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Occurrence of quinolone resistance in *Staphylococcus aureus* from nosocomial infection

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SUMMARY

Among 63 *Staphylococcus aureus* isolates (one isolate per one patient) counted from infections (from August to November 1991) in hospital T., eight exhibited resistance to fluoroquinolones. Seven of these quinolone-resistant isolates were multiply- and methicillin-resistant *S. aureus* (QR-MRSA). The results of phage-, plasmid- and genotyping (pulsed field electrophoresis) revealed that six different strain-clones of these MRSA were spread in the hospital. *In vitro* spontaneous mutants resistant to fluoroquinolones are 10–100-fold more frequent in MRSA than in other *S. aureus* when selected on isosensitest-agar containing 1 μg/ml of ciprofloxacin. However, the same mutant frequencies were found in strain 8325-4 with and without the mecA-determinant. The resistance phenotype was stable over 30 generations of subculture in nutrient broth as well in natural quinolone resistant MRSA as in mutants of other types of *S. aureus* selected *in vitro*. The phenotypic association of quinolone resistance and MRSA is rather likely due to a higher frequency of spontaneous resistant mutants which are present in natural populations of MRSA. Data of chemotherapy prior to the isolation of *S. aureus* show that three of seven patients from whom QR-MRSA were isolated were treated with a quinolone. In eight cases of infections with non-MRSA and quinolone treatment the isolated *S. aureus* strains were *in vitro* sensitive to quinolones.

INTRODUCTION

Fluoroquinolone inhibitors of DNA gyrase exert a broad antibacterial activity which is extremely high against Enterbacteriaceae and lower against Gram-positive bacteria as e.g. staphylococci [1] with no difference between methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive strains. The *in vitro* activity in relation to the pharmacokinetic behaviour [2] was one of the reasons for a clinical use of the newer quinolones in cases of staphylococcal infection [3]. There is always cross resistance between different quinolones which is mainly due to mutations affecting the gyr A-gene. The corresponding amino acid substitutions occurring in a definite region of the gyrase A protein of ciprofloxacin-resistant clinical isolates of *S. aureus* are analogous to those in quinolone-resistant *Escherichia coli* [4]. Also an efflux mechanism has been

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described for norfloxacin-resistant *S. aureus* [5]. The concentrations of quinolones at which resistant mutants of bacteria can be isolated *in vitro* are 8–16-fold lower than the MIC's for quinolones after subcultivation of these mutants [6]. Reports on the emergence of quinolone-resistant clinical isolates of *S. aureus* and *Pseudomonas aeruginosa* together with therapeutic use correspond to these observations [7, 8]. The emergence of quinolone resistance is of particular concern in the case of MRSA for which relatively few antimicrobial agents are still effective. The aim of this study was to analyze whether quinolone resistant *S. aureus* from various kinds of infections in a regional hospital was due to an outbreak of infection caused by intrahospital spread of one particular strain or if this resistance occurred in different strains, and if quinolone resistance was associated with other resistance characters.

MATERIALS AND METHODS

Staphylococcal strains. The wild strains were cultivated from different kinds of clinical specimen on sheep-blood-agar. For strain 8325-4 med A (BB 270) see [9], for strains 1309 and 108/83, 253/86 and 524/86 see [10].

Typing of S. aureus. Phage typing was performed using the international basic set [11] and two sets of experimental phages (for details see [12]. Plasmid-profiles were established by use of the 'boiling method' [13].

Resistance determinations. Minimal inhibitory concentrations (MIC) were determined by microdilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS); M7-A, Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. For detection of resistance to oxacillin 1 ml of isosensitest-broth containing 20 μg of NaCl and 2 μg of oxacillin were inoculated with about 10^7 colony-forming units from a preculture in nutrient broth. The tube was incubated at 36 °C for 18–20 h.

