JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2009, p. 2751–2758 Vol. 47, No. 9
0095-1137/09/$08.00+0 doi:10.1128/JCM.00456-09
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Evaluation of Quinolones for Use in Detection of Determinants of Acquired Quinolone Resistance, Including the New Transmissible Resistance Mechanisms *qnrA*, *qnrB*, *qnrS*, and *aac*(6′)*Ib-cr*, in *Escherichia coli* and *Salmonella enterica* and Determinations of Wild-Type Distributions[▽]

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Received 4 March 2009/Returned for modification 2 May 2009/Accepted 25 June 2009

Fluoroquinolone resistance in members of the *Enterobacteriaceae* family is mostly due to mutations in the quinolone resistance-determining regions of the topoisomerase genes. However, transferable genes encoding quinolone resistance have recently been described. The current methods for susceptibility testing are not adapted to the detection of new resistance determinants, which confer low levels of resistance. The aim of this study was to compare the ability of the screening of the different quinolones by disk diffusion assays and MIC determinations to detect fluoroquinolone resistance. Sixty-nine *Escherichia coli* strains and 62 *Salmonella* strains, including strains fully susceptible to quinolones, nalidixic acid-resistant strains, strains with resistance to fluoroquinolones (resistant to nalidixic acid), and strains showing low-level resistance to fluoroquinolones conferred by transferable quinolone resistance genes, including *qnrA*, *qnrB*, *qnrS*, and *aac*(6′)*Ib-cr*, were selected. Disk diffusion assays and MIC determinations by the agar dilution method were performed, according to CLSI standards, with nalidixic acid, flumequine, oxolinic acid, ciprofloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin, and levofloxacin. The MIC of levofloxacin was determined by an Etest. The results showed a trimodal distribution of the MICs for both *E. coli* and *Salmonella*. The MIC distributions for the isolates varied with the compounds tested. Screening for nalidixic acid resistance by MIC testing or disk diffusion assay was not efficient for the detection of some of the isolates carrying *qnr* and *aac*(6′)*Ib-cr*. Transferable resistance genes would best be detected by testing for the MIC of ciprofloxacin or norfloxacin, as testing for the MICs of the other compounds would fail to detect isolates carrying *aac*(6′)*Ib-cr* because the enzyme produced is able to reduce the activities of these two compounds only due to their chemical structures. In conclusion, screening with nalidixic acid is efficient for the detection of mutants, but it is not so efficient for the detection of *qnr* and *aac*(6′)*Ib-cr*. Detection would be maximized by screening with either ciprofloxacin or norfloxacin by both MIC determination and disk diffusion assays. Furthermore, a low concentration of ciprofloxacin (1 μg) in the disks seemed to increase the sensitivity of the disk diffusion assay.

Fluoroquinolone resistance in members of the *Enterobacteriaceae* family has until recently been attributed to mutations in the gyrase and topoisomerase genes quinolone resistance-determining regions (QRDRs). Stepwise increases in resistance have been described for both *Escherichia coli* and *Salmonella enterica* species as a result of the accumulation of topoisomerase mutations (14). Efflux pump mechanisms were also described to act in resistance alone or in combination with decreased levels of expression of outer membrane porins (20, 21, 27). However, in the late 1990s, Martinez-Martinez et al. found a new plasmid-mediated mechanism, *qnrA1*, that is able to increase the MICs of quinolones through a target protection mechanism (18). Other related *qnr* genes (*qnrA*, *qnrB*, *qnrC*, and *qnrS*) and numerous variants have been described (13, 15, 16). Furthermore, a new resistance mechanism, *aac*(6′)*Ib-cr*, based on the enzymatic modification of some fluoroquinolones was described (23). However, the reduction in the susceptibility to fluoroquinolones might not be observed when other quinolones, such as enrofloxacin, pefloxacin, gemifloxacin, and levofloxacin, are tested (22, 23).

The actual screening methods include nalidixic acid as the first screening drug, since the sensitivity of the MIC test is higher for the detection of first-step mutants. However, the use of nalidixic acid might not be appropriate for the detection of these recently described resistance determinants that do not affect the MIC of nalidixic acid in the same way that mutations in the QRDRs of topoisomerases do. Furthermore, the breakpoints for quinolones for the detection of reduced susceptibility in surveillance programs have been under discussion to improve the detection of resistance determinants (5, 7).

The methods for the detection of recently described transferable resistance are not well established, and if susceptibility testing is performed according to the CLSI guidelines and interpretations (8, 9, 10), the isolates carrying these resistant mechanisms would be classified as susceptible since they me-

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[▽] Published ahead of print on 1 July 2009.

