, to commonly used biocides and to find out the distribution of the *qacEΔ1*and *qacE* gene markers in *P. aeruginosa* isolates and their correlation to antimicrobial resistance.

Materials and Methodology

In this study, one hundred and thirty-six clinical isolates of *P. aeruginosa* were used. The isolates were isolated from different specimens submitted to the Microbiology Laboratory, Clinical Pathology Department, Ain Shams University Hospital for routine culture. All the isolates were characterized and identified according to standard techniques [14]. Isolates were examined for antimicrobial susceptibility using the disk diffusion method as described by Kirby Bauer according to the CLSI [15].

The antibiotic panel included : piperacillin (PC,100 μg), ticarcillin/clavulinic acid (TC,75/10 μg), piperacillin/ tazobactam (PT, 100 μg/10 μg), cefoperazone (CZ, 30 μg), cefotaxime (CFO, 30 μg), cefepime (CPM, 30 μg), ceftriaxone (CT, 30 μg), ceftazidime (CAZ, 30 μg), imipenem (IMP, 10 μg), amikacin (AK, 30 μg), gentamicin (GN, 10 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEF, 5 μg), and chloramphenicol (CMP, 30 μg). Resistance to each antibiotic was recorded, and isolates resistant to one antimicrobial agent in the three or more anti-pseudomonal antimicrobial classes (penicillin/cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides) were defined as MDR [16].

The MDR isolates (n=38) plus 14 sensitive isolates were tested for biocide susceptibility by the broth macro-dilution method. Fresh colonies obtained after overnight subculture were suspended in 10 ml 0.85% saline to prepare the inoculum. The inoculum suspension was adjusted to match the turbidity of 0.5 McFarland standards [15].

The biocides selected for testing were the following chemical disinfectants/antiseptics recommended for patient care items and instruments: sodium dichloroisocyanurate (sodium troclosene) (SaniTab tablets 4.72gm); sodium hypochlorite solution (10% w/v, pH 7); formalin (37% w/v); aqueous povidone–iodine (10% w/v, PVP-I_2) solution; chlorhexidine digluconate (0.3%)/cetrimide (3%) (Savlon) and phenol (10% w/v) solution. Broth macro-dilution

method was used to determine the MICs of the biocides as described by Mazzola *et al.*,[17], starting from the initial concentrations, as shown in Table 1. Each disinfectant solution was diluted by the serial twofold dilution method using Mueller Hinton broth. All tubes except the negative control tube were inoculated with 0.1 ml of the prepared inoculum adjusted to 0.5 McFarland standards. The positive control tube contained 0.1 ml inoculum in 1 ml Mueller Hinton broth with evidence of bacterial growth (turbidity). The negative control tube contained Mueller Hinton broth and the disinfectant to be tested with no evidence of bacterial growth (clear). The tubes were incubated at 37°C for 18–24 hrs. After the incubation period, the tubes were examined and the MIC was identified as the lowest concentration of the disinfectant had no visible bacterial growth.

Table 1. Biocide Agents, their Started Concentrations and Dilutions Used for MIC Test

Chemical agents	Commercial name	Recommended dilution	Starting concentration	Dilution
Chlorhexidine(Ch)/ Cetrimide (C)	Savlon	3.3%	3.3 % (0.3% Ch, 3% C)	1.65 %, 0.825 %, 0.4125 % and 0.206 %
Povidone-iodine	Betadine	5%	10 %	5 %, 2.5 %, 1.25 % and 0.625 %
Sodium hypochlorite	Sodium hypochlorite	0.5%	10 %	5%, 2.5 %, 1.25 %, 0.625% and 0.312%
Sodium troclosene	Sanitab	0.3%	10 %	5%, 2.5 %, 1.25 %, 0.625 %, 0.312 %, 0.156 % and 0.078 %
Phenol	Phenol	1-5%	10 %	5 %, 2.5 %, 1.25 %, 0.625 %, 0.312 % and 0.156 %
Formalin	Formalin	0.2-8%	9.25 %	4.625 %, 2.312 %, 1.156 %, 0.578 %, 0.289 %, 0.145 %, 0.0725% , 0.036 % and 0.018 %

