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Detection a New Antiseptic Resistant Variant of *qac* Gene in Some Multi Drug Resistant *Staphylococcus aureus* Isolated from Different Clinical Sources

Noor Saber ***Nuha Joseph Kandala*** [*] ***Hiba Abdulameer Mohammed***

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Abstract:

The increasing use of antiseptic compounds creates selective pressure cause emergence of antiseptic resistance among *Staphylococcus aureus* .Resistance mechanism of antiseptic is driven mainly by multi drug resistant (MDR) efflux protein.Sixty five isolates of *S.aureus*were collected from different clinical sources and subjected to 11 antibiotics most of them are recognized by efflux systems as extruded substrates. Range of efflux activity was estimated using cartwheel method. Simultaneous discrimination of antiseptic coding genes (*qac*A/B, *smr* and *nor*A)as well as *nuc* and *mec*A genes among multidrug resistant*S.aureus*(MRSA) isolates was preformed using multiplex PCR assay , 61 isolatesamong 65 were positive to*nuc*and *mec*A genes, 58 of them were positive to *nor*A, 14 of them were positive to *qac*A/B and only two were positive to *smr*. All isolates detected with *qac*A/B characterized by fluoroquinolones resistant and most of them show strong efflux activity at cartwheel assay, all of the 14 isolates positive *qac*A/B were sequenced to differentiate between variants depending on position 323 (aspartic in QacA, alanine in QacB), 3 of them harbored asparagines amino acid at position 323 and considered to be a new variants that reported for the first time.

Keywords: Efflux pump , Multiplex PCR , *Staphylococcus aureus*, Transmembrane domain,

Introduction:

Antiseptic efflux pumps possessed by *S.aureus* belong mainly to major facilitator superfamily (MFS) and small multi drug resistance family (SMR) and harbored by plasmid- or chromosomally based located (1,2). Staphylococcal efflux determinants can be specialized to export one type of drug such as TetK efflux pump or transport vast array of unrelated compound (antiseptics, dyes, and antibiotics) included mainly chromosomal NorA efflux protein (3). Plasmid encoded proteins Quaternary Ammonium Compunds (QacA) and (QacB)(4) and Small Multidrug resistance family*(smr)* (5) .Effluxes machinery belong to MFS and SMR family that uses secondary transporting system depend on ion moving (H+, K+) in electrochemical concentration gradients called proton motive force as energy source involved in transporting substrate from inside bacteria cell to surrounding environment to reduc intracellular concentration of toxic compound(1 , 6 ,7).

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
[*]Corresponding author: nuhakasto@yahoo.com

The multi-drug resistance (MDR) pattern efflux pump that belongs to efflux family when consists of different number of monomers, 4 segments (Smr), 12 segments flouroquinoline efflux transporter protein (*nor*A) and 14 segment (QacA, QacB) called transmembrane helices(8,9).Efflux pump is classified as one of the mechanisms contributes to MDR phenotype in methicillin resistant *S.aureus* since resistance of antibiotics-biocides is interface (10).Chromosomal active efflux mediated by NorA extrude multiple targets included quaternary ammonium compounds (tetraphenylphosphonium bromide, cetrimide, benzelkonium chloride), fluoroquinolones antibiotics (Norfloxacin, enoxacin, ofloxacin, ciprofloxacin) and intercalating dyes(11,12) and (13).Although the chromosomal NorA transporter possesses higher recognizing target ability than plasmid based effluxes, QacA/B efflux pump with 14 α-helix transmembrane segments displayed exceptionally wide range of substrate specificity, *qac*A shared great homology; genetic resemblance with *qac*B which distorted in 6 positionS only Single nucleotide polymorphisms (SNPs) at codon 323 provide non-conserved change in amino acid (Aspartic in QacA protein to Alanine in QacB protein) which clarifies their specificity differences

(6,14),now *qac*A and *qac*B are called *qac*A/B genes since they share high homology (5),some early observations hypothesized that plasmid based-antiseptic resistance can be found as part of *S.aureus* chromosome since it is harbored by transposon (6).Biocides resistance in *S.aureus* also contributed by *smr*; historically known (*qac*C, *ebr*and *qac*D) which confers resistance to some quaternary ammonium compound and ethidium bromide (15,6).As far as we know , the current study is the first report that was investigated the occurrence of antiseptic resistance determinates among Methicilin Resistant *Staphylococcus aureus* (MRSA) isolates collected from different clinical cases.