TABLE 1. *Escherichia coli* and *Salmonella enterica* strains included in this study[a]

Strain	No. of strains	Serotype (no. of isolates)	Origin	Resistance status	Resistance gene or mutation	Reference
E. coli, susceptible	31	NA	Swine	S	None	7
E. coli 1 mut	29	NA	Swine	1 mut	One mutation in *gyrA* gene	7
E. coli, resistant	5	NA	Swine	2 mut	Two or more mutations in *gyrA* and *parC* or *parE* gene	7
E. coli H88	1	NA	Human	*qnr*	*qnrS1*	7
E. coli H93	1	NA	Human	*qnr*	*qnrA1*	7
E. coli KAM 3	1	NA	Sewage	*qnr*	*qnrS2*	4
E. coli E12	1	NA	Human-blood	*aac*(6′)*Ib-cr*	*aac*(6′)*Ib-cr*	11
Salmonella, susceptible	29	Typhimurium (3)	Human, cattle, poultry, swine	S	None	1, 2
Salmonella, susceptible		Enteritidis (5)				
Salmonella, susceptible		Schwarzengrund (5)				
Salmonella, susceptible		Bovismorbificans (2)				
Salmonella, susceptible		Anatum (1)				
Salmonella, susceptible		Corvallis (5)				
Salmonella, susceptible		Dublin (3)				
Salmonella, susceptible		Mbandaka (2)				
Salmonella, 1 mut	18	Typhimurium (8)	Cattle, poultry swine	1 mut	One mutation in *gyrA*	28
Salmonella, 1 mut		Enteritidis (5)				
Salmonella, 1 mut		Dublin (5)				
Salmonella, resistant	5	Schwarzengrund (5)	Human, poultry	2 mut	Mutation in *gyrA* or *parC* gene	1
Salmonella qnrS1	5	Corvallis (4) Bovismorbificans (1)	Human, poultry, beef	*qnr*	*qnrS1*	6, 12
Salmonella qnrB5	2	Berta (2)	Human	*qnr*	*qnrB5*	12
Salmonella qnrB2	1	Mbandaka (1)	Human	*qnr*	*qnrB2*	12
Salmonella qnrS2	1	Anatum (1)	Human	*qnr*	*qnrS2*	12
Salmonella aac(6′)*Ib-cr*	1	Typhimurium (1)	Human	*aac*(6′)*Ib-cr*	*aac*(6′)*Ib-cr*	29

[a] S, susceptible; 1 mut, strains with one amino acid substitution in the QRDR of *gyrA*; 2 mut, resistant strains containing at least two amino acid substitutions of the topoisomerase genes; *qnr*, strains carrying a *qnr* gene; *aac*(6′)*Ib-cr*, strains carrying an *aac*(6′)*Ib-cr* gene; NA, not applicable.

diate a reduction in susceptibility which is below the actual clinical breakpoints, although their clinical importance is still unclear.

Given their transferability and the possibility that they cause increases in resistance that might affect the clinical response to treatment, the detection of quinolone resistance should routinely be performed. Therefore, laboratories will have to adapt the detection methods that they use. The detection of quinolone resistance might be facilitated through the selection of the drugs that are the most adequate for use in susceptibility tests and by the recognition of the expected phenotypes, isolates with which can then be further studied for their genetic backgrounds.

As part of this study, we measured the MICs and inhibition zone diameters for a panel of quinolones used in human and veterinary medicine to observe the distribution of quinolone-susceptible isolates and isolates harboring different mechanisms of resistance. This information could be useful for the optimization of quinolone resistance detection and the establishment of cutoff values and the interpretative criteria to be used for the detection of quinolone resistance for clinical and surveillance purposes.

MATERIALS AND METHODS

Bacterial isolates. Our strain collection comprised two subsets consisting of 69 *Escherichia coli* isolates and 62 *Salmonella* isolates. Each of these subsets included susceptible isolates; well-characterized nalidixic-acid resistant strains with one mutation in the QRDRs of the topoisomerase genes; and low-level-resistant strains with either *qnrA*, *qnrB*, or *qnrS* and *aac*(6′)*Ib-cr*. The strains either were from our collections or were previously described in the literature, and they were obtained from the respective authors and included in the assays as controls (Table 1).

Antimicrobial substance. The antimicrobial substances used in the assays were obtained either as powder for the dilution assays or as disks for the disk diffusion assays, as described in Table 2.

MIC testing. MIC determinations were performed according to the standards of the CLSI (8, 10) by agar dilution assays for nalidixic acid, flumequine, oxolinic acid, ciprofloxacin, enrofloxacin, marbofloxacin, norfloxacin, and ofloxacin. Susceptibility to levofloxacin was determined by Etest (AB Biodisk, Solna, Sweden). Quality control was performed for every determination by testing *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853.

Disk diffusion testing. Antimicrobial susceptibility testing by disk diffusion was performed according to the standards of the CLSI (8, 9) for nalidixic acid, flumequine, oxolinic acid, ciprofloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin, and levofloxacin. Quality control was performed in parallel by testing *E. coli* ATCC 25922.

RESULTS

In general, the results of the MIC determinations and the disk diffusion assays gave a trimodal distribution with distinct susceptible and resistant isolates, as well as isolates in between with reduced susceptibility. The distributions of the intermediate resistance levels varied, depending on the resistance mechanism present (a single *gyrA* mutation or the presence of transferable quinolone resistance-conferring genes) and the drug tested (Tables 3 to 6).

