

Original Research Article

Activity of some clinically used biocides to commonly isolated bacterial pathogens from tertiary hospitals in Nigeria

ABSTRACT

Aim: There has not been any critical assessment to the efficacy and choice of biocides/disinfectants use in various tertiary hospitals in Nigeria despite several reports of high level bacterial resistance to antibiotics. The aim of this study was to determine the activities of some commonly used biocides against bacterial isolates from tertiary hospitals in Nigeria.

Study Design: This was a cross sectional study.

Methods: Fifty three non-duplicate bacterial isolates were obtained from different tertiary hospitals. The susceptibility to conventional antibiotics was determined by disc diffusion method, while **minimumminimal** inhibitory concentration**s** (MIC**s**), of a range of biocides **waswere** determined using **the** microtitre broth and agar diffusion methods including minimal bactericidal concentration**s**, MBC**s**.

Results: High level resistance to third generation antibiotics was obtained for Gram negative bacteria, 52.2%. *S. aureus* were found to be resistant to cefoxitin, **indicating an indication of presence of** methicillin resistance **exists among these strains. Similarly,** **The** MIC showed *S. aureus* to be more resistant to all the biocides **used**, while sodium hypochlorite was more effective against all the strains, 0.5 – 2%. Ethanol and triclosan were less effective even at the popular 70% for ethanol. All test compounds appeared to

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24 be bactericidal with MBC/MIC ratios of >4. There was no statistical difference between
25 diffusion and dilution techniques in determining the susceptibility of the isolates to
26 biocides except for *P. aeruginosa*.

27 **Conclusion:** Sodium hypochlorite and chloroxylenol are the biocides of choice in
28 hospital disinfection according to this study. Ethanol and sometimes chlorhexidine
29 should be used in higher concentrations compared to the current usage in order to
30 achieve the requisite efficacy.

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31 **Keywords:** Biocides, Bacteria, Susceptibility, Resistance, Nigeria

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33 1. INTRODUCTION

34 The issues relating to biocide resistance are considered to have a very low profile and
35 priority unlike antibiotics resistance [1]. Biocides have been far less studied than
36 antibiotics in terms of efficacy, mechanisms of action and epidemiology of bacterial
37 resistance. Despite this paucity of information on many biocides, current knowledge still
38 indicates that bacteria possess a number of mechanisms to survive exposure to a
39 biocide. Some of these mechanisms include; efflux pumps, permeability changes and
40 biofilms also confer resistance to antibiotics [2, 3]. Other mechanisms have also been
41 described, such as mutation and by-pass of metabolism [4]. It was also recognized that
42 some environmental factors contribute to a reduced susceptibility to biocides including
43 new factors such as bacterial swarming, motility are emerging as contributing to cross-
44 resistance [5].

45 The concentration of a biocide is the most important factor that affects its efficacy. In
46 most cases bacterial resistance emerged following the improper storage or use of the
47 formulations, resulting in a decrease in the effective concentration [6]. To improve
48 hygiene measures or reduce nosocomial infections to the barest minimum in hospitals
49 and its environs there is need to increase the use of biocides for effective control of
50 these organisms. Recently, most chemical based disinfectants are now incorporated
51 into many personal care products that are used at home or hospitals; these include
52 cosmetic, hand sanitizers, lotions [7]. In the health care setting, bacterial resistance to
53 biocides has long been reported with compound such as: chlorhexidine [8], quaternary
54 ammonium compounds; bisphenol, and triclosan [9, 10].

55 There has been an increase in bacteria resistance to biocides and antibiotics and this
56 has led to the development of multidrug resistant strains that are the major causes of
57 hospital acquired infections [11]. Biocides, unlike antibiotics lack selective toxicity, and
58 most do not have a distinct bacterial cell target upon which to act except for triclosan
59 and chlorhexidine [12]. In Nigeria, there have been several reports of high level bacterial
60 resistance to antibiotics including Gram negative bacteria to carbapenem the last resort

61 antibiotic [13] and **methicillin resistance *Staphylococcus aureus* (MRSA)** with a

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62 prevalence of 41.4% [14]. Therefore, it is imperative to study the activities of common
63 available biocides against some of these multidrug resistant bacterial isolates in order to

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64 have background information for **the use of these biocides in our hospitals and various**

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65 homes since routine biocide susceptibility testing is not popular or practicable as
66 obtained in antibiotics. Moreover, there is little or no current information on the activities

67 of available biocides **toon** these isolates in Nigeria. The aim of this study was to

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determine the activities of some common available biocides against bacterial isolates from tertiary hospitals in Nigeria.

