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ORIGINAL ARTICLE

Distribution of accessory gene regulator (agr) system and the prevalence of linezolid and mupirocin resistance in biofilm producer/non producer *Staphylococcus aureus* in Sohag University Hospitals

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Staph aureus, Biofilm, agr genes, antibiotic resistance

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ABSTRACT

Background: *Staphylococcus aureus is an important human pathogen with biofilm formation that increases antimicrobial resistance which impairs successful treatment of infections.* ***Objective****: The study aimed to identify the biofilm formation of Staphylococcus aureus and its relationship with antibiotic resistance, detection of agr (accessory gene regulator) genes among them and mupirocin, linezolid resistance.* ***Methodology:*** *swabs from infected wounds were cultured and confirmed by vitek 2 Gram positive identification cards (GP ID), Antibiotic susceptibility was assessed using disc diffusion, biofilm development was achieved through tissue culture plate technique then molecular detection of mup A gene, agr genes.* ***Results:*** *Among 114 Staph aureus isolates, 88 (77.19%) were resistant to Cefoxitin, 97 (85.09%) of the isolates were positive for biofilm, with 42.11% being weak, 22.81% moderate, and 18.42% high. a significant association between Tetracycline and rifampin resistance and levels of biofilm development with (P value=0.001 and 0.036). AGR I was present in 44.74%, AGR II was in 19.3%, AGR III was in 26.32% and AGR IV was in 8.77% of the isolated bacteria, MupA was positive in 82(71.93%) of the isolates. AGR genes were significantly present in Cefoxitin-MRSA, gentamicin, and trimethoprim sulfamethoxazole resistance with p values of 0.007, 0.018 and 0.001.****Conclusion****: agr genes are present with a higher percentage in Staph aureus that forms biofilms, They influence the development of biofilms, which increases antibiotic resistance, studying these genes and developing antagonistic effects can regain sensitivity of antibiotics also mupirocin resistance is present which necessitate its wise description not to lose its effectiveness in eradication of staphylococci nasal carriage*.

INTRODUCTION

Staphylococcus aureus is a common pathogen capable of producing both community and nosocomial infections. The severity of infections expands from mild skin and soft tissue infections to more complicated ones, such as bacteremia, osteomyelitis, and endocarditis[1].

Many multidrug-resistant bacteria form biofilms to survive in unsuitable conditions and use them as a defense mechanism. Biofilms are sessile microbial colonies surrounded by extracellular matrix and attached to non-living or living subjects. They are associated with resistance to antibiotics and the production of virulence factors [2].

Formation of biofilm is controlled by multiple regulatory systems or regulators e.g. the quorum sensing mechanism of *Staph. aureus* that includes the accessory gene regulator (*agr*) system which responds to cell density-dependent stimuli. This quorum-sensing mechanism up regulates the expression of secreted virulence factors and down regulates the expression of surface virulence factors at high cell densities. The activity of the *agr* system involves two transcripts, RNAII and RNAIII, where RNAII encodes four proteins that generate the *agr* sensing mechanism and their activation leads to RNAIII (the effector molecule of the *agr* locus) production that controls the presence of many virulence genes expressed [3].

Staphylococcus aureus develops resistance to antibiotics. This has been attributed mainly to the the procurement of different genes through the transmission of genes horizontally, modifications to drug binding sites, and the increased expression of efflux pumps [4].

Various antibiotics, including teicoplanin and vancomycin (glycopeptides), are used in clinically accepted technique to treat MRSA infection. Nevertheless, a recent variety of MRSA has developed glycopeptide medication resistance, making infection treatment problematic. Additional antibiotics used as second-line therapy for MRSA include co-trimoxazole, fusidic acid, clindamycin, mupirocin, and linezolid. However, Mupirocin resistance has increased globally

due to its frequent usage[5].so, this study aims to characterize biofilm forming *Staphylococcus aureus* phenotypically to determine its Link to medication resistance , Also identify the predominance of distinct kinds of agr (accessory gene regulator) genes within them and prevalence of mupirocin, linezolid resistance among them.

