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Diclofenac Sodium Inhibits the Gene Expression of the *norB* Efflux Pump in Clinical Methicillin-Resistant *Staphylococcus aureus*

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Abstract:

Background. Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant public health challenge because of its ability to resist multiple antibiotics, primarily through efflux pump mechanisms and the expression of resistance genes. Recent studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, may play a role in modulating bacterial resistance mechanisms.**Aim.** To investigate the impact of diclofenac sodium (Olfen) on the expression of the efflux pump gene *norB* in MRSA isolates and evaluate its potential for enhancing antibiotic efficacy.**Methods.** A total of 125 clinical samples were collected and analyzed for the presence of *Staphylococcus aureus* via phenotypic and molecular identification methods. Antibiotic susceptibility was assessed via the Kirby–Bauer disk diffusion method, whereas the minimum inhibitory concentration (MIC) was determined via the resazurin-based microplate dilution assay. The expression of efflux pump genes was quantified before and after diclofenac sodium treatment via quantitative real-time PCR (RT–qPCR) and normalized to that of the housekeeping gene 16SrRNA.**Results.** PCR analysis confirmed the presence of the *norB* gene in 77% of the MRSA isolates. RT–qPCR analysis demonstrated significant downregulation of *norB* expression following diclofenac sodium treatment. These findings suggest that diclofenac sodium may interfere with efflux pump activity, potentially restoring bacterial susceptibility to antibiotics.**Conclusion.** These findings indicate that diclofenac sodium can modulate efflux pump gene expression in MRSA, reducing bacterial resistance mechanisms. This study highlights the potential of NSAIDs as adjunctive agents for combating antibiotic-resistant infections.

Keywords: Diclofenac sodium, gene expression, efflux pump, MRSA, RT–PCR

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a significant public health challenge globally, especially because of its capacity to cause infections that range from mild skin conditions to life-threatening diseases. This bacterium, which

is frequently found in the normal flora of the skin and upper respiratory tract, has a remarkable ability to acquire resistance mechanisms against multiple antibiotics, posing substantial treatment hurdles (1). Staphylococci are a group of bacteria commonly found in various environmentswhere they can be a source of contamination (2). Methicillin-resistant S. aureus (MRSA) is particularly challenging due to its resistance to beta-lactam antibiotics (3). The dissemination of methicillin resistance occurs via horizontal gene transfer of *staphylococcal* cassette chromosome (SCCmec) elements and other genetic mutations (4). Staphylococcus aureus is a bacterial pathogen associated with various infections, and its ability to form biofilms contributes significantly to its virulence. (5). Efflux pumps play a crucial role in antimicrobial resistance by expelling antibiotics from bacterial cells, reducing their effectiveness (6). Efflux pumps in bacteria are categorized into five major families: the ATP-binding cassette (ABC), major facilitator superfamily (MFS), resistance-nodulation-division (RND), small multidrug resistance (SMR), and multidrug and toxin extrusion (MATE) families (7). These pumps, powered either by ATP hydrolysis or chemical gradients, play pivotal roles in mediating multidrug resistance (8). In particular, the efflux systems of MRSA can extrude various classes of antibiotics, complicating treatment strategies (9). In addition to traditional antibiotics, alternative approaches are being explored to combat MRSA infections. Diclofenac sodium is a widely used nonsteroidal anti-inflammatory drug (NSAID) that is associated with bacterial resistance and has promising antibacterial properties (10). Its ability to inhibit biofilm formation and target bacterial virulence factors has been reported, suggesting its potential as an adjunctive antivirulence therapy (11). In the present study, we investigated the effect of diclofenac sodium (olfen), an efflux pump inhibitor of the *norB* gene, on antibiotic resistance in methicillin-resistant *S. aureus* via RT–PCR as a synergistic agent.

