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Research Article

Prevalence of microbial contamination and disinfectant-resistant *P. aeruginosa* in bottled drinking water samples tested at the National Institute for Food Control in 2024-2025

Pham Van Quan[1], Nguyen Thi Thanh Huyen[1], Tran Quynh Anh[2]*, Ninh Thi Hanh[1], Vu Thi Quy[2]
[1]*National Institute for Food Control, Hanoi, Vietnam*
[2]*Hanoi Medical University, Hanoi, Vietnam*

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Abstract

Bottled drinking water (BDW) is increasingly consumed in Vietnam; however, the risk of microbial contamination remains a significant public health concern. In particular, *Pseudomonas aeruginosa* (*P. aeruginosa)* is not only an opportunistic pathogen but also exhibits resistance to common disinfectants used in production processes. This study aimed to describe the current status of microbial contamination according to QCVN 6-1:2010/BYT and the presence of heterotrophic plate counts (HPC) in 70 BDW samples (19–21 L) tested at the National Institute for Food Control between October 2024 and April 2025. Results showed that 28.57% (20/70) of samples failed at least one microbiological parameter, with *P. aeruginosa* accounting for the highest contamination rate (25.71%, 18/70). Moreover, 52.86% (37/70) of samples exceeded the 500 CFU/mL limit for heterotrophic bacteria. Antimicrobial susceptibility testing revealed that 83% (15/18) of *P. aeruginosa* isolates were resistant to benzalkonium chloride (BKC) and 44% (7/18) were resistant to didecyldimethylammonium chloride (DDAC). The presence of *qacE* and *qacEΔ1* genes was strongly associated with the resistance phenotype, particularly in strains harboring both genes. These findings indicate that large-volume BDW poses a high risk of microbial contamination, with *P. aeruginosa* being both a prevalent contaminant and notably resistant to commonly used disinfectants, suggesting the potential dissemination of resistance mechanisms within production and consumer environments.

Keywords: *Bottled drinking water, microorganisms, P. aeruginosa, disinfectants, heterotrophic plate counts (HPC).*

1. INTRODUCTION

Water plays a vital role in metabolic processes, as most biochemical reactions in the human body involve water as a solvent for numerous dissolved substances. Clean water improves quality of life and contributes significantly to the prevention of poisoning and infectious diseases, particularly gastrointestinal disorders such as diarrhea, typhoid fever, cholera, and dysentery. However, according to recent statistics from the Institute of Occupational and Environmental Health, approximately 17 million people in Vietnam are currently using unsafe and untreated water sources, such as rainwater or groundwater. Therefore, water safety has become an increasingly pressing public health concern.

In recent years, the consumption of bottled drinking water (BDW) has become widespread in schools, offices, institutions, factories, and households due to its convenience for direct use without boiling. In fact, the quality of BDW has a substantial impact on human health. Several studies have reported high rates of microbial contamination in BDW samples. For instance, a study by Nguyen Vu Thuan *et al.* (2023) found that 70.7% of BDW samples failed to meet microbiological standards [1]; Nguyen Thi Ngoc Duyen (2023) in Khanh Hoa province reported a non-compliance rate of 73.1% [2]; and Pham Van Hung (2020) in Hanoi observed a rate

*Corresponding author: Tran Quynh Anh (E-mail: tranquynhanh@hmu.edu.vn)
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of 33.0% [3]. Hanoi and Ho Chi Minh City, being major economic and cultural centers, attract a large number of migrants from surrounding regions, leading to population growth and an increasing demand for BDW. Therefore, ensuring the quality control of BDW is an urgent and essential task for safeguarding public health.

Moreover, many international studies monitoring the microbiological quality of BDW have highlighted the importance of heterotrophic plate counts (HPC) as a key indicator [4]. The use of HPC provides a more comprehensive understanding of contamination levels, as increased bacterial counts often signal potential hygiene deficiencies during production or storage.

