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Detection of Chlorhexidine Resistant Genes in Methicillin Resistant *Staphylococcus aureus* in Mansoura University Hospitals

Salma O. Ghanim, [1], Heba E. Eldegla [2], and Gamal M. Abdel-Fattah[1]

[1]Department of Botany, Faculty of Science, Mansoura University, Egypt

[2]Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Egypt.

Corresponding author: *Salma Osama Khalid Ghanim*E-mail: Salmaghanim70@gmail.com

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Abstract Methicillin Resistant *Staphylococcus aureus* (MRSA) is the main reason for severe human infections in hospital settings as well as in the community. **Objective:** This study aims to detect chlorhexidine resistance of MRSA by phenotypic and genotypic methods. **Results:** Fifty MRSA strains were isolated from different hospitals of Mansoura University over a period of one year from December 2021 to November 2022.The antimicrobial susceptibility test was performed by the disk diffusion method. The highest resistance was shown to Penicillin (100%) and Ceftazidime (100%) and the highest susceptibility was shown to Vancomycin (72%).The minimum inhibitory concentration (MIC) towards chlorhexidine (CHG) was determined by the two-fold microdilution broth method. Reduced susceptibility to CHG at MIC ≥4 mg/L was detected in 48% of isolates (24/50). Polymerase Chain Reaction was used to detect qac genes (norA, qacA/B1, qacA/B2 and smr). The most frequently detected genes were smr in 24 isolates (100%), norA in 12 isolates (50%), and qacA/B2 in 11 isolates (45.8%), with a low prevalence of qacA/B1 (1 isolate; 4.16%).

Keywords: MRSA, Quaternary Ammonium Compounds, antiseptic resistance.

Introduction

Staphylococcus aureus strains have spread worldwide and connected with adverse patient outcomes, longer hospital stays, a major source of dangerous human infections (nosocomial infections) in both community and healthcare settings [1]. The success of *S. aureus* as a human pathogen in health care is highly associated with its multidrug resistance [2]. Starting in 1950s, *S. aureus* evolved resistance to mostly all categories of antibiotics. A decade later, this bacterium has been able to resist methicillin because of PBP2a protein [3].

Resistance often develops from cellular changes that decrease the accumulation of antiseptics, including cell wall alteration such as increasing its thickness which limits the uptake of biocides or expression of efflux mechanisms [4-7].

The use of biocides (including disinfectants and antiseptics) clinically play a main role in limiting nosocomial infections. In hospitals and other healthcare facilities, a wide range of biocides, including quaternary ammonium compounds (QACs) like benzalkonium chloride and divalent cations like chlorhexidine digluconate, are frequently utilised [8].

The activity of multidrug efflux pumps is the known mechanism of resistance to antibiotics and plays a major role multi drug resistance (MDR) phenotypes [6]. In *S. aureus*, various multidrug efflux pumps have been identified and were encoded by chromosomal genes (norA, norB, norC, mepA, mdeA, sepA and sdrM) and genes located on plasmids (e.g. qacA/B, smr) [2].

Usually, reduced susceptibility to either antibiotics or biocides can be conferred by chromosomally encoded multidrug efflux pumps, while the plasmid-encoded pumps can contribute to reduced susceptibility to biocides only [6]. The multidrug efflux pump QacA and the closely related QacB are two examples

which use the proton-motive force to expel a wide range of monovalent and divalent cationic compounds from MRSA [9] [10].

Long-term use of antiseptics containing QACs may increase the prevalence of MRSA isolates which harbor qac genes [11] . This study aimed to detect the antiseptic resistance of MRSA by phenotypic and genotypic methods.

Material and method

Sample collection and culture conditions:

Staphylococcus aureus strains were collected from Mansoura university hospitals and grown on Blood agar for blood and sputum samples, Mannitol salt agar (MSA) for nasal swab samples and CLED agar for urine samples and incubated aerobically at 37ºC overnight. *S. aureus* was recognized using traditional techniques (colony morphology, Gram stain, catalase activity, growth on mannitol salt agar, DNase test, and tube coagulase) [12] [9].

Identification of MRSA by Cefoxitin Disc Diffusion Method:

The isolates were confirmed to be methicillin resistant using a cefoxitin disc diffusion test with a 30 µg disc. According to the Clinical and Laboratory Standards Institute, zone sizes were interpreted., (resistant if ≤ 21mm) [13].

