WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 4, Issue 4, 346-356. Research Article ISSN 2277– 7105

IDENTIFICATION OF AN EFFLUX PUMP GENE, *NORA*, IN METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN BAGHDAD

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Article Received on
28 Jan 2015,

Revised on 23 Feb 2015,
Accepted on 19 March 2015

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ABSTRACT

A total of 75 clinical isolates of Methicillin-resistant *Staphylococcus aureus*(MRSA) were collected from various infections from different hospitals in Baghdad. The susceptibility to different antibiotics was evaluated by disk diffusion method. Results showed that all isolates were resistant to ceftriaxone, cloxacillin and aztreonam. The resistance to ciprofloxacin and norfloxacin were 17.33 % and 20% respectively. The minimum inhibitory concentrations (MICs) for ciprofloxacin and norfloxacin ranged from 4 to 1024 µg/ml for both Antimicrobial agents. Ciprofloxacin resistant isolates were tested for efflux pumps by using Ethidium Bromide-Agar Cartweel Methode (EtBrCW), the results showed that 52.9% of isolates give positive result. Polymerase Chain Reaction (PCR) technique was performed done using specific primer targeting the specific sequences of the *norA* gene, The results showed that *norA* found in 47% of isolates. Nucleotide sequence for *norA* gene in 9 isolates were determined , results revealed consistency reaching up to 90 % as compared Nitrogen bases sequence of the *norA* gene present in the *Staphylococcus aureus* strain in NCBI . Detection of gene expression was performed done by using q RT-PCR technique after RNA extraction and cDNA syenthesis, the result showed that gene expression were various among isolates, and the gene expression increase with increasing MIC of ciprofloxacin and norfloxacin. The effect of biosurfactant (from *Lactobacillus rhamnosus*) as efflux pumps inhibitors was tested by detection of MIC for ciprofloxacin alone and mixed with

biosurfactant , the result showed ability of biosurfactant in decreasing the MIC to two or four-fold in the presence of inhibitor. This refer to biosurfactant effective material against NorA efflux pump.

KEYWORDS: Methicillin-resistant *S. aureus* (MRSA), efflux pumps, NorA gene.

INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA) includes those strains that have acquired a gene giving them resistance to methicillin and essentially all other beta-lactam antibiotics(Lee, 2003). MRSA is a very strong pathogen developing infections which are acquired from hospitals and communities and cause abroad spectrum of diseases from mild skin infections up to the very severely invasive ones (Dinges *et al* ., 2000).

Bacteria can use different mechanisms of resistance to antimicrobials, which include modification of the antimicrobials; changing of the target of the antimicrobials ; reduction of the intracellular concentration of the antimicrobials by the efflux of the antimicrobials from the cell. Efflux-mediated resistance has been gathering more interest, are able to extrude several, unrelated classes of antimicrobial compounds from the cell (Costa *et al*., 2013).

S.aureus encodes several multidrug resistance(MDR)efflux pumps, among which NorA has been extensively examined. NorA appears to export a variety of structurally unrelated drugs, such as fluoroquinolones, ethidium bromide, cetrimide, benzalkonium chloride, tetra phenyl phosphonium bromide, and acriflavine (Kaatz and Seo,1995).

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* (Kaatz *et al*.,2003). Discovery of Efflux pumps Inhibitors (EPIs) is a promising approach to deal with Multidrug Efflux Systems that may improve clinical performance of antibiotics and chemotherapeutic agents (Lomoveskay *et al*., 2001). The potential for developing broad acting EPIs is exemplified by reserpine that effectively inhibits both bacterial and mammalian ABC-system P-glycoprotein (P-gp), as well as by biricodar (VX-710), timcodar (VX-853), and verapamil (Tegos *et al* ., 2011).

The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made real-time PCR technology an appealing alternative to conventional culture-based or immunoassay-based testing methods used in the clinical microbiology for diagnosing many infectious diseases (Espy *et al* ., 2006). The aims of this study were to determine the prevalence of efflux Pump Gene, *norA*, Associated with Fluoroquinolone Resistance in *Staphylococcus aureus* Isolated from the patients admitted to Some Iraqi medical centers in Baghdad , and study the effect of biosurfactant as a Nor A inhibitor.

MATERIAL AND METHODS

Bacterial Isolates

Bacterial isolates were recovered from blood, wound, urine, ear and respiratory tract from patients admitted to Some Iraqi medical centers in Baghdad between 1/9/2013 to 1/1/2014. The isolates were identified by their colony characteristic, gram-stain and confirmed by the pattern of biochemical profiles using Vitec, 2 system.