In addition, contamination with *P. aeruginosa* in BDW is relatively common in Vietnam. Several studies have shown that *P. aeruginosa* contamination rates are typically higher than those of other bacteria found in BDW products. Quaternary ammonium compounds (QACs) are a group of broad-spectrum antimicrobial agents widely used in various fields, including food production, healthcare facilities, and water treatment (such as washing reusable bottles and treating source water). However, the increasing use of disinfectants containing QACs has raised concerns regarding the emergence of disinfectant-resistant bacteria. Some studies have reported the presence of resistance genes in *P. aeruginosa*, such as the works of Detmar Kücken *et al.* (2000) [5] and Mohammadreza Mahzounieh *et al.* (2015) [6]. Furthermore, *P. aeruginosa*'s ability to form biofilms contributes to its persistence and prevalence as a contaminant in water production systems.

In Vietnam, research on this topic remains limited. Therefore, this study was conducted on BDW samples analyzed at the National Institute for Food Control in 2024, aiming to describe the current situation of microbial contamination and determine the prevalence of *P. aeruginosa* resistant to disinfectants in these samples.

2. MATERIALS AND METHODS

2.1. Study materials

Materials: Bottled drinking water (BDW) samples with volumes of 19–21 liters, collected at the National Institute for Food Control using a convenience sampling method.

The sample size was calculated using the standard formula for a cross-sectional study.

$$N = Z^2_{(1-\alpha/2)} \; x \; \frac{p\,(1-q)}{d^2} \quad (1)$$

$Z_{(1-\alpha/2)} = 1.96$, corresponding to a significance level of p = 0.05.

p = 0.787, representing the proportion of contaminated bottled drinking water products in the Central Highlands region in 2021 [7].

d = 0.1, the acceptable absolute error.

By substituting these values into the sample size calculation formula, the required sample size was determined to be n = 65. Considering an additional 8–10% allowance for potential sample loss, the final sample size selected for the study was n = 70.

2.2. Equipment, chemicals, and reference materials

2.2.1. Equipment and instruments

A six-branch stainless-steel microbiological filtration system (Microsart e.jet Fluid Pump, 4 L/min – Sartorius, Germany); membrane filters with pore sizes of 0.2/0.45 µm (Cellulose Nitrate Filter – Sartorius, Germany); autoclave sterilization unit (ALP, Japan); incubator with adjustable temperature control at 36 ± 2°C (Memmert, Germany); pH meter with an accuracy of ± 0.1 at 20°C and 25°C; and 96-well V-bottom microplates (Corning, USA).

2.2.2. Chemicals

Thiosulfate–Tryptose Agar (Oxoid, UK); Chromogenic Coliform Agar (Oxoid, UK); Slanetz and Bartley Medium (Oxoid, UK); Pseudomonas Agar Base (Oxoid, UK); Brain Heart Infusion – BKC BHI (Merck, Germany); PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, USA); Primers (IDT, USA).

2.2.3. Reference standards

The reference standards included benzalkonium chloride and didecyldimethylammonium chloride obtained from LGC, Germany.

2.3. Study design

The study was a cross-sectional descriptive study with the samples tested at the National Institute for Food Control from October 2024 to April 2025.

2.4. Research methods

2.4.1. Sampling technique

Sampling of bottled water plays a crucial role in the process of microbiological analysis and food safety quality assessment. The collected samples were required to remain intact in their original packaging, within their expiration dates, and of sufficient volume for analysis.

Before analysis, samples were thoroughly mixed by vigorous shaking to ensure an even distribution of microorganisms. The bottle caps and surrounding areas were sterilized with 70% ethanol, and the caps were opened using sterile scissors. During sampling, the initial portion of water was discarded, and the sample was then taken from the main flow and transferred into sterile 1.5 L bottles for filtration and analysis. The entire sampling and transfer process was conducted in a cleanroom environment to ensure aseptic conditions and accuracy of analytical results [8].

