

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 minimal inhibitory concentrations (MICs), of a range of biocides were determined using the microtitre broth and agar diffusion methods including minimal bactericidal concentrations, MBCs.

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, an indication of presence of methicillin resistance. The MIC showed *S. aureus* to be more resistant to all the biocides, 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 be bactericidal with MBC/MIC ratios of >4. There was no

24 statistical difference between diffusion and dilution techniques in determining the
25 susceptibility of the isolates to biocides except for *P. aeruginosa*.

26 **Conclusion:** sodium hypochlorite and chloroxlenol are the biocides of choice in
27 hospital disinfection. Ethanol and sometimes chlorhexidine should be used in higher
28 concentrations compared to the current usage in order to achieve the requisite efficacy.

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

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31 1. INTRODUCTION

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

43 The concentration of a biocide is the most important factor that affects its efficacy. In
44 most cases bacterial resistance emerged following the improper storage or use of the

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

There has been an increase in bacteria resistance to biocides and antibiotics and this has led to the development of multidrug resistant strains that are the major causes of hospital acquired infections [11]. Biocides, unlike antibiotics lack selective toxicity, and most do not have a distinct bacterial cell target upon which to act except for triclosan and chlorhexidine [12]. In Nigeria, there have been several reports of high level bacterial resistance to antibiotics including Gram negative bacteria to carbapenem the last resort antibiotic [13] and MRSA with a prevalence of 41.4% [14]. Therefore, it is imperative to study the activities of common available biocides against some of these multidrug resistant bacterial isolates in order to have background information for use of these biocides in our hospitals and various homes since routine biocide susceptibility testing is not popular or practicable as obtained in antibiotics. Moreover, there is little or no current information on the activities of available biocides on these isolates in Nigeria. The aim of this study was to determine the activities of some common available biocides against bacterial isolates from tertiary hospitals in Nigeria.

67

68 MATERIALS AND METHODS

69 2.1 Bacterial strains

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

77 2.2 Chemicals

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

82 ***Determination of susceptibility to conventional antibiotics***

83 The antibiotic susceptibility testing was determined using disc diffusion method on
84 Muller Hinton (MH) agar as described by Clinical Laboratory Standards Institute, CLSI
85 [15]. Gram- positive and negative antibiotic discs from Oxoid, UK were used for the
86 study, these include erythromycin (15 µg); cefoxitin (30 µg); mupirocin (200 µg);
87 vancomycin (30 µg); tetracycline (30 µg); linezolid (30 µg); teicoplanin (30 µg);

88 clindamycin (2 µg) and gentamicin (30 µg) as well as Gram negative antibiotic discs,
89 aztreonam (30 µg); tigecycline (15 µg); amoxycillin (20 µg); ceftriazone (30 µg);
90 ceftazidime (30 µg); imipenem (10 µg); meropenem (10 µg); ciprofloxacin (5 µg).
91 Bacteria inoculum was prepared to match 0.5 McFarland standards and was tested
92 against all the aforementioned antibiotics. Sterile swab stick was used to streak the
93 surface of Muller Hinton agar plate, after which the antibiotic discs were placed on the
94 surface of Muller Hinton plate and incubated at 37°C overnight. The zones of inhibition
95 was measured and interpreted as sensitive and resistant based on the guidelines of
96 Clinical Laboratory Standards Institute, CLSI [15].

97 **2.3 Determination of minimum inhibitory concentrations**

98 **2.3.1 Diffusion technique**

99 This was done using the punch hole diffusion method to determine the minimum
100 inhibitory concentrations of the isolates against the biocides. In this technique, several
101 punch holes were made in agar plates that have been previously seeded with the test
102 organisms. These punched holes were then filled with varying concentrations of the
103 biocides to be tested. The working stock of the various biocides used included ethanol
104 (100%), sodium hypochlorite (6%), chlorhexidine (4%), chloroxylenol (2.4%) and
105 triclosan (4%). The plates were then incubated at 37°C overnight, and the zones of
106 growth inhibition were measured in millimeters using transparent metric rule. Various
107 controls were set up; positive control organisms were run alongside the tests using *E.*
108 *coli* ATCC 25922 for *Enterobacteriaceae*, *P. aeruginosa* ATCC 27853 for *P. aeruginosa*
109 and ATCC 25923 for *S. aureus*, while sterility control was set up with least

concentrations of the various biocides without organisms, viability control by inoculating the test strains in the broth without biocide. All MIC determinations were repeated three times in independent experiments.

