

ANTISEPTICS RESISTANCE GENES (*QACA/B*, *SMR*) DETECTION AND EXPRESSION IN *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

This study was aimed to investigate the resistance of *S. aureus* to different antiseptics. This research indicates that the resistance of *S. aureus* to antiseptics is due to possessing either the *smr* gene or the *qacA/B* genes that associated with decreased susceptibility to antiseptics there for this study aimed to determine the frequencies of *S. aureus* chloroxylenol resistant isolates and the presence of the previous genes in these isolates as well as the effect of chloroxylenol on the expression of these genes. 189 clinical isolates isolated from skin infections identified as *S. aureus* in Baghdad by microscopical and biochemical tests. The chloroxylenol resistance *S. aureus* isolates was identified and chloroxylenol MIC was evaluated for these isolates. Antiseptic resistance genes (*qacA/B*, *smr*) were detected by PCR method and the results revealed that 21(84%) out of 25 isolates harbored *qacA/B* gene. While the *smr* gene was not demonstrated in any isolates. Furthermore, the chloroxylenol had no effect on *qacA/B* gene expression in these isolates.

Keywords: chloroxylenol, disinfectants, skin and soft tissue infections.

جواد وعتبة

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الكشف والتعبير عن جينات مقاومة المطهرات (*qacA/B, smr*) في بكتريا المكورات العنقودية الذهبيةنوال محمد عتبة²أصالة أركان جواد¹

استاذ

باحث

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كلية الفارابي الجامعة

المستخلص

أشارت النتائج إلى أن مقاومة بكتريا المكورة العنقودية الذهبية للمطهرات المختلفة ترجع إلى امتلاك إما جين *smr* أو جينات *qacA / B* المرتبطة بانخفاض الحساسية للمطهرات لهذه الدراسة وسط تحديد ترددات عزلات المكورات العنقودية الذهبية المقاومة للديتول ووجود الجينات السابقة في هذه العزلات وكذلك تأثير الكلوروكسيلينول على التعبير عن هذه الجينات. 189 عزلة سريرية تم عزلها من إصابات الجلد المختلفة و تم تحديدها بالمكورات العنقودية الذهبية في بغداد بواسطة الاختبارات المجهرية والكيميائية الحيوية. تم التعرف على عزلات المكورات العنقودية الذهبية المقاومة للديتول وتحديد تركيزه الأدنى لهذه العزلات. تم الكشف عن جينات مقاومة المطهرات (*smr*, *qacA / B*) بطريقة تفاعل البوليميراز المتسلسل وأظهرت النتائج أن 21 (84%) من 25 عزلة تحتوي على جين *qacA / B*. بينما لم يظهر الجين *smr* في أي عزلات. علاوة على ذلك، لم يكن للديتول أي تأثير على التعبير الجيني *qacA/B* في هذه العزلات.

الكلمات المفتاحية: كلوروكسيلينول، المطهرات، مضخات تدفق الادوية المتعددة، التهابات الجلد والانسجة

INTRODUCTION

Staphylococcus aureus is a gram positive bacteria that grows in the form of clusters with 0.5 to 1.5µm in diameter portrayed by individual cocci since division of cell happens in more than one plane, these bacteria are non-motile, non-spore forming. (9). *S. aureus* are considered as a major human pathogen that causes a wide range of clinical infections. Disinfectants and antiseptics play a major role in control and the prevention of these infections. Dettol(chloroxylenol) are widely used for disinfection, antisepsis, and cleaning in Iraq. *S. aureus* colonizes both artificial and animate tissue in humans causing chronic persistent infections that are difficult to cure. It is a leading cause of wounds, skin and soft tissue infections, bacteremia, and infective endocarditis as well as osteoarticular, pleuropulmonary, and device-related infections. The annual incidence of *S. aureus* bacteremia in the United States is 38.2 to 45.7 per 100,000 person-years; elsewhere in the industrialized world, the incidence is approximately 10 to 30 per 100,000 person-years (19,26). Antibiotics have become the key factor for the rise of drug-resistant strains of groups of microorganisms. Almost all synthetic or semi-synthetic antimicrobials presented with many side effects. To resolve such a problematic various plant-therapeutic and-antimicrobial compounds have the most attention. Especially when these substances have no or minimal side effects (14). Although strains of *S. aureus* have been emerged and developed resistance to some antibiotics, some strains still sensitive to more commonly known antibiotics. Methicillin resistant *staphylococcus aureus* (MRSA) are resistant to antibiotic methicillin and others related antibiotics (1). Accordingly, one of the significant characteristics of *S. aureus*, its ability to secrete toxins which involving in severity diseases that including: exfoliative toxins, toxic shock syndrome toxin-1 and staphylococcal enterotoxins which implicated in staphylococcal scalded skin syndrome, toxic shock syndrome and staphylococcal food poisoning (SFP), respectively (28). Diarrhoea caused by different enteropathogens has been

