Polytechnic Journal. 2022. 12(2) 85-91
ISSN:
http://journals.epu.edu.iq/index.php/polytechnic

RESEARCH ARTICLE

Effects of Subminimal Inhibitory Concentrations of Chlorhexidine on the Chlorhexidine Resistance and Biofilm Formation in Clinical Drug-resistant *Acinetobacter baumannii* Isolates

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Received: 1 Jul 2022
Accepted: 16 Aug 2022
Published: 1 February. 2023

DOI10.25156/ptj.v12n2y2022.pp85-91

ABSTRACT

Background and objectives: Multi-drug resistant microorganisms have caused a remarkable increase in hospital-acquired infections (HAI) during the previous few decades. Moreover, microbiological contamination of antiseptics and disinfectants has been recorded in medical settings.

Methods: A total of 61 clinically relevant *Acinetobacter baumannii* isolates were collected from various clinical specimens in hospitals in Erbil city/Iraq between September 2021 and March 2022. The isolates were identified using VITEK-2 compact system. Antimicrobial susceptibility testing was carried out for a total of 16 different therapeutically relevant antibiotics in accordance with the criteria of the Clinical Laboratory Standards Institute (CLSI) using the above mentioned system. A spectrophotometer was used to determine the optical density at 600 nm (OD_{600nm}) for biofilm evaluation. Wilcoxon signed ranks test used to determine the effect of sub-MIC of CHX on the CHX-MIC and biofilm formation.

Results: the study showed that there is a significant difference in CHX-MIC and biofilm formation before and after 7 days of incubation of *Acinetobacter baumannii* in sub-MIC of CHX ($p<0.05$). Exposure of *Acinetobacter baumannii* to sub-lethal CHX concentrations develops inducible resistance to lethal CHX doses. The biofilm formation ability of isolates also increased after using sub-lethal dose of CHX.

Keywords: *Acinetobacter baumannii*, Chlorhexidine, Biofilm, Multiresistant.

INTRODUCTION

Multi-drug resistance microorganisms have caused a remarkable increase in hospital-acquired infections (HAI) during the previous few decades. Moreover, microbiological contamination of antiseptics and disinfectants has been recorded in medical settings (Amsalu et al., 2020). Nonetheless, the environmental transmission of antibiotic-resistant bacteria has been established all through hospital outbreaks (Nath et al., 2020). Furthermore, antibiotic resistance has been demonstrated in nosocomial microorganisms isolates from the healthcare setting (Jain et al., 2021).

Chlorhexidine (CHX) as a biguanide compound has been widely used as disinfectants, mouth rinses, in health facilities and during surgery (Cieplik et al., 2019). CHX is quite slightly soluble in water, therefore it's usually combined with gluconate or acetate to make water-soluble salts. CHX possesses antibacterial, antifungal, and even antiviral properties (Hashemi et al., 2019).

CHX acts on bacterial cell through disrupting the cell membrane (Tokajuk et al., 2017). The positive charge of CHX molecules attaches to the phosphate group that have a negative charge on the bacterial cell wall, causing the osmotic balance to be disrupted, this increases cell wall permeability, allowing CHX molecules to penetrate the bacteria, resulting in cytoplasmic leaking and death of cells (Balaure and Grumezescu, 2020). It has been found that CHX has both bacteriostatic and bactericidal properties, when used in low and high concentrations (Akhlaghi et al., 2019).

CHX is particularly effective towards Gram-positive bacteria, although it also acts on Gram-negative bacteria and fungi, and it can be combined with a number of antibiotics (Caruso et al., 2020). Unfortunately, frequently uses in wide range of CHX in a clinical and residential settings for many years has put selection pressure on dangerous bacteria and has expedited the development of resistant strains, including *Acinetobacter baumanii* (Biswas et al., 2019, Röhner et al., 2020).

Bacterial resistance to disinfectants could be an intrinsic characteristic, or it could result from chromosomal genetic mutation or from the acquiring of genetic material (biocide resistant genes (BRGs) such as qac and cepA genes) in the form of transposons or plasmids (Cieplik et al., 2019).

The reemergence of microbial resistance to antiseptics especially CHX, as well as the potential link between antiseptics, antibiotic resistance and biofilm formation is a serious concern: the phenomenon could result in the failure in disinfecting aseptic technique, as well as spread of nosocomial pathogens that are both disinfectant and antibiotic resistant (Chiang et al., 2018).

