Original Article

Evaluation of the Bactericidal Activity of Some Disinfectant Agents against Carbapenem-Resistant *Klebsiella pneumoniae* Isolates

Ali Öztürk, Özlem Tunçcan Güzel[1], Olkar Abdulmajed[2], Merve Erdoğan[3], Ramazan Kozan[4], Kayhan Çağlar[5], Ayşe Kalkancı[5]

Department of Medical Microbiology, Faculty of Medicine, Niğde Ömer Halisdemir University, Niğde, Turkey, [1]Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Gazi University, Ankara, Turkey, [2]Department of Medical Microbiology, Faculty of Medicine, Yüksek İhtisas University, Ankara, Turkey, [3]Department of Medical Microbiology, Faculty of Medicine, SANKO University, Gaziantep, Turkey, [4]Department of General Surgery, Faculty of Medicine, Gazi University, Ankara, Turkey, [5]Department of Medical Microbiology, Faculty of Medicine, Gazi University, Ankara, Turkey

Abstract

Aim: This study is aimed to determine the disinfectant activity of Derdevice plus Y® and I&D Sept® against carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates. **Materials and Methods:** The bactericidal activity of Derdevice plus Y® and I&D Sept® were tested *in vitro* under clean and dirty conditions by a quantitative suspension test according to EN 13727. The effectiveness of the disinfectants was compared with 10% sodium hypochlorite (NaOCl), 7% benzalkonium chloride (BC), 2% chlorhexidine digluconate (CHX), and 70% ethyl alcohol (EtOH). After 1, 5, and 60 min of contact with the biocides, the colony-forming units were counted, and the logarithmic reduction factor was determined. **Results:** A dilution of 1/300 of Derdevice plus Y® disinfectant showed bactericidal activity against clinical isolates and standard strains (growth reduction was ≥ 5 $\log_{10}$) after 5 and 60 min contact times and under clean and dirty conditions. A 100% concentration of I&D Sept® showed a bactericidal effect within the contact time (60 min) under clean and dirty conditions with the reductions of ≥ 5 $\log_{10}$ and ≥ 3 $\log_{10}$, respectively. Standard biocides such as BC, CHX, NaOCl, and EtOH showed marked effects after various contact times and the conditions onto tested strains. **Conclusions:** The results of our study confirm that the biocides of Derdevice plus Y® and I&D Sept® used in our hospital were found to be effective against *K. pneumoniae* isolates.

Keywords: Bactericidal activity, disinfectant, EN 13727, *Klebsiella pneumoniae*, quantitative suspension test

Introduction

Resistant bacteria are spreading fast worldwide and becoming an increasing health problem.[1] Especially the increase of infections caused by multidrug-resistant Gram-negative (MRGN) bacteria has become a clinical problem worldwide. MRGN bacteria are responsible for serious infections and have important effects on morbidity and mortality.[1,2] *Klebsiella pneumoniae* is one of the most common bacteria leading to the infections, has caused healthcare-associated urinary tract infections in the patients whose immune system is suppressed, hospital-based pneumonia and serious infections, including intra-abdominal ones. *K. pneumoniae* infection has become a much bigger concern since it is inclined to be "Multi Drug-Resistant" (e.g., carbapenem-resistance), making the treatment of infections more difficult.[3,4]

Nosocomial infections or by actual naming, healthcare-associated infections (HCAI), cause long-term admission to hospitals with high morbidity, mortality and treatment problems, and require taking effective measures.[5] The principles of preventing and controlling HCAI are surveillance, hand hygiene, disinfection, sterilization, patient isolation, and cleanness. The efficacy testing of disinfectant agents is cumbersome. Application

Access this article online

Quick Response Code:

Website: www.ijehe.org

DOI: 10.4103/ijehe.ijehe_15_20

Address for correspondence: Dr. Ali Öztürk, Department of Medical Microbiology, Faculty of Medicine, Nigde Ömer Halisdemir University, 51240 Nigde, Turkey.
E-mail: ali.81ozturk@gmail.com

How to cite this article: Öztürk A, Güzel ÖT, Abdulmajed O, Erdoğan M, Kozan R, Çağlar K, *et al.* Evaluation of the bactericidal activity of some disinfectant agents against carbapenem-resistant *Klebsiella pneumoniae* isolates. Int J Env Health Eng 2020;9:17.

