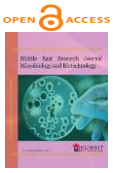

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Effect of Disinfectants Exposure on the Susceptibility of Antibiotics, Disinfectants, Heavy Metals and Biochemical Profile for *Staphylococcus aureus* Isolated from Caesarean Wounds

Mohemid Maddallah Al-Jebouri[1]*, Hana Salman Al-Bayati[2]
[1]Department of Medical Laboratory Technology, Al-Qalam University, Kirkuk, Iraq
[2]Department of Microbiology, College of Medicine, University of Tikrit, Tikrit, Iraq

Abstract: ***Background*:** Contact with disinfectants in sublethal condition allows survival and multiplication of bacteria. It has been found that serial passage of bacteria through diluted disinfectants not only increase minimal inhibitory concentrations, but also brings about phenotypic changes in their antibiogram Contact with disinfectants in sublethal condition allows survival and multiplication of bacteria. It has been found that serial passage of bacteria through diluted disinfectants not only increase minimal inhibitory concentrations (MICs), but also brings about phenotypic changes in their antibiogram. ***Materials and Methods*:** The present work was conducted on 500 patients with caesarean sections. Wound swab was taken on the third postoperative day from hospitalized patients and on the seventh postoperative day from patients attended outpatient clinic. *Staphylococcus aureus* was isolated and conventionally identified. A doubling dilutions of each antimicrobial agent like antibiotic, disinfectant and heavy metal in Muller-Hinton agar plates were done and *S. aureus* susceptibility to these agents was estimated. Selected antibiotic resistance and biochemical characters were concluded following bacterial exposure to serial dilutions of disinfectants. ***Results*:** It was found that different resistance levels to antimicrobial agents ranged from 4-1024, 2-1024 and 1-1024 µg/ml for antibiotics, disinfectants and heavy metals respectively. Exposure of bacteria to serial dilutions of disinfectants induced changes in their biotyping like urease and antibiotic resistance like gentamicin. There was a significant variation in MICs values of most antibiotics tested and ranged from 4-1024 µg/ml. This exposure led to an increase in MICs of disinfectants for the strains tested e.g. MIC of cetrimide for the first strain was 4 µg/ml in the first exposure, and then became 16 µg/ml in the second exposure. The mercury was the most effective metals in the killing of *S. aureus*. ***Conclusions*:** This analysis shows that disinfectant exposure caused progressive and multidimensional changes in both biochemical traits and antibiotic resistance, with the biggest jump was clearly seen between after first and after second exposures.

Keywords: Caesarean Wound, *S. Aureus*, Disinfectant Exposure, Resistance, Antibiotics, Disinfectants, Heavy Metals, Biochemical Traits.

Research Paper

***Corresponding Author:**
Mohemid Maddallah Al-Jebouri
Department of Medical Laboratory Technology, Al-Qalam University College, Kirkuk, Iraq

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INTRODUCTION

Contact with disinfectants in sublethal condition allows survival and multiplication of bacteria. It has been found that serial passage of bacteria through diluted disinfectants not only increase minimal inhibitory concentrations, but also brings about phenotypic changes in their antibiogram [1-4]. In a study conducted by Sivaji *et al*., [3], on the effect of disinfectant exposure, they found that *S. aureus* strains become resistant to many antibiotics after growth in series of diluted disinfectants [5, 6]. The mechanisms have not been fully studied and suggested that disinfectants probably altered target sites in bacterial ribosomes making it selectively less susceptible to certain antibiotics [6]. They suggested that enzymes involved in peptidoglycan synthesis might be destroyed causing resistance to penicillins and cephalosporins. Destruction of periplasmic enzymes by groups of disinfectants is also another contributing factors [7-10]. The most commonly used disinfectant in microbiology laboratory and hospitals are ethanol, Dettol,

chlorohexidine and soap [6]. Ethanol, as a dehydrating agent causes cell membrane damage, denaturalization of protein and cell lyses [11]. Dettol affect bacterial cells by denaturation of protein and also act on the cytoplasmic membrane of microorganisms. Bleach with a main constituent of sodium hypochlorite effect can be achieved by oxidizing of the cell of microorganism of attaching essential cell component including protein, lipid and DNA, while hibitane (chlorohexidine) acts by disruption of membranes, precipitation of proteins and inactivation of enzymes [12]. The role of antibiotics in propagating of antibiotic resistance has been extensively studied, other emerging evidence demonstrates the contribution of environmental pollutants, such as microplastics and heavy metals, to this phenomenon. It was previously thought that these pollutants, to be passive contaminants, were playing an active role in driving the evolution of microbial resistance dynamics and amplifying the major threats posed by antimicrobial resistance toward public and environmental health [13, 14]. Resistance genes codifying to Co, Ni (e.g., *rcn*A), and Co, Zn, Cd (*czc*A) have been already identified as dominant in metal-polluted samples, mostly co-existed with resistance to beta-lactams e.g., (*bla*CTX-M, *bla*NDM-1), macrolides (*erm*A, *mef*A, *mph*A), tetracyclines (*tet*A, *tet*M, *tet*W), and other antibiotics [9-15]. The present study was performed to determine the effect of disinfectants exposure on the antibiotics, disinfectants, heavy metals sensitivity and resistance patterns and on selected biochemical characters of *S. aureus.*

MATERIALS AND METHODS

Patients

This study was carried out in teaching hospital of Tikrit. The present work was conducted on 500 patients with caesarean sections. Three hundreds were hospitalized and the other two hundreds were non-hospitalized attended the outpatients clinic after operation for removal of stitches. Their ages ranged from 15-45 years. The majority of these patients were from rural areas or referred to this hospital from other town's hospitals. The acceptance for participation in the present study was taken from all the participants whose native language is Arabic. They were not mentally retarded and they were completely healthy considering hearing and speaking. A pilot study to ensure the questionnaire and identification of the most frequent pathogens causing caesarean wound infection was carried out on 50 patients with caesarean section before the start of the present study.

Sampling

Wound swab was taken on the third postoperative day from hospitalized patients and on the seventh postoperative day from patients attended outpatient clinic. Samples were taken by using sterile cotton swabs moistened with nutrient broth carried in test tubes contained 2 ml broth liquid [4]. Wound swabs were enriched in nutrient broth at 37 ^{0}C for 18 hours. Each sample was sub-cultured on mannitol salt agar and incubated at 37 ^{0}C for 24 hours. Pure cultures were obtained after isolation on appropriate selective media. The suspected colonies were purified twice then sub-cultured on nutrient agar slants and kept at 4 ^{0}C for full identification [10].

