Correlation between the activity of different fluoroquinolones and the presence of mechanisms of quinolone resistance in epidemiologically related and unrelated strains of methicillin-susceptible and -resistant *Staphylococcus aureus*

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Objective To study the activity of five different fluoroquinolones against 22 epidemiologically related and unrelated strains of *Staphylococcus aureus* (13 methicillin-resistant (MRSA) strains and nine methicillin-susceptible (MSSA) strains) in which the mechanisms of quinolone resistance are also investigated.

Methods The MICs of the different fluoroquinolones were determined by the microdilution method, in the presence and absence of reserpine. The quinolone resistance-determining regions of the *gyrA*, *gyrB*, *grlA* and *grlB* genes were amplified and sequenced to establish the presence of mutations. The molecular epidemiology of the 22 strains was performed by low-frequency restriction analysis of chromosomal DNA with *Sma*I.

Results MSSA strains showed lower homology than MRSA strains, in which only two clones were seen. Trovafloxacin showed the best activity against these clinical isolates of *S. aureus*, since strains carrying one amino acid change in both GyrA and GrlA subunits remained susceptible to this antimicrobial agent. Furthermore, trovafloxacin did not seem to be a substrate for NorA.

Conclusion Trovafloxacin was the most active quinolone tested against *S. aureus* strains, followed by levofloxacin and sparfloxacin, whereas ciprofloxacin and norfloxacin were the least active quinolones, in both the presence and absence of reserpine. Epidemiologically related *S. aureus* strains presented different mechanisms of quinolone resistance, suggesting a divergent evolution of the same clone. Finally, 16 *S. aureus* strains with a ciprofloxacin plus reserpine MIC $\geq$ 1 mg/L already showed a mutation in the *grlA* gene. This MIC may be useful as a marker of mutation in this gene, contraindicating the use of this quinolone, since a second mutation may develop during treatment.

Keywords Fluoroquinolones, resistance, *S. aureus*

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INTRODUCTION

Methicillin-susceptible (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) are the most frequently isolated Gram-positive bacteria causing infection. Despite the availability of potent antimicrobial agents and improved public health conditions, *S. aureus* has remained a major human pathogen which colonizes and infects both hospitalized patients with decreased host defenses and healthy, immunologically competent people in the community [1].

At the time of their introduction, fluoroquinolones showed good activity against MSSA and

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MRSA [2]. Therefore, they were considered as alternative therapy to treat infections caused by MRSA [3,4]. However, the sporadic emergence of quinolone resistance with the use of the new fluoroquinolones has been reported [5,6] in *S. aureus* isolated from the skin flora of patients during ciprofloxacin therapy as well as during the treatment of MRSA carriers [7]. Thornsberry [8] found that most of the MSSA strains were susceptible (95.7%) or moderately susceptible (1.4%) to ciprofloxacin, whereas 76.4% of the MRSA strains were resistant to this antibiotic. The increase in ciprofloxacin-resistant MRSA may be due to selective pressure created not only by ciprofloxacin but also by β-lactams, macrolides, lincosamides, chloramphenicol and aminoglycosides, since MRSA are almost invariably multiresistant [9].

Fluoroquinolones act by inhibiting either DNA gyrase and/or topoisomerase IV [10–12]. In *S. aureus*, several reports have shown that topoisomerase IV is the primary target for quinolones [13,14]. The biochemical mechanisms of quinolone resistance in *S. aureus* have been classified into two groups: (1) mutations at the A subunit of the DNA gyrase [15–19] and at the A subunit of the topoisomerase IV [13–15]; and (2) fluoroquinolone efflux in the cytoplasmatic membrane [20,21]. The described mutations of the *gyrA* gene, which encodes the A subunit of DNA gyrase, related to quinolone resistance, affect the amino acid codons Ser84, Ser85 and Glu88 [15–19]. The quinolone mutations in the *grlA* gene, encoding the A subunit of topoisomerase IV, are located in the amino acid codons Ser80, Glu84 and Ala116 [13–15]. Most of these reports also showed amino acid substitutions in the B subunits of the DNA gyrase and/or the topoisomerase IV [14,17,18], encoded by the *gyrB* and *grlB* genes, respectively, which have not been directly associated with fluoroquinolone resistance.