METHODOLOGY

This cross-sectional study was conducted at Sohag University Hospital at General and Plastic Surgery, Medical Microbiology and Immunology Departments from January 2023 to February 2024.

Inclusion criteria: Patients admitted at General and Plastic Surgery Departments during the period of the study with signs of surgical wound infection or infected burn with pus or exudate.

Exclusion criteria: Samples with other organisms rather than *staph aureus* or mixed infections.

Informed consent was taken from all the patients Listed in the study or their relatives.

Ethical consideration: The study has been permitted by the Faculty Ethical Committee with IRB number: Soh-Med-24-01-05MD.

The study included 265 patients with surgical site infections from general surgery and plastic surgery departments at Sohag university hospital. *S. aureus* was present in 114 of the clinical samples. Basic demographic and clinical information was recorded.

Sample collection:

The samples collected for the study were wound swabs from the surgical wounds and burns, the specimens were collected aseptically. The area around the surgical wound was cleaned with 70% ethyl alcohol and the exudates was collected from the depth of the wound using 2 sterile cotton swabs, almost care was taken not to touch the surrounding tissues to prevent contamination of the swab from resident flora then the samples were immediately sent to the laboratory.

Sample processing:

The samples were processed as soon as it reached the laboratory which includes the following: One swab was utilised for Gram staining, then smear was examined for the presence of bacteria and cellular elements using light microscope. The second swab was inoculated on (Nutrient, Mannitol salt agar, OXOID). These plates underwent incubation at 37°C for 24-28 hrs.

Biochemical reactions:

Catalase test and coagulase test were done to isolate that appeared golden yellow on nutrient agar and yellow discoloration on mannitol salt agar, then automated confirmation of the genus and species was detected by VITEK 2 compact (BioMérieux, France) using GP ID.

Test for sensitivity to antibiotics:

The sensitivity of *S. aureus* isolates to diverse antimicrobials were determined by the modified Kirby-Bauer method, readings were assessed in light of CLSI 2023, the following antibiotics were used: cefoxitin (30µg), Mupirocin (200µg), Gentamicin (10µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Erythromycin (15 µg), Clindamycin (2 µg), Linezolid (30µg), Tetracycline (30µg), Rifampicin (5 µg), trimethoprim/sulfamethoxazole (5 µg).

Biofilm detection:

Using tissue culture plates, biofilm formation by *Staphylococcus aureus* was identified according to Sultan and Nabiel [6].

Molecular detection of agr and mup A genes by conventional PCR:

Extracted DNA was collected from freshly subcultured bacteria by boiling technique in accordance with Tarchouna et al. [7], extracted DNA was stored at -20 for subsequent use.

Conventional PCR was done in thermal cycler (biometra, germany) to agr genes (agr I, agrII,agr IIIandagr IV),mupirocin resistance gene(mupA), sequences of primers (invitrogens by thermofischer) , cycling condition and amplicon size are as mentiond by [8]for agr and [5] for mupA gene as in **table 1.** Each PCR reaction was adjusted to a total volume of 25 µl using the following mixture: 12.5 µl of cosmo red Master Mix for PCR [2x] (Willowfort, Uk), 1 µl of forward primer, 1 µl of reverse primer , 3 µl of template DNA then the reaction was adjusted to 25 µl water lacking nuclease. Negative control tubes were also included without DNA template. After amplification, 10µl of the PCR mixture was analyzed by gel electrophoresis (2% agarose in Trisacetate-EDTA using ethidium bromide to stain). The Gene Ruler DNA ladder with 100 base pairs (invitrogen, Thermo fisher) was used as a DNA size marker. visualization of bands performed by DNA gel documentation system.

Table 1: Sequences of the amplified genes

Target genes	Primer name	Primer sequence (5'–3')	Amplicon size (bp)	Ref.
Pan-*agr (*PANFORWARD)		F-ATG CAC ATG GTG CAC ATG C		8
AgrI	AgrI	R-GTC ACA AGT ACT ATA AGC TGC GAT	441	
AgrII	AgrII	R-TAT TAC TAA TTG AAA AGT GGC CAT AGC	575	
AgrIII	AgrIII	R-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323	
AgrIV	AgrIV	R-CGA TAA TGC CGT AAT ACC CG	659	
MUP A	MUP A	F ; 5'-TGA CAA TAG AAA AGG ACA GG-3 '	190	9
		R ; 5'-CTA ATT CAA CTG GTA AGC C-3.		

