ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1999, p. 2051–2055 Vol. 43, No. 8
0066-4804/99/$04.00+0

Comparative In Vitro Activities of Ciprofloxacin, Clinafloxacin, Gatifloxacin, Levofloxacin, Moxifloxacin, and Trovafloxacin against *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes* Clinical Isolates with Alterations in GyrA and ParC Proteins

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Received 15 March 1999/Returned for modification 4 May 1999/Accepted 24 May 1999

The in vitro activities of ciprofloxacin, clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin were tested against 72 ciprofloxacin-resistant and 28 ciprofloxacin-susceptible isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes*. Irrespective of the alterations in GyrA and ParC proteins, clinafloxacin exhibited greater activity than all other fluoroquinolones tested against *K. pneumoniae* and *E. aerogenes*.

Fluoroquinolones are broad-spectrum antimicrobial agents. However, resistance to fluoroquinolones has been increasingly reported in many bacterial species, including those of the *Enterobacteriaceae* (1, 3, 12). The main resistance mechanism in these bacteria is an alteration of the GyrA subunit of DNA gyrase (5, 11, 20, 21). Mutations in *parC*, which encodes the ParC subunit of topoisomerase IV, seem to play a secondary role in a great variety of enterobacterial species, including *Klebsiella pneumoniae* and *Enterobacter cloacae* (4, 6–8, 15, 16); however, they have not yet been investigated in *Klebsiella oxytoca* and *Enterobacter aerogenes*.

The purpose of this study was to compare the in vitro activities of ciprofloxacin, clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against isolates of *Klebsiella* spp. and *Enterobacter* spp. with characterized *gyrA* and *parC* genes and to analyze the prevalence of alterations in those genes within a representative European strain collection of these species.

The isolates tested were sent from 24 European university hospitals participating in the European SENTRY Antimicrobial Surveillance Program, which is implemented in 13 European countries (13, 19). Only one isolate per patient was submitted. Between April 1997 and October 1998, 465 *K. pneumoniae* isolates, 148 *K. oxytoca* isolates, and 501 *Enterobacter* sp. isolates were collected. Of these, 4.7% of *K. pneumoniae*, 2.8% of *K. oxytoca*, and 10.8% of *Enterobacter* spp. were intermediately or fully resistant to ciprofloxacin (MICs greater than 1 μg/ml). Most of these ciprofloxacin-resistant isolates (22 *K. pneumoniae*, 4 *K. oxytoca*, 22 *E. cloacae*, and 24 *E. aerogenes* isolates) as well as some randomly selected ciprofloxacin-susceptible isolates were included in the study. Ciprofloxacin-resistant *K. pneumoniae* isolates were found in six countries, including Greece (6 isolates of 39), France (1 of 83), Italy (3 of 29), Poland (6 of 18), Spain (3 of 68), and Turkey (3 of 31). Ciprofloxacin-resistant *K. oxytoca* isolates were found in Belgium (1 of 9 isolates), Greece (1 of 7), and Italy (2 of 15). Ciprofloxacin-resistant isolates of *E. cloacae* were found in Austria (1 of 7 isolates), Belgium (1 of 6), France (7 of 63), Germany (9 of 40), Italy (2 of 27), and Portugal (2 of 21). Ciprofloxacin-resistant *E. aerogenes* isolates were found in Austria (1 of 3 isolates), Belgium (4 of 8), France (7 of 54), Germany (4 of 6), Greece (2 of 23), Italy (3 of 14), Portugal (2 of 8), and the United Kingdom (1 of 6). Approximately half of the ciprofloxacin-resistant isolates came from blood cultures; the rest were isolated from urinary tract infections, wound infections, and nosocomial pneumonia.

MICs were determined by a broth microdilution method defined by the National Committee for Clinical Laboratory Standards (17).

