

Exploring antibacterial activity of diclofenac sodium and determining the phenotypic and genotypic analysis of some efflux pump genes in methicillin-resistant *Staphylococcus aureus*

Shahad N. Abdullah and Zina H. Shehab*

Department of Biology, College of Science for Women, University of Baghdad, Iraq

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Abstract

Multidrug-resistant (MDR) *Staphylococcus aureus* is becoming a remarkable crisis because of the potential of this bacterium to develop resistance to many groups of antibiotics. This study investigated the antibacterial effect of diclofenac sodium (Olfen) and the phenotypic and genotypic detection of two classes of efflux pumps among (MDR) *Staphylococcus aureus*. Methicillin-resistant *Staphylococcus aureus* (MRSA) can be identified via culture and biochemical examinations and confirmed via the use of the specific primers *16SrRNA* and the *mecA* gene. Antibiotic susceptibility tests were performed via the disc diffusion method against ten antibiotics. The results revealed the highest resistance of the *S. aureus* isolates to cefoxitin (55.55%), and the resistance of the isolates gradually decreased with erythromycin (38.88%), doxycycline (30.55%), norfloxacin (25%), ciprofloxacin (19.44%), clindamycin (16.66%), rifampin and trimethoprim-sulfamethoxazole (11.11%), and chloramphenicol (8.33%). Diclofenac sodium, a non-steroidal anti-inflammatory drug (NSAID), showed distinct antibacterial activity against some MRSA isolates at 125 µg/ml. The efflux pump phenotype was identified via the Ethidium Bromide - Cart Wheel (EtBr-CW) method. The results indicated that 10 isolates (27%) had efflux pumps, and polymerase chain reaction was used to look at the genotypic efflux pump genes. The findings revealed that 34 (94%) MRSA strains presented the *mdeA* and *mepA* genes, whereas 28 (77%) *norB* genes were found.

Keywords: Antibiotic resistance, Efflux pump, EtBr-CW method, diclofenac sodium, MRSA.

1. Introduction

Staphylococcus aureus is an important infectious pathogen in the health sector and communities. It causes various infections ranging from simple to life-threatening ones (Rasheed and Hussein, 2021). Typically, this bacterium is found in the body's regular flora. It is present in the upper respiratory system and on the skin (Sabbar *et al.*, 2023). Opportunistic bacteria are responsible for a variety of infectious diseases, including infections contracted in hospitals and communities. Methicillin-resistant *S. aureus* (MRSA) can cause skin infections, urinary tract infections (UTIs), bloodstream infections, food poisoning, and respiratory disorders by generating enterotoxins and alpha pore-forming toxins that kill host cells and tissues. The virulence of *S. aureus* infections is influenced by several factors (Scudiero *et al.*, 2020). *Staphylococcus aureus* exhibits a variety of antibiotic resistance mechanisms, such as target site mutation, antibiotic efflux systems, enzymatic drug inactivation, and changes in antibiotic permeability (Foster, 2017). Penicillin-binding protein 2a (PBP2a), which is encoded by *mecA* and has a poor affinity for methicillin and other β -lactams, is the cause of methicillin and oxacillin resistance. Moreover, staphylococcal cassette chromosome element (*SCCmec*) horizontal gene transfer and

chromosomal alterations that change drug binding sites might result in methicillin-resistant *S. aureus* (Jang, 2016). Many commonly used antibiotics, including β -lactamases, fluoroquinolones, aminoglycosides, macrolides, and lincosamides, frequently cause resistance in MRSA (El-Baz *et al.*, 2021 and Youssef *et al.*, 2021). One of the main mechanisms of antibiotic resistance is efflux pumps (Saber *et al.*, 2019). Following their attachment to substrates often antibiotics these membrane proteins aggressively catalyze the outward translocation of the substrates, lowering their intracellular concentrations and, hence, the efficacy of the therapy (Pu, *et al.*, 2017). The efflux systems are divided into two groups according to the energy-obtaining method used. While secondary efflux pumps obtain energy from chemical gradients created by protons or ions, primary efflux pumps obtain energy from the hydrolysis of ATP molecules (Li *et al.*, 2015). Certain bacterial efflux systems can only extrude one class of antibiotics; however, multidrug-resistant (MDR) bacterial efflux systems can extrude multiple classes (Sharma *et al.*, 2016 and Hassanzadeh *et al.*, 2017). The ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance nodulation cell division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxin extrusion (MATE) family are the five primary families of efflux pumps found in prokaryotes (Blair *et al.*, 2014). According to recent studies, both *S. aureus* infection

* Corresponding author. e-mail: zinahs_bio@cs.w.uobaghdad.edu.iq.

and influenza virus infection increase the risk of pneumonia and death (Jia *et al.*, 2019 and Al-Mayahi, 2021). Diclofenac sodium is a non-steroidal anti-inflammatory medication (NSAID) that has FDA approval. It was used to treat *S. aureus* osteomyelitis as an antivirulence agent in conjunction with antibiotics (Abdel-Karim *et al.* 2022). The aim of the current study, we determined the presence of several chromosomal efflux pump genes (*mdeA*, *mepA* and *norB*) by phenotypic and genotypic methods and their relationship with antibiotic resistance in methicillin-resistant *S. aureus* and explored the impact of diclofenac sodium (Olfen) as a synergistic antibacterial agent at a first attempt in Iraq.