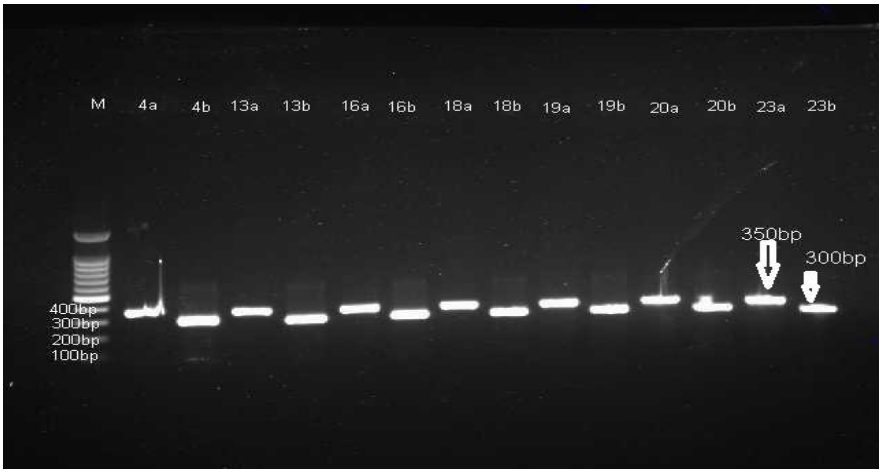

Fig. 1- Gel electrophoresis of the PCR amplified product for detection of *qacE* and *qacEΔ1* genes. M: 100bp DNA ladder, lanes 4a, 13a, 16a, 18a, 19a, 20a and 23a positive samples for *qacE* gene (350 bp); lanes 4b, 13b, 16b, 18b, 19b, 20b and 23b: positive samples for *qacEΔ1* gene (300 bp)

The *qacEΔ1* and *qacE* resistance genes were amplified by Polymerase chain reaction (PCR). DNAs were extracted using a Genomic DNA Extraction Kit (Fermentas; Life Science, Waltham, MA) in accordance with manufacturer instructions. To amplify *qacE* gene, PCR was done using 10 pmol from each forward (5`-CCCGAATTCATGAAAGGCTGGCTT-3′) and reverse (5′-TAAGCTTTCACCATGGCGTCGG-3′) specific *qacE* primers, which amplify 350 bp fragments (Figure 1). The *qacEΔ1* gene was detected by using the same PCR conditions, with the exception of 10 pmol from each forward (5′-TAGCGAGGGCTTTACTAAGC-3′) and reverse (5′-ATTCAGAATGCCGAACACCG-3′) specific *qacEΔ1* primers, which amplify 300 bp fragments (Figure 1). The primers, described by Kücken *et al.* [18] and Wang *et al*. [19], were prepared by Invitrogen (Grand Island, NY). To perform the PCR, GoTaq Green Master Mix (Promega, Madison, WI) was used in the PCR assay. DNA was amplified according to the following conditions;120s of denaturation at 93°C followed by 35 cycles of 30s at 93°C; 30s at 55°C; 60s at 72°C; and finally, 5 min at 72°C. The PCR products were separated through a 1.5% agarose gel by electrophoresis. The Chi-square test was applied to analyze results.

Results

Fourteen (10.3%) out of 136 isolates of *P. aeruginosa* were susceptible to all antibiotics used, while, 89.7 % of the isolates were resistant to one or more antimicrobial agents. With respect to the total number of resistant strains, 34.4% were MDR. Table 2 shows the antibiotic susceptibility patterns of the 136 isolates of *P. aeruginosa*.

Imipenem was the most effective antibiotic tested against *P. aeruginosa* isolates and showed the maximum sensitivity (66.2%), followed by amikacin (63.2%) and ciprofloxacin (55.1%), whereas the *P. aeruginosa* isolates were highly resistant to chloramphenicol (78%), followed by cefepime (75.7%) then ceftriaxone (64.7%).

One hundred and twenty two *P. aeruginosa* isolates were categorized into 41 different antibiotypes. Table 3 presents antibiotypes 1–11, including 68 strains that were the most prevalent antibiotypes. Thirty-eight MDR *P. aeruginosa* isolates were subjected to biocide susceptibility testing and were categorized as antibiotypes 1, 2, 4, and 7 (Table 3).

In the present study, both sensitive and MDR *P. aeruginosa* isolates were effectively inhibited by the user's defined concentrations of PVP-I_2, Savlon, phenol, and formalin. However, the clinical MDR *P. aeruginosa* had statistically significant MICs to PVP-I_2 and Savlon ($p<0.05$). No significant differences in formalin or phenol MICs were found between the two groups. A low formalin concentration, 0.018%, effectively inhibited the growth of all of the sensitive *P. aeruginosa* and more than 60% of the MDR *P. aeruginosa* strains. On the other hand, both *P. aeruginosa* groups were inhibited by a phenol concentration ≤1.25%. Both *P. aeruginosa* groups had MICs exceeding the user's defined concentration of sodium hypochlorite and SaniTab. The MDR *P. aeruginosa* had statistically significant MICs to sodium hypochlorite and SaniTab ($p<0.05$).

