

# Effect of Chlorhexidine and Sodium Hypochlorite on *Staphylococcus aureus* Biofilm

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## Abstract

Biofilm is a matrix in which a microorganism encases in it and survives environmental stresses. It help the organisms to resist the antibiotics and disinfectant, chronic biofilm associated infection lead to significant increase in morbidity and mortality especially patient with indwelling medical devices. The objective of this research was to analyze the effectiveness of chlorhexidine, sodium hypochlorite and antimicrobial activity of methicillin and vancomycin against biofilm of isolated strains of *Staphylococcus aureus* isolated from different clinical samples. The results revealed that most biofilm strains were sensitive to vancomycin, some strains were sensitive some were moderate resist and some were resist to methicillin. In comparing different concentrations (0.3%, 0.2%, 0.15% and 0.075%) of chlorhexidine among time interval (1 min, 3 min and 5 min) concentrations showed significant decrease in biofilm formation in association with time; P value (0.001, 0.001, 0.000 and 0.000, respectively). Different concentrations (5%, 4%, 2.5% and 1.25%) of sodium hypochlorite also tested through the same time intervals; concentrations showed significant decrease in biofilm in association with time; P value (0.000, 0.000, 0.000 and 0.000, respectively).

**Keywords:** *Staphylococcus aureus*; Biofilm; Chlorhexidine; Sodium hypochlorite; Congo red agar.

## Introduction

A biofilm are cells stick to a surface or other cells and produce matrix. This matrix is composed of extracellular polymeric substances (EPS) which is: extracellular DNA, protein, polysaccharide and host factors [1], which encase the cells within the sticky matrix and facilitate living in extreme environments. Chronic biofilm associated infection caused by *Staphylococcus aureus* often lead to significant increase in morbidity and mortality particularly when associated with medical indwelling device. It causes chronic wound infection, chronic urinary tract infection (UTI), cystic fibrosis pneumonia, chronic otitis media, chronic rhinosininitis, osteomyelitis, periodontitis, and recurrent tonsillitis [2]. Biofilm formation allow non-spore forming soil bacteria to colonize surrounding habitat and to survive common environmental stresses as

nutrition limitation [3]. Biofilm development can be divided into three stages: attachment of the cells to a surface, growth of the cells into a sessile biofilm colony, detachment of cells from the colony into the surrounding media [4].

Disinfectant are chemical agents used to inactivate all recognized microorganisms, the mode of action of disinfectant depend on biocide used, potential target sites in Gram positive or Gram negative bacteria are the cell wall or outer membrane, cytoplasmic membrane, functional and structural protein, DNA, RNA and other cytosolic component. Although biocide treatment eliminates most surface contamination some microorganisms may survive and give rise to public health problem [5]. The wide spread use of antiseptic result in cross resistance to antibiotic [6].

Sodium hypochlorite NaOCl is non-specific proteolytic, fungicidal and bactericidal agent, it is strongly alkaline and hypertonic, although it action is more pronounced on necrotic tissue, NaOCl also exhibit toxicity on all living tissues depending on the concentration used (varying from 0.5%-6%) and time of exposure.

Chlorhexidine (CHX) is cationic bisbiguanide that is stable as a salt (chlorhexidine gluconate) it use in concentration ranging from 0.2%-2% as endodontic irrigant, it is antimicrobial agent active against viruses, fungi and bacteria [7].

Thus, the aim of this study was to evaluate effectiveness of sodium hypochlorite and chlorhexidine to reduce *Staphylococcus aureus* biofilm biomass.

## Material and Methods

Cross sectional study was conducted in 70 *Staphylococcus aureus* isolated from different samples from different hospitals in Khartoum state. The practical work was conducted in Alzeim Al-azhari University during the period March to May 2017 and the data were analyzed using SPSS 21.

## Identification

The isolates of *Staphylococcus aureus* were cultured on sterile blood agar, MacConkey agar, nutrient and mannitol salt agar. Then the identification based on cultural characteristic, microscopic examination and biochemical characteristic.

## Detection of biofilm

### Congo red agar

The congo red agar prepared by 37 g/l from Brain heart infusion broth, 50 g/l from sucrose, 10 g/l agar and 0.8 g/l from congo red. The Brain heart infusion and sucrose and agar prepared separately from the congo red. The congo red and BHI autoclaved at 121°C for 21 min, then after cooling to 55°C the congo red is added to the BHI and poured in plates. Fresh growth of organisms was inoculated in the plates and incubated at 37°C for 24-48 h. The culture is triplicate. Positive biofilm strains appear in black color [8].

## Antimicrobial susceptibility test

### Kuerbey baeur method

Sensitivity done to methicillin and vancomycin.

**Table 1** Showed frequencies and association of biofilm and vancomycin resistant among strains of *staphylococcus aureus*; p value 0.664, chi-square test was used to calculate p value, p value less than 0.05 considered significant.