recognized as a major clinical problem for calves worldwide. Among these bacteria *Salmonella typhimurium*, *Clostridium perfringens* and *Staphylococcus aureus* are believed to be the major microbial causes of diarrhea in calves (2). The emergence and rise of antibiotic and antiseptic resistance among staphylococci are a burden in health care facilities and communities (25) leading to increase mortality and morbidity in humans, highlighting the importance of infection control practices and the key role of biocides in healthcare. However, the widespread use of biocides in hospitals has led to concerns about the emergence of disinfectant-resistant bacteria (16). Large amounts of biocides are consumed within the different settings, including the medical environment where they are used for disinfection, antisepsis, and cleaning (25). One of these antiseptics commonly used in Iraq is chloroxylenol (Dettol), its effect through the denaturation of protein and also acts on the cytoplasmic membrane of microorganisms (3). The plasmid-borne resistance genes *qacA/B* and *smr* encode multidrug efflux pumps that yield high-level resistance to quaternary ammonium compounds, chlorhexidine, chloroxylenol and other antiseptic compounds commonly used. The *qacA/B* genes are linked to higher minimal inhibitory concentrations (MICs) and antiseptic agent tolerance (10). Which was integrated into the cytoplasmic membrane via transmembrane segments and contained distinct subsets of amino acid residues involved in substrate recognition and binding (5). Research has shown that antiseptic exposure is linked to the carriage of *qac* and *smr* genes in *Staphylococcus* (11). This study aimed to determine the frequencies of *S. aureus* chloroxylenol resistance isolates prevalence and the presence of *qacA/B* and *smr* in chloroxylenol resistance *S. aureus* Iraqi isolate. as well as the effect of chloroxylenol on the expression of these genes. And the detection of the presence of *qacA/B* and *smr* in *S.aureus* was done by the nucleotidic sequences of the *tqi* amplicons, were shown to be highly associated with the 16S rDNA gene sequences in a phylogenetic tree (8).

MATERIALS AND METHODS

Ethical statement : All participants agreed to provide the investigator with swab specimens.

Informed and written consent was obtained from all participants in the study approved by the College of Science Research Ethics Committee at University of Baghdad (reference number; CSEC/0920/0060).

Specimen collection

One hundred and eighty- nine different clinical specimens suspected as *S. aureus* were collected from patients attending hospitals, private clinics and from private clinical laboratories in Baghdad/Iraq. The collection of specimens (wounds, burn, and boils) were accomplished by rotating a sterile moistened swab with sterile normal saline, placed in brain-heart infusion broth (BHI) as a transport medium, and incubated overnight at 37°C. The specimens were re-cultured on blood agar, and mannitol salt agar. Isolates of *S. aureus* were identified in accordance to the morphological features on laboratory culture media and biochemical tests. Staphylococcus species was diagnosed by using Gram stain , colony shape, oxidase , catalase, growth on manitol salt agar, growth at 15 and 45 Co. according to their morphological, physiological and biochemical properties (12).

Estimation of chloroxylenol minimum inhibitory concentration : The concentration of chloroxylenol in Dettol bottle used in this

study was 4.85%. MICs of chloroxylenol were detected by a modified agar dilution method according to the recommendations of Clinical and Laboratory Standards Institute (CLSI)(CLSI, 2020). From a pure overnight culture, at least 3-4 colonies were picked and suspended entirely in about 5 ml normal saline using a vortex. Broth culture was visually compared with the McFarland 0.5 standard. The inoculum suspension (200µl) was spotted onto Muller Hinton agar surface. Subsequently, the inoculum spots were dried and plates were positioned upside down prior to incubation, then all plates were incubated overnight at 37°C. The MIC was read as the lowest concentration without visible growth (17).

Detection of *qaqA/B*, *smr* , and *tpi* in *S.aureus* isolates

DNA was extracted using ABIOPure™ Total DNA kit (ABIOPure, USA) from 25 *S. aureus* isolates resistant to chloroxylenol. Conventional PCR technique was carried out to amplify fragments of *tpi*, *qaqA/B*, and *smr* using specific primer pairs (Table 1).The PCR reaction protocol listed in (Table 2) was carried out in 20µl as a final volume.PCR products were analyzed in 1.5% agarose gels by electrophoresis (6).