NorA is one of the efflux systems related to fluoroquinolone resistance [20,21], and it seems that the increase in the level of resistance provided by NorA is due to overexpression of the gene associated with a mutation in the promoter region [22].

The main aim of this study was to determine the activity of new fluoroquinolones against epidemiologically related and unrelated quinolone-resistant strains of *S. aureus* (MRSA and MSSA), in which the mechanisms of quinolone resistance have been investigated in depth.

MATERIALS AND METHODS

Bacterial strains

In total, 22 clinical isolates of MSSA and MRSA were randomly chosen from 249 strains isolated during 1994 and 1995. The clinical isolates of *S. aureus* were obtained from different samples submitted to the Clinical Microbiology Laboratory at the Hospital Clinic of Barcelona, Spain. Nine strains of 22 were methicillin susceptible, whereas 13 were resistant to this antimicrobial agent.

Susceptibility testing

Susceptibility testing was performed by a microdilution method for ciprofloxacin (Bayer, Leverkusen, Germany), norfloxacin (Merck Sharp and Dohme, West Point, Pa, USA), sparfloxacin (Rhone-Poulenc, Vitry, France), levofloxacin (Hoechst Marion Roussell, Romainville, France) and trovafloxacin (Pfizer Ltd, Sandwich, UK), in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) [23]. To evaluate the action of the efflux systems, the MIC of each antimicrobial agent was determined in the presence and absence of 25 mg/L of reserpine (Sigma, St Louis, USA), an efflux pump inhibitor.

To determine the susceptibility to methicillin, oxacillin was used as a marker. A strain was considered resistant when the MIC was greater than 4 mg/L. In this case, the MIC was determined by an agar dilution method, according to the guidelines of the NCCLS [23].

Amplification of the quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *grlA* and *grlB* genes by PCR

Four sets of oligonucleotides, gyrA1–5′-ATG GCT GAA TTA CCT CAA TC-3′ and gyrA2–5′-GTG TG-A TTT TAG TCA TAC GC-3′, grlA1–5′-CAG TCG G-TG ATG TTA TTG GT-3′ and grlA2–5′-CCT TGA ATA ATA CCA CCA GT-3′, gyrB1–5′-GAA GCT G-CT ACG CAT GAA-3′ and gyrB2–5′-GCT CCA TC-C ICA TCG GCA TC-3′, and grlB1–5′-GIG AAG CI-G CAC GTA A-3′ and grlB2–5′-TCI GTA TCI GCA TCA GTC AT-3′, were used to amplify the *gyrA*, *grlA*, *gyrB* and *grlB* genes, respectively. Briefly, five colonies were resuspended in 300 μL of TE buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA), and 10 units of lysostaphin were added. This bacterial suspension was incubated at 37 °C for 15 min.

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After this, DNA was precipitated with 300 μL of isopropanol, centrifuged for 15 min, washed with 70% ethanol, and finally resuspended in 100 μL of sterile water. Two microliters of this suspension was added to 23 μL of distilled sterile water, and finally 25 μL of reaction mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3 mM magnesium chloride, gelatin (0.1 w/v), 400 μM deoxynucleoside triphosphates, 1 μM of each primer and 2 U of *Taq* polymerase (Gibco, Life Technologies Inc., Gaithersburg, MD, USA) were added. Each reaction was overlaid with a drop of mineral oil, and amplified with the following temperature profiles: (1) 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, for *gyrA* and *grlA*; and (2) 30 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min for *gyrB* and *grlB*.

The amplified DNA products were resolved in 2% agarose gel containing 0.5 mg/L of ethidium bromide.

Sequencing of the PCR product

The DNA product obtained from PCR was purified with the Quiaquick spin PCR purification kit according to the manufacturer's instructions (Quiagen Inc., Cathsworth, CA, USA). The sample was then directly processed for DNA sequencing using the *Taq* DyeDeoxiTerminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA, USA) and analyzed in an automatic DNA sequencer (Abi Prism 377, Perkin Elmer, Foster City, CA, USA).

