



## Original article

# Characterization of sparfloxacin-resistant mutants of *Staphylococcus aureus* obtained in vitro

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## Abstract

A sparfloxacin-susceptible clinical isolate of *Staphylococcus aureus* was grown in increased concentrations of sparfloxacin. The presence of mutations in *gyrA*, *gyrB*, *grlA* and *grlB* genes was analyzed. The primary point mutation was located in the *gyrA* gene (Glu-88 to Lys). Two further mutation steps appeared in the amino acid change Ser-80 to Tyr in GrlA. No mutations occurred in the *gyrB* or *grlB* genes. Efflux pumps involved in the increase of resistance were also found to affect norfloxacin and ciprofloxacin. This effect may be related to NorA. An overexpression of NorA, may be associated with the increase of the MIC of norfloxacin from 32 mg/l to > 200 mg/l in the final mutant. The MICs levels of sparfloxacin were affected by unknown mechanism. © 2001 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

**Keywords:** *S. aureus*; Sparfloxacin; Quinolones; Resistance; GyrA; Efflux pumps

## 1. Introduction

New fluoroquinolones are broad-spectrum antibacterial agents that showed good activity against Gram-negative bacteria [1–4] and *Staphylococcus aureus* at the time of their introduction [5].

The continuous rise in fluoroquinolone resistance levels in both Gram-negative and Gram-positive microorganisms has led to a decrease in the effectiveness of these antibacterial agents [6–8]. However, new quinolones such as sparfloxacin, trovafloxacin or clinafloxacin are especially active against Gram-positive bacteria [1,3,9,10].

Fluoroquinolones act by inhibiting the activity of the type II topoisomerases [11–13]. In prokaryotes two different enzymes are included in this subfamily of the topoisomerases: DNA-Gyrase and Topoisomerase IV. These two enzymes have great similarity. They are

tetrameric enzymes, composed of two subunits A (GyrA in the case of the DNA-Gyrase and *parC* [also named GrlA in *S. aureus*] in the case of the Topoisomerase IV), and two subunits B (GyrB in the case of the DNA-Gyrase and ParE [also named GrlB in *S. aureus*] in the case of the Topoisomerase IV). Moreover, the A and B subunits of these two enzymes possess a high sequence homology.

It is well established that the primary quinolone-target in Gram-negative microorganisms is the DNA-Gyrase [14], whereas in Gram-positive microorganisms it is the Topoisomerase IV [15–17]. However, in a previous study (Sierra et al., unpublished results) with clinical isolates of *S. aureus*, the results suggested that amino acid substitutions in GrlA without any in GyrA does not affect the MIC of sparfloxacin. Recent reports showed that the first point mutation for sparfloxacin in *Streptococcus pneumoniae* was at *gyrA* [18,19]. However, a study developed by Pan and Fisher [20] showed that independent of the location of the first point mutation, sparfloxacin has a greater affinity to Topoisomerase IV than to DNA-Gyrase in Gram-positive microorganisms.

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The most relevant quinolone-resistance development mechanism is the presence of mutations in the targets, especially in their respective A subunits [7,8,15–17,21–24]. However, other mechanisms involved in the acquisition of quinolone-resistance have been described, such as the presence of efflux pumps. In *S. aureus* norfloxacin resistance mediated by NorA, a specific efflux pump classified as a 12-TMS multidrug efflux protein [25], is well established [26–28]. Two ways have been described to explain the increase in MIC of quinolones associated with NorA; an increase in the expression of this efflux pump, or the presence of mutations that may affect the affinity of NorA for the quinolones (or other molecules) [22,29].

The main aim of this study was to establish the primary point mutation affecting sparfloxacin in a *S. aureus* clinical isolate and determine the possible relevance of reserpine-inhibited efflux pumps or other mechanisms in an in vitro sparfloxacin-resistant mutant of *S. aureus*.

## 2. Materials and methods

### 2.1. Microorganism

A quinolone-susceptible *S. aureus*, isolated from a clinical sample at the Microbiology Laboratory of the Hospital Clinic of Barcelona, was used for this study.

### 2.2. Growth of the strains with increasing sparfloxacin concentration

The selected strain was initially grown in Mueller-Hinton agar media with 0.03 µg/ml of sparfloxacin. One colony was both kept frozen and regrown with 0.06 µg/ml of sparfloxacin. The procedure was repeated five times with an exponential increase of the sparfloxacin concentration at each step until a concentration of 4 µg/ml of sparfloxacin was reached.

### 2.3. Minimal inhibitory concentration

The minimal inhibitory concentrations (MIC) of norfloxacin, ciprofloxacin and sparfloxacin were measured using the E-test method, following the instructions given by the manufacturer (AB-Biodisk, Solna, Sweden). The effect of reserpine on the MIC was also evaluated in Mueller-Hinton agar supplemented with 25 µg/ml of reserpine.