Table 1. Primers used to detect *qaqA/B*, *smr* gene and *tpi* (18). ,(MLST Database)

Target gene primers	Primer sequencing 5' — 3'	Product size (bp)
<i>tpi</i> -F <i>tpi</i> -R	TCG TTC ATT CTG AAC GTC GTG AA TTT GCA CCT TCT AAC AAT TGT AC	475
<i>qaqA/B</i> - F <i>qaqA/B</i> - R	GTTGGAGCTGGTATGGCTTC CGTTTGCAAGCTGTTTTATCC	292
<i>smr</i> - F <i>smr</i> -R	ATGCCTTATATTTATTTAATAATAGCC ATGCGATGTTCCGAAAATGTTTAAC	321

Table2. The PCR reaction protocol (20),(MLST Database).

Steps	Temperature °C	Time	Cycle
Initial Denaturation	95	05:00min.	1
Denaturation	95	00:30 Sec.	
Annealing	55	00:30Sec.	30
Extension	72	00:30Sec.	
Final extension	72	07:00min.	1
Hold	10	10:00 min.	

Annealing temp.for *qaqA/B*, *smr*, and *tpi* is 55°

Effect of chloroxylenol on the gene expression of *qaqA/B* , *smr*, and *tpi* genes: RNA was extracted from *S.aureus* isolates before and after treatment with chloroxylenol

at sub MIC for 24hrs according to the protocol of TRIzol™ (24). qPCR was performed by GoTaq® 1-Step RT-qPCR Master Mix (15). The thermocycling protocol utilized to estimate the gene expression of

qacA/B and smr is summarized in (table 4). The results were normalized to the level of tpi expression. Tpi housekeeping gene was used because it is more accurate in diagnosis of S.

aureus than the classical methods of S. aureus diagnosis, and it was also used to calculate the gene expression of the examined genes.

Table 3. program that used for qacA/B, smr, and tpi genes expression in the qPCR.

Steps	Temperature °C	Time	Cycle
RT.enzymeactivation	37	15:00 min.	
Initial denaturation	95	10:00 min.	1
Denaturation	95	15 Sec.	
Annealing	55	30 Sec.	
Extension	72	30 Sec.	40

RESULTS AND DISCUSSION

chloroxylenol revealed antibacterial activity at different concentrations and the results showed that the chloroxylenol MIC was 0.303% for one isolate, 0.152% for 14 isolates, 0.076% for 10 isolates and 0.038% for 19 isolates. As shown in (table 4) Saber in 2019 found that the MIC of S.aureus isolates was >625µg/ml for chloroxylenol (22). In 1993 Dawaf demonstrated that MIC values of chloroxylenol for S.aureus was 128µg/ml respectively (7). In this study, all investigated 25 S.aureus isolates demonstrated 100% harbored the housekeeping gene (tpi) (Figure 1). Identification of S.aureus by using tpi gene is more accurate than bacteriological and biochemical assays. 21(84%) out of 25 isolates harbored qacA/B gene (Figure 2). More likely, the rest four isolates employed other genes to resist the chloroxylenol. While the smr gene

was not located in any isolate (Figure 3). The prevalence of qac and smr genes around the world is extremely diverse. In 2020 AlKhazraji found that qacA/B1 gene found in 2.5%, qacA/B2 in 5%, and smr gene was not detected in any S.aureus isolates collected from Baghdad (4). Saber in 2019 indicated that out of Sixty-five isolates of S. aureus from Baghdad were collected from different clinical sources, 14 of them were positive to qacA/B and only two were positive to smr gene (22). While Ignak in 2017 revealed the prevalence of qacA/B and smr genes in S.aureus isolates 10.3%, and 13.8%, respectively in Turkey(13).While, in Malaysia Antiseptic genes in S. aureus isolates (qacA/B and smr) were identified in 83.3% and 1.6%, respectively (23).In Iran, the frequency of qacA/B and smr genes among clinical isolates was 19.4% and 45.2%, respectively (21).