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71 MATERIALS AND METHODS

72 2.1 Bacterial strains

73 A total of 53 bacterial strains were collected from varying clinical specimens from 4
 74 tertiary hospitals in Nigeria. These strains included 23 *Staphylococcus aureus*; 15
 75 *Klebsiella pneumoniae*; 6 *Pseudomonas* species and 9 *Escherichia coli* which were
 76 clinical isolates obtained from varying clinical specimens such as urine, blood, vaginal
 77 swab, wound swab, etc submitted to diagnostic laboratory of Medical Microbiology
 78 departments of these hospitals (Table 1). The distribution of the 53 bacterial strains
 79 used in the study is presented in Table 1.

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80 2.2 Chemicals

81 Antibiotics and biocides, sodium hypochlorite (NaClO) solution; chlorhexidine (CHX)
 82 were all purchased from Sigma-Aldrich, UK. representatives in different countries.
 83 Disinfectants, ethanol was purchased from VWR Chemicals, France; chloroxylenol
 84 (C₈H₉ClO) was purchased obtained from Reckitt Benckiser, UK and triclosan from
 85 CIBA, Germany.

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86 Determination of susceptibility to conventional antibiotics

87 The antibiotic susceptibility testing was determined using disc diffusion method on
 88 Muller Hinton (MH) agar, Oxoid UK as described by Clinical Laboratory Standards

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89 Institute, CLSI [15]. Gram- positive and negative antibiotic discs from Oxoid, UK were
 90 used for the study, these included erythromycin (15 µg); cefoxitin (30 µg); mupirocin
 91 (200 µg); vancomycin (30 µg); tetracycline (30 µg); linezolid (30 µg); teicoplanin (30 µg);
 92 clindamycin (2 µg) and gentamicin (30 µg) as well as Gram negative antibiotic discs,
 93 aztreonam (30 µg); tigecycline (15 µg); amoxycillin (20 µg); ceftriazone (30 µg);
 94 ceftazidime (30 µg); imipenem (10 µg); meropenem (10 µg); ciprofloxacin (5 µg).
 95 Bacteria inoculum was prepared to match 0.5 McFarland standards and was tested
 96 against all the **mentioned** antibiotics. Sterile swab stick was used to streak the
 97 surface of Muller Hinton agar plate, after which the antibiotic discs were placed on the
 98 surface of Muller Hinton plate and incubated at 37°C overnight. The zones of inhibition
 99 was measured and interpreted as sensitive and resistant based on the guidelines of
 100 **Clinical Laboratory Standards Institute, CLSI [15].**

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101 2.3 Determination of minimum inhibitory concentrations

102 2.3.1 Diffusion technique

103 **This was done using the p**unch hole diffusion method **was employed** to determine
 104 the minimum inhibitory concentrations of the isolates against the biocides. **Several**
 105 **this technique, several** punch holes were made in agar plates that have been
 106 previously seeded with the test organisms. These punched holes were then filled with
 107 varying concentrations of **the** biocides to be tested. The working stock of the various
 108 biocides used included ethanol (100%), sodium hypochlorite (6%), chlorhexidine (4%),
 109 chloroxylenol (2.4%) and triclosan (4%). **These stocks were previously prepared using**
 110 **sterile distilled water except for triclosan where 1 M sodium hydroxide was used.** The

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plates were **then** incubated at 37°C overnight, and the zones of **growth** inhibition were measured in millimeters using transparent metric rule. Various controls were set **up**; **positive control**; **control** organisms were run alongside the tests using *E. coli* ATCC 25922 for *Enterobacteriaceae*, *P. aeruginosa* ATCC 27853 for *P. aeruginosa* and ATCC 25923 for *S. aureus*, while sterility control was set up with least concentrations of the various biocides without organisms, viability control **contained by inoculating** the test strains in the broth without biocide. **All MIC determinations** **This was** **were** repeated three times in independent experiments.