Restriction fragment analysis of genomic DNA. For DNA-preparation, cells were grown in 5 ml of trypticase soy broth overnight at 37 °C. Cells were harvested from 1·5 ml of this culture and suspended in PIV buffer (1 M-NaCl, 10 mM Tris HCl, pH 8·0). The cells were transferred to an Eppendorf-tube, centrifuged and resuspended in PIV so that 150 μl PIV resulted in a pellet of 5 μl. 200 μl of this suspension were mixed with 200 μl of 1·2% LMP-agarose in PIV, pipetted into a plug mould (Bio Rad) and allowed to solidify. For lysis one plug was placed in 1 ml of fresh lysis solution (6 mM Tris HCl, pH 8·0, 10 mM EDTA, pH 8·0, 0·2% deoxycholate, 0·5% laurylsarcosine, 100 μg/ml of lysozyme, 50 μg/ml of lysostaphin and 50 μg/ml of RNase) and incubated overnight at 37 °C with gentle shaking. The plugs were washed twice with ES-buffer (0·5 m EDTA, 1% laurylsarcosine) and incubated in ESP-buffer (ES-buffer + 1 mg/ml of proteinase K) at 50 °C with gentle shaking. The plugs were washed three times for at least 30 min with 14 ml of TE (10 mM Tris HCl, pH 7·5, 0·1 mM EDTA). For digestion with restriction endonuclease SmaI one plug was placed in 100 μl of reaction buffer and 1 μl of enzyme (Boehringer) and incubated overnight at 25 °C. The reaction was stopped by adding 50 μl of 0·5 m EDTA. The plugs were then placed into the wells of a 1% agarose gel (Sigma agarose type II, medium EEO) in 0·1 m Tris HCl, 0·1 m borate, 0·4 mM EDTA-buffer. The gel was run in the same buffer using the

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Table 1. *Characteristics of ciprofloxacin-resistant* S. aureus (*MIC* > 2 *μg/ml*)

Grouping of strains (number of infections caused)	Phage-pattern*	Crystal violet-type	Haemolysine type	Resistance phenotype†	Plasmid profile	Genotyping pattern (Fig. 1)	Clinical origin
1. (3)	a 75, 77, +RTD b 630, +RTD c 92	C	A	Pn Ox Tc Sm Em Tp/Su Gm Sm Am Cp Hg	20; 1, 8; 1, 6	A	1 Catheter tip 2 Wound swab
2. (2)	a 75, 77 RTD b 630 RTD c 92 RTD	C	B	Pn Ox Tc Em Tp/Su Sm Cp	18	B	Wound swab Decubital ulcus
3. (2)	a 75, 77 RTD b 630 RTD c 92 RTD	C	A	Pn Ox Tc Em Tp/Su Gm Am Cp	18; 1, 8	C	2 Wound swab
4. (1)	a 85 RTD b 630 RTD c 91, 92 RTD	C	A	Pn Ox Cm Tc Em Tp/Su Gm Sm Am Fs Cp	17, 5; 2, 0	D	Wound swab
5. (1)	a 85, 100 RTD b 630 RTD c 92, +RTD	C	A	Ox Cm Tc Sm Gm Am Em Tp/Su Cp	Without plasmids	E	Wound swab
6. (1)	a (79), 85, 100 RTD b 617, 626, +100 RTD c 89, 92, 100 RTD	C	A	Pn Ox Tc Em Sm Gm Cp	17; 1, 8	F	Wound swab
7. (1)	a 75, 77, 84, 85, +RTD b 618, 623, 629, 630 RTD c 89, 90, 92 RTD	C	—	Pn Tc Em Tp/Sa Gm Am Cp	21, 5	G	Wound swab
8. (1)	52, 100 RTD	C	A	Pn Cp	20	H	Wound swab

* a, International basic set for phage typing staphylococci; b, experimental phages 616, 617, 618, 620, 622, 629, 630; c, experimental phages 88, 89, 90, 91, 92, 93.

† Pn, penicillin; Ox, oxacillin; Cm, chloramphenicol; Tc, oxytetracycline; Gm, gentamicin; Am, amikacin; Sm, streptomycin; Em, erythromycin; Tp/Su, trimethoprim/sulphonamide; Cp, ciprofloxacin.

CHEF-DRII-System from Bio Rad at about 200 V; first with pulse time from 5 sec to 60 sec for 15 h and second from 60 sec to 90 sec for another 15 h.