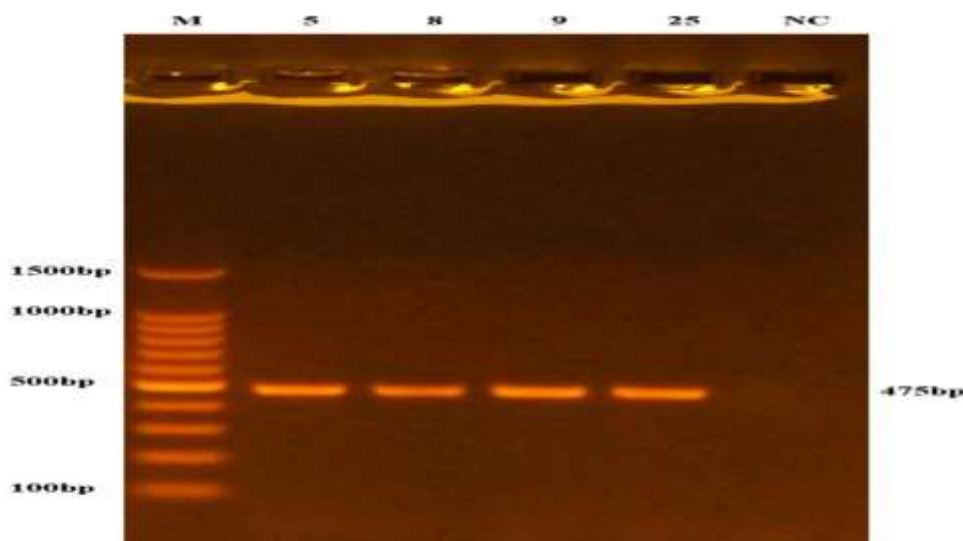


Figure 1. Results of the amplification of tpi gene of S.aureus isolates were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker, electrical power was turned on at 100v/mAmp for 60min. Lanes 5, 8, 9 and 25 resemble PCR products

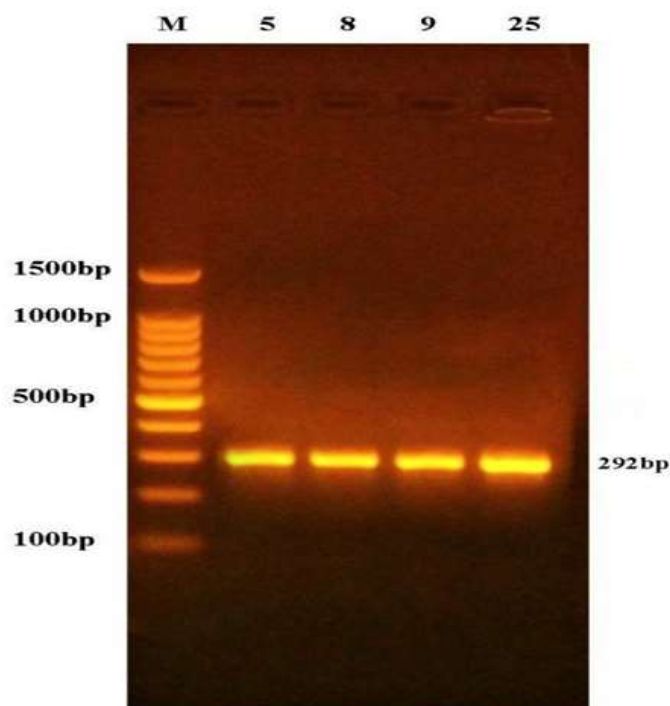


Figure 2. Results of the amplification of qacA/B gene of S.aureus isolates were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker, electrical power was turned on at 100v/mAmp for 60min. Lanes 5, 8, 9 and 25 resemble PCR products

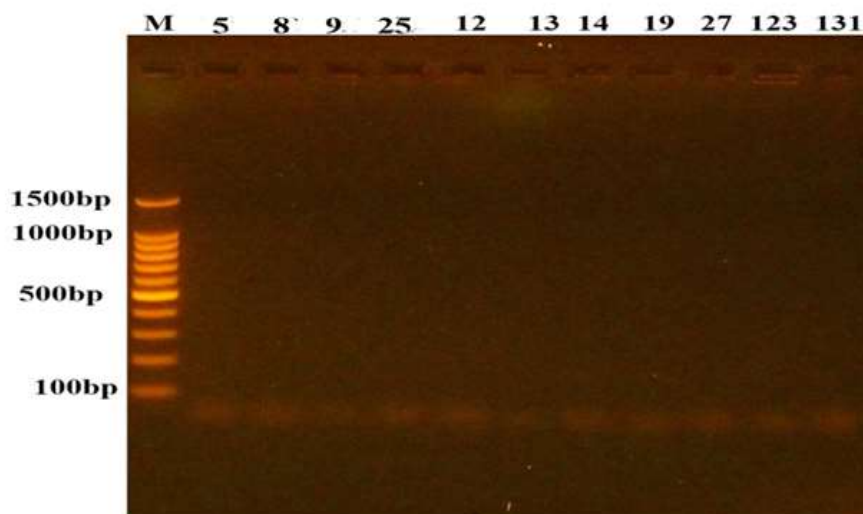


Figure 3. Results of the amplification of smr gene of S. aureus isolates were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker, electrical power was turned on at 100v/mAmp for 60min. Lanes 5-131 resemble PCR products

