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Medical Advances and Case Report

Vol. 5 (2), pp. 22 – 30, March, 2017

Adequacy of hibitane® on some nosocomial confines of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*

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Abstract

The effectiveness of the disinfectant hibitane on some microorganisms associated with nosocomial infections were assessed at various use-dilutions of 1: 10, 1: 100, 1: 200 and 1: 2000 constituted using aqueous ethanol and 10% serum solutions. The shapes of the death curves were qualitatively similar for all the organisms exhibiting an initial shoulder (lag), the duration of which depended on hibitane® concentration used and this was closely followed by an exponential order of death. Alcoholic solutions were much more potent than aqueous solutions. Activity of the disinfectant was maintained in the presence of potable water and serum though to a lesser extent than deionized water. The order of resistance to the disinfectant was *Escherichia coli* (MBC 0.00008 - 0.0003) > *Staphyloccocus aureus* (MBC 0.00007 - 0.00026) > *Candida albicans* MBC 0.00006 - 0.00022).

Keywords: Hibitane®, nosocomial infections, exponential death, *Escherichia coli, Staphyloccocus aureus, Candida albicans*.

INTRODUCTION

Hibitane® is a disinfectant that has been used successfully in the treatment of mouth, skin, and soft tissue infections as well as in decontamination and disinfection of slaughter houses and healthcare settings (Elmahmood and Doughari, 2007). The chemical consti-tuents of hibitane® include chlorhexidine gluconate, 5% nonyl-phenol/ethylene oxide condensate, isopropyl alco-hol, carmoisinea (E12) linolylacetate, d-gluconol-actone. The antimicrobial properties of chorhexidne were first reported by Davis et al. (1954). Its mode of action has been extensively studied (Rye and Wiseman, 1968; Russel et al., 1979; Rutala 1996). Hibitane® has been used extensively in hospital during surgery, in the labora-tories, disinfection of wards and dressing of wounds. The disinfectant is often diluted for these various purposes in practice using tap water or in some cases even water from wells contrary to sterile distilled water as often recommended by the manufacturers. This water often contains dissolved substances, impurities and other organic substances that might interfere with the efficacy of the disinfectant.

Microorganisms are not often found in pure cultures, buten closed in proteinaceous materials in the hospital environment such as serum, blood, faeces, sputum, pus and milk. These provide coatings that protect the micro-organisms from being destroyed at certain concentrations. Sterile distilled water on the other hand is devoid of impurities such as cationic and anionic substances that might likely interact with the active component in the formulation that would eventually decrease the activity of the disinfectant (Russel et al., 1979). Extensive studies on the mechanism of cell death in the presence of microbicidal concentrations of phenols, chlorhexidine, and other surface active agents have been documented. The mode of action of these compounds had been found to be due to their adverse effects on cellular permeability leading to inhibition of enzymes and leakage of intracellular materials (Judis, 1962; Alwood and Hugo, 1971; Hugo and Bloomfield, 1971; Lamikanra and Alwood, 1977). Thus the cytoplasmic membrane and its components are considered to be the main sites of action of hibitane; however, this needs to be confirmed. It is therefore important to determine the concentrations in practice that will be more effective on the pathogenic microbes associated with hospital environments if adequate control is to be achieved. Even though chorhexidine (trade name hibitane®) has been extensively used in hospitals, studies were often not carried out on micro-organisms associated with hospital based infections. This study was aimed at assessing the effectiveness of hibi-

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tane® against some microorganisms associated with nosocomial infections under conditions of use using sterile tap water (STW), sterile deionized water (SDW) and serum as diluents. STW was used to in order to simulate usual hospital practices and to find out the effect of impurities on the activity of the disinfectant, SDW was used as a positive control devoid of both impurities and microorganisms, while serum was employed to find out the effect of proteinaceous matter on activity of the disinfectant.

MATERIALS AND METHODS

Source of disinfectant

Hibitane® (Tubifoam Limited, UK) was procured from Mahmuda Pharmaceutical Chemist Shop, Yola, Nigeria. All nutrient and suspending media were of Oxoid grade and prepared according to manufacturer's instructions.

Selection of patients for specimen collection

Patients were identified based on analysis of responses to questionnaires issued to Doctors, Nurses and Laboratory Staff of Federal Medical Centre Yola, Adamawa State, Nigeria about patients hospitalized for reasons other than infections associated with the test organisms, but for disease conditions such as surgical wounds, catheter sites, blood-related disease conditions, urinary tract infections (UTI) and respiratory tract infections (RT). Specimens from such patients were collected and screened for the presence of the selected organisms and patients found harboring such organisms at initial stages of hospitalization were excluded from the study. Those patients whose samples did not yield the test organisms were further observed during their prolonged hospital stay (48 - 72 h) and appropriate samples were then collected for microbiological investigation. The prolonged period was to ensure that organisms encountered on such patients might likely be hospital based (nosocomial) pathogens acquired during hospital stay.