Received: 21-02-2020, **Accepted:** 30-05-2020, **Published:** 31-12-2020

method and application concentrations are problematic. The clinical impact of the activity tests is highly controversial. Activity test results are far from being correlated with daily hospital procedures.[6,7]

Evaluation of the biocidal activities of the chemical disinfectants and antiseptic agents or "biocides" depends on the fact that they can be applied under defined conditions, and those logarithmic reductions are detected in the number of the microorganisms according to the European Standards (European Norms = EN).[8]

This study aims to test *in vitro* the bactericidal activity of some disinfectant and antiseptic agents on carbapenem-resistant *K. pneumoniae* (CRKP) strains isolated from the Hospital of the Medical Faculty of Gazi University by quantitative suspension method according to the European standard EN 13727.

Materials and Methods

Clinical strains

In this study, seven CRKP isolates, which were obtained from the culture collection of the Microbiology Laboratory of Medical Faculty of Gazi University, were used. Reference strains according to EN 13727 standard, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, and *Escherichia coli* NCTC 10538 were used as well. The identification of *K. pneumoniae* isolates was performed by using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Vitek-MS, bioMerieux, France).

Biocides

The list of the biocides used in this study is given in Table 1. In our study, the used disinfectants were "Derdevice plus Y®" (7.8% alkyl dimethyl benzyl ammonium chloride [ADBAC] 3.8% didecyl dimethyl ammonium chloride [DDAC], Deren Ilac, Turkey) and "I&D Sept®" (70% ethanol, 0.5% 2-propanol, DTH Health Services, Turkey), which were frequently used in our hospital. According to the method and recommendations of the EN 13727 standard, at least one of the concentrations must be effective in disinfection (application dosage) and at least one ineffective concentration. Therefore, two different dilutions were tested in the study. The effectiveness of the disinfectants used was compared with 10% sodium hypochlorite (NaOCl) (Sigma, USA), 7% benzalkonium chloride (BC)(Sigma, Denmark), 2% chlorhexidine digluconate (CHX) (Sigma, Spain) and 70% ethyl alcohol (EtOH) (Merck, Germany).

Table 1: Biocides and its dilutions

Name of biocide	Intended purpose	Test dilution
Derdevice plus Y®	Floor and surface disinfectant	1/300 and 1/1200
I&D Sept®	Hand and skin disinfectant	100 % and 10%
NaOCl	Floor and surface disinfectant	10%
BC	Disinfectant and antiseptic	7%
CHX	Antiseptic	2%
EtOH	Antiseptic	70%

NaOCl: Sodium hypochlorite, BC: Benzalkonium chloride, CHX: Chlorhexidine digluconate, EtOH: Ethyl alcohol

Preparing bacterial suspensions

The microorganisms archived at − 20°C were cultured onto Tryptic Soy Agar (TSA, Merck, Germany) medium and were incubated at 37°C for 24 h. The bacterial suspensions were prepared by McFarland standard in the concentrations of 1.5–5.0 × 10^8 CFU/mL in the diluent solution (consisted of 1 g tryptone and 8.5 g NaCl in 1000 ml of H_2O). Final test concentrations were adjusted to 1.5–5.0 × 10^7 CFU/mL, as specified in EN 13727 protocol.