2. Materials and Methods

2.1. Bacterial isolates and identification of *Staphylococcus aureus*

One hundred and twenty-five samples were collected from various sources from patients who visited some Baghdad hospitals. These samples were Wound 37 (29,6%), Urine 55 (44%), Burn 8 (6,4%), Sputum 9 (7,2%), Nasal swap 6 (4,8%), Throat swap 7 (5,6%) and Cerebro-Spinal Fluid (CSF) 3 (2,4%), which were selected between the ages of 15 and 60 and represented both genders. Each sample was immediately inoculated in blood agar and incubated for 24 hours at 37°C. Gram stain and biochemical tests were used, and growth on mannitol salt agar (MSA) was used as a selective medium (Nuaimi *et al.*, 2023). Next, the PCR technique for genotypic detection via the *16S rRNA* gene was used.

2.2. Antibiotic susceptibility test.

To assess the antibiotic susceptibility of *S. aureus* isolates on Mueller Hinton agar (Hi-media), the Kirby-Bauer method was employed for eleven antibiotic discs (Mehrotra *et al.*, 2000). The plates were incubated at 37°C for eighteen hours. The diameter of the inhibitory zone was evaluated following the incubation period at the Clinical and Laboratory Standards Institute (CLSI 2024). The following antibiotics were examined in this study: cefoxitin (FOX: 30 µg), ciprofloxacin (CIP:5µg), erythromycin (ERY:15µg) and norfloxacin (NOR: 10µg). Clindamycin (CD:10µg), doxycycline (DOX: 30µg), nitrofurantion (F:30µg), chloramphenicol(C:30µg), rifampin (RIF: 5µg), and trimethoprim-sulfamethoxazole (SXT: 1.25/23.75 µg).

2.3. Phenotypic methicillin resistance detection

All identified *S. aureus* isolates were screened for methicillin resistance phenotypically via the cefoxitin disk diffusion test, which is alternative to a 30 µg disk on Mueller Hinton agar for methicillin. Cefoxitin (methicillin) was found to be resistant to inhibition zones < 21 mm in size but susceptible to those > 21 mm in size (CLSI 2024).

2.4. The antibacterial activity of diclofenac sodium was evaluated according to the MIC via the microtiter plate method (MTP)

The assay was designed to test the minimum inhibitory concentrations (MICs) of diclofenac sodium in the range of concentrations (1000,500,250,125,26.5,31.25) tested against several multidrug-resistant isolates of MRSA, according to the guidelines recommended by the CLSI

(2024) document and (Elshikh *et al.*, 2016). A 96-well microtiter plate supplemented with resazurin dye in Mueller Hinton broth (MHB).

2.5. Ethidium bromide qualitative efflux detection via the cart-wheel method.

The efflux of ethidium bromide was measured via the cartwheel(EtBr-CW) method. After the bacterial isolates were cultured overnight at 37°C, the cell concentration was increased the next day to 0.5 of the McFarland standard. A cartwheel-shaped pattern was created by dividing Tryptone soy agar with varying concentrations of ethidium bromide (0, 1, 2 and 4) mg/L via radial lines. After the bacterial isolates were swabbed with a sterile cotton swab, they were incubated for 16 hours at 37°C. A UV transilluminator was used to evaluate the colonies on tryptone soy agar plates. Martins *et al.*, 2013). Positive efflux activity was defined as the isolates' response to ethidium bromide exceeding 2 mg/mL, whereas moderate efflux activity was defined as the isolates' response to ethidium bromide equal to 2 mg/mL. Furthermore, isolates with ethidium bromide concentrations of 1 µg/mL or lower were regarded as negative.

2.6. Molecular identification by polymerase chain reaction (PCR)

A commercial genomic DNA purification kit (Promega, USA) was used to extract the genomic DNA of the *S. aureus* isolates. DNA samples from *S. aureus* isolates were used for this investigation to identify and identify efflux pump genes. By comparing their molecular weight on a DNA ladder and analyzing the bands via gel electrophoresis, the PCR results were verified. The primers used are listed in Table 1 of our previous study (Hamel *et al.*, 2021).

