Molecular epidemiology of quinolone resistance and comparative in vitro activities of new quinolones against European Staphylococcus aureus isolates

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Abstract

New fluoroquinolones (FQ) may possibly be used as alternative therapeutic options for Staphylococcus aureus infections. Our objectives were: (1) to define the in vitro activities of seven FQs in a collection of 434 methicillin-susceptible and 457 methicillin-resistant S. aureus from 23 European university hospitals; (2) to characterise the prevalence of mutations in the grlA and gyrA genes in all ciprofloxacin-resistant (n = 433) isolates of S. aureus; (3) to determine the percentage of ciprofloxacin-resistant S. aureus strains with measurable quinolone efflux. Methods: (1) The in vitro activities of different FQs were determined by microdilution tests. (2) PCR-amplified DNA was sequenced. (3) Ciprofloxacin minimum inhibitory concentrations (MIC) were determined in the presence and absence of reserpine, which inhibits efflux pumps. Results: (1) Irrespective of the methicillin resistance of the isolates, sitafloxacin and clinafloxacin showed the best in vitro activities. (2) All ciprofloxacin-resistant isolates exhibited GrlA alterations, namely Ser-80 → Phe or Tyr or Glu-84 → Lys or Ala-116 → Glu or Pro or a combination of Ser-80 → Phe and Glu-84 → Val. These alterations in GrlA were combined with alterations in GyrA, namely Ser-84 → Leu or Lys or Glu-88 → Lys or Val. (3) Reserpine reduced ciprofloxacin MIC values in ca. 30% of the clinical isolates tested. Conclusions: (1) This current European overview of mutations involved in FQ resistance demonstrates that only a limited number of classical mutations in grlA and gyrA contributed to resistance in clinical isolates. (2) An efflux pump is involved in ca. 30% of ciprofloxacin-resistant S. aureus isolates. (3) Sitafloxacin and clinafloxacin are two very promising new FQs with good anti-staphylococcal activity. New FQs, perhaps in combination with efflux pump inhibitors, might play a role in the treatment of S. aureus infections. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Fluoroquinolones (FQs) are inhibitors of DNA topoisomerases, mainly DNA gyrase and topoiso-

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merase IV. In most Gram-negative bacteria, DNA gyrase is the primary target for FQs, but in Gram-positive micro-organisms, topoisomerase IV seems to be the main target for most FQs. Nevertheless, target specificities can alter with various drugs, and some molecules, such as sparfloxacin, have been shown to mainly target gyrase in some Gram-positive bacteria [1–4].

As part of the topoisomerase reaction mechanism, gyrase and topoisomerase IV transiently break the DNA backbone and pass a double strand of DNA through those breaks. The quinolones trap an intermediate during this reaction, leaving the topoisomerases attached to the DNA as part of drug-enzyme-DNA complexes in which the DNA probably remains broken. The number of such complexes formed with gyrase correlates well with the extent to which overall DNA synthesis is inhibited, consistent with the idea that the complexes block the movement of DNA through the DNA replication machinery. Cell death apparently results from release of double-stranded DNA breaks from drug-enzyme-DNA complexes which are located throughout the chromosome. Quinolone-resistant mutations are expected to prevent complex formation, since they allow DNA synthesis and cell growth to occur in the presence of quinolone [2,5,6].

Mutations associated with increased resistance to quinolones in *Staphylococcus aureus* have been documented in conserved regions of *grlA* and *grlB*, genes encoding DNA topoisomerase IV, and in conserved regions of *gyrA* and *gyrB*, genes encoding DNA gyrase. These conserved regions are referred to as the quinolone-resistance-determining region (QRDR). Mutations in *grlA* and *gyrA* are the most frequently encountered changes associated with quinolone resistance in *S. aureus*. In addition to these mutations, *S. aureus* also have multi-drug efflux pumps like NorA that contribute to decreased fluoroquinolone susceptibility [1,3,4,7–9].

Because of the inherent limitations of ciprofloxacin and other available quinolones in the treatment of staphylococcal infections, there is a need for the development of new quinolones with increased anti-staphylococcal potency. Clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, trovafloxacin, and sitafloxacin are recently developed newer fluoroquinolones with a broad antimicrobial spectrum and minimal inhibitory concentrations (MICs) within clinically achievable levels in blood for some *S. aureus* isolates that are resistant to ciprofloxacin [2,5,6,10–12]. The clinical utility of these newer fluoroquinolones for the treatment of infections caused by ciprofloxacin-resistant Gram-positive cocci has yet to be established, however.

The purposes of the present investigation were:

1. to define the in vitro activities of seven FQs in a collection of 434 methicillin-susceptible and 457 methicillin-resistant *S. aureus* from 20 European university hospitals;
2. to characterise the prevalence and manner of mutations in the *grlA* and *gyrA* genes in all ciprofloxacin-resistant (n = 433) isolates of *S. aureus*, and
3. to determine the percentage of ciprofloxacin-resistant *S. aureus* strains with measurable quinolone efflux.