Statistical analysis

Statistical analysis was carried out by SPSS v26 (IBM Inc., Chicago, IL, USA). Shapiro-Wilks test and histograms were used to evaluate the normality of the data distribution. Quantitative parametric data were presented as mean and standard deviation (SD). Qualitative variables were presented as frequency and percentage (%) and analyzed using the Chi-square or Fisher's exact test when appropriate. A two-tailed P value less than 0.05 was deemed statistically significant.

RESULTS

The study was done on 114 patients to detect mupirocin and linezolid resistance among *Staph aureus* causing hospital acquired infections to rationalize the use of them in treatment, and to identify the AGR gene distribution in biofilm producers/ non producers *S. aureus* strains Obtained from clinical specimens.

Demographic data among the patients under study:

Age ranged from 19 to 74 years with a mean value (± SD) of 38.9 ± (12.46) years. Sixty (52.63%) patients were male, and 54 (47.37%) patients were females. Regarding the departments, 88 (77.19%) patients were from general surgery department, and 26 (22.81%) patients were from plastic surgery department. Regarding specimens, 88 (77.19%) were from infected wound, and 26 (22.81%) were from infected burn. Regarding risk factors, liver diseases were present in 14 (12.28%) followed by hypertension 12 (10.53%) and diabetes was present in 11 (9.65%) of patients as demonstrated in table (2).

Table 2: Demographic data of the studied patients

		(n=114)
Age (years)	Mean ± SD	38.9 ± 12.46
	Range	19 - 74
Sex	Male	60 (52.63%)
	Female	54 (47.37%)
Department	General Surgery	88 (77.19%)
	Plastic surgery	26 (22.81%)
Specimen	Wound	88 (77.19%)
	Burn	26 (22.81%)
Risk factors:		
Hypertension		12 (10.53%)
Liver diseases		14 (12.28%)
Diabetes		11 (9.65%)
No		77 (67.54%)

Antibiotic sensitivity profile of the isolated *Staph aureus*:

Cefoxitin resistance (MRSA) was present in77.19%of patients followed by 66.67%erythromycin and clindamycin resistance, 65.79% rifampin resistance, 64.04%trimethoprim sulfamethoxazole, 58.77% of isolates were gentamycin resistant, regarding linezolid,6.14% were resistant and mupirocin 21.93% between the isolates were resistant as demonstrated in table(3).

Table 3: Antibiotic resistance of the isolated staphylococci.

		(n=114)
Cefoxitin (MRSA)	(MRSA)Resistant	88 (77.19%)
	(MSSA) Sensitive	26 (22.81%)
Tetracycline	Resistant	61 (53.51%)
	Sensitive	53 (46.49%)
Chloramphenicol	Resistant	16 (14.04%)
	Sensitive	98 (85.96%)
Gentamicin	Resistant	67 (58.77%)
	Sensitive	47 (41.23%)
Erythromycin	Resistant	76 (66.67%)
	Sensitive	38 (33.33%)
Clindamycin	Resistant	76 (66.67%)
	Sensitive	38 (33.33%)
Ciprofloxacin	Resistant	65 (57.02%)
	Sensitive	49 (42.98%)
Rifampin	Resistant	75 (65.79%)
	Sensitive	39 (34.21%)
Trimethoprim sulfamethoxazole	Resistant	73 (64.04%)
	Sensitive	41 (35.96%)
Linezolid	Resistant	7 (6.14%)
	Sensitive	107 (93.86%)
Mupirocin	Resistant	25 (21.93%)
	Sensitive	89 (78.07%)

Detection of Biofilm development among isolated *Staph aureus* using TCP method.

Regarding biofilm development among the isolated bacteria, 97(85.09%) of the isolates were positive of them 48 (42.11%) were weak, 26 (22.81%) were moderate, and 21 (18.42%) were high table (4).

Table 4: Biofilm formation of the isolated *Staph aureus*.