Table 1. Primers used in this study

Primers name	Primer's sequence 5' 3'	Product size (bp)	Reference
<i>16S rRNA</i>	AACCTACCTATAAGACTGGG	578	(Kumar <i>et al.</i> , 2020)
	CATTCACCCTACACATGG		
<i>mecA</i>	ACTGCTATCCACCTCAAAC	163	(Mehrotra <i>et al.</i> , 2000)
	CTGGTGAAGTTGTAATCTGG		
<i>norB</i>	TCGCCTTCAACCCATCAAC	236	Shamkhi <i>et al.</i> , 2019)
	GGCGTAGGAGATGATGGTCA		
<i>mdeA</i>	TATGGCGATTGTTGTTTTACTAC	173	(Ahmed and Al-Daraghi, 2022)
	AACCGTGTGCATTCATTCTGG		
<i>mepA</i>	GCAGTTATCATGTCTATCGGCG	240	(Patel <i>et al.</i> , 2010)
	TGCACCTTGAAAAATGGCCA		

The PCR components of 25 µl of master mix, 4µl of extracted template DNA, 11 µl forward and 11µl reverse primers, and 6.5 µl of nuclease-free water. In a PCR thermal cycler, the mixture was amplified by first denaturing at 94°C for five minutes, and then amplifying it 35 times (denaturing it for 20 seconds at 94°C, annealing it for 45 seconds at 55, 57,54,55 and 55) °C respectively for genes in Table (1), and elongating it for 1 minute at 72°C.

Following a final extension at 72°C for 5 minutes, the PCR products were run on a 1.5% agarose gel at 70 volts for 80 minutes. For the *16S rRNA* gene, ethidium bromide dye was used, which was then observed under an ultraviolet transilluminator and photographed. For the *mecA* gene, the mixture was amplified by first denaturing at 94°C for five minutes, after with the amplification process involved 35 cycles: denaturation at 94°C for 2 minutes, annealing at 57°C for 2 minutes, and elongation at 72°C for 1 minute. The final extension was conducted for 7 minutes at 72°C, and 90 minutes at 70 volts were used to run the PCR products on a 1.5% agarose gel via an ultraviolet transilluminator, ethidium bromide dye, and photography.

2.7. Statistical analysis

The Statistical Packages of Social Sciences-SPSS (2019) program was used to detect the effects of different groups and factors on the study parameters. The chi-square test was used to compare significant differences between percentages (0.05 and 0.01 probability) in this study (SPSS, 2019).

3. Results and Discussion

3.1. Isolation and identification of *Staphylococcus aureus*

A total of 125 clinical samples were collected from Baghdad hospitals. The presence of beta-hemolytic colonies on blood agar and yellow (golden) colonies resulting from the fermentation of mannitol sugar, which turns phenol red golden, are indicators of samples produced directly on mannitol salt agar and blood agar. As a selective medium, these samples also exhibit tolerance to elevated salt concentrations of MSA, and the isolates were examined through typical biochemical examinations. Both the coagulase and catalase responses in these samples were positive. However, the results of oxidase testing were negative. In addition, culture, morphology and biochemistry revealed that these isolates *S. aureus* (Tille, 2017). The results revealed that only 36 (24%) samples had typical biochemical testing and morphological characteristics peculiar to *Staphylococcus aureus*. The isolates proportions were as follows: burn 2 (5.5%), sputum 3 (8.3%), nasal swab 2 (5.5%), throat swab 2 (5.5%), wound 8 (22.2%), urine 18 (50%), and CSF 1 (2.7%). Our findings concur with those of Ahmed and Al-Daraghi (2022), who reported that fifteen *S. aureus* isolates were isolated from UTIs and nine from wounds. The variation in the prevalence rates of isolates is due to several factors, including differences in sample collection methods and sample sizes. Likewise, differences in isolation and diagnosis methods and the purpose of the study.

3.2. Molecular identification of MRSA

An effective method for identifying and detecting bacteria is the amplification of DNA from phylogenetically distinct bacteria by focusing on specific regions of the *16S rRNA* gene. (Vestergaard *et al.*, 2019). This gene's bacterial DNA was amplified via the PCR technique in a monoplex pattern via certain primers under ideal conditions. The results of the PCR via agarose gel electrophoresis revealed that 36 (100%) *S. aureus* isolates were 100% positive for the *16S rRNA* gene (578 bp), as

shown in Figure (1). Our findings agree with those of (Shamkhi *et al.*, 2019), who also reported that 100% of clinical *S. aureus* isolates were positive for the *16S rRNA* gene.

