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ORIGINAL ARTICLE

Evaluation of agar dilution and broth microdilution methods to determine the disinfectant susceptibility

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A variety of disinfectants have been widely used in veterinary hygiene, food industries and environments, which could induce the development of bacterial resistance to disinfectants. The methods used to investigate antimicrobial effects of disinfectant vary considerably among studies, making comparisons difficult. In this study, agar dilution and broth microdilution methods were used to compare the antimicrobial activities of four quaternary ammonium compounds (QACs) against foodborne and zoonotic pathogens. The potential relationship between the presence of QACs resistance genes and phenotypic resistance to QACs was also investigated. Our results indicated that the minimum inhibitory concentrations (MICs) determined by two methods might be different depended upon different QACs and bacteria applied. Regardless of the testing methods, *Klebsiella pneumoniae* was more tolerant among Gram-negative strains to four QACs, followed by *Salmonella* and *Escherichia coli*. The agreement between MICs obtained by the two methods was good, for benzalkonium chloride (78.15%), didecyldimethylammonium chloride (DDAC) (82.35%), cetylpyridinium chloride (CTPC) (97.48%) and cetyltrimethylammonium bromide (CTAB) (99.16%), respectively. Among all Gram-negative bacteria, 94.55% ($n=52$) of *qacEΔ1*-positive strains showed higher MICs (512 mg l^{-1}) to CTAB. The *qacEΔ1* gene was highly associated ($P<0.05$) with the high MICs of QACs ($\geqslant$512 mg l^{-1}). In addition, DDAC remained as the most effective disinfectant against both Gram-positive and Gram-negative bacteria. This is the first study that compared the agar dilution and broth microdilution methods to assess the antimicrobial activity of QACs. The study demonstrated the need to standardize method that would be used in evaluating QACs antimicrobial properties in the future.
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INTRODUCTION

Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC) and didecyldimethylammonium chloride (DDAC), are common disinfectants used in controlling contamination within health-care facilities, veterinary practices and food manufacturing facilities because of their little irritation, low corrosiveness and toxicity, yet high antimicrobial efficacy over a wide pH range.[1–3,4] However, the excessive use of QACs in these sensitive environments may impose selective pressures contributing to the emergence of disinfectant-resistant microbes[4,5] and also induces directed selection of antimicrobial resistant bacteria.[6]

Given the global emergence of antimicrobial resistance bacteria and the widespread use of QACs, it is crucial to evaluate available QACs antimicrobial susceptibility techniques to best determine a benchmark method for use in a variety of bacterial species.[7] Currently, there is no standardized method for quantifying the antimicrobial activity of QACs using minimum inhibitory concentrations (MICs), which are *in vitro* susceptibility tests using dilution methods (agar dilution or broth microdilution) standardized by the Clinical and Laboratory Standards Institute.[8] By categorizing bacterial isolates as susceptible, intermediate or resistant and monitoring these trends over time, observed changes in MICs could indicate the gradual development of antimicrobial resistance in those strains.

The molecular characterization of QACs resistance is also poorly understood, however, five QAC resistance genes, *qacE*, *qacEΔ1*, *qacF*, *qacG* and *sugE(p)*, have been identified on mobile genetic elements like plasmids and integrons in Gram-negative organisms.[9–11] These genes belong to the SMR (small multidrug resistance) family and are key epidemiological factors associated with QAC resistance.[12] The rapid spread of QAC resistance between different types of bacteria is mainly caused by the location of these resistance genes, which encode proteins conferring efflux-mediated resistance to QACs on mobile genetic elements.[10,13] The *qacE* gene is located in the 3′-CS (conserved segment) of class 1 integrons in Gram-negative bacteria, whereas *qacEΔ1* is a defective version of *qacE* which has been associated with an increased MIC to benzalkonium chloride.[14] With 67.8% similarity

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to *qacE, qacF* also contributes to the resistance to QACs.[11,14] When the *qacG* gene is carried by class 1 integrons,[15] the *sugE(p)* gene is commonly located on an IncA/C multidrug resistance (MDR) plasmid and confers high MICs to cetylpyridinium chloride.[16,17]

The objectives of this study were to compare the agar dilution and broth microdilution methods for determining MICs of QACs against foodborne and zoonotic pathogens, and to investigate how the presence of QACs resistance genes in strains relates to their phenotypic resistance.