2.4.2. Study variables

Proportion of bottled drinking water samples contaminated with *P. aeruginosa*, *Coliforms*, *E. coli*, *Streptococcus faecalis*, and Clostridia in finished products.

Proportion of total heterotrophic plate count (HPC) bacteria isolated from bottled drinking water samples.

Proportion of *P. aeruginosa* isolates resistant to disinfectants in bottled drinking water samples.

2.4.3. Analytical methods

- Microbiological analysis techniques: Detection and enumeration of *E. coli* and Coliform bacteria were performed according to TCVN 6187-1:2019 [9]. Detection and enumeration of *P. aeruginosa* followed TCVN 8881:2011 [10]. Isolation and quantification of *Streptococcus faecalis* was carried out in accordance with TCVN 6189-2:2009 [11]. Detection and enumeration of sulfite-reducing Clostridia spores were based on TCVN 6191-2:1996 [12]. Quantification of total heterotrophic microorganisms (HPC) was conducted following ISO 6222:1999 [13].

- Determination of minimum inhibitory concentrations (MIC) for disinfectants: The MIC of the two quaternary ammonium compounds (QACs) - benzalkonium chloride (BZK) and didecyldimethylammonium chloride (DDAC) - were determined in 96-well polystyrene microtiter plates using the microdilution method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [14].

Initially, pure bacterial colonies were cultured in tryptic soy broth (TSB) at 37°C for 18–24 h to achieve a cell density of approximately 10^8–10^9 CFU/mL. The *P. aeruginosa* suspension was then adjusted to 10^6 CFU/mL before proceeding with subsequent experimental steps.

After preparing the bacterial suspension, 200 µL of the QAC disinfectant solution was added to the first well of each row. Simultaneously, 100 µL of tryptic soy broth (TSB) was added to the subsequent eight wells. The antimicrobial solution was then serially twofold diluted by transferring 100 µL from the first well to the second, followed by transferring 100 µL from the second to the third, and so on, resulting in decreasing QAC concentrations from 256 µg/mL in the first well to 2 µg/mL in the eighth well of each row (**Figure 1**).

The positive control consisted of 100 µL of bacterial culture mixed with 100 µL of TSB, while the negative control represented the interaction between TSB and the disinfectant, containing 100 µL of QAC and 100 µL of TSB. The microplates were then incubated at 37°C for 24 h to assess bacterial growth based on turbidity observation [15]. In this study, the MIC value was defined as the lowest concentration of the antimicrobial agent that inhibited the visible growth of *P. aeruginosa* (**Figure 1**).

PCR Amplification of Target Genes: Genomic DNA was extracted from the enriched cultures of isolated colonies using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's protocol. The primer pairs for amplification of the target genes *qacE* and *qacEΔ1* were adopted from the study by Zeinab H. Helal *et al*. (2015) [16], as shown in **Table 1**. The reaction components and thermal cycling conditions for qacE and qacEΔ1 genes are presented in **Table 2**.

