



# Detection the Prevalence of Some Chromosomal Efflux Pump Genes in Methicillin Resistant *Staphylococcus aureus* Isolated from Iraqi Patients

Ghufran J. Shamkhi<sup>1</sup> , Shurook M. K. Saadedin<sup>2</sup> , Kifah Ahmed Jassim<sup>3</sup>

<sup>1</sup>Wassit University / College of Science.

<sup>2</sup> Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / University of Baghdad / Baghdad / Iraq.

<sup>3</sup>Ministry of Health / Central Public Health Laboratory / Baghdad / Iraq.

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**Abstract:** Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with multi drug resistance infections and high levels of illness and the efflux pump has vital role in multi drug resistance for antimicrobial agent in *S. aureus*. Four hundred and thirty nine clinical specimens (burns, blood, wounds) were collected from patients attending different hospitals in Baghdad and Wassit City. *S. aureus* has been identified using biochemical and molecular methods. Molecular method was depended on PCR in detection specific genes, *16S rRNA* to diagnos *staphylococcus* genus and *mecA* to diagnos *S.aureus* species. The results demonstrated that 168 isolates belonged to *S. aureus* , 96 isolates of them were related to Methicillin resistance *S.aureus* (MRSA). The isolation results showed that burns 51 (53.13%), blood 18 (18.75%) and wounds 27 (28.13%) isolated out of 96 (100%). The aim of this study is to investigate the prevalence of number of chromosomal efflux pumps genes including (*norA*, *norB*, *norC*, *mdeA* and *mepA*). The results indicated the presence of *mdeA* gene in all isolates 96 (100 %) of MRSA. The *mepA* gene has been recorded the second highest prevalence present in 89 (92.71%) isolates. The *norA* and *norB* genes were present in 77 (80.21%) and 54 (56.25%) respectively of isolates, while the gene *norC* was present in 17 (17.81%) of the total isolates. In conclusion, our results showed the role of *16S rRNA* for molecular detection *Staphylococcus* genus and using *mecA* gene in *S.aureus* bacteria considered one of the most important features in the description of MRSA isolates. The results of detection efflux pump genes demonstrated the presence of *MdeA* gene in all multidrug resistant local isolates of *S.aureus* and also high prevalence of other genes especially *MepA* gene.

**Keywords:** MRSA, Efflux pump, *norA*, *norB*, *norC*, *mdeA* , *mepA* .

**Corresponding author:** (Email: ghufran.alhajj@yahoo.com).

## Introduction

Since methicillin resistant *S. aureus* (MRSA) description in the early 1960s, MRSA has become a major public health issue because of the worldwide spread of several clones. In 1970s, *S. aureus* strains have become resistant to most penicillinase-stable penicillins (1). Methicillin-resistance is in staphylococci conferred by the carriage of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*)(2). Efflux

pumps transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms (3). Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multiple

drug resistance (MDR) (4). Efflux pumps are found in almost all bacterial species and genes encoding this class of proteins can be located on chromosomes or plasmids. Efflux pumps are classified into five families: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily], and the multidrug and toxic compound extrusion (MATE) family (5,6). Except for the RND superfamily which is only found in Gram-negative bacteria, efflux systems of the other four families: MFS, ABC, SMR and MATE are widely distributed in both Gram-positive and negative bacteria (7). *norA* is a member of the major facilitator superfamily. It is a chromosomally encoded protein with 12 transmembrane-spanning segments, it is a proton motive force (PMF) dependent multidrug (MDR) efflux pump in *Staphylococcus aureus* (8). *norA* is a 388 amino acid protein and appears to export a variety of structurally unrelated drugs, such as fluoroquinolones, ethidium bromide, cetrимide, benzalkonium chloride, tetraphenyl phosphonium bromide, and acriflavine (9). The efflux pump *norB* is a MFS proton-driven efflux pump composed by 463 amino acids, with 12 transmembrane segments. *NorB* confers resistance to some of the *norA* substrates, such as hydrophilic fluoroquinolones (norfloxacin and ciprofloxacin), biocides (tetraphenylphosphonium and cetrимide) and the dye ethidium bromide, as well as to non-*norA* substrates, such as the hydrophobic fluoroquinolones moxifloxacin and sparfloxacin, and to