Determination of MIC and disk diffusion assay results with nalidixic acid (30-μg disks). Determination of the MIC for nalidixic acid allowed the clear separation of susceptible isolates and those carrying one mutation or multiple mutations in the QRDRs of the topoisomerase genes (MICs $\geq$ 32 mg/liter). However, screening with this drug did not allow the

TABLE 2. Origins of antimicrobial drugs and disks used in agar dilution and disk diffusion assays

Quinolone	Agar dilution		Disk content(s) (μg [reference]) in disk diffusion assay
	Reference	Range of concn (mg/liter) tested	
Nalidixic acid	N-5035 (Sigma)	1–512	30 (CT0032; Oxoid)
Flumequine[a]	F7016 (Sigma)	0.06–32	30 (CT0666; Oxoid)
Oxolinic acid[a]	O0877 (Sigma)	0.03–32	2 (CT0181; Oxoid)
Ciprofloxacin	Lot 455985/1 (Fluka)	0.008–16	5 (CT0425; Oxoid), 1 (CT0623; Oxoid)
Enrofloxacin[b]	PT no. R-177-3 (Bayer)	0.008–16	5 (CT0639; Oxoid)
Marbofloxacin	44357 (Vetoquinol)	0.008–16	5 (356-7628; Bio-Rad)
Norfloxacin	N9890 (Sigma)	0.008–16	10 (CT0434; Oxoid)
Ofloxacin	O 08757 (Sigma)	0.008–16	5 (CT446; Oxoid)
Levofloxacin	51002748 (Etest, AB Biodisk)		5 (CT1587; Oxoid)

[a] 0.1 M NaOH was added dropwise for dissolution in water.
[b] 1 M NaOH was added dropwise for dissolution in water.

detection of all isolates carrying transferable resistance determinants.

The results of the nalidixic acid disk diffusion assay allowed the good separation of the wild-type isolates and those containing mutations in the QRDRs of the topoisomerase genes but not the strains carrying transferable resistance genes.

Determination of MIC and disk diffusion assay results with flumequine (30-μg disks). Determination of the MIC for

TABLE 3. Frequency distribution of MICs of selected quinolones for the *Escherichia coli* isolates tested[a]

Drug	Status	No. of isolates with the following MIC (mg/liter):																	
		0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024
Nalidixic acid	S								6	21	4								
	2 mut																		5
	1 mut													**2**	7	19	1		
	qnr or *aac*(6′)*Ib-cr*									1	1		*1*			1			
Flumequine	S					1	9	21								NT	NT	NT	
	2 mut														5				
	1 mut										18[b]	11							
	qnr or *aac*(6′)*Ib-cr*							1		1		1		1					
Oxolinic acid	S					5	23	3											
	2 mut														5				
	1 mut									2	23	4							
	qnr or *aac*(6′)*Ib-cr*						1		1	1		1							
Ciprofloxacin	S	30	1																
	2 mut							1	2		**1**	1							
	1 mut			1	6[b]	20	2												
	qnr or *aac*(6′)*Ib-cr*			1		1	1	1											
Enrofloxacin	S		6	17	7														
	2 mut									**2**	1	1		1					
	1 mut					1	14[b]	*13*	1										
	qnr or *aac*(6′)*Ib-cr*			1			1[b]		1	**1**									
Marbofloxacin	S		10	21															
	2 mut									3		1	1						
	1 mut					3	9	17											
	qnr or *aac*(6′)*Ib-cr*			1			1	1		1									
Norfloxacin	S			1	29	1													
	2 mut										2	*1*	**1**	1					
	1 mut						1	27[b]	1										
	qnr or *aac*(6′)*Ib-cr*						1	1[b]	1	1									
Ofloxacin	S			2	27	2													
	2 mut										*3*	**1**	1						
	1 mut						1	12[b]	16										
	qnr or *aac*(6′)*Ib-cr*				1			1[b]	1		*1*								
Levofloxacin	S	1	14	14	2														
	2 mut								2	*1*	**1**			1					
	1 mut					2	24	3[b]											
	qnr or *aac*(6′)*Ib-cr*			1			1	1[b]	1										

[a] S, susceptible; 1 mut, strains with one amino acid substitution in the QRDR of *gyrA*; 2 mut, resistant strains containing at least two amino acid substitutions of the topoisomerase genes; *qnr*, strains carrying a *qnr* gene; *aac*(6′)*Ib-cr*, strains carrying an *aac*(6′)*Ib-cr* gene. Boldface numbers correspond to the CLSI breakpoints for resistance, and italic numbers correspond to the CLSI breakpoints for intermediate susceptibility.
[b] EUCAST cutoff value. When the EUCAST cutoff value coincides with the CLSI breakpoint, the cutoff was omitted.

TABLE 4. Frequency distribution of MICs of selected quinolones for the *Salmonella enterica* isolates tested[a]

Drug	Status	No. of isolates with the following MIC (mg/liter):																	
		0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024
Nalidixic acid	S									3	25	1							
	2 mut																	5	
	1 mut															1	7	8	
	qnr or *aac*(6′)*Ib-cr*											1	*5*	**4**					
Flumequine	S							20	9										
	2 mut														5				
	1 mut											11	5	2					
	qnr or *aac*(6′)*Ib-cr*									1	2[b]	7							
Oxolinic acid	S					14	15												
	2 mut													5					
	1 mut									7	8	2	1						
	qnr or *aac*(6′)*Ib-cr*							1	2	7									
Ciprofloxacin[c]	S	12	17																
	2 mut											3	2						
	1 mut				6	6[b]	5	1											
	qnr or *aac*(6′)*Ib-cr*					21[b]	7	1											
Enrofloxacin	S			3	25	1													
	2 mut													5					
	1 mut						1	*14*	1	**2**									
	qnr or *aac*(6′)*Ib-cr*					1			3	**6**									
Marbofloxacin	S			23	6														
	2 mut											3	2						
	1 mut					1	7	8	2										
	qnr or *aac*(6′)*Ib-cr*					1		5	4										
Norfloxacin[d]	S				13	16													
	2 mut													5					
	1 mut						1	7	8	2									
	qnr or *aac*(6′)*Ib-cr*								6	4									
Ofloxacin[e]	S				9	20													
	2 mut												1	4					
	1 mut							8[b]	8	2									
	qnr or *aac*(6′)*Ib-cr*						1		2	7									
Levofloxacin[c]	S			20	9														
	2 mut											1	3	1					
	1 mut					2	10	4[b]	2										
	qnr or *aac*(6′)*Ib-cr*					1		8[b]	1										