Materials and methods

Bacterial isolates and identification of *Staphylococcus aureus*

Between June 2024 and October 2024, a total of 125 clinical samples were collected from patients visiting various hospitals in Baghdad. These samples included wounds (37; 29.6%), urine (55; 44%), burns (8; 6.4%), sputum (9; 7.2%), nasal swabs (6; 4.8%), throat swabs (7; 5.6%), and cerebrospinal fluid (CSF) (3; 2.4%). The patients ranged in age from 15--60 years and represented both genders. Each sample was promptly cultured on blood agar and incubated at 37°C for 24 hours. Identification was carried out via Gram staining, biochemical tests, and growth analysis on mannitol salt agar (MSA), a selective medium (12).

Antibiotic susceptibility test.

The antibiotic susceptibility of the *Staphylococcus aureus* isolates was evaluated via the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA) (Hi-Media). Eleven antibiotic discs

were tested in this study (13). The agar plates were incubated at 37°C for 18 hours, and the diameters of the inhibition zones were measured in accordance with the Clinical and Laboratory Standards Institute (CLSI 2024) (14). The antibiotics tested included cefoxitin (FOX: 30 µg), ciprofloxacin (CIP: 5 µg), erythromycin (ERY: 15 µg), norfloxacin (NOR: 10 µg), azithromycin (AZM: 15 µg), doxycycline (DOX: 30 µg), nitrofurantoin (NIT: 300 µg), clindamycin (CLI: 2 µg), chloramphenicol (CHL: 30 µg), rifampin (RIF: 5 µg), and trimethoprim-sulfamethoxazole (SXT: 1.25/23.75 µg).

Phenotypic methicillin resistance detection

All identified *Staphylococcus aureus* isolates were phenotypically screened for methicillin resistance via the cefoxitin disk diffusion test. For this method, a 30 µg cefoxitin disk on Mueller–Hinton agar was used as a substitute for methicillin. Isolates were classified as resistant if the inhibition zone measured less than 21 mm, and as susceptible if the zone exceeded 21 mm. (15).

Assessment of the antibacterial activity of diclofenac sodium via the microtiter plate (MTP) method for MIC determination

In accordance with the CLSI (2024) (14), an assay was created to determine the minimum inhibitory concentrations (MICs) of diclofenac sodium against certain multidrug-resistant isolates of MRSA via a resazurin-based microplate broth dilution assay. The antibiotic concentrations of the drug diclofenac sodium (75000 µg/ml) and Mueller–Hinton broth (MHB) spiked with resazurin dye were applied to a 96-well microtiter plat.

Molecular identification by PCR

Genomic DNA from *Staphylococcus aureus* isolates was extracted via a commercial DNA purification kit (Promega, USA). The extracted DNA samples were utilized to identify efflux pump genes. The PCR results were validated by comparing the molecular weights of the DNA bands against a DNA ladder and analyzing the bands through gel electrophoresis. The primers used in the PCR analysis were selected on the basis of the specifications outlined in Tables (1) and (2) of this study.

Gel electrophoresis protocol

The genes were detected through agarose gel electrophoresis of the amplified PCR product via a 1.5% agarose gel (70 $^{V/cm2}$ for 80 minutes) and stained with ethidium bromide.

Table (1): Primers used in this study

Primers name	Primer's sequence 5′ ⟶3′	Product size (bp)	Reference
16S rRNA	AACCTACCTATAAGACTGGG	578	(16)
	CATTTCACCGCTACACATGG		
mecA	ACTGCTATCCACCCTCAAAC	163	(17)
	CTGGTGAAGTTGTAATCTGG		
norB	TCGCCTTCAACACCATCAAC	236	(18)
	GGCGTAGGAGATGATGGTCA		
16S rRNA	TGTCGTGAGATGTTGGG	270	(19)
	CGATTCCAGCTTCATGT		

Table (2): PCR products for all primers.