		(n=114)
Biofilm	Positive	97 (85.09%)
	Negative	19 (16.66%)
Positive biofilm	Weak	48 (42.11%)
	Moderate	26 (22.81%)
	High	21 (18.42%)

The association between the degree of biofilm development and antibiotic resistance.

There was a significant association between Tetracycline and rifampin resistance and degree of biofilm development with (***P value***=0.001 and 0.036 respectively) as in table (5)

Table 5: The association between the degree of biofilm formation and antibiotic resistance

		Weak (n=48)	**Moderate (n=26)**	**High (n=21)**	**Non biofilm (n=19)**	**P value**
Cefoxitin-MRSA	Resistant	38 (79.17%)	24 (50%)	13 (27.08%)	13 (27.08%)	0.069
Tetracycline	Resistant	34 (70.83%)	10 (20.83%)	10 (20.83%)	4 (8.33%)	**0.001***
Chloramphenicol	Resistant	8 (16.67%)	2 (4.17%)	3 (6.25%)	3 (6.25%)	0.755
Gentamicin	Resistant	32 (66.67%)	10 (20.83%)	14 (29.17%)	11 (22.92%)	0.102
Erythromycin	Resistant	35 (72.92%)	14 (29.17%)	15 (31.25%)	12 (25%)	0.378
Clindamycin	Resistant	36 (75%)	14 (29.17%)	13 (27.08%)	13 (27.08%)	0.300
Ciprofloxacin	Resistant	29 (60.42%)	12 (25%)	10 (20.83%)	14 (29.17%)	0.222
Rifampin	Resistant	36 (75%)	11 (22.92%)	15 (31.25%)	13 (27.08%)	**0.036***
Trimethoprim sulfamethoxazole	Resistant	16 (33.33%)	8 (16.67%)	10 (20.83%)	7 (14.58%)	0.638
linezolid	Resistant	4 (8.33%)	3 (6.25%)	0 (0%)	0 (0%)	0.228
Mupirocin	Resistant	12 (25%)	2 (4.17%)	6 (12.5%)	5 (10.42%)	0.251

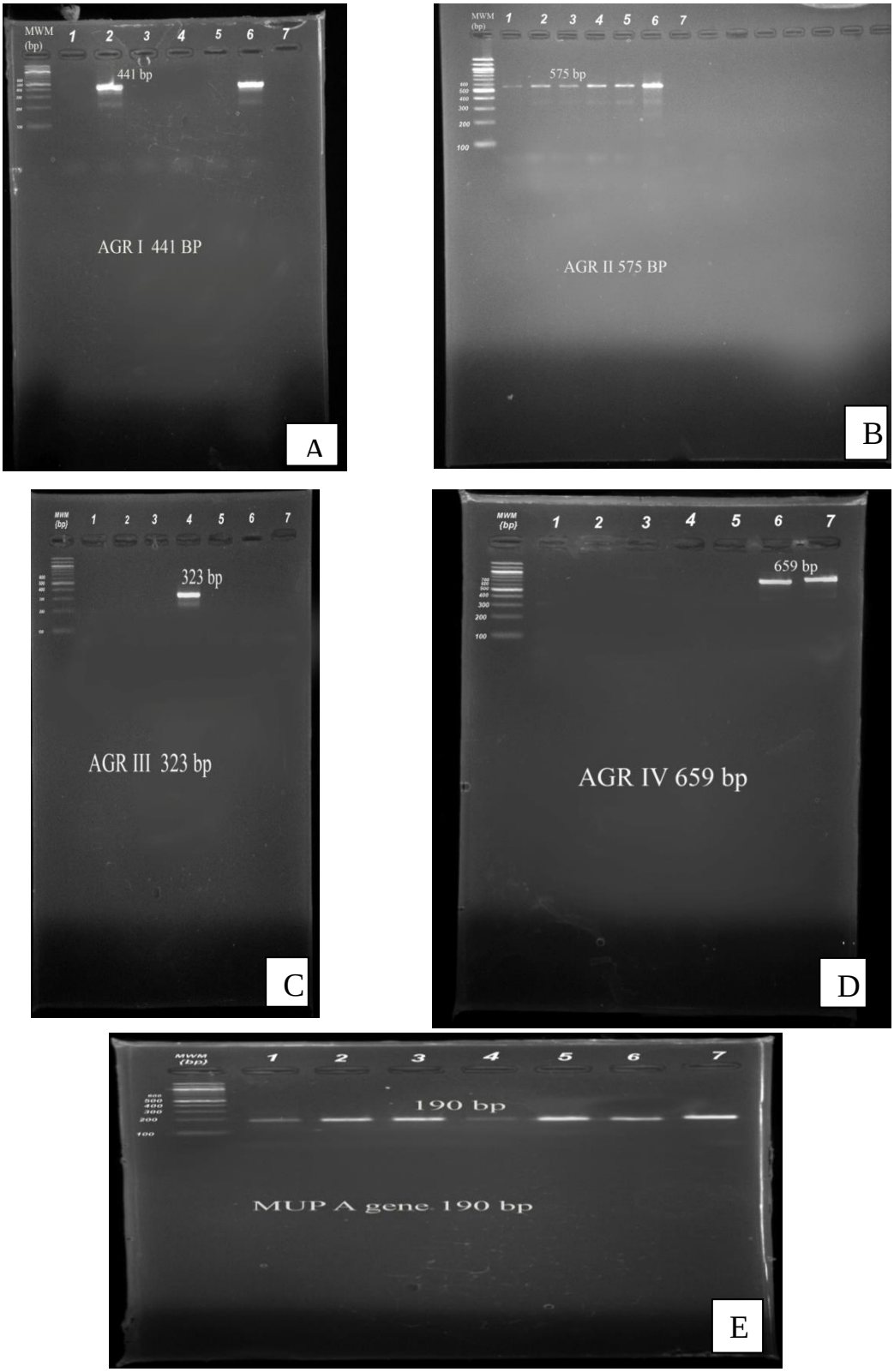
Molecular detection of agr genes and mup A genes by conventional pcr.

