Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 4
Open Access

NorA efflux pump gene overexpression in *Staphylococcus aureus* isolated from human and animal infection

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Cite this paper as: Khilood Hamdan Fahad, Jenan Nadhim Sadeq, Azhar Abdulsada , Ola Hakim Khudhair (2024) NorA efflux pump gene overexpression in Staphylococcus aureus isolated from human and animal infection. *Frontiers in Health Informatics, 13* (4), 997-1004

Abstract

Bacterial resistance to antibiotics is a highly challenging health related problem that affects both patients and economy. This resistance can be permitted by different mechanisms, such as norA pumping system that can remove antibiotics out of the bacterial cell. The present study was conducted to identify *norA* efflux pump (EP) gene in *S. aureus* isolated from human and animal infection. The study involved the recruitment of 60 infection samples distributed into 30 human infected wounds and 30 animal infection samples. These samples were examined using regular bacterial cultivation and biochemical assays, employing the EtBr-agar cartwheel method, a fluorescent-dye based technique, and recruiting a qRT-PCR method. The results revealed the presence of the bacterial *norA* gene in the bacterial isolates in mRNA-fold changes of 62.2 and 10.5 for human and animal samples, respectively. The present study result indicates high existence of antibacterial resistance induced by the norA pumping system with a higher rate in human samples compared to these from the animal samples.
Keywords: Antibiotic resistance, *norA* gene, *S. aureus*.

Introduction

Over the years the emergence and spread of AR in S. aureus have become a major concern in clinical settings. (1). *S. aureus* has developed various mechanisms to evade the action of antibiotics rendering them ineffective in treating infections caused by this bacterium. One of the most prominent mechanisms is the acquisition of resistance genes through horizontal gene transfer. *S. aureus* can obtain resistance genes from other bacteria allowing it to produce enzymes that inactivate antibiotics or modify their targets. For instance the acquisition of the mecA gene is responsible for methicillin resistance which is a major challenge in the handling of *S. aureus* infections (2).

Furthermore *S. aureus* can also develop resistance through the mutation of its own genetic material. This can occur in mutation of antibiotic target genes, such as antibiotic uptake or efflux. Additionally the formation of biofilms by *S. aureus* contributes to its ability to resist antibiotics (3). The AR in *S. aureus* have significant implications in both biological and biomedical sciences. From a biological perspective understanding the mechanisms of AR in this bacterium can provide valuable insights into the evolution of resistance in other pathogens. The study of *S. aureus* can serve as a model system to investigate the genetic and molecular basis of AR which can help in the development of new strategies to combat resistance in other bacterial species (4,5). This efflux mechanism enables Staphylococcus to survive in the presence of antibiotics that would otherwise inhibit bacterial growth (6-8).

Increased expression of the norA gene leads to higher levels of the EP resulting in enhanced AR. Studies

have shown that clinical isolates of *S. aureus* with elevated norA expression exhibit reduced susceptibility to multiple antibiotics leading to treatment failures and the need for alternative therapeutic options (9,10).

The present study was conducted to identify *norA* EP gene in *S. aureus* isolated from human and animal infection.

Materials and Methods

Samples collection

Thirty transport media swab samples were collected from wounds of patients at the Microbiology Laboratory, Al-Diwaniyah Hospital, Al-Qadisiyah Province, Iraq, and another thirty animal infection samples were collected at Al-Diwanyiah Veterinary Hospital. The samples were transferred to the Microbiology Laboratory, College of Veterinary Medicine, University ofAl-Qadisiyah.

Bacterial isolation method

Conventional bacterial isolation methods were used to isolate bacteria from human and animal infection.

EtBr-agar Cartwheel method

The EtBr-agar Cartwheel method was performed following mthods described by Martins et al., (11). Briefly, bacterial growth was place onto ethidium bromide enriched Muller-Hinton media. Incubation at 37°C for 24hrs. Later, the mixture was examined by UV light. No fluorescence from bacteria means that the bacteria developed norA pump.

