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Original Article

Evaluation of Gene Expression of *nor*A and *nor*B Gene in Ciproflaxin and Levofloxacin Resistant *Staphylococcus aureus*

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Abstract

The presence of efflux pumps genes in *Staphylococcus aureus*, such as *nor*A and *nor*B, is critical for ciprofloxacin and levofloxacin resistance. This study examined the efflux pump gene expression and activity in ciprofloxacin and levofloxacin-resistant *S. aureus* strains. Twenty clinical samples of wounds and burns were collected. *S. aureus* strains were tested using specific culture media. Antibiotic susceptibility testing was done using the disc diffusion method. After determining the disc diffusion method of ciprofloxacin and levofloxacin, Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were found in ten of the twenty clinical samples. The susceptibility of *S. aureus* in the study revealed 40% ciprofloxacin resistance and 20% levofloxacin resistance. The gene expression of *nor*A and *nor*B efflux pump genes was assessed using Real-Time PCR. The *nor* A gene was detected in all ciprofloxacin-resistant pathogens, and *nor*A gene expression increased in samples treated with ciprofloxacin compared to samples not treated with ciprofloxacin results of a real-time PCR test. The *nor*B gene was detected in resistant strains, and its expression increased, as was the case with the *nor*A gene. The fold of gene expression of *nor*B gene for the ten isolates ranged from (12.082 to 42.81 fold) and also this result was higher than the fold of *nor*A gene (0.0036-34.05 fold). The research study discovered that efflux pump genes play a crucial role in ciprofloxacin and levofloxacin resistance. Also, when employed as a housekeeping gene in gene expression, the *16S rRNA* gene produced excellent results.

Keywords: Gene expression, *nor*A and *nor*B, *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is responsible for a wide range of infections worldwide. One of the most common causes of nosocomial and community-acquired infections is methicillin-resistant *S. aureus.* Fluoroquinolones are a class of antibiotics used to treat infections caused by *Staphylococcus aureus.* Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacteria with a 2.2-2.8 Mbp circular chromosome and a wide range of extrachromosomal components (1). The genome of *Staphylococcus aureus* contains virulence factors, antibiotic resistance genes, and multi-drug efflux genes, each of which plays an important role (2). In hospitals around the world, *Staphylococcus aureus* is an opportunistic infective factor, and the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) has become a severe issue in recent years. Fluoroquinolone medicines, like ciprofloxacin, are commonly used to treat MRSA-related infections. Unfortunately, after receiving ciprofloxacin to treat MRSA-related illnesses, these bacteria quickly grew resistant to the antibiotic. (3, 4). There are different mechanisms of antibiotic resistance in *S. aureus*. The efflux pumps, which release antibiotics and decrease their intracellular concentration, are one of the most important processes (5, 6). The existence of efflux pumps in *S. aureus* strains is one of the mechanisms of ciprofloxacin resistance (7). Antibiotics, antiseptic chemicals, dyes, and detergents are among the items

transported by these pumps. As a result, they play an essential role in developing multi-drug resistance. The efflux pump genes are generally found on chromosomes and are conserved among strains. The existence of efflux pumps in *Staphylococcus aureus* is one of the critical mechanisms of resistance to antibiotics, and one of the fundamental mechanisms of resistance is the existence of efflux pumps in this bacteria (8, 9). The five main groups of bacterial efflux pumps based on amino acid similarity are 1- Major Facilitator Super Family (MFS), 2- ATP-binding cassette (ABC),3- Resistance-Nodulation Division (RND), 4- Small Multidrug Resistance (SMR), 5- Multidrug and Toxic Compound Extrusion (MATE) (10, 11). *nor*A gene is a member of (efflux pumps) MFS family. It is a protein with 388 amino acids and consists of 12 components that move across the plasma membrane, which is 24% similar to the efflux pump Tet (A) found in *E. coli* bacteria (12, 13). The gene expression of the *nor*A efflux pump is variable in different strains, according to Jo and Ahn (14).

The current study will look into the antibiotic resistance pattern, the presence of efflux pumps, and the function and gene expression of *neither* A and *nor*B efflux pumps in clinical strains of ciprofloxacin-resistant *strains Staphylococcus aureus*, isolates from hospitals in Iraq.