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 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. 50 µl of biocide from column 2-11 was double diluted and 50 µl was removed from column 11 ensuring that column 12 was not touched still with the aid of the multi-channel pipette.

An overnight broth culture of the organisms was diluted to 10^4 orgs/ml. The microtitre plates covered with sterile lid, incubated at 37°C and examined for growth after overnight incubation. All MIC determinations were repeated three times in independent experiments.

2.4 Determination of minimum bactericidal concentrations

The minimum bactericidal concentrations were determined by inoculating 50 µl aliquot of all suspensions where no visible bacterial growths were observed on LB agar and

131 incubated at 37°C overnight. MBC was recorded as highest dilution where there were
132 no growths.

133 **2.5 Statistics analysis**

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

138 **3.0 Results**

139 **3.1 Antibiotics susceptibility**

140 The susceptibility patterns of all the strains to conventional antibiotics were shown in
141 Table 2 in order to confirm the level of resistance to these antibiotics; panel I is for the
142 Gram negative bacteria while panel II *S. aureus*. Several differences in antimicrobial
143 susceptibilities were observed, high level resistance to third generation antibiotics was
144 obtained for Gram negative bacteria; 100% resistance to ceftriaxone, ceftazidime
145 86.7%. Fluoroquinolone antibiotic, ciprofloxacin also showed similar pattern with 76.7%
146 resistance including tigecycline 70% resistance. The sensitivity was found to be highest
147 in imipenem, only 6.7% resistance. For *S. aureus*, 52.2% were found to be resistant to
148 ceftazidime, an indication of presence of methicillin resistance. Overall, these patterns of
149 susceptibilities depict a multidrug resistance strains.

150 **3.2 Biocides susceptibility**

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

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

167

4.0 Discussion

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

All the bacterial isolates were resistant to chlorhexidine at MIC >4%, similar scenario was obtained for ethanol at 70%; no activity was observed until the susceptibility was repeated at increased concentrations from 80% and above. It would be recalled that 70% ethanol has been the mainstay for ensuring antisepsis on the skin or in clinical settings for swabbing animate or inanimate objects. The 70% came into being due essentially to the fact that 100% alcohol cannot be used as a disinfectant because of its volatility and denaturation of external membrane proteins only. In order to prevent

190 breeding biocide resistant strains we need to increase the concentration of ethanol as
191 disinfectant/biocide to 80 – 85% as a matter of emergency with little or no economic
192 cost in view of the global high level bacteria resistance to antimicrobial agents rather
193 than adhering strictly to the conventional 70% which is still the current practice
194 worldwide. Already use of biocide in higher concentrations is inevitable due to the
195 decrease in susceptibility as observed in this study and elsewhere [17]. Likewise, in
196 antibiotics there had been cases where combined therapy or increased concentration of
197 drugs is recommended solely to avoid emergence of resistant strains and this can be
198 emulated for biocides. Moorer reported that a mixture of 70% ethanol or isopropanol
199 diluted in water was effective against a wide spectrum of bacteria [18].

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

209 Because there is no standardized susceptibility testing techniques and/or critical
210 concentrations for disinfectants to classify bacterial susceptibility to biocides like in
211 antibiotics, we included two methods for susceptibility testing in order to determine

which method best suit 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

References

1. Cookson BD, Bolton MC, Platt JH. Chlorhexidine resistance in methicillin resistance *Staphylococcus aureus* or just an elevated MIC? An *In vitro* and *In vivo* assessment. *Antimicrob Agents Chemother* 2005;35:1997-2002.
2. Bailey AM, Constantinidou C, Ivens A, Garvey MJ, Webber MA, et al. Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *J Antimicrob Chemother* 2009;64:973–985.
3. Daniels C, Ramos JL. Adaptive drug resistance mediated by root-nodulation-cell division efflux pumps. *Clin Microbiol Infect* 2009;15(1):32-36.
4. Webber MA, Coldham NG, Woodward MJ, Piddock LJV. Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother* 2008;62:92–97.
5. Lai S, Tremblay J, Deziel E. Swarming motility: A multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* 2009;11:126-136.