AGR geneI was present in 51 (44.74%), AGR gene II was in 22 (19.3%), AGR gene III was in 30 (26.32%) and AGR gene IV was in 10 (8.77%) of the isolated bacteria, MupA was positive in 82(71.93%) of the isolates as shown in table 6.

Table 6: AGR and Mup A genes in the isolated *Staph aureus*

		(n=114)
AGR gene I	Positive	51 (44.74%)
	Negative	63 (55.26%)
AGR gene II	Positive	22 (19.3%)
	Negative	92 (80.7%)
AGR gene III	Positive	30 (26.32%)
	Negative	84 (73.68%)
AGR gene IV	Positive	10 (8.77%)
	Negative	104 (91.23%)
Mup A gene	Positive	82 (71.93%)
	Negative	32 (28.07%)

AGR: Accessory gene regulator
Mup A: Mupirocin A gene



Figure(1): gel electrophoresis showing: A:agr I gene (441bp)
B: agr II (575 bp) C: agr III (323bp) D: agr IV (659bp)E: mup A gene (190 bp).

Association between the degree of biofilm formation and agr alleles.

AGR genes were significantly associated with weak and high degree of biofilm with ***p values*** of 0.036 and 0.001 respectively as in table 7.

Table 7: Association between the degree of biofilm formation and agr alleles.

	Weak (n=48)	Moderate (n=26)	High (n=21)	Non biofilm (n=19)
AGR alle I	16 (33.33%)	9 (18.75%)	14 (29.17%)	8 (16.67%)
AGR alle II	8 (16.67%)	7 (14.58%)	3 (6.25%)	3 (6.25%)
AGR alle III	12 (25%)	6 (12.5%)	7 (14.58%)	7 (14.58%)
AGR alle IV	5 (10.42%)	2 (4.17%)	4 (8.33%)	2 (4.17%)
P value	**0.036***	0.130	**0.001***	0.070

Association of AGR I, II , III , IV and antibiotic resistance of the *Staph aureus*.

AGR genes were significantly present in Cefoxitin-(MRSA), gentamicin, and trimethoprim sulfamethoxazole resistance with ***p values*** of 0.007, 0.018 and 0.001 respectively as in table 8.