		Vancomycin		Total
		Sensitive	Moderate resist	
Biofilm	Positive	10 (17.2%)	2 (16.7%)	12 (17.1%)
	Negative	48 (82.8%)	10 (83.3%)	58 (82.9%)
Total		58 (100.0%)	12 (100.0%)	70 (100.0%)

**Table 2** Showed frequencies and association of biofilm and methicillin resistant among strains of *staphylococcus aureus*; P value 0.664, chi-square test was used to calculate P value, P value less than 0.05 considered significant.

		Methicillin			Total
		Sensitive	Moderate resist	Resist	
Biofilm	Positive	5 (23.8%)	4 (19.0%)	3 (10.7%)	12 (17.1%)
	Negative	16 (76.2%)	17 (81.0%)	25 (89.3%)	58 (82.9%)
Total		21 (100.0%)	21 (100.0%)	28 (100.0%)	70 (100.0%)

## Results

The biofilm was detected in 12 (17.1%) out of all 70 strains of *Staphylococcus aureus* 58 (82.6%) of the species were sensitive to vancomycin, whereas 12 (17.4%) were moderate resistant. Among all 58 vancomycin sensitive strains, 10 (17.2%) were formed biofilm and considering the 12 moderate resistant strains, there were 2 (16.7%) of them formed biofilm. There was insignificant association between biofilm formation and vancomycin resistant in *Staphylococcus aureus* (P-value 0.664), as showed in **Table 1**.

The biofilm was detected in 12 (17.1%) out of all 70 strains of *Staphylococcus aureus*. 21 (30%) of the all species were sensitive to methicillin, whereas 21 (30%) of the species were moderate resist and 28 (40%) were resist. Among sensitive strains 5

## Detection the effect of chlorhexidine and sodium hypochlorite disinfectant on biofilm

24 h culture of *Staphylococcus aureus* suspended in sterile normal saline then diluted in 5 ml of sterile Brain heart infusion broth (BHI). 1 ml of bacterial suspensions were added to tubes and incubated at 37°C for 24 h to allow the build of biofilm in the tube. Then carefully remove the suspension from each tube by aspiration and refilled the tubes by one of the different concentrations; chlorhexidine (0.3%, 0.2%, 0.15%, 0.075%) and sodium hypochlorite (5%, 4%, 2.5%, 1.25%) and incubate for different time intervals (1 min, 3 min and 5 min). then remove the disinfectants and wash the tube by phosphate buffer saline(PBS); and stain it by 0.1% crystal violet for 20 min, remove the stain and wash the excess stain by PBS and fill the tubes by 95% ethylic alcohol and read the optical density by colorimeter [7].

(23.8%) were formed biofilm, 4 (19%) strains formed biofilm in moderate resist and 3 (10.7%) in resistance strains. There was insignificant association between biofilm formation and methicillin resistant in *Staphylococcus aureus* showed in **Table 2**.

With concentration of 0.3%, chlorhexidine showed significant decrease in biofilm formation in association with time (P value 0.001). Same results were observed with concentrations of 0.2%, 0.15% and 0.075% with P value 0.001, 0.000 and 0.000, respectively, as in **Tables 3 and 4**.

**Table 3** Shows statistics and mean differences of chlorhexidine 0.3%, 0.2%, 0.15% and 0.075% among three time interval subgroups; one way anova test was used to calculate P value, P value less than 0.05 considered significant.

		Mean $\pm$ Std. Deviation	Minimum- Maximum	P value
Chlorhexidine 0.3%	Chlorhexidine 1 min	85.00 $\pm$ 56.165	30-250	0.001
	Chlorhexidine 3 min	73.33 $\pm$ 41.729	11-154	
	Chlorhexidine 5 min	17.33 $\pm$ 3.393	10-24	
Chlorhexidine 0.2%	Chlorhexidine 1 min	73.58 $\pm$ 49.170	40-220	0.001
	Chlorhexidine 3 min	75.92 $\pm$ 46.903	8-182	
	Chlorhexidine 5 min	18.75 $\pm$ 3.019	12-23	
Chlorhexidine 0.15%	Chlorhexidine 1 min	109.08 $\pm$ 58.287	50-250	0.000
	Chlorhexidine 3 min	91.75 $\pm$ 62.005	12-210	
	Chlorhexidine 5 min	17.33 $\pm$ 2.871	11-21	
Chlorhexidine 0.075%	Chlorhexidine 1 min	117.83 $\pm$ 56.937	70-270	0.000
	Chlorhexidine 3 min	82.58 $\pm$ 47.779	13-175	
	Chlorhexidine 5 min	22.00 $\pm$ 5.427	10-28	

**Table 4** Shows multiple mean differences of chlorhexidine 0.3%, 0.2%, 0.15% and 0.075% among three time interval subgroups; ISD test was used to calculate P value, P value less than 0.05 considered significant.

