

## An investigation of the bactericidal activity of chlorhexidine digluconate against multidrug-resistant hospital isolates

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**Background/aim:** Hospital infections are among the most prominent medical problems around the world. Using proper biocides in an appropriate way is critically important in overcoming this problem. Several reports have suggested that microorganisms may develop resistance or reduce their susceptibility to biocides, similar to the case with antibiotics. In this study we aimed to determine the antimicrobial activity of chlorhexidine digluconate against clinical isolates.

**Materials and methods:** The susceptibility of 120 hospital isolated strains of 7 bacterial genera against chlorhexidine digluconate was determined by agar dilution test, using minimum inhibitory concentration (MIC) values and the EN 1040 Basic Bactericidal Activity Test to determine the bactericidal activity. According to MIC values, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were found to be less susceptible to chlorhexidine digluconate.

**Results:** Quantitative suspension test results showed that 4% chlorhexidine digluconate was effective against antibiotic resistant and susceptible bacteria after 5 min of contact time and can be safely used in our hospital. However, concentrations below 4% chlorhexidine digluconate caused a decrease in bactericidal activity, especially for *Staphylococcus aureus* and *P. aeruginosa*.

**Conclusion:** It is crucial to use biocides at appropriate concentrations and to perform surveillance studies to trace resistance or low susceptibility patterns of *S. aureus*, *P. aeruginosa*, and other hospital isolates.

**Key words:** Biocides, resistance, chlorhexidine digluconate, quantitative suspension test, EN 1040

### 1. Introduction

Disinfectants are chemical agents used to kill microorganisms on surfaces or in order to eliminate them from the environment. Some of these chemical agents, which have been used to prevent or limit microbial infection on the skin, are called antiseptic or topical antimicrobial. On the other hand, there are chemical agents that have been used as preservatives against microbial contamination by adding them into pharmaceuticals, cosmetics, and other products. Those chemical substances with antiseptic, disinfectant, and/or preservative activity have been defined as biocides. As an example, chlorhexidine salts and quaternary ammonium compounds can be used for these 3 purposes while others, like glutaraldehyde, and ortho-phthalaldehyde, are mainly used for the disinfection of endoscopes (1–3). The disinfectant used in this study, chlorhexidine digluconate, has a biguanide structure, low toxicity, low tolerability, and a wide antimicrobial spectrum (2,4).

Biocide resistance, similar to antibiotic resistance, is described as microbial growth when bacteria are tested with in-use concentrations. Furthermore, resistance or insusceptibility to biocides can be either intrinsic, as a result of natural characteristics of microorganisms, or it can be acquired. Acquired resistance to biocides may arise from mutation and horizontal transfer of genetic material such as plasmids or transposons (2,3,5–8). Efflux pumps are common mechanisms of acquired resistance to chlorhexidine digluconate. By means of this mechanism, not only chlorhexidine but also other chemical substances are excluded from the cell, which can therefore also lead to resistance to antibiotics (3,4,7). Antimicrobial effectiveness of chlorhexidine may differ within pathogenic bacteria. Horner et al. (4) classified chlorhexidine and bacterial interactions into 4 different groups. Chlorhexidine tolerance is described as when bacterial growth is inhibited but bacteria are not killed at bacteriostatic concentrations (4 mg/L). On the other

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hand, chlorhexidine resistance is described as survival of bacteria at bactericidal concentrations (40 000 mg/L) (4).

Hospital infections are one of the main problems in Turkey, as well as around the world. In order to prevent these infections, it is of great importance to determine which microorganisms are responsible. Moreover, relevant biocides must be chosen and proper disinfection and sterilization applications need to be ensured. The diversity of clinical isolates and the different options in using biocides, varying the bacterial susceptibility profile, may cause problems in disinfection and antiseptis implementations in hospitals. To this end, it is important to conduct susceptibility tests for biocides that have been widely used against problematic, multidrug-resistant microorganisms (e.g., MRSA) in order to prevent the development of hospital infections (7,9,10).