Source of microorganisms, culture conditions and selection of test and control strains

The clinical samples (urine, blood, wound and catheter) were cultured on MacConkey agar (MCA, Oxoid) for the isolation of *Escherichia coli*, and MCA and Mannitol Salt agar (MSA, Oxoid) for the isolation of *Staphylococus aureus* and on Saboraud Dextrose Agar (SDA, Oxoid) for the isolation of *Candida albicans.* All cultures were incubated at 37ºC for 24 h. Identification of the isolates were based on standard biochemical tests as described by Cheesbrough (2002). Bacterial isolates and those of *C. albicans* were subjected to antimicrobial susceptibility tests on Mueller Hinton agar (MHA, Oxoid) as described by Gupter et al. (2004) and Archibald et al. (2004). Based on susceptibility results obtained, the organisms were grouped into resistant or susceptible strains accordingly. Resistant organisms were those that showed stable resistance to more than 3 antibiotics and it is from this group that the test organisms *S. aureus* (SA1), *E. coli* (EC1) and *C. albicans* (CA1) were selected. Those organisms that showed stable susceptibility to all the drugs tested were regarded as susceptible and from these the control strains *S. aureus (*SA2), *E. coli* (EC2) and *C. albicans* (CA2) were selected.

Preparation of use-dilutions of disinfectant solutions

Various use-concentrations of the disinfectant solutions were carried out as practiced in the hospitals using sterile distilled water (SDW), sterile tap water (STW) and ethanol. Concentrations of 0.1 v/v and 0.01 v/v were prepared to obtain 1: 10 and 1: 100 aqueous use-dilutions each of both SDW and STW respectively, and concentrations of 0.005 v/v and 0.0005 v/v to obtain use-dilutions of 1: 200 and 1: 2000 respectively for hibitane® use-dilution in 95% ethanol as specified by the manufacturer. The effect of these various concentrations against the test organisms were then determined.

Effects of 1:10 aqueous use-dilutions of hibitane®

To 2 ml of hibitane, 16 ml of sterile deionized water (SDW) was added in a 50 ml sterile conical flask and 2 ml of exponentially growing cultures of *S. aureus* (SA1) containing 2 x 10^8 cells/ml and the flask content shaken vigorously. One milliliter (1 ml) of the mixture was immediately transferred from the flask into a sterile test tube containing 9 ml of 2% v/v tween 80 and 1% soy lecithin to inactivate chorhexidine component of hibitane so that the number of surviving cells will not be killed so as to allow counting of viable cells) and the mixture homogenized on a Gallenkamp whirl mixer and then allowed to stand for 1 min (Acheampong et al., 1988). Subsequent dilutions of the cell suspensions in trypton soy broth to obtain countable colonies were made and 1 ml of the final dilution was immediately cultured on nutrient agar plates using the pour plate technique. Culture of the cell suspensions were repeated after every 5 min interval for 30 min. The colonies were counted using the Gallenkamp colony counter after 48 h of incubation (Acheampong et al. 1988; El-mahmood and Doughari, 2007). This procedure was followed for each of the other five organisms, except but Saboraud Dextrose Agar (SDA) was used instead of nutrient agar (NA) for *C. albicans* (CA1 and CA2). The viability of the untreated (control) organisms was also determined at 0 min and at 30 min. Graphs of log Nt/No versus time were plotted. For the 1:10 use-dilutions of hibitane® in sterile deionized water (STW), the same procedure was used except that STW was used in place of SDW.

Effects of 1:100 aqueous use-dilutions of hibitane® on microorganisms

To 17.8 ml of SDW, 0.2 ml of the undiluted solution of hibitane® and 2 ml of cell suspension of *S. aureus* (SA1) of inoculum density 2.0 x 10^8 cells/ml added and mixed on a Gallenkamp whirl mixer. Other experimental details were carried out as earlier described for 1:10 aqueous dilutions. For the 1: 100 aqueous use- dilutions of hibitane® in STW, the same procedure was used except that STW was used instead of SDW.

Effects of 1:200 alcoholic use-dilutions of hibitane® on the microorganisms

To 17.9 ml of 95% ethanol, 0.10 ml of hibitane® and 2 ml of 2.0 x 10^8 cells/ml exponentially growing cells of *S. aureus* (SA1) was added in a 50 ml sterile conical flask, and the flask vigorously shaken to mix the contents. The effect of 1: 200 alcoholic use-dilution on the test bacteria was then determined as described for 1: 10 aqueous use-dilutions. The same procedure was also carried on the other 5 test bacteria.