Quantitative Reverse Transcription Real-Time PCR

The qRT-PCR technique was performed for identifying the *norA*-mRNA fold change in the bacterial growth from humans and animals. The method was based on steps mentioned by Ibrahim and Shehan (12). The total RNA was extracted using easy-BLUE™ Total RNA Extraction Kit). The kit results were evaluated using a NanoDrop. DNase I treatment was performed to restrict the presence of any amount of DNA as 1µl DNase per 10µl (1µg) RNA was used. The DNase-I treated RNA samples were cDNA-synthesized by utilizing the M-MLV Reverse Transcriptase kit as 8µl (100ng/µl), 1µl random hexamer primer, and 1µl DEPC H2O were employed. The steps in Table 1 show the next procedures.

Table 1: CDNA synthesis

RT master mix	**µL**
RT master mix	10
M-MLV RTase (200µ)	1
5X M-MLV RTase reaction buffer	4
100mM DTT	2
dNTP	2
RNase inhibitor	1
Total	20

The thermocycler conditions were 1hr, 42°C cDNA synthesis and 5mins, 95°C heat inactivation.

Real-Time PCR (qPCR) master mix preparation

The GoTaq® qPCR Master Mix Kit was utilized to prepare the RT-PCR master mix using SYBER green dye.

The RT-PCR primers were designed and purchased from ScientificResercher, Co. Ltd from Iraq (Table 2).

Table 2: RT-PCR primers

Gene	Sequence (5'-3')		Product Size	NCBI Reference code
norA gene	F	AAGCTCGTCAATTCCAGTGG	96 bp	D90119.1
	R	TGGTGCATGTGATGACGTTG		
gyrB gene	F	GCAGCGTATTAGAGAGCTTGC	111 bp	M86227.1
	R	ATACCGCCCTCATAGTGATAGG		

The standard qPCR master mix protocol was 5µL (10ng) cDNA template, 1 µL for each (10pmol) F or R primer, 10 µL qPCR Master Mix, and 3 µl nuclease free water in a total volume of 20µl. The CFX96 Real-Time PCR system was used after using the thermal conditions

Table 3: qPCR thermocycler conditions

qPCR step	°C	Seconds (s)	Numbers of cycles
Denaturation (1st)	95	600	1
Denaturation (2nd)	95	20	40
Annealing\Extension Detection(scan)	60	30	
Melting	65-95		1

Data analysis of qPCR

The data were analyzed as (fold change) depending the (Livak method) by Livak and Schmittgen (12).

Results

The Et-Br cartwheel method showed the existence of the norA pumping system in the bacteria tested (Figure 1).

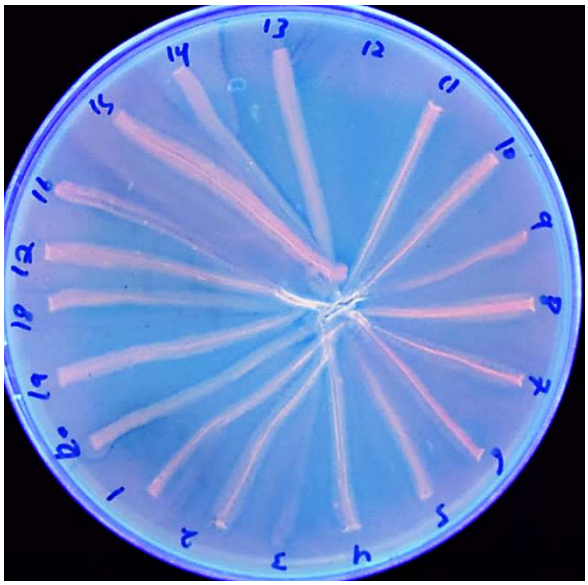
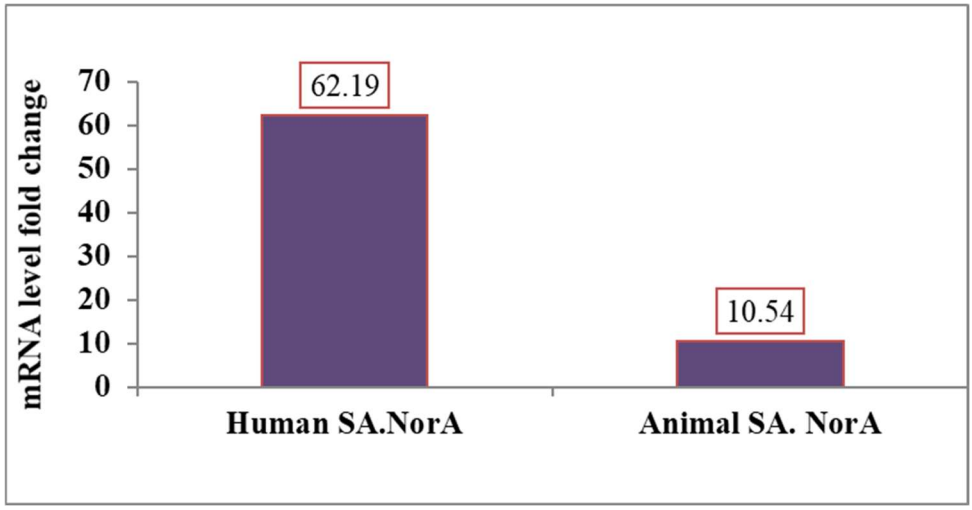