[a] S, susceptible; 1 mut, strains with one amino acid substitution in the QRDR of *gyrA*; 2 mut, resistant strains containing at least two amino acid substitutions of the topoisomerase genes; *qnr*, strains carrying a *qnr* gene; *aac*(6′)*Ib-cr*, strains carrying an *aac*(6′)*Ib-cr* gene. Boldface numbers correspond to the CLSI breakpoints for resistance, and italic numbers correspond to the CLSI breakpoints for intermediate susceptibility.

[b] EUCAST cutoff value. When the EUCAST cutoff value coincides with the CLSI breakpoint, the cutoff was omitted.

[c] The cutoff for resistance is 4 mg/liter, and the cutoff for intermediate susceptibility is 2 mg/liter.

[d] The cutoff for resistance is 32 mg/liter, and the cutoff for intermediate susceptibility is 16 mg/liter.

[e] The cutoff for resistance is 8 mg/liter, and the cutoff for intermediate susceptibility is 4 mg/liter.

flumequine allowed the clear separation of susceptible isolates and those carrying transferable genes or mutations in the QRDRs of the topoisomerase genes. However, screening with this drug did not allow the detection of one *E. coli* strain carrying *aac*(6′)*Ib-cr* (MIC = 0.5 mg/liter).

The results of the flumequine disk diffusion assay allowed the good separation of wild-type isolates and those containing *qnr* genes or mutations in the QRDRs of topoisomerase genes; however, as for the MIC determination, it did include the *E. coli* isolate carrying *aac*(6′)*Ib-cr* (inhibition zone diameter, 31 mm) in the susceptible population.

Determination of MIC and disk diffusion assay results with oxolinic acid (2-μg disks). Determination of the MIC for oxolinic acid allowed the clear separation of the susceptible *E. coli* and *Salmonella* isolates (MICs, ≤0.5 mg/liter and ≤0.25 mg/liter, respectively) from those carrying *qnr* genes or mutations in the QRDRs of the topoisomerase genes, but it did not allow the detection of the *E. coli* strain harboring *aac*(6′)*Ib-cr* (MIC = 0.25 mg/liter).

The results of the oxolinic acid disk diffusion assay allowed the good separation of the wild-type isolates and those containing *qnr* or mutations in the QRDRs of the topoisomerase genes; however, the *E. coli* isolate carrying *aac*(6′)*Ib-cr* and a *Salmonella* isolate carrying *qnr* were classified in the group of susceptible isolates.

Determination of MIC and disk diffusion assay results with ciprofloxacin (1- and 5-μg disks). Determination of the MIC for ciprofloxacin allowed the separation of the susceptible isolates and most isolates carrying mutations in the QRDRs of the topoisomerase genes by use of EUCAST cutoff values of 0.06 mg/liter for *E. coli* and 0.12 mg/liter for *Salmonella*. However, the MIC determination did not allow the separation of one *E. coli* strain with a first mutation in the *gyrA* gene and another strain containing *aac*(6′)*Ib-cr* (MIC =

TABLE 5. Frequency distribution of disk diffusion assay results for selected quinolones and *Escherichia coli* isolates tested

Drug (concn [μg])	Status[a]	No. of isolates with the following inhibition zone diam (mm):									
		≥40	37–39	34–36	31–33	29–30	26–28	24–25	21–23	16–20	13–15	≤12
Nalidixic acid (30)	S				1	5	23	2				
	2 mut											5
	1 mut											29
	qnr or *aac*(6′)*Ib-cr*						1	1			1	1
Flumequine (30)	S			11	16	3	1					5
	2 mut											
	1 mut								8	20	1	
	qnr or *aac*(6′)*Ib-cr*				1			1		1		1
Oxolinic acid (2)	S				1	18	12					
	2 mut											5
	1 mut										2	27
	qnr or *aac*(6′)*Ib-cr*							1		1	1	1
Ciprofloxacin (5)	S	9	14	7	1							
	2 mut										3	2
	1 mut			1	15	8	5					
	qnr or *aac*(6′)*Ib-cr*				1	2	1					
Ciprofloxacin (1)	S			13	14	3	1					
	2 mut											5
	1 mut					1	4	16	8			
	qnr or *aac*(6′)*Ib-cr*							1	1	2		
Enrofloxacin (5)	S		3	22	6							
	2 mut									2		3
	1 mut						17	10	2			
	qnr or *aac*(6′)*Ib-cr*				1		1	1		1		
Marbofloxacin (5)	S	4	13	14								
	2 mut								1	2		2
	1 mut				4	13	11	1				
	qnr or *aac*(6′)*Ib-cr*		1			1	1		1			
Norfloxacin (10)	S	2	4	22	3							
	2 mut									2	1	2
	1 mut				1	16	12					
	qnr or *aac*(6′)*Ib-cr*					2	1	1				
Ofloxacin (5)	S		1	13	15	2						
	2 mut									2		3
	1 mut					1	16	8	4			
	qnr or *aac*(6′)*Ib-cr*				1		1	1	1			
Levofloxacin (5)	S	1	3	22	5							
	2 mut									3		2
	1 mut				3	17	8	1				
	qnr or *aac*(6′)*Ib-cr*			1			2	1				