2. Materials and methods

2.1. Bacterial isolates

The in vitro activity of seven FQs was tested against 434 methicillin-susceptible *S. aureus* (MSSA) and 457 methicillin-resistant *S. aureus* (MRSA) isolates deriving from 23 university hospitals in 13 European countries. These isolates were collected between April 1997 and July 1998. The strains originated from patients with bacteraemia, nosocomial pneumonia, wound infections, or urinary tract infections. Only one isolate per patient, which was considered clinically significant according to local criteria, was submitted. Upon receipt, isolates were subcultured onto blood agar to ensure purity. Isolate identity was confirmed, if necessary, using a Vitek system (bioMérieux, France).

From these 891 isolates, all ciprofloxacin-resistant strains (n = 433) (minimum inhibitory concentration (MIC) > 4 mg l⁻¹), comprising 420 MRSA and 13 MSSA were analysed for mutations in the QRDR of the *grlA* and *gyrA* genes.

2.1.1. Participating countries and hospitals

These included Austria (Krankenhaus der Elisa-
bethinen, Linz), Belgium (Hôpital Erasme, Brussels), France (Hôpital St. Joseph, Paris; Hôpital de la Pitié-Salpêtrière, Paris; Hôpital Eduard Herriot, Lyon; A. Calmette Hôpital, Lille), Germany (University Hospital Freiburg, Freiburg; University Hospital Düsseldorf, Düsseldorf), Greece (National University of Athens, Athens), Italy (University Hospital of Genoa, Genoa; University Hospital of Rome, Rome), The Netherlands (University Hospital Utrecht, Utrecht), Poland (Jagiellonian University Hospital, Cracow; University Hospital Warsaw, Warsaw), Portugal (University Hospital of Coimbra, Coimbra), Spain (University Hospital of Sevilla, Sevilla; Hospital Ramon y Cajal, Madrid; Hospital de Bellvitge, Barcelona), Switzerland (CHUV, Lausanne), the UK (St. Thomas’s Hospital Medical School, London), and Turkey (University Hospitals in Ankara, Ankara, University Hospital in Istanbul, Istanbul).

2.1.2. Susceptibility testing

Antimicrobial susceptibility testing of isolates was performed using a reference broth microdilution method according to the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [13], with an inoculum of approximately $10^5$ cfu ml$^{-1}$ and doubling dilutions of FQs ranging from $0.008$ to $16$ mg l$^{-1}$. The seven FQs were provided by the respective manufacturers.

2.2. Polymerase chain reaction, oligonucleotide primers and sequencing

Protocols for the amplification and subsequent sequencing of *grlA* and *gyrA* have been described previously [12].

2.3. Influence of reserpine on ciprofloxacin MIC values

MICs of ciprofloxacin for all ciprofloxacin-resistant *S. aureus* strains were determined, as described by Neyfakh et al. [14,15], using microdilution plates containing 1:2 serial dilutions of the antibacterial drugs in 100 μl of LB medium (inoculum, $2 \times 10^5$ exponential-phase cells, incubation, 12 h at 37°C). All experiments were carried out in the absence or presence of reserpine (20 mg l$^{-1}$). After the incubation period bacterial growth was assessed by observing medium turbidity. For each isolate tested a MIC value ratio was calculated by dividing the obtained values in the absence of reserpine by the values obtained in the presence of reserpine.

3. Results and discussion

3.1. In vitro activities of different FQs

The differences between the in vitro potencies of the seven FQs tested against MSSA were smaller than those against MRSA. Based on the MIC values determined for MSSA, sitafortoxacin, clinafloxacin, and trovafloxacin exhibited the best in vitro potencies with MIC$\text{MIC}_{90}$ values of 0.03 mg l$^{-1}$, followed by moxifloxacin (MIC$\text{MIC}_{90}$ 0.06 mg l$^{-1}$), gatifloxacin (MIC$\text{MIC}_{90}$ 0.12 mg l$^{-1}$), levofloxacin (MIC$\text{MIC}_{90}$ 0.25 mg l$^{-1}$) and finally ciprofloxacin as the least active compound tested (MIC$\text{MIC}_{90}$ 0.5 mg l$^{-1}$). While 99.8% of the MSSA are inhibited by a MIC of 1 mg l$^{-1}$ for sitafortoxacin, this value decreases to 96.3% for ciprofloxacin.

Sitaflaxacin was the most active agent against MRSA, being two times more active than clinafloxacin, and at least eight times more active than the other quinolones. The MIC$\text{MIC}_{90}$ values for sitafortoxacin, clinafloxacin, moxifloxacin, gatifloxacin, trovafloxacin, levofloxacin, and ciprofloxacin against MRSA were 0.5 mg l$^{-1}$, 1 mg l$^{-1}$, 4 mg l$^{-1}$, 4 mg l$^{-1}$, 8 mg l$^{-1}$, 16 mg l$^{-1}$ and $>16$ mg l$^{-1}$, respectively. Of the 457 MRSA tested 95.7% were resistant to ciprofloxacin, 55.4% to gatifloxacin, 44.4% to levofloxacin, 27.1% to moxifloxacin, whereas only 0.4% were resistant to clinafloxacin and 0.2% to sitafortoxacin (MIC $\approx 4$ mg l$^{-1}$). Fig. 1 illustrates the MIC distribution of the different FQs in all the 891 *S. aureus* isolates tested. Overall, sitafortoxacin and clinafloxacin exhibited the best in vitro activities against MSSA and MRSA, with MIC ranges of $\leq 0.008$–8 mg l$^{-1}$ and $\leq 0.008$–4 mg l$^{-1}$, respectively. Thus, these two FQs are two promising new compounds with good anti-staphylococcal activity.