tetracycline (10). The overexpression of *norB* was associated with an increased resistance to *norB* substrates (11). *MepA* is encoded by the chromosomal gene *mepA* and it was the first multidrug transporter from the MATE family to be described in *S. aureus*. This 451 amino acid protein has 12 transmembrane segments. *MepA* was found to be associated with a MDR phenotype, conferring low-level resistance to quaternary ammonium compounds, such as benzalkonium chloride, cetrимide, dequalinium, tetraphenyl-phosphonium, pentamidine and the dye ethidium bromide (12). The efflux pump *norC* is a 462 amino acid protein with 12 transmembrane segments that belongs to the MFS and shares 61% identity with *norB* (11). *NorC* is associated with low-level resistance towards hydrophilic and hydrophobic fluoroquinolones, such as ciprofloxacin, moxifloxacin and garenoxacin, and to the dye rhodamine. Studies have indicated that the wild-type expression of *norC* is apparently not sufficient to affect the susceptibility towards these compounds, and that low-level resistance is achieved through *norC* overexpression (13).

## Material and methods

### Bacterial isolates

**Standard strain:** Used in this study was *staphylococcus aureus* ATCC- 25923 obtained from central public health laboratory.

**Bacterial isolates and Identification:** Four hundred and thirty nine samples from different sources (wound, burns, and blood) were included in this study. The clinical

samples were collected from different patients attending Baghdad hospitals (Al Kindy, Al Yarmouk and Ibn Al-Balady) and Wassit hospitals (Al Zahraa and Al Kramah) using sterile swabs, placed into a transport medium and were cultured immediately on blood agar and brain heart infusion agar. *S. aureus* was identified depending on the morphological features on culture media and biochemical tests according to Bergey's manual(14). All colonies from primary cultures were purified by subculture on brain-heart infusion (BHI) agar and then re-cultured on mannitol salt agar (15-17).

### Molecular Identification

**Genomic DNA Extraction:** The genomic DNA of *S. aureus* isolates was extracted using Genomic DNA Mini kit supplemented by the manufacturing company (Geneaid, Korea).

**Molecular Identification of isolates:** Conventional PCR was used for detection the specific genes, *16S rRNA* gene for identification of the *Staphylococcus* genus and *mecA* gene for identification of the Methicillin resistance *S. aureus*. Primeres sequences and expected amplicon sizes of *16s RNA* and *mecA* genes is shown in Table (1).

**Efflux pump genes detection by Conventional PCR:** Uniplex PCR was used to amplify five efflux pump genes (*mdeA*, *mepA*, *norA*, *norB* and *norC*) related to resistance against antibiotic. A primer stock solution was prepared from the lyophilized primers according to manufacturing of (Alpha DNA company, Canada). The set of 5 primers with the Sequences and expected amplicon sizes is shown in Table (1).

Table (1): The primers sequences and expected amplicon size of efflux pump genes.

Name of Primer	Sequence	Expected amplicon Size (bp)	References
<i>16SrRNA</i>	F5-TCACCGTAGCATGCTGATCT-3	195	This study
	R5-AGGTGGGGATGACGTCAAAT-3		
<i>MecA</i>	F5-AACAGGTGAATTATTAGCACTTGTAAG-3	170	18
	R5-ATTGCTGTTAATATTTTTTGAGTTGAA-3		
<i>NorA</i>	F5- ATGAATAAACAGATTTTTGT- 3	1167	19
	R5- CTACATATTTTGTTCCTTCA- 3		
<i>NorB</i>	F5- TCGCCTTCAACACCATCAAC- 3	236	This study
	R5- GGCGTAGGAGATGATGGTCA - 3		
<i>NorC</i>	F5- GCGGGAGTGTGTTCTTCATC - 3	441	This study
	R5- CTGGAGGAAGGTGTTGAAGC - 3		
<i>MdeA</i>	F5- TATGGCGATTGTTGTTTTACTAC - 3	1072	20
	R5- AACCGTGTGCATTCATTTCTGG - 3		
<i>MepA</i>	F5- GCAGTTATCATGTCTATCGGCG -3	240	21
	R5- TGCACCTTGTAATAATGGCCA -3		