Effects of 1:2000 alcoholic use-dilutions of hibitane® on the microorganisms

To 17.99 ml of 95% ethanol was added 0.010 ml of hibitane® and 2

Table 1. Number of viable cells after 10 min of treatment with different recommended use dilutions of hibitane® (chlorhexidine gluconate)

	SA1				SA2				EC1				EC2				CA1				CA2			
Conc(v/v)	**10min**		**30min**		**10min**		**30min**		**10min**		**30min**		**10min**		**30min**		**10min**		**30min**		**10min**		**30min**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
1: 10 SDW	$1.3x10^6$	0.7	$3.8x10^3$	0.0020	$1.0x10^6$	0.5	$1.7x10^3$	0.0010	$1.3x10^6$	0.7	$3.9x0^3$	0.002	$1.1x10^6$	0.6	$2.6x10^3$	0.0010	$9.3x10^5$	0.3	$1.1x10^3$	0.0005	$5.9x10^5$	0.3	$5.2x10^2$	0.0003
		0.9		0.0030		0.7		0.0010		1.2		0.003		0.9		0.0030		0.3		0.0008		0.3		0.0005
STW	$1.9x10^6$		$5.1x10^3$		$1.4x10^6$		$2.3x10^3$		$2.3x10^6$		$6.9x0^3$		$1.8x10^6$		$5.4x10^3$		$1.0x10^6$		$1.6x10^3$		$6.8x10^5$		$1.1x10^3$	
1:100 SDW	$3.1x10^6$	1.6	$1.5x10^4$	0.0070	$2.6x10^6$	1.3	$1.5x10^4$	0.0080	$3.5x10^6$	1.7	$1.6x0^4$	0.008	$3.4x10^6$	1.7	$1.3x10^4$	0.0060	$1.6x10^6$	0.9	$5.6x10^3$	0.0030	$1.3x10^6$	0.7	$5.3x10^3$	0.0030
		3.6		0.0200		2.9		0.0200		4.7		0.030		3.8		0.0200		1.7		0.0100		1.3		0.0080
STW	$7.2x10^6$		$3.7x10^4$		$5.8x10^6$		$4.7x10^4$		$9.4x10^6$		$5.1x0^4$		$7.6x10^6$		$4.9x10^4$		$3.3x10^6$		$2.6x10^4$		$2.6x10^6$		$1.5x10^4$	
1:200ET	$6.7x10^5$	0.3	$1.0x10^3$	0.0005	$6.2x10^5$	0.3	$5.2x10^2$	0.0003	$7.7x10^5$	0.4	$1.7x0^3$	0.001	$7.6x10^5$	0.4	$2.1x10^3$	0.0010	$4.1x10^5$	0.2	$6.5x10^2$	0.0003	$3.0x10^5$	0.2	$2.5x10^2$	0.0001
1:2000		2.6		0.0100		2.1		0.0100		3.3		0.010		2.1		0.0100		0.9		0.0400		0.9		0.0030
ET	$5.2x10^6$		$2.1x10^4$		$4.1x10^6$		$2.8x10^4$		$6.6x10^7$		$2.3x0^4$		$4.2x10^6$		$2.0x10^4$		$1.9x10^6$		$8.2x10^3$		$1.8x10^6$		$6.9x10^3$	

Key: N = number of viable cells; % = percentage of number of viable cells; SA1 = *S. aureus*; SA2 = *S. aureus*; EC1 = *E. coli;* EC2 = *E. coli;* CA1 = *C. albicans;* CA2 = *C. albicans*

ml of cell suspension of *S. aureus* (SA1) containing cell density of 2.0×10^8 cells/ml in a 50 ml sterile conical flask. The effect of 1: 2000 ethanol use-dilution on the test bacteria was then determined as described for 1:10 aqueous use dilutions were. The same procedure was also carried out for the other 5 test bacteria.

Determination of death reduction times (DRT)

The DRT is the time for 90% reduction in number of viable cells, and the values were higher in STW followed by SDW and lowest in ethanolic dilution. Extrapolation of the regression lines to the log Nt/No axis and the difference in the intercepts of the extrapolated lines gives the log10 extrapolation numbers on the multiplicity of the process.

The log10 extrapolation numbers gives the numbers of molecules of hibitane® required to act on one cell at that particular concentration to cause death (Cove and Holland, 1983) . The death rates, the slopes of the killing curves, the DRT, the length of the shoulders and the log10 extrapolation numbers are measurements of resistance of the cells to the disinfectant hibitane®. The determination of these values had made it possible to compare the resistance of different organisms at the same concentration of an agent or the resistance of one particular organism at different concentrations of an agent (Elmahmood, 2006). These organisms have been used as test organisms for the investigation of the activity of some antibiotics, phenolic compounds, biguanides and various other agents, though such differences in susceptibility had not always been documented.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of hibitane® against the test organisms

The broth dilution technique was used to determine the minimum inhibitory concentration of the test organisms (Elmahmood and Doughari, 2007). Briefly, graded volumes of 1: 100 (0.01 v/v) hibitane® (1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4 and 3.6 ml) were added to 5 ml double strength Nutrient Broth (NB, Oxoid) and calculated volumes of sterile deionized water (SDW, 3.6, 3.4, 3.2, 3.0, 2.8, 2.6, 2.4, 2.2, 2.0, 1.8, 1.6, 1.4 and 1.2 ml)were also added and mixed. To each of the test tubes were added 0.2 ml of 1.0×10^4 cells ml^{-1} of culture suspension of *S. aureus* (NS1) to make up to 10 ml in each of the test tubes and the contents properly mixed on a Gallenkamp Whirl