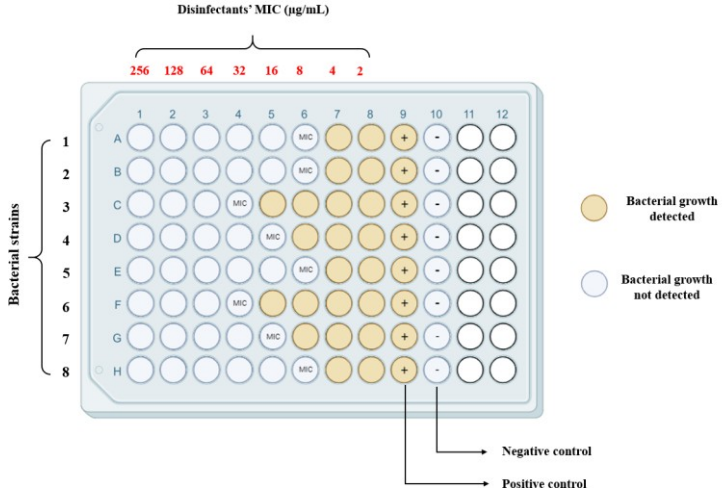

Figure 1. *Determination of MIC for disinfectants*

Table 1. *Specific primer sequences for qacE and qacEΔ1 genes*

Primer sequence	Product size	Target gene
Forward primer qacE-For: 5`- CCCGAATTCATGAAAGGCTGGCTT-3′ Reverse primer qacE-Rev: 5′-TAAGCTTTCACCATGGCGTCGG-3′	350 bp	qacE
Forward primer qacEΔ1-For: 5′- TAGCGAGGGCTTTACTAAGC-3′ Reverse primer qacEΔ1-Rev: 5′-TAAGCTTTCACCATGGCGTCGG-3′	300 bp	qacEΔ1

Table 2. *Reaction components and thermal cycling conditions for qacE and qacEΔ1 genes*

Reaction components	Promega GoTaq master mix, 2X	1X
	Template DNA (100 – 200 ng)	100 - 200 ng
	Forward primer (10 µM)	0.4 µM
	Reverse primer (10 µM)	0.4 µM
	Add nuclease-free water to	25 µL
Thermal cycling conditions		
Step 1	Initial denaturation	120 s at 93°C
Step 2	35 cycles consisting of:	
	Denaturation	30 s at 93°C
	Annealing	30 s at 55°C
	Extension	60 s at72°C
Step 3	Final extension	300 s at 72°C

PCR amplification products were electrophoresed for 47 min at 110 V on a 1.5% (w/v) agarose gel containing RedSafe dye (INtRON Biotechnology, code: 21141). The molecular size marker used for electrophoresis analysis was the GeneRuler 50 bp DNA Ladder (Thermo Fisher Scientific).

2.4.4. Criteria for evaluation of analytical results

Microbiological criteria for bottled drinking water followed QCVN 6-1:2010/BYT issued by the Ministry of Health [17]. For heterotrophic plate counts (HPC), the limit of 500 CFU/mL was applied according to the recommendation of the U.S. Environmental Protection Agency (EPA) [18].

Following the classification proposed by Nicola L. Cowley *et al.* [19], *P. aeruginosa* isolates were divided into two groups based on their tolerance to QACs: isolates with MIC values of 2–16 µg/mL were considered susceptible, while those with MIC values of 16–128 µg/mL were classified as reduced-susceptibility or resistant strains.

3. RESULTS AND DISCUSSION

3.1. Results

A total of 70 bottled drinking water samples were collected and analyzed for microbiological indicators at the Department of Microbiology and Genetic Modification, National Institute for Food Control, during 2024–2025. The findings are presented in **Figure 2**.

Based on the results presented in **Figure 2**, a total of 20 out of 70 bottled drinking water samples (28.57%) failed to meet microbiological standards (contaminated with at least one of the five microbial indicators according to QCVN 6-1:2010/BYT). Meanwhile, 50 out of 70 samples (71.43%) met all microbiological requirements.

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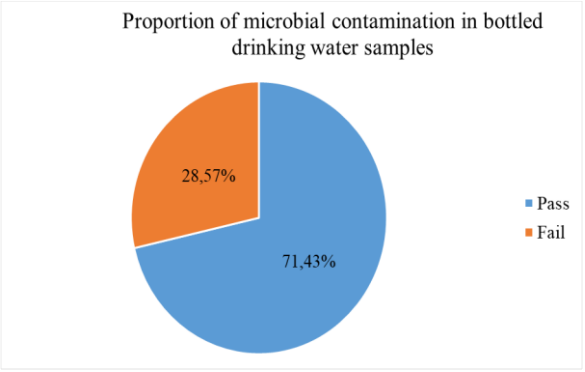

Figure 2. *Proportion of microbial contamination in bottled drinking water samples*