Components	Volume (µl)
Master Mix	12.5
Forward-primer (10 pmol/µl)	1
Reverse-primer (10 pmol/µl)	1
Nuclease Free Water	6.5
DNA	4
Total volume	25

Table (3): PCR steps *for 16S rRNA*

primer	Steps	Temp. (°C)	Duration	Cycles
16SrRNA	Initial denaturation	94	5 min	1
	Denaturation	94	20 sec	35
	Annealing	55	45 sec	
	Elongation	72	45 sec	
	Final extension	72	10 m	1

Table (4): PCR steps for *mecA*

Primer	Steps	Temp. (°C)	Duration	Cycles
mecA	Initial denaturation	94	5 min	1
	Denaturation	94	2 min	35
	Annealing	57	2 min	
	Elongation	72	1 min	
	Final extension	72	7 min	1

Table (5): PCR steps for *norB*

Primers	Steps	Temp. (°C)	Duration	Cycles
norB	Initial denaturation	94	5 min	1
	Denaturation	94	1 min	25
	Annealing	54	45 sec	
	Elongation	72	1 min	
	Final extension	72	5 min	1

Table (6): RT–qPCR program

Stage	Temp. (°C)	Period	No. cycle
RT. Enzyme Activation	37	15 min.	1
Initial Denaturation	95	5 min	1
Denaturation	95	30 sec	40
Annealing	52	30 sec	
	54		
	60		
Extension	72	30 sec	

Efflux pump gene expression

Quantitative real-time PCR was used to measure the gene expression of *norB* to examine the impact of diclofenac sodium on efflux pump genes in *S. aureus* isolates. Both before and following MIC treatment of the microorganisms. The primers used for efflux pumps are listed in Table (1). To normalize the target genes' mRNA levels, the expression of the housekeeping gene *16SrRNA* (19) was assessed for gene expression.

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 (20).

Results:

Isolation and identification of *Staphylococcus aureus*

A total of 125 clinical samples were collected from various hospitals in Baghdad. Beta-hemolytic colonies on blood agar and yellow (golden) colonies resulting from the fermentation of mannitol sugar, which turns phenol red to golden, were observed in samples cultured directly on mannitol salt agar (MSA). As a selective medium, MSA also demonstrated the tolerance of the isolates to high salt concentrations.

These isolates were further analyzed via standard biochemical tests. The results showed positive reactions for both coagulase and catalase, whereas the oxidase test results were negative. Cultural, morphological, and biochemical analyses identified the isolates as *Staphylococcus aureus* (21).

However, only 36 isolates (24%) presented the typical biochemical and morphological characteristics of *S. aureus*. The distribution of these samples was 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%).

Molecular identification of MRSA.

An effective approach for identifying and detecting bacteria involves amplifying DNA from phylogenetically distinct bacteria by targeting specific regions of the 16S rRNA gene (23). In this study, bacterial DNA for this gene was amplified via the PCR technique in a monoplex format with specific primers under optimal conditions.

The results of the PCR analysis via agarose gel electrophoresis revealed that all 36 (100%) *S. aureus* isolates were positive for the 16S rRNA gene (578 bp), as illustrated in Figure (1).

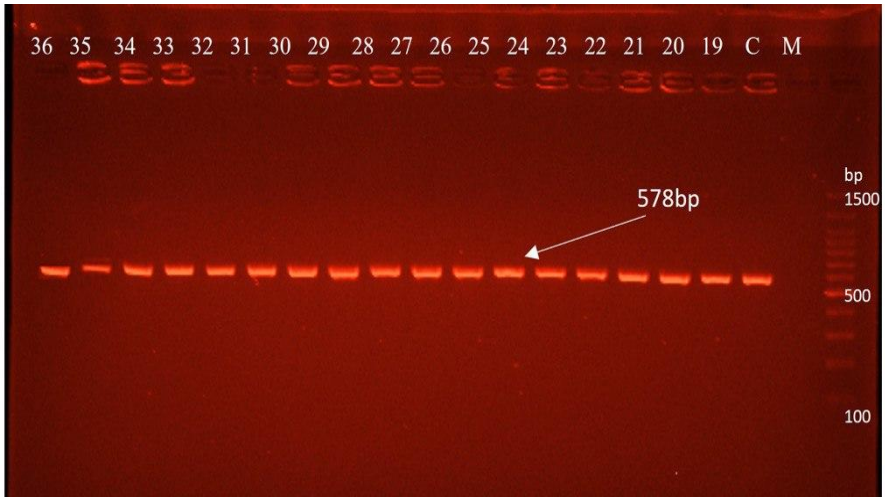

Figure (1): The *16SrRNA* gene (578 bp) was identified via ethidium bromide-stained agarose gel electrophoresis of the amplified PCR product in lanes 19–36 on 1.5% agarose at 70 V^{cm2} for 80 min. M: Marker DNA ladder (100 bp); C: Negative control.

