Original Article

Effect of Benzalkonium Chloride and Chlorhexidine on the Antibiotic Susceptibility of *Pseudomonas aeruginosa*

Thitinan Phutthilertmethawee[1], Potjanee Srimanote[2], Pholawat Tingpej[1*]

Abstract

Introduction: The transmission of *Pseudomonas aeruginosa* can be controlled using biocides such as benzalkonium chloride (BKC) and chlorhexidine (CHX). However, debates continue over whether biocide exposure contributes to the development of antibiotic resistance.

Objectives: To investigate the impact of BKC and CHX exposure on the development of antibiotic resistance in *P. aeruginosa*.

Methods: The minimal inhibitory concentrations (MICs) of *P. aeruginosa* PAO1 (ATCC27853) and 15 clinical isolates against BKC and CHX were determined. The bacteria were grown in media containing subinhibitory concentrations (SICs) of these biocides for five consecutive passages. MICs for ciprofloxacin, ceftazidime, and gentamicin, as well as their susceptibility status, were compared before and after exposure.

Results: The MIC of BKC ranged from 0.002% to 0.047%, while the MIC of CHX was 0.001%, both well below their recommended concentrations. After exposure to BKC and CHX, most strains showed minimal or no changes in MIC values for ciprofloxacin, ceftazidime, and gentamicin, with no significant changes in antibiotic susceptibility. A few strains, such as SP275, P330, SP119, SP546, and SP48, exhibited more than twofold antibiotic MIC increases, but these changes were not statistically significant. After CHX exposure, strain P10 showed significant MIC changes without altering its susceptibility status to ciprofloxacin or ceftazidime.

Conclusions: Exposure to SICs of BKC and CHX increased antibiotic MICs in a minority of *P. aeruginosa* strains without affecting their antibiotic susceptibility status. This research provides valuable data supporting the use of biocides in infection control in both healthcare settings and the general environment.

Keywords: *Pseudomonas aeruginosa*, Benzalkonium chloride, Chlorhexidine, Biocides, Antibiotic resistance

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[1] Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani, Thailand
[*] **Corresponding Author:** Pholawat Tingpej, MD, PhD, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Thailand, Tel. +668 9797 8101, Email: tpholawa@tu.ac.th

Introduction

Healthcare-associated infections have become a significant issue in global public health.[1, 2] One of the common pathogens responsible for hospital-acquired infections is *P. aeruginosa*.[3, 4] This notorious pathogen plays a crucial role in causing infections in various systems of the body.[5] It is often found in patients with reduced immune levels, those undergoing chemotherapy, and hospitalized patients. Additionally, *P. aeruginosa* commonly exhibits resistance to antibiotics, making it necessary for World Health Organizations to prioritize it as one of the pathogens of critical concern that urgently requires research and development of new antibiotics.[6] Therefore, it is important to investigate factors that may contribute to drug resistance of this pathogen.

The mitigation of hospital-acquired infections relies on a collaborative approach involving hand hygiene practices, thorough cleaning and disinfection, stringent sterilization of medical equipment, and appropriate use of personal protective equipment. These measures should be complemented by robust surveillance for infection outbreaks, patientisolation and quarantine protocols, and prompt investigation when outbreaks occur.[7]

One strategy for controlling pathogen outbreaks in hospitals involves the cleaning and disinfecting of environmental surfaces prone to pathogen dissemination or transmission. Currently, biocides, which include disinfectants for general surfaces and antiseptics for human tissue, are increasingly utilized for this purpose. Commonly used biocides include benzalkonium chloride (BKC) and chlorhexidine (CHX).[8]

While one approach to mitigate hospital-acquired infections involves the application of biocides for environmental disinfection, ongoing debates persist regarding the potential role of biocide utilization in promoting antibiotic resistance.[9, 10] The present study thus aimed to investigate the effects of biocide exposure, specifically BKC and CHX, on the alteration of susceptibility profiles of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin by comparing minimal inhibitory concentration (MIC) values before and after exposure. These antibiotics were chosen to represent different major classes of antibiotics: ciprofloxacin (a fluoroquinolone) inhibits DNA gyrase, ceftazidime (a cephalosporin) targets bacterial cell wall synthesis, and gentamicin (an aminoglycoside) interferes with protein synthesis. This selection enables a broader assessment of how biocide exposure might affect bacterial resistance across different antibiotic classes. Understanding the factors contributing to this resistance is crucial for more effective infection prevention and control in hospitals.

