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Gatifloxacin, Moxifloxacin, and Balofloxacin Resistance due to Mutations in the *gyrA* and *parC* Genes of *Staphylococcus epidermidis* Strains Isolated from Patients with Endophthalmitis, Corneal Ulcers and Conjunctivitis

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Key Words
Moxifloxacin resistance · Gatifloxacin resistance · Balofloxacin resistance · *Staphylococcus epidermidis* · *gyrA* · *parC*

Abstract
Aims: *Staphylococcus epidermidis* is considered a commensal bacterium; however, it is frequently isolated from ocular infections showing a multidrug resistance. Ciprofloxacin-resistant strains have been isolated from ocular infections; however, resistance to quinolone, such as gatifloxacin and moxifloxacin, is not often studied, consequently the resistance mechanism is unknown. Our aim was to address the quinolone resistance and to explore the resistance mechanism in *S. epidermidis* strains isolated from ocular infections. ***Methods:*** *S. epidermidis* strains were isolated from patients with conjunctivitis (n = 23), endophthalmitis (n = 14) and corneal ulcers (n = 7). Minimum inhibition concentrations were determined by broth and agar dilution methods for moxifloxacin, gatifloxacin, balofloxacin, rufloxacin and pazufloxacin. Mutations were identified by sequencing the *gyrA* and *parC* genes, and their expression was determined by reverse transcriptase polymerase chain reaction. ***Results:*** We found that 13.6% (6/44) of the strains were quinolone resistant. In endophthalmitis, 21.4% were gatifloxacin, moxifloxacin and balofloxacin resistant. In corneal ulcers, 14.2, 14.2 and 28.5% were gatifloxacin, moxifloxacin and balofloxacin resistant, respectively, and in conjunctivitis only 4.3% were gatifloxacin resistant. The 6 strains with quinolone resistance showed mutations at Ser84Phe for the *gyrA* gene, and Ser80Phe for the *parC* gene. Gatifloxacin did not change the expression levels of *gyrA* and *parC* genes. ***Conclusion:*** *S. epidermidis* strains isolated from three ocular pathologies were gatifloxacin and moxifloxacin resistant due to mutations on the *gyrA* and *parC* genes.

Introduction

The prevalence of multidrug-resistant strains of *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) has increased worldwide; consequently it is necessary to find new effective agents. Strains of *Staphylococcus* have shown increased resistance to β-lactam com-

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Table 1. Sequence of oligonucleotides to amplify the quinolone resistance-determining region of the *gyrA* and *parC* genes

Oligo-nucleo-tide name	Sequence 5′ to >3′	PCR product size bp
gyrAF	TGGCTGAATTACCTCAATCA	280
gyrAR	GCCATTCTTACCATTGCTT	
parCF	ACTATTCGCAATGTATTCAAGTGGG	350
parCR	TGGTTCCAAAGTTGTGTCATCATAG	

pounds. In the early 1970s, 70–85% of *S. aureus* isolates were penicillin and methicillin resistant [1] and, particularly in this strain, often accompanied by resistance to other antimicrobial agents including quinolones [2]. Antibacterial fluoroquinolones, e.g. ciprofloxacin, have been effective for the treatment of staphylococcal infections, especially those caused by methicillin-resistant strains [3–5]. Unfortunately, the widespread use of these agents has led to a notorious increase in the resistance, specifically to *S. aureus* and *Staphylococcus epidermidis* [6–9]. However, quinolone resistance of *Streptococcus pneumoniae* and *Haemophilus influenzae* has rarely been reported [10–13].

Fluoroquinolones act by inhibiting the homologous type II topoisomerases, DNA gyrase and DNA topoisomerase IV, which control DNA topology and are vital for chromosome function and replication. Each of these enzymes is a tetramer composed of two subunits: GyrA and GyrB forming the A_2B_2 complex in DNA gyrase, and ParC and ParE forming the C_2E_2 complex in DNA topoisomerase IV. Amino acid substitutions on any subunit of either gyrase or topoisomerase IV have the potential to increase fluoroquinolone resistance in *S. pneumoniae* [14]. Alterations in DNA gyrase have been involved in quinolone resistance in S. *aureus* and S. *epidermidis* [15, 16].