The study aimed to evaluate the MIC of CHX among *Acinetobacter baumannii* isolates, and to assess the effect of sub-MIC of CHX on the MIC of CHX and biofilm formation.

Materials AND METHODS

The study was conducted in accordance with Hawler Medical University's ethical commitment, and all participants had given their verbal permission to participate. A total of 61 clinically relevant *Acinetobacter baumannii* specimens (sputum, blood, pus, wound swab, urine, and body fluids (pleural fluid and cerebrospinal fluid) were collected from various clinical units and ICUs in hospitals in Erbil city/Iraq (West Emergency Hospital, Rzgary Teaching Hospital, Erbil Central Laboratory, Maryamana Private Hospital, PAR Private Hospital, and Bio Laboratory) between September 2021 and March 2022.

2.1 Materials

All the bacteriological culture media used in this study for bacterial cultivation were bought from the company Becton, Dickinson (BD BBL, USA). The broths and the agar plates were made in accordance with the instructions provided by the manufacturer. They were autoclaved for 15 minutes at a temperature of 121 °C. The laboratory works were performed with proper devices and tools uses in microbiological laboratories at private Maryamana Hospital in Erbil City.

2.2 Methods

2.2.1 Identification of *Acinetobacter baumannii*

In different medical laboratories all of the isolates were put through screening and identification using the VITEK-2 compact system (BioMérieux, Marcy L'Etoile, France). The procedure followed the instructions provided by the manufacturer. The method of identification is considered phenotypic identification, and it relies on biochemical responses to determine the identities of the isolates. The collected specimens were inoculated on MacConkey agar plates before being placed into an incubator at 37 °C overnight. Following incubation, a single colony was removed and suspended within the saline solution. VITEK Densichek (BioMérieux, France) photometric instrument was used to adjust the turbidity of the sample suspension using 0.45% VITEK-saline solution in order to conform to the McFarland 0.5 standard. The VITEK-2 compact system was then carefully loaded with the VITEK-2 ID-GN (Gram Negative) card as well as the bacterial suspension tubes. (Şimşek and Demir, 2020).

2.2.2 Antimicrobial Susceptibility Testing (AST)

Following identification of *Acinetobacter baumannii* isolates, VITEK-2 compact system was used to test the antimicrobial susceptibility pattern of the isolates for a total of 16 different therapeutically relevant antibiotics using specified (AST N326 and AST N327) in accordance with the criteria of the Clinical Laboratory Standards Institute (CLSI) including: cefepime (FEP: 30 µg), ceftazidime (CAZ: 30 µg), cefotaxime (CTX: 30 µg), meropenem (MEM: 10 µg), imipenem (IPM: 10 µg), levofloxacin (LVX: 5 µg), ciprofloxacin (CIP: 5 µg), netilmicin

(NET: 10 µg), piperacillin-tazobactam (TZP: 100/10 µg), piperacillin (PIP:100 µg), tobramycin(TOB: 10 µg), tigecycline (TIG: 15 µg), tetracycline (TE:30µg), gentamicin (GN: 10 µg), trimethoprim/sulfamethoxazole (SXT:1.25/23.75µg), and colistin (CL, 10 µg) (Clinical and Institute, 2015).

The Multidrug- resistant (MDR), Extensively drug- resistant (XDR), and Pandrug -resistant (PDR) strains were detected as per criteria described by (Magiorakos et al., 2012) as classified below: -

MDR: resistant to at least one agent in three or more than three antimicrobial categories, XDR: resistant to at least one agent in all but one antimicrobial categories,

PDR: resistant to all antimicrobial agents.

2.2.3 Estimation of MIC-CHX

Following identification of *Acinetobacter baumannii* isolates and antimicrobial susceptibility testing, 10 tubes contained 1 mL Brain heart infusion broth (BHI) were prepared making a two-fold dilution series of CHX (1024 µg/mL, 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, and 2 µg/mL), then 100 µL bacterial suspension (McFarland 0.5 standard) added to each tube. Positive and negative controls were prepared; the positive control contained the BHI and bacterial suspension without adding the CHX, while the negative control contained only the BHI without bacterial suspension and CHX, then all tubes incubated 24 hrs at 37 °C. Later, the tubes were checked for the determination of the MIC of CHX against *Acinetobacter baumannii*. The minimal inhibitory concentration (MIC) was determined to be the lowest concentration that can inhibit observable growth (Apisarnthanarak et al., 2014).