Prepared bacterial DNA was used as the template for PCR amplification of the *gyrA* and *parC* portions homologous to the quinolone resistance-determining region (QRDR) of *Escherichia coli* (14, 21). Primers *gyrA-A* (5′-CGCGTACTATACG CCATGAACGTA-3′) and *gyrA-C* (5′-ACCGTTGATCACTT CGGTCAGG-3′) were used for *Klebsiella* spp. Primers *parC-A* (5′-CTGAATGCCAGCGCCAAATT-3′) and *parC-C* (5′-TG CGGTGGAATATCGGTCGC-3′) were used for *K. pneumoniae*. Primers *parC-F1* (5′-ATGGACCGTGCGTTGCCGTTT AT-3′) and *parC-R1* (5′-CGGCAATACCGGTGGTGCCGT T-3′) were used for *K. oxytoca*. For the amplification of *gyrA* and *parC* of *Enterobacter* spp., we used the primers previously defined by Deguchi et al. (8).

PCR conditions were as described previously (18), with an annealing temperature of 55°C for *Enterobacter* spp. and 50°C for *Klebsiella* spp. PCR-amplified DNA was sequenced by the dye terminator method in both directions using the Ready Reaction Dye Terminator Cycle sequencing kit (Perkin-Elmer).

Sequences with no mutations were identified on the basis of their being identical to the published sequences of the *gyrA* and the *parC* genes (7–9, 20). Strain NCTC49141 was sequenced for *parC* as a reference strain for *K. oxytoca*.

The amino acid changes identified in *Klebsiella* spp. and *Enterobacter* spp. GyrA and ParC proteins are shown in Table 1.

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TABLE 1. Amino acid changes within GyrA and ParC and corresponding MICs of six fluoroquinolones

Organism	Amino acid change(s) in:		Drug	No. of isolates for which the MIC (μg/ml) was:									
	GyrA	ParC		<0.06	0.12	0.25	0.5	1	2	4	8	16	>32
Klebsiella pneumoniae	None	None	Ciprofloxacin	11	1								
			Levofloxacin	11	1								
			Gatifloxacin	11	1								
			Trovafloxacin	12									
			Moxifloxacin	4	8								
			Clinafloxacin	12									
	Ser-83→Tyr		Ciprofloxacin						4	3	2		
			Levofloxacin					1	4	3	1		
			Gatifloxacin					3	4	1	1		
			Trovafloxacin					2	4	2	1		
			Moxifloxacin					1	5	2		1	
			Clinafloxacin		1	4	3		1				
	Ser-83→Phe		Ciprofloxacin						2				
			Levofloxacin						2				
			Gatifloxacin					1	1				
			Trovafloxacin						2				
			Moxifloxacin					1		1			
			Clinafloxacin			1	1						
	Ser-83→Phe, Asp-87→Tyr		Ciprofloxacin							1			
			Levofloxacin							1			
			Gatifloxacin					1					
			Trovafloxacin							1			
			Moxifloxacin					1					
			Clinafloxacin				1						
	Ser-83→Ile	Ser-80→Ile	Ciprofloxacin										1
			Levofloxacin									1	
			Gatifloxacin										1
			Trovafloxacin										1
			Moxifloxacin										1
			Clinafloxacin							1			
	Ser-83→Phe	Ser-80→Ile	Ciprofloxacin						2	2			
			Levofloxacin					1	3				
			Gatifloxacin					1	3				
			Trovafloxacin					2	1	1			
			Moxifloxacin					1	3				
			Clinafloxacin			2	2						
	Ser-83→Phe, Asp-87→Asn	Ser-80→Ile	Ciprofloxacin									1	1
			Levofloxacin							1	1		
			Gatifloxacin								2		
			Trovafloxacin							1	1		
			Moxifloxacin								1	1	
			Clinafloxacin					1	1				
	Ser-83→Tyr, Asp-87→Tyr	Ser-80→Ile	Ciprofloxacin										1
			Levofloxacin										1
			Gatifloxacin										1
			Trovafloxacin										1
			Moxifloxacin										1
			Clinafloxacin							1			
	Ser-83→Phe, Asp-87→Asn	Glu-84→Lys	Ciprofloxacin										2
			Levofloxacin										2
			Gatifloxacin										2
			Trovafloxacin										2
			Moxifloxacin										2
			Clinafloxacin								1	1	
Klebsiella oxytoca	None	None	Ciprofloxacin	3		1							
			Levofloxacin	3		1							
			Gatifloxacin	3			1						
			Trovafloxacin	3		1							
			Moxifloxacin	3			1						
			Clinafloxacin	3	1								
	Thr-83→Ile		Ciprofloxacin						1		1		
			Levofloxacin						2				
			Gatifloxacin						2				
			Trovafloxacin						1		1		