### 2.4. Amplification and DNA sequencing of quinolone resistance-determining region (QRDR) in *gyrA*, *gyrB*, *grlA* and *grlB* genes

The DNA was extracted as described by Zambardi et

al. [30], with some modifications. Briefly, one colony was incubated in 25 µl of water with 0.1 mg/ml of lysostaphin at 37 °C for 30 min and then boiled for 30 min at 100 °C. Thereafter, 25 µl of the PCR mixture containing 20 mM Tris-HCl (pH 8.8), 100 mM potassium chloride, 3.0 mM magnesium chloride, gelatin (0.1% wt./vol.), 400 µM deoxynucleoside triphosphates and 1 µM of each primer was added. The corresponding set of primers were: 5'-ATGGCTGAATTACCTCAATC-3' and 5'-GTGTGATTTTAGTCATACGC-3' to amplify a fragment of 398 bp of the *gyrA* gene, from base 1 to 397; 5'-GAA GCT GCT ACG CAT GAA-3' and 5'-GCT CCA TCC ICA TCG GCA TC-3' to amplify a fragment of 680 bp of the *gyrB* gene, from position 862 to position 1541; 5'-CAGTCGGTGATGTTATTGGT-3' and 5'-CCTTGAATAATAC-CACCAGT-3' to amplify a fragment of 469 bp of the *grlA* gene, from position 197 to position 665; 5'-GIG AAG CIG CAC GTA A-3' and 5'-TCI GTA TCI GCA TCA GTC AT-3' to amplify a fragment of 363 bp of the *grlB* gene, from position 1157 to 1520. Both the concentrations of the PCR mixture and the amplification conditions have been previously described [24]. The amplified DNA products were resolved by electrophoresis in agarose (2% w/v) gels containing 0.5 µg/ml of ethidium bromide. PCR products were recovered and directly sequenced using the Thermosequenase Dye Terminator Sequencing kit (Amersham, Cleveland, OH). The sequence analysis was performed in an automatic DNA sequencer (Abi Prism 377, Perkin Elmer, Foster City, CA).

### 2.5. Amplification and sequence of the NorA gene

The NorA gene was amplified and identified using the primers and following the procedures previously described [31]. The amplified products were recovered and directly sequenced following the methodology described above.

## 3. Results

Mutations in the QRDR of the four subunits of DNA-Gyrase and Topoisomerase IV as well as the MIC of norfloxacin, ciprofloxacin and sparfloxacin in the absence or presence of reserpine were analyzed in an in vitro obtained sparfloxacin-resistant mutant of *S. aureus*. (Table 1).

In the second step of mutation, the strains obtained (4-32A and 4-32B) did not show an increase in the MIC of sparfloxacin and only a small increase in the MIC of norfloxacin (1.5 mg/l in the parent strain, 2 mg/l in the 4-32B strain). This latter increase did not occur in the presence of reserpine (MIC of parent and 4-32B strain

Table 1  
Quinolone susceptibility of the parent and sparfloxacin obtained strains of *S. aureus* and changes in GyrA and GrlA proteins

Strain	Selection <sup>b</sup>	MIC (mg/l)						Amino acid changes				R.I. <sup>c</sup>			Other mech. <sup>a</sup>		
		N	N <sup>+</sup>	C	C <sup>+</sup>	S	S <sup>+</sup>	GyrA		GrlA		N	C	S	N	C	S
4.32	Clinical	1.5	0.75	0.38	0.064	0.094	0.064	Ser-84	Glu-88	Ser-80	Glu-84	x2	x6	x1.5	–	–	–
4.32A	0.03	1	0.5	0.5	0.094	0.047	0.047	–	–	–	–	x2	x5.4	NI <sup>c</sup>	x-1.5	x1.5	x-1.4
4.32B	0.06	2	0.75	0.75	0.125	0.094	0.047	–	–	–	–	x2.6	x6	x2	x1.5	x1.3	NI
4.32C	0.125	4	1	0.75	0.125	0.25	0.25	–	–	–	–	x4	x6	N.I.	x1.3	NI	x5.3
4.32D	0.25	4	1.5	0.75	0.125	0.25	0.25	–	–	–	–	x2.6	x6	N.I.	x1.5	NI	NI
4.32E	1	4	1.5	1.5	0.38	1	0.75	–	Lys	–	–	x2.6	x4	x1.3	–	–	–
4.32F	2	8	3	2	0.38	2	1	–	Lys	–	–	x2.6	x5.3	x2	–	–	–
4.32H	4	>256	32	>32	>32	>32	>32	–	Lys	Tyr	–	x>8	ND <sup>d</sup>	ND	–	–	–

N, Norfloxacin; C, Ciprofloxacin; S, Sparfloxacin; +/–, Presence of 25 µg/ml of reserpine.

