Effect of selected disinfectants on biofilm-forming clinical isolates of *Staphylococcus aureus* in Lagos State, Nigeria

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Summary

Background and Aims: Staphylococcus aureus is one of the most important pathogens of public health concern and a leading cause of nosocomial infections. In this study, we evaluated the effect of routinely used disinfectants in hospitals for surface decontamination on biofilm-forming *S. aureus*.

Materials and Methods: forty-eight *S. aureus* isolates were phenotypically evaluated for biofilm formation using the Tissue Culture Plate (TCP) technique. Effect of disinfectants (Dettol®, Izal®, Jik® and Savlon®) on biofilm was tested and time-kill kinetics evaluated. PCR was used to confirm the identity of *S. aureus* using species-specific primers.

Results: biofilm formation assay revealed that 15 (31.2%) of the isolates formed biofilm with 7 (14.5%) and 8 (16.6%) considered as strong and moderate biofilm formers, respectively. Biofilm formation was time-dependent ($p<0.0001$). Jik® was significantly effective ($p<0.0001$) as it disrupted biofilm formed in all 15 (100%) isolates, followed by Izal® 13 (86.6%), Savlon® 11 (73.3%) and Dettol® 9 (60%). Time-kill kinetics of the four disinfectants revealed Dettol®, Jik® and Savlon® achieved total (100%), (7 $\log_{10}$) lethality against isolates within 1 h contact time while Izal® attained complete lethality at 6 h contact time.

Conclusions: of the four disinfectants evaluated Jik®, a chlorine-based formulation, was more effective in destroying biofilm-forming *S. aureus*. The need to use effective disinfectants in sanitization is imperative to facilitate the control and prevention of hospital and community-acquired infections.

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Introduction

Staphylococcus aureus is the etiology of numerous infections, ranging from minor infections of the skin to serious postoperative wound infections, bacteremia, and necrotizing pneumonia [20]. The skin and nasopharynx are the major sites of colonization, and *S. aureus* is also known to be implicated in local infections of the nose, urethra, vagina, and gastrointestinal tract. This bacterium colonizes and infects hospitalized patients as well as healthy individuals in the community. *S. aureus* capacity to breach the host immune response and cause disease is attributed to an extensive repertoire of both known and unknown virulence factors, including efflux pump activity, biofilm formation ability, *etc*. [10]. Efflux pumps are largely conserved in bacteria for self-defense and can be a potential target for effective antimicrobial therapy to treat infectious diseases caused by multidrug-resistant bacteria. Active extrusion of antibiotics and other substances toxic to the cell is well known to be a successful resistance mechanism deployed by various antibiotic-resistant bacteria, including Methicillin-Resistant *S. aureus* (MRSA), to survive the deleterious effect of antimicrobials [8,22]. The survivability of *S. aureus* to the selective pressure of antimicrobials and its implications in the emergence and spread of nosocomial infections has also been attributed to the survival strategy of colonization at the surfaces and growth as biofilm communities embedded in a gel-like polysaccharide matrix [22,18]. In order to control infectious diseases and prevent transmission of infec-

tious pathogens from contaminated surfaces and medical equipment to patients, disinfectants which are agents that kill or inhibit the growth and development of microorganisms are routinely employed in disinfection [3,23,21]. *S. aureus* is one of the most problematic pathogens, being the second most common pathogen that causes nosocomial infection, and special attention has been given to surface disinfection in order to curb its transmission from surfaces in hospital environment [9].

S. aureus grows and form biofilms on surfaces and medical devices producing an extracellular polymeric matrix that provides coverage to the embedded cells against adverse conditions including tolerance to disinfectants. The bactericidal efficacy of disinfectants on biofilms is much lower compared to the efficacy of the same disinfectants against planktonic cells [4]. In Nigeria, a number of disinfectants are routinely used in houses and hospitals for hygienic purposes. However, oftentimes some disinfectants are adulterated and their efficacy compromised. This current study evaluates the effectiveness and time-kill kinetics of four disinfectants routinely used in tertiary hospitals in Lagos State, Nigeria for disinfection on biofilm-forming *S. aureus* isolates.