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TABLE 1—*Continued*

Organism	Amino acid change(s) in:		Drug	No. of isolates for which the MIC (μg/ml) was:									
	GyrA	ParC		<0.06	0.12	0.25	0.5	1	2	4	8	16	>32
			Moxifloxacin							2			
			Clinafloxacin			2							
	Thr-83→Ile	Ser-80→Arg	Ciprofloxacin								1		
			Levofloxacin							1			
			Gatifloxacin								1		
			Trovafloxacin									1	
			Moxifloxacin								1		
			Clinafloxacin				1						
	Thr-83→Ile, Asp-87→Gly	Ser-80→Ile	Ciprofloxacin									1	
			Levofloxacin								1		
			Gatifloxacin								1		
			Trovafloxacin									1	
			Moxifloxacin								1		
			Clinafloxacin					1					
Enterobacter cloacae	None	None	Ciprofloxacin	6									
			Levofloxacin	1	5								
			Gatifloxacin	5	1								
			Trovafloxacin	5	1								
			Moxifloxacin	3	3								
			Clinafloxacin	6									
	Ser-83→Phe	Ser-80→Ile	Ciprofloxacin										1
			Levofloxacin										1
			Gatifloxacin										1
			Trovafloxacin										1
			Moxifloxacin										1
			Clinafloxacin								1		
	Ser-83→Tyr, Asp-87→Asn	Ser-80→Ile	Ciprofloxacin									2	3
			Levofloxacin									2	3
			Gatifloxacin								3	1	1
			Trovafloxacin									2	3
			Moxifloxacin									3	2
			Clinafloxacin						2	3			
	Ser-83→Phe, Asp-87→Gly	Ser-80→Ile	Ciprofloxacin									2	2
			Levofloxacin									2	2
			Gatifloxacin								2	2	
			Trovafloxacin									1	3
			Moxifloxacin									2	2
			Clinafloxacin						3	1			
	Ser-83→Tyr, Asp-87→Gly	Ser-80→Ile	Ciprofloxacin									5	4
			Levofloxacin								4	5	
			Gatifloxacin							1	6	2	
			Trovafloxacin									3	6
			Moxifloxacin								1	7	2
			Clinafloxacin					1	6	2			
	Ser-83→Tyr, Asp-87→His	Ser-80→Ile	Ciprofloxacin								1	2	
			Levofloxacin							1	2		
			Gatifloxacin							2	1		
			Trovafloxacin								1	2	
			Moxifloxacin								1	2	
			Clinafloxacin					1	2				
Enterobacter aerogenes	None	None	Ciprofloxacin				3	3					
			Levofloxacin			3	1	2					
			Gatifloxacin			3	3						
			Trovafloxacin			3	3						
			Moxifloxacin			4	2						
			Clinafloxacin		4	2							
	Ser-83→Ile	Ser-80→Ile	Ciprofloxacin								1	7	2
			Levofloxacin							1	6	3	
			Gatifloxacin								7	2	1
			Trovafloxacin									6	4
			Moxifloxacin									7	3
			Clinafloxacin				6	3		1			