Investigation of the *mecA* gene:

Methicillin-resistant *S. aureus* was identified through detection of the *mecA* gene, a specific genetic marker (24). PCR was employed to amplify the bacterial DNA of this gene in a monoplex format under specific primer conditions.

The amplification results, which revealed a 163 bp *mecA* gene product, were visualized via agarose gel electrophoresis and documented with a UV transilluminator. Among the clinical *S. aureus* isolates, 97.22% tested positive for the *mecA* gene.

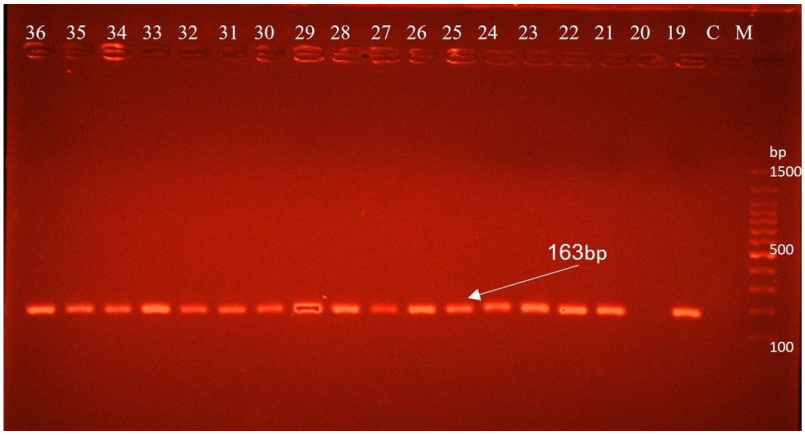

Figure (2): For *mecA* gene (163 bp) identification, the amplified PCR product was electrophoresed on 1.5% agarose at 70V ^{cm2} for 75 minutes via lanes 19–36 of the ethidium bromide-stained agarose gel; M: marker DNA ladder (100 bp); and C: negative control.

Antibiotic resistance profiles in *Staphylococcus aureus* isolates

The antibiotic resistance profiles of the *S. aureus* isolates were analyzed. Among the 36 *S. aureus* isolates, 11 (23%) were identified as multidrug-resistant (MDR).The highest resistance was observed against cefoxitin (FOX) in 20 isolates (55.55%). The resistance levels decreased progressively for erythromycin (ERY) in 14 isolates (38.88%) and doxycycline (DOX) in 11 isolates (30.55%).

Conversely, most isolates showed high sensitivity to nitrofurantoin (100%), chloramphenicol (91.66%), and rifampin (88.88%). The sensitivity levels gradually declined for ciprofloxacin (69.44%), doxycycline and norfloxacin (66.66%), and cefoxitin (44.44%).A high degree of resistance to various antibiotic classes in the majority of *S. aureus* isolates is illustrated in Table (7).

Table (7): Percentages of antibiotic susceptibility rates of 36 *S. aureus* isolates to 10 antibiotic agents.

Antibiotic	S	I	R	
	N (%) n=36			P value
Cefoxitin	16 (44.44)	0	20 (55.55)	0.0001 **
Ciprofloxacin	25 (69.44)	3(8.33)	8 (22.22)	0.0001 **
Doxycycline	24(66.66)	1 (2.77)	11 (30.55)	0.0001 **
Norfloxacin	24 (66.66)	3(8.33)	9 (25)	0.0001 **
Nitrofuration	36 (100)	0	0	0.0001 **
Clindamycin	27 (75)	3 (8.33)	6 (16.66)	0.0001 **
Erythromycin	14 (38.88)	8(22.22)	14 (38.88)	0.363 NS
Chloramphenicol	33 (91.66)	0	3 (8.33)	0.0001 **
Rifampin	32 (88.88)	0	4 (11.11)	0.0001 **
Trimethoprime-sulfamethoxazole	31 (86.11)	1 (2.77)	4 (11.11)	0.0001 **
P value	0.0001 **	0.0025 **	0.0001 **	---
** ($P \leq 0.01$).				