Materials and Methods

Bacterial strains

Forty-eight *S. aureus* isolates were obtained from stock at the Molecular Epidemiology Laboratory at Molecular Biology and Biotechnology Department, Nigerian Institute of Research (NIMR), Yaba, Lagos State, Nigeria. These isolates were previously isolated from the urine samples of non-pregnant women, within the age range of 20-50 years, presenting with urinary tract infection in tertiary hospitals in Lagos State [22]. Isolates were inoculated into 2 mL of freshly prepared Brain Heart Infusion (BHI) (Oxoid, Basingstoke, UK) broth and incubated at 37°C for 24 h for resuscitation. Isolates were confirmed to be *S. aureus* by streaking bacterial broth culture onto Mannitol Salt Agar (MSA) (Himedia, Mumbai, India) plates and incubated at 37°C for 24 h. This was followed by standard biochemical tests, including Gram, coagulase, catalase, oxidase, urease, DNase, and novobiocin susceptibility, as described by Cheesbrough [6], and molecular characterization by PCR using *S. aureus* species-specific primers Sa-fib F- 5'-AATTGCGTCAACAGCAGATGCGAG-3'and Sa-fib R-5'-GGACGTGCACCATATTCGAATGTACC-3' [24]. A 20 µL reaction containing 4µL (5x) FIREPol master mix (7.5 mM $MgCl_2$, 1 mM dNTPs, 0.4M Tris-HCl, 0.1M (NH4)2SO4, 0.1% Tween-20, FIREPol DNA Polymerase) (Solis BioDyne, Tartu, Estonia), 0.6µL forward primer, 0.6µL reverse primer, 4 µL DNA template and 10.8 µL nuclease-free water was used. Polymerase Chain Reaction (PCR) cycling parameter was initial denaturation at 95°C for 5 minutes and 30 cycles of denaturation at 95°C for 30 seconds, annealing temperature at 58°C for 40 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 10 minutes. PCR was carried out in a Master cycler Vapor Protect thermo cycler (Eppendorf AG, Hamburg, Germany). The PCR products were loaded on a 1.5% agarose gel stained with ethidium bromide and electrophoresed in a 0.5 x Tris Borate EDTA (TBE) at 100v for 60 minutes. It was run in parallel with a 100 bp ladder molecular weight marker (Solis BioDyne). After electrophoresis, gels were viewed under a UV transilluminator fitted with a camera (Cleaver Scientific Ltd., Rugby, UK).

Selection of disinfectants

Four registered disinfectants assayed in this study were purchased from the University of Lagos Pharmacy and included Dettol® (chloroxylenol) (Reckitt Benckiser Nigeria Ltd., Agbara, Nigeria), Izal® (phenol) (Nath Peters Hygeian Ltd., Andhra Pradesh, India.), Savlon® (chlorhexidine gluconate) (Johnson & Johnson (Pty) Ltd., London, UK) and Jik® (Hypochlorite) (Reckitt Benckiser (Nigeria) Ltd.). The disinfectants were selected based on wide acceptability and frequency of use in the hospitals.