MATERIALS AND METHODS

Quaternary ammonium compounds

QACs used in this study included benzalkonium chloride (BC; Chengdu Best-Reagent Company, Chengdu, China), cetyltrimethylammonium bromide (CTAB; Chengdu Best-Reagent Company), DDAC and cetylpyridinium chloride (CTPC; J&K China Chemical ltd, Beijing, China).

Bacterial strains

A total of 119 bacterial strains were included in the study (Supplementary Table S1). Fifty-five isolates were zoonotic, including 23 *Salmonella* from living chicken, 14 *Escherichia coli*, 13 *Klebsiella pneumoniae* and 5 *Staphylococcus aureus* from swine. Fifty-six isolates were from retail meats, including 24 *Salmonella* and 7 *E. coli* from retail chicken, 11 *E. coli*, 9 *K. pneumonia* and 5 *S. aureus* from pork. Six additional *Salmonella* serotypes were also tested, including *S. pullorum* JBL527 and *S. enteritidis* CY3377 (obtained from China Veterinary Culture Collection Center (CVCC)) and *S. enteritidis* ATCC 13076, *S. pullorum* ATCC13036, *S. gallinarum* ATCC 9184 and *S. dublin* ATCC 15480 (obtained from ATCC). *E. coli* ATCC 10536 and *St. aureus* ATCC 25923 were used as quality control strains. All isolates were stored in trypticase soy broth containing 15% glycerol at −80 °C until use.

Determination of MICs of QACs

MICs of disinfectants in these isolates were determined using agar dilution and broth microdilution as described by the Clinical and Laboratory Standards Institute.[8] The range of concentrations used to determine the MICs of all disinfectants was 0.125–1024 mg l^{-1}. All experiments were run in triplicate. Bacterial suspensions were prepared by suspending 3–5 individual overnight colonies from trypticase soy agar plates into 3 ml of 0.9% saline, equivalent to the turbidity of a 0.5 McFarland standard.

For agar dilution, the suspensions were further diluted 1:10 with 0.9% saline before inoculation. Bacterial suspensions were delivered to the surface of Mueller-Hinton agar plates using a multipoint inoculator (MIT-60 P; Sakuma Seisakusho, Tokyo, Japan). The final inoculum was ~10^4 CFU ml^{-1}. Plates were incubated at 37 °C for 24 h. The MICs of QACs were recorded as the lowest concentration of QACs that completely inhibited the bacterial growth on the agar plate.

For broth microdilution, the 0.5 McFarland inoculum suspensions were further diluted 1:100 in Mueller-Hinton broth before inoculation. The prepared 96-well microtiter test plates contained Mueller-Hinton broth with a twofold concentration of QACs solution and were inoculated with 50 µl of the suspended culture to a final inoculum density of 10^5 CFU ml^{-1} per wells. The 96-well microtiter plates were sealed using a perforated plate seal and incubated at 37 °C for 24 h. The MICs of QACs were recorded as the lowest concentration of QACs where no visible growth was observed in the wells of the microtiter plates.

PCR amplification

The primers used to amplify *sugE(p)*, *qacEΔ1*, *qacE*, *qacF* and *qacG* genes in Gram-negative strains and *qacA/B*, *qacG*, *qacJ*, *qacH*, *smr (qacC+qacD)* genes in Gram-positive strains are shown in Table 1.

DNA templates were prepared by suspending overnight culture in 600 µl of Milli-Q water, heating at 100 °C for 10 min (*S. aureus* was heated at 100 °C for 20 min), and centrifuging at 13 000 r.p.m. for 5 min. The supernatant was used as the template. Each 25 µl PCR mastermix consisted of 2.5 µl template, 5 µl 5× PCR buffer, 1.5-mM $MgCl_2$, 200-µM dNTP, 0.4-µM primers and 1.25 -U Taq DNA polymerase (Shanghai Shenggong, Shanghai, China). PCR assays were run in a DNA thermal cycler (Bio-Rad, Hercules, CA, USA), and included positive and negative amplification controls selected from previously described *E. coli* isolates.[18] Amplified PCR products were analyzed on 1.5–2.0% (w/v) agarose gels, and confirmed by sequencing (Shanghai Shenggong). All results were confirmed by at least two independent experiments.