The minimal inhibitory concentration (MIC) of diclofenac sodium should be determined.

The inhibitory effects of diclofenac sodium at various concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/mL) on eight MDR methicillin-resistant *S. aureus* isolates were examined via a resazurin-based microplate broth dilution assay. Resazurin, an indicator dye, can alter color in response to the metabolic activity of living cells, which is

the basis for the assay shown in Figure (3). Resazurin turns pink when bacterial growth occurs because the metabolic activity of the bacteria reduces the hue of the dye (33).One of the most standardized techniques for testing antibiotics does not require a spectrophotometer because, unlike the conventional assay, the color change may be observed visually (34).The results indicate that diclofenac sodium inhibits methicillin-resistant *Staphylococcus aureus* (MRSA) in a dose-dependent manner, as determined via a resazurin-based microplate broth dilution assay. The concentrations tested ranged from 1000 µg/mL to 31.25 µg/mL.The term "MIC" (Minimum Inhibitory Concentration) appears under concentrations of 1000 µg/mL, 500 µg/mL, 250 µg/mL, and possibly lower concentrations. These findings suggest that diclofenac sodium effectively inhibited MRSA at these higher concentrations.

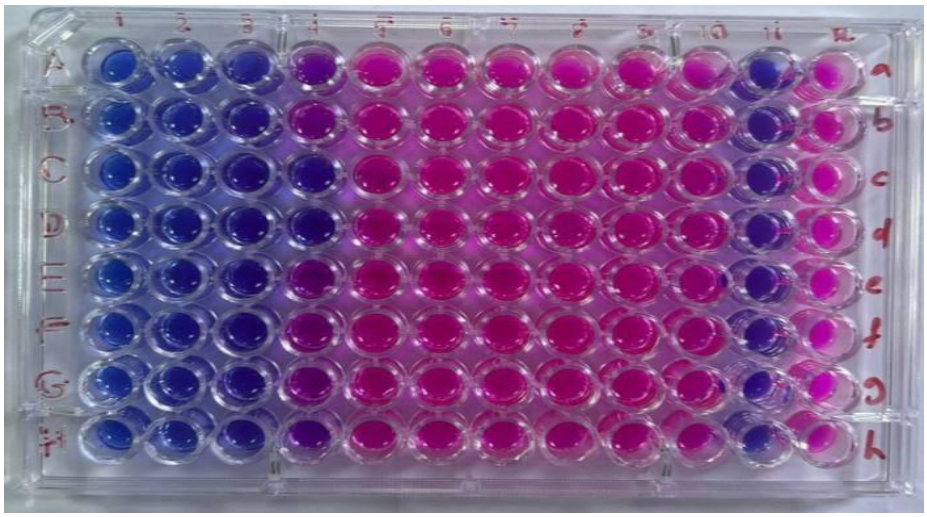

Figure (3): Diclofenac sodium minimum inhibitory concentration of eight MDR *S. aureus* strains determined via the resazurin-based method. Row (11) represents the negative control, which shows the natural color of resazurin (blue/purple). Row 3 (A, E, F, G, H), a positive control, was changed to a reduced form (pink). Well A and F of each raw sample contained diclofenac sodium at 1000–1.25 µg/ml, respectively.

Detection of the *norB* efflux pump gene in multidrug-resistant *S. aureus* isolates via PCR

The efflux pump is one of the many chromosomally encoded resistance mechanisms found in bacteria. To better understand the role of the efflux pump gene *norB* in resistance, PCR amplification was used in this work to examine 36 MDR (multidrug-resistant) *S.aureus* isolates.Twenty-eight (77%) of these isolates had positive *norB* gene tests.