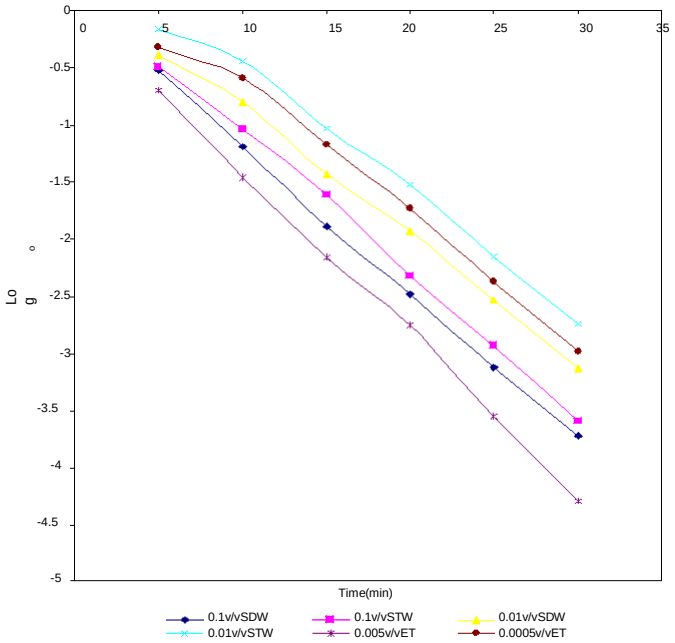

Figure 1a. Effects of used dilutions of hibitane (chlorhexidine gluconate) on the viability of *S. aureus* (SA1) in various diluents incubated at 37^{o}C.

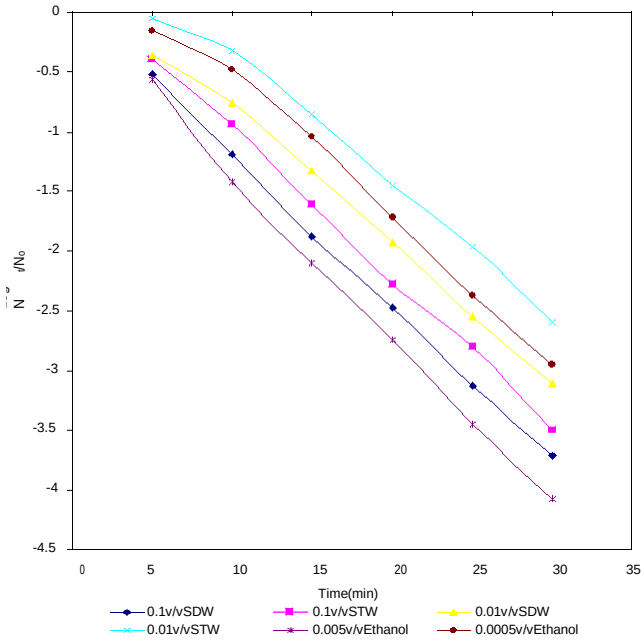

Figure 2a. Effects of used dilutions of chlorhexidine gluconate on the viability of *E. coli* (EC1) in various diluents incubated at 37^{o}C.

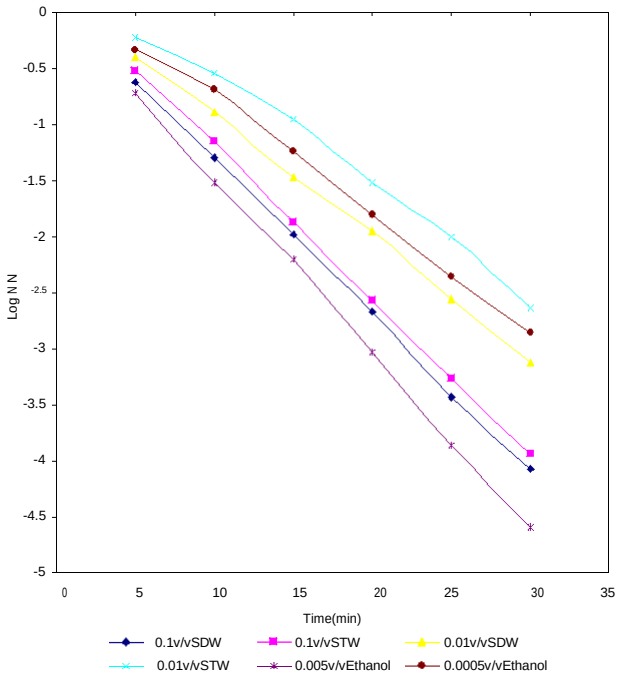

Figure1b. Effects of used dilutions of chlorhexidine gluconate on the viability of *S. aureus* (SA2) in various diluents incubated at 37^{o}C