Evaluation of biofilm formation by the Tissue Culture Plate Method

Quantitative determination of biofilm formation was performed according to Christensen *et al*. [7] with slight modification by incubating at two different temperatures (25°C and 37°C) for two different time intervals (24 hours and 48 hours). To evaluate biofilm-forming potential, the bacteria isolates were cultured in Brain Heart Infusion (BHI) broth and incubated for 24 h at 37°C. The bacterial culture was then diluted (1:100) in fresh BHI broth. Sterile broth served as a negative control, while *S. aureus* ATCC 29213 and *S. aureus* ATCC 35556 served as biofilm-negative and biofilm-positive control, respectively. The wells of a 96 -microtiter plate were then filled with 0.2 mL of the diluted culture and incubated for 24 h at 25°C, 48 h at 25°C, 24 h at 37°C and 48 h at 37°C. The wells were washed 3 times with distilled water, dried in an inverted position, and stained with 0.5% (p:v) crystal violet solution. The adherent cells were resuspended in 95% glacial acetic acid (33% v/v) solution and the absorbance measured at wave length 620 nm using an ELISA auto-reader (EZ reader 400; Biochrom, Holliston, USA). The experiment was performed in triplicates. The average OD values of the sterile medium were calculated and subtracted from all test values [19]. The results were interpreted, and data obtained was used to classify biofilm formation into three categories: a) non-adhering, with an optical density less than 0.120; b) moderately adhering, with an optical density greater than 0.120, but less than or equal to 0.240, and c) strongly adhering, with an optical density greater than 0.240.

Effects of disinfectants on biofilm formation

Evaluation of the effect of disinfectants on biofilm formation was performed according to Kara *et al*. [16] with slight modification in the duration of incubation of isolates. Fifteen isolates that showed consistency in biofilm formation were selected for treatment with disinfectants. After the formation of a 24 h young biofilm by the Tissue Culture Plate (TCP) technique, the 96-well microplate was rinsed 3 times with sterile distilled water and dried. Then, 0.2 mL of the disinfectants; Dettol®, Izal®, Savlon®, and Jik®, diluted as described by EL Mahmood and Doughari, [11] was added to the biofilm. The microplate was incubated for 24 h and 48 h contact time at 25°C. After incubation, the wells of the microplate were carefully rinsed, dried, and stained with crystal violet according to the standard technique. The Optical Density (OD) was measured at 620 nm by the ELISA auto reader (EZ reader 400; Biochrom).

Time-kill assay of disinfectants on planktonic *S. aureus*

S. aureus isolate A58 was used for time-kill assay since the biofilm formed by the isolate was significantly disrupted by all disinfectants. Time-kill assay was performed as described by White *et al.* [29] and Aiyegoro *et al.* [2] with some modifications

regarding the counting of viable cells. One hundred microlitres (0.1 mL) of each of the four disinfectants diluted according to EL Mahmood and Doughari [11] were individually dispensed into 0.9 mL Mueller Hinton Broth (MHB) (Oxoid, Basingstoke, UK). Test tubes of MHB without disinfectants were used as growth controls. Inoculum suspensions with approximately $1.5x10^8$ CFU/mL (0.5 McFarland Standard) of exponentially growing bacterial cells were used to inoculate 0.1 mL volumes of both test and control tubes. The cultures were then incubated in a Grant GLS400 shaker (Grant Instruments, Cambridge, England) at 37°C for 1, 2, 4, 6, and 24 h. After each interval, ten-fold serial dilutions were prepared with Phosphate-Buffered Saline (PBS), and 0.1 mL samples were pipetted onto Mueller Hinton Agar (MHA) (Oxoid) plates in duplicate. Colony counts were performed after 18h incubation at 37°C. Plates with 30-300 colonies were used for these counts, and the kill rate was determined using the Log reduction formula:

Log reduction = $\log_{10}$

(Where A, Baseline count CFU/mL and B, count after test (reduction after test) CFU/mL)

Time-kill profile was evaluated by plotting $\log_{10}$ viable counts (CFU/mL) against time. Bactericidal activity was defined as a $\geq 3\log_{10}$ decrease in CFU/mL of the initial microbial population, while bacteriostatic activity was defined as a $<3\log_{10}$ decrease in CFU/mL.

Statistical analysis

Data generated was entered into Graphpad Prism 8.0 (GraphPad Software, La Jolla, CA, USA), and Analysis Of Variance (ANOVA) was used in comparing means. A p-value <0.05 was considered as significant.