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2.3.2 Dilution techniques

The minimum inhibitory concentrations of a range of biocides and antibiotics were determined, defined as the lowest biocide or antibiotic concentration that inhibit bacterial growth or no visible growth using the microtitre broth dilutions as recommended by **Clinical Laboratory Standards Institute**, CLSI [15]. **The working stocks of the various biocides used were as described above.** Using a multi-stopper pipette, 50 µl of Mueller- Hinton broth was added to **microtiter plate** wells 2-12 **while broth was not added to wells A1-H1. With a multichannel pipette**, 50 µl of biocide diluted in broth (at twice the required final concentration) was added to column A1-H1 and A2-**H2 using the working stocks previously described above.** 50 µl of biocide from column 2-11 was double diluted **while** **and** 50 µl was removed from column **11 ensuring that column 12 was not touched still with the aid of the multi-channel pipette.**

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50 µl of Anan overnight broth culture of the organisms **already was** diluted to 10⁴ orgs/ml **was added**. The microtitre plates **were** covered with sterile lid, incubated at

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133 37°C and examined for growth after overnight incubation. All MIC determination **s was**
 134 **werealso** repeated three times in independent experiments.

135 2.4 Determination of minimum bactericidal concentrations

136 **The m**Minimum bactericidal concentration **s waswere** determined by inoculating 50 µl
 137 aliquot of all suspensions where no visible bacterial growths were observed on LB agar.
 138 **Sigma-Aldrich UK** and incubated at 37°C overnight. MBC **was** recorded as highest
 139 dilution where there **waswere** no **bacterial** growths.

140 2.5 Statistics analysis

141 Data were analysed using statistical package within the Microsoft Excel and Epi-info
 142 software from Centre for Disease control and prevention, USA. Student *t*-test was used
 143 to determine the statistical significance. **The** *P* value less than 0.05 was considered to
 144 be significant.

145 3.0 Results

146 3.1 Antibiotics susceptibility

147 The susceptibility patterns of all the strains to conventional antibiotics were shown in
 148 Table 2 **in order to confirm the level of resistance to these antibiotics**; panel I is for
 149 **the** Gram negative bacteria while panel II *S. aureus*. Several differences in antimicrobial
 150 susceptibility **yes patterns** were observed, high level resistance to third generation
 151 antibiotics was obtained for Gram negative bacteria; 100% **and 87%** resistance to
 152 ceftriaxone **and** ceftazidime **respectively86.7%**. Fluoroquinolone antibiotic, ciprofloxacin

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also showed similar pattern with 76.7% resistance including tigecycline 70% resistance.

The sensitivity was found to be highest in imipenem, only with 6.7% resistance. For *S. aureus*, 52.2% were found to be resistant to ceftazidime, an indication of presence of methicillin resistance. Overall, these patterns of susceptibility depict a multidrug resistance strains.

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3.2 Biocides susceptibility

The number of strains inhibited with different concentrations of various biocides (sodium hypochlorite, chloroxylenol, triclosan, chlorhexidine, and ethanol) using agar diffusion and broth microtiter dilution techniques were shown in Table 3. Sodium hypochlorite was more effective against all the strains compared to the other biocides, 0.5 – 2%, while ethanol and triclosan were less effective even at the popular 70% for ethanol normally used for antiseptics. *S. aureus* showed 100% resistance to ethanol even at 100% concentration while some levels of activities were obtained at 80 – 90% for other bacteria. *P. aeruginosa* was found to be 100% resistant to triclosan at all concentrations. The minimum bactericidal concentration for all the biocides was determined. In most cases, all test compounds appeared to be bactericidal with MBC/MIC ratios of >4 (Table 4).

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Diffusion technique appeared to be a better technique than the dilution technique in determining the MICs of biocides in this study, however it was not statistically significant except for *Pseudomonas aeruginosa* for certain biocides such as sodium hypochlorite; chlorhexidine; chloroxylenol and *S. aureus* with sodium hypochlorite ($P < 0.05$). These were repeated severally for reproducibility.