Antibiotic Susceptibility Testing

A loopful growth from isolates of *Staphylococcus aureus* were inoculated into nutrient broth and incubated at 37 ^{0}C for 18 hours. The bacterial suspensions were diluted with ringer solution. The proportion of dilution was 1:1000 (6). Diluted bacterial suspension were poured onto the surface of the Muller-Hinton agar plates. The excess of bacterial suspensions were discarded using Pasteur pipette and plates were left for one hour at room temperature to dry. The antibiotic discs which are shown in Table 1 were selectively applied by using sterile forceps which was flamed after being cleansed with alcohol. The plates were incubated at 37 ^{0}C for overnight. The size of zones of inhibition were measured from edge of disc to the edge of inhibition of growth and the result was compared with standard diameter of inhibition zones for each antibiotic utilizing the method of Bauer *et al*., [11]. The following standard strain *Staphylococcus aureus* ATCC25923 was used as a reference strain.

Table 1: Antibiotics used in susceptibility testing

Antibiotic	Code	Concentration(µg/disc)	Manufacturer
Chloramphenicol	C	30	Oxoid *
Streptomycin	S	10	Oxoid
Gentamicin	CN	10	Oxoid
Tetracycline	TE	30	Oxoid
Erythromycin	E	15	Oxoid
Penicillin G	P	10	Oxoid
Tobramycin	TOB	10	Oxoid
Nitrofurantoin	F	300	Oxoid
Rifampicin	RF	30	Oxoid
Fucidin	FU	30	Leo**
Ampicillin	AP	2	SDI***
Cloxacillin	CX	5	SDI

Antibiotic	Code	Concentration(µg/disc)	Manufacturer
Clindamycin	CL	2	SDI
Trimethoprim	TMP	5	SDI
Benzathine penicillin	Bp	6	SDI

*= Oxoid Ltd., Britain; **= Leo Pharmaceutical Products, Denmark; ***= SDI, Samarra Drug Industry, Samarra, Iraq.

Determination of Minimal Inhibitory Concentration of Antibiotics for *Staphylococcus Aureus*

A doubling dilutions of each antibiotic in Muller-Hinton agar plates were done as shown in Table 1. A loopful of each isolate was inoculated into tubes containing nutrient broth and incubated at 37 ^{0}C overnight. Dilution of broth culture was done up to 100 fold with nutrient broth, All plates were inoculated with diluted broth cultures of *S. aureus* and incubated at 37 ^{0}C for 24 hours. The results were read to the end of visible growth [6-12]. Plates containing no antibiotic were included in each batch of incubated plates as control.

Table 2: Antibiotics used in the minimal inhibitory concentration testing of *Staphylococcus aureus*

Antibiotic	Code	Solvent	Diluent	Concentration (µg/ml)
Ampicillin	AP	Phosphate buffer(pH 6)	Phosphate buffer (pH 7)	0.5-512
Tetracycline	TE	Distilled water	Distilled water	0.5-512
Chloramphenicol	C	Absolute alcohol	Distilled water	0.5-256
Rifampicin	RF	Dimethyl sulphoxide	Phosphate buffer (pH 7)	0.5-256
Cephaloridine	CR	Phosphate buffer(pH 6)	Distilled water	0.5-256
Nitrofurantoin	F	Distilled water	Distilled water	0.5-256
Trimethoprim	TMP	10% NaOH	Distilled water	0.5-1024
Sulphamethoxazole	SMZ	10% NaOH	Distilled water	0.5-1024

Determination of Minimal Inhibitory Concentration of Heavy Metals for *Staphylococcus Aureus*

The method used in this test was the same as that used for determination of minimal inhibitory concentration (MIC) of antibiotics which described before. Table 3 shows all types of heavy metals used, doubled concentrations of each heavy metal and their manufacturer [1].

Table 3: Heavy metals used in susceptibility of *Staphylococcus aureus* isolated from caesarean wounds.

Metal	Chemical compound	Concentration(µg/ml)	Manufacturer
Lead(pb $^{2+}$)	Pb9Ch3Coo)2	2- 1024	British drugs Home (BDH)
Zinc(Zn^{2+})	ZnSO4.7H2O	8-512	Hopkin and Williams, UK
Cadmium(Cd^{2+})	CdCl2.1/2 H2O	2-256	BDH
Mercury(Hg^{2+})	HgCl2	0.5-128	Hopkin and Williams

Determination of Minimal Inhibitory Concentration of Disinfectants for *Staphylococcus Aureus*

The method used was the same as that used for antibiotics and heavy metals [6]. Table 4 shows the types of disinfectants used, doubled concentrations of each disinfectant and their manufacturer. Eleven strains of *S. aureus* were selectively exposed to five types of chemical disinfectants in concentrations shown in the Table 4. The series of disinfectants exposure was as follow: hibitane, Dettol, cetavlon, savlon and providine-iodine. All strains were inoculated into nutrient broth and incubated at 37 ^{0}C for three hours producing a growth yield of $10^6 – 10^7$ cells/ml. Plates of Mueller-Hinton agar containing doubling dilutions of disinfectants were prepared. Strains in broth cultures were subcultured by streaking onto Mueller-Hinton agar plates containing the first disinfectant and incubated at 37 ^{0}C for 18 hours. Colony from subminimal inhibitory concentration (SMIC) was picked up and inoculated into nutrient broth and incubated at 37 ^{0}C for three hours. The identity of strains was checked by subculturing on blood agar, Gram stain and coagulase test (6) and the same was done for the remaining disinfectants. The process of exposure was repeated on media containing the second disinfectants [6]. The strains then kept on nutrient agar slant at 4 ^{0}C for performing biochemical and antibiotic susceptibility testing. The second exposure to the same disinfectants was repeated for the same strains as it was done in the first exposure. The strains after second exposure were kept on nutrient agar slants at 4 ^{0}C for performing the second biochemical and antibiotic susceptibility testing.