Then all tubes incubated up to 7 days at 37 °C as preparation for the next experiments (The study designed to incubate all the bacteria isolates in sub-lethal doses of CHX, for up to 7 days, in order to determine if this disinfectant would lead to changes in susceptibility if exposed for prolonged periods to chlorhexidine).

2.2.4 Effect of sub-MIC of CHX on CHX-MIC

When the MIC of CHX determined, 100 µL of bacterial suspension (McFarland 0.5 standard) prepared from the tube following the MIC (incubated 7 days) and once more added to a 10 tubes contained BHI and serially diluted CHX (1024 µg/mL, 512 µg/mL, 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, and 2 µg/mL), all samples were incubated at 37 °C for overnight, then tubes were observed to evaluate the difference in MIC values of CHX before and after keeping the bacteria in sub-MIC of CHX for a week.

2.2.5 Effect of sub-MIC of CHX on the Biofilm Formation

The same tubes that used in the above experiment were also used to evaluation of the effect the sub-MIC of CHX on the biofilm formation. The tubes were handled carefully while being rinsed three times with 1 mL of phosphate buffered saline (PBS), after which they were allowed to dry in an inverted position before being stained with 1% crystal violet for 30 minutes. In order to dissolve the crystal violet, the tubes were given one additional washing in 1 mL of 95% ethanol. A spectrophotometer was used to determine the optical density at 600 nm (OD_{600nm}) (King et al., 2009).

For figuring out the biofilm formation, the following values were used to estimate the degree of biofilm:

Non-biofilm producer: OD < ODC-ve

Weak biofilm producer: ODC-ve ≤ OD<2 ODC-ve

Medium biofilm producer: 2 ODC-ve ≤ OD<4 ODC-ve

Strong biofilm producer: 4 ODC-ve ≤OD

2.2.6 Statical Analysis

The statistical analysis of the data was performed using SPSS statistics (version 24.0). The discrepancies between the variables were described using descriptive statistics. The data were presented as frequency and percentages. Wilcoxon Signed Ranks Test was used to calculate the significances. *P* values < 0.05 were considered as statistically significant.

RESULTS

A total of 61 isolates of *Acinetobacter baumannii* were collected from different hospitals in Erbil city during the study period. *Acinetobacter baumannii* was isolated from 32 sputum (52.46%), 10 blood (16.39%), 8 wound swab (13.11%), 4 urine (6.56%), 3 cerebrospinal fluid (CSF) (4.92%), 2 central venous (CV) line (3.28%), and 2 Pleural fluid (3.28%) specimens (Fig 1).

Out of 61 isolates, only 2 (3.28%) were considered as PDR, while 8 (13.12%) were MDR, and 50 (83.6%) were XDR (Fig 2).

Regarding the Antimicrobial susceptibility testing (AST), all the isolates 61(100%) were resistant to (CTX 30mg) and (CIP 5mg), while only 2(3.28%) of the isolates were resistant to (CL 10mg). The extent of antimicrobial susceptibility test (AST) for 16 relevant antibiotics is presented in table 1.

3.1. Estimation of MIC-CHX

The results of the current study revealed the mean MIC of CHX for isolates was (46.951 ± 11.68) µg/mL. Out of 61 isolates the CHX-MIC of 31 (50.82%) was 32 µg/mL, and 13 (21.31%) isolates showed 16 µg/mL CHX-MIC, while, 12 (19.67%), 3 (4.92%), and 2 (3.28%) isolates were 64,128, and 256 µg/mL respectively table 2.

3.2. Effect of Sub-MIC of CHX on the CHX-MIC

Wilcoxon signed ranks test used to determine the effect of sub-MIC-CHX on the CHX-MIC of *Acinetobacter baumannii*; results showed a statistically significant difference between the two means (*P*-value=0.047), where the mean of CHX-MIC after one week incubation of isolates in sub-MIC of CHX (53.51) was higher than the mean of CHX-MIC (46.95) before the incubation period as showed in Table 3.