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UNDER PEER REVIEW

176 4.0 Discussion

177 In Nigeria, there are several reports of high level bacterial resistance to antibiotics in
 178 Gram negative bacteria including last resort antibiotic such as carbapenem [13] and
 179 MRSA with a prevalence of 41.4% [14], and yet to the best of our knowledge, there
 180 has not been any critical assessment of the efficacy and choice of biocides/disinfectants
 181 use in our various tertiary hospitals. A great number of disinfectants are used in the
 182 health care setting including sodium hypochlorite, triclosan, chloroxylenol, ethanol, and
 183 chlorhexidine. These agents are chemicals that are capable of rendering harmless or
 184 exerting a controlling effect on any harmful microorganism by chemical means
 185 meanwhile, the goal of disinfection is to reduce the risk of endemic and epidemic
 186 nosocomial infections in hospital patients and limiting its spread [16, 17]. Majority of the
 187 isolates wereare resistant to more than three different types of antibiotics including
 188 carbapenem for the Gram negative bacteria depicting multidrug resistance isolates,
 189 cefoxitin and teicoplanin for *S. aureus* an indication of methicillin resistant *S. aureus*,
 190 MRSA.

191 All the bacterial isolates were resistant to chlorhexidine at MIC >4%, similarly scenario
 192 was obtained for ethanol at 70%; no activity was obtainedobserved for ethanol at
 193 70% until the susceptibility was repeated at increased concentration s was increased
 194 to from 80% and above. It would be recalled that 70% ethanol has been the mainstay
 195 for ensuring antisepsis on the skin or in clinical settings for swabbing animate or
 196 inanimate objects. The 70% came into being due essentially due to the fact that 100%
 197 alcohol cannot be used as a disinfectant because of its volatility and denaturation of

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external membrane proteins only. In order to prevent breeding biocide resistant strains we need to increase the concentration of ethanol as disinfectant/biocide to 80 – 85% as a matter of emergency with little or no economic cost in view of the global high level bacteria resistance to antimicrobial agents rather than adhering strictly to the conventional 70% which is still the current practice worldwide. Already use of biocide in higher concentrations is inevitable due to the decrease in susceptibility as observed in this study and elsewhere [1718]. Likewise, in antibiotics there had been cases where combined antibiotic therapy or increased concentration of drugs isare recommended solely to avoid emergence of resistant strains and this can be emulated for biocides. Moorer reported that a mixture of 70% ethanol or isopropanol diluted in water was effective against a wide spectrum of bacteria [1819].

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MIC range of 0.5 - 1% sodium hypochlorite was required to inhibit Gram negative bacteria; such as *E. coli*, *K. pneumoniae* and *P. aeruginosa*, while MIC range of 1 - 3% was more effective to inhibit the growth of *S. aureus*. The MIC range of 3 - 6% was required for chlorhexidine to work effectively; most Gram negative bacteria were inhibited at 5% while *S. aureus* strains were prevented essentially at 3%, while 0.15 - 1.2% was obtained for chloroxylonol. The minimum bactericidal concentrations range for most of the biocides arewere greater than four times hence they may not be regarded as being very effective because an agent is usually regarded as bactericidal if the MBC is no more than four times the MIC.

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Because there is no standardized susceptibility testing techniques and/or critical concentrations for disinfectants to classify bacterial susceptibility to biocides like in

antibiotics, we included two methods for susceptibility testing in order to determine which method best suits biocide(s). We discovered that there was no difference between disc diffusion and broth microdilution except for *P. aeruginosa* but we do not understand why this is so because the results were products of repeated independent experiments.

Conclusion

The more appropriate disinfectants for hospital disinfection are sodium hypochlorite and chloroxylenol. Ethanol should be used in higher concentration more than the conventional 70% in current usage in order to achieve the requisite efficacy in routine surface disinfection particularly in hospitals and environs.