Materials and Method:

Collection and Characterization of *S.aureus*Isolates

The 65 clinical samples were collected from different sites of patients attending AL – WassetyHospital, Al-Yarmouk Hospital, kindy Hospital and Al-Karama hospital in Baghdad, these samples were distributed as follows: food ulcers(44 isolate) , urinary tract infection(12 isolate), nasal(5 isolates), oral (2 isolates) and wound(2 isolates). These samples were collected from January 2017 to March 2017. Sixty one isolate were identified as *S.aureus*, these isolates were characterized according to Bergey's manual of Systematic Bacteriology (16).

Antibiotics Susceptibility Test

Resistance pattern of collected isolates were tested against 11 antibiotics and most of them were resisted by efflux pump including cefoxitin(FOX-30 µg), meropenem (MEM-10µg), ceftriaxone (CRO-30µg), norfloxacin (NOR-10µg), levofloxacin (LEV-5µg), ciprofloxacin (CIP-5µg), erythromycin (E-15µg), tetracycline (T-10µg),trimethoprim (TM-5µg), tigicycline (TGC-15µg)and Vancomycin(VA- 30)using Kirby-Bauer method.

Cartwheel Test

Conventional Ethidium bromide-agar evaluation procedure(17)was used to evaluate the expression level of efflux transporter protein within sixty one isolates; to achieve this purpose multiple concentration of trypton soy agar containing ethidium bromide plate were prepared (0, 0.25, 0.5, 1, 2 and 4) mg/l .

Minimum Inhibitory Concentration(MIC)

Stock solutions of benzylkonium chloride (50%), chlorohexidine glucanoate (4%), cetrimide and chloroxylenol prepared according to (18).

DNA Extraction and Multiplex PCR

All bacterial isolates were activated on 10 ml of LauriaBertani **(**LB) broth containing 0.2mg/ml of ampicillin, and incubated at 37°C for 18hr,Genomic DNA was extracted using salting out method (19). Plasmid DNA was extracted efficiently using PureYield™ Plasmid Miniprep System (Promega/USA). Simultaneous discrimination using multiplex PCR was accomplished to provide rapid efflux pumps detection and initial diagnosis of methicillin resistant *S.aureus* in one PCR run.The ideal reaction annealing temperature of (*nuc*, *nor*A and *qac*A/B-1) primers mixture was 54ºC while annealing temperature for *mec*A was 60 ºC for 45 seconds in the presence of 50 ng genomic DNA as template in one PCR program using two temperatures (54,60)ºC Table1, Second Multiplex PCR run was achieved using plasmid DNA as template to detect efflux pumps genes (*qac*A/B and *smr*) harbored by plasmid only, two primers were used to target two different regionsin*qac*A/B and one primer was used to detect *smr* gene, Optimum amplification parameters for primers *qac*A/B-1, *qac*A/B-2 and *smr* were 57ºC for 45 seconds using only plasmid DNA, Table 2, primers sequences are listed in Table 3.

Table 1. The program of *qac*A/B, *nor*A, *mec*A and *nuc* primers in PCR analysis with two different temperature.

Steps	Temperature °C	Time	No. of cycles
Initial denaturation	94	5min	1 cycle
Denaturation	94	45sec	
Annealing	**54**	45sec	35 cycles
Annealing	**60**		
Extension	72	45sec	
Denaturation	94	45sec	
Final extension	72	7min	1cycle

Table 2. PCR program for qacA/B-1, qacA/B-2 and smr primers.