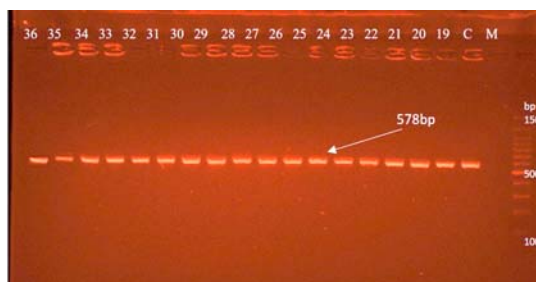


Figure 1: The *16S rRNA* gene (578bp) was identified via ethidium bromide-stained agarose gel electrophoresis of the amplified PCR product in lanes 19–36 on 1.5% agarose (80 min at 70 volts); M: marker DNA ladder (1500 bp); and C: negative control.

3.3. Investigation of the *mecA* gene

Methicillin-resistant *S. aureus* (MRSA) was detected via the *mecA* gene, a proprietary genetic marker. (Kadhun and Abood, 2022). PCR was used to amplify the bacterial DNA of this gene in a monoplex pattern under certain primer conditions. Figure (2) shows the 163 bp result of *mecA* gene amplification, which was confirmed by agarose gel electrophoresis and recorded on a camera with an ultraviolet transilluminator of clinical *S. aureus* isolates had positive *mecA* gene tests (97.22%). These results disagree with research by Kadhun and Abood, (2022) who identified the *mecA* gene in 100% of clinical *S. aureus* isolates.

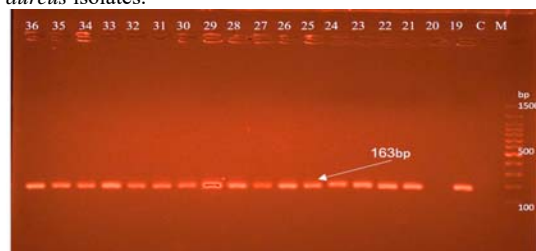


Figure 2: Identification of the *mecA* gene (163 bp), the amplified PCR product was electrophoresed on 1.5% agarose for 90 minutes at 70 volts in lanes 19–36 of the ethidium bromide-stained agarose gel; M: marker DNA ladder (1500 bp); and C: negative control.

3.4. Antibiotic resistance profiles in *Staphylococcus aureus* isolates

Antibiotic resistance summaries were studied for *S. aureus* isolates. Thirty-six *S. aureus* isolates, only 11 (30.55%), were determined to be MDR. The results revealed the greatest resistance of the *S. aureus* isolates to cefoxitin (FOX) 20 (55.55%). The resistance of the isolates gradually decreased, followed by erythromycin (ERY) 14(38.88%), doxycycline (DOX) 11(30.55%), norfloxacin (NOR) 9(25%), ciprofloxacin (CIP) 8(22.22%). Most of the isolates were highly sensitive to nitrofurantoin (F) 0(100%), chloramphenicol(C) (91.66%), rifampicin(RIF)(88.88%), and trimethoprim-sulfamethoxazole(STX) (86.11%), and clindamycin(CD) (75%), and the sensitivity of the isolates gradually decreased to other antibiotics. A high proportion of resistance levels to the various antibiotic classes found in the majority of *S. aureus* isolates is depicted in Figure (3).

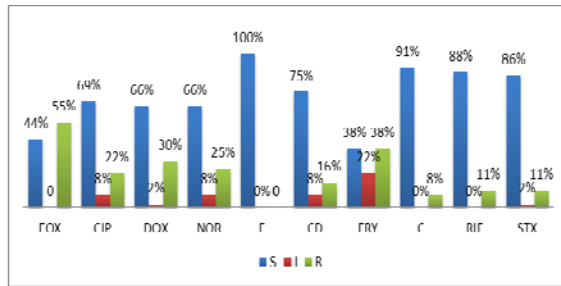


Figure (3) Percentages of antibiotic susceptibility rates of 36 *S. aureus* isolates to ten antibiotic agents.