Antibiotic Susceptibility testing

The susceptibility of the isolates was determined against 8 antibiotic discs ; including: Penicillin, Ceftazidime, Azithromycin, Erythromycin, Tetracycline, Ciprofloxacin, Amikacin and Clindamycin; by Kirby-bauer disc diffusion technique using Mueller-Hinton agar [14] with the exception of vancomycin which was detected by MIC determination using microdilution broth method according to the Clinical and Laboratory Standards Institute (CLSI) [15] [9].

The antibiotic discs were purchased from Oxoid, England.

Determination of minimum inhibitory concentration (MIC) of the strains towards chlorhexidine

Following the recommendations of the Clinical and Laboratory Standards Institute (CLSI), the MIC of the study strains for chlorhexidine (CHG) was measured using the microdilution broth method [15].

Detection of quaternary ammonium compounds (QACs) resistance genes in MRSA isolates by multiplex PCR

DNA extraction for PCR:

DNA extraction was carried out using the modified colony direct method [16]. Briefly, one colony on an agar plate was lightly touched with a toothpick, mixed with 100 µl of sterile distilled water and heated for 10 min. at 99°C. Centrifugation was done at 30,000 g for 1 minute then 3 µl of supernatant were used as PCR templates. The PCR reaction was performed in a volume of 25 µl [9].

PCR amplification.

PCR was used to amplify QACs resistance genes (qacA/B, smr, and norA) with the primers illustrated in Table 1. The initial denaturation step was perfomed at 96 °C for 3 min, 25 cycles of 94 °C for 20 s, 53 °C for 20 s and 72 °C for 20 s, and the final extension step at 72 °C for 5 min [17-19].

PCR products were analyzed by 1.5% (w/v) agarose gels electrophoresis (Promega). Ethidium bromide (Promega) was added to the agarose gel for staining bands. UV illumination was used to visualise the bands [9].

Table 1 Primer sequences and their product size

Target gene	5-3(Primer sequence)		Product size (bp)
qacA/B1	AATCCCACCTACTAAAGCAG	F1	630
	GCTGCATTTATGACAATGTTTG	R1	
qacA/B2	GCAGAAAGTGCAGAGTTCG	F2	361
	CCAGTCCAATCATGCCTG	R2	
smr	GCCATAAGTACTGAAGTTATTGGA	F	157
	GACTACGGTTGTTAAGACTAAACCT	R	
norA	GGCGGTATATTTGGGGCACT	F	314
	ACGCACCTGCGATTAAAGGA	R	

Results

Collection of samples and isolation of MRSA isolates

In this study, Fifty clinical MRSA isolates were collected from different Mansoura University Hospitals. Each sample was cultured on a specific medium. The isolates were identified as *S. aureus* as shown in the figures (1, 2, 3, and 4).

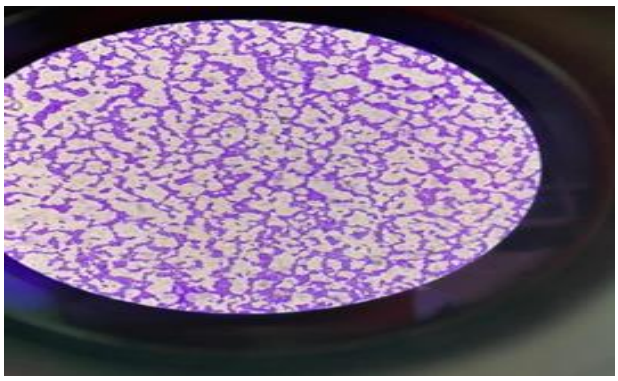


Fig. 1: Microscopic examination of Gram stained film of MRSA (100x magnification by oil immersion lens)

