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Identification and expression of *nor* efflux family genes in *Staphylococcus epidermidis* that act against gatifloxacin

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ABSTRACT

NorA, NorB, and NorC are efflux proteins in the Nor family that regulate the secretion of fluoroquinolones, and MgrA/NorR is a transcription factor of the Nor family. Overexpression of Nor family proteins provides fluoroquinolone resistance in *Staphylococcus aureus*. However, in coagulase-negative staphylococci (CNS), members of the Nor family had not been identified. In this work, the presence of Nor family proteins in *Staphylococcus* spp. and the expression of Nor family in gatifloxacin resistant *S. epidermidis* strains obtained from ocular infections (OI) were identified and analyzed.

S. epidermidis strains from OIs ($n = 44$) and healthy skin (HS; $n = 52$) were isolated. The *nor* family genes were identified in CNS using PCR, sequencing and phylogenetic approaches. *Nor* family expression was determined by RT-PCR. NorA efflux activity was determined using the automated ethidium bromide method.

In-silico analysis showed that *norA*, *mgrA*/*norR*, and "*norB*-like" and "*norC*-like" (*norB*/*norC*) genes are present in CNS. The *nor* family genes were distributed and constitutively expressed in all *S. epidermidis* strains studied. In one gatifloxacin resistant strain isolated from the endophthalmitis, treatment with gatifloxacin induced overexpression of the *norA* gene and resulted in high activity of NorA efflux.

These results indicate that the Nor family of proteins is present in CNS, and the NorA efflux mechanism for gatifloxacin response occurs in at least one strain of *S. epidermidis*, contributing to gatifloxacin resistance.

1. Introduction

The prevalence of *Staphylococcus aureus* strains resistant to multiple drugs and of coagulase-negative staphylococci (CNS) has increased worldwide; consequently, it is necessary to find new effective agents against these microbes. *Staphylococcus* strains have shown increased resistance to beta-lactam compounds [1]. Often, this resistance is accompanied by resistance to other antimicrobial agents such as fluoroquinolones [2]. Ciprofloxacin, for example, was previously effective for the treatment of staphylococcal infections, especially those caused by methicillin resistant strains [3]. Unfortunately, the widespread use of these agents has led to an increase in resistance, specifically in *S. aureus* and *S. epidermidis* strains [4].

Fluoroquinolones, such as ciprofloxacin and gatifloxacin (antibiotics of the first and fourth generation, respectively), act on bacteria by inhibiting DNA gyrase and DNA topoisomerase IV, which control DNA topology and are essential for chromosome function. Bacteria have developed mechanisms of resistance to fluoroquinolones; one mechanism is mutations in the *gyrA* and *parC* genes [5], which encode DNA gyrase and DNA topoisomerase IV, respectively. The bacterial genus *Staphylococcus* frequently uses this mechanism of resistance. Approximately 75% of the fluoroquinolone resistant *S. aureus* clinical isolates contain these mutations [6]. Additionally, 50–59% of the *S. epidermidis* strains that are isolated from urinary tract with resistance to fluoroquinolones have mutations in these genes [7].

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Recently efflux pumps have been considered as another mechanism for gaining fluoroquinolone resistance [8]. Bacterial drug efflux pumps have been categorized into the following five families: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multi-drug and toxic compound extrusion (MATE) family, the small multi-drug resistance (SMR) family and the resistance-nodulation division (RND) superfamily [8]. The efflux pumps for fluoroquinolones in *S. aureus* are members of the MFS superfamily [8]. In *S. aureus*, the *nor* family genes, *norA*, *norB*, and *norC*, are efflux pumps that provide resistance to fluoroquinolones [9–12]. The NorA protein of *S. aureus* confers resistance to a variety of hydrophilic fluoroquinolones [9,10,13]. The NorA protein is able to transport diverse compounds, indicating that it has broad substrate specificity [14,15]. A correlation between an increase in resistance to ciprofloxacin and an increase in *norA* gene transcript levels has been observed [13]. The *norB* gene product is a 49 kDa protein that participates in the resistance to quinolones [11]. The *norC* gene of *S. aureus* is a new efflux pump that also contributes to resistance to quinolones [12].

MgrA/NorR overexpression results in an increase in *norA* expression, suggesting that *norA* gene expression is regulated by the MgrA/NorR global regulatory protein [16]. However, the overexpression of *norA* can be due to mutations in the promoter or the 5′-untranslated region of this gene [17]. Additionally, the two-component regulatory system, such as the arlR–arlS complex, can also be involved in the regulation of the *norA* gene [18,19]. Another transcription factor that was recently found in *S. aureus* is NorG, which is a member of the GntR-like transcriptional regulator family. NorG binds to the promoters of the *norA*, *norB*, and *norC* efflux pump genes. Overexpression of the *norG* gene produces a threefold increase in *norB* mRNA transcripts, which is associated with a fourfold increase in the level of resistance to quinolones [20].