Multidrug-resistant *S. epidermidis* has been isolated with a high prevalence in endophthalmitis, corneal ulcers and conjunctivitis [17]. Although ciprofloxacin is used effectively for the treatment of bacterial keratitis, an increasing number of *S. epidermidis* strains with ciprofloxacin resistance has been reported [18–22]. Moxifloxacin and gatifloxacin are fourth-generation quinolones; strains of *S. epidermidis* isolated from ocular infections (endophthalmitis, corneal ulcers, conjunctivitis) with resistance to these antibiotics have seldom been studied. Thus, this work is focused on determining the frequency of moxifloxacin, gatifloxacin, and balofloxacin resistance in *S. epidermidis* strains isolated from ocular infections and on the establishment of the resistance mechanism.

Methods

Patients

This work is a single-center study in which clinically diagnosed patients with conjunctivitis (n = 23), corneal ulcers (n = 7), and endophthalmitis (n = 14) from the Instituto de Oftalmología 'Conde de Valenciana', Mexico City, were examined. Corneal ulcer and conjunctivitis samples were obtained by scraping and swabbing, respectively. The vitreous samples of patients with endophthalmitis were obtained mainly by vitrectomy. The Research Committees of the Instituto Politécnico Nacional from Mexico City approved this study.

Isolation and Identification

The clinical samples were inoculated directly on chocolate, blood and mannitol salt agar plates. The chocolate agar plate was cultured in a 3% CO_2 atmosphere and all media were incubated at 37°C for 12–48 h. The bacteria were identified by means of the Vitek Jr computerized system (bioMérieux, L'Etoile, France), using the GPS-101 and V-1305 identification cards for Gram-positive bacteria.

Determination of Quinolone Resistance

Agar and broth dilution methods were used to determine the minimum inhibition concentrations (MICs) for gatifloxacin, moxifloxacin, balofloxacin, rufloxacin and pazufloxacin. The procedure was performed according to the Clinical and Laboratory Standards Institute (CLSI/NCCLS) by using agar and broth Mueller-Hinton (Becton Dickinson, Sparks, Md., USA).

Amplification of gyrA *and* parC *Genes by Polymerase Chain Reaction*

Bacterial DNA from strains with or without resistance to quinolones were obtained by using the DNeasy blood and tissue kit (Qiagen, Valencia, Calif., USA). Primers for *gyrA* and *parC* of *S. epidermidis* were designed for amplification of the quinolone resistance-determining region in both genes (table 1). The polymerase chain reactions (PCRs) were performed according to Martinez-Rodriguez et al. [23]. PCR products were purified and sequenced by the Big Dye terminator fluorescence kit (Applied Biosystems, Foster City, Calif., USA).

Expression of gyrA *and* parC *Genes*

Mutant and wild-type strains were grown in tripticase soy agar medium until reaching 0.5 McFarland absorbance without antibiotic. Growing conditions were similar for mutant strains except that 25 μg/ml of gatifloxacin was used. Bacterial cells were harvested and washed twice with PBS and incubated with lysis solution (40% sucrose, 10 mg/l lysozyme) at 37°C for 20 min. Total RNA was obtained by the TRIzol (Invitrogen, Carlsbad, Calif., USA) method and treated with RNAse-free DNAse I (Invitrogen). The reverse transcriptase (RT) reaction was carried out according to Rodríguez-Martínez et al. [23].