Consent

It is not applicable

Ethical Approval

It is not applicable

Acknowledgments

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302 **Table 1. Sources and distribution of bacterial strains**

Source	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	Total
Wound swab	2	4	3	7	16
Eye swab	1	0	0	4	5
HVS	1	0	0	2	3
Blood culture	3	3	0	4	10
Urine	4	2	3	2	11
Sputum	2	0	0	0	2
Catheter tip	1	0	0	0	1
Aspirate	1	0	0	4	5
Total	15	9	6	23	53

303

304 **Table 2. Antibiotic susceptibility pattern of 53 bacterial strains**

Antibiotics (µg/ml)	No. sensitive	% Sensitive	% Resistance
Panel I: Gram negative, 30 isolates			
Ceftazidime (30)	4	13.3	86.7
Ceftriazone (30)	0	0.0	100.0
Amoxycylav (30)	6	20.0	80.0
Ciprofloxacin (5)	7	23.3	76.7
Gentamicin (10)	19	35.9	64.2
Tigecycline (15)	9	30.0	70.0
Imipenem (10)	28	93.3	6.7
Aztreonam (30)	19	63.3	36.7
Panel II: Gram positive, 23 isolates			
Tetracycline (30)	12	52.2	47.8
Cefoxitin (30)	11	47.8	52.2
Linezolid (30)	17	73.9	26.1
Clindamycin (2)	15	62.2	34.8
Teicoplanin (30)	22	95.7	4.4
Erythromycin (15)	17	73.9	26.1

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306 Table 3. Minimum inhibitory concentrations of bacterial isolates to biocides

MICs, %; wt/vol or vol/vol (Range)						Formatted: English (United Kingdom)
Isolate, n	SH (0.12 – 6)	CHX (0.02 – 2.4)	CHD (0.5 – 10)	EtOH (10 – 100)	TRIC (0.01 – 2)	Formatted: Highlight
Panel I: Diffusion						Formatted: Highlight
<i>E. coli</i> , 9	0.25(3) 0.5(4) 1(1) 2(1)	0.02(3) 0.04(1) 0.6(3) 1.2(1) 2.4(1)	5(2) 6(5) >10(2)	75(2) 80(2) 85(1) 95(1) 100(1) >100(2)	0.02(3) 0.25(1) 1(1) >2(4)	Formatted: Highlight
<i>K. pneumoniae</i> , 15	0.25(3) 0.5(7) 1(5)	0.08(1) 0.15(3) 0.3(6) 0.6(2) >2.4(3)	5(14) >10(1)	75(3) 80(2) 85(3) 90(1) 100(2) >100(4)	0.02(1) >2 (14)	Formatted: Highlight
<i>P. aeruginosa</i> , 6	0.12(2) 0.25(4)	1.2(2) 2.4(3) >2.4(1)	5(3) 6(1) >10(2)	75(3) 80(1) >100(2)	>2(6)	Formatted: Highlight
<i>S. aureus</i> , 23	0.12(1) 0.5(3) 1(5) 2(14)	0.02(5) 0.04(4) 0.08(2) 0.15(2) 0.3(2) 0.6(3) 1.2(2) >2.4(3)	3(17) 6(2) 7(3) >10(1)	>100(23)	0.02(6) 0.04(1) 0.08(1) 0.5(2) 1(1) >2(12)	Formatted: Highlight
Panel II: Dilution						Formatted: Highlight
<i>E. coli</i> , 9	0.5(2) 1(5) 2(2)	0.3(1) 0.6(8)	5(1) 6(2) 8(3) 9(2) 10(1)	85(1) 90(3) 95(1) 100(4)	0.02(3) 0.25(1) 1(1) >2(4)	Formatted: Highlight
<i>K. pneumoniae</i> , 15	0.5(9) 1(6)	0.15(1) 0.3(11) 0.6(3)	5(1) 6(2) 8(3) 9(2) 10(1)	85(4) 90(2) 95(4) 100(5)	0.02(1) 2(1) >2(13)	Formatted: Highlight
<i>P. aeruginosa</i> , 6	0.5(5) 1(1)	0.3(1) 0.6(5)	8(4) 9(1) 10(1)	90(3) 95(1) 100(2)	>2(6)	Formatted: Highlight
<i>S. aureus</i> , 23	1(4) 2(10) 3(9)	0.15(5) 0.3(10) 0.6(4) 1.2(4)	6(12) 7(3) 8(5) 9(2) 10(1)	95(5) 100(6) >100(12)	0.02(6) 0.04(1) 0.08(1) 0.5(2) 1(1) >2(12)	Formatted: Highlight