Low-frequency restriction analysis of chromosomal DNA

The DNA preparation for pulsed-field gel electrophoresis (PFGE) was performed as previously described [24] with slight modifications. Briefly, 20 units of lysostaphin was added in the lysis buffer, and the incubation of ESP buffer and restriction mix solution were done overnight. DNA fragments generated by *Sma*I were separated by PFGE using a CHEF-DRII apparatus (Bio-Rad, Richmond, CA, USA). The pulse times were linearly increased over 20 h from 5 to 40 s at 200 V. Strain *S. aureus* NCTC 8325 was used as molecular size DNA marker, since the DNA pattern of this strain was known. Following electrophoresis, the gel was stained with ethidium bromide and photographed.

To analyze the PFGE patterns, software from Bio-Rad was used (Diversity Database, Bio-Rad, Richmond, CA, USA).

RESULTS

Typing of bacterial isolates

The genotyping of *S. aureus* clinical isolates was performed by low-frequency restriction analysis of chromosomal DNA followed by separation of the DNA fragments by PFGE. MRSA strains showed greater homogeneity (Figure 1), while MSSA strains showed more heterogeneity (Figure 2).

The 13 MRSA strains could be distributed into two different clones, clone A with nine strains, and

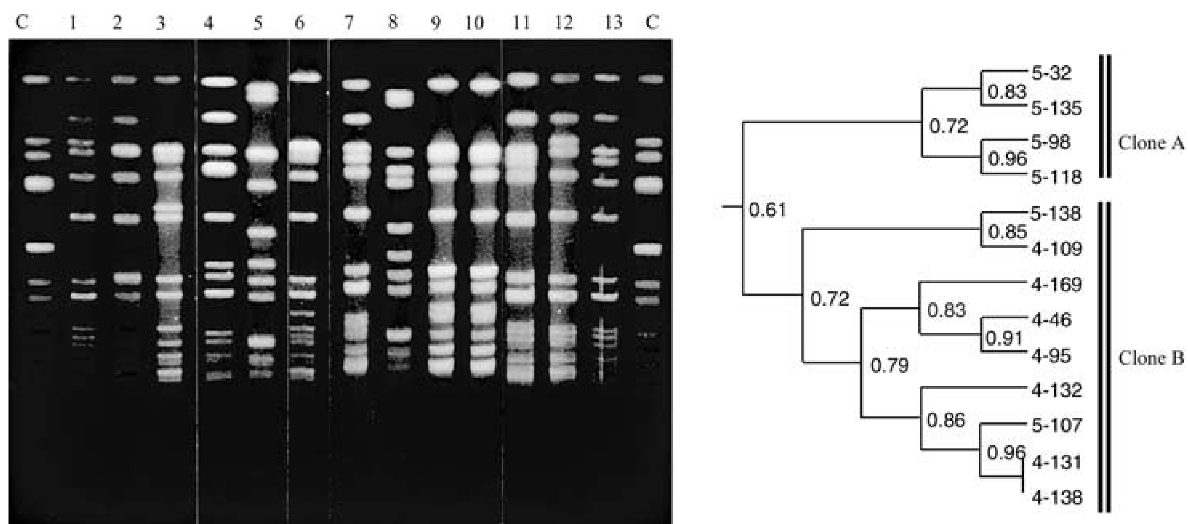

Figure 1 Pulsed-field gel electrophoresis and dendrogram of MRSA strains. Lane C, control strain NCTC; lane 1, strain 5-118; lane 2, strain 4-109; lane 3, strain 5-98; lane 4, strain 4-46; lane 5, strain 5-32; lane 6, strain 4-95; lane 7, strain 4-131; lane 8, strain 4-169; lane 9, strain 4-138; lane 10, strain 4-132; lane 11, strain 5-107; lane 12, strain 5-135; lane 13, strain 5-138.