Table 2. MICs for quinolones of *S. epidermidis* strains from ocular infections

Disease/ antibiotics	Range[a] μg/ml	MIC_{50} μg/ml	MIC_{90} μg/ml	Percent resistance[b]
Endophthalmitis (n = 14)				
Gatifloxacin	0.08–1	1	30	21.4
Moxifloxacin	0.04–1	0.5	25	21.4
Balofloxacin	0.5–2	2	25	21.4
Rufloxacin	1–20	20	40	64.3
Pazufloxacin	1 to >6	2	>80	50
Corneal ulcers (n = 7)				
Gatifloxacin	0.08–0.5	0.5	1	14.2
Moxifloxacin	0.04–1	1	2	14.2
Balofloxacin	<0.08–0.5	0.5	4	28.5
Rufloxacin	2–20	4	>20	85.7
Pazufloxacin	1 to >6	>6	>6	57.4
Conjunctivitis (n = 23)				
Gatifloxacin	0.08–1	0.08	1	4.3
Moxifloxacin	0.04–1	0.04	1	0
Balofloxacin	<0.08–4	0.5	2	0
Rufloxacin	1 to >20	2	>20	34.7
Pazufloxacin	1 to >6	2	>6	39.1

[a] Values for sensitive strains only. In general, ranges for resistant strains were as follows: gatifloxacin 1–40 μg/ml, moxifloxacin 0.04–30 μg/ml, balofloxacin 1–25 μg/ml, rufloxacin 2–40 μg/ml and pazufloxacin 6 to >80 μg/ml.

[b] The definition of a quinolone-resistant strain was according to CLSI/NCCLS for which an MIC ≤0.5 μg/ml is considered quinolone sensitive, MIC = 1 μg/ml is quinolone-intermediate and MIC ≥2 μg/ml quinolone resistant.

Results

Determination of MICs for Quinolones in S. epidermidis *Strains*

As shown in table 2, strains isolated from patients with conjunctivitis were the most sensitive to the different quinolones, showing the lowest values of MIC_{50} and MIC_{90}, while strains of endophthalmitis were the most resistant. The antibiotics rufloxacin and pazufloxacin had minor potency against the strains of isolates studied, with MIC_{50} and MIC_{90} values higher than for other quinolones, indicating that these antibiotics were not effective. In contrast, gatifloxacin, moxifloxacin and balofloxacin were the most effective to strains isolated from corneal ulcers and conjunctivitis but not to endophthalmitis. In accordance with the CLSI/NCCLS manual, we found that 13.6% (6/44) of the strains were quinolone resistant according to their MICs.

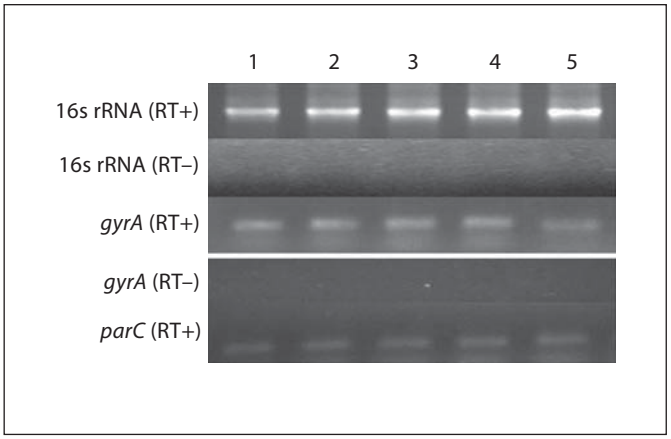

Fig. 1. Expression of *gyrA* and *parC* mRNAs in gatifloxacin-resistant *S. epidermidis* strains. Expression of *gyrA* and *parC* mRNAs by RT-PCR of 93, 98-3 and wild-type strains without gatifloxacin (lanes 1, 3, and 5) and with gatifloxacin (lanes 2, and 4). Expression of 16s rRNA was used as a housekeeping gene for the normalization of RT-PCR. RT– and RT+ consist of an RT reaction without or with MMLV RT enzyme, respectively. The mutant strains were grown with 25 μg/ml of gatifloxacin.