[a] S, susceptible; 1 mut, strains with one amino acid substitution in the QRDR of *gyrA*; 2 mut, resistant strains containing at least two amino acid substitutions of the topoisomerase genes; *qnr*, strains carrying a *qnr* gene; *aac*(6′)*Ib-cr*, strains carrying an *aac*(6′)*Ib-cr* gene.

0.03 mg/liter) because this MIC was in the expected range for wild-type isolates, according to EUCAST (http://www.eucast.org/).

The results of the disk diffusion assay with the 5-μg disks showed a slight overlap of the inhibition zone diameters between the wild-type isolates and those containing resistance mechanisms, whereas the results of the disk diffusion assay with the 1-μg disks showed a better separation of the population of wild-type isolates and isolates containing one or more mutations in the QRDRs of the topoisomerase genes. As described above, the disk diffusion assay with ciprofloxacin and disks with the low concentration allowed the detection of isolates carrying *aac*(6′)*Ib-cr*.

Determination of MIC and disk diffusion assay results with norfloxacin (10 μg disks). Determination of the MIC for norfloxacin allowed the separation of wild-type isolates and those carrying mutations in the QRDRs of the topoisomerase genes or a transferable resistance gene (MICs ≥ 0.25 mg/liter).

The results of the disk diffusion assay showed a slight overlap of the inhibition zone diameters between the wild-type isolates and those containing one or more mutations in the QRDRs of the topoisomerase genes among the *Salmonella* isolates; however, any of the transferable resistance genes could be detected by using a low breakpoint (inhibition zone diameters, ≤32 mm).

Determination of MIC and disk diffusion assay results with enrofloxacin (5-μg disks). Determination of the MIC for enrofloxacin allowed the clear separation of the susceptible isolates and those carrying one or multiple mutations in the QRDRs of the topoisomerase genes and *qnr* genes (MICs, ≥0.125 for *E. coli* and 0.25 mg/liter) for *Salmonella*. However, the MIC determination did not to allow the isolates carrying *aac*(6′)*Ib-cr* to be distinguished from the susceptible isolates.

The results of the disk diffusion assay showed the good separation of the inhibition zone diameters between the wild-type isolates (≥29 mm) and those containing mutations in the QRDRs of the topoisomerase genes or transferable resistance

TABLE 6. Frequency distribution of disk diffusion results for selected quinolones and the *Salmonella enterica* isolates tested

Drug (concn [μg])	Status[a]	No. of isolates with the following inhibition zone diam (mm):										
		≥40	37–39	34–36	31–33	29–30	26–28	24–25	21–23	16–20	13–15	≤12
Nalidixic acid (30)	S						11	12	6	1		
	2 mut											5
	1 mut											18
	qnr or *aac*(6′)*Ib-cr*									1	8	
Flumequine (30)	S			1	17	9	2	1				
	2 mut											5
	1 mut									10	6	2
	qnr or *aac*(6′)*Ib-cr*								2	6	2	
Oxolinic acid (2)	S						4	18	6	2		
	2 mut											5
	1 mut											18
	qnr or *aac*(6′)*Ib-cr*									1		9
Ciprofloxacin (5)	S	7	16	6								
	2 mut											5
	1 mut			2	7	4	5					
	qnr or *aac*(6′)*Ib-cr*					2	8					
Ciprofloxacin (1)	S		2	7	20					1		
	2 mut											5
	1 mut					1	3	3	7	4		
	qnr or *aac*(6′)*Ib-cr*								4	6		
Enrofloxacin (5)	S			6	22	2						5
	2 mut											
	1 mut						4	4	8			
	qnr or *aac*(6′)*Ib-cr*						1			9		
Marbofloxacin (5)	S		9	10	9	1						
	2 mut											5
	1 mut				5	3	7	1	2			
	qnr or *aac*(6′)*Ib-cr*						21	5	2	1		
Norfloxacin (10)	S	1	10	15	2	1	1					
	2 mut				2	2	9	4	1			5
	1 mut						4	5				
	qnr or *aac*(6′)*Ib-cr*						1					
Ofloxacin (5)	S			9	18	2		1				
	2 mut											5
	1 mut						1	4	8	3	2	
	qnr or *aac*(6′)*Ib-cr*								11	1	8	
Levofloxacin (5)	S		9	14	7							
	2 mut											5
	1 mut				1	2	11	2	2			
	qnr or *aac*(6′)*Ib-cr*				1			1	8			

[a] S, susceptible; 1 mut, strains with one amino acid substitution in the QRDR region of *gyrA*; 2 mut, resistant strains containing at least 2 amino acid substitutions of the topoisomerase genes; *qnr*, strains carrying a *qnr* gene; *aac*(6′)*Ib-cr*, strains carrying an *aac*(6′)*Ib-cr* gene.

genes (≤28 mm); however, the *E. coli* isolate carrying *aac*(6′)*Ib-cr* (inhibition zone diameter, 33 mm) would be grouped with the wild-type strains and therefore would not be detected.