Table 4: Disinfectants used in susceptibility testing of *Staphylococcus aureus* isolated from the caesarean wounds

Disinfectant	Scientific name	Chemical concentration	Concentration used(µg/ml)	Manufacturer
Cetrimide	Cetrimide	Pure powder	0.5-128	ICI(Britain)
Hibitane	Chlorhexidine gluconate	5%	0.5-128	ICI (Britain)
Dettol	Chloroxylenol	4.8%	0.5-128	SDI,Samarra, Iraq
Savlon	Chlorhexidine: Cetrimide	3%cetrimide:0.3% Chlorohexidine	0.3cetrimide:0.03 chlorhexidine -76.8 Cetrimide:7.68 chlohexidine	ICI (Britain)
Providine-iodine		10%	0.5-1024	ICI(Britain)

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics such as means, standard deviations, and frequency distributions were computed to summarize the data. To examine the relationships between variables, linear regression and multivariate linear regression models were applied. Where appropriate, interaction terms were included to evaluate the effect modification between predictors. An exponential decay model was used to describe the decreasing trends in the outcome variable over time, and the model was linearized using natural logarithm transformation for compatibility with linear regression frameworks. The significance of individual coefficients in the regression models was assessed using t-tests, with a p-value < 0.05 considered statistically significant. Assumptions of normality, linearity, homoscedasticity, and multicollinearity were checked prior to model interpretation. All graphical outputs and residual diagnostics were also generated using SPSS. In addition, for confirmation of model robustness, selected analyses were repeated using R version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria) [10].

RESULTS

Minimal Inhibitory Concentration of Antibiotics for Strains of *Staphylococcus Aureus*

The minimal inhibitory concentrations of different antibiotics used for strains of *S. aureus* varied (Table 1). In general, MICs were ranged from 4-1024 µg/ml. The MICs of both trimethoprim and sulphamethoxazole were ranged from 256-1024 µg/ml, whereas MICs of rifampicin and nitrofurantoin were low i.e. ranged from 4-64 µg/ml. The rifampicin and nitrofurantoin were the most effective drugs by in vitro testing. The correlation coefficient (R^2) was equal to 0.965 indicating an excellent linear fit in log of MIC space. The red line is the fitted decay, and blue points are observed data (Figure 1).

Table 1: Minimal inhibitory concentration (MIC) of antibiotics for 40 strains of *Staphylococcus aureus* from inpatients with caesarean wound (22 from inpatients and 18 from outpatients)

Antibiotic	Source of strains	No. (%) of strains with minimum inhibitory concentration (µg/ml):									
		2	4	8	16	32	64	128	256	512	1024
Ampicillin	Inpatients	0	0	0	5(22.3)	5(22.7)	0	6(27.3)	4(18.2)	2(9.1)	-
	Outpatients	0	0	2(11.1)	3(16.6)	1(5.5)	3(16.6)	2(11.1)	7(38.8)	0	
	Total	0	0	2(5)	8(20)	6(15)	3(7.5)	8(20)	11(27.5)	2(5)	-
Tetracycline	Inpatients	0	0	0	1(4.5)	4(18.2)	2(9.1)	9(40.9)	6(27.3)	0	-
	Outpatients	0	0	0	2(11.1)	2(11.1)	6(33.3)	4(22.2)	4(22.2)	0	
	Total	0	0	0	3(7.5)	6(15)	8(20)	13(32.5)	10(25)	0	-
Chloramphenicol	Inpatients	0	0	8(36.4)	5(22.7)	3(13.6)	5(22.7)	1(4.5)	0	-	-
	Outpatients	0	0	6(33.3)	5(22.7)	4(22.2)	3(16.6)	0	0		
	Total	0	0	14(35)	10(25)	7(17.5)	8(20)	1(2.5)	0	-	-
Rifampicin	Outpatients	0	7(31.8)	5(22.7)	5(22.7)	2(9.1)	3(13.6)	0	0	-	-
	Outpatients	0	4(22.7)	4(22.7)	3(16.6)	4(22.7)	3(16.6)	0	0		
	Total	0	11(27.5)	9(22.7)	8(20)	6(15)	6(15)	0	0	-	-
Cephaloridine	Inpatients	0	0	5(22.7)	4(18.2)	4(18.2)	7(31.2)	2(9.1)	0	-	-
	Outpatients	0	0	6(33.3)	4(22.2)	2(11.1)	6(33.3)	0			
	Total	0	0	11(27.6)	8(20)	6(15)	13(32.5)	2(5)	0	-	-
Nitrofurantoin	Inpatients	0	0	3(13.6)	10(45.4)	7(31.8)	2(9.1)	0	0	-	-
	Outpatients	0	1(2.5)	1(2.5)	12(66.6)	12(66.6)	0				
	Total	0	1(2.5)	4(10)	22(55)	12(30)	2(5)	0	0	-	-
Trimethoprim	Inpatients	0	0	0	0	0	0	0	1(4.5)	12(54.6)	9(40.9)
	Outpatients	0	0	0	0	0	0	0	2(11.1)	10(55.5)	10(55.5)
	Total	0	0	0	0	0	0	0	3(7.5)	22(55)	19(47.5)
Sulphathiomoxazole	Inpatients	0	0	0	0	0	0	0	2(9.1)	10(45.4)	10(45.4)
	Outpatients	0	0	0	0	0	0	0	4(22.2)	7(38.8)	7(38.8)
	Total	0	0	0	0	0	0	0	6(15)	17(42.5)	17(42.5)

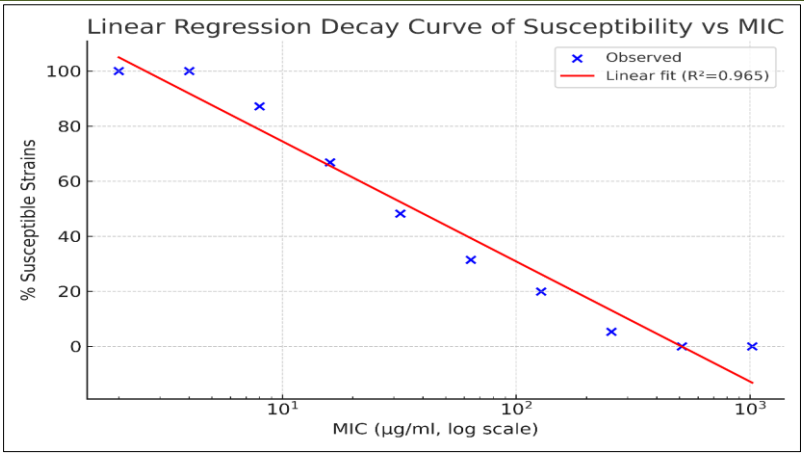

Figure 1: Linear regression decay curve of susceptibility vs minimal inhibitory concentration of antibiotics for *Staphylococcus aureus*

Minimal Inhibitory Concentration of Chemical Disinfectants

Tables 2 revealed that MICs of cetrimide, chlorhexidine, chloroxylenol and providine-iodine ranged between 4-64, 8-128, 32-256 and 256-1024 respectively. Correlation coefficient (R^2) value was 0.961, showing a strong linear relationship between log(MIC) and % susceptible strains. Blue points are observed data whereas the red line is the fitted regression (Figure 2). The MIC study of savlon (cetrimide: chlorhexidine) showed a killing concentration ranged between 2.4:0.24 to 19.2: 1.92 µg/ml as shown in Table 3 and Figure 3.