Preparation of the neutralizer and other chemicals

Neutralizer consisted of 30 g tween 80, 30 g saponin, 1 g L-histidine, 3 g lecithin and 5 g sodium thiosulfate in 1000 ml of diluent solution. For clean conditions, 0.3 g of bovine albumin (BA) was dissolved in 100 ml of diluent solution and was sterilized with membrane filtration. For the testing under dirty conditions, 3 g of BA was prepared in 97 ml of diluent solution and was mixed with 3 ml of sheep erythrocyte suspension. Hard water is a saline solution prepared by mixing 6 ml from solution A (consisted of 19.84 g of magnesium chloride [$MgCl_2$] and 46.24 g of calcium chloride [$CaCl_2$] in 1000 ml of H_2O) and 8 ml from solution B (consisted of 35.02 g of sodium bicarbonate [$NaHCO_3$] in 1000 ml of H_2O) and then it is completed with H_2O to 1000 ml.

The control test (validation)

To control the bactericidal effects of hard water, interfering substances (BA, erythrocytes) and neutralizer agent were used as the validation tests according to EN 13727 standard. Parallel to each disinfection test, three validation (control) procedures were applied for each time as follows. The bacterial suspension prepared for validation tests is described as the validation suspension (Nv). Nv value was prepared between 2.4-8 × 10^4 CFU/ml.

Control A

Aims to test the effect of interfering substance and hard water on the microorganism. For the test, the bacterial suspension of 100 µl of 2.4–8 × 10^4 CFU/ml and 100 µl of interfering substance were added to a tube. After waiting for 2 min, 800 µl of hard water was added to it; and 5 min later, 100 µl was taken from the tube and cultured on TSA plates. When the counted live bacteria number was ≥2.4–8 × 10^4 CFU/ml, it was accepted as significant.

Control B

It is a test done to show if the neutralizer has any bactericidal effects on bacteria. For this purpose, the neutralizer (900 µl) was added to the bacterial suspension (100 µl) and was cultured on TSA plates. It was incubated at 37°C for 24 h, and the colonies were counted. If there was no reduction in the colonial numbers, the results were accepted as significant.

Control C

To show that the neutralizer used in this study neutralized the bactericidal effects of the biocide, the bacterial suspension

(100 µl) was added to the neutralized disinfectant. It was cultured on TSA plates and left for 24 h incubation. If the colony number was ≥2.4–8 × 10^4 CFU/ml, it was accepted as significant.

Specifying the bactericidal activity with the quantitative suspension

In accordance with EN 13727 Protocol, the experiments were done for each bacteria in the contact times of 1, 5, and 60 min, and under clean and dirty conditions. Briefly, 2 ml of bacterial suspension was mixed with 1 ml of interfering substance and waited for 2 min. Then, 8 ml of biocide solution was added on it and was incubated for 1, 5, and 60 min. At the end of the contact time, 1 ml of the solution was taken and transferred into new tubes with 1 ml of hard water. After the mixture in the tube was neutralized for 5 min, 1 ml of it was taken and cultured on the TSA medium. Then, it was incubated for 24 h at 37°C. After incubation, the colony numbers were counted, and the logarithmic reduction factor (LRF) was determined. LRF is the number of bacteria alive after contact with the biocide. According to EN 13727 Standard, when the LRF has a reduction ≥5 $\log_{10}$ in the bacterial number under clean and dirty conditions for floor and surface disinfectant, a reduction ≥5 $\log_{10}$ under clean conditions for hygienic and surgical handwashing disinfectant and a reduction ≥3 $\log_{10}$ under dirty conditions were accepted as significant.[9]

Results

Identification of CRKP strains with MALDI-TOF

A total of seven CRKP strains were identified using the MALDI-TOF Vitek MS system. The spectral peaks are shown in Figure 1. The findings demonstrated that all isolates were found to be CRKP with a similarity rate of 95.6%–99.8%.