In this study, the bactericidal activity of chlorhexidine digluconate against hospital-isolated multidrug-resistant bacteria was determined. We aimed to identify susceptibility profiles of bacteria isolated frequently from several services of our hospital against chlorhexidine digluconate and to collect data in order to monitor the changes in susceptibility over time. The data obtained from this study are of great importance because according to the European Committee for Standardization (CEN) there are not many studies investigating the susceptibility of isolates from our hospital against chlorhexidine digluconate, a commonly used disinfectant/antiseptic agent in our hospital.

## 2. Materials and methods

### 2.1. Microorganisms

The following multidrug-resistant clinical strains used in this study were obtained from the Hacettepe University Clinical Microbiology Laboratory: *Pseudomonas aeruginosa* (n = 22), *Acinetobacter lwoffii* (n = 11), *A. baumannii* (n = 8), *Stenotrophomonas maltophilia* (n = 13), *Klebsiella pneumoniae* (n = 13), *K. oxytoca* (n = 1), *Enterobacter* sp. (n = 15), *Staphylococcus aureus* [methicillin-susceptible (MSSA) (n = 6), methicillin-resistant (MRSA) (n = 15)], and *Enterococcus* sp. (n = 17). These strains were isolated from clinical samples such as urine, pus, bronchoalveolar lavage, and blood that were obtained from the following hospital clinical services: the intensive care unit, neurology, surgery, urology, and internal medicine. *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442 were employed as reference strains.

### 2.2. Identification and determination of antibiotic susceptibility

The identification and determination of antibiotic susceptibility of clinical strains were carried out by a Sceptor automatized identification system (Becton

Dickinson, Sparks, MD, USA). Isolates were stored at  $-20^{\circ}\text{C}$  in a glycerol stock solution.

### 2.3. Determination of minimum inhibitory concentration

The determination of minimum inhibition concentration (MIC) was performed for all of the clinical strains except for *Enterococcus* sp. The MICs of chlorhexidine digluconate (Drogsan, Turkey) were determined by the agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) (11). Chlorhexidine digluconate was prepared prior to the test at concentrations of 0.125–512  $\mu\text{g}/\text{mL}$ .

### 2.4. Determination of bactericidal effect of chlorhexidine digluconate

The bactericidal effect of chlorhexidine digluconate was determined as previously described by the EN 1040 quantitative suspension test method (12). Tested concentrations of chlorhexidine digluconate were 4%, 2%, 0.5%, 0.1%, 0.05%, and 0.02%. Disinfectants solutions were prepared prior to the test using sterile distilled water. The methods for preparing bacterial inoculum and performing the suspension test were described in detail earlier (13). Briefly, a single isolated colony of bacteria was inoculated in tryptic soy broth (TSB, Merck) for 24 h at  $37^{\circ}\text{C}$ . After incubation, the bacterial suspension was centrifuged, and the cell pellets were washed with TSB and adjusted between  $1.5 \times 10^8$  and  $5 \times 10^8$  cfu/mL. In the test, bacterial suspension was added to the disinfection solutions (1:10) for 5 min of contact time at room temperature, and then 1 mL was removed to 9 mL of the neutralizing solution (0.75% w/v lecithin, 5% v/v Tween 80 in TSB) and serially diluted in sterile distilled water. One hundred microliters of each dilution was then inoculated onto tryptic soy agar (TSA, Merck) by the spread plate technique and incubated at  $37^{\circ}\text{C}$  for 24 h. The samples were studied in triplicate. Colony forming units counted from plates that had a colony count between 30 and 300 were taken into account. The test was repeated using sterile distilled water instead of the disinfectant solutions as the control. The reduction factor (RF) was calculated as the expression of the disinfectant efficacy according to the following formula (12,13):

$$\text{RF} = \log_{10} \text{pre-disinfection control} - \log_{10} \text{disinfection control}$$

$\log_{10}$  reductions of 5 or more were taken as an indication of satisfactory bactericidal activity.

### 2.5. Neutralization efficacy test

A neutralization efficacy test was previously performed to determine whether it was appropriate to inactivate the chlorhexidine digluconate. In this study, neutralizer was checked for its neutralizing efficacy on chlorhexidine digluconate at 3 different concentrations (0.5%, 0.05%, and 0.005%) using *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15442. One milliliter of disinfectant solution was added to 9 mL of neutralizer, and then bacterial suspension

containing  $10^8$  cfu/mL was added to this mixture and left for 10 min. Next, 100  $\mu$ L of each dilution was inoculated onto tryptic soy agar plates ( $n = 3$ ). After incubation, colonies were enumerated and expressed as cfu/mL. A control study was conducted without disinfectant solution and the same procedure was repeated. Neutralizer efficacy was calculated according to the formula below (14):

Inactivation effect (IF) =  $1 - (\log_{10} \text{pre-disinfection control} - \log_{10} \text{disinfection control})$

Cases where the IF value was close to 1 were taken as an indication that the neutralizer was effective in neutralizing the disinfectant and that it had no bactericidal activity.