Dependent Variable	(I) Chlorhexidine	(J) Chlorhexidine	P value
Chlorhexidine 0.3%	Chlorhexidine 1 min	Chlorhexidine 3 min	0.485
		Chlorhexidine 5 min	<b>0.000</b>
	Chlorhexidine 3 min	Chlorhexidine 1 min	0.485
		Chlorhexidine 5 min	<b>0.002</b>
	Chlorhexidine 5 min	Chlorhexidine 1 min	0.000
		Chlorhexidine 3 min	<b>0.002</b>
Chlorhexidine 0.2%	Chlorhexidine 1 min	Chlorhexidine 3 min	0.885
		Chlorhexidine 5 min	<b>0.002</b>
	Chlorhexidine 3 min	Chlorhexidine 1 min	0.885
		Chlorhexidine 5 min	<b>0.001</b>
	Chlorhexidine 5 min	Chlorhexidine 1 min	0.002
		Chlorhexidine 3 min	<b>0.001</b>
Chlorhexidine 0.15%	Chlorhexidine 1 min	Chlorhexidine 3 min	0.394
		Chlorhexidine 5 min	<b>0.000</b>
	Chlorhexidine 3 min	Chlorhexidine 1 min	0.394
		Chlorhexidine 5 min	<b>0.001</b>
	Chlorhexidine 5 min	Chlorhexidine 1 min	0.000
		Chlorhexidine 3 min	<b>0.001</b>
Chlorhexidine 0.075%	Chlorhexidine 1 min	Chlorhexidine 3 min	0.053
		Chlorhexidine 5 min	<b>0.000</b>

	Chlorhexidine 3 min	Chlorhexidine 1 min	0.053
		Chlorhexidine 5 min	<b>0.002</b>
	Chlorhexidine 5 min	Chlorhexidine 1 min	0.000
		Chlorhexidine 3 min	<b>0.002</b>

With concentration of 5%, sodium hypochlorite showed significant decrease in biofilm formation in association with time (P value 0.000). Similar results were observed with concentrations of 4%, 2.5% and 1.25% with P value 0.000, 0.000 and 0.000, respectively (**Tables 5 and 6**).

**Table 5** Shows statistics and mean differences of sodium hypochlorite 5%, 4%, 2.5% and 1.25% among three time interval subgroups; one way anova test was used to calculate P value, P value less than 0.05 considered significant.

		Mean ± Std. Deviation	Minimum- Maximum	P value
Sodium hypochlorite 5%	Sodium hypochlorite 1 min	91.42 ± 37.130	40-170	0.000
	Sodium hypochlorite 3 min	34.83 ± 22.663	7-68	
	Sodium hypochlorite 5 min	16.83 ± 2.406	12-20	
Sodium hypochlorite 4%	Sodium hypochlorite 1 min	82.00 ± 30.223	40-130	0.000
	Sodium hypochlorite 3 min	34.17 ± 20.626	4-59	
	Sodium hypochlorite 5 min	17.00 ± 4.243	10-25	
Sodium hypochlorite 2.5%	Sodium hypochlorite 1 min	84.25 ± 38.429	50-190	0.000
	Sodium hypochlorite 3 min	36.75 ± 19.666	10-57	
	Sodium hypochlorite 5 min	19.67 ± 3.257	16-26	
Sodium hypochlorite 1.25%	Sodium hypochlorite 1 min	95.75 ± 36.362	50-170	0.000
	Sodium hypochlorite 3 min	44.58 ± 25.098	8-74	
	Sodium hypochlorite 5 min	42.50 ± 27.367	15-80	

**Table 6** Shows multiple mean differences of sodium hypochlorite 5%, 4%, 2.5% and 1.25% among three time interval subgroups; lsd test was used to calculate p value, p value less than 0.05 considered significant.

Dependent Variable	(I) Sodium hypochlorite	(J) Sodium hypochlorite	P value
Sodium hypochlorite 5%	Sodium hypochlorite 1 min	Sodium hypochlorite 3 min	<b>0.000</b>
		Sodium hypochlorite 5 min	<b>0.000</b>
	Sodium hypochlorite 3 min	Sodium hypochlorite 1 min	<b>0.000</b>
		Sodium hypochlorite 5 min	0.089
	Sodium hypochlorite 5 min	Sodium hypochlorite 1 min	<b>0.000</b>
		Sodium hypochlorite 3 min	0.089
Sodium hypochlorite 4%	Sodium hypochlorite 1 min	Sodium hypochlorite 3 min	<b>0.000</b>
		Sodium hypochlorite 5 min	<b>0.000</b>
	Sodium hypochlorite 3 min	Sodium hypochlorite 1 min	<b>0.000</b>
		Sodium hypochlorite 5 min	0.056
	Sodium hypochlorite 5 min	Sodium hypochlorite 1 min	<b>0.000</b>
		Sodium hypochlorite 3 min	0.056