Specifying the bactericidal activity by the quantitative suspension test

The bactericidal effect of the biocides used in this study was evaluated *in vitro* under clean and dirty conditions against *K. pneumoniae* isolates [Tables 2 and 3] and reference strains [Tables 4 and 5]. A dilution of 1/300 of Derdevice plus Y® disinfectant was found to be bactericidal since the growth reduction was ≥5 $\log_{10}$ in the clinical isolates and standard strains after 5 and 60 min contact times and under clean and dirty conditions. The dilution of 1/1200 Derdevice plus Y® disinfectant had no bactericidal effect (LRF ≤5 $\log_{10}$); and the 100% concentration of I&D Sept® showed bactericidal effect within the contact time (60 s) under clean and dirty conditions with the reductions of ≥5 $\log_{10}$ and ≥3 $\log_{10}$, respectively. The dilution of 10% of I&D Sept® had no bactericidal activity. Well-known biocides such as BC, CHX, EtOH, and NaOCl showed a marked effect after the contact times and the conditions applied to the tested clinical and reference strains.

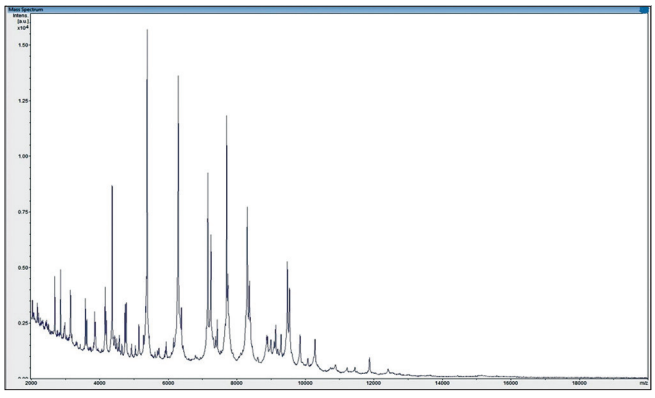

Figure 1: MALDI-TOF Vitek MS spectra of CRKP strain

In this study, we have not seen any toxic and bactericidal effects of the interfering substances. Furthermore, it was clear that neutralizer and hard water did not have any bactericidal activity against tested strains in the validation tests.

Discussion

HCAI cause high morbidity and mortality. Biocides are used on hospital surfaces to prevent HCAIs. The term "biocide" is used to define the antiseptic and disinfectant compounds with protective activity. In health institutions, a number of biocides such as glutaraldehyde, formaldehyde, and chlorine compounds are used.[10,11] According to EN, the quantitative suspension test is probably the best activity test among various possible methods.[11]

There is a wide number of biocidal compounds that have different antimicrobial activities to fight against the pathogen microorganisms. Therefore, it is important to design the antibacterial efficacy of these compounds and the chemical analysis of the active substances they contain.[12]

In the present study, CRKP isolates, which were isolated as the causative agents of HCAIs, were tested. According to EN 13727 Standard, the experiment was performed at different contact times (1, 5, and 60 min) and different application conditions such as clean and dirty conditions and also different biocide concentrations in a quantitative suspension test.

In our study, the effectiveness of the floor and surface disinfectant Derdevice plus Y® and the hand and skin disinfectant I&D Sept® were tested against CRKP isolates. CRKP isolates showed LRF ≥5 $\log_{10}$ in colony numbers in the recommended concentrations (1/300 and 100%, respectively). Both disinfectants showed significant bactericidal effects against tested bacterial strains at 1, 5, and 60 min of exposure. However, Derdevice plus Y® and I&D Sept® biocides at ineffective concentrations (1/1200 and 10%, respectively) were not effective at the same contact times (LRF ≤5 $\log_{10}$).The other biocides, BC, CHX, EtOH, and NaOCl, showed bactericidal effects as expected (LRF ≥5 $\log_{10}$ or ≥3 $\log_{10}$). Our results show that the potent bactericidal activity of these biocides is not reduced in the presence of organic matter contamination. No toxic and bactericidal effect detected of neutralizer, hard water, and protein (BA) against tested strains in the validation tests. Consequently,

Table 2: Bactericidal activity of biocides against *K. pneumoniae* clinical isolates under clean conditions