3.3. Effect of Sub-MIC of CHX on the biofilm formation

Among of 61 collected *Acinetobacter baumannii*, only 6 (9.84%) showed no biofilm formation, while 7 (11.48%) showed weak biofilm, 8 (13.11%) of them were produced moderate biofilm, and 40 (65.57%) considered to be strong biofilm as shown in Figure 3. On the other hand, the tubes with high CHX concentration (1024 µg/mL, 512 µg/mL, 256 µg/mL, and 128 µg/mL) were negative for biofilm production.

The optical density (OD_{600nm}) of samples that contained (64 µg/mL, 32 µg/mL, and 8 µg/mL) of CHX has a statistically significant reduction in biofilm formation when compared to controls (*p*-value <0.05), which revealed reduction in the biofilm production after using such concentrations of CHX. Moreover, the bacterial isolates that incubated in 4 µg/mL and 2 µg/mL CHX showed no reduction in biofilm production, even controversially the mean OD_{600nm} of isolates were more than the mean OD_{600nm} +/controls as represented in Table 4.

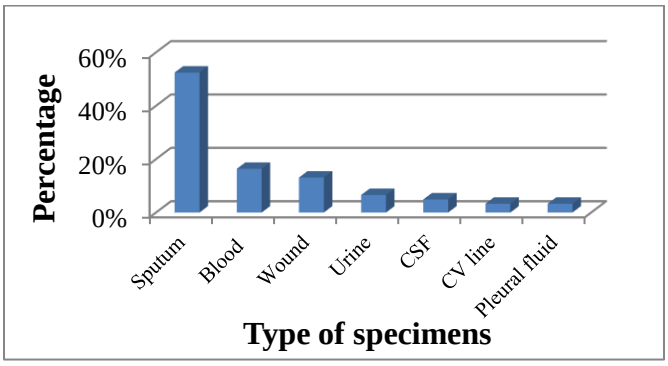

Figure 1: The isolation rate of *Acinetobacter baumannii* isolates from different clinical specimens.

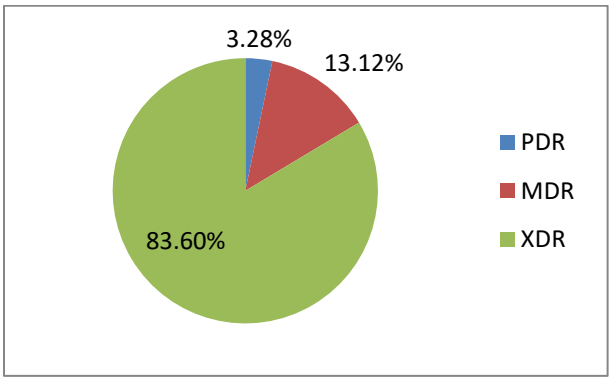

Figure 2: Rate and pattern of drug resistance among *Acinetobacter baumannii* isolates

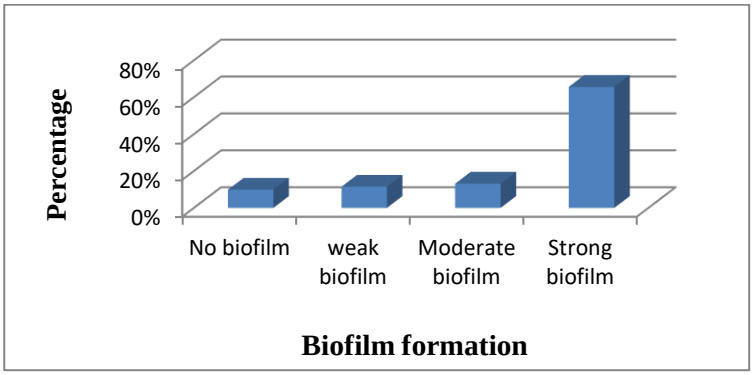

Figure 3: Rate of biofilm formation among 61 *Acinetobacter baumannii* isolates

Table 1: Antimicrobial susceptibility testing (AST) pattern of *Acinetobacter baumannii* isolates