<sup>a</sup> Increase in the MIC not attributable to a reserpine inhibiting mechanisms or to a target mutations.

<sup>b</sup> Concentration of sparfloxacin in the Mueller-Hinton.

<sup>c</sup> N.I., no increase.

<sup>d</sup> N.D., not determined.

<sup>e</sup> R.I., Increase in the MIC attributable to a reserpine inhibiting efflux pumps.

was 0.75 mg/l). The MIC of ciprofloxacin increased from 0.38 to 0.75 mg/l in the absence of reserpine. A similar increase was also found in presence of reserpine.

In the third step (strain selected with 0.125 mg/l of sparfloxacina) no changes were observed in the MIC of ciprofloxacin, in either the presence or absence of reserpine. However the strain 4-32C showed a two-fold increase in the MIC of norfloxacin (MIC 4 µg/ml). This increase was only 1.3-fold in the presence of reserpine. However the MIC in the presence or absence of reserpine was the same for sparfloxacina (0.25 µg/ml); during this step the MIC of sparfloxacina increased 2.6-fold in absence of reserpine and 5.3-fold in the presence of this efflux pump inhibitor.

The fourth selection step did not affect the MIC of the quinolones involved in this study. At this level, previous to the appearance of the target's mutations, the accumulative increase of the MIC of the three quinolones was analyzed, taking in account the reserpine effect ( $\Delta$ MIC in presence of reserpine between strains 4.32D and 4.32) was two-fold for norfloxacin and ciprofloxacin and 3.9-fold for sparfloxacina.

The presence of a substitution at amino acid Glu-88 to Lys of the GyrA protein in the fifth step of selection, resulted in a four-fold increase in the sparfloxacina MIC (only three-fold in the presence of reserpine), with no alteration in the MIC of norfloxacin in the presence or absence of reserpine, and with a two-fold increase in the MIC of ciprofloxacin, both in the presence and absence of reserpine.

In the sixth step of selection a low increase in the MIC of the three quinolones was seen; the MIC of norfloxacin changed from 4 and 1.5 µg/ml in presence and absence of reserpine in the fifth step of selection to 8 and 3 µg/ml in the sixth. The MIC of ciprofloxacin changed from 1.5 µg/ml in the fifth step of selection to 2 mg/l in the absence of reserpine, whereas the MIC in presence of reserpine was the same as the fifth step of selection (0.38 µg/ml). Finally, the MIC of sparfloxacina changed from 1 µg/ml and 0.75 µg/ml in the presence and absence of reserpine in the fifth step of selection to 2 mg/l and 1 mg/l in the sixth, respectively.

In the last selection step, when a concomitant mutation was obtained in *grlA* (Ser-80 to Tyr), the MIC of norfloxacin increased to > 256 µg/ml, and the MIC of ciprofloxacin and sparfloxacina increased to > 32 µg/ml. Reserpine only increased the norfloxacin MIC to 32 mg/l, whereas MICs to ciprofloxacin and sparfloxacina increased to over 32 µg/ml.

All mutant strains were examined for mutations in the *gyrB* and *grlB* genes but none were found.

The level of activity of the reserpine-inhibited efflux-pumps was constant in all the mutants produced, except in the last step mutant in which reserpine produced a greater than eight-fold decrease in the MIC of norfloxacin (the MIC of norfloxacin was ~2–2.6-fold

in the other strains except 4.32C which showed a four-fold decrease in the MIC). Reserpine affected the ciprofloxacin MIC (around six-fold), more than the other two quinolones. Its effect on the MIC of sparfloxacina was negligible (Table 1).

The NorA gene present in the analyzed strain was sequenced and identified as Yoshida-like and showed no differences from the NorA genes of the parent strain or the mutants obtained in vitro.

#### 4. Discussion

It has been shown that the most important mechanism in the acquisition of quinolone resistance is the development of mutations in the quinolone-target molecules [7,8,15–17,21–24]. The primary quinolone-target in Gram-positive microorganisms seems to be Topoisomerase IV [15–17]. Although, it has been suggested that the primary sparfloxacina target in Gram-positive microorganisms might be the DNA-Gyrase [18,19]. However, a study using *S. pneumoniae* by Pan and Fisher [20] showed that this quinolone has a greater affinity to Topoisomerase IV than to DNA-Gyrase.

