ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2000, p. 3229–3231 Vol. 44, No. 11
0066-4804/00/$04.00+0

Development of Resistance to Ciprofloxacin, Rifampin, and Mupirocin in Methicillin-Susceptible and -Resistant *Staphylococcus aureus* Isolates

FRANZ-JOSEF SCHMITZ,[1,2]* AD C. FLUIT,[2] DIETER HAFNER,[3] ANDREAS BEECK,[1] MIRELLA PERDIKOULI,[1] MECHTHILD BOOS,[1] SYBILLE SCHEURING,[1] JAN VERHOEF,[2] KARL KÖHRER,[1] AND CHRISTOF VON EIFF[4]

Institute for Medical Microbiology and Virology[1] and Institute for Pharmacology and Clinical Pharmacology,[3] Heinrich-Heine Universität Düsseldorf, Düsseldorf, and Institute of Medical Microbiology, Westfälische Wilhelms-Universität, Münster,[4] Germany, and Eijkman-Winkler Institute for Medical Microbiology, University Medical Center, Utrecht, The Netherlands[2]

Received 3 March 2000/Returned for modification 3 May 2000/Accepted 11 August 2000

A relationship between resistance to methicillin and resistance to fluoroquinolones, rifampin, and mupirocin has been described for *Staphylococcus aureus*. Differences in resistance rates may be explainable by a higher spontaneous mutation rate (MR) or a faster development of resistance (DIFF) in methicillin-resistant *S. aureus* (MRSA). No differences in MR, DIFF, and mutations in *grlA* and *gyrA* were detected between methicillin-susceptible *S. aureus* and MRSA. The higher resistance rates in MRSA are not the result of hypermutability of target genes or a faster emergence of different mutations and may be the consequence of clonal spread of multiresistant MRSA.

Fluoroquinolone, rifampin, and low-level mupirocin resistances in *Staphylococcus aureus* are chromosomally encoded, based mainly on mutations in the *gyrA* and *grlA* genes (4, 5, 11, 13), the *rpoB* gene (1, 14), and, probably, the *its* gene (3), respectively.

A relationship between resistance to methicillin and resistance to fluoroquinolones, rifampin, and mupirocin has been described previously (9, C. L. C. Wielders, F.-J. Schmitz, J. Verhoef, A. C. Fluit, and the European SENTRY Participants Group, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1236, p. 162, 1999). However, the cause of these differences between methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates has not yet been elucidated. Besides the clonal spread of multiresistant MRSA, differences may be explainable by a higher spontaneous mutation rate (MR) and/or a faster accumulation of possibly different mutations resulting in a quicker development of resistance in MRSA isolates. Thus, this study tries to rule in or out the possibility that MRSA acquires resistance phenotypes as a result of the hypermutability of target genes (6, 8). The so-called mutator strains typically have mutations in genes that control DNA metabolism (6, 8). Hypermutability should be seen for a strain with each of the three antibiotics tested.

We investigated the MRs for 60 MRSA and MSSA isolates, respectively, exposed to the three compounds. In addition, the in vitro activities were measured for all 120 isolates, before and after 10 serial passages in antibiotic-containing medium, followed by sequencing of the target genes (*gyrA*, *grlA*, and *rpoB*) in 10 randomly selected MRSA and MSSA isolates, respectively.

All susceptible isolates were derived from patients at the University Hospital of Düsseldorf, Germany, and exhibited different pulsed-field gel electrophoresis types (10). Two isogenic *S. aureus* strains, BB270 and BB255, with *mecA* insertion-deletion, were also studied (strains kindly provided by B. Berger-Bächi) (2).

The MICs were determined using a broth microdilution method according to NCCLS criteria (7). MR was calculated as described before (12). In order to characterize the emergence of multistep resistance, bacteria were grown overnight in test tubes containing one of the antibiotics and brain heart infusion broth, respectively. Aliquots taken from the test tube containing the highest drug concentration that permitted visible growth (i.e., 0.5× the MIC) were used to inoculate the second set of serial drug dilutions. Following overnight incubation, bacteria were transferred again over a period of 10 days.

Sequencing of the resistance-determining region of *grlA* and *gyrA* (11) and of *rpoB* (1) was performed as described previously.

MR and development of resistance [as the difference of the $\log_2$(MIC) values before and after 10 passages (DIFF)] were statistically analyzed using two tests: a two-way analysis of variance (PROC GLM, SAS-PC 6.12) and a Bonferroni-Dunn a posteriori test for group differences. Both parameters were evaluated as means and 95% confidence intervals (CI). In order to screen for classic mutator strains, MRSA and MSSA isolates were compared with respect to the number of strains showing particularly high MRs and/or high DIFF values for all three drugs tested.