**Primers design:** Three primers had been designed covering whole gene using Primer3 software, by Alpha DNA company.

**Master mix of PCR :**The PCR experiment was carried out using a mixture of specific sets of primers designated for each target gene that

were mixed with the DNA sample (template) and a master mix reagent which contains (Taq polymerase, PCR buffer, MgCl<sub>2</sub> and dNTPs). The final constituent was the nuclease free water. A typical PCR mixture contained 10 µl of lyophilized master mix, 1 µl each of forward and reverse primers, 3 µl of DNA and 5 µl of water adjusted to a total volume of 20. The reaction mixture was mixed and centrifuged for 3 seconds to collect the drops from walls in order to ensure the final volume of 20 µl, and then transferred to a thermal cycler to start reaction according to the steps of the specific program for each gene under study.

### PCR conditions

Detection of *16S rRNA* and *mecA* genes used to identify *S. aureus*. Optimized PCR reaction mixture and amplification program were used as shown in table 2 according to Saruta (22) with some modifications. In this study, uniplex PCR was used to amplify efflux pump genes (*norA*, *norB*, *norC*, *mdeA* and *mepA*) under study to detection of genes encoding efflux pump resistance genes in clinical isolates of methicillin resisting *S. aureus*. For each gene the suitable program of PCR conditions were shown in Table (2).

Table (2): The PCR amplification programme for the genes were used in this study.

Genes	Initial denaturation Temp. (°C) and time	Denaturation Temp. (°C) and time	Primer annealing Temp. (°C) and time	Primer extension 35 cycles	Final extension
<i>16S rRNA</i>	94°C 3 min	94°C 1min	58 °C 1 min	72°C 1 min	72°C 3.5 min
<i>MecA</i>			50 °C 45 sec	72°C 1.5 min	
<i>NorA</i>			52 °C 1 min	72°C 1 min	
<i>NorB</i>			62 °C 45 sec	72°C 1.5 min	
<i>NorC</i>			62 °C 45 sec	72°C 1 min	
<i>MepA</i>					
<i>MdeA</i>					

## Results and discussion

### Detection of *16S rRNA* gene

The PCR products of *16S rRNA* gene (195bp) (Figure 1) exists in all 96 *S.aureus* isolates that were identified by the previous identification methods. The amplifying of DNAs from phylogenetically divergent bacteria by targeting conserved regions of the *16S rRNA* gene have become a powerful tool in detection and identification of bacteria (23). The present findings

suggested that PCR using *16S rRNA* gene was an excellent method for detection of *S.aureus* spp. isolates. These results are in agreement with other studies which mentioned that detection and sequencing of this gene is an effective means for the identification of clinical isolates of *S.aureus* (24, 22). The results of AL-Alak (25) revealed the ability of using 16s and 23s *rRNA* genes for molecular identification of *staphylococcus* clinical isolates and this tool was rapid and accurate with high identification genomic rate (100 %).

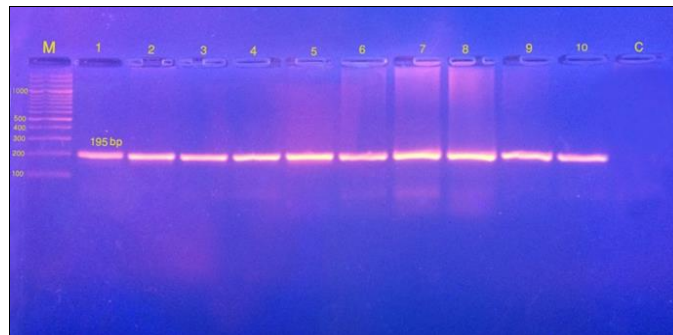


Figure (1): Agarose gel electrophoresis of PCR amplified products for *16S rRNA* gene. lane (M): 100bp DNA ladder, lane (c): Negative control, lane (1-10): amplification PCR products of *16S rRNA* (expected size).