MICs, %; wt/vol or vol/vol (Range)					
Isolate, n	SH (0.12 – 6)	CHX (0.02 – 2.4)	CHD (0.5 – 10)	EtOH (10 – 100)	TRIC (0.01 – 2)
Panel I: Diffusion					
<i>E. coli</i> , 9	0.25(3) 0.5(4) 1(1) 2(1)	0.02(3) 0.04(1) 0.6(3) 1.2(1)	5(2) 6(5) >10(2)	75(2) 80(2) 85(1) 95(1) 100(1) >100(2)	0.02(3) 0.25(1) 1(1) >2(4)
<i>K. pneumoniae</i> , 15	0.25(3) 0.5(7) 1(5) 2.4(1)	0.08(1) 0.15(3) 0.3(6) 0.6(2)	5(14) >10(1)	75(3) 80(2) 85(3) 90(1) 100(2) >100(4)	0.02(1) >2 (14)
<i>P. aeruginosa</i> , 6	0.12(2) 0.25(4)	1.2(2) 2.4(3) >2.4(1)	5(3) 6(1) >10(2)	75(3) 80(1) >100(2)	>2(6)
<i>S. aureus</i> , 23	0.12(1) 0.5(3) 1(5) 2(14)	0.02(5) 0.04(4) 0.08(2) 0.15(2)	3(17) 6(2) 7(3) >10(1)	>100(23)	0.02(6) 0.04(1) 0.08(1) 0.5(2) 1(1) >2(12)
Panel II: Dilution					
<i>E. coli</i> , 9	0.5(2) 1(5) 2(2)	0.3(1) 0.6(8)	5(1) 6(2) 8(3) 9(2) 10(1)	85(1) 90(3) 95(1) 100(4)	0.02(3) 0.25(1) 1(1) >2(4)
<i>K. pneumoniae</i> , 15	0.5(9) 1(6)	0.15(1) 0.3(11) 0.6(3)	5(1) 6(2) 8(3) 9(2) 10(1)	85(4) 90(2) 95(4) 100(5)	0.02(1) 2(1) >2(13)
<i>P. aeruginosa</i> , 6	0.5(5) 1(1)	0.3(1) 0.6(5)	8(4) 9(1) 10(1)	90(3) 95(1) 100(2)	>2(6)
<i>S. aureus</i> , 23	1(4) 2(10) 3(9)	0.15(5) 0.3(10) 0.6(4) 1.2(4)	6(12) 7(3) 8(5) 9(2) 10(1)	95(5) 100(6) >100(12)	0.02(6) 0.04(1) 0.08(1) 0.5(2) 1(1) >2(12)

307 n - number of strains, () - numbers in parentheses are the number or frequency of strains with the MICs by their sides,

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308 SH – sodium hypochlorite, CHX – chlorxylenol, CHD – chlorhexidine, EtOH – ethanol, TRIC – triclosan.

309

UNDER PEER REVIEW

310 **Table 4. Minimum bactericidal concentrations of bacterial isolates to biocides**

Isolate, n	MBC, % (Range)				
	SH (0.12 – 6)	CHX (0.02 – 2.4)	CHD (0.5 – 10)	EtOH (10 – 100)	TRIC (0.01 – 2)
<i>E. coli</i> , 9	4(2) 6(4) >6(3)	2.4(6) >2.4(3)	10(5) >10(4)	95(2) 100(7)	0.25(3) 1(1) >2(5)
<i>K. pneumoniae</i> , 15	6(6) >6(9)	0.3(2) 1.2(6) 2.4(6) >2.4(1)	10(10) >10(5)	95(4) 100(7)	0.25(1) >2 (14)
<i>P. aeruginosa</i> , 6	6(4) >6(2)	0.6(1) 2.4(2) >2.4(3)	10(2) >10(4)	100(6)	>2(6)
<i>S. aureus</i> , 23	3(6) 4(5) 6(12)	0.3(4) 0.6(10) 1.2(9)	7(9) 8(11) 10(3)	>100(23)	0.25(6) 2(2) >2(15)

311 () - numbers in parentheses are the number or frequency of strains with the MBCs by their sides, SH – sodium hypochlorite, CHX – chlorxylenol, CHD –
 312 chlorhexidine, EtOH – ethanol, TRIC – triclosan.

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