The results agreed with of Jabur and Kandala (2022) who reached (6%) resistance to chloramphenicol and no resistance to nitrofurantion; however, they disagreed with those of Doxycyclin (10%) and cefoxitin (87%). However, the results agreed with those of Maharjan *et al.* (2021), who reached (60.8%) resistance to cefoxitin; however, they disagreed with those of chloramphenicol (56.8%). The results disagreed with Hantoosh, (2022), who reached 25% resistance to rifampin and 26% resistance to nitrofurantion, and disagreed with Hamad, (2023), who reached 40.6% resistance to clindamycin while agreeing with norfloxacin (25%), while he reached 21.9% resistance to trimethoprim-sulfamethoxazole. In addition, the results agreed with Belbase *et al.*, who reported that 27.8% of the strains were resistant to erythromycin. In addition to the previously mentioned outcome of Hamad (2023), who reported that isolates of *S. aureus* are sensitive to trimethoprim(78.1%), the results do not agree with those of clindamycin (59.4%), while the results agreed with those of Awayid and Mohammad (2022), who reached 94.2% sensitivity to rifampin but disagreed with those of cefoxitin (0), which also disagreed with Hantoosh (2022), who reached 75% sensitivity to rifampin and 74% sensitivity to nitrofurantion, while the results of Aniba *et al.*(2024), erythromycin (28%), also agreed with those of Saud *et al.* (2023).The prevalence of methicillin-resistant

Staphylococcus aureus (MRSA) drug resistance is due to numerous factors, including inappropriate prescription of antibiotics and self-medication by the use of antibiotics without a prescription. Additionally, hospital infections can spread between patients and staff via a breeding ground for MRSA, and poor hygiene through failure to adhere to proper hygiene practices in hospitals can facilitate the transmission of infection. In addition, genetic mutations lead to the development of antibiotic resistance mechanisms. Finally, the failure to treat with antibiotics before the end of treatment can lead to the persistence of resistant bacteria and a weakened immune system in individuals (Alghamdi *et al.*, 2023., Marciniak *et al.*, 2024).

3.5. Phenotypic detection of efflux pumps

As shown in Figure (4) and Table (2), EtBr-CW method is utilized to phenotypically detect efflux pumps in 36 isolates of *S. aureus* as an easy way to evaluate MDR bacteria for overexpressed efflux pumps by expelling ethidium bromide(EtBr) dye. Fluorescent growth indicates no efflux, whereas isolates with active efflux pumps do not exhibit fluorescent growth. Our work revealed that 10 of the 36 isolates, or approximately 27%, do not show fluorescence at a concentration of 2 mg/L EtBr, which indicates intermediate results for efflux pumps. This finding is consistent with the findings of Baiomy *et al.* (2020), who reported a value of 30%, whereas the results of Silva *et al.* (2021), who reported a value of 34.7%; however, 6 of the 36 isolates that were positive (16%) did not show fluorescence at a concentration of 4 mg/L EtBr, which indicates positive results for the phenotypic detection of efflux pumps, as shown in Figure (3). We considered the 1 mg/L concentration a negative control compared with the positive results of the presence of efflux pumps in *S. aureus*. Similarly, another study revealed that the ratios between average EtBr intermediate and positive EtBr were similar (Costa *et al.*, 2013).

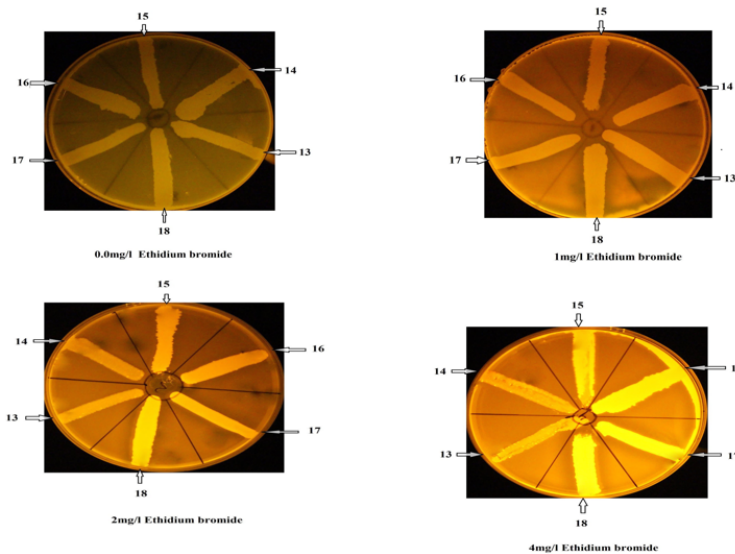


Figure (4): Tryptone soy agar plates containing different concentrations of EtBr were swabbed with *S. aureus* isolates.

In summary, the EtBr-CW approach is simple to implement, takes less time, and can screen a large number of bacterial strains, making it easier to identify isolates

with an MDR phenotype quickly. It can be utilized to identify MDR strains caused by efflux in both gram-

positive and gram-negative clinical isolates. (Martins *et al.*, 2011).

Table (2): Phenotypic detection of *Staphylococcus aureus* pump efflux at varying ethidium bromide dye concentrations in a tryptone soy agar plate.