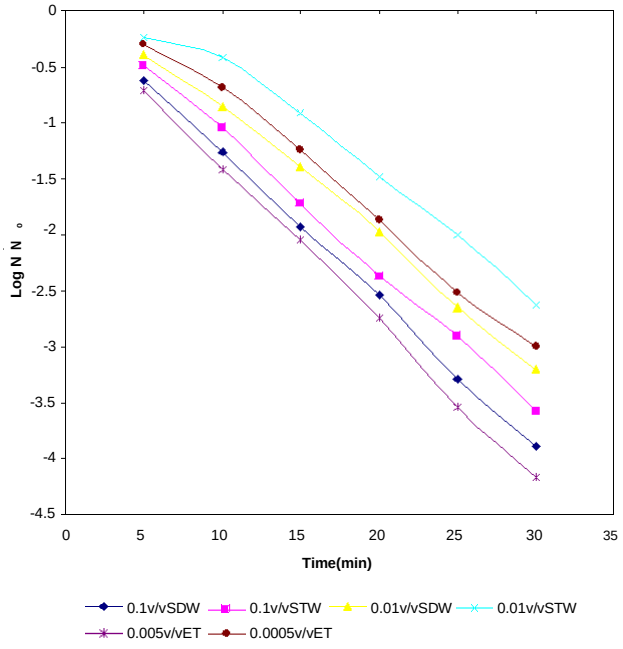

Figure 2b. Effects of used dilutions of chlorhexidine gluconate on the viability of *E. coli* (EC2) in sterile deionized water (SDW) sterile tap water (STW) and 95% ethanol (ET) incubated at 37^{o}C.

Mixer. A test tube containing NB only served as control. All the test tubes were incubated at 37ºC for 24 h and observed for turbidity. The same procedure was repeated for each of the five test organi -

organisms as well as for SDW and STW except that a higher inoculum size of 1.0 x 10^{4}cells ml^{-1} was used. The same procedure was also followed for determination of MIC of chorhexidine in 10% rabbit serum using the two inoculum densities of microbial sus-

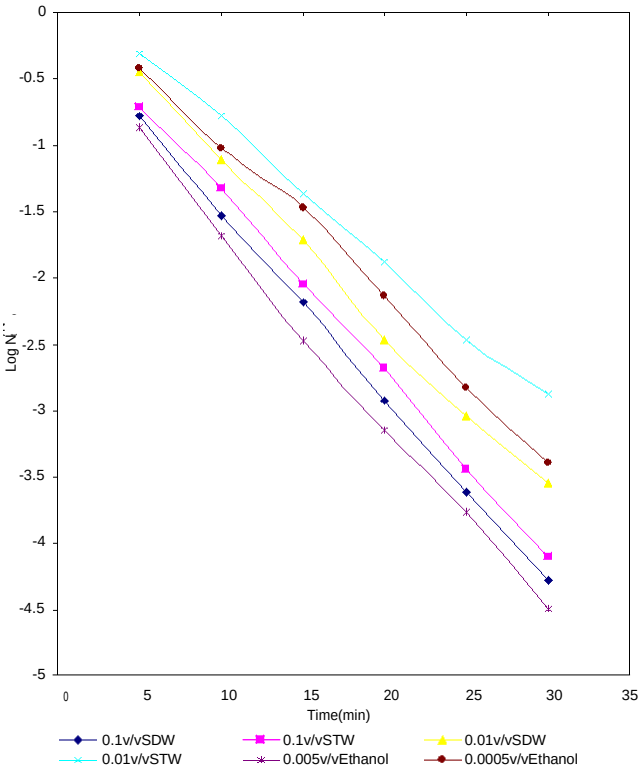

Figure 3a. Effects of used dilutions of chlorhexidine gluconate on the viability of *C. albicans* (CA1) in various diluents incubated at 37^{o}C.

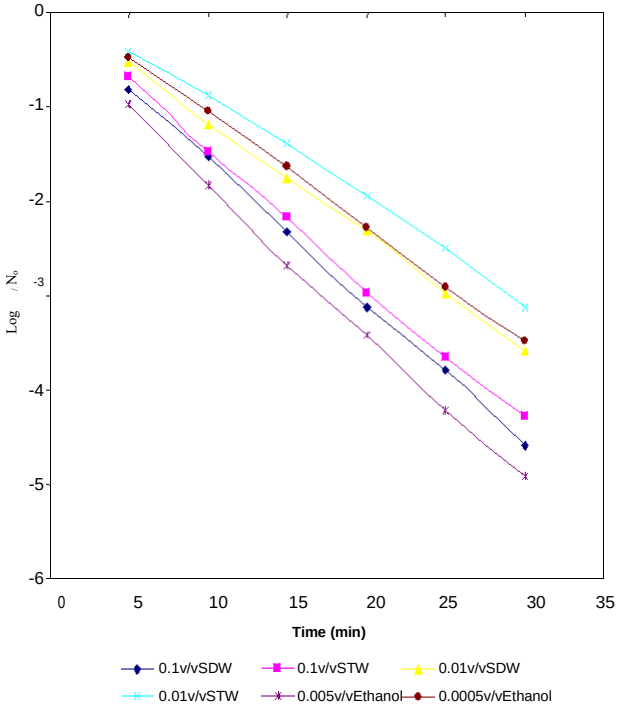

Figure 3b. Effects of used dilutions of chlorhexidine gluconate on the viability of *C. albicans* (CA2) in various diluents incubated at 37^{o}