K. pneumoniae isolates (*n*=7)	Logarithmic reduction								
	Biocide	Derdevice Plus Y®		I and D Sept®		NaOCl	BC	CHX	EtOH
	Dilutions	1/300	1/1200	100%	10%	10%	7%	2%	70%
	Time (min)								
1	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
2	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
3	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
4	1	-	-	5	1	-	-	5	5
	5	5	3	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
5	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	5	3	-	-	5	5	-	-
6	1	-	-	5	1	-	-	5	5
	5	6	2	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
7	1	-	-	5	1	-	-	5	4
	5	6	2	-	-	5	5	-	-
	60	5	3	-	-	5	5	-	-

Table 3: Bactericidal activity of biocides against *K. pneumoniae* clinical isolates under dirty conditions

K. pneumoniae isolates (*n*=7)	Logarithmic reduction								
	Biocide	Derdevice plus Y®		I and D Sept®		NaOCl	BC	CHX	EtOH
	Dilutions	1/300	1/1200	100%	10%	10%	7%	2%	70%
	Time (min)								
1	1	-	-	4	1	-	-	4	3
	5	5	3	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-
2	1	-	-	4	1	-	-	5	5
	5	5	2	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-
3	1	-	-	4	1	-	-	3	4
	5	5	2	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-
4	1	-	-	3	2	-	-	4	4
	5	5	1	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-
5	1	-	-	3	2	-	-	5	5
	5	5	2	-	-	5	5	-	-
	60	5	2	-	-	5	5	-	-
6	1	-	-	4	1	-	-	5	5
	5	5	2	-	-	5	5	-	-
	60	5	2	-	-	5	5	-	-
7	1	-	-	4		-	-	4	4
	5	5	1	-	-	5	5	-	-
	60	5	2	-	-	5	5	-	-

Table 4: Bactericidal activity of biocides against reference strains under clean conditions

Reference strains ($n=3$)	Logarithmic reduction								
	Biocide	Derdevice plus Y®		I and D Sept®		NaOCl	BC	CHX	EtOH
	Dilutions	1/300	1/1200	100%	10%	10%	7%	2%	70%
	Time (min)								
S. aureus ATCC 6538	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	6	3	-	-	5	5	-	-
E. coli ATCC 15442	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	5	2	-	-	5	5	-	-
P. aeruginosa NCTC 10538	1	-	-	5	1	-	-	5	5
	5	6	3	-	-	5	5	-	-
	60	6	2	-	-	5	5	-	-

Table 5: Bactericidal activity of biocides against reference strains under dirty conditions

Reference strains ($n=3$)	Logarithmic reduction								
	Biocide	Derdevice plus Y®		I and D Sept®		NaOCl	BC	CHX	EtOH
	Dilutions	1/300	1/1200	100%	10%	10%	7%	2%	70%
	Time (min)								
S. aureus ATCC 6538	1	-	-	3	1	-	-	4	3
	5	5	2	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-
E. coli ATCC 15442	1	-	-	3	1	-	-	4	5
	5	5	2	-	-	5	5	-	-
	60	5	2	-	-	5	5	-	-
P. aeruginosa. NCTC 10538	1	-	-	3	1	-	-	4	4
	5	5	2	-	-	5	5	-	-
	60	5	1	-	-	5	5	-	-

it is possible to conclude that these disinfectants were not affected by dirty conditions.

A variety of quaternary ammonium compounds (QACs) containing disinfectants can be used in healthcare and industry. Among these QACs, DDAC and ADBAC compounds are commonly used for surface disinfection. There are many investigations about the antimicrobial activities of QACs. Nakipoglu and Gurler investigated bactericidal activities of 16 biocides agents against standard bacteria (*S. aureus* ATCC 6538, *P. aeruginosa* NCTC 6749 and *Bacillus subtilis var niger* ATCC 9372). Among the disinfectants used in the study, "Desam extra®" (surface disinfectant) and "Septoderm spray®" (hand and skin antiseptic) were found to be active against bacterial strains tested.[13] Derdevice plus Y® and I&D Sept® disinfectants that were used in our study contained the same active ingredients (ADBAC and DDAC) like the disinfectants that were used by Nakipoglu and Gurler. In our study, Derdevice plus Y® and I&D Sept® were found to be more effective on standard strains, and resistant *K. pneumoniae* isolates in both clean and dirty conditions and at the concentrations recommended by the manufacturer.