Steps	Temperature °C	Time	NO. of cycles
Initial denaturation	94	5min	1 cycle
Denaturation	94	1min	
Annealing	57	45sec	35 cycles
Extension	72	1min	
Final extension	72	5min	1 cycle

Table3. Primer sequence and amplicon size

Primer	Sequence (5'……3')	Amplicon size (bp)	References
nuc-F	GCGATTGATGGTGATACGGTT	276	(20)
nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
mecA-F	GTGAAGATATACCAAGTGATT	147	
mecA-R	ATGCGCTATAGATTGAAAGGAT		
qacA/B-1R	GCTGCATTTATGACAATGTTTG	630	(21)
qacA/B-1F	AATCCCACCTACTAAAGCAG		
qacA/B-2 F	GCAGAAAGTGCAGAGTTCG	361	
qacA/B-2 R	CCAGTCCAATCATGCCTG		
smr-F	AAACAATGCAACACCTACCACT	157	(22)
smr-R	AACGAAACTACGCCGACTATG		
norA-F	GGCGGTATATTTGGGGCACT	314	(23)
*nor*A-R	ACGCACCTGCGATTAAAGGA		

Sequencing of PCR Product

The amplified products of this study contain 3 diagnostic Single Nucleotide Polymorphysim (SNP) positions in order to differentiate between *qac*A and *qac*B on that basis; the FASTA sequence of both genes were downloaded from sequence database in gene bank then compared with sequence of amplified product by basic local alignment search tool (BLAST) which is available at National center of biological information (NCBI) website, the amplified segment by *qac*A/B-1 primer covering region between nucleotide number 844 and 1454, single nucleotide polymorphism (SNP) genotype assay designed to differentiate *qac*A from *qac*B according to SNPs at position: codon 320 (GCA/GAA), codon 323 (GAT/GCT) and codon 380 (ATG/ACG).

Results and Discussion

Biotyping Antibiotic/Antiseptics Susceptibility

In this study, A total of 65 local isolates were characterized depending on cultural and microscopic characteristics. Morphologically, 61 isolates grow and shows smooth, translucent, creamy, yellow pigmented colonies onmannitol salt agar and fermented mannitol. Microscopically examination showed that the bacterial cells positive for gram stain reaction and appeared as grape like clusters,non sporeformingand non motile.Several biochemical tests were performed, and the results demonstrated that 61 isolates gave positive results for catalase, coagulase, and negative for oxidase test. All isolates subjected to 11 antimicrobial agents.The sensitivity result showed that oxacillin resistant phenotype present in (70.49%) of isolates represented by resistance to cefoxitin, ceftriaxone, meropenem (24) resistant percentage of *S.aureus* isolates to norfloxacin, ciprofloxacin, and levofloxacin was 59.06%.While resistance percentage to tetracycline, erythromycin, trimethoprim, and tigecyline was (63.93, 70.49, 36.06 and 0)% respectively. Cartwheel method was used to estimate the efflux activity within MDR *S.aureus*, All of the 61 isolates were cultured on trypton soy agar containing different concentrations of ethidium bromide (EtBr), absence of florescence from cultured bacterial mass under UV light considered as a clue for positive efflux activity, 38 isolate possessed the phenotypic characters of efflux protein since the resistance of ethidium bromide related to the resistance of antiseptics and designated as main substrate for the study of membrane proteins (25),from these 13 isolate were positive at higher concentration of 2 and 4mg\l of EtBr,while 25 isolates were negative detected with florescence under UV light in the presence of low concentration of EtBr (0.25, 0.5) mg/l (Figure -1 ,2 and 3). In order to confirm cartwheel test result, 8 isolates that showed high efflux activity at 2 and 4 mg of EtBr were tested against cetrimide, chloroxylenol, chlorohexidineglucanoate and benzylkonium chloride, the MIC of these isolates was >625 µg/ml for cetrimide and chloroxylenol, for benzylkonium chloride was >500µg/ml .