Frequencies of ciprofloxacin-resistant mutants in vitro. Cells were grown in 5 ml of trypticase soy broth for 3 h at 37 °C under shaking. 10 μl from this culture was pipetted into 5 ml trypticase-soy-broth and incubated at 37 °C overnight under shaking. Undiluted and appropriate diluted aliquots were placed on isosensitest agar without and with ciprofloxacin (1 μg/ml). After incubation overnight at 37 °C the mutant frequencies were calculated as the quotient from number per ml of ciprofloxacin-resistant mutants and total colony forming units per ml.

Experiment on the stability of resistance to ciprofloxacin. One colony of each investigated strain was picked from an isosensitest-agar-plate containing 4 μg/ml of ciprofloxacin, inoculated into 5 ml of isosensitest-broth (subculture A). The tube was incubated at 37 °C for 18 h. From this culture about 10^4 cells were inoculated into 50 ml of isosensitest-broth and incubated for 18 h at 37 °C (subculture B). Again from subculture B 10^7 cells were inoculated into 50 ml of isosensitest-broth and incubated for 18 h at 37 °C (subculture C). After appropriate dilutions aliquots from subcultures A to C were plated on isosensitest-agar with and without addition of ciprofloxacin (4 μg/ml). From the colony counts on the plates without ciprofloxacin the number of generations of growth was calculated according to [6]. The quotient from the colony counts on plates with ciprofloxacin and those without expresses the stability (1 $\cong$ stable).

RESULTS

Typing of quinolone-resistant and quinolone-sensitive S. aureus

All *S. aureus* isolates from different kinds of infections in hospital T obtained from September to November 1991 were subjected to resistance determination and to typing. Twelve of 67 isolates exhibited resistance to quinolones (18·2%). The MICs for the resistant isolates were for ciprofloxacin 4–64 μg/ml, ofloxacin 4–32 μg/ml and pefloxacin 8–64 μg/ml.

By typing the quinolone-resistant isolates 8 different strains could be differentiated, 7 exhibiting the multiresistance phenotype and 1 only resistant to penicillin and to quinolones (Table 1). Although strains grouped as 1, 2 and 3 exhibited rather similar phage patterns, they clearly differed in their plasmid-profiles and the patterns of SmaI-restriction of their chromosomal DNA and pulsed-field-elecrophoresis (Fig. 1).

The 55 quinolone-sensitive isolates exhibited the whole spectrum of phage-patterns known in *S. aureus* from infections in humans (Table 2). Nine of these isolates were sensitive to the antibiotics tested and 36 resistant to penicillin; other kinds of resistances were more rare and only one strain was resistant to three antibiotics. With regard to the clinical origin there is no difference between the quinolone-resistant and the quinolone-sensitive isolates.

These results indicate that quinolone resistance is more frequently associated with multiply- and methicillin-resistant *S. aureus* than with more sensitive strains. There are two possibilities to explain the association of quinolone-resistance with MRSA. (*a*) Multiply and methicillin-resistant *S. aureus*-strains (MRSA) exhibit a higher frequency of quinolone-resistant mutants (e.g. resistant subpopulations). (*b*) Mutant frequencies for quinolone-resistance are in the same

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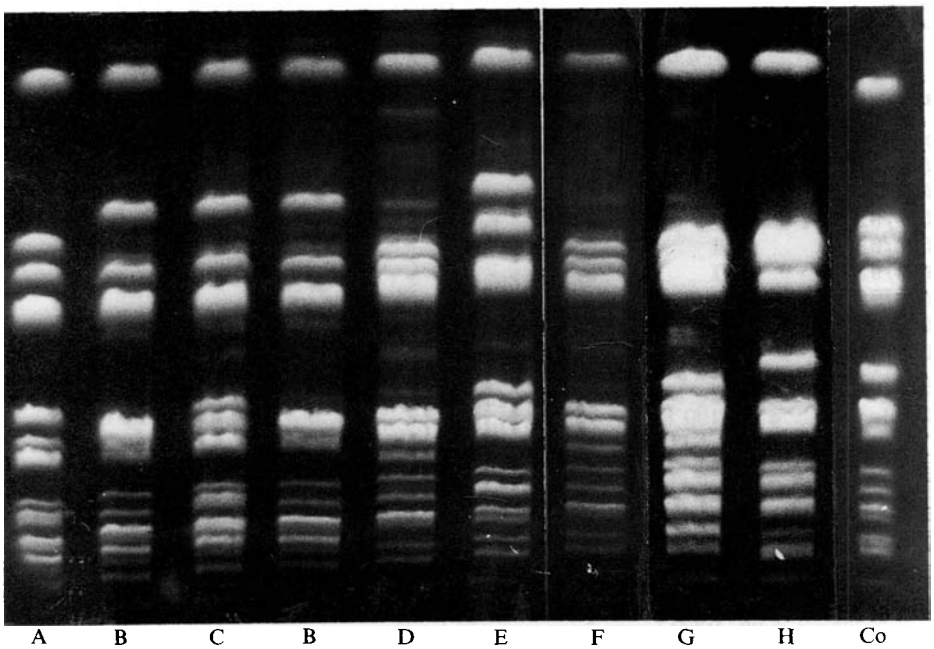