Sodium hypochlorite 2%	Sodium hypochlorite 1 min	Sodium hypochlorite 3 min	0.000
		Sodium hypochlorite 5 min	0.000
	Sodium hypochlorite 3 min	Sodium hypochlorite 1 min	0.000
		Sodium hypochlorite 5 min	0.104
	Sodium hypochlorite 5 min	Sodium hypochlorite 1 min	0.000
		Sodium hypochlorite 3 min	0.104
Sodium hypochlorite 1.25%	Sodium hypochlorite 1 min	Sodium hypochlorite 3 min	0.000
		Sodium hypochlorite 5 min	0.000
	Sodium hypochlorite 3 min	Sodium hypochlorite 1 min	0.000
		Sodium hypochlorite 5 min	0.873
	Sodium hypochlorite 5 min	Sodium hypochlorite 1 min	0.000
		Sodium hypochlorite 3 min	0.873

## Discussion

In this study 70 strains of *Staphylococcus aureus* were tested for vancomycin and methicillin susceptibility, biofilm formation and the effect of sodium hypochlorite and chlorhexidine on biofilm.

In vancomycin susceptibility test 58 (82.6%) were sensitive to vancomycin and 12 (17.4%) were moderate resistance. This finding is lower than work of Hasan et al. [9] they found the frequencies of vancomycin resistance was 37.9%.

From the 58 sensitive strains 10 (17.2%) were formed biofilm, 2 (16.4%) from moderate resistance strains formed biofilm. There was no significance association between biofilm formation and vancomycin resistance in *Staphylococcus aureus*. In study conducted by Bhattacharya et al. [10] they found that 66% of moderate resistance vancomycin was biofilm producer and 100% of vancomycin resistance were biofilm former.

In this study methicillin susceptibility test, 21 (30%) were sensitive to methicillin, whereas 21 (30%) were moderate resist and 28 (40%) were resist. Another study conducted by Ekrami et al. [11] they found the frequency of methicillin resistance was 60%.

Among sensitive strains 5 (23.8%) were formed biofilm, 4 (19%) strains formed biofilm in moderate resist and 3 (10.7%) in resistance strains. There was insignificant association between biofilm formation and methicillin resistant in *Staphylococcus aureus*. Similar study conducted by Kwon et al. [12] showed the relationship between methicillin resistance and biofilm formation, they found that the rate of biofilm positivity was 37.9% for methicillin-resistant strains and 14.3% for methicillin-susceptible strains ( $P < 0.05$ )

In comparing different concentrations (0.3%, 0.2%, 0.15% and 0.075%) of chlorhexidine among time interval (1 min, 3 min and 5 min) 0.3% concentration showed significant decrease in biofilm formation in association with time ( $P$  value 0.001). Similar results were observed with concentrations of 0.2% ( $p$  value 0.001), 0.15% ( $p$  value 0.000) and 0.075% ( $p$  value 0.000),

this indicate inhibitory effect of chlorhexidine on biofilm formation of *Staphylococcus aureus* is affected by time of exposure. This result agree with another study conducted in Belgium by Toté et al. [13] which showed that longer contact time generally increase the antibiofilm activity of chlorhexidine.

Different concentrations (5%, 4%, 2.5% and 1.25%) of sodium hypochlorite also tested through the same time intervals; 5% showed significant decrease in biofilm in association with time ( $p$  value 0.000), same results were observed with concentrations of 4% ( $p$  value 0.000), 2.5% ( $p$  value 0.000), 1.25% ( $p$  value 0.000). The effect of sodium hypochlorite on biofilm also affected by time of contact. Similar study conducted by de Castro Melo et al. [14] and the result was NaOCl, was able to promote a significant reduction on the number of *Staphylococcus aureus* biofilm depending on time of exposure.

The optical densities were increased with the decrease of chlorhexidine and sodium hypochlorite concentrations. This indicates the effect of concentration as factor on inhibition of formation of biofilm of *Staphylococcus aureus* by chlorhexidine and sodium hypochlorite.

## Conclusion

After analyzing the finding of this study it concluded that:

- *Staphylococcus aureus* was sensitive and moderate resistance (17.4%) to vancomycin.
- For methicillin susceptibility test *Staphylococcus aureus* was sensitive, moderate resistance and resistance (40%) to methicillin.
- In both vancomycin (sensitive and moderate resistance) and methicillin (sensitive, moderate resistance and resistance) *Staphylococcus aureus* were formed biofilm.
- Chlorhexidine and sodium hypochlorite were reduced the biofilm of *Staphylococcus aureus* depend on time of contact and concentration of them.

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