Methods

Bacterial strains and cultivation

The *P. aeruginosa* strains used in this study included a standard strain (PAO1; ATCC27853) and 15 strains separated from the clinical samples (Table 1). *P. aeruginosa* strains were cultured on 5% Sheep Blood agar (BioMedia, Nonthaburi, Thailand) at 37°C for 18 hours or inoculated into Tryptic Soy Broth (TSB) (Thermo Fisher Scientific, Waltham, MA, USA), at 37°C for 18 hours. Stock cultures were prepared by transferring the isolated colonies grown on 5% Sheep Blood agar into 15% Glycerol in 2-mL TSB and stored at -80°C. For each experiment, subculturing was performed directly from the stock culture to ensure consistent characteristics of the strains in each experiment.

Table 1 *P. aeruginosa* strains used in this study

Strain	Characteristics
PAO1 ATCC 27853	Standard strain
SP2	Clinical isolate from a sputum sample
SP21	Clinical isolate from a sputum sample
SP30	Clinical isolate from a sputum sample
SP48	Clinical isolate from a sputum sample
SP119	Clinical isolate from a sputum sample
SP275	Clinical isolate from a sputum sample
SP539	Clinical isolate from a sputum sample
SP541	Clinical isolate from a sputum sample
SP546	Clinical isolate from a sputum sample
SP556	Clinical isolate from a sputum sample
H2173	Clinical isolate from a blood sample
P4	Clinical isolate a pus sample
P10	Clinical isolate a pus sample
P327	Clinical isolate a pus sample
P330	Clinical isolate a pus sample

Determination of the minimum inhibitory concentration (MIC) of benzalkonium chloride (BKC) and chlorhexidine (CHX)

The MIC of BKC and CHX was determined using the broth microdilution method, adapted from the Clinical Laboratory Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing.[11] BKC was purchased from Chemipan Corporation, Bangkok, Thailand, and Chlorhexidine digluconate solution was purchased from Sigma, Burlington, MA, USA. *P. aeruginosa* strains were incubated in Mueller Hinton Broth (MHB) (Biokar Diagnostics, Allonne, France) at 37°C for 18 hours. After the incubation period, the bacterial cultures were adjusted to a turbidity equivalent to 0.5 McFarland standard (10^8 CFU/mL) and tested with BKC at an initial concentration of 2% v/v and CHX at an initial concentration of 2% v/v in a 96-well plate. Each well contained 50 microliters of the biocides, followed by a 2-fold serial dilution with MHB. Then, 50 microliters of the adjusted *P. aeruginosa* culture were added to each well (final volume of 100 microliters). Positive control wells contained *P. aeruginosa* PAO1 ATCC27853 in MHB, while negative control wells contained only MHB at a volume of 100 microliters. The inhibition of bacterial growth was recorded, and the MIC was defined as the lowest concentration of the antimicrobial agent that inhibited bacterial growth.

Cultivation in the subinhibitory concentration (SIC) of BKC and CHX

Subinhibitory concentration (SIC) was calculated as half of the MIC values. *P. aeruginosa* isolates were inoculated into MHB containing the SIC of BKC and the SIC of CHX and incubated at 37°C for 18 hours. Subsequently, the cultures were transferred onto Mueller Hinton Agar (MHA) (Himedia, Mumbai, India) plates containing the same concentration of biocides and incubated again at 37°C for 18 hours. This process was repeated four more times. After completion, the remaining cultured bacteria were then subjected to further testing.

Determination of the MIC for ciprofloxacin, ceftazidime and gentamicin using broth microdilution test

The MIC values of ciprofloxacin, ceftazidime, and gentamicin for each *P. aeruginosa* strain were obtained through the broth microdilution test method as recommended in the CLSI guidelines for antimicrobial susceptibility testing M100-S32.[11] Ciprofloxacin and gentamicin were purchased from Sisco Research Laboratories, Mumbai, India, and ceftazidime was purchased from Sigma, Burlington, MA, USA. The MIC testing was conducted for all three antibiotics before the bacteria were exposed to the antimicrobial agents and again after culturing the bacteria under the subinhibitory concentration