Fig 1. Chromosomal digestion pattern of multiply- and methicillin-resistant *S. aureus* strains (SmaI-digestion). The letters at the bottom of the lanes designate digestion patterns as indicated in Table 1. Co, chromosomal digest of strain *S. aureus* 8325-4 as control.

Table 2. *Phenotypical characteristics of ciprofloxacin-sensitive* S. aureus *(MIC < 0·5 μg/ml) and their clinical origin*

Phage-group	Number of isolates	Resistance phenotype	Clinical origin
I	8	Pn	Wound swab
	1	Tc Sm Em	Wound swab
	4	Sensitive	Wound swab
	1	Pn Gm	Pus
	1	Cp	Drain
II	6	Pn	Wound swab
	1	Pn Tc	Wound swab
	3	Pn	Abscess
	1	Pn	Ulcus cruris
	1	Pn	Punctuation
III	6	Pn	Wound swab
	1	Tp/Su Gm	Wound swab
	1	Cm Em	Wound swab
	1	Pn	Pus
I/III (mixed)	1	Pn Tc	Abscess
94, 96	3	Pn	Wound swab
	2	Sensitive	Wound swab
	1	Sensitive	Catheter tip
95	4	Pn	Wound swab
	2	Sm	Wound swab
Nt, 100 RTD	3	Pn	Wound swab
	1	Pn Sm	Wound swab
	2	Sensitive	Wound swab
Total	55		

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Table 3. *Frequencies of spontaneous ciprofloxacin-resistant mutants (selected on isosensitest-agar containing 1 μg/ml ciprofloxacin)*

Strain	Phage-group	Resistance phenotype	Mutant frequencies
Non-MRSA			
559/91	I	Sensitive	3×10^{-10}
565/91	II	Pn	2×10^{-9}
579/91	II	Pn, Tc	Not detectable ($< 5 \times 10^{-10}$)
596/91	III	Pn, Gm	Not detectable ($< 5 \times 10^{-10}$)
603/91	III	Pn	Not detectable ($< 5 \times 10^{-10}$)
572/91	III	Cm, Em	2×10^{-10}
593/91	95	Pn	Not detectable ($< 5 \times 10^{-10}$)
558/91	94, 96	Sensitive	Not detectable ($< 5 \times 10^{-10}$)
MRSA			
1309	a 85, 100 RTD b 618, 623, 625, 630, RTD c 92, RT		$1, 9 \times 10^{-8}$
524/86	a NT, 100 RTD b NT, 100 RTD c 617, 618, 622, 626, 630, RTD		3×10^{-8}
108/83	a NT, 100 RTD b 616, 617, 622, 623, 626, 630, RTD c 89, RTD		1×10^{-8}
253/86	a 54, 77, +RTD b c 90, 91, 92, 100 RTD		5×10^{-8}
8325-4			Not detectable ($< 5 \times 10^{-10}$)
8325-4, mec A (BB270)			Not detectable ($< 5 \times 10^{-10}$)

For phage-patterns a,b,c see Table 1.

range for MRSA and more sensitive strains, but the resistance mutation is more stable in MRSA.

To check possibilities (*a*) and (*b*) the following *in vitro* experiments were performed with ciprofloxacin as a representative of the newer quinolones.

Frequencies of ciprofloxacin-resistant mutants in non-MRSA and in MRSA

For these tests characteristic strains of each phage-group collected from infections in hospital T. were used. As representatives of MRSA we took quinolone-sensitive MRSA from earlier infections in East Germany prior to therapeutic chinolone application.