2. Materials and Methods

2.1. *Staphylococcus aureus* isolates

Ten samples of *Staphylococcus aureus* from wound infections obtained from patients in Baghdad hospitals, Iraq, were chosen for this study. Conventional biochemical assays and molecular approaches were used to identify the clinical isolates.

2.2. Antimicrobial Susceptibility Test

The antimicrobial susceptibility tests of *S. aureus* isolates were disk diffusion methods. According to PCR results, the 10 isolates were multidrug-resistant and contained the *nor*A and *nor*B efflux pump genes. The following antibiotics were tested ciprofloxacin (10 µg) and levofloxacin (5 µg). All antibiotic discs were obtained from Oxoid, the UK. The antibiotic concentrations have been used according to manufacturing instructions. The plates were checked, and inhibition zone widths around antibiotic disks were measured after 18-24 hours of incubation at 37°C. The antimicrobial susceptibility of the examined isolates was analyzed using CLSI breakpoints. According to the method previously mentioned by Magiorakos (15), the determination of multi-drug resistant isolates was doneMagiorakos, Srinivasan (15).

2.3. *nor*A and norB Genes Expression Using RT-PCR Technique

Ten isolates of Ciproflaxin and levofloxacin-resistant *S. aureus* were used in this study, which have different values of disc diffusion method and has the two *nor*A and *nor*B efflux pumps genes. Before and after treatment with the antibiotics (Ciprofloxacin and levofloxacin), the gene expression of the two genes in the resistant isolates was measured. The antibiotics employed in the treatment were ciprofloxacin (10 µg) and levofloxacin (5 µg) to allow bacterial growth with resistance induction. Total RNA was extracted after the *S. aureus* isolate was grown in Mueller Hinton broth (without antibiotics) and incubated overnight at 37°C to detect *neither* A and *nor*B gene expression. Total RNA Isolation according to the manufacturer kit.

Preparation of primers Specific primers were obtained (Table 1) according to the previous studies (16-18) for the detection of gene expression.

Table 1. Sequences of primers that used for gene expression

Primers		Primer sequence	Product size (base pair)	Ref.
Nor A	F	ATCGGTTTAGTAATACCAGTCTTGC	112	16
Nor A	R	GCGATATAATCATTTGAGATAACGC		
Nor B	F	AGCGCGTTGTCTATCTTTCC	213	17
Nor B	R	GCAGGTGGTCTTGCTGATAA		
16S	F	GGACGGGTGAGTAATGTC	193	18
16S	R	TCTCAGACCAGCTAGGGATCG		

Using the SYBR green GoTaq® 2-Step RT-qPCR System, a two-step quantitative real-time PCR assay (QRT-PCR) is performed (Promega, USA). The following master amplification reaction was used with the two-step RT-PCR list in table 2 and the software in table 3, figure 1 to amplify the mRNA fragment 3. This study was carried out to improve cDNA synthesis and annealing temperature.

Table 2. Steps of cDNA synthesis quantitative RT-PCR reaction mix

Steps	°C	h:m:s	Cycle
Annealing	16	00:15:00	1
Extension	37	02:00:00	
Enzyme Inactivation	85	00:05:00	
Hold	72	00:10:00	

Table 3. Thermocycler software for quantitative RT-qPCR using cDNA synthesis

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:15	40
Annealing	55	00:30	
Extension	72	00:30	

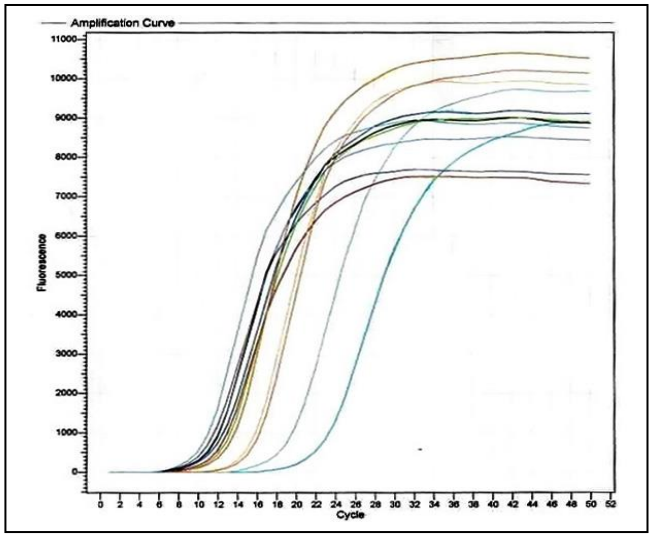

Figure 1. qPCR Application Plot. The photograph was taken directly from a Rotor-Gene qPCR system

2.4. Delta Delta Ct (ΔΔCt) Method

This is the easiest technique since it directly compares Ct values between the target gene and the reference gene (16sr RNA). The selection of a calibrator sample is required for relative quantification.