Isolates code	No.	EtBr_dye concentration		
		1 mg/l	2 mg/l	4 mg/l
St1,St4,St8,St9, St10,St11,St13, St14,St16,St17, St23,St25,St29, St30,St33,St34, St35,St36	17	-	-	-
St3,St6,St31,St18 St24	5	+	+	+
St12,St15,St20, St29	4	+	+	-
St55,St7,St21, St22,St26, St27, St28	7	+	-	-
St32	1	-	+	-
St19	1	-	-	+

A positive result is no fluorescence (+), and a negative result is fluorescence (-)

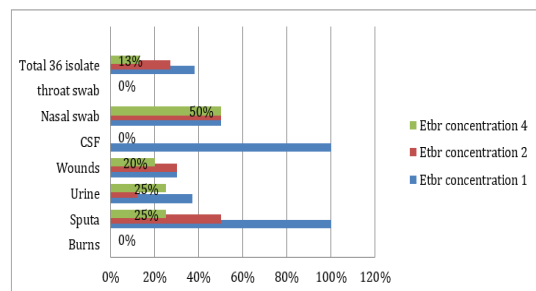


Figure (5): Efflux pump gene prevalence in clinical isolates from different *S. aureus* sources.

Figure (5) shows the relationship between the ability of the isolates to expel ethidium bromide dye and the source of the isolates, where the highest percentage of efflux pumps appeared in 50% of the nasal swab isolates at a concentration of 4 mg/L, followed by 25% of the urine and sputum isolates, 20% of the wound isolates and 15% of the throat swabs; however, these pumps did not appear in the burn or cerebrospinal fluid isolates. This finding reinforces the role of efflux pumps in resistance to antibiotics, disinfectants, and detergents, as well as the importance of this method for detecting efflux pumps in methicillin-resistant isolates. (Costa *et al.*, 2013).

3.6. Detection of some efflux pump genes via PCR

One of the highly distributed chromosomally encoded traits of resistance is the efflux pump. In our study, a PCR amplification approach was used for several efflux pump genes, *norB*, *mdeA* and *mepA*, which were examined in the 36 MDR isolates to further identify the efflux pump as a resistance mechanism of the tested MDR *S. aureus*. In our study, 28 isolates (77%) were positive for the *norA* gene, 34 (94%) were positive for the *mpeA* gene, and 34 (94%) were positive for the *mdeA* gene. In light of the results we obtained above, the genes encoding efflux pumps that are most abundant in the isolates of *S. aureus* were *mdeA* and *mepA*, whereas the result for the *norB* gene was the lowest among these two genes. The authors reported that 60.9%

of the isolates produced from clinical samples from Korean patients were *norB* to be the most often overexpressed MDR efflux pump gene, and in another study, the presence of the *mdeA* gene in their isolates was the equivalent of 61.7%, which disagrees with our results, whereas in the search results of Suma *et al.* (2023), the *mdeA* gene percentage agreed with them, who reached 93.33%, and the *mepA* gene was present in their isolates (93.33%), which is in agreement with the results mentioned above. The *mepA* gene disagreed with the results of Antiabong *et al.*, (2017), who reached 97.9%. The *norB* gene was the lowest percentage that appeared between the *mepA* gene and the *mdeA* gene, which agreed with the findings of Shamkhi *et al.* (2019), who reached 56.25%, which is the lowest percentage of the two genes mentioned.

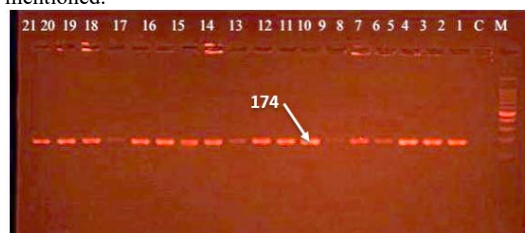


Figure 6: Identification of the *mdeA* gene (174 bp), the amplified PCR product was electrophoresed on 1.5% agarose for 90 minutes at 70 volts in lanes 1–21 of the ethidium bromide-stained in agarose gel; M: marker DNA ladder (100 bp); and C: negative control.



Figure 7: Identification of the *mepA* gene (240 bp), the amplified PCR product was electrophoresed in lanes 1–20 of an ethidium bromide-stained agarose gel on 1.5% agarose for 90 minutes at 70 volts; M: marker DNA ladder (100 bp); and C: negative control.

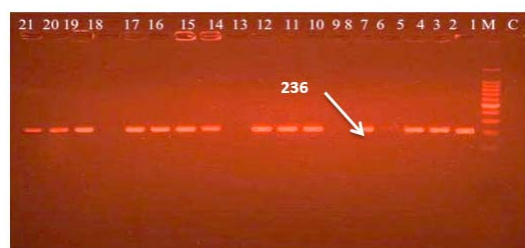


Figure 8: Identification of the *norB* gene (236 bp), the amplified PCR product was electrophoresed in lanes 1–21 of an ethidium bromide-stained agarose gel on 1.5% agarose for 90 minutes at 70 volts; M: marker DNA ladder (100 bp); and C: negative control.