Data analysis

For two dilutions, the MIC_{50} and MIC_{90} values were calculated to be the MICs where 50 and 90% of the bacteria were inhibited. The doubling dilution difference in the MIC was calculated as: log2 (MIC by agar dilution method) -log2 (MIC by microdilution method).[19] MIC agreement was defined as ±1 log2 dilution between the agar dilution and broth microdilution method.[20,21] Agreement between the methods was considered excellent if the MICs were within ±1 doubling dilution for ⩾90% of isolates. Agreement was good if ⩾75% of the MICs were within ±1 doubling dilution. Agreement was poor if <75% of MICs were within ±1 doubling dilution. Exact agreement was determined by the same MIC for each bacterial between agar dilution and microbroth dilution. [20,21]

Chi square or Fisher's exact test and T-test were used to analyze the data using SAS 9.2 (SAS Institute, Cary, NC, USA). A *P*-value <0.05 was considered statistically significant for comparison.

RESULTS

MICs determined by agar dilution and broth microdilution

MICs were obtained by agar dilution and broth microdilution for each bacterial strain and were shown in (Supplementary Table S1). While determining the MICs of individual strains, more noticeable differences were observed in the agar dilution method between the four QACs. Overall, DDAC was found to be more effective than other three QACs, BC, CTAB and CTPC against all the tested bacteria with the lowest MICs generated by both methods. Following DDAC, BC was more effective than CTPC and CTAB with lower MICs measured only

Table 1 Primer sequences for detection of QAC resistance genes

	Gene names	Oligonucleotide primer sequences	Product size (bp)	Reference
G−	*sugE(p)*	GTCTTACGCCAAGCATTATCACTA CAAGGCTCAGCAAACGTGC	190	31
	qacEΔ1	AATCCATCCCTGTCGGTGTT CGCAGCGACTTCCACGATGGGGAT	175	
	qacE	AAGTAATCGCAACATCCG CTACTACACCACTAACTATGAG	258	
	qacF	GTCGTCGCAACTTCCGCACTG CTACTACACCACTAACTATGAG	229	
	qacG	TCGCCTACGCAGTTTGGT AACGCCGCTGATAATGAA	122	
G+	*qacA/B*	GCAGAAAGTGCAGAGTTCG CCAGTCCAATCATGCCTG	361	34
	qacG	TAACTTACGCAACATGGGCA TCAATGGCTTTCTCCAAATAC	155	33
	qacJ	CTTATATTTAGTAATAGCG GATCCAAAAACGTTAAGA	306	4
	qacH	CAAGTTGGGCAGGTTTAGGA TGTGATGATCCGAATGTGTTT	122	33
	smr (qacC+ qacD)	GCCATAAGTACTGAAGTTATTGGA GACTACGGTTGTTAAGAC-TAAACCT	195	34

Abbreviations: G−, Gram-negative bacteria; G+, Gram-positive bacteria; QACs, quaternary ammonium compounds.

by broth microdilution method. And three Gram-positive strains (YM-2, ZHf1 and ATCC29213) were extremely susceptible to all of the four QACs compared with other strains.

The MICs varied depending upon the type of QACs and the bacterial species. Agar dilution MICs for CTAB were lower than or equal to those of broth microdilution MICs in all strains. The MICs of BC to *E. coli* of agar dilution were lower than or equal to those of broth microdilution. Agar dilution MICs for BC to *Salmonella* of agar dilution were higher or equal to those of broth microdilution. Agar dilution MICs for DDAC were lower than or equal to those of broth microdilution MICs in all strains. The MICs of CTPC to *E. coli* and *Staphylococcus* were also lower than those of broth microdilution. Conversely, agar dilution MICs for CTPC were higher or equal to those of broth microdilution MICs for *Salmonella* and *Klebsiella*. Overall, MICs of agar dilution were lower than those of broth microdilution except that MICs of BC and CTPC to *Salmonella* and *Klebsiella* of agar dilution were higher.

Table 2 summarized the MIC_{50} and MIC_{90} data by bacterial groups. The MIC_{50} and the MIC_{90} values of *Salmonella* were the same as *K. pneumoniae* for microdilution method. Compared with Gram-negative bacteria, the *S. aureus* was more sensitive to the QACs, and the MIC_{90} values were the lowest among all the isolates tested.