Determination of MIC and disk diffusion assay results with marbofloxacin (5-μg disks). Determination of the MIC for marbofloxacin allowed the clear separation of the susceptible isolates and those carrying one or multiple mutations in the QRDRs of the topoisomerase genes for both species or in the *qnr* genes (MICs ≥ 0.125 mg/liter). However, the MIC determination did not separate the *E. coli* isolate containing *aac*(6′)*Ib-cr* (MIC = 0.03 mg/liter) from the susceptible isolates.

The results of the disk diffusion assay showed the separation of the inhibition zone diameters of the wild-type isolates and those containing one or more mutations in the QRDRs of the topoisomerase genes of *E. coli* or *Salmonella* (inhibition zone diameters, ≤33 and 32 mm, respectively); however, both isolates carrying *aac*(6′)*Ib-cr* would be grouped with the wild-type strains and therefore would not be detected.

Determination of MIC and disk diffusion with ofloxacin (5-μg disks). Determination of the MIC for ofloxacin allowed the clear separation of the susceptible isolates and those carrying one or multiple mutations in the QRDRs of the topoisomerase genes or *qnr* genes (MICs ≥ 0.25 mg/liter). However, the MIC determination did not separate the *E. coli* isolate carrying *aac*(6′)*Ib-cr* (MIC = 0.06 mg/liter) from the susceptible isolates.

The results of the disk diffusion assay showed the good separation of the inhibition zone diameters of the wild-type isolates (≥30) and those containing one or more mutations in the QRDRs of the topoisomerase genes (≤29 mm) in both species; however, neither isolate carrying *aac*(6′)*Ib-cr* (inhibition zone diameters, 32 and 33 mm for *E. coli* and *Salmonella* isolates, respectively) could be distinguished from the wild-type isolates.

Determination of MIC and disk diffusion assay results with levofloxacin (5-μg disks). Determination of the MIC for levofloxacin by Etest allowed the clear separation of the susceptible isolates and those carrying one or multiple mutations in the

QRDRs of the topoisomerase genes or *qnr* genes (MICs ≥ 0.125 mg/liter). However, the MIC determination did not separate the isolates carrying *aac*(6′)*Ib-cr* (MICs = 0.032 and 0.094 mg/liter) from the susceptible isolates.

The results of the disk diffusion assay showed some overlap of the inhibition zone diameters between the *E. coli* wild-type isolates and isolates containing one or more mutations in the QRDRs of the topoisomerase genes. Furthermore, both *E. coli* and *Salmonella* isolates carrying *aac*(6′)*Ib-cr* (inhibition zone diameters, 35 and 33 mm, respectively) would be grouped with the wild-type strains and therefore would not be detected.

DISCUSSION

The emergence of new mechanisms of quinolone resistance, namely, those caused by the horizontal transfer of resistance genes on mobile genetic elements, are of great concern since they bring new possibilities for the spread of resistance. However, the phenotypes resulting from quinolone resistance caused by topoisomerase gene mutations or by these transferable resistance determinants are diverse, increasing the difficulty of the detection of resistance.

The interpretation of susceptibility testing results has mostly been based on clinical breakpoints, which have been used to predict treatment success or failure; however, these breakpoints have been set by the use of data for strains harboring mutations in the QRDR of the topoisomerase genes and have not yet been adapted to the presence of transferable resistance determinants. These resistance determinants might have an influence on the outcome of treatment because of either the reduction in the level of susceptibility that they cause or an additive effect on the resistance levels conferred by additional topoisomerase gene mutations, which might lead to higher levels of resistance. The use of the epidemiological cutoffs set by EUCAST (www.eucast.org) and the separation of wild-type isolates from those that display resistance mechanisms have recently been adopted by many laboratories, mostly for monitoring purposes. The use of these methods provides a higher sensitivity for the detection of resistance mechanisms.

However, regarding the new mechanisms of resistance to quinolones, the resistance levels that have been observed are the result of acquired resistance genes and vary for the different quinolones, as described previously for isolates carrying *qnr*, *aac*(6′)*Ib-cr*, and *qepA* genes (22–24, 30). Therefore, the detection of resistance requires adaptation of the methods used for the efficient detection of these emerging resistance determinants.