Figure (1): Assessment of efflux pumps activity by Ethidium Bromide agar cartwheel method. Staphylococcus aureus isolates that produced less fluorescence at concentrations 1 µg/ml EtBr were considered to have positive efflux activity, while the more fluorescence at concentrations 1 µg/ml EtBr less efflux activity.

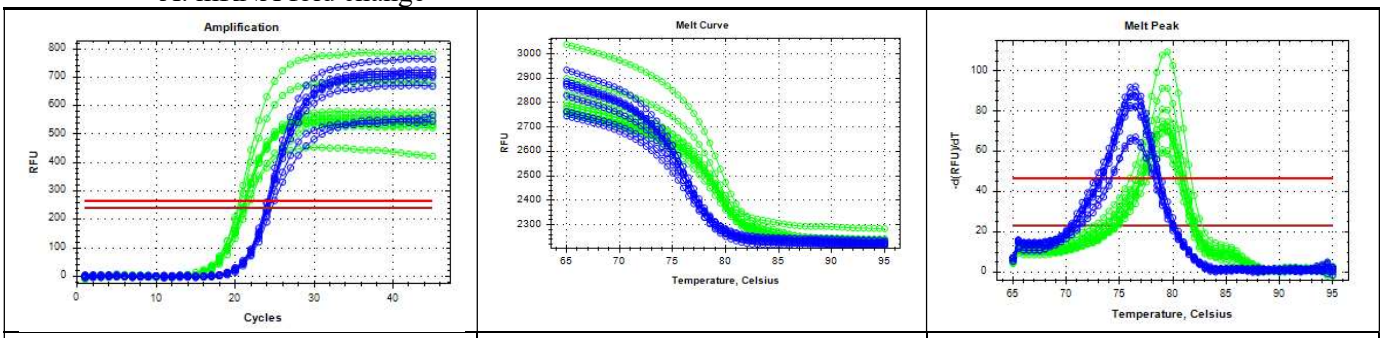
The results revealed the presence of the bacterial *norA* gene in the bacterial isolates in mRNA-fold changes of 62.2 and 10.5 for human and animal samples, respectively, with significant (p<0.0001) differences (Table 4 and figure 2). The table shows the cycle threshold (CT) values for the norA gene and the gyrB gene. The table also shows the ACT values.

Table 4: RT-PCR values of *norA* gene of *S. aureus* isolated from human and animal samples