Agreement between MICs obtained by agar dilution and broth microdilution

To compare the MIC agreement between agar dilution and broth microdilution clearly, exact agreement and log2-transformed MIC data agreement were compared (Table 3). The exact agreement as determined by the same MICs for all bacterial groups were 17.65, 32.77, 49.58 and 22.69% for the DDAC, BC, CTPC and CTAB, respectively. When bacterial groups were defined as being within the ±1 doubling dilution, the combined MICs showed good or excellent MIC agreement for BC (78.15%), DDAC (82.35%), CTPC (97.48%) and CTAB (99.16%), respectively. In addition, *K. pneumoniae*/DDAC and *S. aureus*/DDAC approached excellent agreement (100%) within ±1 doubling dilutions. In contrast to Gram-negatives, the *S. aureus* group showed 100% agreement in DDAC, BC and CTAB testing, but it did not achieve excellent agreement in CTPC testing. Therefore, for the Gram-negatives, four QACs suggested high agreement, and a 100% match was observed for *St. aureus* tested for DDAC, BC and CTAB.

Detection of QACs resistance genes

The QACs resistance genes in each strain and the frequency of QAC resistance genes in different bacterial group were displayed in Supplementary Table S1 and Figure 1. Regardless of the test strains source, the *qacEΔ1* gene was commonly found in all Gram-negative bacteria. The *qacEΔ1* gene was the most frequently present in *E. coli* (69.70%; $n=23$), followed by *K. pneumoniae* (50.00%; $n=11$) and *Salmonella* (39.62%; $n=21$), respectively. The *sugE(p)* gene was found in 6.06% ($n=2$) of *E. coli*, 4.55% ($n=1$) of *K. pneumoniae*, and 1.88% ($n=1$) of *Salmonella*. The *qacF* gene was only detected in *K. pneumoniae* (22.73%; $n=5$) and *E. coli* (3.03%; $n=1$). The *qacE* or *qacG* genes were not detected, and no QACs resistance genes were found in any of Gram-positive bacterial groups. Compared with the control strains, all *qacEΔ1*-positive isolates ($n=55$) showed higher

Table 2 MIC_{50} and MIC_{90} values of four QACs against each bacterial group as determined by agar dilution and broth microdilution.

		MIC_{50} (%) values tested by								*MIC_{90} (%) values tested by*							
		Agar dilution				*Broth microdilution*				*Agar dilution*				*Broth microdilution*			
Bacterial group	*No. of strains*	*DDAC*	*BC*	*CTPC*	*CTAB*	*DDAC*	*BC*	*CTPC*	*CTAB*	*DDAC*	*BC*	*CTPC*	*CTAB*	*DDAC*	*BC*	*CTPC*	*CTAB*
Salmonella	53	16	128	256	256	32	128	256	512	16	256	256	512	32	128	256	512
E. coli	33	8	32	64	256	16	128	128	512	8	512	128	256	32	128	128	512
K. pneumoniae	22	16	256	256	128	32	128	256	256	32	512	512	512	32	128	256	512
S. aureus	11	4	32	128	32	8	64	256	64	8	256	256	128	16	128	256	256

Abbreviations: BC, benzalkonium chloride; CTAB, cetyltrimethylammonium bromide; CTPC, cetylpyridinium chloride; DDAC, didecyldimethylammonium chloride; QACs, quaternary ammonium compounds.

Table 3 Agreements of MICs for four QACs obtained by agar dilution and broth microdilution for different bacterial groups

		Agreement (%) between agar dilution and broth microdilution[a]							
		DDAC		*BC*		*CTPC*		*CTAB*	
Bacterial group	*No. of strains*	*Exact agreement*	*Within ± 1 dilution*	*Exact agreement*	*Within ± 1 dilution*	*Exact agreement*	*Within ± 1 dilution*	*Exact agreement*	*Within ± 1 dilution*
Salmonella	53	15.09	75.47	39.62	88.68	90.57	98.11	35.85	100.00
E. coli	33	18.19	75.76	33.33	72.73	9.09	93.94	6.06	96.97
K. pneumoniae	22	22.73	100.00	18.19	50.00	27.27	100.00	27.27	100.00
S. aureus	11	18.18	100.00	27.27	100.00	18.18	90.90	0.00	100.00
Combined	119	17.65	82.35	32.77	78.15	49.58	97.48	22.69	99.16

Abbreviations: benzalkonium chloride, BC; cetyltrimethylammonium bromide, CTAB; cetylpyridinium chloride, CTPC; didecyldimethylammonium chloride, DDAC; minimum inhibitory concentrations, MICs; QACs, quaternary ammonium compounds.
[a]Exact agreement was determined by the same MIC for each bacterial between agar dilution and microbroth dilution.