For each sample, the Ct between the target gene and the reference gene is first calculated (for the unknown samples and the calibrator sample).

ΔCt = Ct target – Ct reference gene

Then the difference between the unknown's ΔCt and the calibrator's ΔCt is calculated, yielding the ΔΔCt value:

ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator.

The normalized target amount in the sample is equal to $2^{-\Delta\Delta Ct}$, which can be used to compare expression levels between samples (19).

The samples were examined in triplicate and compared to the expression of the *16S rRNA* gene. The relative variations in mRNA expression levels between antibiotic-exposed and antibiotic-unexposed *S. aureus* were determined using the comparative threshold cycle (CT) approach (2-ΔΔCt).

3. Results and Discussion

3.1. Antibiotics Susceptibility Test

Staphylococcus aureus was studied using the disk diffusion method and PCR. The isolate was obtained from the wound and burn infection samples. The susceptibility test results for *S. aureus* in the research show 40% ciprofloxacin resistance and 20% levofloxacin resistance, as shown in figure 2. The *nor*A gene was found in all ciprofloxacin-resistant isolates in a previous investigation. The *nor*A gene was detected inside the chromosome, The chromosomal gene codes for *nor*A (20).

Figure 2. Disk diffusion method of ciprofloxacin (10 µg) and levofloxacin (5 µg)

3.2. Quantitative Real-Time PCR Study of the *nor*A and *nor*B Genes

SYBR green, a fluorescent dye that identifies any double-stranded DNA, including cDNA, is used in real-time PCR quantification in the current experiment. The amplification was measured in Ct (cycle threshold). *16S rRNA* was chosen as the housekeeping gene in this investigation because Different bacterial species have one to multiple copies of the *16S rRNA g*ene. *16S rRNA* gene sequencing is one of the most common methods targeting housekeeping genes to study bacterial phylogeny and genus/species classification.

The use of this gene in molecular investigations is bas ed on the fact that its expression remains consistent in t he cells or tissues under study under various situations (15). In this experiment, a quantitative PCR reaction was carried out using 10 ciprofloxacin and levofloxacin-resistant bacteria with neither A *nor* B genes, respectively.

In this investigation, a quantitative RT-PCR assay was used to examine the mRNA expression of the *nor*A and *nor*B genes by comparing treated and untreated samples of bacterial growth with ciprofloxacin and levofloxacin antibiotics at concentrations in the susceptibility test results of each sample.

The Ct values of gene amplification were recorded using quantitative RT PCR software. As demonstrated in figures 1 and 2, the fold change in gene expression was calculated using the delta Ct value's relative quantification (RQ). Couto found overexpression of the *nor*A efflux pump gene in the presence of inhibitors in a study (16).

The gene expression of *nor*A increased in the sample that had been treated with ciprofloxacin when compared with a sample that had not been treated with ciprofloxacin, and Real-Time PCR was used to check for the existence of the *nor*A pump gene and its expression in ciprofloxacin-resistant bacteria (Figure 3).

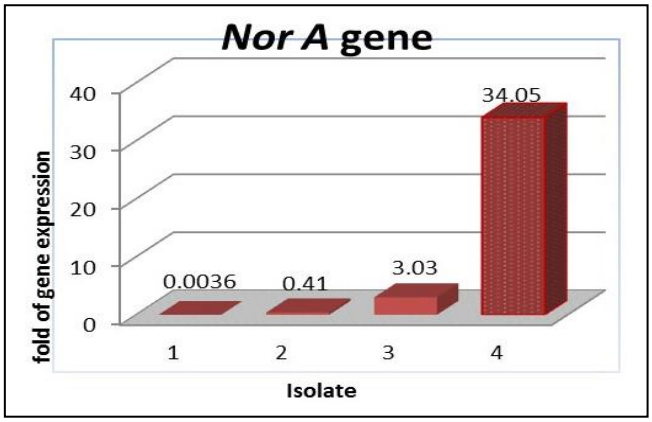

Figure 3. The fold of gene expression of the *norA* gene depending on the ΔΔCt method

Pourmand, Yousefi (18) the *nor*A gene is present in all ciprofloxacin-resistant strains, and its gene expression rises in the presence of the biocide hexahydroquinolone.