Results

Biofilm forming potentials of isolates

The result of the biofilm formation is shown in Table 1. Of the 48 *Staphylococcus aureus* isolates, 19 (40%) were biofilm formers, which comprised 7 strong biofilm formers and 12 moderate biofilm formers after incubation at 25°C for 24 h. Twenty-nine (60%) of them did not form biofilm at 25°C for 24 h. However, after 48 h at the same incubation temperature (25°C), the number of biofilm formers increased as 31 (65%) of the isolates formed biofilm, which comprised 19 (40%) strong biofilm formers, 12 (25%) moderate biofilm formers and 17 (35%) non-biofilm formers. After incubation at 37°C for 24 h, it was observed that 24 (50%) of the isolates formed biofilm, which comprised 10 (21%) strong biofilm formers, 14 (29%) moderate biofilm formers, and 24 (50%) non-biofilm formers. Incubation for 48 h at 37°C showed that 33 (69%) of the isolates formed biofilm, of which 14 (28%) were strong biofilm formers, 19 (40%) were moderate biofilm formers and 15 (31%) were non-biofilm formers. At 48 h, isolates that formed biofilm increased at both incubation temperatures.

Duration of incubation had a significant (p<0.0001) effect on biofilm formation. There was an increase in the number of strong biofilm formers both at 25°C and 37°C incubation temperature from 24 hours to 48 hours. However, there was no significant influence (p=0.3510) of temperature on biofilm forming potential of the isolates.

Effects of disinfectants on biofilm formation by *S. aureus*

The effect of the disinfectants on *S. aureus* biofilm is shown in Figure 1. Overall, Jik® was more effective against biofilm formed by all 15 (100%) isolates at both incubation durations (24 hours and 48 hours) at temperatures 25°C, resulting in a significant reduction in OD. This was followed by Izal®, Savlon® and Dettol® that disrupted biofilm formed by 13 (86.6%), 11 (73.3%) and 9 (60%) isolates, respectively. All biofilms formed by *S. aureus* isolates were significantly (p<0.0001) destroyed by Jik®. Biofilm formed by isolates A24 and A75 were significantly (p<0.0001) destroyed by all disinfectants except Dettol® that had a p-value of 0.5732, while biofilm formed by isolates A14 and A30 were significantly (p<0.0001) destroyed by only Dettol® and Jik®. However, biofilm formed by isolates A3, A59, and A66 showed resistance to all disinfectants except Jik®.

Time-kill kinetic of disinfectants on biofilm-forming *S. aureus*

Time-kill kinetic of the four disinfectants (Dettol® Izal®, Savlon® and Jik®) against biofilm forming *S. aureus* isolate A58 (one of the isolates which had biofilm significantly (p<0.0001) disrupted by all disinfectants) and log reduction of survivor cells are presented in Figure 2. The increase in the population of the control group, as shown by the number of viable counts, indicated that the isolate was exponentially growing from 1-24 h. Time-kill assay of isolate A58 at 1h contact time revealed that Dettol®, Savlon®, and Jik® demonstrated the highest bactericidal ($7\log_{10}$) effect, resulting in total lethality (100% reduction of survivor cells) of isolate. While at 1 h contact time, Izal® (phenol compound) showed a bacteriostatic ($0\log_{10}$) effect on the isolate with an 11.0% reduction of survivor cells but eventually demonstrated progressive lethality from 2-4 h (47.7%-67.7%) contact time and achieved total lethality $7\log_{10}$ (100%) at 6 h.

Discussion

Biofilm formation is a major factor that enhances the survival of bacterial pathogens on surfaces, thereby facilitating their transmission. Biofilm formation by *S. aureus* not only enhances persistence and virulence, but also mediates antibiotic resistance, which makes the infections they cause difficult to treat and manage [27]. In this study, a considerable number of *S. aureus* isolates were biofilm formers characterized by moderate and strong biofilm formers.

Table 1. Biofilm forming potential of *S. aureus* isolates at different incubation time and temperature.