### 3. Results

#### 3.1. Assessment of antibacterial activities

The determination of bactericidal susceptibility to antibiotics was carried out using a Sceptor system. All of the isolates had multiple antibiotic resistance. However, the *Acinetobacter* isolates possessed a higher level of resistance than other gram-negative bacteria.

The MIC values of chlorhexidine digluconate against hospital isolates were investigated using an agar dilution test. The obtained MIC values for all of the isolates are shown in Table 1. According to these results, *S. aureus* including MRSA and *E. coli* isolates had low MIC values, while *P. aeruginosa* and *S. maltophilia* isolates had the highest MIC values.

The bactericidal activity of chlorhexidine digluconate was identified by using the quantitative suspension test. According to the results shown in Tables 2 and 3, all hospital isolates that were studied were found to be susceptible to 4% chlorhexidine digluconate after 5 min of contact time. There was no decrease in the bactericidal activity against the isolates, except for MRSA, in 2% chlorhexidine digluconate (no data available for *P. aeruginosa*). *Acinetobacter* sp., *Enterobacter* sp., *S. maltophilia*, *Klebsiella* sp., and *Enterococcus* sp. isolates were

found to be susceptible in 0.5% chlorhexidine digluconate, whereas 11 *P. aeruginosa*, 14 MRSA, and 5 MSSA isolates were found to be resistant. All of the *Enterococcus* isolates and 9 isolates of *S. maltophilia* were susceptible in 0.02% chlorhexidine digluconate.

Chlorhexidine digluconate at a concentration of 0.02% was active against only 2 *S. aureus* isolates (4.7%), whereas at the same concentration it was active against all *Enterococcus* isolates. This result showed that *S. aureus* isolates (MRSA and MSSA) had a lower level of susceptibility than *Enterococcus* in low concentrations of chlorhexidine digluconate.

#### 3.2. Evaluation of neutralizing efficacy

After conducting the neutralizing efficacy test, it was observed that the neutralizing agent could inactivate chlorhexidine digluconate and had no antibacterial effect on studied bacteria.

### 4. Discussion

Hospital infections constitute a serious problem in Turkey, as well as around the world. Hospital infections rank sixth in the list of causes of death in the United States, where the most reliable data are available (15). The rate of hospital infection in a university hospital in Turkey was found to be 13.4% and 10.9% in 2 surveys conducted monthly in 2 months (16). Achieving control over these hospital infections is extremely important to maintain the safety of patients and hospital staff. For this reason, intensive activity has been implemented in hospitals in order to prevent the development of infections. Among these activities, establishing appropriate disinfection policies and providing their activation play an important role. The core of these policies is to determine the appropriate concentrations, implementation methods, and application areas of disinfectants (7,17). Despite all these activities, infection epidemics caused by the wrong use of biocides have still been reported (18,19).

**Table 1.** Minimum inhibitory concentration characteristics (MIC range, MIC<sub>50</sub>, and MIC<sub>90</sub>) of chlorhexidine digluconate for hospital isolates.

Test strain	N	MIC values ( $\mu$ g/mL)		
		MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>P. aeruginosa</i>	21	16–512	512	512
<i>Klebsiella</i> sp.	14	8–128	32	64
<i>Acinetobacter</i> sp.	19	8–256	32	256
<i>Enterobacter</i> sp.	15	8–128	16	125
<i>S. maltophilia</i>	13	16–512	>512	>512
<i>S. aureus</i>	21	1–4	2	2

**Table 2.** Log<sub>10</sub> reduction values of chlorhexidine digluconate against hospital isolates after 5 min of contact time. \*: 10 of the isolates have a log reduction value of ≥5. \*\*: 3 of the isolates have a log reduction value of ≥5. \*\*\*: 1 of the isolates has a log reduction value of ≥5.