### Detection of *mecA* gene

The *mecA* gene was the private genetic marker for detection of methicillin resistance *S. aureus* (MRSA) (26). MRSA isolates were identified by PCR using specific primer of *mecA* gene. The result shown PCR product size 170 bp (Figure 2). Furthermore methicillin antibiotics disk on plate detected 96 MRSA resistant isolates out of 168 *S. aureus*. *mecA*

codes for a penicillin-binding protein, PBP2a (also called PBP2) which has a lower binding affinity for  $\beta$ -lactam drugs than regular PBPs. PBPs are trans peptidases involved in the construction of the bacterial cell wall. Moreover *mecA* was harbored on the staphylococcal chromosome cassette *mec* (*SCCmec*), which can be transferred between staphylococcal species horizontally (27,28).

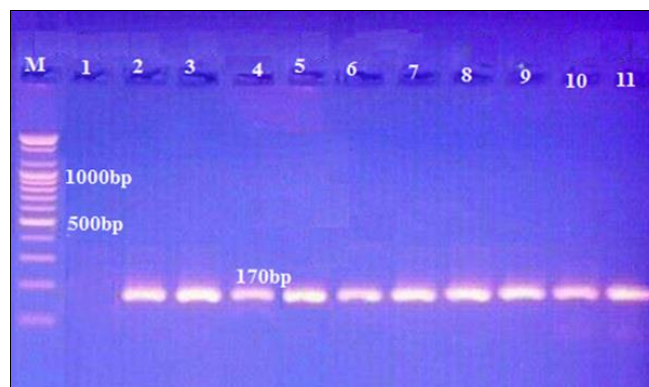


Figure (2): Agarose gel electrophoresis of PCR amplified products for *mecA* gene. Lane (M): 100bp DNA ladder, Lane (1): Negative control, Lane (2-11): PCR product of *mecA* gene.

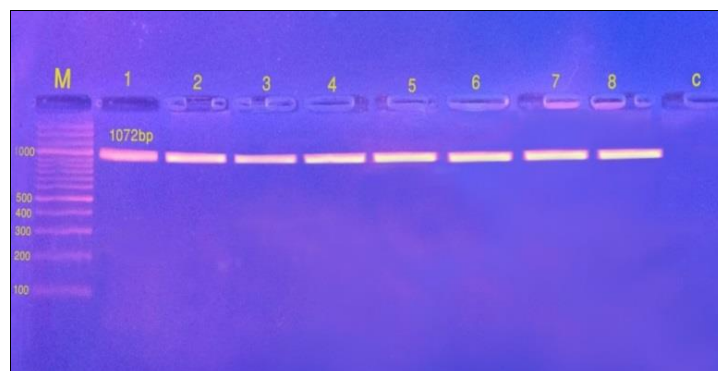
### Detection of efflux pump genes by PCR

DNA extracted sample has been used in order to detect the presence of genes encoding efflux pumps and determination of the prevalence of each gene among *S.aureus* clinical isolates

uniplex polymerase chain reaction (PCR) for each DNA. The PCR reaction included 96 isolates for detection of the chromosomal efflux pump *mdeA*, *mepA*, *norA*, *norB*, *norC* genes. The PCR products have been confirmed by analysis of the bands on gel electrophoresis. PCR products have

been confirmed by comparing their molecular weight with 100 bp DNA Ladder. The results of uniplex PCR reaction for chromosomal efflux pump genes are shown in (Figures 3, 4, 5, 6 and 7). In Iran, from a total of 60 multidrug resistant isolates of *S.aureus*, the *MdeA* genes were detected in 61.7% of isolates (29). A study by (30) in South Africa, a tertiary academic hospital in Pretoria city, demonstrated the distribution of patterns of multidrug resistance. Efflux pump genes *norA* and *norB* were observed in the clinical isolated from *S.aureus* were present in 98.9% from 40 isolate. A study of (31) on 19 isolates of methicillin-resistant *Staphylococcus aureus*. isolated in University Darul Iman, Malaysia,