The distribution of *qacE* and *qacEΔ1* genes is presented in Table 5. Out of 52 *P. aeruginosa* isolates, 13.4% contained *qacE* and 47.2% contained *qacEΔ1*. The prevalence differed between pseudomonas groups: *qacEΔ1* detected in 57.9% of the MDR *P. aeruginosa* isolates and 21.4% of the sensitive strains, and *qacE* detected in 18.4% of the MDR strains and in none of the sensitive strains. Concomitant expression of the *qacE* and *qacEΔ1* genes occurred in seven of 38 (18.4%) of the MDR isolates; no isolates from the sensitive group had concomitant expression of both genes. Table 6 presents the occurrence of *qacEΔ1* and the MICs of the biocides.

Table 2. Antibiotic Susceptibility of Clinical *P. aeruginosa* Isolates

Antibiotic	Resistant		Intermediate		Susceptible	
	Number	%	Number	%	Number	%
Piperacillin	74	54.4	0	0	62	45.6
Piperacillin/tazobactam	74	54.4	0	0	62	45.6
Ticarcillin/ clavulinic acid	84	61.8	1	0.7	51	37.5
Cefoperazone	75	55.1	10	7. 4	51	37.5
Cefotaxime	83	61	14	10.3	39	28.7
Ceftriaxone	88	64.7	7	5.1	41	30.2
Ceftazidime	77	56.6	9	6.6	50	36.8
Cefepime	103	75.7	6	4.4	27	19.9
Imipenem	22	16.1	24	17.7	90	66.2
Amikacin	40	29.4	10	7.4	86	63.2
Gentamicin	55	40.4	20	14.7	61	44.9
Ciprofloxacin	49	36	8	5.9	75	55.1
Levofloxacin	56	41.2	27	19.9	53	38.9
Chloramphenicol	106	78	11	8.1	19	13.9

Table 3. Groups of Clinical *P.aeruginosa* Isolates that Have the Same Antibiotype

Profile	Number of isolates	Resistance pattern
1 (14)	16	PC- PT- TC- CZ-CFO- CT- CAZ- CPM -IMP- AK- GN - CIP- LEF- CMP
2 (13)	12	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - AK- GN - CIP- LEF- CMP
3 (11)	11	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - CIP- LEF- CMP
4 (12)	6	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - AK- GN - LEF- CMP
5 (10)	6	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - GN - CMP
6 (10)	4	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - LEF- CMP
7 (13)	4	PC- PT- TC- CZ-CFO- CT- CAZ- CPM -IMP- AK- GN - LEF- CMP
8 (10)	3	PC- PT- TC- CZ-CFO- CT- CAZ- CPM -IMP – CMP
9 (10)	2	PC- PT- TC- CZ-CFO- CT- CAZ- CPM - CIP- CMP
10 (8)	2	PC- PT- TC-CFO- CAZ- CPM - GN – CMP
11 (4)	2	CFO- CPM -CIP – CMP

Table 4. Distribution of MICs of Biocides among *P. aeruginosa* Isolates

Chemical agent	MIC mg/L	%	*P. aeruginosa* Group		Chi-square	P value (Significant)
			MDR (n=38)	Sensitive (n=14)		
PVP-I_2	50000	5 %	9 (23.6%)	0	23.4804	Significant at $p < 0.05$
	25000	2.5 %	0	7 (50%)		
	12500	1.25 %	15 (39.4%)	3 (21.4%)		
	6250	0.625 %	14 (36.8%)	4 (28.6%)		
Savlon	33000	3.3 %	6 (15.8%)	1 (7.1%)	35.254	Significant at $p < 0.05$
	16500	1.65 %	3 (7.8%)	0		
	8250	0.825 %	6 (15.8%)	0		
	4125	0.412 %	13 (34.2%)	3 (21.4%)		
	2062.5	0.206 %	10 (26.3%)	0		
	1031.25	0.103 %	0	10 (71.4%)		
Hypochlorite	50000	5 %	3 (7.8%)	0	22.618	Significant at $p < 0.05$
	25000	2.5 %	12 (31.5%)	1 (7.1%)		
	12500	1.25 %	17 (44.7%)	3 (21.4%)		
	6250	0.625 %	3 (7.8%)	0		
	3125	0.312 %	3 (7.8%)	10 (78.5%)		
Phenol	12500	1.25 %	7 (13.11%)	2 (14.3%)	5.7191	Not significant at $p < 0.05$
	6250	0.625 %	4 (7.8%)	2 (14.3%)		
	3125	0.312 %	0	1 (7.1%)		
	1562.5	0.156 %	27 (71%)	9 (64.3%)		
Sanitab	100000	10 %	19 (50%)	0	23.7627	Significant at $p < 0.05$
	25000	2.5 %	0	3 (21.4%)		
	12500	1.25 %	7 (13.1%)	2 (14.3%)		
	1562.5	0.156 %	0	3 (21.4%)		
	781.25	0.078 %	12 (31.5%)	6 (42.9%)		
Formalin	578.125	0.578 %	0	0	7.767	Not significant at $p < 0.05$
	289	0.289 %	4 (10.5%)	0		
	144.5	0.144 %	4 (10.5%)	0		
	72.26	0.072 %	4 (10.5%)	0		
	36.132	0.036 %	3 (7.8%)	0		
	18.066	0.018 %	23 (60.5%)	14 (100%)		