This study confirms a previous investigation [12] and extends earlier analyses since this is one of the few comparisons of the in vitro activities of seven
Fig. 1. MIC distribution (μg ml⁻¹) of different fluoroquinolones in European S. aureus isolates (n = 891).
FQs against current S. aureus isolates from European university hospitals.

3.2. Prevalence and manner of GrlA and GyrA alterations in ciprofloxacin-resistant S. aureus

The present study constitutes one of the largest series of ciprofloxacin-resistant S. aureus strains (n = 433) from distinct geographic areas for which partial sequences of the grlA and gyrA gene loci have been obtained.

Within GrlA six single or combined alterations were obtained: Ser-80 → Phe or Tyr, Glu-84 → Lys, Ala-116 → Glu or Pro and the combination of Ser-80 → Phe with Glu-84 → Val.

Within GyrA four single alterations were obtained: Ser-84 → Leu or Lys and Glu-88 → Lys or Val.

Table 1 illustrates the combinations of alterations in GrlA and GyrA that were found in the ciprofloxacin-resistant S. aureus population. In addition the percentage of appearance is given. As illustrated, the combination of Ser-80 → Phe in GrlA with Ser-84 → Leu in GyrA is by far the most prevalent combination of alterations in DNA topoisomerase IV and gyrase in the European ciprofloxacin-resistant S. aureus population. This current European overview of mutations involved in FQ resistance demonstrates that only a limited number of classical mutations in grlA and gyrA contributed to resistance in clinical isolates. The present study confirms previous investigations [1,12,16–19] and extends earlier analyses since this is one of the few investigations in which a large population from European university hospitals was screened for alterations in GrlA and GyrA.

3.3. Analysis of percentage of ciprofloxacin-resistant S. aureus strains with measurable quinolone efflux

The efflux of fluoroquinolones from the cell mostly results in a lower level of resistance than that seen in grlA or gyrA mutants. Since most experiments concerning NorA in S. aureus only used one clinical fluoroquinolone-susceptible strain, S. aureus SA-1199, it is possible that efflux pumps are important only in a limited number of fluoroquinolone-susceptible and -resistant S. aureus isolates [7,14,15]. The plant alkaloid reserpine has been shown to inhibit multidrug transporters like NorA [14,15], increasing the intracellular concentration of fluoroquinolones, thus potentially lowering MIC values.

The presence of reserpine (20 μg ml⁻¹), which by itself does not affect the growth of S. aureus [14], in the incubation medium resulted in up to four-fold decreases in ciprofloxacin MIC values in 139 out of 433 (30.3%) ciprofloxacin-resistant S. aureus isolates. However, in the rest of the isolates tested reserpine had no effect on the MIC values, resulting in MIC ratios of 1 for these strains.

For this there could be several possible explanations. Strains could vary in the extent to which reserpine is able to block NorA or other efflux transporters because of reserpine resistance, as has been reported for Bmr, a related transporter in Bacillus subtilis [20]. Reserpine could also have varying effects because the expression of NorA and/or other as yet unidentified efflux transporters could vary due to regulatory mutations in the region 5’ to norA or to the genes for other reserpine-sensitive transporters or may be localised to other regions of the chromosome, as appears to be the case for a previously reported inducible mutant [9].

The response to reserpine and the reduction in susceptibility do not correlate with the determined MIC and IC₅₀ values in the absence of reserpine or with mutations within the grl or gyr gene loci, as has been shown by us previously [21]. Furthermore, the observed inhibitory effect is not dependent on fluoroquinolone susceptibility (data not shown).

Thus, reserpine as an inhibitor of multi-drug efflux pumps seems to increase the intracellular concentra-
tion of ciprofloxacin in one fourth to one third of the strains tested. This might have therapeutic consequences, if in those isolates an inhibitor of efflux pump activity could be combined with a new quinolone. Reserpine is toxic for man in those concentrations which are needed to inhibit efflux activity. Therefore other efflux inhibitors are needed.

In summary, clinafloxacin and sitafloxacin are two new fluoroquinolones with excellent anti-staphylococcal activity. The combination of Ser-80→Phe in GrlA with Ser-84→Leu in GyrA is by far the most prevalent combination of alterations in DNA topoisomerase IV and gyrase in the European ciprofloxacin-resistant S. aureus population. In one fourth to one third of the ciprofloxacin-resistant strains tested, reserpine slightly decreased MIC values of ciprofloxacin, suggesting the existence of multi-drug efflux pumps which seem to decrease the intracellular ciprofloxacin concentration. New FQs, perhaps in combination with efflux pump inhibitors, might play a role in the treatment of S. aureus infections.

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