Table 8: The association of AGR I, II, III, IV and antibiotic resistance of *Staph aureus*.

		AGR I (n=51)	AGRII (n=22)	AGR III (n=30)	AGR IV (n=10)	P value
Cefoxitin-MRSA	Resistant	25 (21.93%)	19 (16.67%)	23 (20.18%)	6 (5.26%)	**0.007***
Tetracycline	Resistant	23 (20.18%)	15 (13.16%)	15 (13.16%)	6 (5.26%)	0.310
Chloramphenicol	Resistant	4 (3.51%)	2 (1.75%)	5 (4.39%)	0 (0%)	0.401
Gentamicin	Resistant	23 (20.18%)	13 (11.4%)	24 (21.05%)	7 (6.14%)	**0.018***
Erythromycin	Resistant	26 (22.81%)	14 (12.28%)	17 (14.91%)	7 (6.14%)	0.608
Clindamycin	Resistant	31 (27.19%)	18 (15.79%)	24 (21.05%)	5 (4.39%)	0.083
Ciprofloxacin	Resistant	24 (21.05%)	11 (9.65%)	17 (14.91%)	4 (3.51%)	0.777
Rifampin	Resistant	31 (27.19%)	17 (14.91%)	22 (19.3%)	9 (7.89%)	0.197
Trimethoprim sulfamethoxazole	Resistant	11 (9.65%)	17 (14.91%)	24 (21.05%)	5 (4.39%)	**<0.001***
linezolid	Resistant	3 (2.63%)	2 (1.75%)	3 (2.63%)	0 (0%)	0.706
Mupirocin	Resistant	6 (5.26%)	5 (4.39%)	4 (3.51%)	1 (0.88%)	0.631

DISCUSSION

S. aureus is now among the most prominent antibiotic-resistant pathogens in hospitals and communities worldwide[3]. It has four types of *agr* systems, referred to as *agr*I through *agr*IV. Agr activity has the potential to influence *S. aureus* antibiotic resistance and infection type under specific circumstances. For instance, it has previously been shown that agrIV is linked to the presence of exfoliative toxin genes and that agr types I and II are associated with intermediate vancomycin resistance. There is debate, nevertheless, about the involvement of AGR in the pathophysiology of human infections and, in particular, in the use of antibiotics. [9].

One hundred fourteen *Staph. aureus* isolates were gathered for the current study from infected wounds and burns, the age of people under study ranged from 19 to 74 years with a mean value (± SD) of 38.9 ± (12.46) years. Sixty (52.63%) patients were male, and 54 (47.37%) patients were females. Regarding the department, 88 (77.19%) patients were from surgical department, and 26 (22.81%) patients were from plastic department. Our results are near to Adeyanju et al[10] Who discovered that males are more than females and mean age ± SD was 36.0 ± 18.6 but he reported more samples from plastic surgery30 (9.2%) than general surgery 25 (7.7%). Regarding risk factors, liver diseases were found in 14 (12.28%) followed by hypertension 12 (10.53%) and diabetes was present in 11 (9.65%) of patients, also Adeyanju et al., [10] reported that *S. aureus* carriers with underlying bone disease , cardiovascular disease and diabetes mellitus had a higher risk for *S. aureus* nosocomial infection compared with non-carriers affected by these comorbidities.

When studying antibiotic resistance profile of the isolated bacteria;MRSA was found in 77.19% of the isolates followed by 66.67% erythromycin and clindamycin resistance, 65.79% rifampin resistance, 64.04% trimethoprim sulfamethoxazole, 58.77% gentamicin resistance, regarding linezolid, 6.14% were resistant and mupirocin 21.93%of theThe isolates

exhibited resistance. Our results are higher than Saedi et al., [3] who found the incidence of MRSA was (17.1%), erythromycin resistance (52.9%), trimethoprim sulfamethoxazole(4.3%), linezolid(0%) resistance this may be due to abuse in antibiotcs in our community.

Regarding mupirocin, 25 (21.93%) of Isolates showed resistance, and 89 (78.07%) were sensitive. This was consistent with Abdulgader et al., [11] that found low mupirocin resistance rates of 17(21%) among *S. aureus* isolates.