3.7. Antibiotic resistance profile associated with efflux pump genes in MRSA

This study examined the profiles of antibiotic resistance genes in 35 clinical MRSA isolates. The three genes identified in those clinical isolates of *S. aureus* (*norB*, *mdeA* and *mepA*) have been associated primarily with resistance to several drugs encode efflux pumps. The isolates were resistant to nine different antibiotics, namely, ceftaxime (FOX), ciprofloxacin (CIP), erythromycin

(ERY), norfloxacin (NOR), doxycycline (DOX), and trimethoprim-sulfamethoxazole (STX), chloramphenicol (C), rifampin (RIF), clindamycin (CD),

Table (3): The percentages of total genes related to resistance to nine different antibiotics.

Genes	Isolates no. 36	Antibiotic susceptibility									P- value
		FOX	CIP	ERY	NOR	DOX	C	CD	RIF	SXT	
<i>norB</i>	28 (77%)	15 (53%)	6(21%)	13 (46%)	6 (21%)	11 (39%)	2(7%)	4(14%)	3(10%)	2(7%)	0.0001 **
<i>mepA</i>	34 (94%)	20 (58%)	7 (20%)	15 (44%)	7 (20%)	12 (35%)	3(8%)	5(14%)	4(11%)	2(5%)	0.0001 **
<i>mdeA</i>	34 (94%)	19 (55%)	8 (23%)	16(47%)	8 (23%)	11 (32%)	2(5%)	4(11%)	4(11%)	2(5%)	0.0001 **
P value	0.681 NS	0.237 NS	0.794 NS	0.547 NS	0.732 NS	0.904 NS	0.866NS	0.902 NS	0.894 NS	1.00 NS	---

** (P<0.01), NS: Non-Significant.

Table (3) shows the relationships between the presence of resistance genes (*norB*, *mepA*, and *mdeA*) and the extent of their sensitivity or resistance to nine different antibiotics. The *norB* gene is relatively highly resistant to the following antibiotics: FOX: 53%, ERY: 46%, and DOX: 39%, whereas it has low resistance to other antibiotics, such as C (7%) and SXT (7%). The *mepA* gene has greater resistance: FOX: 58%. ERY: 44%. DOX: 35%. However, its resistance to some antibiotics, such as SXT (5%), is also low. Finally, the resistance pattern of the *mdeA* gene is similar to that of *mepA*, with greater

resistance to the following antibiotics: FOX: 55%. ERY: 47%. DOX: 32% and low resistance to antibiotics such as C (5%) and SXT (5%). As an explanation for this, the most prevalent genes are *mepA* and *mdeA* (94%), which indicates that these two genes play major roles in the resistance of isolates to antibiotics. The three genes show significant resistance to FOX and ERY, which means that these antibiotics may be less effective against these isolates. Low resistance to antibiotics such as C and SXT indicates the possibility of using these antibodies as therapeutic options.

Table (4): The percentages of total MDSs and MDRs according to gene distribution.

Isolate sources	Total no. 35	Genes			EtBr Agar concentration			P value
		<i>norB</i>	<i>mepA</i>	<i>mdeA</i>	1 mg/L	2 mg/L	4 mg/L	
Multidrug sensitive (MDS) isolates	24 (68%)	21 (87%)	24(100%)	24 (100%)	13 (54%)	9 (37%)	6 (25%)	0.037 *
Multidrug resistance (MDR) isolates	11 (23%)	7 (63%)	10 (90%)	10 (90%)	3 (27%)	2 (18%)	1 (9%)	0.816 NS
P value	0.0087 **	0.0074 **	0.0082 **	0.0082**	---	0.0367 *	0.078 NS	---

* (P<0.05), ** (P<0.01), NS: Non-Significant.

Table (4) shows the distinctions between multidrug-susceptible (MDS) and multidrug-resistant (MDR) isolates. The percentages of each type of isolate from a total of 35 MRSA isolates are indicated. Gene distribution and percentages of *norB*, *mepA*, and *mdeA* genes in the isolates. All drug-susceptible (MDS) isolates contained the *mepA* and *mdeA* genes (100%), while the *norB* gene was present in 87% of them. For resistant (MDR) isolates, the *norB* gene is present in 63%, and the *mepA* and *mdeA* genes are present in 90%. The table displays the response of the isolates to different concentrations of ethidium bromide (1, 2, and 4 mg/L). The percentage of susceptible isolates (MDS) gradually decreased with increasing concentration (54% at 1 mg/L to 25% at 4 mg/L). Resistant (MDR) isolates showed a similar reduction but were less susceptible (27% at 1 mg/L to 9% at 4 mg/L). The results revealed that resistant isolates (MDR) carry a greater percentage of resistance genes than susceptible isolates (MDS). Increasing the concentration of ethidium bromide led to reduced growth of both types of isolates, but resistant isolates were more resistant to high concentrations than were susceptible isolates.