Test strain	n	Concentrations of chlorhexidine digluconate (%)					
		4	2	0.5	0.1	0.05	0.02
<i>P. aeruginosa</i>	21	6.24	-	4.32*	1.05	-	-
<i>Klebsiella sp.</i>	14	7.05	7.05	7.05	7.05	7.05	-
<i>Acinetobacter sp.</i>	19	6.60	6.60	6.60	3.30	1.60	-
<i>Enterobacter sp.</i>	15	6.82	6.82	6.82	6.82	6.82	4.18
<i>S. maltophilia</i>	13	7.93	7.93	7.93	7.93	7.93	6.53
MRSA	15	7.04	4.06**	3.16	2.75	2.54	2.40
MSSA	6	6.76	6.76	3.89***	3.68	3.48	3.19
<i>Enterococcus sp.</i>	17	6.82	6.82	6.82	6.82	6.82	6.82
<i>S. aureus</i> ATCC 6538	1	6.50	6.50	6.50	6.50	6.50	6.50
<i>P. aeruginosa</i> ATCC 15442	1	6.34	6.34	6.34	6.34	6.34	6.34

**Table 3.** Ratio of susceptible isolates per test strain against chlorhexidine digluconate after 5 min of contact time.

Test strain	n	Concentrations of chlorhexidine digluconate (%)					
		4	2	0.5	0.1	0.05	0.02
<i>P. aeruginosa</i>	21	21/21	-	10/21	2/21	-	-
<i>Klebsiella sp.</i>	14	14/14	14/14	14/14	14/14	14/14	-
<i>Acinetobacter sp.</i>	19	19/19	19/19	19/19	3/19	3/19	-
<i>Enterobacter sp.</i>	15	15/15	15/15	15/15	15/15	15/15	7/15
<i>S. maltophilia</i>	13	13/13	13/13	13/13	13/13	13/13	9/13
MRSA	15	15/15	3/15	1/15	1/15	1/15	1/15
MSSA	6	6/6	6/6	1/6	1/6	1/6	1/6
<i>Enterococcus sp.</i>	17	17/17	17/17	17/17	17/17	17/17	17/17

In our study, the MIC values and bactericidal activities were determined for a total of 120 strains (82 gram-negative and 38 gram-positive) belonging to 7 bacterial types isolated from patients admitted to the Hacettepe University Adult Hospital. The MIC values of chlorhexidine digluconate were specified with the agar dilution method and the bactericidal activity with an EN 1040 basic bactericidal activity test, as recommended by the European Committee for Standardization.

Bacterial resistance can be shown clearly and reliably by determining the MIC values of the antibiotics. Although the MIC values give an indication in the first stage of the antimicrobial activity of the biocides, they fail

to give specific results about the decreasing susceptibility or the resistance of biocides to in-use concentrations. The reason for the latter is that MIC values for biocides, unlike antibiotics, are lower than in in-use concentrations. The fact that the bacteria are growing at this low concentration does not mean that they are resistant to biocides. This should be defined as 'increasing MIC value' or decreasing susceptibility. However, a resistance can be relevant if the logarithmic decrease value is also below 5. This means that it is important to evaluate the bactericidal impacts rather than the inhibitory effects of biocides (4,6,20). Thus, in comparing the results of different studies, the methods employed should also be taken into consideration. The

results of a study where the bacteriostatic activity was determined should not be compared with the results of bactericidal activity. In our study, chlorhexidine digluconate was found to be bactericidally effective against all strains at a concentration of 0.05%, while the MIC<sub>50</sub> and MIC<sub>90</sub> values of the *S. maltophilia* isolates were found to be very high (512 and >512 µg/mL, respectively).

On the other hand, although *S. aureus* isolates have low MIC<sub>50</sub> and MIC<sub>90</sub> values (2 µg/mL), only 2 isolates were found to be susceptible at the 0.05% concentration of chlorhexidine digluconate. In this case, no link was detected between MIC values and bactericidally effective concentration values. However, more research with an extended range of isolates is needed in order to reach a more certain conclusion.