MRs were in the range of 10^{-5} to 10^{-7} for ciprofloxacin, 10^{-6} to 10^{-8} for rifampin, and 10^{-7} to 10^{-8} for mupirocin. MR remained unchanged irrespective of the resistance phenotype for methicillin. Analyzing statistical differences between MSSA and MRSA isolates with respect to MR using 60 isolates in each group aimed at detecting a critical difference of at least half a log unit (ratio = 3.16). For the worst case of a common standard deviation of 0.9 log units, the power for finding a significant difference ($\alpha = 0.05$) was 85%, which is totally adequate for analyses.

* Corresponding author. Mailing address: Institute for Medical Microbiology and Virology, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, Geb. 22.21, D-40225 Düsseldorf, Germany. Phone and fax: 49-2132-72040. E-mail: schmitfj@uni-duesseldorf.de.

TABLE 1. Effect of ciprofloxacin on spontaneous MRs, MICs before and after 10 passages in ciprofloxacin-containing medium, and accumulation of mutations in the *gyrA* and *grlA* genes after 10 passages

Strain	Inoculum (CFU/ml)	Spontaneous MR	MIC of the parent strain (μg/ml)	MIC after 10 passages (μg/ml)	Mutation(s) in gene after 10 passages	
					gyrA	*grlA*
MSSA1	2.0×10^{10}	5.1×10^{-5}	0.25	64	Ser-84→Leu	Ser-80→Phe
MSSA2	1.2×10^{9}	4.9×10^{-6}	0.25	128	Glu-88→Lys	Ser-80→Tyr
MSSA3	5.3×10^{9}	1.6×10^{-7}	0.12	128	Ser-84→Leu	Ser-80→Phe
MSSA4	7.0×10^{9}	1.9×10^{-5}	0.12	256	Glu-88→Lys	Ser-80→Phe, Glu-84→Val
MSSA5	4.6×10^{9}	3.4×10^{-7}	0.25	1,024	Ser-84→Lys	Ser-80→Tyr, Glu-84→Val
MSSA6	1.2×10^{10}	4.5×10^{-6}	0.25	128	Ser-84→Leu	Glu-84→Val
MSSA7	2.2×10^{10}	1.3×10^{-5}	0.06	64	Glu-88→Val	Glu-84→Val
MSSA8	7.3×10^{9}	5.4×10^{-6}	0.12	>1,024	Ser-84→Leu	Ser-80→Tyr, Glu-84→Val
MSSA9	6.5×10^{9}	9.0×10^{-7}	0.25	128	Ser-84→Lys	Ser-80→Phe
MSSA10	8.5×10^{9}	4.7×10^{-5}	0.25	512	Glu-88→Lys	Ser-80→Phe, Glu-84→Val
MRSA1	2.9×10^{10}	7.2×10^{-6}	0.12	64	Ser-84→Leu	Glu-84→Val
MRSA2	9.2×10^{9}	8.5×10^{-6}	0.25	1,024	Ser-84→Lys	Ser-80→Phe, Glu-84→Val
MRSA3	7.3×10^{9}	3.6×10^{-7}	0.12	256	Glu-88→Lys	Ser-80→Phe, Glu-84→Val
MRSA4	6.5×10^{9}	4.6×10^{-5}	0.25	512	Ser-84→Leu	Ser-80→Tyr, Glu-84→Val
MRSA5	5.2×10^{9}	6.3×10^{-6}	0.12	256	Ser-84→Lys	Ser-80→Phe, Glu-84→Val
MRSA6	1.1×10^{10}	7.8×10^{-7}	0.06	128	Glu-88→Val	Ser-80→Tyr
MRSA7	6.9×10^{9}	5.9×10^{-6}	0.06	64	Glu-88→Lys	Glu-84→Val
MRSA8	8.2×10^{9}	6.8×10^{-7}	0.12	1,024	Ser-84→Lys	Ser-80→Phe, Glu-84→Val
MRSA9	5.1×10^{9}	1.4×10^{-5}	0.06	128	Ser-84→Leu	Glu-84→Val
MRSA10	4.9×10^{9}	7.9×10^{-6}	0.12	>1,024	Glu-88→Val	Ser-80→Phe, Glu-84→Val
BB255	4.8×10^{9}	3.8×10^{-6}	0.25	256	Ser-84→Lys	Ser-80→Phe, Glu-84→Val
BB270	5.7×10^{9}	4.1×10^{-6}	0.25	512	Ser-84→Leu	Ser-80→Tyr, Glu-84→Val

Noteworthily, there were statistically significant differences ($P < 0.05$) between the antibiotics tested. The MR was highest for ciprofloxacin (mean, 8.75×10^{-6}; CI, 5.97×10^{-6} and 1.28×10^{-5}) followed by rifampin (mean, 4.74×10^{-7}; CI, 3.27×10^{-7} and 6.68×10^{-7}) and was lowest for mupirocin (mean, 1.55×10^{-7}; CI, 1.19×10^{-7} and 2.02×10^{-7}).