Biofilm generation is connected with the synthesis of extracellular adhesive substances that leads to critical alterations in bacterial development and the expression of genes. There are certain components of biofilm which are: microorganisms, exopolysaccharide slime and surface; Eliminating any of these will terminate biofilm production [12].

Biofilm development was identified in the study we performed using the tissue culture plate technique, 97 (85.09%) among the isolates were positive biofilm of them 21 (18.42%) were high, 26 (22.81%) were moderate and 48 (42.11%) were weak these results are differ Derakhshan et al., [13] who found that 123(100%) isolates created a biofilm, of which 27 (21.9%) formed high biofilms ,69(56%) developed moderate biofilms and 27(21.9%) isolates produced weak biofilms .

Antibiotics susceptibility amongst bacteria forming biofilm is decreased as the presence of biofilm around bacteria prevents antibiotics from reaching them. Also, Biofilm prevents bacteria from entering the host's immune system, which frequently leads to chronic infections that are potentially fatal and difficult to cure due to development of isolates with the capacity to construct strong biofilms and the creation of strains resistant to several drugs [14].

To determine if biofilm development was correlated with resistance to any mentioned antibiotic(s) we compared the biofilm forming capacities among isolates, we detected a significant association between tetracycline and rifampin resistance and both weak and high level of biofilm development with (***P value***s=0.001 and 0.036 respectively). As opposed to Derakhshan et al., [13] who found The strong biofilm producers were more susceptible to the antibiotics than the non-strong but this difference was not significant. In our study resistance was more in weak producers of biofilms than high biofilm producers comparable to the finding of Tania et al., [12].

Molecular detection of agr genes and mup A gene revealed that AGR gene I was present in 51 (44.74%), followed by AGR geneIII was in 30 (26.32%), AGR geneII was in 22 (19.3%), and lastly AGR geneIV was in 10 (8.77%) of the isolated bacteria, MupA was positive in 82(71.93%) among isolates. Our results regarding agr genes were inconsistent to that of Derakhshan et al., [13] who reported that the agr III was present in 55 isolates (44.7%) and agr I in 25 isolates (20.3%). The agr types II and IV were not found, but similar to (Mataraci &Dosler[14] Tania et al.,[12]) who found that agr I was the most prominent followed by agr III with the following results (46.88%) of Isolates were a part of agr-I, followed by agr-III (21.88%), agr-II (6.25%), and agr-IV (6.25%). Regarding mup A gene in our study MupA was positive in 82(71.93%) among isolates higher than Fouad et al. [5] who found 12% among isolates were positive for mupA gene. The higher percent in our study may be due to horizontal gene transfer between bacteria due to lack of proper infection control practice.

When we studied the association between agr genes and degree of biofilm we reported a significant association between weak and high degree of biofilm and agr genes with ***p value***s of 0.036 and 0.001 respectively these results are similar to Usun et al., [15] who found significant association between agr I,III and biofilm production.

Investigations were conducted on any potential statistical relationships between antibiotic resistance and agr genotypes, cefoxitin resistance was more common in agr I than agrIII with ***p value*** 0.007, but gentamycin and trimethoprim sulfamethoxazole resistance were more in agrIII than agr I with ***p value***sof 0.018 and 0.001 respectively. These results are different from Derakhshan et al., [13] who found cefoxitin resistance 12% in agr I.

CONCLUSION

Our results revealed the capability of creating biofilms among isolated *Staph aureus* that also harbor agr system, these isolates have antibiotic resistance to various groups of antibiotic, agr system has a role in biofilm development that has a role in antibiotic resistance, further studying the agr system is vital to counteract The capacity to create biofilm and hence prevent chronicity and prolonged infection. Mupirocin resistance is also increasing which makes an obstacle in eradication of *Staph aureus* carriage.

Recommendation: Multiple *Staph aureus* were isolated from general surgery department, efforts are needed to identify the source and to treat effectively, judicious use of antibiotics specially mupirocin and linezolid to avoid increasingof their resistance. Infection control strategies to stop nosocomial infections from spreading.

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