In this study, we analyzed the distributions of MICs and inhibition zone diameters for both *Salmonella* and *E. coli* isolates separately to exclude the differences inherent in the susceptibility profiles of the two species and to observe the differences in resistance to the different quinolone compounds. We observed differences in drug potencies; the specificities toward substrates, for example, for isolates carrying *aac*(6′)*Ib-cr*; and some differences in the phenotypes of isolates carrying the *qnr* determinants which might be related to their levels of expression of Qnr proteins. The differences observed seem to be related to the structural differences between the quinolones tested. Both quinolones and fluoroquinolones might be useful for the detection of the low-level resistance caused by these resistance determinants if the right breakpoints are set; so far, however, few data for other quinolone drugs are available (3, 19). Nalidixic acid is known to be a very good drug for use for the detection of mutants; however, it would not be useful for the detection of isolates positive for *qnr* or *aac*(6′)*Ib-cr* mutations which might be found to have inhibition zones in the susceptible range. Some quinolone drugs such as oxolinic acid and flumequine showed good results for the detection of *qnr* genes along with mutations in those genes, whereas nalidixic acid was effective at distinguishing only strains harboring mutations. The fluoroquinolones tested showed good results for the detection of *qnr* genes with resistance determinants, but for *aac*(6′)*Ib-cr*, the best options would be either ciprofloxacin or norfloxacin, as expected due to the mechanism of resistance conferred by the enzymatic modification that is specific to these two drugs. However, although we advise the use of one of these drugs for optimization of the detection of strains carrying mutations in *aac*(6′)*Ib-cr*, they might still be undetected even when low breakpoints are used due to the low level of resistance conferred (23).

In this study, both quantitative MIC determination and disk diffusion methods were used to test each of the quinolones. The results of the quantitative determination offer a much better ability to judge resistance levels, especially when strains carrying resistance determinants which confer only slight reductions in susceptibility are tested. For practical and economical reasons, many laboratories perform susceptibility testing by the disk diffusion assay, and therefore, we tried to observe if the inhibition zone diameter distributions obtained by the disk diffusion assay with a panel of quinolones could give some indications of the suitability of this method for the detection of the diverse quinolone resistance determinants. The results indicate, as is already known, that the disk diffusion test is good for the detection of target mutations, but only the results for ciprofloxacin or norfloxacin could be adapted to the detection of mutations in *qnr* and *aac*(6′)*Ib-cr* by the use of lower breakpoints (17, 23, 30). Furthermore, the disk content presents another factor that must be considered. We observed that the use of a 1-μg ciprofloxacin disk reduced the overlap between the results for susceptible strains and strains carrying transferable resistance compared to the results obtained with the traditionally used 5-μg disk.

The results of this study are in agreement with previous knowledge on these resistance determinants, as mutations in *qnr* are known to protect the target the fluoroquinolones (24–26, 30) in general, and mutations in *aac*(6′)*Ib-cr* cause reductions in susceptibility to ciprofloxacin and norfloxacin. These are the only fluoroquinolones which possess the piperazynil amide side chain, which is acetylated by the enzyme encoded by *aac*(6′)*Ib-cr* (17, 23). However, to our knowledge, similar studies have not been performed to optimize screening procedures in order to obtain increased sensitivity when strains that might contain such resistance determinants are selected.

In conclusion, according to the results obtained in this study, it is advisable that quantitative testing of susceptibility to either ciprofloxacin or norfloxacin be performed and that testing of susceptibility to nalidixic acid, which is a marker for target mutations, also be performed. However, if quantitative testing is not possible and disk diffusion is routinely carried out, a reduction in the inhibition zone diameter for fluoroquinolones and the observation of an inhibition zone for nalidixic

acid might indicate the presence of transferable quinolone resistance, while the presence of mutations confers full resistance to nalidixic acid and no inhibition zone is observed.

ACKNOWLEDGMENTS

The data and isolates included in the present study were generously provided by the Danish Integrated Antimicrobial Resistance Monitoring Programme (http://www.danmap.org/) and by the authors referenced in the text, whom we thank. Thanks also go to Hanne-Dorthe Emborg for the help provided with the selection of the strains and Steen Nordentoft for providing the strains from poultry. Thanks also go to Hanne Nørgaard Nielsen and Christina Aaby Svendsen for their excellent technical assistance.

The study was supported by a grant from EU Marie Curie Programme TRAINAU (grant MEST-CT-2004-007819), the Community Reference Laboratory for Antimicrobial Resistance, and grant 274-05-0117 from the Danish Research Agency.

We have no conflicts to declare.