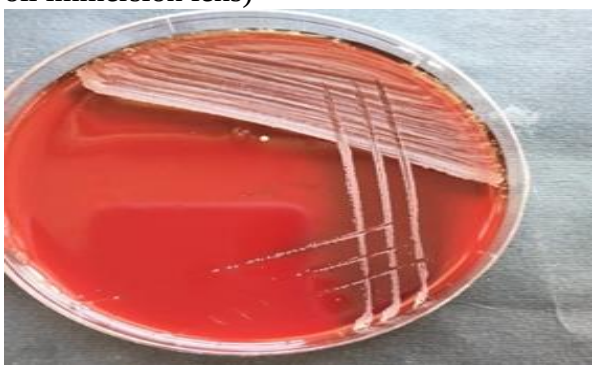


Fig. 2: Growth of MRSA on Blood agar plates

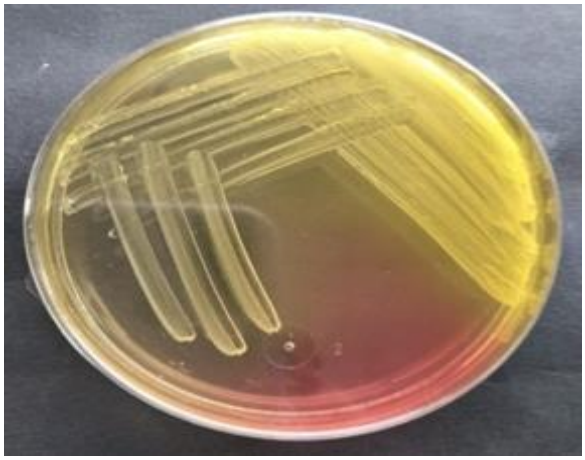


Fig.3: Growth of MRSA on MSA plate

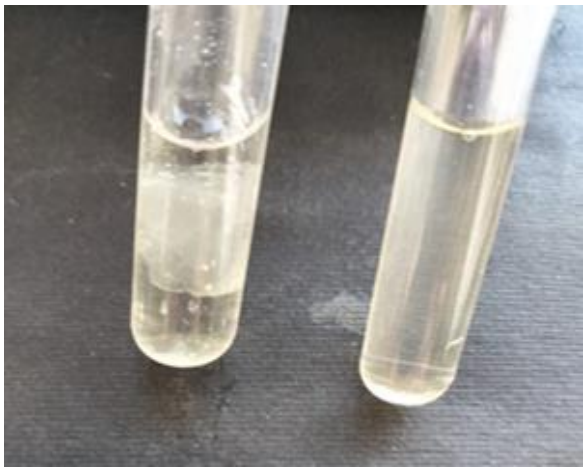


Fig.4: Coagulase-positive MRSA with negative control

Identification of MRSA by Cefoxitin Disc Diffusion Method:

Fifty *S. aureus* isolates were identified to be methicillin- resistant using the cefoxitin disc diffusion test. All the isolates were cefoxitin resistant as shown in figure 5.

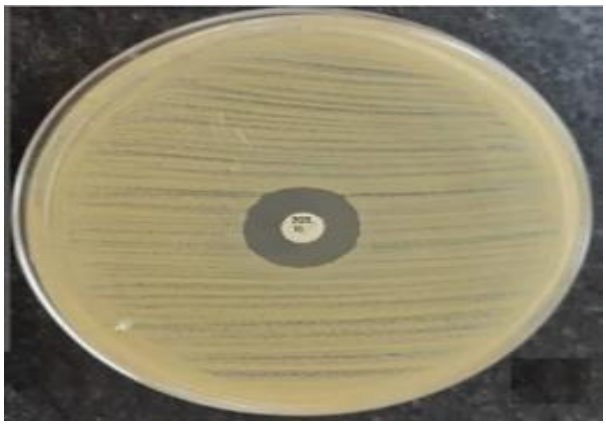


Fig. 5: Cefoxitin disc diffusion test of MRSA strains by Kirby-bauer disc diffusion method.

Antibiotics susceptibility testing

Table (2) and Figure (6) illustrate the antimicrobial susceptibility of fifty MRSA isolates to 10 antibiotics from 9 different antimicrobial groups. The greatest resistance was against Penicillin (100%) and Ceftazidime (100%) and the greatest susceptibility was exhibited to Vancomycin (72%) [9].