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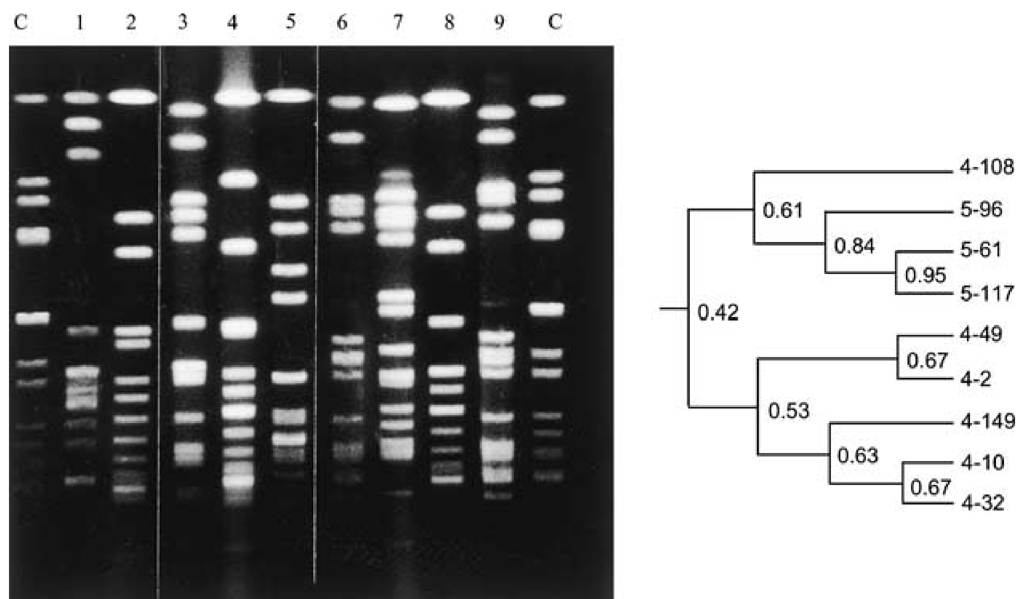

Figure 2 Pulsed-field gel electrophoresis and dendrogram of MSSA strains. Lane C, control strain NCTC; lane 1, strain 4-108; lane 2, strain 5-61; lane 3, strain 4-149; lane 4, strain 5-96; lane 5, strain 4-2; lane 6, strain 4-32; lane 7, strain 4-49; lane 8, strain 5-117; lane 9, strain 4-10.

clone B with the remaining four strains. The methicillin-susceptible strains showed lower homology, and seven different clones were observed; one contained three strains, whereas each of the remaining strains belonged to a specific clone. Less than 0.7 of similarity was considered to indicate that a strain belonged to another clone.

Antimicrobial susceptibility

Table 1 shows the MICs of ciprofloxacin, norfloxacin, sparfloxacin, levofloxacin and trovafloxacin, with and without reserpine, an inhibitor of the NorA efflux pump, and the MIC of oxacillin, against the 22 *S. aureus* strains analyzed in this study. Eight of 22 strains were susceptible to oxacillin and ciprofloxacin, trovafloxacin, levofloxacin. Three of these strains, 4-49, 5-96 and 5-117, were resistant to norfloxacin, whereas one oxacillin-susceptible strain, 4-10, was resistant to ciprofloxacin, norfloxacin and sparfloxacin, with MICs of 4 mg/L, 32 mg/L and 2 mg/L, respectively.

The remaining 13 strains were resistant to oxacillin, ciprofloxacin, norfloxacin and sparfloxacin. Five of these strains showed susceptibility to levofloxacin (MIC 4 mg/L), and the remainder showed resistance to this antimicrobial agent (MIC ranging from 8 mg/L to 16 mg/L). In the case of trovafloxacin, only four strains, with three mutations, two in *grlA* and one in *gyrA*, showed resistance to this antimicrobial agent, with MICs ranging from 8 to 32 mg/L.

Moreover, the MIC_{50} and MIC_{90} of each quinolone were calculated, and are shown in Table 2. Trovafloxacin was at least four-fold more active than ciprofloxacin or levofloxacin and 32-fold more active than norfloxacin. It also had the best activity in the presence of reserpine.