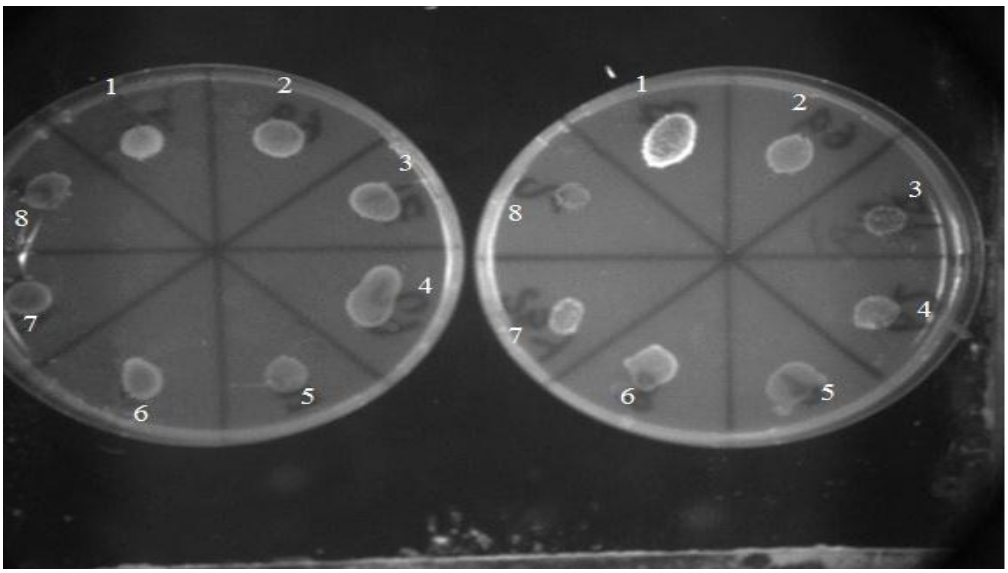


Figure 1. The fluorescence associated with clinical isolates of *S.aureus* on0.25mg/l of EtBr-TSA plates visualized under UV light with gel documentation. All isolates recorded as positive at 0.25mg/l of ethidium bromide except isolates on section 1.

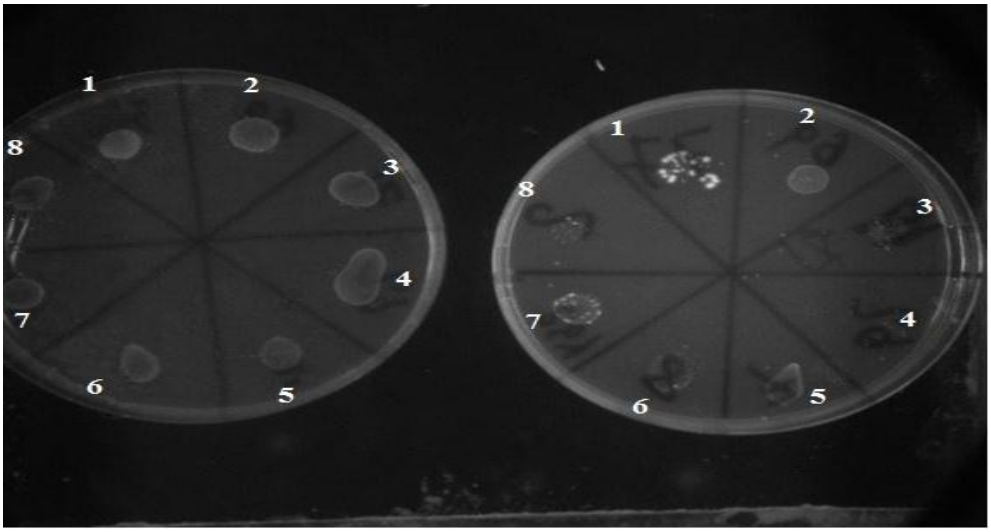
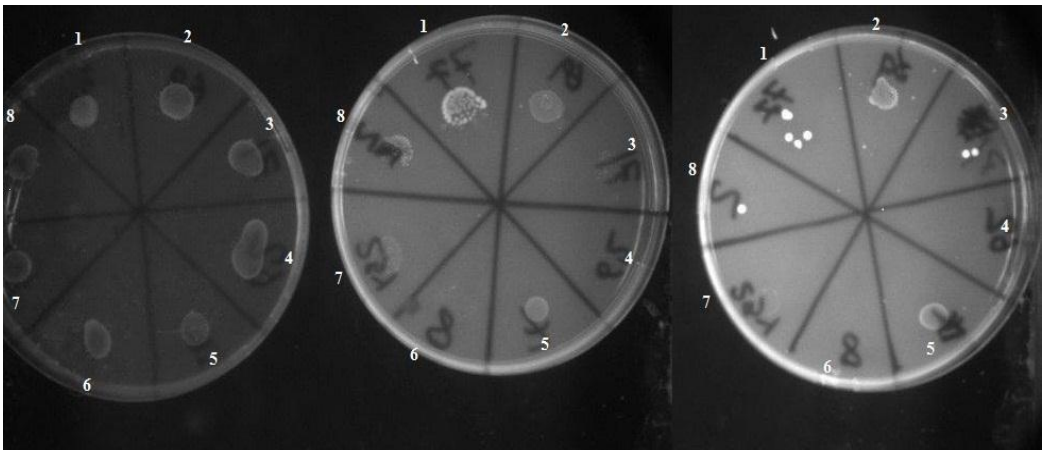