DIFF was also not statistically different between MRSA and MSSA isolates. The same argument concerning the significance and power calculations of statistical differences in MR applies equally well to the detection of differences in DIFF.

However, marked differences ($P < 0.05$) were detected again among the three antibiotics. DIFF was lowest for ciprofloxacin (mean, 9.82; CI, 9.48 and 10.16) followed by mupirocin (mean, 10.43; CI, 10.19 and 10.68) and was highest for rifampin (mean, 14.13; CI, 13.87 and 14.38).

Particularly high MRs for all three drugs were observed for

TABLE 2. Effect of rifampin on spontaneous MRs, MICs before and after 10 passages in rifampin-containing medium, and accumulation of mutations in the *rpoB* gene after 10 passages

Strain	Inoculum (CFU/ml)	Spontaneous MR (μg/ml)	MIC of the parent strain (μg/ml)	MIC after 10 passages (μg/ml)	Mutation(s) in *rpoB* after 10 passages
MSSA1	1.8×10^{10}	4.8×10^{-7}	0.03	512	His-481→Asn
MSSA2	4.3×10^{9}	5.9×10^{-8}	0.03	>1,024	Gln-468→Leu, Ala-477→Val, His-481→Tyr
MSSA3	6.4×10^{9}	2.3×10^{-6}	0.06	>1,024	Gln-468→Arg, His-481→Tyr
MSSA4	5.3×10^{9}	4.8×10^{-7}	0.015	>1,024	Gln-468→Lys, Ala-477→Thr, His-481→Asn
MSSA5	7.3×10^{9}	8.2×10^{-7}	0.03	512	Gln-468→Leu
MSSA6	2.4×10^{10}	7.8×10^{-7}	0.06	1,024	Ala-477→Asp, His-481→Asn
MSSA7	4.3×10^{10}	4.9×10^{-7}	0.03	512	Ser-486→Leu, Ser-529→Leu
MSSA8	3.2×10^{9}	2.3×10^{-8}	0.03	256	Gln-468→Arg, Ser-529→Leu
MSSA9	4.3×10^{9}	5.6×10^{-7}	0.015	1,024	Ala-477→Asp, His-481→Tyr
MSSA10	5.6×10^{9}	5.9×10^{-6}	0.03	1,024	Gln-468→Arg, His-481→Tyr
MRSA1	4.3×10^{10}	7.3×10^{-7}	0.06	>1,024	Ala-477→Asp, His-481→Asn, Ser-529→Leu
MRSA2	6.6×10^{9}	6.6×10^{-8}	0.03	1,024	Ala-477→Asp, His-481→Tyr
MRSA3	6.0×10^{9}	7.3×10^{-7}	0.015	256	His-481→Tyr
MRSA4	5.1×10^{9}	5.8×10^{-6}	0.03	512	Gln-468→Leu, Ser-529→Leu
MRSA5	7.2×10^{9}	4.2×10^{-7}	0.015	>1,024	Gln-468→Arg, Ala-477→Val, His-481→Tyr
MRSA6	4.3×10^{10}	2.3×10^{-7}	0.015	1,024	Ala-477→Thr, His-481→Asn, Ser-529→Leu
MRSA7	6.5×10^{9}	3.9×10^{-8}	0.03	512	His-481→Tyr
MRSA8	8.3×10^{9}	7.9×10^{-7}	0.03	1,024	Ala-477→Asp, His-481→Tyr
MRSA9	5.6×10^{9}	6.5×10^{-8}	0.015	512	His-481→Asn
MRSA10	4.0×10^{9}	6.8×10^{-7}	0.03	512	Gln-468→Lys, Ser-529→Leu
BB255	4.7×10^{9}	6.1×10^{-7}	0.015	512	Ala-477→Val, His-481→Tyr
BB270	6.9×10^{9}	7.9×10^{-7}	0.015	512	Ala-477→Asp, His-481→Asn

only three MSSA and two MRSA isolates (ciprofloxacin, MR, $>4.1 \times 10^{-5}$; mupirocin, MR, $>7.5 \times 10^{-7}$; and rifampin, MR, $>4.1 \times 10^{-6}$). No differences concerning the incidences of mutator strains could be detected between MRSA and MSSA isolates. The incidences (3 to 5%) are similar to those described previously for *Escherichia coli* and *Salmonella* spp. (2.6%) (6).