In this study, the bactericidal activity of the chlorhexidine digluconate was tested at in-use concentrations (4%, 2%, 0.5%, 0.1%, 0.05%, and 0.02%) according to the recommendations of the manufacturer, for close to, or lower than 5 min of contact time. Since chlorhexidine was used in concentrations of 0.05%–0.12% as an antiseptic, the determination of the effectiveness at lower concentrations is also important (4).

In this study, 4% chlorhexidine digluconate was observed to have a bactericidal effect in 5 min of contact time against all studied bacteria. However, as the concentration of chlorhexidine digluconate decreases, the susceptibility of the isolates of *S. aureus* and *P. aeruginosa* in particular increase rapidly. The fact that those bacteria are the ones causing most of the hospital infections makes the especially frequent use of biocides, using chlorhexidine digluconate at appropriate concentrations, even more important. Similarly, in studies done in Turkey, it has been suggested that 4% chlorhexidine digluconate was bactericidally effective on gram-negative and gram-positive bacteria causing hospital infections (21,22). However, Eryılmaz et al. reported that the bactericidal activity of chlorhexidine decreased at low concentrations against *P. aeruginosa* (22). We conducted a similar study in the pediatric hospital of our facilities that indicated that 4% chlorhexidine digluconate was effective against gram-negative bacteria. Thus, it is clear that there is no development of resistance over time (13).

There is no clear evidence that there is a link between antibiotic resistance and biocide resistance and studies still continue in this area (3,5,7,20,23). The fact that antibiotic and biocide resistance mechanisms are similar suggests that there is a possible link between them (3). In most studies, it has been shown that antibiotic resistance does not alter the susceptibility of bacteria to chlorhexidine (3,24). However, there are also studies suggesting that chlorhexidine digluconate susceptibility of multidrug-

resistant gram-negative bacteria has decreased (25,26). In our study, the phenotypic antibiotic resistance profiles of all the isolates used are known. In this respect, there is no evidence that the biocide susceptibility of the antibiotic-resistant bacteria has decreased. The only exception found was for MRSA isolates, where the bactericidal concentration was 40 g/L (4%), while it was 20 g/L (2%) for MSSA isolates. However, subsequently both bacteria groups were susceptible to chlorhexidine digluconate at in-use concentrations. The existence of studies suggesting that the biocide resistances of MRSA and MSSA are different while there are also studies providing the opposite result proves that there is no consensus yet on this issue (4,7). On the other hand, although there is a study showing that *Enterococcus* spp. are more resistant to chlorhexidine digluconate than *S. aureus* isolates, it was shown in this study that the susceptibility of *S. aureus* isolates was lower (26).

Some published studies demonstrate that, in hospitals, the contact of bacteria with biocides at low concentrations can create selective pressure for some isolates, similar to the subinhibitory concentration effects of antibiotics (2,3,20,27). In a recent study, it was also reported that quaternary ammonium compounds used in lower concentrations caused an increase in expression of virulence genes in bacteria (28). Thus, it appears that biocide concentration is a major factor in the development of bacterial resistance. If the surface to be disinfected was not clean and yet to be dried after disinfection, if the disinfectant was prepared at lower concentrations than in-use concentrations, and if the diluted disinfectant was kept longer than suggested by the manufacturer, then a low concentration of biocide is in contact with the bacteria (20). Irrizary et al. showed that chlorhexidine digluconate residues can have a selective effect on MRSA isolates (29). In another study, it was found that subinhibitory concentrations of chlorhexidine digluconate can cause a permanent increase in MIC values of *P. aeruginosa* isolates (30). These findings emphasize how important it is to clean surfaces first before disinfection occurs. It is thereby important to pay attention to possible biofilm formation in wet surfaces. Moreover, using the appropriate concentrations of disinfectant with less residue is particularly recommended against bacteria such as *Acinetobacter* sp. MRSA and *P. aeruginosa* which can survive longer periods in hospital environments and can be exposed to subinhibitory concentrations of biocides.

It has been observed that chlorhexidine digluconate can have bactericidal activity on antibiotic-susceptible and antibiotic-resistant bacteria when treated for 5 min at in-use chlorhexidine digluconate concentrations. However,

there is a need for surveillance studies in order to monitor the possibility of a decrease in susceptibility and/or the development of resistance of bacteria, especially for *S. aureus* and *P. aeruginosa*.

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