Antibiotic susceptibility testing:

The antimicrobial susceptibility was done by using Kirby-Bauer disc diffusion technique on Mueller Hinton agar (Oxoid, England) using overnight culture at a 0.5 McFarland standard followed by incubation at 35c for 16 to 18 h. following Clinical and Laboratory Standards Institute (CLSI) guidelines (2011)with commercially available antimicrobial discs (Bioanalyse/Ankara/Turkey). Isolates were tested against the following antimicrobial agents: Ciprofloxacin(cip) (10µg), Norfloxacin (10µg), Ceftriaxone (CRO) (30 µg), Aztreonam(ATM) (30 µg), Cloxacillin(CX) (10 µg), Cefoxitin (FOX) (30 µg), Cephalexin (CL) (30 µg), Erythromycin(E) (15 µg), Azithromycin(AZM) (15 µg) and Chloramphenicol(C) (10 µg).

Minimal Inhibitory Concentrations

The Minimum inhibitory concentrations (MICs) of ciprofloxacin and Norfloxacin were determined. We used Mueller-Hinton agar with antibiotic concentrations (2-1024) µg/ml according to the guidelines recommended by the CLSI document.

Detection of Efflux pump

Ethidium Bromide-Agar Cartweel Method (EtBrCW) was used to detection of efflux pump in MRSA isolates according to **(**Martins *et al*.,2011).

Molecular Detection of *norA* using PCR technique

All of ciprofloxacin resistant isolates were submitted to the PCR technique to detect *norA* gene. DNA amplification was carried with a Gradient PCR System (TechNet-500 /USA) with a final volume (25 µl). Each reaction contained 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate(dNTP); 1.5 mM MgCl2; 1.5 µl each primer (table 1) ; Template DNA (50ng) ; 1.25 U of *Taq* DNA polymerase. Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker 100bp (Promega/USA) was run with each gel, and the genotype was determined by the size of the amplified product.

RNA isolation, transcriptional profiling, and quantitative RT-PCR

RNA isolation and cDNA preparation from cultures was grown to early stationary growth phase as previously described With some modifications. (Li *et al.*,2007). *S. aureus* (lysostaphin-treated cells) that grown to the exponential or postexponential phase was used to extract total RNA using the RNase mini kit supplemented with DNase (Geneaid, Tailand.).

In the first step, a suitable amount of Total RNA (100 ng) was denatured with (50 pmole) of hexamer primer and variable amount of DEPC for 10 min at 65 ^{o}C then immediately cooled on ice.

In the second step : cDNA was synthesized by using (cDNA synthesis Bioneer, Korea), all mixture from step one was mixed with 4 uL of 5X M-MLV RTase reaction buffer , 2uL of 100mM DTT , 2ul of dNTP, 20 units RNase inhibitor ,1uL of 200 units M-MLV Reverse Transcriptase. The reaction was left for 1 hr at 42 ^{o}C.

In the third step : 100 ng of a step two product (cDNA) was mixed with 2x Taq buffer ,5 pmole of primers and 10 units of Taq DNA polymerase (promega, USA) in a total volume 25 µl. The reaction after brief spin was submitted to the following PCR condition: Initial Denaturation at 95°C for 2 min, Denaturation at 95°C for 15 sec, Annealing 60°C for 20 sec. Polymerization at 72°C for 20 sec , 30 cycles and final Polymerization at72°C for 10 min.

Table 1. Primers used for detecting *norA* genes among MRSA isolates.

Gene	Sequence of forward Primer(5´- 3´)	Sequence of reverse primer (5´- 3´)	Product Size(bp)	Reference
norA	TTTGTTTTCAGTGTCAGAATTTATGTTTG	GGCTTGGTGAAATATCAGCTATTAAAC	140	Patel *et al* (2010)
norA Probe	6FAM-AGGCATAACCATACCAGCACTCATACCACC-BHQ1			
16S rRNA	CCAGCAGCCGCGGTAAT	CGCGCTTTACGCCCAATA	62	Patel *et al* (2010)
16S rRNA *Probe*	CY5-CGTAGGTGGCAAGCGTTATCCGGA-BHQ3			

Biosurfactant as inhibitor of the NorA in MRSA isolates

Biosurfactant produced by locally *Lactobacillus rhamnosus* isolate obtained from Al-Qaralucy(Department of Biology \ College of Science \ Al- Mustansiriya University \ Baghdad\ Iraq) was used as inhibitor of the NorA in MRSA isolates.MIC for ciprofloxacin alone and mixed with biosurfactant was determined.