Determination of Mutations in gyrA *and* parC *Genes*

All strains with or without quinolone resistance were analyzed to detect mutations in *gyrA* and *parC* genes. The nucleotide sequence was translated into amino acids and compared with the amino acid sequence of *S. epidermidis* RP62A and ATCC12228 strains deposited in the GenBank. Of all strains sequenced, only 6 have mutations in these genes. In all the strains, the changes in *gyrA* were on serine 84 for phenylalanine. In addition to these mutations, 2 strains (98-3 and 93) isolated from endophthalmitis also showed a mutation of glutamic acid 88 changing to lysine (table 3).

In the *parC* gene, mutations in serine 80 for phenylalanine were found in all the strains except for strain 1654, where tyrosine was the altered amino acid (table 3; 4 strains had a double mutation for *parC,* including the strains 98-3 and 93 wherein aspartic acid 84 was altered to valine). There is a perfect correlation of quinolone resistance with the mutations, since the 6 mutant strains were the same that showed quinolone resistance.

In order to discard any resistance mediated by mutations induced by treatment with quinolones in the patients, we confirmed that no patient had been treated with quinolones prior to isolating.

Table 3. Mutations in *gyrA* and *parC* genes of quinolone-resistant *S. epidermidis* isolated from ocular infections

Disease/ strain	*gyrA* gene	*parC* gene	Gati-floxacin MIC μg/ml	Moxi-floxacin MIC μg/ml	Balo-floxacin MIC μg/ml	Ruflox-acin MIC μg/ml	Pazu-floxacin MIC μg/ml
Endophthalmitis							
98-3	S84F (TCT to TTT) E88K (GAA to AAA)	S80F (TCT to TTT) D84V (GAT to GTT)	40	30	25	40	>80
93	S84F (TCT to TTT) E88K (GAA to AAA)	S80F (TCT to TTT) D84V (GAT o GTT)	30	25	25	40	>80
214	S84F (TCT to TTT)	S80F(TCT to TTT) D84V (GAT to GTT)	6	2	6	>20	>6
Corneal ulcers							
1654	S84F (TCT to TTT)	S80Y (TCT to TAT) D84V (GAT to GTT)	1	2	4	>20	>6
1948	S84F (TCT to TTT)	S80F (TCT to TTT)	2	0.04	4	2	>6
Conjunctivitis							
105	S84F (TCT to TTT)	S80F (TCT to TTT)	2	1	1	>20	>6

The definition of a quinolone-resistant strain was according to CLSI/NCCLS for which an MIC ≤0.5 μg/ml is considered quinolone sensitive, MIC = 1 μg/ml is quinolone-intermediate and MIC ≥2 μg/ml quinolone resistant.

Expression of gyrA *and* parC *Genes in* S. epidermidis *Strains with Quinolone Resistance*

In order to determine if quinolone affects the expression of *gyrA* and *parC* genes in the mutant strains (98-3 and 93), expression levels of these genes were determined. It was observed that the mutant strains and the wild-type strain showed similar expression levels for both genes in the presence and absence of gatifloxacin (fig. 1).

Discussion

S. epidermidis has been the most frequently isolated bacterium from ocular infections [24–26], and 35–65% of CNS isolated from clinical samples, among them *S. epidermidis*, are resistant to methicillin [27]. Quinolones have emerged as an alternative for treating methicillin-resistant strains. Ciprofloxacin, gatifloxacin and moxifloxacin have been employed clinically; nevertheless *S. epidermidis* strains resistant to these antibiotics have been reported in the eye [18, 21, 28, 29]. We found that 13.6% (6/44) of the strains were quinolone resistant. Our results also show differences in quinolone susceptibility profiles of isolates from different types of infection (table 2), which are in accordance with evidence that molecular typing of nosocomial *S. epidermidis* strains has shown considerable diversity within the *S. epidermidis* population [30–32]. The diversity is observed not only in studies involving isolates from diverse geographic or clinical origins [33, 34] but also in collections which originated from the same hospital [35] (as in our case) and even a single intensive care unit [30].