Results obtained in the present study showed that the primary point mutation was the *gyrA* gene. The studied strain had a substitution of Glu-88 to Lys. Different mutations at amino acid codon Glu-88 of the *gyrA* gene have frequently been described in both clinical and laboratory obtained quinolone-resistant isolates of *S. aureus* [15,22,23]. This mutation is usually seen in isolates that also have a concomitant mutation in *GrlA*. We think this mutation has only been described once previously without any concomitant mutation in the *grlA* gene, in a clinical isolate of *S. aureus* with a ciprofloxacin MIC of 1 mg/l [23]. In a study conducted by Ng et al [17], mutations at amino acid codon Ser-84 of GyrA of *S. aureus* did not produce an increase in the MIC of ciprofloxacin in the absence of a concomitant mutation in the *grlA* gene. This has also been described for sparfloxacina resistant mutants of *S. pneumoniae* obtained in vitro [19]. In these mutants alterations present at amino acid codon Ser-83 (Ser to Phe/Tyr) and produce a four-fold increase in the sparfloxacina MIC, but do not affect the MIC of ciprofloxacin [19]. It is interesting to point out that the single mutation observed at position 88 of GyrA from our strain did not affect the MIC of norfloxacin and only induced a two-fold increase in the MIC of ciprofloxacin. However, this mutation induced either a four-fold increase in the sparfloxacina MIC in the absence of reserpine or a three times increase in the MIC in the presence of this efflux pump inhibitor. These results agree with the role of *GrlA* as a primary ciprofloxacin and norfloxacin target while suggesting that, despite the greater affinity

for Topoisomerase IV than for DNA-gyrase [20], the first point mutation for sparfloxacin raised is GyrA, as the DNA-gyrase is a primary sparfloxacin-target in *S. aureus*.

When a concomitant mutation was found in *grlA* (amino acid change from Ser-80 to Tyr), the MIC of the three quinolones was greatly increased (from 1 µg/ml to > 32 µg/ml in the case of sparfloxacin). That seems to suggest that a concomitant mutation both in *gyrA* and *grlA* is necessary to develop a higher level of resistance to this antibacterial agent.

The NorA encoding sequence found in this study was identical to that described by Yoshida et al. [32]. Two different sequences, with a homology of 95.12% in amino acids have been found encoding NorA [32,33], the sequence described by Yoshida being the most prevalent in different studies [31,34].

In order to discard the role of NorA as the cause of quinolone-resistance development in the strains which we studied, the MIC of the three quinolones was measured in the presence of 25 mg/l of reserpine, a NorA efflux pump inhibitor [35]. The results showed that NorA does not affect sparfloxacin. This agrees with the low activity of this efflux pump over hydrophobic quinolones, such as sparfloxacin which has been described previously [32,36].

In our mutated strains, the effect of the NorA-like efflux pumps remained stable and identical to the parenteral strain. There, the effect over the norfloxacin MIC was already 2–2.6-fold (except for strain 4.32C which was four-fold), around six-fold over ciprofloxacin MIC, but very slight for sparfloxacin. Only the last strain (4.32H) showed an important effect of NorA, or other reserpine-inhibited efflux pumps, on the MIC of norfloxacin (its MIC in presence of reserpine decreased more than eight-fold). Due to the fact that no mutations were found in the NorA gene sequence of any of the in vitro obtained mutants when compared with the parenteral strain and the published sequence, this result suggests that this later strain showed an overexpression of this efflux pump, or an alteration in the level of expression or in the affinity to quinolones of an unknown efflux pump able to be inhibited by reserpine [36].

The results obtained strongly suggest the presence of an unknown mechanism able to confer a low resistance level to sparfloxacin. Thus, the analysis of the increase of the MIC between the parenteral strain and the strain 4.32D (the last mutant without target mutations) show that whereas only a two-fold increase in the MIC of norfloxacin or ciprofloxacin remained without explanation, in the case of sparfloxacin a four-fold increase in the MIC was not attributable to a know mechanism (Table 1). Moreover, this increase in the sparfloxacin MIC was obtained between the second and third step of mutation when the MIC of sparfloxacin in the presence

of reserpine increased five-fold, and this increase of the MIC was not associated either with changes in any of the targets or with the NorA activity. At the next step of mutation the MIC of sparfloxacin remained stable and unvaried. A possible explanation might be the presence of a non reserpine-inhibited efflux pump able to pump out sparfloxacin.

A quinolone-susceptible strain of *S. aureus* was chosen to study the role of NorA and the involvement of mutations in the *gyrA*, *gyrB*, *grlA* and *grlB* genes in the acquisition of sparfloxacin resistance. Our results show that the sparfloxacin MIC is not affected by the expression of NorA or other reserpine-inhibited efflux pumps and suggest that GyrA could be the primary target for this specific quinolone, despite the greater affinity for the Topoisomerase IV. An as yet unknown mechanism able to affect the activity of sparfloxacin has been suggested.

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