The MICs of norfloxacin and ciprofloxacin, which are hydrophilic quinolones, were decreased by the presence of reserpine, while trovafloxacin, levofloxacin and sparfloxacin, which are hydrophobic quinolones, showed no change in the MIC values (Table 1). The presence of the activity of NorA on norfloxacin is shown in Table 3. Ten of 22 strains (45.4%), four MSSA and six MRSA, possessed increased activity of NorA (activity of NorA was considered when the MIC was reduced by at least two dilutions), and in the presence of reserpine their MICs were reduced at least four-fold.

Detection of *gyrA*, *gyrB*, *grlA* and *grlB* mutations by PCR and DNA sequencing

The PCR products from the amplification of the QRDR of the *gyrA*, *gyrB*, *grlA* and *grlB* genes

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Table 1 MICs of different fluoroquinolones against 22 *S. aureus* clinical isolates

	Ciprofloxacin		Norfloxacin		Sparfloxacin		Levofloxacin		Trovafloxacin		
Strain	**−R**	**+R**	**−R**	**+R**	**−R**	**+R**	**−R**	**+R**	**−R**	**+R**	**Oxacillin**
MSSA											
4-108	0.06	0.03	0.25	0.25	0.03	0.03	0.12	0.12	0.016	0.03	0.25
5-61	0.06	0.03	0.5	0.25	0.016	0.016	0.12	0.06	0.016	0.008	0.25
4-2	0.25	0.12	1	0.5	0.06	0.06	0.25	0.12	0.016	0.016	0.5
4-149	0.25	0.12	2	0.5	0.06	0.06	0.25	0.12	0.06	0.03	0.5
4-32	0.5	0.25	2	1	0.06	0.06	0.25	0.25	0.06	0.06	0.5
5-96	1	1	16	8	0.06	0.06	0.5	0.5	0.06	0.06	0.25
4-49	2	0.5	32	4	0.12	0.06	1	0.5	0.12	0.06	0.5
5-117	1	1	16	4	0.03	0.06	0.5	0.5	0.06	0.12	0.5
4-10	4	2	32	16	2	2	4	2	1	1	0.5
MRSA											
5-135	16	16	64	64	8	8	8	8	1	2	256
4-131	16	4	64	16	8	4	8	4	2	1	>256
4-138	8	8	64	32	4	4	4	4	0.5	1	>256
5-107	8	4	64	32	4	4	4	4	2	2	256
4-169	8	8	32	32	4	4	8	8	1	1	>256
5-118	8	4	64	16	4	4	4	4	2	2	8
5-98	16	4	64	16	4	4	4	4	2	1	32
4-132	16	4	64	16	16	16	32	16	8	8	>256
4-109	32	32	128	128	8	16	16	32	32	32	16
5-32	64	32	>512	128	8	8	16	8	1	1	64
5-138	64	32	512	128	16	16	32	32	8	16	>256
4-46	64	16	>512	128	8	8	16	8	2	2	24
4-95	128	128	>512	>512	16	16	32	32	8	8	128

Quinolones were tested in the presence and absence of 25 mg/L reserpine (R).

showed the expected sizes of 398 bp, 680 bp, 469 bp, and 363 bp, respectively. The PCR products were sequenced in order to detect mutations in the QRDR. The results are shown in Table 4.

Six of 22 strains, all of them MSSA strains, were susceptible to all the tested quinolones, and did not present any mutation in the QRDR of the different targets. The MICs of ciprofloxacin ranged between 0.006 and 2 mg/L, while the MICs of trovafloxacin were between 0.008 and 0.06 mg/L. Two strains, also MSSA strains, had one mutation at position Ser80 of the *grlA* gene which generated a change from Ser to Phe. These strains had MICs of ciprofloxacin of 1 and 2 mg/L, respectively, and MICs of trovafloxacin of 0.06 and 0.12 mg/L, respectively. One MSSA strain and nine MRSA strains had two mutations, one in the *grlA* gene and one in the *gyrA* gene. The amino acid change in *grlA* was the same as that mentioned above, while in the *gyrA* gene the mutation was at position Ser84, producing a change from Ser to Leu. The MICs of ciprofloxacin and trovafloxacin for these strains ranged from 8 to 64 mg/L and from 1 to 2 mg/L, respectively. Finally, the remaining four MRSA strains had three mutations, the two above-mentioned mutations, plus another mutation at the amino acid codon Glu84 of the *grlA* gene, changing from Glu to Gly in three