As presenrted in **Table 3**, among all 70 bottled drinking water samples analyzed, the number of samples failing each microbiological criterion under QCVN 6-1:2010/BYT, ranked from highest to lowest, was as follows: 18 samples failed the *P. aeruginosa* criterion, accounting for the highest proportion at 25.71%; 4 samples failed the Coliform criterion (5.71%); and 1 sample failed the *Streptococcus faecalis* criterion (1.43%). All 70 samples met the requirements for *E. coli* and sulfite-reducing Clostridia spores. Regarding the HPC indicator, 37 out of 70 samples (52.86%) had heterotrophic bacterial counts exceeding 500 CFU/mL. The number of samples in which no microorganisms were detected or in which counts were below 500 CFU/mL was 33 samples (47.14%). These results demonstrate that the non-compliance rate for total heterotrophic bacteria (HPC) in large-volume bottled drinking water (19–21 L) is notably high (52.86%, 37/70).

Table 3. *Rates of microbial contamination in bottled drinking water products by microbial indicator*

Microbiological indicator	Number of compliant samples		Number of non-compliant samples	
	n	Percentage %	n	Percentage %
Total Coliforms	66	94.29	4	5.71
E. coli	70	100	0	0
Streptococcus faecalis	69	98.57	1	1.43
P. aeruginosa	52	74.29	18	25.71
Sulfite-reducing anaerobic spores	70	100	0	0

According to the results presented in **Table 4**, the MIC values of *P. aeruginosa* isolates against the disinfectants BKC and DDAC ranged from 2–128 µg/mL, encompassing both susceptible and reduced-susceptibility/resistant strains.

Table 4. *Proportion of P. aeruginosa resistant to disinfectants*

Disinfectant	Percentage (%) (n = 18)	
	Susceptible (MIC: 2-16 µg/mL)	***Reduced susceptibility / Resistant (MIC: 16-128 µg/mL)***
BKC	17% (3/18)	83% (15/18)
DDAC	56% (10/18)	44% (8/18)

For benzalkonium chloride **(**BKC**)**, 17% (3/18) of the isolates were classified as susceptible. The remaining 83% (15/18) exhibited reduced susceptibility or resistance. Among these 15 isolates, MIC values of 3 isolates were recorded at 16 µg/mL, while the remaining 12 isolates showed MIC values of 32 µg/mL.

For didecyldimethylammonium chloride (DDAC), 56% (10/18) of the isolates were susceptible and 44% (8/18) showed reduced susceptibility or resistance. Among these 8 isolates, 6 isolates exhibited MIC values of 16 µg/mL, and the remaining 2 isolates had MIC values of 32 µg/mL.

The findings indicate that the levels of resistance and reduced susceptibility to the disinfectants benzalkonium chloride (BKC) and didecyldimethylammonium chloride (DDAC) among *P. aeruginosa* isolates are closely associated with the presence of QAC resistance genes-specifically *qacE*, *qacEΔ1*, or both-as shown in **Table 5.**

Table 5. *Proportion of P. aeruginosa isolates carrying disinfectant resistance genes*

Gene	BKC		DDAC	
	Susceptible	Reduced susceptibility / Resistant	Susceptible	Reduced susceptibility / Resistant
qacE (n=5)	0%(0)	100% (5)	40% (2)	60% (3)
*qacE*Δ1 (n=10)	20% (2)	80% (8)	40% (4)	60% (6)
Both genes (n = 4)	00%(0)	100%(4)	25% (1)	75%(3)
No gene detected (n = 7)	29% (2)	71% (5)	71% (5)	29% (2)

For BKC, resistance was observed across nearly all groups, with the highest rates in isolates carrying both *qacE* and *qacEΔ1*: 100% of these strains exhibited reduced susceptibility or resistance. Among isolates carrying only *qacE* or only *qacEΔ1*, resistance rates also remained high (100% and 80%, respectively). Notably, even in isolates lacking qac genes, 71% still showed reduced susceptibility or resistance to BKC, suggesting the possible presence of additional resistance mechanisms beyond the qac gene family.