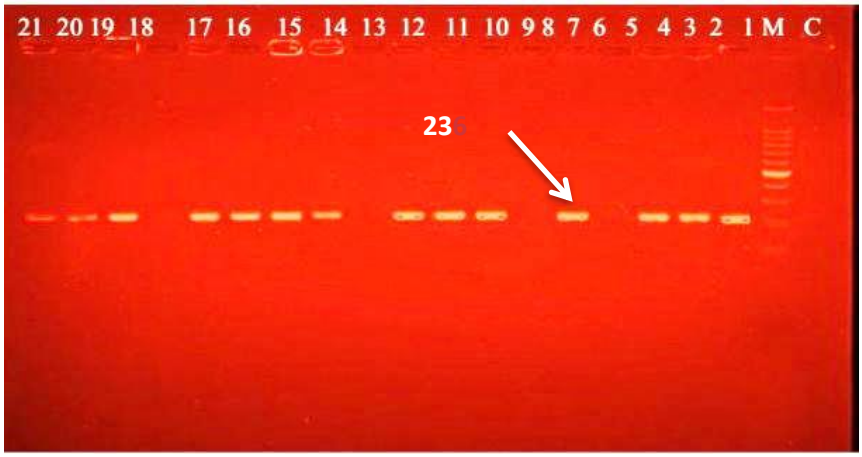

Figure (4): For the *norB* gene (236 bp), the amplified PCR product was electrophoresed in lanes 1–21 of an ethidium bromide-stained agarose gel on 1.5% agarose (70 V cm2 for 90 minutes); M: marker DNA ladder (100 bp); and C: negative control.

Gene expression of the efflux pump gene *norB* by RT–qPCR:

Total genomic RNA was extracted under highly precise conditions via TRIzoL and a ready kit from 28 *norB*-positive isolates. The RNA concentration was quantified via a Quantus fluorometer, and the extracted RNA concentrations ranged from 42.2 to 97.9 ng/µL. The extracted RNA was immediately converted into cDNA.This experiment aimed to quantify the expression of the *norB* gene in two distinct isolates of *S. aureus*. RT-qPCR was used to detect gene expression. The gene expression levels were measured via the fold change (2^-ΔCt) method, and the ΔCt value was normalized against the housekeeping gene stau-16S.The results revealed that before treatment with diclofenac sodium, the fold expression ratio was 1.000 for both the first and second isolates. After treatment, the gene expression values decreased to 0.016 for the first isolate and 0.002 for the second isolate.The results of the fold change analysis revealed a decrease in efflux pump gene expression in the treated samples compared with the control samples.