Table 2 MIC_{50} and MIC_{90} in the presence and absence of reserpine

	Ciprofloxacin	Norfloxacin	Sparfloxacin	Levofloxacin	Trovafloxacin
CMI_{50}	8	64	4	4	1
CMI_{50}[a]	4	16	4	4	1
CMI_{90}	64	>512	8	16	8
CMI_{90}[a]	32	128	16	32	8

[a]MIC in the presence of 25 mg/L of reserpine.

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Table 3 Relationship between the quinolone-resistant mechanism and the MIC of norfloxacin

No. of strains	*gyrA*		*grlA*		*gyrB* (*n*)	*grlB* (*n*)	NorA (*n*)	Range of norfloxacin MIC Activity of	
	S 84	E 88	S 80	E84				−R	+R
6	—	—	—	—	E317 → D(2[a])	E422 → D(2[a])	+(2)	4–32	0.5–4
							−(4)	0.25–2	0.25–1
2	—	—	F	—	—	—	+(1)	16	4
							−(1)	16	8
10	L	—	F	—	L461 → F (1)	H478 → Y (1)	+(5)	64 to >512	16–128
					E435 → Q (1)		−(5)	16–64	16–64
3	L	–	F	G	—	—	+(2)	64–512	16–128
							−(1)	128	128
1	L	–	F	K	—	—	−(1)	>512	512

n, no. of strains.
[a]These two strains possessed the same mutations and did not show NorA activity. They do not belong to the same clone.
R, reserpine.

Table 4 Amino acid substitutions at quinolone protein targets

Strain	GyrA		GyrB	GrlA		GrlB
MSSA						
wt	S 84	E 88	—	S 80	E 84	—
4–108	–	–	—	–	–	—
5–61	–	–	E317(r)=D	–	–	E422(r)=D
4–2	–	–	E317(r)=D	–	–	E422(r)=D
4–149	–	–	—	–	–	—
4–32	–	–	—	–	–	—
5–96	–	–	—	F	–	—
4–49	–	–	—	–	–	—
5–117	–	–	—	F	–	—
4–10	L	–	—	F	–	H478(r)Y
MRSA						
5–135	L	–	—	F	–	—
4–131	L	–	L461(r)F	F	–	—
4–138	L	–	—	F	–	—
5–107	L	–	—	F	–	—
4–169	L	–	—	F	–	—
5–118	L	–	—	F	–	—
5–98	L	–	—	F	–	—
4–132	L	–	—	F	G	—
4–109	L	–	—	F	G	—
5–32	L	–	—	F	–	—
5–138	L	–	—	F	G	—
4–46	L	–	E435(r)Q	F	–	—
4–95	L	–	—	F	K	—

strains and from Glu to Lys in the remaining strain. The MICs of ciprofloxacin ranged from 16 to 128 mg/L, and the MICs of trovafloxacin ranged from 8 to 32 mg/L.

Mutations in the *grlB* and the *gyrB* genes (Table 4) were found in only five of 22 strains, and none could be associated with an increase in resistance. Two of these strains (strains 5-61 and 4-2) had the same mutations in both genes, producing a change from Glu317 to Asp in GyrB and Glu422 to Asp in GrlB; one strain (strain 4-10) had one mutation in *grlB*, His478 to Tyr; and the remaining two strains possessed only one mutation in *gyrB* (strain 4-131, Leu461 to Phe; and strain 4-46; Glu435 to Gln).