Table 2: Minimal inhibitory concentration (MIC) of disinfectants for 40 strains of Staphylococcus aureus isolated from patients with caesarean wounds (22 from inpatients and 18 from outpatients)

Disinfectant	Source of strains	No.(%) of strains with minimum inhibitory concentration (µg/ml):									
		2	4	8	16	32	64	128	256	512	1024
Cetrimide	Inpatients	0	2(2.1)	5(22.7)	4(18.2)	9(40.9)	2(2.1)	0	-	-	-
	Outpatients	0	4(22.2)	3(16.6)	6(33.3)	5(27.7)	0				
	Total	0	6(15)	8(20)	10(25)	14(35)	2(5)	0			
Chlorhexidine	Inpatients	0	0	6(27.3)	4(18.2)	4(18.2)	7(31.8)	1(4.1)	-	-	-
	Outpatients	0	0	5(27.7)	7(38.8)	4(22.2)	2(11.1)	0			
	Total	0	0	11(27.5)	11(27.5)	8(20)	9(22.5)	1(2.5)	-	-	-
Chloroxylenol	Inpatients	0	0	0	0	1(4.5)	16(72.7)	1(4.5)	4(18.2)	-	-
	Outpatients	0	0	0	0	4(22.2)	10(55.5)	3(16.6)	1(5.5)		
	Total	0	0	0	0	5(12.5)	26(65)	4(10)	5(12.5)	-	-
Providine-iodine	Inpatients	0	0	0	0	0	0	0	1(4.5)	8(36.4)	13(59.1)
	Outpatients	0	0	0	0	0	0	0	3(16.6)	9(50)	6(33.3)
	Total	0	0	0	0	0	0	0	4(10)	17(42.5)	19(47.5)

(-), not tested.

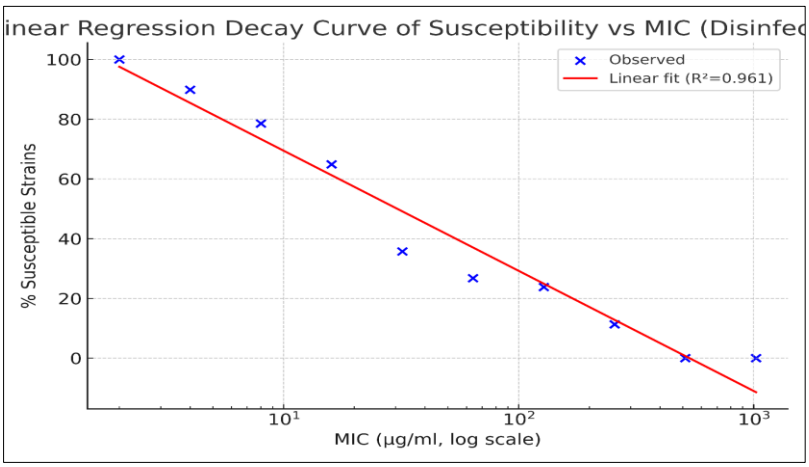

Figure 2: Linear regression decay curve of susceptibility vs minimal inhibitory concentration of disinfectants for *Staphylococcus aureus*.

The MIC study of savlon (cetrimide: chlorhexidine) showed a killing concentration ranged between 2.4:0.24 to 19.2: 1.92 µg/ml as shown in Table 3. The correlation coefficient (R^2) value was 0.874, showing a moderately strong linear relationship between log (MIC) and % susceptible strains. Blue points represent observed values whereas red line shows linear fit (Figure 3).

Table 3: Minimal inhibitory concentration of savlon (3% cetrimide: 0.3% chlorhexidine) for 40 strains of *Staphylococcus aureus* from patients with caesarean wound

Source of strains	Number of strains	No. (%) of strains with minimum inhibitory concentrations (µg/ml):								
		0.3:0.03	0.6:0.06	1.2:0.12	2.4:0.24	4.8:0.48	9.6:0.96	19.2:1.92	38.4:3.84	76.8:7.68
Inpatients	22	0	0	0	0	5(22.7)	6(27.2)	11(50)	0	0
Outpatients	18	0	0	0	2(11.1)	3(16.6)	5(27.7)	8(44.4)	0	0
Total	40	0	0	0	2(5)	8(20)	11(27.5)	19(47.5)	0	0

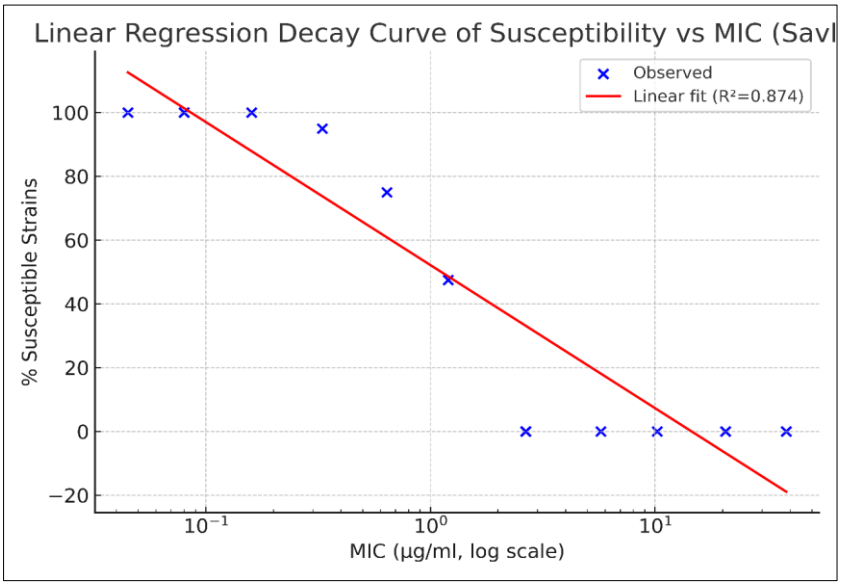

Figure 3: Linear regression decay curve of susceptibility vs minimal inhibitory concentration of savlon for *Staphylococcus aureus.*

Minimal Inhibitory Concentration of Heavy Metals

Table 4 shows the minimal inhibitory concentrations of heavy metals for strains of *Staphylococcus aureus* tested. It was found that MICs of lead ranged between 64 and 1024 µg/ml, whereas MICs of other heavy metals used such as zinc, cadmium and mercury were 32-256, 8-64 and 1-16 µg/ml respectively. The mercury was the most effective metals in the killing of *S. aureus*. Lead correlation coefficient R value which equal to 0.53 revealed a weak to moderate fit and shows gradual susceptibility, but the zinc R^2 value was 0.85 which indicated a strong fit with steep decline in susceptibility with MIC increase. Cadmium heavy metal estimation showed R^2 with 0.84 value indicated a strong fit similar to zinc. The R^2 value of mercury was 0.66 indicated a moderate fit with more variable decline pattern (Figure 4).