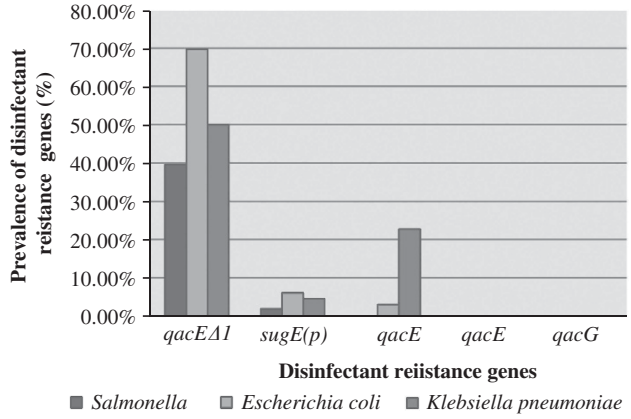

Figure 1 Percentage of QAC resistance genes in different bacterial groups.

MICs to CTAB for broth microdilution. Among these *qacEΔ1*-positive strains, 94.55% ($n=52$) of the MICs for CTAB testing were 512 $mg\,l^{-1}$. The higher MICs of QAC were significantly associated with *qacEΔ1* genes ($P<0.05$).

DISCUSSIONS

In this study, two dilution (broth microdilution and agar dilution) methods were used to assess microbial resistance to QACs against 119 foodborne and zoonotic pathogens. The results indicated that the MICs might be different depending on bacterial species, testing methods and the type of QACs.

Antimicrobial susceptibility testing is essential for effective control of pathogens. Moreover, MICs help in monitoring development of resistance and are relevant for the determination of optimal pharmacodynamic dosage.[21,22] Regardless of testing methods, *K. pneumoniae* was the most tolerant Gram-negative to four QACs, followed by *Salmonella* and *E. coli*. With both methods, DDAC showed stronger activities than the other three QACs against both Gram-negative and Gram-positive bacterial groups, which suggests that DDAC might be a better disinfectant. In fact, a previous study showed that DDAC demonstrated the best biocidal activity.[23] The antimicrobial activity of QACs has been reported against a wide range of foodborne and zoonotic pathogens using either broth or agar based methods.[5,24] Ioannou *et al.*,[25] showed that the BC and DDAC MICs of *S. aureus* were different when the broth concentration ranged from 1×10^5 to 1×10^9 CFU ml^{-1}, with the MICs between 0.4 and 1.8 $mg\,l^{-1}$ tested by agar dilution method. The increase in MICs at cell concentrations of 1×10^8 CFU ml^{-1} could be attributed to an increasing reservoir of cellular negatively charged teichoic acids, which could interact with the cationic biocide molecules reducing the available dose in solution.[25] Meanwhile, the antimicrobial activities of QACs differed with the temperature and pH changed by agar leading to reducing activity.[26,27] However, the effect of DDAC was little influenced by temperature.[23] Although much attention has been paid to employing the antimicrobial effects of QACs in food, domestic, agricultural and health-care facilities, there are no standardized methods to assess their antimicrobial activities.[1,2] The lack of standardization MIC test of QACs makes it very difficult to discuss trends in susceptibility. To our knowledge, this is the first study that compared the agar dilution and broth microdilution methods to assess the antimicrobial activity of QACs and could provide a reference to compare various results using different MIC determining methods.