DISCUSSION

S. aureus is the most important Gram-positive nosocomial pathogen and is also a frequent cause

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of nosocomial outbreaks. Resistance of staphylococci to several antimicrobial agents may contribute to their ability to survive in a hospital environment and spread among patients, although there is an alternative view that they spread because they possess the property of what has been called epidemigenicity, rather than because they are antibiotic resistant. This is the case with MRSA [25–27]. Low-frequency restriction analysis of chromosomal DNA is a powerful technique frequently used for typing *S. aureus.* MSSA strains, in our study, showed genetic heterogeneity on analysis with this technique, with a genetic distance from 0.42 to 0.95, whereas in MRSA strains only two different clones were found. Most of the reports about quinolone and MRSA have described the spread of one or two clones among five to eight different clones present in a hospital, and these epidemic clones of MRSA have the potential to spread because of resistance to antibiotics [25–27].

Different quinolones were tested in order to establish their activity against *S. aureus*. Older quinolones like ciprofloxacin, norfloxacin and sparfloxacin had high levels of resistance—63%, 77% and 63%, respectively—while newer quinolones had different activity rates, levofloxacin showing 40% resistance and trovafloxacin showing the best activity, with only 18% of resistant strains. When reserpine was added, the resistance levels of sparfloxacin and trovafloxacin were the same, while those of the other quinolones decreased, being 59% in the case of ciprofloxacin, 63% for norfloxacin and 37% for levofloxacin. Trovafloxacin had the best activity against these clinical isolates. It is interesting to note that all the strains with an MIC of ciprofloxacin plus reserpine $\geq$1 mg/L presented at least one amino acid change, although testing more strains with a MIC of ciprofloxacin in the presence of reserpine $\geq$1 mg/L is needed to confirm the usefulness of this MIC as a marker of mutations in target genes. Therefore, when an *S. aureus* strain with a MIC of ciprofloxacin in the presence of reserpine $\geq$1 mg/L is isolated, we can consider that it has at least one mutation at the amino acid codon Ser80 of the *grlA* gene. Thus the probability of the development of a second mutation and an increase in the MIC over the breakpoint during treatment with a quinolone is very high, and thus its use is not recommended [28].

Nowadays, it is accepted that both DNA gyrase and topoisomerase IV are the main protein targets for quinolones [10–13]. Mutational alterations in the *gyrA* and the *grlA* genes encoding the A subunits of DNA gyrase and topoisomerase IV, respectively, have been shown to be the main mechanism of quinolone resistance acquisition in a large number of species, including *Escherichia coli* [17,29–31]. In *S. aureus*, the most frequent substitutions observed were in codons Ser84 and Glu88 in the *gyrA* gene, while in the *grlA* gene the substitutions were in codons Ser80 and Glu84, although other changes have been detected [10,14,17–19]. Recently, several mutations outside the QRDR of the *grlA* gene associated with quinolone resistance affecting amino acid positions Arg43, Pro157 and Ala176 have been described [32]. In our study, amino acid codons Pro157 and Ala176 were included in the amplified and sequenced DNA region, and no mutations were found in these positions. A few mutations affecting the *gyrB* and *grlB* genes have also been found to be associated with quinolone resistance in *S. aureus*.

In our study, among MSSA, the highest ciprofloxacin MIC found was 8 mg/L (strain 4-10), which correlated with a triple mutation in the *gyrA*, *grlA* and *grlB* genes, even though mutations in the *grlB* or *gyrB* genes do not play an important role in the acquisition of quinolone resistance. Among the MRSA, the highest ciprofloxacin MIC found was 128 mg/L (strain 4-95), which is correlated with a triple mutation: Ser84 to Leu in the *gyrA* gene and Ser80 to Phe plus Glu84 to Lys in the *grlA* gene.

There were two MSSA strains with the same MIC for ciprofloxacin of 1 mg/L (strains 5-96 and 5-117) with a mutation in the *grlA* gene, whereas another strain (strain 4-49) without mutations had a higher MIC for ciprofloxacin (2 mg/L). This difference could be explained by overexpression of an efflux pump. In fact, when reserpine was added to determine the MIC of ciprofloxacin for the strain 4-49, it decreased four-fold (0.5 mg/L). The same occurred with norfloxacin. Therefore, this efflux pump could be NorA, which confers resistance to hydrophilic quinolones, such as norfloxacin [20,21]. This is an example of epidemiologically unrelated strains with different mechanisms of resistance but with approximately the same MIC.