REFERENCES

1. **Aarestrup, F. M., R. S. Hendriksen, J. Lockett, K. Gay, K. Teates, P. F. McDermott, D. G. White, H. Hasman, G. Sorensen, A. Bangtrakulnonth, S. Pornreongwong, C. Pulsrikarn, F. J. Angulo, and P. Gerner-Smidt.** 2007. International spread of multidrug-resistant *Salmonella* Schwarzengrund in food products. Emerg. Infect. Dis. **13:**726–731.
2. **Archambault, M., P. Petrov, R. S. Hendriksen, G. Asseva, A. Bangtrakulnonth, H. Hasman, and F. M. Aarestrup.** 2006. Molecular characterization and occurrence of extended-spectrum beta-lactamase resistance genes among *Salmonella enterica* serovar Corvallis from Thailand, Bulgaria, and Denmark. Microb. Drug Resist. **12:**192–198.
3. **Becnel, B. L., M. J. Maynard, S. K. Morgan-Linnell, L. B. Horton, R. Sucgang, R. J. Hamill, J. R. Jimenez, J. Versalovic, D. Steffen, and L. Zechiedrich.** 2009. Relationships among ciprofloxacin, gatifloxacin, levofloxacin, and norfloxacin MICs for fluoroquinolone-resistant *Escherichia coli* clinical isolates. Antimicrob. Agents Chemother. **53:**229–234.
4. **Bonemann, G., M. Stiens, A. Puhler, and A. Schluter.** 2006. Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, *qnrS2*, isolated from the bacterial community of a wastewater treatment plant. Antimicrob. Agents Chemother. **50:**3075–3080.
5. **Cavaco, L. M., and F. M. Aarestrup.** 2008. Evaluation of quinolones for detection of quinolone resistance including the new transmissible resistance mechanisms (*qnrA*, *qnrB*, *qnrS*, and *aac*(6′)*Ib-cr*) in *Escherichia coli* and *Salmonella enterica* and determinations of wild type distributions, abstr. B73, p. 58. Abstr., ASM Conf. Antimicrob. Resist. Zoonotic Bacteria Foodborne Pathogens.
6. **Cavaco, L. M., R. S. Hendriksen, and F. M. Aarestrup.** 2007. Plasmid-mediated quinolone resistance determinant *qnrS1* detected in *Salmonella enterica* serovar Corvallis strains isolated in Denmark and Thailand. J. Antimicrob. Chemother. **60:**704–706.
7. **Cavaco, L. M., N. Frimodt-Moller, H. Hasman, L. Guardabassi, L. Nielsen, and F. M. Aarestrup.** 2008. Prevalence of quinolone resistance mechanisms and associations to minimum inhibitory concentrations in quinolone-resistant *Escherichia coli* isolated from humans and swine in Denmark. Microb. Drug Resist. **14:**163–169.
8. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 3rd ed. M31-A3, vol. 28, no. 8. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial disk susceptibility tests; approved standard, 9th ed. M2-A9, vol. 26, no. 1. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th ed. M7-A7, vol. 26, no. 2. Clinical and Laboratory Standards Institute, Wayne, PA.
11. **Corkill, J. E., J. J. Anson, and C. A. Hart.** 2005. High prevalence of the plasmid-mediated quinolone resistance determinant *qnrA* in multidrug-resistant *Enterobacteriaceae* from blood cultures in Liverpool, UK. J. Antimicrob. Chemother. **56:**1115–1117.
12. **Gay, K., A. Robicsek, J. Strahilevitz, C. H. Park, G. Jacoby, T. J. Barrett, F. Medalla, T. M. Chiller, and D. C. Hooper.** 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin. Infect. Dis. **43:**297–304.
13. **Hata, M., M. Suzuki, M. Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae.** 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob. Agents Chemother. **49:**801–803.
14. **Hopkins, K. L., R. H. Davies, and E. J. Threlfall.** 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. Int. J. Antimicrob. Agents **25:**358–373.
15. **Jacoby, G., V. Cattoir, D. Hooper, L. Martinez-Martinez, P. Nordmann, A. Pascual, L. Poirel, and M. Wang.** 2008. *qnr* gene nomenclature. Antimicrob. Agents Chemother. **52:**2297–2299.
16. **Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, H. Oh, A. Robicsek, and D. C. Hooper.** 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50:**1178–1182.
17. **Luzzaro, F.** 2008. Fluoroquinolones and gram-negative bacteria: antimicrobial activity and mechanisms of resistance. Infez. Med. **16**(Suppl. 2)**:**5–11. (In Italian.)
18. **Martinez-Martinez, L., A. Pascual, and G. A. Jacoby.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:**797–799.
19. **Morgan-Linnell, S. K., B. L. Becnel, D. Steffen, and L. Zechiedrich.** 2009. Mechanisms accounting for fluoroquinolone resistance in *Escherichia coli* clinical isolates. Antimicrob. Agents Chemother. **53:**235–241.
20. **Oethinger, M., W. V. Kern, A. S. Jellen-Ritter, L. M. McMurry, and S. B. Levy.** 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. Antimicrob. Agents Chemother. **44:**10–13.
21. **Piddock, L. J.** 1999. Mechanisms of fluoroquinolone resistance: an update 1994–1998. Drugs **58**(Suppl. 2)**:**11–18.
22. **Robicsek, A., G. A. Jacoby, and D. C. Hooper.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. **6:**629–640.
23. **Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. Hye Park, K. Bush, and D. C. Hooper.** 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat. Med. **12:**83–88.
24. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA **99:**5638–5642.
25. **Tran, J. H., G. A. Jacoby, and D. C. Hooper.** 2005. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. Antimicrob. Agents Chemother. **49:**118–125.
26. **Tran, J. H., G. A. Jacoby, and D. C. Hooper.** 2005. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. Antimicrob. Agents Chemother. **49:**3050–3052.
27. **Wang, H., J. L. Dzink-Fox, M. Chen, and S. B. Levy.** 2001. Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations. Antimicrob. Agents Chemother. **45:**1515–1521.
28. **Wiuff, C., M. Madsen, D. L. Baggesen, and F. M. Aarestrup.** 2000. Quinolone resistance among *Salmonella enterica* from cattle, broilers, and swine in Denmark. Microb. Drug Resist. **6:**11–17.
29. **Xia, S., R. S. Hendriksen, Z. Xie, L. Huang, J. Zhang, W. Guo, B. Xu, L. Ran, and F. M. Aarestrup.** 2008. Molecular characterization and antimicrobial susceptibility of *Salmonella* from infections in humans in Henan Province, China. J. Clin. Microbiol. **47:**401–409.
30. **Yamane, K., J. Wachino, S. Suzuki, and Y. Arakawa.** 2008. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. Antimicrob. Agents Chemother. **52:**1564–1566.