pensions. For determination of MBC, two loopfuls of broth cultures of each of the test organisms were collected from the MIC tubes that did not show any turbidity after the 24 h period of incubation at 37ºC and inoculated on Nutrient agar (NA, Oxoid) (for *E. coli* and *S. aureus*) and Saboraud Dextrose Agar (SDA) plates (for *C. albicans*). The culture plates were then incubated at 37ºC for 48 h and observed for growth (Waterworth, 1978; Baldry, 1984).

RESULTS AND DISCUSSION

Table 1 showed the proportion of the isolates surviving after 10min and 30min of contact with use dilutions of hibitane®. For *S. aureus* (SA1), only 0.7% survived in SDW and 0.9% in STW for the 1:10 aqueous dilution, while 1.6% survived in SDW and 3.6% in STW for the 1:100 aqueous dilution. For the alcoholic dilution of 1:200, the number of cells declined to 0.30% and 2.6%for the 1:2000 alcoholic dilution. For *S. aureus* (SA1), 0.002% survived in SDW and 0.003% survived in STW for the 1:10 and 0.007% survived in SDW and 0.002% in STW for the 1:100 aqueous use-dilutions of hibitane. From the results only 0.01% of the test organisms survived in the 1:200 and 0.0005% in the 1:2000 alcoholic use- dilutions (Table 1). This pattern of loss of viability was similar for the other 5 organisms. The results also revealed that the number of cells decreased gradually in such a manner that the logarithm of the number of surviving cells plotted against time falls on a descending straight line with a negative slope (Figure 1a and b). This agrees with earlier reports of Esselen and Pflug, (1956); Acheampong et al. (1988) and El-mahmood (2006) on other pathogenic bacteria. This might mean that there were no subpopulation of cells resistant to the disinfectant hibitane® in both sets of resistant and sensitive test organisms. The result is however contrary to results of studies conducted on another disinfectant benzoyl peroxide against some cutaneous nosocomial pathogens (Cove and Holland, 1983). However, the increase in death rates when sterility was approaching may be due to the difficulty in detecting low number of viable cells and or due to carry over of the agents into the recovery medium since the excess amount increases the concentration of the agent in the medium beyond the level that can be tolerated by the pathogens.

The effect of the various use-dilutions of hibitane® on the viability of the organisms were shown in Figure 1a (*S. aureus* SA1), Figure 1b (*S. aureus* SA2), Figure 2a (*E. coli* EC1), Figure 2b (*E. coli* EC2), Figure 3a (*C. albicans* CA1) and Figure 3b (*C. albicans* CA2). The curves were plotted as log Nt/No against time ($p < 0.05$; using two tailed t test, SPSS). Extrapolation of the graph to the log Nt/No axis gives the extrapolation numbers. All the curves exhibited some initial shoulders before the exponential order of death depending on the use-dilutions. In all the graphs, the organisms exhibited a lag (L) phase, the duration of which depended on the concentration of hibitane. This suggests that such a concentration of hibitane® had no immediate lethal effects

Table 2. Death rates (K-min-1) of the organisms treated with ethanolic (ET) and aqueous use-dilutions of hibitane® (chlorhexidine gluconate) in various diluents.

Conc(v/v)	Death Rates (K-min^{-1})																	
	SA1			SA2			EC1			EC2			CA1			CA2		
	ET	SDW	STW	ET	SDW	STW	ET	SDW	STW	ET	SDW	STW	ET	SDW	STW	ET	SDW	STW
1:10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		0.03	0.30		0.32	0.28		0.30	0.30		0.30	0.30		0.32	0.31		0.37	0.35
1:100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		0.26	0.26		0.24	0.22		0.27	0.26		0.26	0.24		0.31	0.25		0.26	0.25
1:200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.29			0.35			0.30			0.31			0.34			0.36		
1:2000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.26			0.26			0.29			0.27			0.26			0.28		

Key; N = number of viable cells; % = percentage of number of viable cells; SA1 = S. aureus; SA2 = S. aureus; EC1 = E. coli; EC2 = E. coli; CA1 = C. albicans; CA2 = C. albicans; SDW = sterile deionized water; STW = sterile tap water ET = Ethanol; - = not carried out.

Table 3. Slope (M) (min) of the curves and decimal reduction time (DRT) (min) of the organisms treated with use-dilutions of hibitane® (chlorhexidine gluconate) in various diluents.