Figure 2. Positive and negative efflux pump related to *S.aureus* isolates at 0.5mg\l ofEtBr-TSA plates visualized under UV light with gel documentation.



Control plate **2**mg/l 4mg/l

Figure 3. Efflux pumps over expression displayed by *S.aureus* isolates represented in section (2, 3, 5, and 7), this isolates extruded chromophore efficiently.

Detection of Antiseptic Coding Genes in MRSA Isolates

Qacs protein (A/B,Smr) encoded by determinants harbored on large plasmids or small plasmids in case of *smr* gene, or in some cases such genes encoded by chromosomal DNA as result of transposon (26). Two multiplex PCR programs were used to detect the *qac*A/B gene within genomic DNA and for plasmid DNA, the first assay used genomic DNA as template, all isolates were positive (100%) for *nuc*and *mec*A with gene bands molecular weight 267bp and 147bp respectively. Fifty six isolates (91.80%) documented as positive for *nor*A (314bp) and only fourteen isolates of *S.aureus*were positive to *qac*A/B (22.95%) with a band has molecular weight of 630 bp as shown in Figure 4.

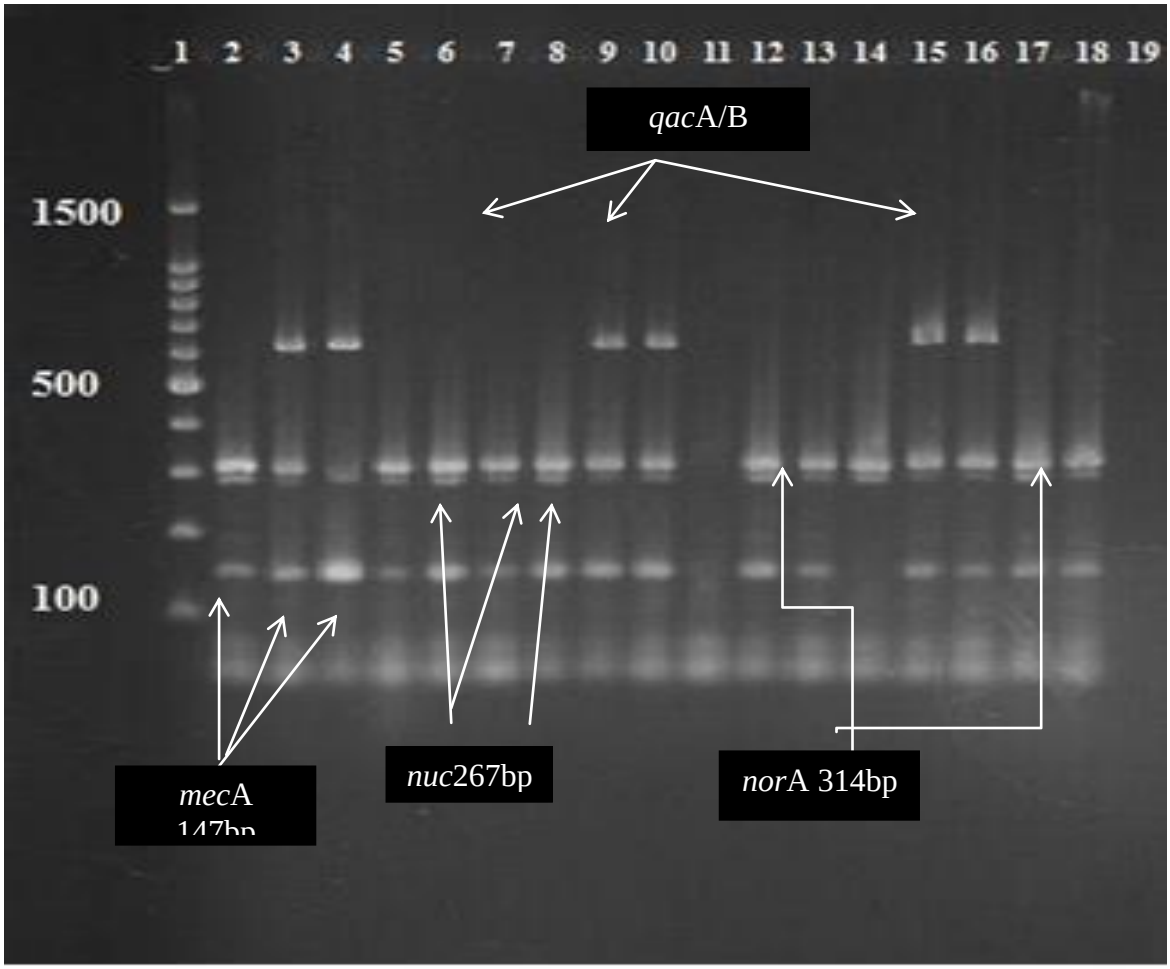


Figure 4. Agarosre gel electrophoresis of multiplex PCR amplification for efflux pumps (*nor*A and *qac*A/B) genes in MRSA isolates diagnosed by (*nuc* and *mec*A) and visualized by UV light after staining by ethidium bromide. Line 1: represent ladder (100-1500 bp), line 11: reactors without template, line 19: negative control, the *qac*A/B 630bp line (3,4,9,10,15,12) and lines (3,4,5,6,7,8,9,11,12,13) *nor*A 310bp, *mec*A (147bp) and nuc (267bp) in all lines except line 11 and 19 .