of BKC and CHX. Specifically, an 18-hour culture in MHB was diluted to a 0.5 McFarland standard (10^8 CFU/mL) and then further diluted 1:100 to achieve a concentration of 10^6 CFU/mL, and 50 µL of this suspension was inoculated into each well of the microdilution plate, resulting in a final bacterial concentration of approximately 5 x 10^5 CFU/mL. Two-fold serial dilutions of the antibiotics were prepared and added to each well to achieve a final volume of 100 µL. Each test was repeated three times, and the results were reported as the average MIC values obtained for all tests. The interpretation of antibiotic susceptibility status was performed according to the criteria defined by CLSI, where "S" indicated susceptibility to the antibiotic, "I" indicated intermediate resistance, and "R" indicated resistance to the antibiotic.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10.3.0 for Windows. MIC values before and after exposure to BKC or CHX were compared using the paired t-test and the Wilcoxon signed-rank test, with p-values < 0.05 considered statistically significant.

Results

MIC of BKC and CHX

The MIC values of BKC and CHX, which were the minimal concentrations of biocides that inhibited the growth of *P. aeruginosa*, and the SIC values, which were half of the MIC, are shown in Table 2.

In this experiment, the MIC values of both BKC and CHX were found to be lower than the recommended concentrations of both biocides. The typical concentration for microbial eradication of BKC ranges from 0.1% to 1%. However, this study demonstrated that concentrations as low as 5 to 50 times lesser than the recommended range still successfully inhibited the growth of *P. aeruginosa*. Similarly, for CHX, which is commonly used at a concentration of 0.5% to 4% for skin disinfection and surgical site preparation, this study found that a minimum concentration of 0.001% was sufficient to inhibit microbial growth.

The findings illustrate that the utilization of both BKC and CHX at their recommended concentrations exhibits potent bactericidal efficacy due to their MIC values being significantly far below the recommended concentrations. No resistance to biocides was detected in this study.

Table 2 MIC and SIC of BKC and CHX

Strains	BKC			CHX		
	MIC (µg/mL)	SIC (µg/mL)	Chosen concentration for culture	MIC (µg/mL)	SIC (µg/mL)	Chosen concentration for culture
PAO1	0.023	0.0117	0.008	0.001	0.0005	0.0005
H2173	0.008	0.0039	0.004	0.001	0.0005	0.0005
P4	0.008	0.0039	0.004	0.001	0.0005	0.0005
P10	0.016	0.0078	0.008	0.001	0.0005	0.0005
P327	0.002	0.0010	0.004	0.001	0.0005	0.0005
P330	0.008	0.0039	0.004	0.001	0.0005	0.0005
SP2	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP21	0.047	0.0234	0.008	0.001	0.0005	0.0005
SP30	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP48	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP119	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP275	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP539	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP541	0.008	0.0039	0.004	0.001	0.0005	0.0005
SP546	0.016	0.0078	0.008	0.001	0.0005	0.0005
SP556	0.016	0.0078	0.008	0.001	0.0005	0.0005

Abbreviations: MIC, Minimal inhibitory concentration; SIC, Subinhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine

Culturing of *P. aeruginosa* in media containing SIC of BKC and CHX

For BKC, we initially chose concentrations based on the SIC levels of each strain. However, it was observed that when culturing *P. aeruginosa* isolates in media containing BKC at those concentrations for 18 hours, strains PAO1 ATCC27853 and SP21 failed to grow. As a result, the final concentration used for culturing these strains was reduced to 0.008 µg/mL alongside other strains, and a concentration of 0.004 µg/mL for the other strains as shown in Table 2.

For CHX, it was found that the MIC values for all strains were the same, at 0.001 µg/mL. This resulted in a calculated SIC value of 0.0005 µg/mL. When culturing *P. aeruginosa* with this concentration, all strains were able to grow. Therefore, we selected a concentration of 0.0005% for CHX for evaluating its effect on *P. aeruginosa.*

Effect of BKC on the susceptibility of *P. aeruginosa* to antibiotics

The MIC values and levels of antibiotic susceptibility to ciprofloxacin, ceftazidime and gentamicin of *P. aeruginosa* were compared before and after exposure to SIC of BKC. These comparisons are shown in Table 3. Overall, it was found that the majority of tested *P. aeruginosa* strains did not exhibit significant changes in MIC values or showed only minimal changes (not exceeding twice the original MIC values), with almost all changes being statistically insignificant. Furthermore, there was no change in the antibiotic susceptibility status after exposure to subinhibitory concentration of BKC.