Table 4: Minimal inhibitory concentration (MIC) of heavy metals for 40 strains of *Staphylococcus aureus* isolated from patients with caesarean wound (22 from inpatients and 18 from outpatients)

Metal	Source of strains	No. (%) of strains with minimum inhibitory concentrations(µg/ml):											
		0.5	1	2	4	8	16	32	64	128	256	512	1024
Lead	Inpatients	-	-	0	0	0	0	0	0	1(4.1)	2(9.1)	5(22.7)	14(63.5)
	Outpatients			0	0	0	0	0	2(11.1)	1(5.5)	2(11.1)	7(38.8)	6(33.3)
	Total	-	-	0	0	0	0	0	2(5)	2(5)	4(10)	12(30)	20(50)
Zinc	Inpatients	-	-	-	-	0	0	2(9.1)	6(27.3)	11(50)	3(13.6)	0	-
	Outpatients					0	0	3(16.6)	9(50)	6(33.3)	0	0	
	Total	-	-	-	-	0	0	5(12.5)	15(37.5)	17(42.5)	3(7.5)	0	-
Cadi mium	Inpatients	-	-	0	0	1(4.1)	7(31.8)	10(45.4)	4(18.2)	0	0	-	-
	Outpatients			0	0	1(5.5)	11(61.1)	5(27.7)	1(5.5)	0	0		
	Total	-	-	0	0	2(5)	18(45)	15(37.5)	5(12.5)	0	0	-	-
Merc ury	Inpatients	0	0	7(31.8)	9(40.9)	5(22.7)	1(4.1)	0	0	0	-	-	-
	Outpatients	0	2(11.1)	5(27.7)	8(44.4)	3(16.6)	0						
	Total	0	2(5)	12(30)	17(42.5)	8(20)	1(2.5)	0	0	0	-	-	-

(-), not tested.

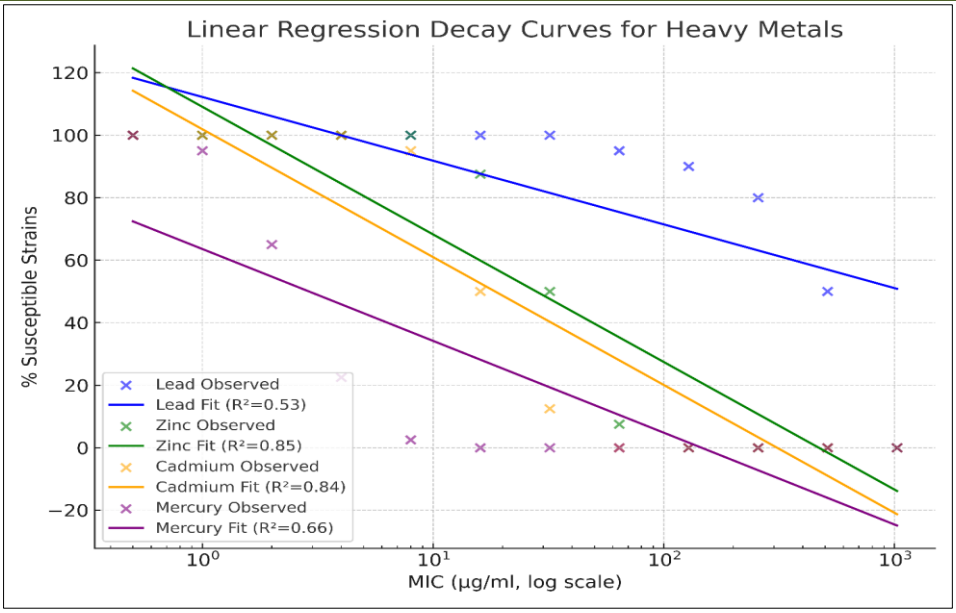

Figure 4: shows linear regression decay curves for different heavy metals for *Staphylococcus aureus*

The Effect of Disinfectant Exposure on *Staphylococcus Aureus*

Eleven strains of *S. aureus* were selected and exposed twice to series of diluted disinfectants (Table 5). This exposure led to an increase in MICs of disinfectants for the strains tested e.g. MIC of cetrimide for the first strain was 4 µg/ml in the first exposure, and then became 16 µg/ml in the second exposure. Other strains showed variable MICs after first and second exposures to disinfectants. Univeriate analysis of variance (ANOVA) revealed that the F value of the first exposure was 31.86 which indicated a P value less than 0.005 with a very high significant effect indicating different minimal inhibitory concentrations between disinfectants. ANOVA test of the data of second exposure revealed F test value of 29.81 with P value less than 0.005 indicating highly significant differences which were persisted among disinfectants after second exposure. Hibitane, dettol and cetrimide revealed large increases in MICs after repeated exposure i.e. loss of susceptibility. Savlon did no show a significant change and suggested a stability in MIC. Providine-iodine (PI) showed a moderate elevation nearing statistical significant in earlier paired tests. Figure 5 shows that the Chi-squared value was more than 0.9 indicating very strong fit with steeper decline in susceptibility as MIC increases. The second exposure R^2 value was 0.934 indicated strong fit also indicating reduced susceptibility after repeated exposure. The parallel shift between blue and red lines in Figure 5 suggest a consistent resistance development across the MIC spectrum.