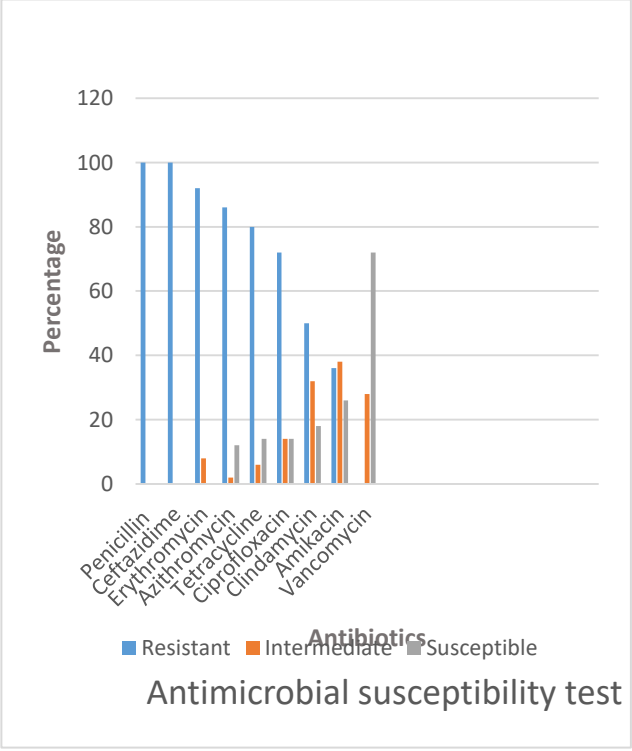


Fig.6: Antimicrobial susceptibility test of 50 MRSA isolates

Table (2): Antimicrobial susceptibility test of 50 MRSA isolates

Antimicrobial classes	Antibiotics	Symbol	Resistance (R)		Intermediate (I)		Susceptible (S)	
			No	%	No.	%	No	%
Glycopeptides	Vancomycin	VA	0	0	14	28	36	72
Aminoglycosides	Amikacin	AK	18	36	19	38	13	26
cephalosporins	Ceftazidime	CAZ	50	100	0	0	0	0
Penicillins	Penicillin	P	50	100	0	0	0	0
Macrolides	Erythromycin	E	46	92	4	8	0	0
Macrolides	Azithromycin	AM	43	86	1	2	6	12
Tetracyclines	Tetracycline	TE	40	80	3	6	7	14
Fluoroquinolones	Ciprofloxacin	CIP	36	72	7	14	7	14
Lincosamides	Clindamycin	CD	25	50	16	32	9	18

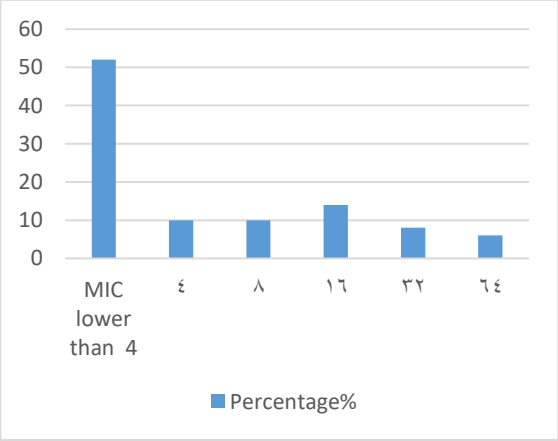
Determination of minimum inhibitory concentration (MIC) of the strains towards chlorhexidine

Reduced susceptibility to CHG at MIC ≥4 mg/L was found in 48% of isolates (24/50). The prevalence of CHG MICs between the 50 MRSA isolates was summarized in Table 6. Reduced susceptibility to CHG was most common with MIC concentration of 16 mg/L as shown in table 3 and figure 7.

Table 3: Distribution of CHG MICs among 50 MRSA isolates

MIC concentration (mg/L)	No. (%)
MIC < 4	26 (52)
4	5 (10)
8	5 (10)
16	7 (14)
32	4 (8)
64	3 (6)
Total	50 (100)

Fig. 7: Distribution of CHG MICs among 50 MRSA isolates



Detection of quaternary ammonium compounds (QACs) resistance genes in MRSA isolates by multiplex PCR

QACs resistance genes were found in the 24 MRSA isolates with decreased susceptibility to chlorhexidine (CHG) at CHG MIC ≥ 4 mg/L. The most commonly found genes were smr in 24 isolates (100%), norA in 12 isolates (50%), and qacA/B2 in 11 isolates (45.8%), with a low prevalence of qacA/B1 (1 isolate; 4.16%) [9].

The incidence of two genes together was detected in 2 isolates (4%), including qacA/B1+ smr in 1 isolate (4.16%) and norA+ smr in 1 isolate (4.16%); moreover, 11 isolates (45.8%) had three genes (qacA/B2 + norA + smr) (Table 7). It was noticed that MRSA strains with QAC genes (qacA/B2, norA and smr genes) have higher CHG MICs (Table 4) and (figure8, 9).