Also compatible with Saiful, who tested 19 MRSA strains, 16 of which included the *nor*A gene, all of which had an effective efflux pump (21). Our findings are consistent with Tavakoli, Sahebjamee (22), who showed that over-expression of efflux pumps genes in ciprofloxacin-resistant isolates plays a critical role in resistance to ciprofloxacin and so the study of gene regulators is reasonably necessary.

Our observations are similar to the Huet, Raygada (23) research which used Real-Time PCR to examine 9 isolate that was ciprofloxacin-resistant MRSA strains for the existence of efflux pumps and expression in the presence of low antibiotic doses. Furthermore, as shown in figure 4, the fold of gene expression of the *nor*B gene for the 10 isolates ranged from (12.082 to 42.81 fold).

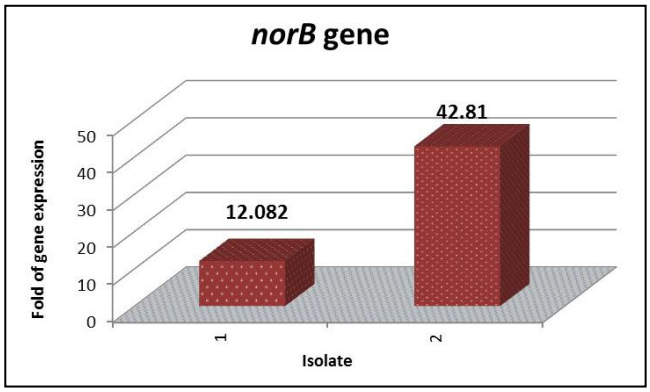

Figure 4. Fold of gene expression of *nor*B gene depending on ΔΔCt method

The fold of expression for the *nor*A gene ranged from (0.0036-34.05 fold) in figure 2 .when we compared the susceptibility test values of the isolates to the result in gene expression to the fold of expression was compatible

It was observed that the susceptibility test values and gene expression for the efflux pump genes correlated. The relationship between antibiotic resistance and the gene expression of efflux pump genes was investigated by our isolates for this research. The results indicated the contribution of these genes to the multi-drug resistance of our local isolates (24, 25).

3.3. Gene Expression of *16S rRNA*

The housekeeping gene used in the present study, the Ct values of *16S rRNA*, revealed that there were no significant variations among treated and not treated samples, and this reference gene was used to eliminate variation from one sample to the next; it is necessary to find reference genes that are consistently expressed because variations in reference gene expression might lead to false positives or hiding true positives (26). The *16S rRNA* gene is a functional control gene because of the slight differences in gene fold expression between treated and untreated samples. The fold of this gene was convergent values (19.52-12.5 fold) under varied antibiotic doses, according to the reference gene expression experiment results. Gene expression of the current study supported the use of *16S rRNA* (193 bp) as a reference gene for MRSA strains; as previously stated, S. aureus is one of the most pathogenic bacteria in hospitals that spread quickly over the world. Most beta-lactam antibiotics are ineffective against this bacteria (27, 28). In the findings of Moreno-Flores, Potel-Alvarellos (29), it was found that MRSA strains are resistant to quinolones. In the United States, Motallebi, Jabalameli (12) found that three months after taking ciprofloxacin, ciprofloxacin-resistant MRSA bacteria formed.

The efflux pump genes (neither A *nor* B) were present in all isolates that resist ciprofloxacin and levofloxacin, and the expression increased when compared with the sample that was untreated with antibiotics. The *nor*B gene expression exhibited the highest fold of gene expression. The results showed that when used as a housekeeping gene in a gene expression experiment, the *16S rRNA* gene (193 bp) produced perfect results with minimum change in different conditions.

Authors' Contribution

Study concept and design: N. S. L.
Acquisition of data: W. Y. S.
Analysis and interpretation of data: A. A. A.
Drafting of the manuscript: R. J. O.
Critical revision of the manuscript for important intellectual content: N. S. L.
Statistical analysis: W. Y. S.
Administrative, technical, and material support: A. A. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

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