Biofilm forming potential	Incubation time and temperature			
	24 h/25°C	48 h/25°C	24 h/37°C	48 h/37°C
SBF	7 (17%)	19 (40%)	10 (21%)	14 (28%)
MBF	12 (25%)	12 (25%)	14 (29%)	19 (40%)
NBF	29 (60%)	17 (35%)	24 (50%)	15 (31%)

SBF, strong biofilm formers; MBF, moderate biofilm formers; NBF, non-biofilm formers.

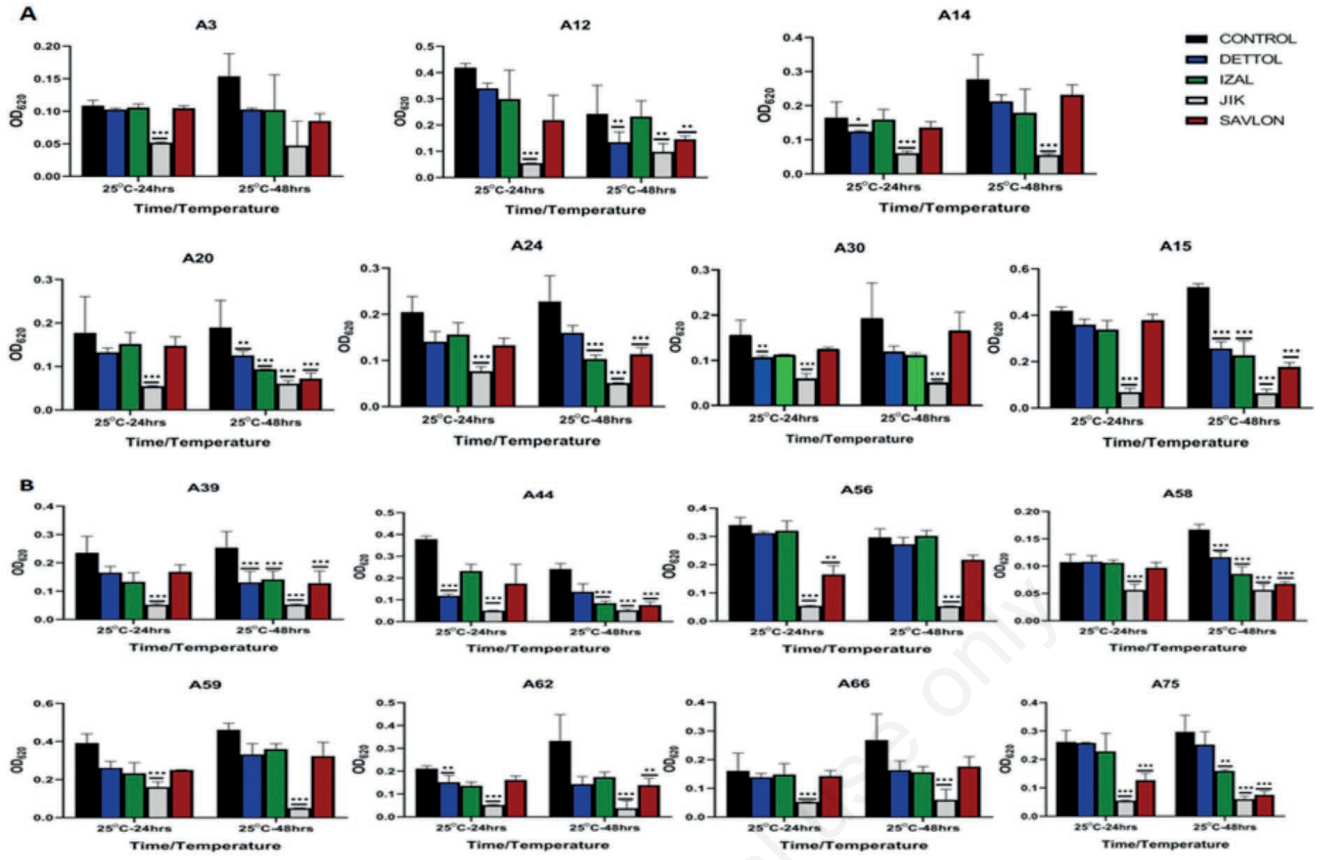

Figure 1. Effect of disinfectants on biofilm formed by *S. aureus* isolates. The effect of Jik® on biofilm formed by all the isolates was significant (***p<0.0001).