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TABLE 1—*Continued*

Organism	Amino acid change(s) in:		Drug	No. of isolates for which the MIC (μg/ml) was:									
	GyrA	ParC		<0.06	0.12	0.25	0.5	1	2	4	8	16	>32
	Ser-83→Ile	Ser-80→Arg	Ciprofloxacin							1			
			Levofloxacin							1			
			Gatifloxacin							1			
			Trovafloxacin								1		
			Moxifloxacin								1		
			Clinafloxacin				1						
	Ser-83→Tyr	Ser-80→Ile	Ciprofloxacin										2
			Levofloxacin									1	1
			Gatifloxacin								1	1	
			Trovafloxacin										2
			Moxifloxacin									1	1
			Clinafloxacin						1	1			
	Asp-72→Glu, Ser-83→Ile	Ser-80→Ile	Ciprofloxacin									9	1
			Levofloxacin								3	7	
			Gatifloxacin							2	6	2	
			Trovafloxacin									2	8
			Moxifloxacin								2	7	1
			Clinafloxacin					8	2				
	Ala-67→Ser, Ser-83→Ile, Asp-87→Asn, Ile-78→Val	Ser-80→Ile	Ciprofloxacin										1
			Levofloxacin										1
			Gatifloxacin									1	
			Trovafloxacin										1
			Moxifloxacin										1
			Clinafloxacin						1				

Most of the ciprofloxacin-resistant *K. pneumoniae* isolates demonstrated an amino acid change within the GyrA protein from Ser-83 to Tyr or Phe, as previously reported (7, 20), and one showed a change from Ser-83 to Ile. All *K. oxytoca* isolates showed a change from Thr-83 to Ile, in agreement with Weigel et al. (20). Similarly, all resistant isolates of *E. cloacae* showed a change from Ser-83 to Phe or Tyr, as described previously (8, 20), and isolates of *E. aerogenes* showed a change from Ser-83 to Ile (20) or Tyr. Six resistant *K. pneumoniae* isolates also showed a change from Asp-87 to Asn (7, 20) or Tyr, while one *K. oxytoca* isolate showed a newly described change from Asp-87 to Gly. An Asp-87 change to Asn, Gly, or His was also found in 21 *E. cloacae* isolates (8), while one *E. aerogenes* isolate showed a newly described change from Asp-87 to Asn. Other amino acid changes were also observed in Ala-67, Asp-72, and Ile-78 in *E. aerogenes* (Table 1).

With regard to the ParC protein, all ciprofloxacin-resistant *Enterobacter* isolates showed a change from Ser-80 to Ile or Arg. In contrast, only 10 *K. pneumoniae* isolates showed a change, 8 from Ser-80 to Ile and 2 from Glu-84 to Lys; the other 12 had no mutation in this gene. Only two of the four *K. oxytoca* isolates showed an amino acid change, from Ser-80 to Arg or Ile.

We confirmed the pattern of mutations previously described for *Klebsiella* spp. (7) with some isolates having only mutations in *gyrA*. In contrast to Japanese (8) and American (20) isolates, all European ciprofloxacin-resistant *Enterobacter* spp. isolates showed an alteration in ParC together with amino acid changes in GyrA. Since we sequenced the QRDR of nearly all ciprofloxacin-resistant *Enterobacter* sp. isolates found in this study, our results may reflect a shift toward isolates with more mutations in the present European populations of these species or a geographic difference with Japan and the United States.

Mutations or combinations of mutations found within the *gyrA* or *parC* genes and the corresponding MICs of each of the quinolones tested are shown in Table 1. Isolates with no identifiable mutations within the *gyrA* or *parC* genes demonstrated MICs of ciprofloxacin of ≤0.06 μg/ml for *K. pneumoniae*, ≤0.06 to 0.25 μg/ml for *K. oxytoca*, ≤0.06 μg/ml for *E. cloacae*, and 0.5 to 1 μg/ml for *E. aerogenes*. For the ciprofloxacin-susceptible isolates, however, there was no clear difference in efficacy between the different quinolones for the concentrations tested.