- 239 6. Russell AD. Introduction of biocide in to clinical practice and the impact on
240 antibiotic resistant bacteria. J Appl Microbiol 2002;92:121-135.
- 241 7. Buffet-Bataillon S, Tattevin P, Bonnaure-Mallet M, Jolivet-Gougeon A.
242 Emergence of resistance to antibacterial agents: the role of quaternary
243 ammonium compounds — a critical review. Int J Antimicrob Agents
244 2012;39(5):381–389.
- 245 8. Stickler DJ. Chlorhexidine resistance in *Proteus mirabilis*. J Clin Pathol
246 2003;92(Supp1):163-70.
- 247 9. Heath RJ, Yu YT, Shapiro S, Olson E, Rock CO. Broad spectrum antimicrobial
248 biocides target the FabI component of fatty acid biosynthesis. J Biol Chem
249 1998;273:30316–30320.
- 250 10. Bamber AL, Neal TJ. An assessment of triclosan susceptibility in methicillin-
251 resistant and methicillin sensitive *Staphylococcus aureus*. J Hosp Infect
252 1999;41:107-109.
- 253 11. Chapman JS. Disinfectant resistance mechanisms, cross-resistance, and co-
254 resistance. Int Biodeterior Biodegradation 2003;5(4):271-276.
- 255 12. Komljenovic I, Marquardt D, Harroun TA, Sternin E. Location of chlorhexidine in
256 DMPC model membranes: a neutron diffraction study. Chem Phys Lipids
257 2010;163:480–487.
- 258 13. Ogbolu DO, Webber MA. High level and novel mechanisms of carbapenem
259 resistance in Gram negative bacteria from tertiary hospitals in Nigeria. Int J
260 Antimicrob Agents 2014;43:412-417.
- 261 14. Alli OAT, Ogbolu DO, Mustapha JO, Akinbami R, Ajayi AO. The non-association
262 of Panton-Valentine leukocidin and mecA genes in the genome of
263 *Staphylococcus aureus* from hospitals in South Western Nigeria. Indian J Med
264 Microbiol 2012;30:159-164.
- 265 15. Clinical Laboratory Standards Institute. Performance standards for antimicrobial
266 susceptibility testing: Twenty fifth informational supplement M100-S25 CLSI
267 Wayne, PA, USA 2015.
- 268 16. Condell O, Iversen C, Cooney S, Power KA, Walsh C, Burgess C, Fanning S.
269 Efficacy of biocides used in the modern food industry to control *Salmonella*
270 enterica, and links between biocide tolerance and resistance to clinically relevant
271 antimicrobial compound. Appl Environ Microbiol 2012;78(9):3087–3097.
- 272 17. Akinkunmi EO, Lamikanra A. Susceptibility of community associated methicillin
273 resistant *Staphylococcus aureus* isolated from faeces to antiseptics. J Infect Dev
274 Ctries 2012;6(4):317-324.
- 275 18. Moorer WR. Antiviral activity of alcohol for surface disinfection. Int J Dent hyg
276 2003;1(3):138-142.
- 277

278 **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

279

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

Antibiotics (µg/ml)	No. sensitive	% Sensitive	% Resistance
Panel I: Gram negative			
Ceftazidime (30)	4	13.3	86.7
Ceftriazone (30)	0	0.0	100.0
Amoxyclav (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			
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

281

282 **Table 3. Minimum inhibitory concentrations of bacterial isolates to biocides**

Isolate, n	MICs, %; wt/vol or vol/vol (Range)				
	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) 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)
<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)
<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) 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)
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)

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

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

285

286 **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)

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