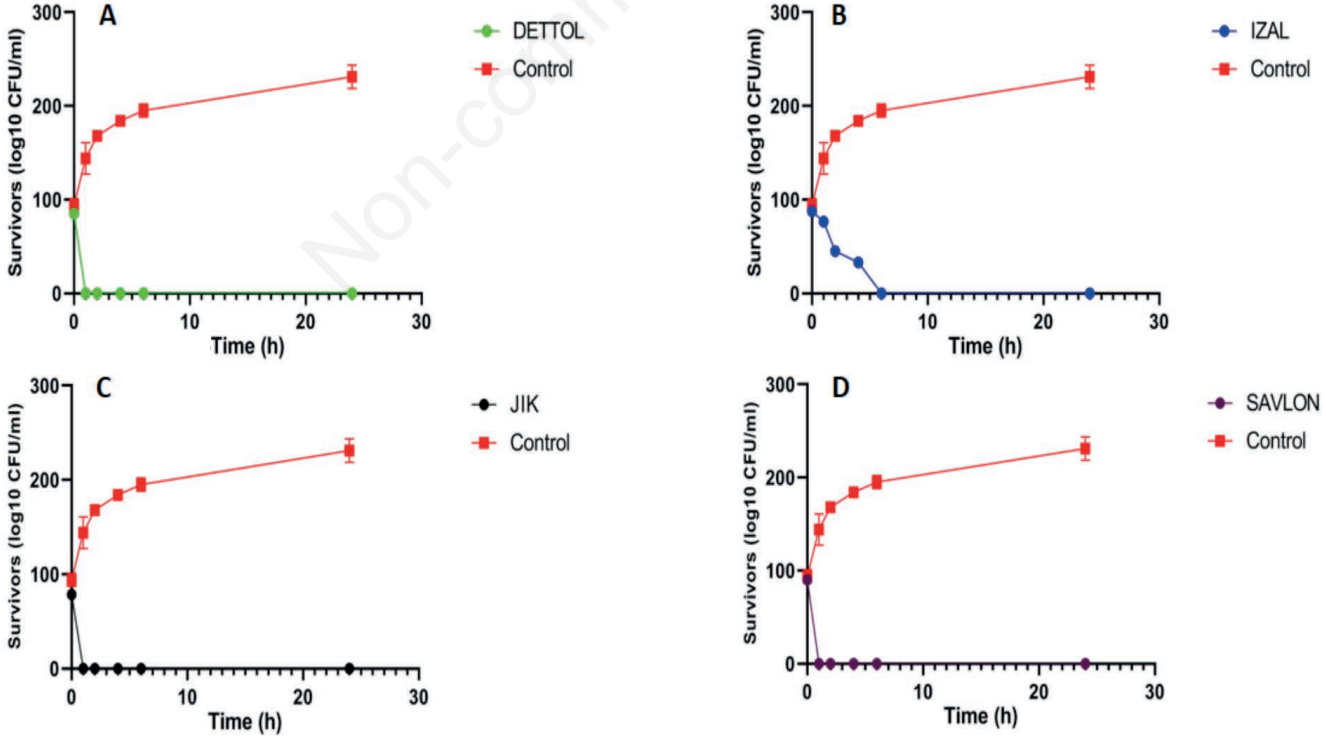

Figure 2. Time-kill profile for *S. aureus* in Mueller-Hinton broth during treatment with A) Dettol®, B) Izal®, C) Jik®, D) Savlon®. Dettol®, Savlon®, and Jik® had a total lethality of $7\log_{10}$ within 1 h contact time with isolate, while Izal® achieved a total lethality of $7\log_{10}$ at 6 h.