Conc. (v/v)	DM	SA1		SA2		EC1		EC2		CA1		CA2	
		M	DRT	M	DRT	M	DRT	M	DRT	M	DRT	M	DRT
1:10	SDW	-0.129	7.75	-0.137	7.30	-0.128	7.81	-0.128	7.81	-0.139	7.19	-0.158	6.33
	STW	-0.129	7.75	-0.142	7.04	-0.135	7.41	-0.132	7.85	-0.135	7.41	-0.150	6.67
1:100	SDW	-0.113	8.85	-0.106	9.43	-0.116	8.62	-0.112	8.93	-0.136	7.35	-0.115	8.70
	STW	-0.108	9.26	-0.097	10.31	-0.112	8.93	-0.106	9.43	-0.110	9.09	-0.106	9.43
1:200	ET	-0.127	7.87	-0.152	6.58	-0.130	7.69	-0.133	7.52	-0.146	6.85	-0.158	9.33
1:2000	ET	-0.114	8.77	-0.111	9.01	-0.124	8.07	-0.118	8.48	-0.111	9.01	-0.123	8.3

Key; DM = dilution medium; SA1 = *S. aureus*; SA2 = *S. aureus*; EC1 = *E. coli*; EC2 = *E. coli*; CA1 = *C. albicans*; CA2 = *C. albicans*; SDW = sterile deionized water; STW = sterile tap water ET = Ethanol; N/A = not applicable.

on the cells, probably due to low concentration of the disinfectant (Figure 1 - 3). The presence of the lag phase (shoulders) in the microbicidal concentration of the disinfectant benzoyl per-oxide has earlier been reported on other patho-gens (Meynell and Meynell, 1970; Cove and Holland, 1983; Acheampong et al., 1988) . The presence of the shoulders was attributed by these workers to the non uniform distribution of these cells as single cells, but rather as clumps. However, the extremely low value of the shoulder at the lower use- dilutions of hibitane® especially for the sensitive pathogens *S. aureus* (SA2), *E. coli* (EC2) and *C. albicans* (CA2) in SDW and ethanol did not support the suggestions of these researchers (Figure 2a and 2b)

Knowledge of the kinetics of loss of viability of a microbial population treated with antimicrobial

Table 4. Lag (L) (min) and the log extrapolation numbers (E) (min) of use-dilutions of hibitane® (chlorhexidine gluconate) against the organisms in the various diluents.

CONC (V/V)	DM	Lag and the log extrapolation number											
		SA1		SA2		EC1		EC2		CA1		CA2	
		L	E	L	E	L	E	L	E	L	E	L	E
1:10	SDW	1.63	0.20	1.14	0.20	1.63	0.30	1.46	0.20	0.96	0.10	0.56	0.06
	STW	2.63	0.30	1.64	0.25	2.64	0.40	2.13	0.30	1.66	0.20	0.95	0.10
1:100	SDW	3.62	0.40	4.11	0.40	3.60	0.45	3.11	0.45	2.14	0.30	1.31	0.20
	STW	5.11	0.50	5.60	0.55	6.63	0.75	5.62	0.65	4.11	0.50	2.13	0.30
1:200	ET	1.13	0.15	0.65	0.10	1.46	0.20	0.63	0.08	0.55	0.06	0.36	0.04
1:2000	ET	5.11	0.50	5.11	0.45	5.12	0.55	4.10	0.55	3.60	0.45	2.13	0.30

Key: DM = Dilution Medium, ET = Ethanol, L = Lag and E= log Extrapolation number; SA1 = *S. aureus*; SA2 = *S. aureus*; EC1 = *E. coli*; EC2 = *E. coli*; CA1 = *C. albicans*; CA2 = *C. albicans.*

agents had been used to predict and control disinfection and sterilization procedures (Hugo, 1967) . The ability of microorganisms to develop resistance to agents whose action is broad spectrum is limited.

However agents whose action is limited to interference with a single point in the biosynthetic pathway in a cell may be rendered less effective if by one or another of the processes of adaptation, an alternative route for the formation of the inhibited end product can arise (Hugo, 1967; Brown et al., 2003). Variations in the use-dilutions of hibitane® and the type of diluents used affected the kinetics of cell death with respect to the length of the shoulder, the DRT and the gradient of the killing curves. Garnett and Brown (1964) had reported that there was no single concentration of an antimicrobial agent at which all the cells in a suspension would be killed instantaneously. The process of death occurs chiefly as a function of time within a range of concentration. Cove and Holland (1983) suggested that for a complete killing of the cells in a suspension, a sufficiently high concentration of the disinfectant molecule must be in contact with the cells for a time longer than the shoulder before exponential order of death. In this study, ethanolic dilutions (MIC 0.00006 - 0.00016) have shorter shoulders closely followed by SDW (MIC 0.00009 - 0.00024), then STW (0.0012 - 0.0026). The results showed that *E. coli* (MBC 0.00008- 0.0003) is least susceptible, closely followed by *S. aureus* (MBC 0.00007 - 0.00026) and then *C. albicans* (MBC 0.00006 - 0.00022) (Table 5).