In contrast, resistance to DDAC was generally lower, although a similar trend was observed. The proportion of resistant isolates in the qac-negative group was 29%, increasing to 60% in isolates carrying a single resistance gene, and reaching 75% in isolates harboring both *qacE* and *qacEΔ1*.

Genomic DNA extracted from all 18 *P. aeruginosa* isolates was subjected to PCR amplification. Two primer pairs (*qacE*-For/Rev and *qacEΔ1*-For/Rev) were used to amplify the target genes associated with resistance to QACs, including *qacE* and *qacEΔ1*. The results showed that each well on the electrophoresis gel displayed a distinct band corresponding to the expected sizes of *qacE* (350 bp) and *qacEΔ1* (300 bp) (**Figure 3**).

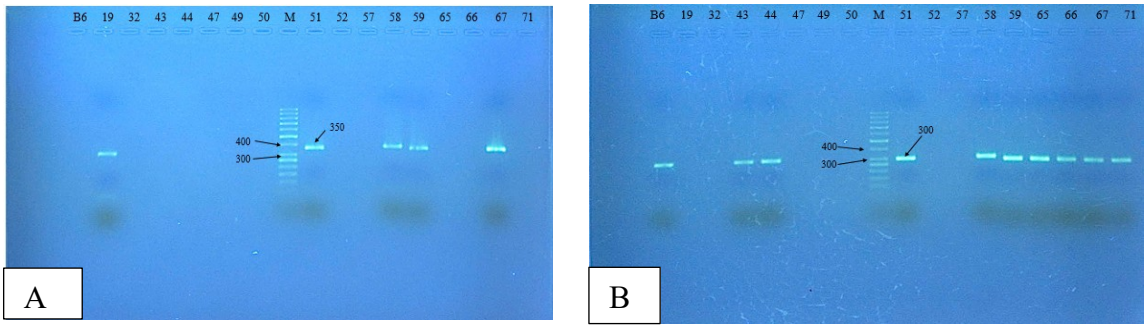

Figure 3. *Electrophoresis image of PCR products on 2.0% agarose gel from several P. aeruginosa isolates. (A) qacE gene (350 bp); (B) qacEΔ1 gene (300 bp); B6–71: amplified DNA products; M: molecular size marker*

The presence of these bands confirmed successful DNA extraction and PCR amplification of the target genes in all 18 *P. aeruginosa* isolates. Additionally, based on the electrophoresis results, *qacEΔ1*-positive strains accounted for a high proportion of the total isolates included in this study.

3.2. Discussion

The findings of this study showed that 28.57% of bottled drinking water (BDW) samples failed to meet microbiological standards according to QCVN 6-1:2010/BYT. Among the detected contaminants, *P. aeruginosa* exhibited the highest rate of occurrence (25.71%), substantially exceeding other indicators such as *Coliforms* (5.71%) and *Streptococcus faecalis* (1.43%). Meanwhile, all samples met the requirements for *E. coli* and sulfite-reducing anaerobic bacterial spores. The contamination rate of *P. aeruginosa* observed in this study (25.71%) is consistent with findings reported by Do Manh Hung (2016) in Hung Yen, with a rate of 25.0% [20]. Multiple studies have similarly documented *P. aeruginosa* as one of the most frequently detected contaminants in BDW. For instance, the study by Nguyen Thi Ngoc Duyen on contamination control at bottled water production facilities in Khanh Hoa province (2023) reported high contamination rates for two organisms: *P. aeruginosa* (68.8%, 64/93) and *Coliform*s (46.2%, 43/93) [21].