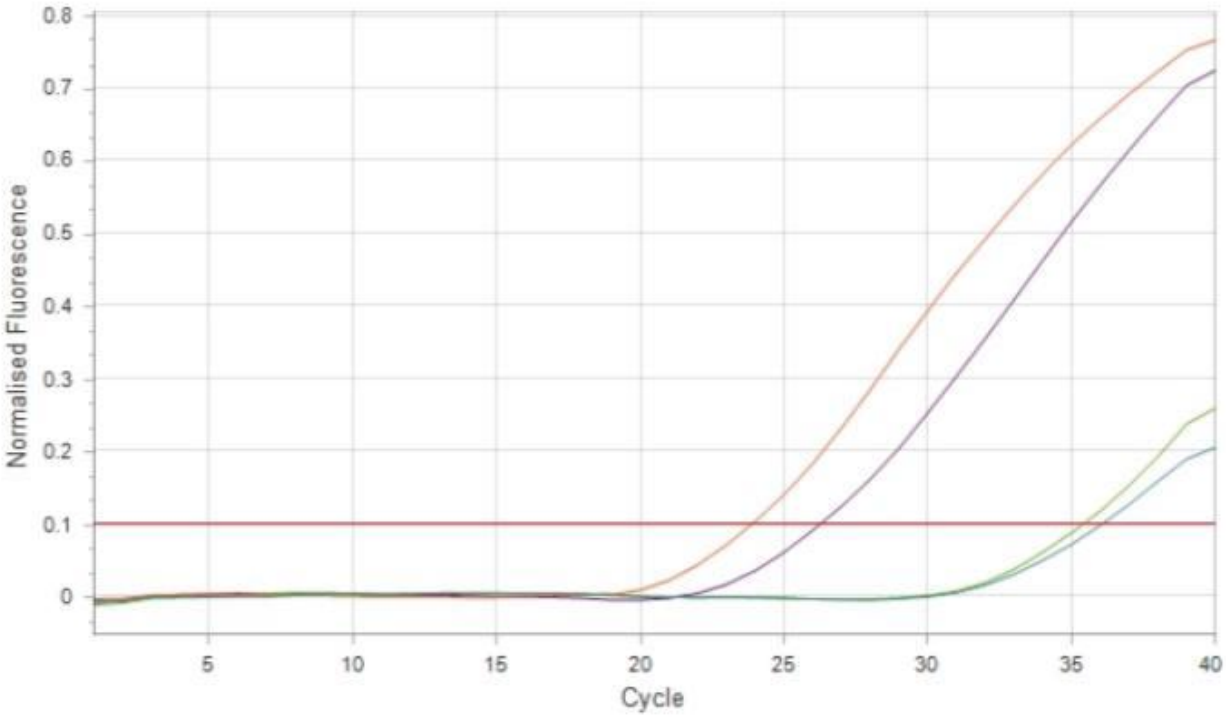

Figure (5)-: Gene expression of the efflux pump gene *norB* by qRT–PCR

Discussion:

Isolation and identification of *Staphylococcus aureus*

Some of our findings align with those of Ahmed and Al-Daraghi (22), who reported 15% *S. aureus* isolates in UTI cases and 9% in wound infections.The variation in the prevalence rates of isolates is due to several factors, including differences in sample collection methods and sample sizes, as larger samples tend to provide more realistic conclusions. Likewise, differences in isolation and diagnosis methods and the purpose of the study, in addition to temporal and geographic variation, may affect bacterial communities and their environmental interactions.

Molecular identification of MRSA

These findings are consistent with those of Shamkhi et al. (18), who reported that 100% of clinical *S. aureus* isolates tested positive for the 16S rRNA gene, and 96 isolates were isolated.This is because the 16S rRNA gene is used to confirm the diagnosis of MRSA and distinguish it from other isolates that are more closely related to it, such as *Staphylococcus epidermidis*.

Investigation of the mecA Gene

These findings contrast with those of the study by Kadhum and Abood (24), which reported the presence of the mecA gene in 100% of clinical *S. aureus* isolates.

Antibiotic Resistance Profiles in *Staphylococcus aureus* Isolates

The observed resistance and sensitivity patterns indicate variability in the effectiveness of different antibiotic classes against *S. aureus* isolates. The high resistance to cefoxitin and the presence of multidrug-resistant isolates suggest the persistence of resistant strains, whereas the high sensitivity to nitrofurantoin, chloramphenicol, and rifampin indicates that these antibiotics remain effective against most isolates tested.The findings of this study largely align with those of previous studies on methicillin-resistant *Staphylococcus aureus* (MRSA) resistance patterns, although some discrepancies were observed. Jabur and Kandala (25) reported 6% resistance to chloramphenicol and no resistance to nitrofurantoin, which aligns with the findings of the present study. However, differences were noted in doxycycline resistance (10%) and cefoxitin resistance (87%). Similarly, Maharjan et al. (26) reported 60.8% resistance to cefoxitin, but their chloramphenicol resistance rate was 56.8%, which differed from the results of this study. Awayid and Mohammad (27) reported 5.8% resistance to rifampin, although they reported 100% cefoxitin resistance, which contrasts with these findings. Aniba et al. (28) reported that 9% of isolates were resistant to trimethoprim-sulfamethoxazole, which is consistent with the findings of this study. However, Hantoosh (29) reported 25% resistance to rifampin and 26% resistance to nitrofurantoin, which diverges from these results. Furthermore, Hamad (30) reported 40.6% resistance to clindamycin, which differed from the results of this study, but their 25% norfloxacin resistance and 21.9% trimethoprim-sulfamethoxazole resistance rates were in agreement. Saud et al. (31) reported a resistance rate of 29.4% for ciprofloxacin, which is consistent with the present findings, whereas Belbase et al. (32) reported an erythromycin resistance rate of 27.8%, which aligns with the findings of this study. Partial agreement with the findings of Maharjan et al. 2021 (26), who reported 100% sensitivity to nitrofurantoin and 94% sensitivity to chloramphenicol, although their doxycycline resistance (90%) and cefoxitin resistance (13%) differed. Similarly, Awayid and Mohammad (27) reported 94.2% rifampin sensitivity but 0% cefoxitin resistance, which contrasts with the findings of this study. Hantoosh et al. (29) reported 75% rifampin sensitivity and 74% nitrofurantoin sensitivity, differing from the present findings. Finally, Aniba et al. (28) reported 28% erythromycin resistance, whereas Saud et al. (31) reported 70.6% ciprofloxacin resistance, highlighting variations in resistance patterns. These findings emphasize the impact of antibiotic misuse, poor infection control, and genetic mutations in driving MRSA resistance.