Reichel *et al.* studied the efficacy of surface disinfectants against resistant Gram-negative bacteria.[2] Among the five surface disinfectants used, Kohrsolin® FF contained glutaral, benzyl alkyl dimethylammonium chlorides, and didecyldimethylammonium chloride as the active compounds. The efficacy of the disinfectants used was examined only in dirty conditions, according to EN 13727: 2012 procedure. The results showed that all disinfectants used in the study had bactericidal effects (≥5 $\log_{10}$ reductions) against all tested strains (ATCC strains and clinical isolates with and without multidrug resistance), which support our findings.

Montagna *et al*. investigated the activity of 2% DDAC disinfectant by Disc Diffusion Method against 187 clinical bacterial isolates. The microorganisms were considered sensitive when the inhibition zone diameter was >8 mm overall. As a result, DDAC disinfectant was found to be ineffective against Gram-negative strains (including *K. pneumoniae*: 58 strains, of which 30 were susceptible and 28 were resistant to carbapenem), but showed significant activity against *S. aureus* ($n = 40$) and *Enterococcus faecalis* ($n = 30$) with an inhibition zone of 13-14.4 mm and 13 mm, respectively.[14]

Wu *et al*. used agar dilution and microdilution methods to compare the antibacterial activities of four QACs against *Salmonella*, *E. coli*, *K. pneumoniae,* and *S. aureus*. BC, cetyltrimethylammonium bromide (CTAB), didecyldimethylammonium chloride (DDAC) and cetylpyridinium chloride (CTPK) were used in the study. MIC values of DDAC were lower than other disinfectants used against bacterial strains. Thus, they reported that DDAC is more effective than other disinfectants.[15]

Tyski *et al*. evaluated the antimicrobial activities of 14 disinfectants containing various chemically-active substances by quantitative suspension test according to EN standards. The disinfectant called Laudamonium® (benzyl alkyl ammonium chloride 9.99 g/100 g) was used in the study as a surface disinfectant. Results showed that Laudamonium® was effective against ATCC strains (≥5 $\log_{10}$ reductions). In the same study by Tyski, the antiseptic called Sterisol Preop® (ethanol 70% and isopropyl alcohol 10%) was also tested. According to the results, Sterisol Preop® antiseptic was found to be effective against bacterial strains tested with ≥5 $\log_{10}$ reductions.[8]

This study emphasizes the importance of performing *in vitro* bactericidal activity tests in relevant simulations. In this regard, the main objective of the current study is to determine the effective concentrations and proper contact time of biocides to ensure that those biocides are used in our hospital settings (e.g., intensive care units, surgical departments). Thus, the emergence of new resistant microorganisms may be prevented.

This study has some limitations, such as a limited number of tested strains and the use of a single method. Thus, future studies would ideally analyze an increased number of bacterial strains and evaluate different analytical methods such as quantitative carrier tests.

Conclusions

The biocides used in this study were found to be effective against clinical bacterial isolates. For this reason, it can be used in our hospital, especially in intensive care units for disinfection purposes. When the formulation was diluted more than the recommended concentration of the biocides, this dilution step caused ineffectiveness. Since the susceptibility of clinically-isolated bacteria to disinfectant agents might be varied, the efficacy of disinfectants should be evaluated under simulated conditions to prevent nosocomial infections and possible risks associated with resistant microorganisms.

In future, it will be interesting to evaluate the efficacy of these biocides on virulence factors of resistant microorganisms such as biofilm.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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