Table 5: The effect of subminimal inhibitory concentration (SMIC) of chemical disinfectants for 11 strains of *Staphylococcus aureus* from patients with caesarean wound

Strains code	Subminimal inhibitory concentration(SMIC) of chemical disinfectants for first exposure(µg/ml)					Subminimal inhibitory concentration(SMIC) of chemical disinfectants for second exposure(µg/ml)				
	Hibitane	Dettol	Cetrimide	Savlon	PI	Hibitane	Dettol	Cetrimide	Savlon	PI
1	16	64	4	41.2:0.12	64	64	128	16	1.2:0.12	64
2	4	32	8	0.6:0.06	64	8	64	8	0.6:0.06	128
3	16	32	4	1.2:0.12	64	16	64	8	1.2:0.12	128
4	8	64	4	0.6:0.06	64	16	128	16	0.6:0.06	128
5	16	32	4	1.2:0.12	32	32	32	4	2.4:0.24	128
6	16	32	4	1.2:0.12	64	64	64	4	1.2:0.12	64
7	8	32	8	1.2:0.12	32	32	64	8	1.2:0.12	64
8	8	32	4	0.6:0.06	128	32	64	16	1.2:0.12	128
9	8	32	4	0.6:0.06	32	32	64	16	1.2:0.12	64
10	16	32	8	1.2:0.12	64	32	64	16	2.4:0.24	64
11	16	32	8	1.2:0.12	64	16	32	16	2.4:0.24	128

PI, providine-iodine

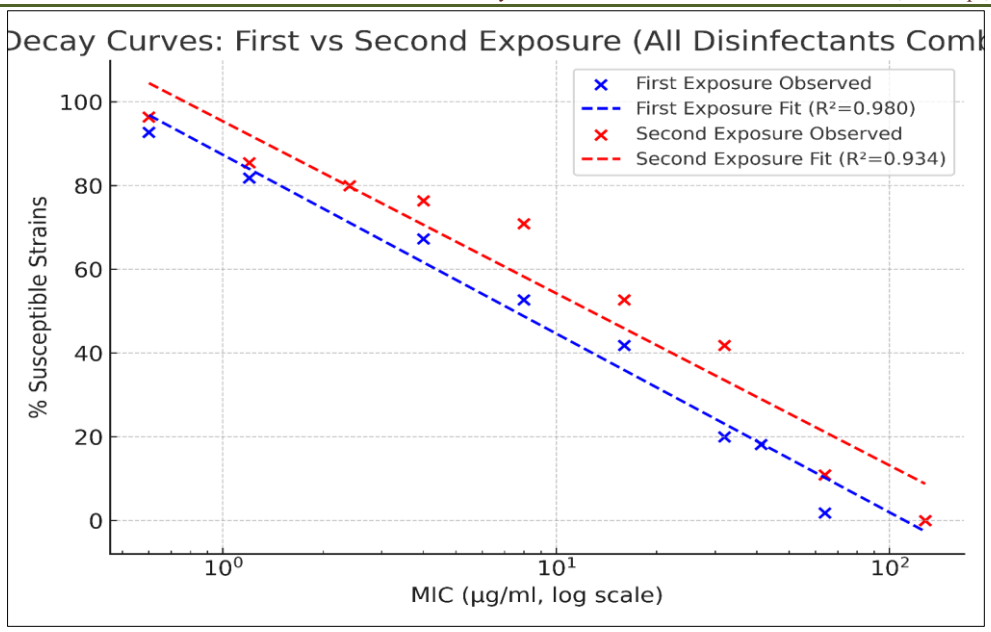

Figure 5: Linear regression decay curves of first and second exposure for all disinfectants combined for *Staphylococcus aureus*.

The Effect of Disinfectant Exposure on Biochemical and Antibiotic Resistance Characters of *Staphylococcus Aureus*

The biochemical characters of disinfectant-exposed strains changed particularly after the second exposure and the negativity of testing increased for urease, Voges-Proskauer, fermentation of lactose, mannitol and arginine hydrolase test (Table 6). There was also an increase in the antibiotic resistance after the first exposure which was higher after the second exposure (Table 6), e.g. there were only four strains resistance to streptomycin before first exposure and the number of streptomycin-resistant strains become seven and eleven after first and second exposures respectively. Susceptibility of all strains tested to nitrofurantoin and penicillin G did not change after exposures. Principal component analysis (PCA) analysis was utilized to simplify complex data by reducing the number of variables while retaining most of the original data's variation. This programme was applied on the biochemical and antibiotic resistance profiles from Table 6, comparing before, after first, and after second exposures. PC1 explained 75% of the total variation which mainly driven by antibiotic resistance profile changes, the before and after Second stages were far apart indicating substantial cumulative effect. PC2 demonstrated 25%, reflecting minor shifts in biochemical test profiles, and after first exposure the change was intermediate but shifted towards before, suggesting partial changes before final shift after second exposure. This analysis shows that disinfectant exposure caused progressive and multidimensional changes in both biochemical traits and antibiotic resistance, with the biggest jump was clearly seen between after first and after second exposures (Table 6, Figure 6).

Table 6: The effect of disinfectant exposure for on 11 strains of *Staphylococcus aureus* on biochemical and antibiotic susceptibility tests

Before exposure	After first exposure	After second exposure
Biochemical tests		
U(9)*, Coag(11), DNase(11), VP(7), M(9), L(11), Arg(9)	U(7), Coag(11), DNase(11), VP(6), M(8), L(10), Arg(7)	U(3), Coag(11), DNase(11) VP(4), M(5), L(7),Arg(3)
Antibiotic resistance		
P(11)**, TE(5), AP(11), TMP(11), S(4), C(11), E(11), CL(5), CX(7), CN(5), FU(0), BP(11), RF(3), F(0)	P(11), TR(9), AP(11), TMP(11), S(7), C(10), E(11), CL(5), CX(10), CN(7), FU(0), BP(11), RF(4), F(0)	P(11), TE(11), AP(11), TMP(11, S(11), C(11), E(11), CL(10), CX(10), CN(11), FU(5), BP(11), RF(6), F(0)

(*), number of positive strains; (**), number of resistant strains. Biochemical tests: U, urease; Coag, coagulase; DNase, deoxyribonuclease; VP, Voges-Proskauer; M, mannitol; L, lactose. Antibiotic resistance drugs: P, penicillin; TE, tetracycline; AP, ampicillin; TMP, trimethoprim; S, streptomycin; C, chloramphenicol; CL, clindamycin; CX, cloxacillin; CN, gentamicin; FU, Fucidin; BP, benzathine penicillin; RF, rifampicin; F, nitrofurantoin.