3.8. The minimal inhibitory concentration (MIC) of diclofenac sodium

The inhibitory effects of diclofenac sodium at various concentrations (1000-500-250-125- 62.5 -32.25) tested against eight MDR strains that were methicillin-resistant

encoded *mecA* gene and also encoded three efflux pump genes after PCR amplification. The isolates code no. (3,7,8,15,20,30,32,36) were examined via a resazurin based microplate broth dilution assay. The color of resazurin an indicator dye can be altered in response to the metabolic activity of living cells, which is the basis for the assay. Resazurin turns pink when bacterial growth occurs because the metabolic activity of the bacteria reduces the hue of the dye (Belbase *et al.*, 2017). As one of the most standardized techniques for testing antibiotics, it does not require a spectrophotometer because, unlike the conventional assay, the color change may be observed visually (Teh *et al.*, 2017). Several studies have shown that several non-steroidal anti-inflammatory drugs (NSAIDs) have strong antibacterial qualities. Diclofenac sodium, in particular, has strong antibacterial activity against both gram positive and gram negative bacteria (Chan *et al.*, 2017). The results of this investigation revealed that the lowest inhibitory concentration of olfen varied between 250 and 125 µg/ml. Olfen, which has analgesic, antipyretic, and anti-inflammatory qualities, has demonstrated antibacterial activity. With MICs ranging from 50 µg/ml to 200 µg/ml for gram negative bacteria and even lower for some gram-positive bacteria, this non-steroidal anti-inflammatory medication has demonstrated antimicrobial action against a variety of gram positive and gram negative bacteria in recent years (Dutta *et al.*, 2007). These variations in the MICs of this drug may be caused by the

different strain types used in different studies or by methodological factors such as the culture media used, the research methods, and the minimum inhibitory concentrations (MICs) of oflen against all tested gram positive bacteria (Chan *et al.*, 2017), which disagrees with the results we obtained. This may be attributed to the fact that the isolates are more resistant and to other reasons, such as the incorrect use of this type of analgesic drug when suffering from bacterial infections. This clearly reveals antibacterial metabolic activity. The effect of diclofenac sodium on the inhibition of bacteria, specifically *S. aureus*, affects many virulence factors in bacteria and this has been confirmed by many studies (Silva *et al.*, 2021). The purpose of this comparison was to clarify the extent of the inhibitory effect of this drug on virulence factors. In particular, for the bacteria *Staphylococcus aureus*. The exact mechanism of antibacterial activity of diclofenac remains to be elucidated. Studies have proposed different mechanism of action like inhibition of bacterial DNA synthesis, impairment of membrane activity, anti-plasmid activity, DNA synthesis and cell envelope, down-regulation of efflux pumps (Dastidar *et al.*, 2000).

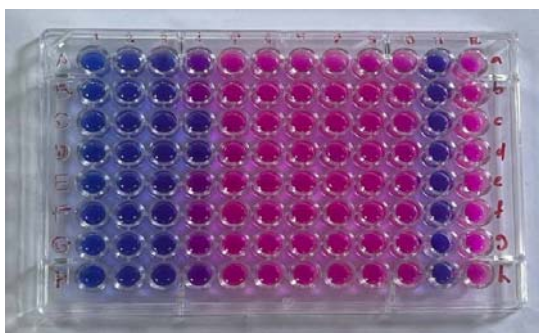


Figure (9): Diclofenac sodium minimum inhibitory concentration of eight MDR *S. aureus* strains determined via the resazurin-based method. Row (11) represents the negative control, which shows the natural color of resazurin (blue/purple). Row 12 represents a positive control, was changed to a reduced form (pink). Wells A-F of each raw isolate contained diclofenac sodium at 1000–31.25 µg/ml, respectively.

4. Conclusion

Application of NSAIDs, such as diclofenac sodium, could serve as adjunctive agents in combating antibiotic resistance as the first study in Iraq against MRSA isolates. Diclofenac sodium might be considered as antibacterial and anti-efflux pump inhibitors agent for the growth inhibition of MRSA isolates harboring for some efflux pump genes. This points to the need for further researches into adjunctive therapies like another NSAIDs or efflux pump inhibitors.

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Conflicts of Competing Interest

The authors declare that they have no potential conflicts of interest.

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