After exposing to BKC at the subinhibitory concentration, only SP275 showed more than a twofold increase in MIC values of ciprofloxacin. However, this change was not statistically significant, and it did not alter the susceptibility status; therefore, SP275 remained susceptible to ciprofloxacin after exposure to BKC. Additionally, strain P10 exhibited a statistically significant decrease in MIC value by 0.1-fold. However, this change did not result in a change in the susceptibility level of the strain P10. Four strains including PAO1 ATCC27853, P330, SP119, and SP546 exhibited more than a twofold increase in MIC values of ceftazidime, but these increases were not statistically significant, and there were no changes in the susceptibility status of these four strains. SP48 showed more than a twofold increase in MIC values of gentamicin. However, this change was also not statistically significant and did not alter the susceptibility status to gentamicin.

Effect of CHX on the susceptibility of *P. aeruginosa* to antibiotics

The MIC values and the levels of antibiotic susceptibility to ciprofloxacin, ceftazidime and gentamicin of *P. aeruginosa* were compared before and after exposure to SIC of CHX (Table 4). Similarly to the results with BKC, it is observed that the majority of *P. aeruginosa* tested strains did not exhibit changes in MIC values or showed minimal changes (not exceeding twice the original MIC value), and these changes were mostly not statistically significant.

Only one strain, SP48, exhibited more than a twofold increase in MIC to ciprofloxacin, but this change was not statistically significant and did not alter the susceptibility status. Interestingly, strain P10 showed a statistically significant decrease in MIC to ciprofloxacin by 0.6-fold. The strain P10 also exhibited a statistically significant increase in MIC to ceftazidime by more than twofold (2.4-fold), yet this did not result in a change in susceptibility status to ceftazidime of this strain. Strain H2173 showed more than a twofold increase in MIC to gentamicin, but this change was not statistically significant and did not lead to a change in susceptibility status to gentamicin.

Table 3 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to BKC

Strains	Ciprofloxacin (µg/ml)			Ceftazidime (µg/ml)			Gentamicin (µg/ml)		
	Baseline	BKC	Fold Change	Baseline	BKC	Fold Change	Baseline	BKC	Fold Change
	Susceptibility level			Susceptibility level			Susceptibility level		
PAO1	0.094	0.104	1.1	0.50	2	4.0	1	1.3	1.3
	S --> S			S --> S			S --> S		
H2173	0.063	0.063	1.0	2	2	1.0	0.25	0.25	1.0
	S --> S			S --> S			S --> S		
P4	0.063	0.063	1.0	1.5	1	0.7	2	1	0.5
	S --> S			S --> S			S --> S		
P10	0.094	0.013	0.1*	0.83	1	1.2	1	1.3	1.3
	S --> S			S --> S			S --> S		
P330	0.063	0.125	2.0	1	4	4.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP2	0.047	0.052	1.1	1.5	4	2.7	1	1.3	1.3
	S --> S			S --> S			S --> S		
SP30	0.031	0.063	2.0	2	2	1.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP48	0.063	0.125	2.0	6	6	1.0	1	4	4.0
	S --> S			S --> S			S --> S		
SP119	0.063	0.063	1.0	1	4	4.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP275	0.063	0.250	4.0	2	4	2.0	0.75	1	1.3
	S --> S			S --> S			S --> S		
SP539	0.125	0.125	1.0	1.5	2	1.3	2	4	2.0
	S --> S			S --> S			S --> S		
SP541	0.125	0.125	1.0	4	4	1.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP546	0.063	0.063	1.0	1.5	8	5.3	1	2	2.0
	S --> S			S --> S			S --> S		
P327	8	8	1.0	>256	>256	N/A	32	32	1.0
	R --> R			R --> R			R --> R		
SP21	32	32	1.0	8	4	0.5	256	>256	N/A
	R --> R			S --> S			R --> R		
SP556	16	16	1.0	>256	>256	N/A	256	>256	N/A
	R --> R			R --> R			R --> R		

Abbreviations: MIC, Minimal inhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine; S, Susceptible; R, Resistance; *, $p < 0.05$

Table 4 Comparison of MIC and susceptibility status of *P. aeruginosa* to ciprofloxacin, ceftazidime and gentamicin before and after exposure to CHX