The minimal inhibitory concentration (MIC) of diclofenac sodium should be determined.

The reduction or lack of inhibition at lower concentrations (below 125 µg/mL) implies that the drug's bacteriostatic or bactericidal activity diminishes as the concentration decreases.

These findings align with those of previous studies showing that nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, have antimicrobial effects on various bacterial strains, including MRSA. This inhibition might be due to interference with bacterial DNA synthesis, membrane integrity, or protein function, as previously proposed in similar research (36).The role of diclofenac sodium as an antibacterial agent involves not only its ability to target inflammation but also its interference with bacterial survival mechanisms, such as efflux pump inhibition, which are vital for multidrug resistance in pathogens such as *Staphylococcus aureus*. By blocking these pumps, the intracellular accumulation of antibiotics increases, thereby restoring their efficacy (16).

Detection of the *norB* efflux pump gene in multidrug-resistant *S. aureus* isolates via PCR

We found previous studies similar to our study in terms of the presence of the gene. Hassanzadeh et al. (35) reported a prevalence rate of 60.9% for the *norB* gene, which is comparable to the findings of the present study.

Gene expression of the efflux pump gene norB by RT–qPCR:

The *norB* gene expression significantly decreased after treatment with diclofenac sodium, suggesting an inhibitory effect on efflux pump activity. These results suggest that diclofenac sodium has a potential role in reducing antibiotic resistance in MRSA by inhibiting the expression of efflux pump NSAIDs, such as diclofenac sodium, could serve as adjunctive agents in combating antibiotic resistance.

genes, particularly *norB*. This inhibition can increase the effectiveness of antibiotics by preventing bacteria from expelling antimicrobial agents.Abdel-Karim et al. (36) investigated the impact of diclofenac sodium on efflux pump activity in *Staphylococcus aureus*. The findings revealed that diclofenac sodium significantly inhibited efflux pump activity, leading to a decrease in the expression of efflux pump genes such as *norA*, *tetK*, and *fexA*. This inhibition enhances bacterial susceptibility to antibiotics, suggesting that diclofenac sodium could be a promising adjunct in antimicrobial therapy.In another study similar to the present study, the researcher revealed the influence of the drug on the gene expression of some types of bacteria, such as *P. mirabilis*. The experiment demonstrated the effectiveness of the drug as an antibacterial agent by reducing bacterial gene expression when exposed to the drug (37).

Conclusion:

This study demonstrated the potential of diclofenac sodium in modulating the expression of the *norB* efflux pump gene in methicillin-resistant *Staphylococcus aureus* (MRSA). These results indicate that diclofenac sodium effectively downregulates the expression of the *norB* gene, suggesting its role as an efflux pump inhibitor. This downregulation could increase the effectiveness of antibiotics by reducing bacterial resistance mechanisms, specifically those related to efflux pump activity. These findings support the idea that

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