This study showed that the agreement obtained by the two methods was good or excellent which was in contrast with the data reported by other researchers. A series of studies have compared the two dilution methods, finding the two methods agreed poorly on the MICs obtained.[28,29] Our results indicated that the MICs might be different depending upon the type of QACs and bacteria applied. The results revealed that with all strains, the agar dilution MICs of CTAB and DDAC were lower than those with broth microdilution. Likewise, the agar dilution MICs of CTPC for *E. coli and S. aureus* were also lower than those of broth microdilution. However, the agar dilution MICs of BC and CTPC for *Salmonella* and *K. pneumoniae* were higher than those of broth microdilution, which was in accordance with previous studies.[28,29] Thus, we found that agar dilution MICs were lower than those of broth microdilution except that MICs of BC and CTPC to *Salmonella* and *Klebsiella* of agar dilution were higher. This may be due to different saturation level of QACs in solid and liquid medium.[25,28] In addition, the broth microdilution using commercially prepared antimicrobial panels may be both cost and time efficient while yielding results comparable with agar dilution.[21] Although the agreement between the two dilution methods was very high, we recommend that agar dilution method could be used as a screening method for preliminary MIC determination, and the broth microdilution could be conducted parallel to confirm the results of the agar dilution method.

The association between antimicrobial resistance and the presence of different QAC resistance genes was investigated. The *qacEΔ1* gene was present in 39.62–69.70% of the isolates, followed by *qacF* (3.03–22.73%) and *sugE(p)* (1.88–6.06%). The *qacE* and *qacG* genes were not detected in all isolates as reported in a previous study.[30] However, mobile element encoded QAC genes were relatively low as reported by previous studies. Only 27% and 0% of *Salmonella* were positive for *qacEΔ1* and *qacE* genes, respectively.[30] In an analysis of 103 Gram-negative bacteria, Kucken *et al.*[9] reported that *qacEΔ1* was detected in 10% of isolates and *qacE* was only in one isolate. *qacF* and *qacG* were only present in 1.8% ($n=10$) and 0.4% ($n=2$), respectively.[31] These genes have been reported to be located in class 1 integrons.[14,15,32] The five QAC resistance genes, *qacE*, *qacEΔ1*, *qacF*[9], *qacG*[15] and *sugE(p)*[33] are located in mobile genetic elements contributing to resistance to QACs and linked (co-existed) with different antibiotic resistance genes. Most notably, *qacEΔ1* were highly associated ($P<0.05$) with high MICs of disinfectant. Studies have shown that *qacEΔ1* gene is common in enteric bacteria, and is located at the 3′-conserved segment of class 1 integrons that carry *sul*1 (sulfonamide resistance determinant).[9] These resistance genes encode efflux conferring resistance to QACs via an electrochemical proton gradient. Interestingly, we also found that the MICs value of some clinical isolates to QACs were high in *qacEΔ1* negative strains. Except for five mobile genetic elements mediated QAC resistance genes (*qacE, qacEΔ1, qacF, qacG* and *sugE(p)*), there might be other resistance genes showed cross-resistance to disinfectant.[14,15,32] Therefore, the strains negative for *qacEΔ1* gene with antibiotic resistance may also have high MIC of QACs due to having resistance genes cross-resistant to disinfectants.[33] The *sugE* gene has demonstrated resistance to CTPC, CTAB and BC, while the *qacEΔ1* gene conferred host resistance to different QACs[31] and co-resistance to antibiotics[9] The QAC resistance of bacteria was associated with antibiotic resistance, and the *qac* and *sugE(p)* disinfectant resistance genes were highly associated with multidrug resistance phenotypes. [30]Therefore, using QACs could provide selection pressure for strains with acquired resistance to antibiotics. Our study showed that these genes were not only commonly present in Gram-negative but also associated with reducing susceptibility to the QACs. However, among the four disinfectants tested, no matter of the sources of isolates, resistance status, and

genotype combinations of QAC resistance genes, DDAC remained as the most effective disinfectant against different bacteria.

In conclusion, this study indicated that MICs values varied depending upon bacterial genus, testing methods and the QACs used. *K. pneumoniae* was the most resistant among Gram-negative strains to four QACs, followed by *Salmonella* and *E. coli*. The agreement between MICs obtained by the two methods showed good or excellent MIC agreement. In addition, DDAC remained as the most effective disinfectant against different bacteria. Notably, *qacEΔ1* gene was highly associated ($P<0.05$) with high MICs of disinfectant. This is the first study that compared the agar dilution and broth microdilution methods to assess the antimicrobial activity of QACs. The study also demonstrated the need of a standardized method that would be used in evaluating QACs antimicrobial properties in the future.

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