Our observation of mutations at serine 84 of the *gyrA* gene and phenylalanine 80 of the *parC* gene in parallel with quinolone resistance is the same as those reported for nonocular infections [36–38]. Similarly to *S. aureus,* CNS strains from nonocular infections have amino acid changes at Ser80 and Asp84 in the *grlA* gene, and changes at Ser84 and Glu88 in the *gyrA* gene. In the *gyrA* gene of *S. epidermidis,* only changes at Ser80Phe or Ser80Tyr were found, while *S. hominis* and *S. haemolyticus* have Ser80Val or Ser80Leu amino acid changes. No mutations in the *gyrB* nor *grlB* genes in any strain of *S. epidermidis* were found [38]. In this study, we did not analyze alterations in the *gyrB* and *parE* genes, therefore, we cannot exclude the possibility that alterations of these genes could also contribute to quinolone resistance. It is interesting to highlight that other *Staphylococcus* species also have the same *gyrA* gene mutation at Ser84. This is the case for *S. epidermidis, S. haemolyticus, S. hominis, S. ca-*

pris and *S. simulans*. All these strains have a high homology (85%) in the quinolone resistance-determining region of the *gyrA* gene [39] indicating that this is a hotspot for these mutations.

We do not discard the possibility that other mechanisms could be contributing to quinolone resistance, such as the mechanism by quinolone resistance genes coded in plasmids, or the efflux of quinolone. This assumption is supported in strains 214, 1654, 1948 and 105 (all from different origins) with different MICs but with identical mutations (table 3). Other species have already been studied with the same mechanisms as *S. pneumoniae,* where moxifloxacin resistance occurs by the efflux of moxifloxacin from the bacterial cell in addition to the mutations in DNA gyrase and topoisomerase IV [14]. Similarly, *S. aureus* and CNS also have an active efflux mechanism that contributes substantially to the resistance phenotype [38]. Another resistance mechanism is that mediated by plasmids that encode quinolone resistance genes such as the *qnrS* gene for *Salmonella enterica* [40]. Another possible mechanism for quinolone resistance is the expression level of the *gyrA* and *parC* genes. We found that gatifloxacin did not induce overexpression of the *gyrA* and *parC* genes in the mutant strains, which indicates that quinolone resistance is not due to their expression levels and therefore the mutations did not change the expression of these genes.

Experiments performed in vitro have demonstrated that a mechanism for acquiring resistance is by selective pressure with antibiotics; i.e., double mutations in *gyrA* and *parC* genes are obtained after serial passage of *S. pneumoniae* treated with moxifloxacin [14] or *S. aureus* treated with gatifloxacin [41]. We found that 2 of 6 strains have a double mutation in the *gyrA* gene, and 4 of 6 strains in the *parC* gene though none of the 6 patients at the clinic were under treatment with quinolones. This result suggests that selective pressure was not the reason for generation of these mutants. A possible explanation for this phenomenon might be that strains become resistant to quinolones in a hospital by horizontal transference of genes among bacteria of the same species or even between different species that share the same habitat. A study demonstrated that approximately half of the *S. epidermidis* isolates from the normal human conjunctiva have mutations in the *gyrA* and *parC* genes and that these strains are gatifloxacin, and moxifloxacin resistant [29], indicating that in the normal ocular surface, strains already exist with quinolone resistance capable of infecting the eye.

In summary, this work provides evidence that the quinolone resistance of *S. epidermidis* strains isolated from patients with endophthalmitis, corneal ulcers and conjunctivitis is due to mutations in the *gyrA* and *parC* genes. Our results suggest that alternatives to the treatment of ocular infections by *S. epidermidis* with gatifloxacin, moxifloxacin or balofloxacin should be considered, since 13.6% of the strains are resistant to these antibiotics.

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