In *Klebsiella* spp., an alteration in Ser-83 of GyrA alone was associated with a MIC of ciprofloxacin of ≥2 μg/ml. Additional changes in either Asp-87 of GyrA or Ser-80 of ParC generally increased the MICs. This illustrates the major role played by alterations in amino acid Ser-83 of GyrA and the secondary role of additional changes, as previously reported (4, 6, 15, 16, 21).

In *Enterobacter* spp., combined amino acid changes in GyrA do not seem to result in higher MICs compared to single changes. Furthermore, as we observed only isolates with changes in both GyrA and ParC, the impact of changes in ParC on MICs, in addition to changes in GyrA, is difficult to define.

Since we have not examined the GyrB and ParE subunits, the possibility that some isolates have alterations in these proteins cannot be excluded.

This study is one of the few in which the in vitro activities of the newer fluoroquinolones have been simultaneously compared for *Klebsiella* and *Enterobacter* isolates with defined alterations in *gyrA* and *parC*. For all ciprofloxacin-resistant isolates tested, clinafloxacin MICs were generally two dilution steps lower than those of the second active quinolone tested. This very general observation is true for all combinations of mutations in *gyrA* and *parC*. New quinolones with improved in vitro activity should be active against both ciprofloxacin-susceptible and ciprofloxacin-resistant isolates. Based on a breakpoint of >1 μg/ml, 16 to 22 *K. pneumoniae* isolates were resistant to gatifloxacin, trovafloxacin, moxifloxacin, levofloxacin, and ciprofloxacin, whereas only 7 were clinafloxacin resistant. No *K. oxytoca* isolate was resistant to clinafloxacin, but four

were resistant to the other quinolones. Six *E. aerogenes* isolates were clinafloxacin resistant, while 24 were resistant to the other quinolones. Finally, 20 *E. cloacae* isolates were resistant to clinafloxacin and 22 were resistant to the other quinolones under study. The number of isolates with MICs of <1 μg/ml was significantly greater for clinafloxacin than for the other quinolones, according to the chi-square test, for *K. pneumoniae* ($P < 0.02$) and for *E. aerogenes* ($P < 0.001$). No difference was found for *E. cloacae*, and due to the low number of isolates the test was not performed for *K. oxytoca*.

This improved in vitro activity against isolates with alterations in GyrA and ParC might be related to the interaction of the C-8 chlorine atom of this compound with the gyrase enzyme (10).

In summary, clinafloxacin shows the best in vitro activity against all species tested, independent of the genetic constitution of the *gyrA* and *parC* genes. These results echo the improved efficacy of clinafloxacin reported previously for genetically undefined isolates of these species (2). Clinafloxacin may, therefore, be of clinical value in the therapy of *Klebsiella* spp. and *E. aerogenes* infections, especially those caused by resistant strains with alterations in GyrA and ParC. However, it seems to have no improved value against *E. cloacae*.

Nucleotide sequence accession number. The partial sequence of the *parC* gene of *K. oxytoca* NCTC49191 was assigned EMBL accession number AJ133197.

We are grateful to Babak Aliyary Ghraboghly for technical help in sequencing. We thank Marita Hautvast, Mirjam Klootwijk, Karlijn Kusters, and Stefan de Vaal for their expert technical assistance.

S.B. was supported by a European Community Human Capital Mobility Grant. This work was funded in part by Bristol-Myers Squibb Pharmaceuticals via the SENTRY Antimicrobial Surveillance Program.

We thank the following members of the SENTRY participants group for referring isolates from their institutes for use in this study: Jacques Acar, Rogelio Martin Alvarez, Fernando Baquero, Jacques Bille, Dario Costa, René Courcol, Franz Daschner, Jérome Etienne, Gary French, Fred Goldstein, Deniz Gür, Ulrich Hadding, Piotr Heczko, Waleria Hryniewicz, Vincent Jarlier, Volkan Korten, Nikos Legakis, Carlo Mancini, Helmut Mittermayer, Evilio Perea, Gian-Carlo Schito, Marc Struelens, and Serhat Unal.

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