DNA sequence analysis: The DNA fragments for sequencing were obtained by PCR amplification , the fragments of each PCR products were sequenced with the set of primers by (Macrogen Co., USA). The program (BioEdit Pro.version: 7.0.0) was used for bioinformatic analysis of nucleotide sequences.

RESULTS AND DISCUSSION

A total of 75 clinical isolates of Methicillin-resistant *S. aureus*(MRSA) isolated from various infections including 60 isolates from wounds, 5 isolates from blood,5 isolates from urine, 3 isolates from pus and 2 isolates from otitis media from different hospitals in Baghdad. All isolates were characterized according to (Brooks *et al*.,2001) . Cultural ,Morphological , Biochemical characteristics and using CHROMO agar MRSA, revealed that these isolates being Methicillin resistant *S.aureus* (MRSA).(Table 2)

Table 2: No. of MRSA isolates according to source of samples.

Sources of samples	No. of isolates
Wound	60
Blood	5
Urine	5
Pus	3
Otitis media	2
Total	75

The susceptibility to different antibiotics was evaluated by disk diffusion method. Results showed that all isolates were resistant to ceftriaxone, cloxacillin and aztreonam. The resistance to ciprofloxacin and norfloxacin were 17.33 % and 20% respectively (Tabl 3). The minimum inhibitory concentrations (MICs) for ciprofloxacin and norfloxacin ranged from 4 to 1024 µg/ml for both antibiotics.

First recognized in 1960, methicillin-resistant *Staphylococcus aureus* (MRSA) was considered to be a medical oddity. Now, MRSA is the most common nosocomial bacterial pathogen isolated in many parts of the world (Grundmann *et al*.,2006). Fluoroquinolone resistance develops as a result of spontaneous chromosomal mutations in topoisomerase IV or DNA gyrase, or by the multidrug efflux pump (Lowy, 2003). The rapid development of ciprofloxacin resistance due to excretion of this antimicrobial agent into the sweat might be involved in the development of multi resistant coagulase negative Staphylococci and possibly other skin bacteria in hospitals and in communities with high use of ciprofloxacin or related drugs (Høiby *et al* .,1997).

Table: 3. Susceptibity of 75 MRSA isolates to the Antimicrobials.

NO	Antimicrobials		Rate of resistant
1-	Ciprofloxacin	10µg	**17.33**
2-	Norfloxacin	10µg	**20**
3-	Ceftriaxone	30 µg	**100**
4-	Aztreonam	30 µg	**100**
5-	Cloxacillin	10µg	**100**
6-	Chloramphenicol	10µg	**29.33**
7-	Azithromycin	15 µg	**37.33**
8-	Erythromycin	15 µg	**40**
9-	Cephalexin	30 µg	**64**
10-	Cefoxitin	10µg	**66.6**

Ciprofloxacin resistant isolates were tested for efflux pumps by using Ethidium Bromide-Agar Cartweel Methode (EtBrCW), the results showed that 52.9% of isolates give positive result.

Polymerase Chain Reaction (PCR) technique was performed using the specific primers targeting to the specific sequences of the *norA* gene, The results showed that *norA* found in 47% of isolates. Nucleotide sequence for *norA* gene in 9 isolates were determined, results revealed consistency reaching up to 90 % as compared with Nitrogen bases sequence of the *norA* gene present in the *Staphylococcus aureus* strain in NCBI.

The *norA* gene is found in all of the total genome sequences of *Staphylococcus aureus* strains currently available in the GenBank. Initially, *norA* was thought to specifically efflux the quinolone drug norfloxacin, but subsequently was found to confer resistance to a number of antimicrobials, including chloramphenicol (Brown and Skurray,2001 ; Hassan *et al*.,2007).

The expression of *norA* is regulated by *mgrA*, a member of a *marR* group of transcriptional regulators(Truong-Bolduc *et al*.,2005). A study by DeMarco *et al*. reported the occurrence of 49% of strains showing increased efflux activity within a collection of 232 bloodstream *S. aureus* isolates. The increased efflux activity present in those isolates was correlated with increased resistance to fluoroquinolones, biocides and dyes.