Strains	Ciprofloxacin (µg/ml)			Ceftazidime (µg/ml)			Gentamicin (µg/ml)		
	Baseline	CHX	Fold Change	Baseline	CHX	Fold Change	Baseline	CHX	Fold Change
	Susceptibility level			Susceptibility level			Susceptibility level		
PAO1	0.094	0.125	1.3	0.50	1	2.0	1	0.8	0.8
	S --> S			S --> S			S --> S		
H2173	0.063	0.052	0.8	2	2	1.0	0.25	1	4.0
	S --> S			S --> S			S --> S		
P4	0.063	0.063	1.0	1.5	2	1.3	2	2	1.0
	S --> S			S --> S			S --> S		
P10	0.094	0.052	0.6*	0.83	2	2.4*	1	1.7	1.7
	S --> S			S --> S			S --> S		
P330	0.063	0.063	1.0	1	2	2.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP2	0.047	0.094	2.0	1.5	4	2.0	1	1.3	1.3
	S --> S			S --> S			S --> S		
SP30	0.031	0.063	2.0	2	2	1.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP48	0.063	0.500	8.0	6	8	1.3	1	1	1.0
	S --> S			S --> S			S --> S		
SP119	0.063	0.031	0.5	1	2	2.0	1	1	1.0
	S --> S			S --> S			S --> S		
SP275	0.063	0.063	1.0	2	2	1.0	0.75	1	1.3
	S --> S			S --> S			S --> S		
SP539	0.125	0.094	0.8	1.5	2	1.3	2	2	1.0
	S --> S			S --> S			S --> S		
SP541	0.125	0.125	1.0	4	4	1.0	1	2	2.0
	S --> S			S --> S			S --> S		
SP546	0.063	0.063	1.0	1.5	2	1.3	1	2	2.0
	S --> S			S --> S			S --> S		
P327	8	8	1.0	>256	>256	N/A	32	64	2.0
	R --> R			S --> S			S --> S		
SP21	32	32	1.0	8	4	0.5	256	>256	N/A
	R --> R			S --> S			R --> R		
SP556	16	32	2.0	>256	>256	N/A	256	>256	N/A
	R --> R			R --> R			R --> R		

Abbreviations: MIC, Minimal inhibitory concentration; BKC, Benzalkonium chloride; CHX, Chlorhexidine; S, Susceptible; R, Resistance; *, $p < 0.05$

Discussion

Healthcare-associated infections represent a significant global public health issue, with *P. aeruginosa* as a common pathogen responsible for hospital-acquired infections. This notorious organism often exhibits multidrug resistance, employing various mechanisms such as target modification, enzyme-mediated drug inactivation, inhibition of drug uptake, and enhanced drug efflux.[12] Given its prevalence and resistance, *P. aeruginosa* poses a critical concern necessitating research and development of new antibiotics. Therefore, investigating the factors contributing to the drug resistance of this pathogen is essential.

One method to control hospital-acquired infection is the use of biocides for cleaning purposes. Biocides are substances that are used to kill or inhibit the growth of microorganisms, including bacteria, viruses, and fungi.[13] They are commonly employed in hospitals and healthcare facilities to disinfect surfaces and equipment, thereby reducing the risk of transmission of pathogens and preventing infections.

However, despite their widespread use, there is ongoing debate and controversy surrounding the potential effects of biocides on antibiotic resistance.[14, 15] Some studies suggest that the use of biocides may contribute to the development of antibiotic resistance in bacteria.[10] This could occur through mechanisms such as cross-resistance, where exposure to biocides leads to the selection of bacteria that are also resistant to antibiotics.[16]

Previous studies have found that the use of biocides such as BKC and CHX supports antibiotic resistance in various pathogens.[17, 18] *Salmonella enterica* and *Escherichia coli* have been shown to exhibit resistance to biocides and demonstrate cross-resistance to antibiotics.[19] Reports also indicate a correlation between *Acinetobacter* spp. with high MIC values for biocides and increased antibiotic resistance.[20] Two *P. aeruginosa* strains, PAO1 and OO14, expressed cross-resistance between BKC and antibiotics, including chloramphenicol and polymyxin B.[21] Considering the mechanisms of drug resistance, *P. aeruginosa* utilizes similar mechanisms for both antibiotic and biocide resistance. This involves reducing the permeability of the cell membrane, as well as employing active efflux to pump substances out, thereby conferring resistance to BKC, CHX, and other biocides.[22] This mechanism is similar to that used for antibiotic resistance. Therefore, it is speculated that biocide stimulation may increase the activation of the bacterial drug resistance mechanism, leading to the development of more drug-resistant strains. A recent study has further demonstrated that the subpopulation of *P. aeruginosa* isolates resistant to BKC showed significant cross-resistance to multiple antibiotics, including fluoroquinolones, cephalosporins, and aminoglycosides, highlighting the implications of biocide exposure on antibiotic resistance development.[23]

On the other hand, other research suggests that the relationship between biocide use and antibiotic resistance is complex and may not always lead to increased resistance.[24] Factors such as the specific biocide used, the concentration and frequency of use, and the genetic makeup of the bacteria being targeted can all influence the outcome.