Antimicrobial agent	R	I	S
FEP 30 µg	60(98.36%)	0(0.00%)	1(1.64%)
CAZ 30 µg	58(95.08%)	0(0.00%)	3(4.92%)
CTX 30 µg	61(100%)	0(0.00%)	0(0.0%)
MEM 10 µg	59(96.72%)	0(0.00%)	2(3.28%)
IPM 10 µg	59(96.72%)	0(0.00%)	2(3.28%)
LVX 5 µg	60(98.36%)	1(1.64%)	0(0.0%)
CIP 5 µg	61(100%)	0(0.00%)	0(0.00%)
NET 10 µg	50(81.97%)	5(8.20%)	6(9.83%)
TZP 100/10 µg	58(95.08%)	2(3.28%)	1(1.64%)
PIP 100 µg	59(96.72%)	1(1.64%)	1(1.64%)
TOB 10 µg	54(88.52%)	3(4.92%)	4(6.56%)
TIG 15 µg	43(70.49%)	8(13.12%)	10(16.39%)
TE 30µg	55(90.16)	3(4.92%)	3(4.92%)
GN 10 µg	58(95.08%)	1(1.64%)	2(3.28%)
SXT 1.25/23.75µg	60(98.36%)	0(0.00%)	1(1.64%)
CL 10 µg	2(3.28%)	0(0.00%)	59(96.72%)

R: resistant, I: Intermediate, S: susceptible

Table 2: CHX-MIC of *Acinetobacter baumannii* isolates

Rate of isolates	CHX-MIC
31(50.82%)	32
13(21.3%)	16
12(19.67%)	64
3(4.92%)	128
2(3.28%)	256

Table 3: Effect of sub-MIC of CHX on the CHX-MIC pre-incubated *Acinetobacter baumannii*

Wilcoxon Signed Ranks Test

	Mean	N	SD	Z	p-value
CHX-MIC $_{before}$	46.95	61	46.544	-1.990	0.047*
CHX-MIC $_{after}$	53.51	61	47.990		

Table 4: Effect of sub-MIC on biofilm formation among *Acinetobacter baumannii* isolates and control

Wilcoxon Signed Ranks Test

OD_{600nm} CHX Conc.	Mean	N	SD	Z	P-value
Test 64 µg/mL	0.016	5	0.008	-2.023	0.043*
Control	0.046	5	0.009		
Test 32 µg/mL	0.016	17	0.009	-2.896	0.004**
Control	0.035	17	0.022		
Test 16 µg/mL	0.029	48	0.032	-4.080	0.000**
Control	0.038	48	0.023		
Test 8 µg/mL	0.033	61	0.045	-2.866	0.004**
Test $_{m}$ Control	0.033	61	0.024		
Test 4 µg/mL	0.037	61	0.022		
Control	0.033	61	0.024		
Test 2 µg/mL	0.041	61	0.023		
Control	0.033	61	0.024		

DISCUSSION

In a certain specialized hospital unit, including medical ICUs, the risk of infection outbreaks caused by strains of *Acinetobacter baumannii* that are resistant to numerous antibiotic classes is extremely high (Rangel et al., 2021). Because of their infection with MDR strains, patients are required to use "last-line" antibiotics such as colistin, polymyxin B, or tigecycline, this is the most significant impact of the infection. Infections caused by MDR *Acinetobacter baumannii* are typically more common in immunocompromised patients, patients who are taking antibiotics with a broad spectrum of activity, patients who have underlying illnesses, and patients who have undergone invasive operations (Salehi et al., 2018).

In the current study the main source of *Acinetobacter baumannii* was sputum 32 (52.46%), the second main source was blood specimens 10 (16.39%), wound swab 8 (13.11%), urine 4 (6.56%), cerebrospinal fluid (CSF) 3 (4.92%), central venous line (CV line) 2 (3.28%), and Pleural fluid 2 (3.28%) respectively were also sources for *Acinetobacter baumannii*. These results are consistent with finding of similar study in Erbil /Iraq, (Smai, 2020), who showed the most common isolates were detected in sputum 20 (51.3%). In contrast, other study conducted in Baghdad/Iraq, showed the most *Acinetobacter baumannii* isolates collected from burns (36.5%) and surgical wounds (34.1%) (Sehree et al., 2021). On the other hand, (Al-Ghazaly and Tuwaij, 2022) found that most *Acinetobacter baumannii* in Najaf /Iraq isolated from wound swab (37.5%).