The effectiveness of hibitane® was not affected in the presence of tap water and serum, though decreased to a lesser degree compared to those of ethanol and SDW. It has earlier been reported that organic matter such as serum and presence of impurities in tap water, cell concentration and nature and the type of organism (cell wall composition) has been reported to reduce the effectiveness of antimicrobial agents, hence affecting the MBC value (Bean, 1967; Hugo, 1967; Gelinas and Gaulet, 1983; Lynn and Hugo, 1983). Table 2 shows the rates of deaths of the organisms treated with various concentrations of used dilutions of the disinfectant. Result shows that the rate of death of *S. aureus* (SA1) was faster (-0.032 and -0.26) in SDW than in STW (-0.30 and -0.25) for the 1:10 and 1: 100 aqueous use-dilutions respectively. For the ethanolic used- dilution, the death rate was slower (-0.29) for the 1:200 and but slightly faster (-0.26) for the 1:2000 use-dilutions. A similar pattern of rates of death was observed for all the other 5 organisms.

The slopes (M) of the curves and the Decimal Reduction Times (DRT) of the cell cultures are shown in Table 3. For *S. aureus* (SA1), at 1:10 aqueous use-dilution, the M was -0.129 and DRT 7.75 min in SDW and M -0.127 and DRT 7.75 min in STW. For the 1:100 use-dilutions, M was -0.113 and DRT 8.85 min in SDW, while M was - 0.108 and DRT 9.26 in STW. For the alcoholic dilutions of 1:200, M was -0.127 and DRT 7.87 min, while for the 1:200 ethanolic dilutions, M was -0.114 and DRT was 8.77 min. The shoulder (L) and log10 extrapolation number (E) of the cultures are shown in Table 4. For *S .aureus* (SA1), the L was 1.63 min and E was 0.20 in SDW, and L was 2.63 min and E was 0.30 in STW for the 1:10 aqueous use-dilution. While for the 1:100 aqueous use-dilutions, L was 0.3.62 min and E was 0.40 in SDW and L was 5.11 min and L 0.50 in STW. For the ethanolic use-dilutions, L was 1.13 min and E 0.15 for the 1:200 dilution, while L was 5.11 min and E 0.50 for the 1: 2000 use-dilution. This trend was the same for the other 5 organisms. The MBC values of the disinfectant (hibitane) in the various dilution mediums (STW, SDW and ET) at inoculum densities of $1x10^3$ and $1x\ 10^7$ cells/ml are shown in Table 5. The result showed that in all the various use-dilutions, SDW demonstrated the least value while the 10 % serum alcoholic use dilutions demonstrated the highest MBC values.

Conclusion

Hibitane® in this study had demonstrated rapid microbicidal properties against *E. coli, S. aureus* and C*. albicans*, all of which are very important nosocomial

Table 5. Minimum bactericidal concentration values of hibitane® (chlorhexidin gluconate) against the organisms.

Organism	Dilution medium	Inoculum size $1.0x10^3$ cells ml^{-1}	Inoculum size $1.0x10^7$ cells ml^{-1}
SA1	SDW	0.00016	0.00018
	STW	0.00018	0.00022
	10% Serum	0.0002	0.00026
	95% Ethanol	0.0008	0.00014
	95% Ethanol plus 10% Serum	0.0001	0.00018
SA2	SDW	0.0001	0.00016
	STW	0.00012	0.00018
	10% Serum	0.00016	0.00020
	95% Ethanol	0.00005	0.00010
	95% Ethanol plus 10% Serum	0.00007	0.00013
EC1	SDW	0.00018	0.00024
	STW	0.0002	0.00026
	10% Serum	0.00022	0.00030
	95% Ethanol	0.00013	0.00016
	95% Ethanol plus 10% Serum	0.00016	0.00020
EC2	SDW	0.00012	0.00020
	STW	0.00014	0.00022
	10% Serum	0.00016	0.00024
	95% Ethanol	0.00006	0.00012
	95% Ethanol plus 10% Serum	0.00008	0.00014
CA1	SDW	0.00014	0.00020
	STW	0.00016	0.00020
	10% Serum	0.00018	0.00022
	95% Ethanol	0.00012	0.00013
	95% Ethanol plus 10% Serum	0.00009	0.00016
CA2	SDW	0.00009	0.00014
	STW	0.00012	0.00016
	10% Serum	0.00014	0.00018
	95% Ethanol	0.00014	0.00010
	95% Ethanol plus 10% Serum	0.00006	0.00012

Key; SA1 = *S. aureus*; SA2 = *S. aureus*; EC1 = *E. coli*; EC2 = *E. coli*; CA1 = *C. albicans*; CA2 = *C. albicans*; SDW = sterile deionized water; STW = sterile tap water.

pathogens, the three most important pathogens that frequently show above average resistance to many antibiotics, antiseptics and disinfectants. From the results of this study therefore, it is recommended that either the alcohollic or the lower use aqueous use-dilution should be used as diluents in hospitals instead of potable tap water since microorganisms are not usually found in pure culture but enveloped in proteinaceous material like serum. Further studies however needs to be carried out in order to investigate the mechanism of action hibitane against *E. coli, S. aureus, C. albicans* and other group of nosocomial pathogenic microorganisms.

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