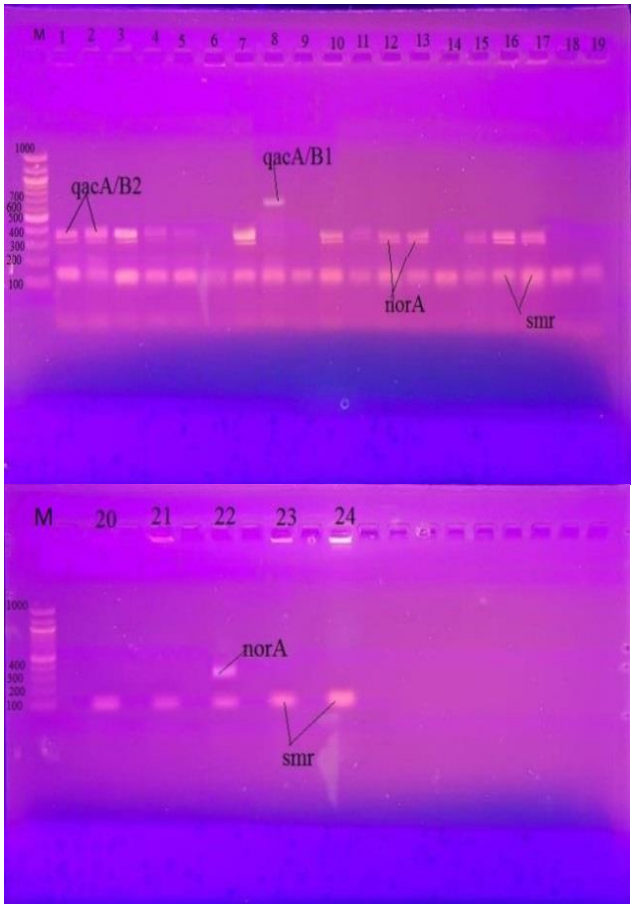
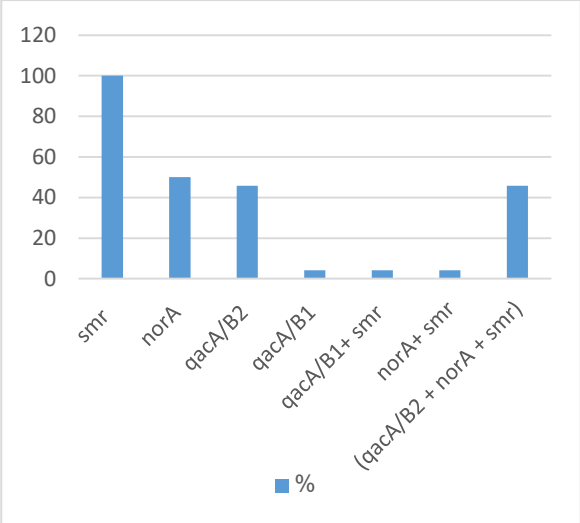


Fig.8: Agarose gel electrophoresis of the Multiplex PCR for the qacA / B1, qacA / B2, smr and norA genes for 24 CHG-resistant MRSA strains. M is the ladder DNA [9].

Table 4: Frequency of QACs resistance genes detected by Multiplex PCR among MRSA isolates with MIC ≥ 4 mg/L (n = 24).

Gene	No. (%)
smr	24 (100)
norA	12 (50)
qacA/B2	11 (45.8)
qacA/B1	1 (4.16)
qacA/B1+ smr	1 (4.16)
norA+ smr	1 (4.16)
(qacA/B2 + norA + smr)	11 (45.8)
Total	24 (100)

Fig.9: Percentage of QACs resistant genes detected by Multiplex PCR in 24 MRSA isolates with MIC ≥ 4 mg/L



Discussion

qac genes, which are antiseptic resistant genes, are so named because of their main resistance action against QACs. In our study, 55% of the MRSA cases were female population. This was similar to the findings of **Duza** [20].

The antibiotic susceptibility test of MRSA isolates in this study exhibited a high resistance against Penicillin (100%) and Ceftazidime (100%) [9]. This consisted with the results of **AlKhazraji *et al.*** [21]. The resistance to Erythromycin, Azithromycin, Tetracycline, and Ciprofloxacin were 92%, 86%, 80%, and 72%, respectively. These findings were near the findings of **Rahimi** [22].