Currently, multi-drug resistant *S. epidermidis* strains have been isolated with high prevalence in samples of endophthalmitis, corneal ulcers, and conjunctivitis [21]. Fluoroquinolone resistant *S. epidermidis* isolated from clinical samples has emerged rapidly because of the frequent and repeated use of these antibiotics [22]. Although ciprofloxacin has been used effectively for the treatment of bacterial keratitis, many *S. epidermidis* strains that are resistant to ciprofloxacin have recently been isolated [23]. Fortunately, the use of fourth-generation fluoroquinolones, such as gatifloxacin, has partially solved this problem. However, approximately 10% of the *S. epidermidis* strains isolated from ocular infections (OI) are resistant to gatifloxacin. These strains contain diverse mutations in the *gyrA* and *parC* genes [24]. Additionally, strains with identical mutations in *gyrA* and *parC* genes have different minimum inhibitory concentrations (MIC) for gatifloxacin (10-fold difference), suggesting that other factors, such as the genetic background or the *nor* efflux genes, are relevant in the resistance phenotypes [24]. The participation of the Nor family in fluoroquinolone resistance in coagulase-negative staphylococci (CNS) has not been explored. Only the *norA* gene has been detected in the *S. epidermidis* genome [25]. However, its contribution to fluoroquinolone resistance remains unknown. Thus, the present work is focused on the identification of *nor* family genes in CNS and determining the participation of the Nor efflux mechanism for gatifloxacin resistance in *S. epidermidis* strains isolated from OIs.

2. Materials and methods

2.1. Strains

S. epidermidis strains were isolated from patients with conjunctivitis ($n = 23$), corneal ulcers ($n = 7$), or endophthalmitis ($n = 14$). Corneal ulcers and conjunctivitis samples were obtained by scraping and swabbing, respectively. The vitreous samples of patients with endophthalmitis were obtained mainly by vitrectomy. *S. epidermidis* strains were obtained from healthy skin (HS) by swabbing (HS; $n = 52$). Isolation and identification of bacterial strains were performed according to the methods described by Juárez-Verdalles [21]. This study followed the tenets of the Declaration of Helsinki, and it was approved by the ethics review board from our institution. All patients agreed to their participation in the study.

Table 1
Primer sequences.

Gene	Nucleotide sequence 5′ → 3′	Molecular size (bp)
norA	Sense GCTATTATCGGTGGAGGCGTG Antisense TTTGCTTCTTTACGGCGTGAC	435
norB/norC	Sense GCAACTAACCTTGGATGGCG Antisense ACGGTCAAGGCACTTCCGA	564
mgrA/norR	Sense ATGTCTGAACAACATAATTTAAAAG Antisense TTACTTTTCTTTTGTTTCGATAAATGC	445
norA promoter	Sense AAAGTTTTTCAAAATGATTGTC Antisense CCTAAGTCACTACCTTTTAATCC	281

2.2. Determination of gatifloxacin resistance

The MIC of gatifloxacin was performed according to the methods from the Clinical and Laboratory Standards Institute (CLSI/NCCLS). A strain with MIC $\geq$ 2 mg/L was considered resistant.

2.3. Identification of nor family genes in Staphylococcus species by in silico analysis

Using the *S. epidermidis* RP62A genome database (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gse) and the *S. epidermidis* RP62A transporter proteins database (TransportDB-Transporter Protein Analysis Database.mht), the orthologous genes and amino acid sequences corresponding to each *norA*, *norB*, *norC*, *norG* and *mgrA/norR* family member were examined in the *S. aureus* N315 strain.

In a similar way, the putative orthologous genes from *S. aureus* MW2, COL, RF122, MRSA252, JH9, Mu50, MSSA476, USA300FPR3757; *S. epidermidis* ATCC12228; *S. capitis* SK14; *S. haemolyticus* JCSC1435; *S. hominis* SK119; *S. carnosus* TM300; and *S. saprophyticus* ATCC15305 strains were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov).

All nucleotide and amino acid sequences for the Nor family in the *Staphylococcus* spp. were aligned with ClustalX and MUSCLE, respectively, using SeaView 2.4 with the default alignment parameters adjustments [26]. The similitude and identity matrixes were computed using the MatGAT4.50.2 software [27]. Maximum likelihood analyses were performed using ATGC Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). The LG model was selected for the tree analysis. The confidence at each node was assessed by 1000 bootstrap replicates.

Prediction of the motif sequences was performed with PROSITE (http://www.expasy.org) [28], Pfam (http://pfam.sanger.ac.uk/), and the conserved domains and protein classification from the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The protein internal sequence repeats were detected by the TRUST repeat detection method [29].