After RNA extraction and cDNA syenthesis(Fig 1 and Fig 2) detection of gene expression was performed by Real time-PCR technique. The result showed that gene expression was various among isolates, isolate (No. 5) showed higher expression compared with other isolates (fig. 3 B) and the gene expression increases with increasing MIC of ciprofloxacin and norfloxacin.

The effect of biosurfactant (from *Lactobacillus rhamnosus*) as efflux pumps inhibitors was tested by detection of MIC for ciprofloxacin alone and mixed with biosurfactant , the result showed ability of biosurfactant in decreasing the MIC to two or four-fold in the presence of inhibitor this refer to biosurfactant effective material against NorA efflux pump.

Fig (1): PCR product (140bp) for Quinolone resistance protein norA gene with primers and probe, *Staphylococcus aureus* subsp. *aureus* LGA251, GenBank: FR821779.1

Fig (2): Alignments of 16S ribosomal RNA gene, showing PCR product (62bp) with probe. *Staphylococcus aureus* subsp. *aureus* GenBank: X84731.1.

(A)

Sample No.	ΔΔCt	ΔCt-value (s=√(sTarget2+sNormalization2)
1	1.466667	3.261390297
2	0.1	2.357965225
3	-0.36667	0.152752523
4	-0.06667	1.539480432
5	-2.8	5.896043871
6(Wild)	0.4	0.964365076
7	2.393333	0.594754851
8	-0.71	0.118462371
9	-0.23333	0.456106713
10	-0.6	0.45181117
11	-1.45667	0.505601951
12	-0.57667	1.558086433
13	-0.78667	1.202164714

(B)

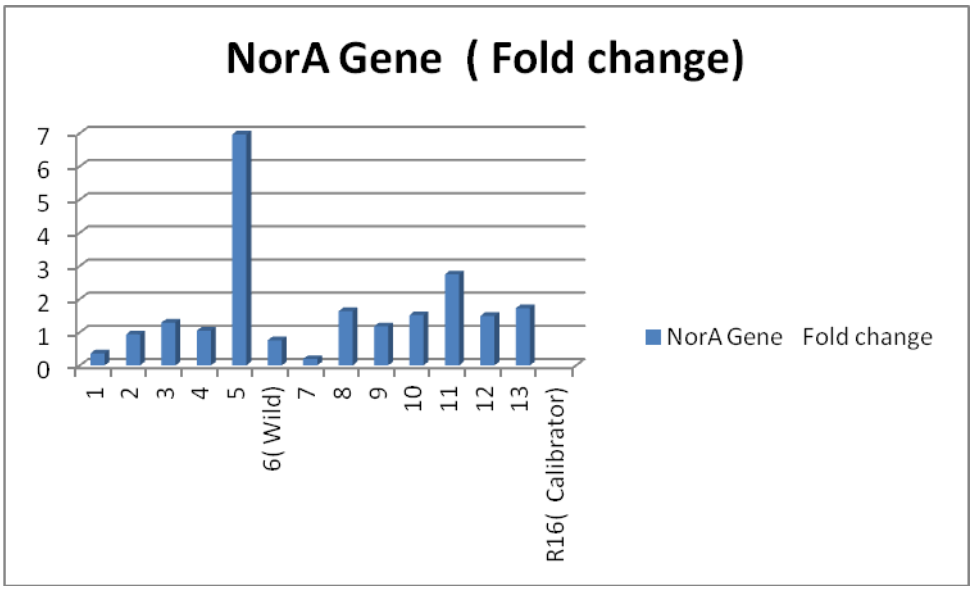

Fig (3): Gene expression of NorA gene measured by real time PCR using FAM and Cy5 filters (A) Expression of NorA gene compared with control(16S ribosomal RNA) Each sample has been replicated triple times and the average calculated using the formula: ΔΔCt=(ΔCtTest sample- ΔCtCalibrator), relative quantification (ΔΔCt method), (B): The fold-change has been calculated by the formula 2-ΔΔCt with ΔΔCt+s and ΔΔCt-s.

One plausible alternative is the combination of conventional antimicrobial agents/antibiotics with small molecules that block MES known as multidrug efflux pump inhibitors (EPIs). An array of approaches in academic and industrial research settings, varying from high-

throughput screening (HTS) ventures to bioassay guided purification and determination, have yielded a number of promising EPIs in a series of pathogenic systems. This synergistic discovery platform has been exploited in translational directions beyond the potentiation of conventional antimicrobial treatments.(Tegos *etal* ., 2011)

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