The highest susceptibility was shown Vancomycin (72%) followed by Amikacin (26%) and Clindamycin (18%). This suggests that these antibiotics may be used for treating MRSA infections.

The antiseptic resistant genes have been broadly dispersed, particularly in staphylococcal species [23].

According to various geographic regions, the frequency of these genes differs with a vast range varying from 10% up to 80% [23-26]. The position of QACs resistant genes reported in different MRSA clones may impact their incidence rates, as these genes are commonly carried on plasmids causing their quick transfer, while in some MRSA clones, another genes carried on the chromosome control resistance to QACs [27].

In this study, a reduction in susceptibility to chlorhexidine was detected in 48% of isolates detected by CHG MIC ≥ 4 mg/L. Chlorhexidine resistance genes were found in the 24 MRSA isolates with decreased sensitivity to CHG. The most abundantly observed genes were smr in 24 isolates (100%), norA in 12 isolates (50%), and qacA/B2 in 11 isolates (45.8%), with a low prevalence of qacA/B1 (1 isolate; 4.16%) [9].

In our study, *smr* was the predominant resistance gene, like the finding of **Vali *et al*** [26]. In contrast to our study, *qacA/B* was the predominant resistance gene in previous studies [28, 29].

Also the occurrence of *qac* and *smr* became more frequently reported than previously [28] [9]. Although *qacA/B* causes resistance to a wider range of biocides than *smr* [30, 31].

When the antiseptic susceptibility to a QAC known as acriflavine and gene distributions of MRSA isolates were investigated in Japan in 1992, 71.4% of the MRSA showed resistance to this antiseptic. Also, qacA/B and smr were determined in 10.2% and 20.4 % of the isolates, respectively. However, In 1999, qacA/B and smr were found in 47.9% and 3%, respectively [32].

In Europe between 1997 and 1999, qacA/B and smr were detected at 62.6% and 6.4%, respectively, in MRSA strains [28].

These findings strongly imply that antiseptic-resistant genes are disseminated rapidly in MRSA. Consequently, monitoring MRSA that is resistant to antiseptics could reveal crucial details about how to prevent nosocomial infection.

Conclusion

These findings strongly imply that antiseptic resistant genes are disseminated rapidly in MRSA. Consequently, monitoring MRSA that is resistant to antiseptics could reveal crucial details about how to prevent nosocomial infection.

References

1. De Lencastre, H., D. Oliveira, and A. Tomasz, (2007) Antibiotic resistant Staphylococcus aureus: a paradigm of adaptive power. Current opinion in microbiology,. **10**(**5**): p. 428-435.
2. Lee, J.-H., et al.,(2011) Low concentrations of honey reduce biofilm formation, quorum sensing, and virulence in Escherichia coli O157: H7. Biofouling,. **27(10)**: p. 1095-1104.
3. Cooper, R., L. Jenkins, and R. Rowlands, (2011).Inhibition of biofilms through the use of manuka honey. Wounds UK, **7(1):** p. 24-32.
4. Gilbert, P. and A.J. McBain, (2003) Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clinical microbiology reviews,. **16(2)**: p. 189-208.
5. Piddock, L.J., (2006).Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical microbiology reviews, **19**(2): p. 382-402.
6. Poole, K., (2007) Efflux pumps as antimicrobial resistance mechanisms. Annals of medicine,. **39**(**3**): p. 162-176.
7. Russell, A., (2003) Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. The Lancet infectious diseases,. **3(12)**: p. 794-803.
8. Noguchi, N., et al.,(2005) Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes qacA/B and smr of methicillin-resistant Staphylococcus aureus isolated in Asia during 1998 and 1999. *Journal of medical microbiology,*. **54(6)**: p. 557-565.
9. Ghanim, S.O., et al., (2023).Molecular study of Chlorhexidine resistance in Methicillin Resistant Staphylococcus aureus. *Egyptian Journal of Basic and Applied Sciences*, **10(1)**: p. 493-502.
10. Brown, M.H. and R.A. Skurray, (2001).Staphylococcal multidrug efflux protein QacA. *Journal of molecular microbiology and biotechnology*, **3(2)**: p. 163-170.
11. Shi, G.S., (2016).Effects of long term use of contact lens disinfecting solutions on the presence of bacteria-harbouring antiseptic-resistance genes in the conjunctival sac, eyelid and on the lens and lens accessories of orthokeratology lens wearers.
12. Forbes, B.A., D.F. Sahm, and A.S. Weissfeld,(2007) Diagnostic microbiology.: Mosby St Louis.
13. Humphries, R., et al., (2021) Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. *Journal of clinical microbiology,*. **59(12)**: p. e00213-21.
14. Bauer, A., (1966) Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology,*. **45(3)**: p. 149-158.
15. Wayne, P., (2006).Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A7. Clinical and Laboratory Standards Institute,
16. Tsuchizaki, N., J. Ishikawa, and K. Hotta, (2000) Colony PCR for rapid detection of antibiotic resistance genes in MRSA and enterococci. *The Japanese Journal of Antibiotics,*. **53**(6): p. 422-429.
17. Huet, A.A., et al., (2008) Multidrug efflux pump overexpression in Staphylococcus aureus after single and multiple in vitro exposures to biocides and dyes. Microbiology,. **154(10)**: p. 3144-3153.
18. Zmantar, T., et al., (2011) Detection of macrolide and disinfectant resistance genes in clinical Staphylococcus aureus and coagulase-negative staphylococci. BMC research notes,. **4(1)**: p. 1-9.
19. Heir, E., G. Sundheim, and A. Holck, (1995) Resistance to quaternary ammonium compounds in Staphylococcus spp. isolated from the food industry and nucleotide sequence of the resistance