Globally, the prevalence of MDR, XDR and, in particular, PDR *Acinetobacter baumannii* are growing, restricting treatment choices for infected patients (Theuretzbacher, 2017). In the current study the majority of isolates were XDR 50 (83.6%). Our results are supported by study conducted by (Aziz and Al-Jubori, 2017) in AL- Najaf as they isolated (12.5%) PDR, (16.7%) MDR and (70.8%) XDR *Acinetobacter baumanni*. Another study from Tehran, Iran found out of 62 isolates of *Acinetobacter baumanni*, 36 (50%) were considered MDR, 17 (27.5%) XDR and 9 (14.5%) PDR (Sobouti et al., 2020). Furthermore, study from European countries could classify 65 isolates of *Acinetobacter baumanni* to the 22 (33.84%) XDR, and 20 (30.76%) PDR. (Nowak et al., 2017)

The mean of CHX-MIC in the current study was (46.951 ± 11.68) µg/mL,

these results are very close to the study from Ukraine, which estimated CHX-MIC (45.07±23.28). A study from Malaysia by (A'shimi et al., 2019) showed the Majority of the isolates (43%) has MIC values (>50 µg/mL).

Further to above-mentioned, the bacterial efflux pump is thought to be involved in CHX resistance in *Acinetobacter baumannii* (Fuangthong et al., 2011). The current study showed that the MIC of CHX is differ before and after the experiment that mean using sub-MIC of CHX lead to increase in the MIC of CHX among isolates. These findings agreed with some other studies, those who concluded the extent of CHX exposure led to a rise in the CHX minimum inhibitory concentrations for *Acinetobacter baumannii*. (Apisarnthanarak et al., 2014). In addition, study by (Fuangthong et al., 2011) revealed that exposing *Acinetobacter* sp. (strain ADP1) to CHX at sub-lethal doses results in the conferral of inducible resistance to CHX at lethal concentrations. Furthermore, some evidence suggests that CHX resistance among gram-negative bacteria has been steadily rising in recent years (Liu et al., 2017, Ortega-Peña et al., 2017). Due to the high frequency of qac genes in *Acinetobacter baumannii*, its susceptibility to the antiseptics is drastically reduced (Liu et al., 2017).

Our findings showed that among 61 collected *Acinetobacter baumannii* isolates, 6 (9.84%) were no biofilm formers, 7 (11.48%) were weak biofilm formers, 8 (13.11%) of them were produced moderate biofilm, and 40 (65.57%) were strong biofilm formers, although a study by (A'shimi et al., 2019) concluded that the vast majority of the isolates produced biofilms of either a moderate (n = 51/100) or a weak (n = 45/100) biofilms , with only a few (n = 4/100) producing strong biofilms.

Furthermore, our results indicated that the concentration of CHX >8 µg/mL effective against the formation of biofilm, but when the concentrations dropped to (4 and 2 µg/mL) the CHX may induce the production of biofilms. Studies explained the biofilm producer strains of *Acinetobacter baumannii*, who possess class I integrons are resistant to biocide (Rajamohan et al., 2009). The data revealed a link between class1 integrons, biofilm formation, and biocide resistant persistence, implying that biofilm-forming abilities may be a key role in *Acinetobacter baumannii* persistence in the clinical setting. (Houari and Di Martino, 2007) concluded that at standard in-use doses, CHX inhibits the production of biofilms in a variety of bacterial species. However, the stimulation of biofilm formation in the *Staphylococcus epidermidis* CIP53124 strain in the presence of biocides at sub-MIC levels raises concerns about the improper use of cationic antiseptics.

CONCLUSION

The current study was intended to assess the effect of sub-MIC of CHX on the resistance of *Acinetobacter baumannii* to CHX and biofilm formation. Exposure of *Acinetobacter baumannii* to sub-lethal CHX concentrations develops inducible resistance to lethal CHX doses. Furthermore, the biofilm formation ability of isolates also increased after using sub-lethal dose of CHX. Accordingly the utilization of CHX in medical settings needs to be carried out very carefully, as using the appropriate concentration is able to prevent the growth of bacteria and reduce production of biofilm, but on the other hand, using the sub-MICs of CHX can cause resistance to this chemical, which means that we may aspect a more significant problem in the future in disinfecting procedures.

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