On the other hand, epidemiologically related strains, such as strains 4-46 and 4-95, belonging to the same clone, but with different mechanisms of resistance, can be found. The former showed a

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substitution in GyrB (E435 to Q), whereas strain 4-95 did not. However, the latter strain had a substitution in GrlA (E84 to K) that strain 4-46 did not have. Furthermore, strain 4-46 showed reduced ciprofloxacin and norfloxacin MICs when reserpine was added, whereas strain 4-95 did not. The above-mentioned mutations affected the MIC of trovafloxacin differently in these strains, the MIC being higher for strain 4-95. These results suggest divergent evolution of the same clone associated with the acquisition of different mechanisms of quinolone resistance (overexpression of efflux pump or *grlA* mutations). In Gram-negative bacteria, especially *E. coli*, the first step in quinolone resistance appears to be a mutation in protein targets, and only bacteria with a high level of quinolone resistance show enhanced expression of efflux pumps, such as AcrAB [33–35]. However, a more extensive study in Gram-positive bacteria is required to find whether overexpression of efflux pump or *grlA* mutations are the first step in quinolone resistance acquisition.

It is interesting to note that the MIC of sparfloxacin did not increase until a mutation in the *gyrA* gene appeared. This suggests that, in this case, the DNA gyrase was the primary target for sparfloxacin, because there were two MSSA strains (5-117 and 5-96) with a mutation in the *grlA* gene (Ser80→Phe) with an MIC of sparfloxacin below the breakpoint. Some recent studies have shown that the primary quinolone protein target could be changed, depending on the structure or the hydrophobicity of quinolones [36].

Among the antimicrobial agents tested, trovafloxacin presented the best activity against MSSA and MRSA strains, even when reserpine was added, and only the last four strains with three mutations were resistant to this antimicrobial agent. These results suggest that trovafloxacin was not affected by the NorA efflux pump.

In our study, it was seen that in over 50% of all the clinical isolates of *S. aureus*, the MIC of some quinolones, such as norfloxacin, decreased in the presence of reserpine. Similar results were found by Schmitz et al. [37], with about 30% of the ciprofloxacin-resistant strains showing decreased resistance to ciprofloxacin in the presence of reserpine. In all cases, the different MICs could be attributed to the different level of expression of NorA (studies in progress). Furthermore, in a previous study with these strains, the *norA* gene was analyzed in order to establish the prevalence of the different sequences encoding this gene [38]. It was seen that the sequence like that described by Yoshida et al. [20] was more prevalent (18 of 23 strains, 78%) than the sequence described by Kaatz et al. [21] (five of 23 strains, 22%). Moreover, efflux systems other than NorA and not inhibited by reserpine may exist [39], and may explain the different MICs of trovafloxacin, such as in strains 4-95 and 4-109. These strains had MICs for trovafloxacin of 8 and 32 mg/L, respectively, and carried the same mutations, two in the *grlA* gene and one in the *gyrA* gene. The MIC of trovafloxacin for these strains did not decrease in the presence of reserpine, showing that trovafloxacin is not a substrate for this kind of efflux pump, and suggesting the presence of a non-reserpine-affected efflux pump that can pump trovafloxacin out of the cell, although mutations outside the QRDR cannot be ruled out.

The molecular mechanism of overexpression of the *norA* gene remains unknown. It has been suggested that a single mutation in the promoter region could be responsible [22]. Recently, a new region upstream from the promoter was described by Fournier et al. [40] as a regulatory region, where one unknown protein of 18 kDa could bind and regulate the transcription of the *norA* gene by a two-component regulatory system (ArlS–ArlR). Therefore, a mutation in a regulatory region or in the genes coding for these two regulatory proteins may also explain the overexpression of the *norA* gene.

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