Abdulrahim *et al.* [1] similarly, reported moderate and strong biofilm-forming *S. aureus* strains isolated from clinical samples, including urine, at the National Orthopedic Hospital in Kano, Nigeria. In Hungary, *S. aureus* isolates from different healthcare facilities were reported by Tahaei *et al*. [25] to be biofilm formers. The prevalence of biofilm-forming *S. aureus* pervading clinical samples indicates the high risk of hospital environments being contaminated and serving as a portal for onward transmission to patients and hospital personnel.

Infection prevention and control has environmental cleaning as a pivotal strategy [5]. The use of disinfectants in the routine cleaning of the hospital environment is a global practice. However, the effectiveness of disinfectants used could be compromised by biofilm formation. There is a paucity of information on the effectiveness of routinely used disinfectants on biofilm-forming *S. aureus* in Nigeria. Our study revealed that Jik® (Sodium hypochlorite) was the most potent disinfectant, showing a significant disruption ($p<0.0001$) of *S. aureus* biofilm than Izal® (Phenolic compound), Dettol® (Chloroxylenol) and Savlon® (Chlorhexidine Gluconate and Cetrimide). In contrast to the report of Iniguez-Moreno *et al*. [14], peracetic acid was more effective against biofilm-forming *S. aureus* and *Salmonella spp*. isolates compared to sodium hypochlorite tested in Brazil. Although no peracetic acid formulation was tested in this study, chlorine-based disinfectants such as sodium hypochlorite have been reported to be more potent. Lineback *et al*. [17] reported hydrogen peroxide and sodium hypochlorite disinfectants to have significantly higher bacteriocidal effects against biofilm-forming *S. aureus* and *Pseudomonas aeruginosa* isolates. The mechanism of action of sodium hypochlorite is not known in entirety, as a strong oxidizing agent, it has the ability to interfere with numerous structural and functional components of the cell wall integrity and metabolic activities of bacteria [26]. The loss of activity/potency of sodium hypochlorite can be attributed to the release of hypochlorous acid on exposure to light during product formulation, packaging or storage. The low bacteriocidal efficacy of Dettol® observed in this study is in line with Oleghe *et al.* [21] who reported a moderate bacteriocidal efficacy of Dettol® in Edo, Nigeria. Like phenol, chloroxylenol (Dettol®) is a membrane-active agent that, when adsorbed into the biofilm, depending on the quantity adsorbed, results in depletion of the biofilm, inhibition of growth and metabolic activities or loss of viability [13].

Time-kill kinetics of the four disinfectants revealed Dettol®, Jik®, and Savlon® showed the highest bactericidal with $7\log_{10}$ reduction of planktonically growing *S. aureus* isolates and achieved total lethality (100%) within 1 h contact time. This correlates with the findings of Eyo *et al.* [12] and Inyang *et al.* [15] both reported total kill of bacterial isolates studied within 1 h contact time by Dettol®, Jik®, and Savlon®. However, Izal® (Phenolic compound) achieved complete lethality with $7\log_{10}$ reduction at 6 h contact time. This finding shows a correlation with the report from a previous study carried out by Uchejeso [28], which revealed Izal® attained total lethality after 12 h contact time. Apart from factors such as poor storage condition and method of application, interference of components of broth with the active chemical of disinfectants, blockade of adsorption site necessary for disinfectant activity *etc.* could be responsible for the less rapid lethality of isolate associated with Izal® in this study.

Limitations

This study did not determine the level of adhesion and structure of *S. aureus* biofilms and the effect of treatment on the structure using scanning electron microscopy, which could be considered as a limitation of the study. However, data available from the study can help inform policy.

Conclusions

Of the four disinfectants evaluated Jik®, a chlorine-based formulation, was more effective in destroying biofilm-forming and planktonically growing *S. aureus*. The need to use effective disinfectants in sanitization is imperative to facilitate the control and prevention of hospital and community-acquired infections.

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