Among 36 isolates positive to phenotypic detection of efflux pump only two were negative to *nor*A,although the role of *nor*A in antiseptic resistance, this study found out that *nor*A gene present even among isolates without efflux activity, such condition possibly resulted fromnegative regulation of *nor*A by the global regulator *mgr*A; member of *mar*R group of regulator (2). This highly incidence level of *nor*A mainly because that *nor*A likes other chromosomally encoded efflux determinants; are highly conserved and should be predominant in all *S.aureus* isolates (11).As a result, this study predicted prevalence of antiseptic and antibiotic resistant determinant (*nor*A) in Iraqi clinical MDR *S.aureus* isolates, The emergence of *qac*A/B gene was limited to only fourteen MRSA isolates among the selected samples (22.95%) with unique band 630bp; Twelve of them showed efflux activity in phenotypic examination and most of them were also positive to *nor*A. The screening of the second run targeted two region of *qac*A/B by two primers (630bp and 361bp) and *smr* gene (157bp) among the plasmids of all selected samples as represented in Fig. 5.

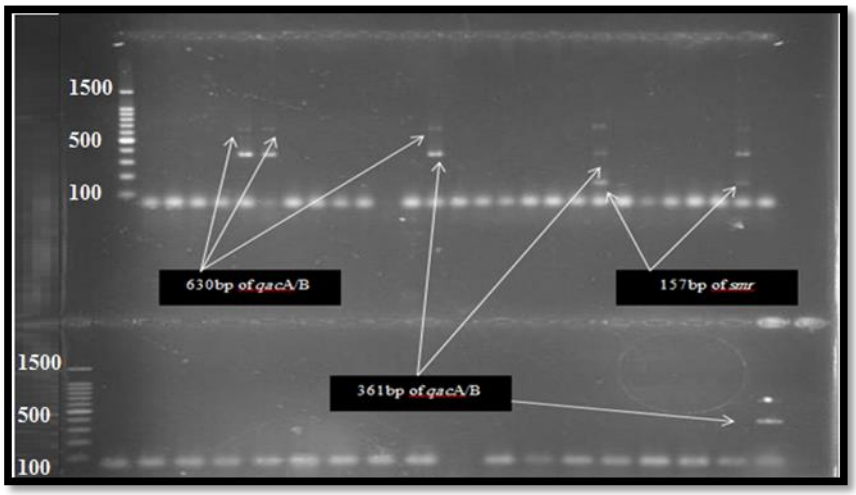


Figure 5. Detection of *qac*A/B and *smr* among plasmid DNA by triplex PCR visualized by U.V light after electrophoresis and staining for 20m in EtBr solution (2% agarose).