revealed the presence of *mdeA* in 18 isolates, while 16 of them carried *norA* gene. The *mdeA* gene was assumed to be very ancient, widespread in the bacterial genome and could possess a different function other than drug efflux (30). Another study by (20) in Pennsylvania has demonstrated the presence of a novel MDR protein, *MdeA*, in the major human pathogen *S. aureus* and the *mdeA* gene was present in the genomes of all six strains of *S. aureus* examined and this result was concordant with our result. *Nor* family pumps can also extrude other chemical compounds, such as ethidium bromide, cetrinide, and tetraphenylphosphonium (20).



**Figure (3):** Agarose gel electrophoresis of PCR products for the efflux pump gene *mdeA* in size (1072)bp. Lane M: 100bp DNA ladder; lanes 1-8: PCR products of *mdeA* gene; lan 9: Negative control. (70V for 2h).



**Figure (4):** Agarose gel electrophoresis of PCR products for the efflux pump gene *mepA* in size (240)bp . Lane M: 100bp DNA ladder; lanes 1-10: PCR products of *mepA* gene; lan C: Negative control. (70V for 2h).



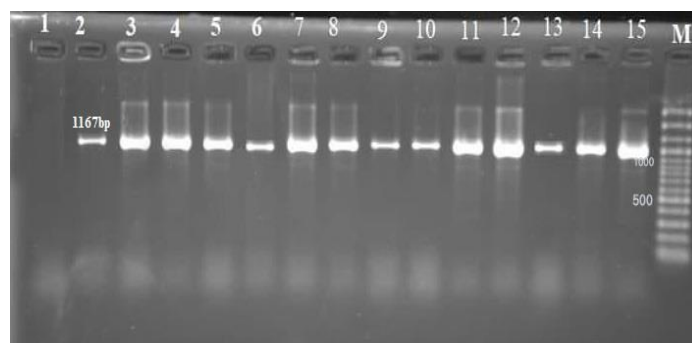


Figure (5): Agarose gel electrophoresis of PCR products for the efflux pump *norA* gene in size (1167)bp . Lane M: 100bp DNA ladder; lanes 2-15: PCR products of *norA* gene; lan 1: Negative control. (70V for 2h).

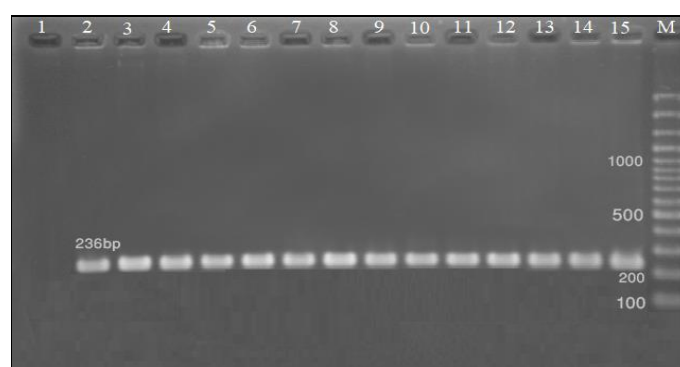


Figure (6): Agarose gel electrophoresis of PCR products for the efflux pump *norB* gene in size (236)bp. Lane M: 100bp DNA ladder; lanes 2-15: PCR products of *norB* gene; lane 1: Negative control. (70V for 2h).