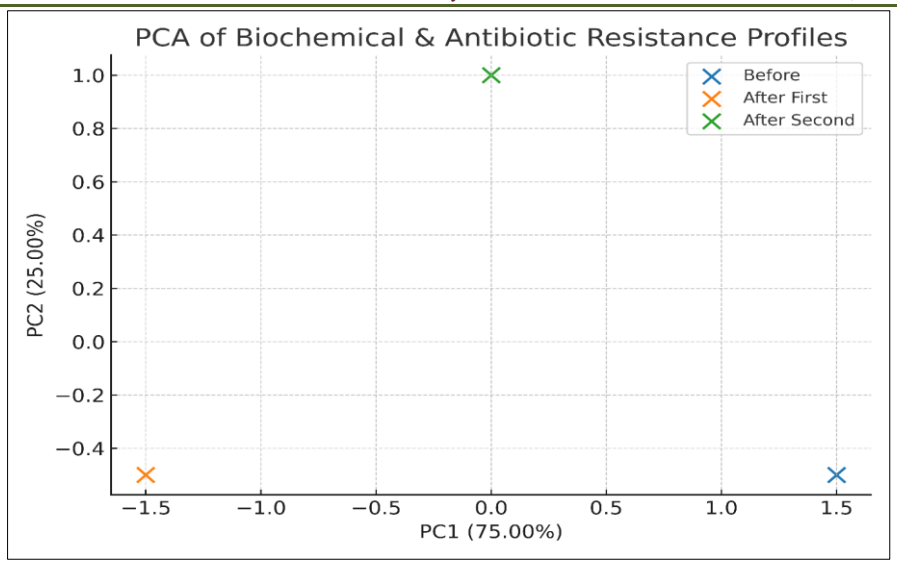

Figure 6: Principal component analysis (PCA) of biochemical and antibiotic resistance profiles

DISCUSSION

There was a significant variation in MICs values of most antibiotics tested and ranged from 4-1024 µg/ml. Al-Najjar found that MICs for 15 antibiotics against ten sensitive strains of *S. aureus* ranged from 5-90 µglml [13]. Rifampicin and nitrofurantoin were the most effective drugs and their MICs ranged from 4-64 µg/ml for each. However, Al-Jebouri in a study on minimal bactericidal concentration (MBCs) and minimal inhibitory concentration (MICs) of many antibiotics against strains of *S. aureus* found that gentamicin was the most effective drug against these organisms, and their MBCs were much lower than that of tetracycline and chloramphenicol [6]. The problem of hospital cross-infection due to contamination of disinfectants has been recognized [2]. The passage of bacteria through diluted disinfectants may not only bring about phenotypic changes in their antibiograms [4], but it might lead also to changes in phage typing [10-16]. Minimal inhibitory concentrations of the antimicrobial agents including antibiotics, disinfectants and heavy metals for different strains were markedly differ between strains. Passage through disinfectants induced susceptibility to most of the antibiotics in many of the strains of *S. aureus* in the present study. It was concluded that, in the case of *Escherichia coli*, the loss of phosphatases and heptose in the lipopolysaccharide resulted in increased susceptibility to antimicrobial agents including antibiotics, such as novobiocin, spiramycin and actinomycin D [3]. In the present study, the mechanisms of antibiotic resistance in these variants are still under investigation. One hypothesis would be that MICs were ranged from 4-1024 µg/ml. The MICs of both trimethoprim and sulphamethoxazole were ranged from 256-1024 µg/ml, whereas MICs of rifampicin and nitrofurantoin were low i.e. ranged from 4-64 µg/ml. The rifampicin and nitrofurantoin were the most effective drugs by in vitro testing. The disinfectants altered the target site in the bacterial ribosome, making it less susceptible to neomycin and kanamycin but not to gentamicin or amikacin. Sivaji, Mandal &Agarwal [3], observed that disinfectant derived variants of *Staphylococcus aureus* became resistant to streptomycin but not to gentamicin or kanamycin. Destruction of periplasmic enzymes by disinfectants has also been investigated [16-19]. Decreased absorption of antibiotics by bacteria can also be another contributory factor to the resistance. However, micro-organisms exposed to subinhibitory concentrations of antimicrobials would be under adaptative response or develop other resistance mechanisms in order to overcome this selective pressure of antimicrobial exposure [20]. These mechanisms are enabling the dissemination of antimicrobial resistance genes and alteration of antimicrobial susceptibility profiles [21]. However, the changes in susceptibility do not indicate development of resistance [22- 23], because tolerance and/or adaptation as a phenotypic display may be considered instead [24]. Hence, cross-resistance might be proposed when the use of biocides drives selective pressure towards antibacterial resistance concerning some microbial subpopulations [25]. The concerned stress induces an adaptative response which protects pathogens, by producing cellular changes that may affect the original antimicrobial susceptibility pattern [26]. This status is particularly important in healthcare requirements and infrastructures where contamination acts a significant effect in healthcare-associated infections [27]. Furthermore, comprehensive efforts, including basic infection control education, improved selection, use of products and practical training are important to decrease harmful cleaning and disinfection exposures without decreasing the effectiveness of infection prevention [28]. On the other hand, the evolution of antibiotic-resistant microorganisms after treatment with sub-MICs of different disinfectants has been worked out elsewhere [27]. The results of previous studies suggested that exposure to minimal inhibitory dosages of various disinfectants can induce antibiotic resistance among

clinical bacteria isolates through either natural selection mechanisms or enforcement of acquiring resistance changes to antibiotics as an adaptation to the new environment [6]. Moreover, in contrast the present data revealed that the exposure of antibiotic-resistant bacteria to disinfectants induced sensitivity to these therapeutic agents. Inducing antibiotic sensitivity might be due to anatomical, genetic, and physiological changes occurring in bacterial cells when they are under disinfectants exposure pressure. It has been reported previously that the losses of phosphatases and heptose in the liposaccharide of *E.coli* lead (Figure 1) to increased susceptibility to antibiotics [17]. However, the correlation coefficient (R^2) was equal to 0.965 indicating an excellent linear fit in log of MIC space. The red line is the fitted decay, and blue points are observed data (Figure 1). Thus, the use of suitable bactericidal concentrations of various disinfectants should be decided by the infection prevention and control specialists who are responsible and directly involved in infection control program standards in health care settings. However, precautions should be taken regarding the use of appropriate concentrations as recommended by the manufacturers, specially for prevention of infections caused by antibiotic-resistant bacteria [28, 29]. The changing susceptibility of *S. aureus* utilizing MICs following exposure to disinfectants has been carried out, and and demonstrated an elevation in minimal inhibitory concentration for certain strains of bacteria under present study. The growing considerations about the dissemination of biocide resistance and cross resistance with antibiotics among pathogenic bacteria have been suggested. It is clear that isolates of hospitals particularly of *S. aureus, P. aeruginosa* and *E.coli* should be under continuous surveillance and possible mechanisms associated with disinfectant-resistance should be control by infection policy particularly among hospitals where immunocompromised patients are resident [30]. On the other hand, hospitals represent a large and significant reservoir of microorganisms, including multidrug-resistant pathogens such as *S. aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *P. aeruginosa*, many of them carrying antibiotic and heavy metal resistance genes (HMRGs) (20). The large use of antibiotics and disinfectants in hospitals might lead to formation of bacterial biofilms of the resistant pathogens on surfaces of medical devices and hospital wastewater, allowing and promoting the co-selection of resistance genes, since some genetic characters can be carried at the same time for heavy metals, antibiotics and biocides in bacteria such as *P. aeruginosa*, facilitating dissemination in the environment through the wastewater flow [27, 28].