The first run for multiplex PCR for screening of *qac*A/B gene revealed fourteen positive isolates, however the second run detected only nine positive isolates. This study argued that *qac*A / B in these five isolates located on the chromosome. Such condition was reported by (27)in which *qac*A/B could be found as a part of the chromosome of clinical *S.aureus* isolates. The *smr* gene detected in two isolates only in the first and seconed run of multiplex PCR, and these isolates were also positive for *mec*A, *nor*A and *qac*A/B. This is the first study in Iraq that investigated prevalence level of antiseptic/disinfectant resistant determinants among local *S.aureus* isolates. Among the 14 isolates that detected with *qac*A/B gene, 12 isolates of them were previously documented as positive to efflux pumps with cartwheel test; and only two isolates were negative but possesses MDR pattern, (Table -2).

Table 2. Efflux/antibiotic resistance pattern among *S.aureus* isolates positive to *qac*A/B.

ID	Positive EA at cartwheel test (mg/l)	CNL	TM	T	E
1	P (4)	R	R	R	R
2	P (1)	R	R	R	R
3	P (4)	R	R	R	R
4	N	R	R	S	S
5	P (4)	R	R	R	R
6	P (2)	R	R	R	R
7	P (2)	R	R	R	R
8	P (1)	R	R	R	R
9	P (2)	R	R	R	R
10	P (2)	R	S	S	S
11	P (0.5)	R	S	S	S
12	P (4)	R	R	R	R
13	P (4)	R	R	R	R
14	N	R	R	R	R

EA: efflux activity, P: positive, N: Negative, CNL: Ciprofloxacin, Norfloxacin, Levofloxacin, TM: trimethoprim, T: tetracycline, E: erythromycin.

All isolates with antiseptic resistant *qac*A/B gene characterized by fluroquinulones resistance, *mec*A positive and resistance to cefoxitine, the incidence of *qac*A/B and *smr* in MRSA isolates only is comprehensible since multi-resistance is handled by plasmids as revealed by (28).Seven isolates were identical 100% to the reference sequences of *qac*A gene five of them *were* amplified from plasmid and two amplified from chromosome, other 6 isolates were identical 99% to *qac*A reference sequences, only one isolate has 85% identity to reference sequences of *qac*A.Multiple SNPs at different lociof*qac*A were recorded at NCBI (Table-3).The change should be (A>G) to converet ATG codon coding for asp to AAT codon coding for asn at position 323which is responsible for specific differences between QacA and QacB a new SNP in *qac*A gene at this position (A>G) was detected leading to amino acid change (Asp to Asn). As a result, this study detected a new variant form of antiseptic resistant *qac gene* (called *qac*A′ by this study) in addition to *qac*A and *qac*Bwhich are

carried by three isolates; two isolates were collected from foot ulcer of diabetic patients and one was obtained from patient with UTI.

Table 3: Detection of new SNPs among positive *qac*AS.*aureus* isolates.