No. sample	CT (NorA)	CT (rpoB)	ΔCT: T	ΔCT: C	ΔΔCT	Fold change (2^-ΔCT)
Human isolate No.1	21.32	27.23	-5.91	-0.46	-5.45	43.71
Human isolate No.2	21.59	27.59	-6.00	-0.46	-5.54	46.53
Human isolate No.3	20.99	27.55	-6.56	-0.46	-6.10	68.59
Human isolate No.4	21.05	27.34	-6.29	-0.46	-5.83	56.89
Human isolate No.5	20.46	27.42	-6.96	-0.46	-6.50	90.51
Human isolate No.6	21.04	27.35	-6.31	-0.46	-5.85	57.68
Human isolate No.7	21.29	27.99	-6.70	-0.46	-6.24	75.58
Human isolate No.8	21.03	27.62	-6.59	-0.46	-6.13	70.03
Human isolate No.9	20.86	27.47	-6.61	-0.46	-6.15	71.01
Human isolate No.10	21.09	26.92	-5.83	-0.46	-5.37	41.36
Animal isolate No.1	24.75	27.45	-2.70	-0.46	-2.24	4.72
Animal isolate No.2	23.29	27.64	-4.35	-0.46	-3.89	14.83
Animal isolate No.3	23.00	27.59	-4.59	-0.46	-4.13	17.51
Animal isolate No.4	23.29	27.34	-4.05	-0.46	-3.59	12.04
Animal isolate No.5	24.31	27.55	-3.24	-0.46	-2.78	6.87
Animal isolate No.6	23.11	27.34	-4.23	-0.46	-3.77	13.64
Animal isolate No.7	23.25	27.48	-4.23	-0.46	-3.77	13.64
Animal isolate No.8	24.38	27.35	-2.97	-0.46	-2.51	5.70
Animal isolate No.9	24.04	27.92	-3.88	-0.46	-3.42	10.70
Animal isolate No.10	24.63	27.62	-2.99	-0.46	-2.53	5.78
Control	26.26	26.72	-0.46			



A: mRNA fold change



B. qPCR amplification plots *norA* gene (green) and housekeeping gyrB gene (blue)	C: melt curve of qPCR amplification plots *norA* gene (green) and housekeeping gyrB gene (blue)	D: melt peak of qPCR amplification plots *norA* gene (green) and housekeeping gyrB gene (blue)

Fig 2: RT-PCR findings of the norA gene f *S. aureus* isolated from human and animal samples.

Discussion

The norA EP plays a crucial role in the development of multidrug resistance in S. aureus. By actively pumping out antibiotics from the bacterial cell this pump reduces the intracellular concentration of the drugs making them less effective in inhibiting bacterial growth. This mechanism of resistance is often referred to as efflux-mediated resistance. Several studies have provided evidence of the association between norA expression and AR in S. aureus. For example a study by Smith et al. (13-19) demonstrated that the overexpression of norA in clinical isolates of S. aureus resulted in increased resistance to multiple antibiotics including fluoroquinolones and ethidium bromide.

A study by Li et al. (20-25) investigated the effect of combining the EPI phenylalanine-arginine β-naphthylamide (PAβN) with fluoroquinolones against S. aureus strains overexpressing norA. The results showed that the combination of PAβN and fluoroquinolones significantly enhanced the antibacterial activity of the antibiotics suggesting the potential of EPIs as adjuvants in the treatment of S. aureus infections.

Numerous studies have investigated the functional aspects of the norA EP in Staphylococcus. For instance a study by Archer et al. (26) demonstrated that overexpression of the norA gene leads to increased resistance to fluoroquinolones in *S. aureus*. The researchers found that the norA EP actively pumps out fluoroquinolones from the bacterial cell thereby preventing their accumulation and promoting resistance. These findings were further supported by the work of Kaatz et al. (27) who observed a similar phenomenon in methicillin-resistant *S. aureus*.

Several authors have contributed to the understanding of the norA EP in Staphylococcus. In a study by Truong-Bolduc et al. (28) the researchers investigated the impact of norA expression on the susceptibility of Staphylococcus epidermidis biofilms to antibiotics. Their results reported that increased expression of norA in biofilm-associated cells significantly reduced the susceptibility to antibiotics emphasizing the role of the norA EP in biofilm-mediated AR.

Furthermore the work of Coster et al. (29) focused on the regulation of the norA EP in *S. aureus*. The researchers identified a two-component regulatory system MgrA/MgrR that controls the expression of norA. Their results showed that the MgrA/MgrR system acts as a positive regulator of norA leading to increased EP activity and subsequent AR (30-37).

The present study result indicates high existence of antibacterial resistance induced by the norA pumping system with a higher rate in human samples compared to these from the animal samples.

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