Interestingly, the current study compared MIC values and antibiotic susceptibility levels of *P. aeruginosa* clinical isolates and PAO1 before and after exposure to the subinhibitory concentrations of BKC and CHX. Across both experimental conditions, the majority of tested strains did not exhibit significant changes in MIC values, with most alterations being non-statistically significant. Moreover, none of *P. aeruginosa* strains demonstrated the shifts in their antibiotic susceptibility status following exposure to the subinhibitory concentrations of BKC and CHX. Notably, the strain P10 exhibited a statistically significant increase in MIC to ceftazidime by more than twofold (2.4-fold) after being exposed to CHX. This unique observation suggests a possible inducement of resistance to ceftazidime due to CHX exposure. We also observed that exposure to BKC and CHX caused the strain P10 to exhibit a significant decrease in MIC to ciprofloxacin by 0.1-fold and 0.6-fold, respectively. However, it is important to note that this observation was limited to a single isolate out of the 16 tested strains, and it did not result in a change in the susceptibility status to antibiotics of this particular strain.

Our findings elucidated that the level of antibiotic resistance of *P. aeruginosa* after being cultured in subinhibitory concentration did not differ compared to the baseline antibiotic resistance level of the strain. These results were unexpected because it was anticipated that when bacteria adapted to become resistant to biocides, they would also increase their resistance to antibiotics.

While no significant changes were observed, they are consistent with the findings in various reports. Thomas et al. conducted experiments using *P. aeruginosa* NCIMB 10421, wherein they induced resistance to chlorhexidine diacetate and subsequently tested for antibiotic resistance. They found no cross-resistance between biocides and antibiotics.[24, 25] This aligns with the findings of Cole et al., who studied cross-resistance in over 1200 strains of various pathogens collected from patients and household settings, dividing them into two groups: those exposed to biocides and those not exposed.[26] They concluded that there was no correlation between antibiotic resistance and biocide exposure.[26]

Considering the conflicting findings in these reports, it should be cautioned when applying laboratory findings to clinical practice. The discovery of a relationship between biocide tolerance and antibiotic resistance is often tested in laboratory settings, and its application to practical medical contexts should be approached with caution.[22]

Despite contradictory conclusions in previous reports, the risk of developing biocide-resistant bacteria is minimal.[9] This is because the actual concentrations of biocides used are higher than those required to inhibit bacterial growth by several times, which aligns with the findings of this study (Table 2).

This study has notable limitations, including a small number of clinical and multidrug-resistant isolates, which may restrict the applicability of our findings. Nevertheless, the overall results provide important insights into the complex interplay between antimicrobial agents and bacterial resistance mechanisms.

In conclusion, while exposure to subinhibitory concentrations of BKC and CHX did not significantly alter the overall antibiotic susceptibility profile of the tested strains, the observed increase in MIC of ceftazidime in strain P10 after exposure to CHX suggests that certain isolates may develop resistance mechanisms upon biocide exposure. These findings emphasize the need for further research to elucidate the underlying mechanisms driving antimicrobial resistance in *P. aeruginosa.* Future studies with larger and more diverse sample sizes are essential for developing effective intervention strategies against hospital-acquired infections.

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Compliance with Ethics Requirements

The current work was approved by the Thammasat University Biosafety Committee, and the approval number is 018/2564.

Conflict of Interest

We declare that there are no conflicts of interest associated with this research.

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Author Contributions

T. Phutthilertmethawee, P. Srimanote, P. Tingpej contributed to the conception and design of the study. T. Phutthilertmethawee performed data acquisition. T. Phutthilertmethawee, P. Tingpej performed data analysis, and interpretation. P. Tingpej drafted the manuscript. All authors critically revised the manuscript and approved the final version for publication.

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