P. aeruginosa is not only an opportunistic pathogen, particularly dangerous for immunocompromised individuals, but also a microorganism capable of surviving in low-nutrient water environments. It can persist in water distribution systems and filtration equipment even after treatment, especially when hygiene practices are inadequate, biofilms are present, or large containers (19–21 L) are not thoroughly sanitized. The presence of *P. aeruginosa* in bottled water has also been documented in many other countries; for example, up to 50% of bottled water samples from various brands in Sri Lanka tested positive for this organism [22]. This underscores the global challenge of microbiological control in the bottled water industry, requiring not only proper treatment of source water but also strict adherence to hygienic practices in storage, bottling, and distribution.

Coliforms and Clostridia were detected at lower rates (5.71% and 1.43%, respectively). Although *E. coli* and sulfite-reducing Clostridia spores were absent from the samples, the detection of *Coliforms* remains a significant hygiene indicator, reflecting potential shortcomings in sanitation or processing and signaling an increased risk of contamination. These indicator organisms are strictly regulated in drinking water standards because they reflect overall sanitary conditions and may signal the possible presence of other enteric pathogens.

Currently in Vietnam, HPC is not a mandatory parameter for BDW under QCVN 6-1:2010/BYT. However, the proportion of samples exceeding the recommended HPC limit was notably high. Elevated HPC levels may result from insufficient water treatment or inadequate sanitary conditions during bottling, storage, and distribution. In particular, large-volume containers (19–21 L) may accumulate microbial biomass over time due to repeated reuse and improper cleaning. Although HPC does not specifically represent pathogenic microorganisms, it is an important indicator of overall microbiological quality and treatment efficiency. According to WHO (2017), high HPC levels may increase the likelihood of opportunistic pathogens persisting in drinking water, especially when combined with factors such as unstable storage temperatures or prolonged use after opening.

P. aeruginosa is a Gram-negative, aerobic bacillus capable of surviving under harsh environmental conditions and commonly found in inadequately maintained water systems. It can cause opportunistic infections, particularly in immunocompromised individuals. Importantly, *P. aeruginosa* can form biofilms and exhibit resistance to numerous disinfectants and antibiotics, supported by mechanisms such as multidrug efflux pumps and resistance-associated genes including *qacE* and *qacEΔ1*. The detection of these genes in the isolates is crucial in understanding their potential resistance to quaternary ammonium compounds (QACs), particularly BKC and DDAC. All strains carrying both genes (n = 4) showed complete resistance to BKC, suggesting that the co-occurrence of *qacE* and *qacEΔ1* may enhance resistance. Previous studies have also noted that *qacE/qacEΔ1* genes are often located on plasmid-associated gene cassettes, which may facilitate their spread across bacterial species [23].

Notably, 71% of isolates lacking qac genes still exhibited reduced susceptibility or resistance to BKC, indicating the presence of alternative resistance mechanisms, such as upregulation of intrinsic efflux pumps (e.g., MexAB-OprM) or alterations in membrane lipid composition. Furthermore, biofilms are known to reduce disinfectant efficacy by restricting penetration and creating a protective environment for embedded cells [24].

Compared to BKC, DDAC showed greater effectiveness against *P. aeruginosa*, with 56% of isolates remaining susceptible, whereas only 17% were susceptible to BKC. This suggests that DDAC may be a more

suitable choice for water system disinfection. Previous studies have also reported that DDAC can penetrate bacterial membranes more effectively than BKC and is less affected by biofilm structures [25]. However, the proportion of DDAC-resistant isolates remained considerable (44%), particularly in the group carrying both resistance genes (75%). This highlights the risk that excessive or improper use of QAC-based disinfectants may increase selective pressure and drive the emergence of disinfectant-resistant microorganisms-an issue increasingly recognized in public health and food industry settings.

4. CONCLUSION

The high prevalence of *P. aeruginosa* in bottled drinking water, together with its substantial resistance to the disinfectants BKC and DDAC, indicates the potential for persistent contamination within production environments. The presence of disinfectant-resistant strains increases the likelihood of recurrent contamination and complicates control efforts, posing significant challenges for hygiene assurance and public health protection.

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