Table 5. Incidence of *qacE* and *qacEΔ1* Genes among Isolates of *P. aeruginosa*.

Gene		MDR (n=38)	Sensitive (n=14)	Chi-square	P value
qacE	Positive	7	0	2.9801	Significant at $p < 0.05$
	Negative	31	14		
	Total	38	14		
qacEΔ1	Positive	22	3	5.4499	Not significant at $p < 0.05$
	Negative	16	11		
	Total	38	14		
qacE+qacEΔ1	Positive	7	0		
	Negative	31	14		
	Total	38	14		

Table 6. MIC of Biocides and Association with *qacEΔ1* Gene

Chemical agents	MIC mg/ml	%	Positive strains of qacEΔ1 (%)	Negative strains of qacEΔ1 (%)	Total
PVP-I_2	50000	5%	4 (44.4%)	5 (55.6%)	9
	25000	2.5%	2 (28.6%)	5 (71.4%)	7
	12500	1.25%	12 (66.7%)	6 (33.3%)	18
	6250	0.625%	7 (38.8%)	11(61.2%)	18
Savlon	33000	3.3%	4 (57.1%)	3 (42.9%)	7
	16500	1.65%	2 (66.7%)	1(33.3%)	3
	8250	0.825%	4 (66.7%)	2 (33.3%)	6
	4125	0.412	10 (62.5%)	6 (37.5%)	16
	2062.5	0.206	5 (50%)	5 (50%)	10
	1031.25	0.103	0 (0%0)	10 (100%)	10
Hypochlorite	50000	5%	2 (66.7%)	1(33.3%)	3
	25000	2.5%	10 (76.9%)	3 (23.1%)	13
	12500	1.25%	11 (55%)	9 (45%)	20
	6250	0.625%	1 (33.3%)	2 (66.7%)	3
	3125	0.312	1(7.7%)	12 (92.3%)	13
Phenol	12500	1.25%	7 (77.7%)	2 (22.3%)	9
	6250	0.625%	1 (16.6%)	5 (83.4%)	6
	3215	0.312%	1 (100%)	0 (0%)	1
	1562.5	0.156%	16 (44.4%)	20 (55.6%)	36
Sanitab	100000	10%	9 (47.3%)	10 (52.7%)	19
	25000	2.5%	1 (33.3%)	2 (66.7%)	3
	12500	1.25%	7 (77.7%)	2 (22.3%)	9
	1562.5	0.156%	0 (0%)	3 (100%)	3
	781.25	0.078%	8 (44.4%)	10 (55.6%)	18
Formalin	289	0.289%	2 (50%)	2 (50%)	4
	144.5	0.144%	4 (100%)	0 (0%)	4
	72.26	0.072%	4 (100%)	0 (0%)	4
	36.123	0.036%	3 (100%)	0 (0%)	3
	18.066	0.018%	12 (32.4%)	25 (67.6)	37

Discussion

In recent years, *P. aeruginosa* emerged in Egypt and it is seen mainly in hospital acquired infections [20, 21, 22]. The standard definition of MDR is an acquired non-susceptibility to at least one agent in three or more antimicrobial categories [16]. In our study, we demonstrated that 34.4 % of the *P. aeruginosa* isolates were MDR. Similar reports of MDR *P. aeruginosa* have been documented previously [21, 23, 24].