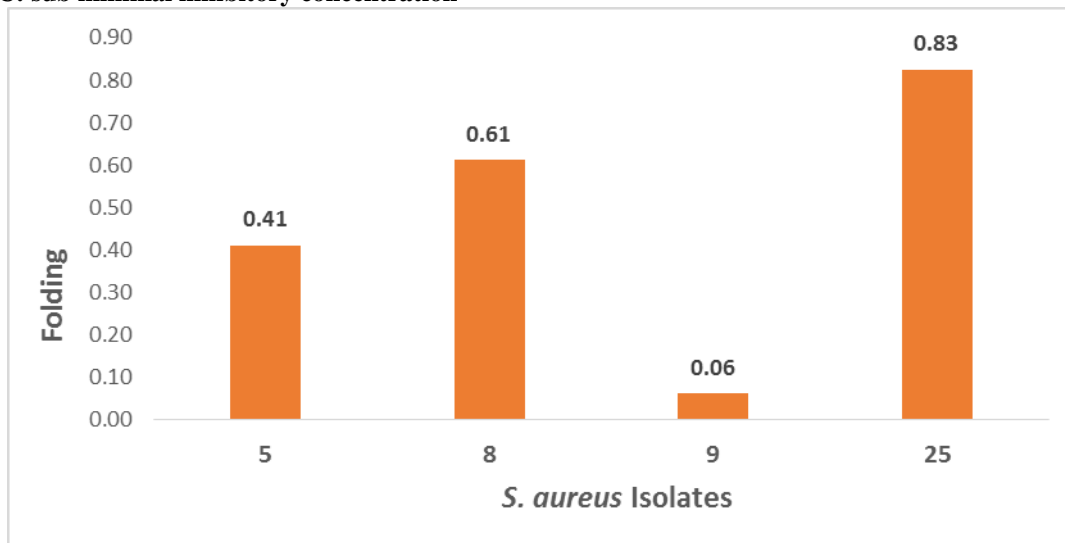
Four S. aureus isolates were chosen randomly to assess the impact of chloroxylenol at sub MIC (Table 4) on the gene expression of qacA/B. The results depicted in Figure 4 demonstrated that the qacA/B gene expression

was down- regulated in the four isolates. Accordingly, the present findings suggested a contribution of additional gene(s) might participate in the resistance of chloroxylenol S. aureus

Table 4. chloroxylenol sub-MIC of selected isolates in current study.

<i>S. aureus</i> isolate	Sub MIC* (%)
S5	0.075
S8	0.303
S9	0.151
S25	0.151

*sub MIC: sub minimal inhibitory concentration

**Figure 4. qac A/B gene expression in *S. aureus* isolates**

Antiseptic resistance genes (*qacA/B*, *smr*, *qacG*, *qacH*, and *qacJ*), encoding multidrug efflux pumps which are carried by plasmids, were identified in *Staphylococcus* genus for the first time. Antiseptic resistance gene studies of *qacA/B* and *smr* mostly focused on *S. aureus* (27). Many studies have shown both the presence of plasmid-mediated antiseptic resistance genes such as *qacA/B*, *smr*, *qacG*, *qacH*, and *qacJ* and reduced susceptibility to antiseptic agents. Other researchers have reported that plasmids carry antiseptic resistance genes together with antibiotic resistance genes and contribute to the development of resistance in pathogens (13). Marked difference in the frequency and type of antiseptic resistance genes between countries or even between different hospitals in the same country, encourage us to determine the frequencies of *qacA/B* and *smr* genes in Iraqi clinical *S. aureus* isolates treated with chloroxylenol and to evaluate the expression of them. *qacA/B* gene was more frequent in *S. aureus* local isolate (84%) than *smr* gene and may be play a role in bacteria resistance to chloroxylenol. Further surveillance and research are necessary to better understand the effect and the role that the antiseptic resistance organisms play in the hospitals and in the community infections.

CONCLUSION

This study confirmed that the presence of *qacA/B* gene in some *S. aureus* in Iraqi isolates resistance to chloroxylenol, although chloroxylenol revealed no effect of on *qacA/B* gene expression in these isolates. However, the resistance to chloroxylenol may be achieved by another efflux pump mediated genes other than *qacA/B* or other factors. We found that the topical using of chloroxylenol as antiseptic for disinfection *S. aureus* and other nosocomial infection were effective on bacterial prevent or inhibition. but we could not consider chloroxylenol as an excellent antiseptic because it's could eradicate the bacterial infection only in moderate relative expression.

Declaration of conflicting interests

Authors declare no conflict of interests

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