Isolate number	Collection source	Origin of amplification	unrecorded SNP detected	Amino acid change	Position number	Identified (%) and Gaps	NCBI Locus Code
1	Foot ulcer	Plasmid	-	-	-	(100) and 0 gaps	
2	Foot ulcer	Plasmid	-	-	-	(100) and 0 gaps	
3	Foot ulcer	Plasmid	-	-	-	(100) and 0 gaps	
4	Foot ulcer	Chromosome	-	-	-	(100) and 0 gaps	
5	Foot ulcer	Plasmid	-	-	-	(100) and 0 gaps	
6	Foot ulcer	Plasmid	TTT>ATT	F>I	352	(99) and 0 gaps	
7	Foot ulcer	Plasmid	TTT>ATT	F>I	352	(99) and 0 gaps	LC335718
			AAT>GAT	N>D	323		
8	Foot ulcer	Chromosome	AAT>GAT	N>D	323	(99) and 0 gaps	LC335719
			AAG>GAG	K>E	408		
9	Foot ulcer	Plasmid	-	-	-	(100) and 0 gaps	
10	Foot ulcer	Chromosome	-	-	-	(100) and 0 gaps	
11	Foot ulcer	Chromosome	TTT>ATT	F>I	352	(99) and 0 gaps	LC335717
12	UTI	Plasmid	TGT>TAT	C>Y	358	(99) and 0 gaps	LC335720
			GTT>GCT	V>A	378		
13	UTI	Plasmid	AAT>GAT,	N>D,	323,	(99) and 0 gaps	LC335721
			AAA>GAA,	K>E , K>E	393, 407		
			AAA>GAA				
14	UTI	Chromosome	-	-	-	(85) and 4 gaps	

Conclusion:

The resistance of MRSA isolates to disinfectant and antiseptic compounds mediated mainly by plasmids *qac*A/B and *smr* determinants and by chromosomal *nor*A gene, The occurrence of such determinants occupied by MDR resistant pattern in MRSA isolates toward multiple targets leading to cross-resistance between antibiotic-antiseptics in local isolates.Consenquantly these isolates must be detected by efflux activities and their genotypic coding factors. In this study, a new *qac*determinant variant spreading among MDR *S.aureus* clinical isolates was detected.

Conflicts of Interest: None.

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التحري عن المورث الجديدالمقاوم للمطهربين عزلات المكورات العنقودية الذهبية المتعددة المقاومة للمضادات الحيوية والمعزولة من مصادرسريرية مختلفة

نور صابر **نهى جوزيف قندلا** **هبة عبد الامير محمد**

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة:

يودي الاستخدام المتزايد للمركب المطهرالى خلق ضخ انتقائي وظهورمقاومة للمطهرات بين المكورت العنقودية الذهبية،وآلية المقاومة للمطهرمدفوعة بشكل رئيسي من قبل بروتينات مضخات التدفق المتعددة المقاومة للادوية proteinMDR efflux . جمعت 65من عزلات المكورات العنقودية الذهبية من مصادر سريرية مختلفة واخضعت جميع العزلات الى اختبار الحساسية لـ 11من المضادات الحيوية المنتخبة على اساس المقاومة لمضخات التدفق . كشف عن الفعالية لمضخات التدفق باستخدام cartwheel method.وتم التحري عن المورثات المشفرة لمقاومة المطهرات(*qac*A/B, *smr* and *nor*A) والمورثات المشفرة لـ*nuc* و*mec*A في عزلات المكورات العنقودية الذهبية المتعددة المقاومة للمضادات الحيوية باستخدام تقنية تفاعل البلمرة المتسلسل المتعدد. واظهرت النتائج ان 61من مجموع 65 من عزلاتالمكورات العنقودية الذهبية ذي نتيجة موجبة للمورثاتnuc و58،mecAمنها كانت موجبة لـnorA ،14 عزلة الىqacA / Bواثنين فقط كانت إيجابيةلـSMR. ، و18 من عزلات*S.aureus* التي اعطت نتيجة موجبة للمورث*nor* A كانت 14 عزلة منهامقاومة لمجموعة الفلوروكينولونات موجبة لمضخات التدفق *qac*A/B ومنها عزلتين اعطت نتيجة موجبة للمورث *smr* المشفر لمضخة التدفق.جميع العزلات التي اظهرت ايجابية مع المورثات qacA / B تميزت بمقاومة الفلوروكينولونات واظهرت فعالية ضخ قوية في اختبار الـ cartwheel method ،درست تسلسلات 14 عزلة موجبة لـ qacA / B للتمييزبين المتغيرات حسب الموضع (الاسبارتك فيQacAوالالنين في QacB)، امتلكت ثلاث من العزلات حمض أميني الأسباراجين في الموضع 323 واعتبر نوع جديد اكتشف لاول مرة.

الكلمات المفتاحية: مضخات التدفق، تفاعل البلمرة المتسلسل المتعدد، المكورات العنقودية الذهبية، محدد نواقل الغشاء .