High DIFF values for all three compounds in parallel were observed for four MSSA and five MRSA isolates (ciprofloxacin, DIFF of >12; mupirocin, DIFF of >11; rifampin, DIFF of >14). In no isolate were a high MR and a high DIFF similarly detected. Thus, DIFF is not exclusively dependent on MR but seems also to be dependent on other factors, e.g., efflux pump efficacy of the isolates or speed of accumulation and stability of mutations.

The development of ciprofloxacin resistance in *S. aureus* requires multiple mutations and is mainly associated with alterations at codons 80 and 84 of GrlA and 84 and 88 of GyrA (Table 1) (4, 11, 13).

In contrast, the development of rifampin resistance does not necessarily require multiple or stepwise mutations (1). Rifampin resistance in *S. aureus* is mainly associated with alterations at codons 486, 477, and 481 of RpoB (Table 2).

Notably, the observed mutations responsible for ciprofloxacin and rifampin resistances differed between the isogenic strains. This phenomenon, however, seems to be simply the result of randomly occurring mutational events and not to be related to resistance type.

In summary, no differences in MR and DIFF for ciprofloxacin, rifampin, and mupirocin were detected between MSSA and MRSA isolates, although considerable variations within each group of clinical isolates were observable. The results for the three antibiotics tested illustrate that the higher resistance rates in MRSA are not the result of hypermutability of target genes or a faster emergence of resistance or appearance of different mutations after serial passages with antibiotic pressure but might rather be the consequence of clonal spread of multiresistant MRSA.

REFERENCES

1. **Aubry-Damon, H., C. J. Soussy, and P. Courvalin.** 1998. Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **42:**2590–2594.
2. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. J. Bacteriol. **154:**479–487.
3. **Cookson, B.** 1998. The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. J. Antimicrob. Chemother. **41:**11–18.
4. **Ferrero, L., B. Cameron, and J. Crouzet.** 1995. Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39:**1554–1558.
5. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updates **2:**38–55.
6. **LeClerc, J. E., B. Li, W. L. Payne, and T. E. Gebula.** 1996. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science **274:** 1208–1211.
7. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Laboratory Standards, Wayne, Pa.
8. **Oliver, A., R. Canton, P. Campo, F. Baquero, and J. Blazquez.** 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288:**1251–1253.
9. **Schmitz, F.-J., E. Lindenlauf, B. Hofmann, A. C. Fluit, J. Verhoef, H.-P. Heinz, and M. E. Jones.** 1998. The prevalence of low and high-level mupirocin-resistance in staphylococci from 19 European hospitals. J. Antimicrob. Chemother. **42:**489–495.
10. **Schmitz, F.-J., M. Steiert, H.-V. Tichy, B. Hofmann, J. Verhoef, H. P. Heinz, K. Köhrer, and M. E. Jones.** 1998. Typing of methicillin-resistant *Staphylococcus aureus* isolates from Düsseldorf by six genotypic methods. J. Med. Microbiol. **47:**341–351.
11. **Schmitz, F.-J., B. Hofmann, B. Hansen, S. Scheuring, M. Lückefahr, M. Klootwijk, J. Verhoef, A. Fluit, H. P. Heinz, K. Köhrer, and M. E. Jones.** 1998. Relationship between ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin and moxifloxacin MICs and with mutations in *grlA*, *grlB*, *gyrA* and *gyrB* in 116 unrelated clinical isolates of *Staphylococcus aureus*. J. Antimicrob. Chemother. **41:**481–484.
12. **Schmitz, F.-J., C. von Eiff, M. Gondolf, A. C. Fluit, J. Verhoef, G. Peters, U. Hadding, H.-P. Heinz, and M. E. Jones.** 1999. *Staphylococcus aureus* small colony variants: rate of selection and MIC-values compared to wild-type strains, using ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin and moxifloxacin. Clin. Microbiol. Infect. **5:**376–378.
13. **Schmitz, F.-J., A. C. Fluit, S. Brisse, J. Verhoef, K. Köhrer, and D. Milatovic.** 1999. Molecular epidemiology of quinolone resistance and comparative in-vitro activities of new quinolones against European *Staphylococcus aureus* isolates. FEMS Immunol. Med. Microbiol. **26:**281–287.
14. **Wichelhaus, T. A., V. Schäfer, V. Brade, and B. Böddinghaus.** 1999. Molecular characterization of *rpoB* mutations conferring cross-resistance to rifamycins on methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **43:**2813–2816.