The present study revealed that MICs of lead ranged between 64 and 1024 µg/ml, whereas MICs of other heavy metals used such as zinc, cadmium and mercury were 32-256, 8-64 and 1-16 µg/ml respectively. The mercury was the most effective metals in the killing of *S. aureus*. Lead correlation coefficient R value which equal to 0.53 revealed a weak to moderate fit and shows gradual susceptibility, but the zinc R^2 value was 0.85 which indicated a strong fit with steep decline in susceptibility with MIC increase. Cadmium heavy metal estimation showed R^2 with 0.84 value indicated a strong fit similar to zinc. The R^2 value of mercury was 0.66 indicated a moderate fit with more variable decline pattern. Moreover, Heavy metals are highly used in essential physiological functions or to cause toxic effects on microorganisms. At low concentrations, metals such as zinc (Zn) and copper (Cu) act as enzymatic cofactors involved in the regulation of antioxidant functions, but others such as cadmium (Cd) and mercury (Hg), even their trace amounts, can interfere with protein synthesis and enzymatic function leading to negative effects on viability and growth [26].

Several studies have linked exposure to heavy metals (HMs) with the selection of antibiotic resistance breakers (ARBs). However, epidemiological studies examining this relationship are scarce. It has been reported [25] reported that multidrug-resistant *Acenetobacter baumannii* (MDRAb) was the main bacteria isolated from wounded US soldiers during the Iraq War. In our area conflict, high levels of heavy metals (Hg, Zn, Cu, Ni, Pb and Cr) were traced in soil and debris, which may have favored resistance to these metals and antibiotics, contributing to severe infections particularly in hospitals. Furthermore, in 2021, Eggers *et al*., [26], found that, in a human population from urban areas with high heavy metal pollution (Wisconsin, USA), elevated urinary Pb levels correlated with a higher prevalence of antibiotic resistance breakers (ARBs), including methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), fluoroquinolone-resistant Gram-negative bacilli (RGNB) and *Clostridium difficile*. The presence of multiple antimicrobial agents in the environment like heavy metals, antibiotics, polycyclic aromatic hydrocarbons (PAHs), microplastics, nanoparticles and residues from disinfectants and personal care products facilitates the selection and spread of resistance genes due to plasmids, transposons and integrons, facilitating their mobilization through horizontal gene transfer (HGT), which involves the exchange of genic materials between the same or different microbial species [7-9].

Biochemical characters of eleven selected strains of *S. aureus* were changed markedly after exposure to disinfectants e.g. there were nine strains of *S. aureus* showed urease activity before exposure to disinfectants, then the number of urease -producing strains became seven and then three after the first and after the second exposure respectively. The present study showed that disinfectant exposure caused progressive and multidimensional changes in both biochemical traits and antibiotic resistance, with the biggest jump was clearly seen between after first and after second exposures. The same change was almost found by other workers who found that the number of urease positive strains were six before the first disinfectant exposure and

all strains became urease negative after the first and second exposure to a series of disinfectants [6]. These changes might be due to disturbance in the transport mechanism from and to the bacterial cell as reported by Bergman who found that exposure of *E.coli* strains to thiolutine lead to an inhibition of carbon utilization by exposed strains [23]. Furthermore, it was found elsewhere that disinfectants exposure lead to destruction of periplasmic enzymes and consequently changes in the biochemical characters of the exposed strains [30-37]. The growing concerns about the development of biocide resistance and cross-resistance with antibiotics among pseudomonads have been suggested. It is clear that clinical isolates particularly of clinical bacterial isolates should be under continuous surveillance and possible mechanisms associated with disinfectant-resistance should be further investigated particularly among hospitals where patients who are mostly immunocompromised are resident[38-47]. However, the present findings revealed that exposure of some hospital *Staphylococcus* to disinfectants could change the antibiotic sensitivity pattern, and this might lead to the erroneous conclusion that strains are unrelated. In these circumstances other typing methods are required.

CONCLUSION

The minimal inhibitory concentrations (MICs) of different antibiotics used for strains of *S. aureus* were varied and ranged from 4-1024 µg/ml. The rifampicin and nitrofurantoin were the most effective drugs by in vitro testing. Correlation coefficient (R^2) value of disinfectants was 0.961, showing a strong linear relationship between log (MIC) and % susceptible strains. It was found that MICs of lead ranged between 64 and 1024 µg/ml, whereas MICs of other heavy metals used such as zinc, cadmium and mercury were 32-256, 8-64 and 1-16 µg/ml respectively. The mercury was the most effective metals in the killing of *S. aureus*. This analysis shows that disinfectant exposure caused progressive and multidimensional changes in both biochemical traits and antibiotic resistance, with the biggest jump was clearly seen between after first and after second exposures. Biochemical characters of eleven selected strains of *S. aureus* were changed markedly after exposure to disinfectants e.g. there were nine strains of *S. aureus* showed urease activity before exposure to disinfectants, then the number of urease - producing strains became seven and then three after the first and after the second exposure respectively. The present study showed that disinfectant exposure caused progressive and multidimensional changes in both biochemical traits and antibiotic resistance, with the biggest jump was clearly seen between after first and after second exposures.

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Statement of Ethics

All the procedures involving human participation were conducted in strict accordance with ethical standards of Institutional Research Committee, Department of Scientific Research, Tikrit University as well as the 1964 Helsinki Declaration and its subsequent amendments or equivalent ethical norms.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest Statement: The author declares that he has no conflicts of interest, financial or otherwise.

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Author Contributions

Mohemid Maddallah Al-Jebouri, suggested the protocol, reading, correction and supervision of the study; Hana Salman Al-Bayati, collection and analyses of data and manuscript draft writing.

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