The resistance to commonly used antimicrobial agents by *P. aeruginosa* is reportedly increasing worldwide and is considered a public health threat [21, 25, 26]. In this study *P. aeruginosa* isolates were highly resistant to all antibiotic tested except imipenem and amikacin. The effectiveness of imipenem and amikacin against *P. aeruginosa* has also been reported by other studies [8, 21, 24, 27, 28, 29]. The resistance pattern of *P. aeruginosa* to quinolones observed in the present study was similar to other results reported [21, 24]. We noticed that, among cephalosporin, a high resistance rate was exhibited against cefepime and cefotriaxone, which was consistent with other reports [20, 21, 30]. In contrast, earlier studies have reported cefepime to be the most effective antimicrobial agent against *P. aeruginosa* [31, 32-33]. However, this contradiction may be due to the continuous evolution of MDR strains worldwide.

Biocides have been widely used to minimize the spread of infections by MDR organisms [34,

35], however the emerging resistance to biocides has prompted the need for more research into biocide efficacy [27, 36]. Although *P. aeruginosa* resistance to biocides has been extensively studied, this is the first study to report on biocide activity against this bacterium in Egypt.

Formaldehyde, phenols, PVP-I_2, Savlon, and chloride-releasing agents are widely used as disinfectants in hospitals. In this study, we verified that formalin, phenols, PVP-I_2, and Savlon were effective when tested against clinical isolates of *P. aeruginosa* according to the user's defined concentration. We found that formalin and phenol were the most effective biocidic agents in the present study due to the fact that no significant differences were found between the sensitive and MDR *P. aeruginosa* groups regarding the MICs of formalin and phenol.

Lower concentrations of Savlon were found to be effective against 92.8% and 84.2% of the drug-susceptible and MDR isolates of *P. aeruginosa* respectively. Comparable findings were reported in studies conducted in Nigeria and Ethiopia [28, 37]. We demonstrated that lower dilutions of PVP-I_2 were effective against 100% of the drug-sensitive group and 76.4% of the MDR group. Clinical MDR *P. aeruginosa* had statistically significant MICs to PVP-I_2 and Savlon ($p<0.05$).

This data proved that multidrug resistant *Pseudomonas aeruginosa* are not necessarily more resistant to biocides like Savlon, Povidone-iodine, phenol and formalin than antibiotic sensitive strains.

In this study, resistance was observed with some biocide formulations at the dilution prescribed by their manufactures. Studies in USA and Brazil reported that the recommended user dilution with 0.5% of sodium hypochlorite was effective against 92.2% and 86%, of *P. aeruginosa* isolates respectively [3. 38]. In contrast, in our study 69.2 % and 59.6 % of 52 *P. aeruginosa* isolates examined were resistant to the user's defined concentrations of hypochlorite and SaniTab respectively. This may be attributed to the extensive use of this type of disinfectant in the routine infection control in Egypt.

Both *qacA* and *qacE* were the first reported *qac*-mediated resistance genes to be involved in the efflux-based system [39, 40]. Subsequently, an energy-dependent efflux pump that relies on the proton motive force was assured as a mechanism of resistance conferred by the qac genes [41, 42]. The clinical importance of qac-mediated resistance is primarily attributed to biocides resistance [43].

We examined the presence of the *qacE* and *qacEΔ1* genes in 52 *P. aeruginosa* clinical isolates. The percentages of positive strains for *qacE* and *qacEΔ1* among the studied *P. aeruginosa* were 13.5% and 48% respectively. This corresponds to other reports which have recorded higher percentages of *qacEΔ1* compared to *qacE* [10, 18, 27]. In our study, there were statistical significant differences in detection of the *qacEΔ1* gene between the MDR and susceptible strains. The *qacEΔ1* gene was found in 22 of 38 (57.8%) MDR isolates and in three of 14 (21.4%) sensitive strains. However, there was no significant difference in detection of the *qacE* gene between the MDR and drug-sensitive *P. aeruginosa* isolates. Our results indicated that the presence of the *qacEΔ1* gene is well correlated with multidrug resistance, consistent with the results of other studies [19, 27]. The results of our experiments agree with the results of other groups [10, 44] in which all of the *qacE* positive strains were also positive for *qacEΔ1*.

In conclusion, we observed biocide resistance in MDR *P. aeruginosa* isolates examined in this study. Our data indicates that *qacEΔ1 and qacE* are not directly correlated with increased biocide MICs; however the *qacEΔ1 gene was* detected at higher percentages in less susceptible isolates of *P. aeruginosa*. As aforementioned, the *qacE* gene was detected much less frequently and invariable between MDR and sensitive *P aeruginosa*. Our study verifies that the presence of Qac genes alone does not necessarily correlate with increased resistance but Qac genes in combination with other mechanisms might be contributed to the development of cationic antiseptics resistant and the survival of bacteria in toxic environments. Future work should be done to determine the degree to which Qac genes correlate with biocide resistance in other MDR Gram negative bacteria.

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