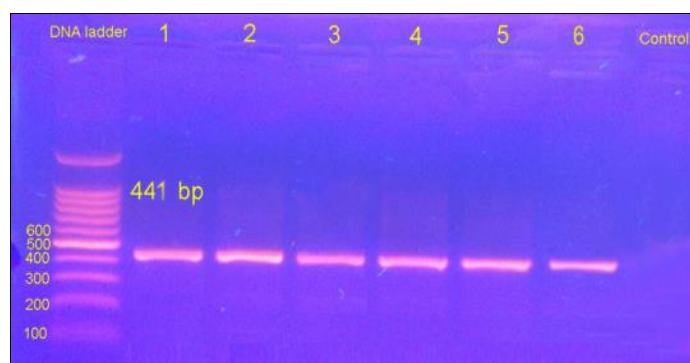


Figure (7): Agarose gel electrophoresis of PCR products for the efflux pump *norC* gene in size (441)bp. Lane M: 100bp DNA ladder; lanes 1-6: products of *norC* gene; lan control: Negative control. (70V for 2h).

The results of this study revealed the dominant of *MdeA* gene among methicillin resistance *S.aureus* isolates (MRSA) isolated from burns, wounds, blood infections, and prevalence of this gene in these isolates was 100 %. The results also demonstrated the presence of *mepA* gene in 92.71% of the MRSA

isolates. From all 96 MRSA isolates, the number of isolates which carried the gene *norA* were 77 isolates (80.21%), the presence of *norB* gene in 56.25%, while the lowest percentage in these genes was recorded for *norC* gene (17.81 %), ( high significant differences at  $P < 0.01$ ).as shown in Table (3).

Table (3): The distribution of efflux pump genes *S.aureus* isolates.

Type of efflux pump gene	<i>S.aureus</i> isolates (MDR)		Chi Square ( $\chi^2$ )
	Positive isolates no.(%)	Negative isolates no.(%)	
<i>NorA</i> gene	77 (80.21)	19 (19.79)	13.28 **
<i>NorB</i> gene	54 (56.25)	42 (43.75)	5.02 *
<i>NorC</i> gene	17 (17.81)	69 (82.19)	13.53 **
<i>MepA</i> gene	89 (92.71)	7 (7.29)	13.95 **
<i>MdeA</i> gene	96 (100)	0 (0.00)	15.00 **

\* (P<0.05), \*\* (P<0.01).

According to the study by (28) which reported that the genes encoded chromosomally MDR efflux pumps *norA*, *norB*, *norC*, *mepA* and *mdeA* in *S. aureus*, are widely present in different strains and the presence of efflux pump genes was assessed by PCR and the highest frequency was related to *mdeA* (61.7%) and in addition, the frequency of *mepA*, *norA*, *norB* and *norC* were 60%, 41.7%, 41.7% and 41.7%, respectively. A study by (32) assessed high prevalence of MDR genes in *S. aureus*, collection was very high for *mepA* (89.4%) and *norA* (86.4%). Another study optioned by (33) showed the high frequency of *norA* was found in 100% of ciprofloxacin resistance while the *norB* genes were found in and 83% among *S.aureus* isolates . In *S. aureus*, the chromosomally encoded the MDR pumps *norA*, *norB*, and *norC* are widely present in different strains and identified based on their ability to confer resistance to quinolones. In addition to hydrophilic (norfloxacin and ciprofloxacin) and hydrophobic (moxifloxacin and sparfloxacin) quinolones . Different roles that bacterial MDR pumps may have, including resistance to antimicrobial compounds produced by hosts or other bacterial species, virulence, detoxification and intercellular signal trafficking and the main role of MDR pumps appears to be detoxification of

intracellular antibiotics rather than resistance to external ones (34).

## Conclusions

Our study confirmed the role of *16S rRNA* for molecular detection *S.aureus* at the level of genus and one hundred percentage accuracy in detecting MRSA and using *mecA* gene in *S.aureus* bacteria considered one of the most important features in the description of MRSA isolates. According to the results of the present study , most local clinical isolates of MRSA, carrying the efflux pump genes and the efflux pumps system plays a vital role in multidrug resistance in clinical *S.aureus* isolates. The results of detection efflux pump genes demonstrated the presence of *mdeA* gene in all multidrug resistant local isolates of *S.aureus* and also high prevalence of other genes especially *mepA* gene. This might be indicated to the significant role of these genes in the resistance mechanism agansit methicillin.

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