As shown in Table 3, MRSA exhibit a 10–100-fold higher frequency of ciprofloxacin resistant mutants than non-MRSA. Although rather unlikely from the well known mechanisms of methicillin-resistance in *S. aureus* (mec A codes PBP2 with reduced affinity [12]) we checked strain 8325-4 and its methicillin-resistant transductant possessing the mec A determinant for mutant frequencies without finding a difference.

Stability of ciprofloxacin-resistant mutants in vitro *and of ciprofloxacin-resistance in MRSA*

These experiments were designed in such a way that 30–35 generations of bacterial growth were included with tests on loss of resistance after the 15th and after the 30–35th generation. For ciprofloxacin-resistant mutants of strains

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Table 4. *Calculated chemotherapy prior to the isolation of* S. aureus *strains in 43 patients*

S. aureus isolated	Number of patients: Treated with a quinolone	Treated with other antibiotics	No antibiotic treatment
QR-MRSA	3	1 doxycyclin 1 cephalexin 1 ampicillin/sulbactam†	1
Other *S. aureus*, quinolone-sensitive	8	9	19

* Ciprofloxacin and ofloxacin.
† Multiply resistant, non-MRSA.

559/91, 565/91 and 572/91 according to Table 3 as well as for strains 504/91, 705/91, 581/91, 590/91, 707/91, 715/91, 580/91 and 589/91 as representative of groups 1–8 according to Table 1, no difference in colony-forming units on isosensitest-agar with and without 4 μg/ml of ciprofloxacin was recorded after subcultivation.

Quinolone therapy and occurrence of quinolone-resistant S. aureus

For 43 patients data for chemotherapeutic measures prior to the isolation of *S. aureus* were accessible. As shown in Table 4, quinolone-resistant MRSA (QR-MRSA) occurred in three patients receiving a quinolone therapy but also in three patients receiving other antibiotics against which these strains were resistant and in one patient without antibiotic treatment. Also *S. aureus* resistant to penicillin only or sensitive to the antibiotics checked were isolated from patients treated with quinolones as well as from those treated with other antibiotics and with no chemotherapy (Table 4).

DISCUSSION

Development of quinolone-resistance in MRSA was already reported during the investigational period of this antibiotic; there are reports for this development during the course of chemotherapy [15, 16]. According to the report by Smith and co-workers [17], the majority of patients with quinolone-resistant MRSA (QR-MRSA) did not receive this antibiotic. Our limited data show that QR-MRSA can also be isolated from patients without quinolone therapy. These strains have probably acquired quinolone resistance through previous treatment of other patients, or they have been imported into the hospital from other clinical settings. There are also previous reports of an epidemic inter- and intra-hospital spread of QR-MRSA [18].

In hospital T the quinolone-resistant *S. aureus*-isolates belonged to eight different strains, six of them MRSA; thus dissemination of one particular CR-MRSA-strain can be excluded. An independent occurrence of quinolone-resistant mutants in different MRSA clones is more likely. Ragnaud and colleagues [19]

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reported treatment failures with pefloxacin and isolation of correspondingly resistant isolates more often for MRSA than for other staphylococci.

As evident from the data presented in this paper, in MRSA-populations quinolone-resistant mutants are more frequent than in other *S. aureus*. The reasons for this are unknown and subject to further investigations. It seems rather unlikely that MRSA exhibit higher mutant frequencies in general; the initial killing of MRSA by quinolones may be slow, thus permitting a more efficient expression of the mutant phenotype. Also the possibility for the isolation of resistant mutants based on an efflux mechanism has to be taken into consideration. Once established, quinolone-resistant mutants of *S. aureus* seem to be quite stable and can be disseminated in the absence of quinolones.

There are indications of an increasing frequency of nosocomial infections with MRSA in Europe. Since quinolone-resistance is developing comparably fast in this group of *S. aureus* and, as also evident from this study, quinolone-resistance seems to be already frequent among MRSA [20, 21], the broader clinical use (e.g. for prophylactic regimes) of the newer quinolones should be in parallel with a constant monitoring of staphylococcal resistance patterns in order to avoid selection and spread of MRSA. Caution is necessary if quinolones are used for treatment of MRSA infection and appropriate combinations should be considered in order to avoid selection for resistance.

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