plasmid pST827. *Journal of Applied Bacteriology,*. **79(2)**: p. 149-156.
20. Duza, S.S.,(2021) Evaluation of Virulence Properties of Staphylococcus aureus Isolated from A Tertiary Level Hospital of Dhaka City., University of Dhaka.
21. AlKhazraji, N.Z., A.S. Al Jubouri, and M.F. Al Ma, (2020).Detection of Antiseptic Resistant Genes and Biofilm Formation in Multidrug Resistant Staphylococcus aureus in Baghdad Hospitals. *Iraqi journal of biotechnology*, **19(2)**.
22. Rahimi, F., (2016) Characterization of resistance to aminoglycosides in methicillin-resistant Staphylococcus aureus strains isolated from a tertiary care hospital in Tehran, *Iran. Jundishapur journal of microbiology,*. **9(1)**.
23. Jaglic, Z. and D. Cervinkova, (2012) Genetic basis of resistance to quaternary ammonium compounds--the qac genes and their role: a review. Veterinarni Medicina,. **57(6)**.
24. Miyazaki, N.H.T., et al., (2007) The presence of qacA/B gene in Brazilian methicillin-resistant Staphylococcus aureus. Memorias do Instituto Oswaldo Cruz,. **102**: p. 539-540.
25. Smith, K., C.G. Gemmell, and I.S. Hunter, (2008) The association between biocide tolerance and the presence or absence of qac genes among hospital-acquired and community-acquired MRSA isolates. *Journal of antimicrobial chemotherapy,*. **61(1)**: p. 78-84.
26. Vali, L., et al., (2008) Frequency of biocide resistance genes, antibiotic resistance and the effect of chlorhexidine exposure on clinical methicillin-resistant Staphylococcus aureus isolates. *Journal of antimicrobial chemotherapy,*. **61(3)**: p. 524-532.
27. Pulido, R.P. and A. Gálvez, (2013) Biocide tolerance in bacteria. *International Journal of Food Microbiology,*. **162**: p. 13-25.
28. Mayer, S., et al., (2001) Distribution of the antiseptic resistance genes qacA, qacB and qacC in 497 methicillin-resistant and-susceptible European isolates of Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy,*. **47(6)**: p. 896-897.
29. Noguchi, N., et al., (2002).Frequency and genetic characterization of multidrug-resistant mutants of Staphylococcus aureus after selection with individual antiseptics and fluoroquinolones. Biological and Pharmaceutical Bulletin, **25(9)**: p. 1129-1132.
30. Heir, E., G. Sundheim, and A.L. Holck, (1998) The Staphylococcus qacH gene product: a new member of the SMR family encoding multidrug resistance. FEMS microbiology letters,. **163(1)**: p. 49-56.
31. Leelaporn, A., et al., (1994) Multidrug resistance to antiseptics and disinfectants in coagulase-negative staphylococci. *Journal of Medical Microbiology,*. **40(3)**: p. 214-220.
32. Noguchi, N., et al., (1999) Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant Staphylococcus aureus. FEMS microbiology letters,. **172**(2): p. 247-253.