Spontaneous Quinolone Resistance in the Zoonotic Serovar of *Vibrio vulnificus*[∇][†]

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This work demonstrates that *Vibrio vulnificus* biotype 2, serovar E, an eel pathogen able to infect humans, can become resistant to quinolone by specific mutations in *gyrA* (substitution of isoleucine for serine at position 83) and to some fluoroquinolones by additional mutations in *parC* (substitution of lysine for serine at position 85). Thus, to avoid the selection of resistant strains that are potentially pathogenic for humans, antibiotics other than quinolones must be used to treat vibriosis on farms.

Vibrio vulnificus is an aquatic bacterium from warm and tropical ecosystems that causes vibriosis in humans and fish (http://www.cdc.gov/nczved/dfbmd/disease_listing/vibriov_gi.html) (33). The species is heterogeneous and has been subdivided into three biotypes and more than eight serovars (6, 15, 33; our unpublished results). While biotypes 1 and 3 are innocuous for fish, biotype 2 can infect nonimmune fish, mainly eels, by colonizing the gills, invading the bloodstream, and causing death by septicemia (23). The disease is rapidly transmitted through water and can result in significant economic losses to fish farmers. Surviving eels are immune to the disease and can act as carriers, transmitting vibriosis between farms. Interestingly, biotype 2 isolates belonging to serovar E have been isolated from human infections, suggesting that serovar E is zoonotic (2). This serovar is also the most virulent for fish and has been responsible for the closure of several farms due to massive losses of fish. A vaccine, named Vulnivaccine, has been developed from serovar E isolates and has been successfully tested in the field (14). Although the vaccine provides fish with long-term protection from vibriosis, at present its use is restricted to Spain. For this reason, in many fish farms around the world, vibriosis is treated with antibiotics, which are usually added to the food or water.

Quinolones are considered the most effective antibiotics against human and fish vibriosis (19, 21, 31). These antibiotics can persist for a long time in the environment (20), which could favor the emergence of resistant strains under selective pressure. In fact, spontaneous resistances to quinolones by chromosomal mutations have been described for some gram-negative bacteria (10, 11, 17, 24, 25, 26). Therefore, improper antibiotic treatment of eel vibriosis or inadequate residue elimination at farms could favor the emergence of human-pathogenic serovar E strains resistant to quinolones by spontaneous mutations. Thus, the main objective of the present work was to find out if the zoonotic serovar of biotype 2 can become quin-olone resistant under selective pressure and determine the molecular basis of this resistance.

Very few reports on resistance to antibiotics in *V. vulnificus* have been published; most of them have been performed with biotype 1 isolates. For this reason, the first task of this study was to determine the antibiotic resistance patterns in a wide collection of *V. vulnificus* strains belonging to the three biotypes that had been isolated worldwide from different sources (see Table S1 in the supplemental material). Isolates were screened for antimicrobial susceptibility to the antibiotics listed in Table S1 in the supplemental material by the agar diffusion disk procedure of Bauer et al. (5), according to the standard guideline (9). The resistance pattern found for each isolate is shown in Table S1 in the supplemental material. Less than 14% of isolates were sensitive to all the antibiotics tested, and more than 65% were resistant to more than one antibiotic, irrespective of their biotypes or serovars. The most frequent resistances were to ampicillin-sulbactam (SAM; 65.6% of the strains) and nitrofurantoin (F; 60.8% of the strains), and the least frequent were to tetracycline (12%) and oxytetracycline (8%). In addition, 15% of the strains were resistant to nalidixic acid (NAL) and oxolinic acid (OA), and 75% of these strains came from fish farms (see Table S1 in the supplemental material). Thus, high percentages of strains of the three biotypes were shown to be resistant to one or more antibiotics, with percentages similar to those found in nonbiotyped environmental *V. vulnificus* isolates from Asia and North America (4, 27, 34). In those studies, resistance to antibiotics could not be related to human contamination. However, the percentage of quinolone-resistant strains found in our study is higher than that reported in other ones, probably due to the inclusion of fish farm isolates, where the majority of quinolone-resistant strains were concentrated. This fact suggests that quinolone resistance could be related to human contamination due to the improper use of these drugs in therapy against fish diseases, as has been previously suggested (18, 20). Although no specific resistance pattern was associated with particular biotypes or serovars, we found certain differences in resistance distribution, as shown in Table 1. In this respect, biotype 3 displayed the narrowest spectrum of resistances and biotype 1 the widest. The latter biotype encompassed the highest number of strains with multiresistance (see Table S1 in the supplemental mate-

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TABLE 1. Percentage of resistant strains distributed by biotypes and serovars

V. vulnificus	No. of isolates	Resistance distribution (%) for indicated antibiotic[a]								
		SAM	CTX	E	NAL	F	OT	OA	SXT-TMP	TE
Biotype 1	49	75.5	24.5	14.3	30.6	83.7	8.2	30.6	28.6	8.2
Biotype 2 (whole)	72	58.3	13.9	12.5	4.2	47.2	9.7	4.2	4.2	13.9
Biotype 2										
Serovar E	36	30.3	12.1	3	9.1	27.3	15.2	9.1	3	21.2
Serovar A	23	100	9.1	18.2	0	77.3	0	0	9.1	4.6
Nontypeable	8	29	14.3	25	0	57.1	14.3	0	0	14.3
Serovar I	5	100	20	20	0	20	20	0	0	0
Biotype 3	5	100	0	20	0	80	0	0	0	20

[a] CTX, cefotaxime; E, erythromycin; OT, oxytetracycline; SXT-TMP, sulfamethoxazole-trimethoprim; TE, tetracycline.

rial). Within biotype 2, there were differences among serovars, with quinolone resistance being restricted to the zoonotic serovar (Table 1).

The origin of resistance to quinolones in the zoonotic serovar was further investigated. To this end, spontaneous mutants of sensitive strains were selected from colonies growing within the inhibition halo around OA or NAL disks. Two strains (strain CG100 of biotype 1 and strain CECT 4604 of biotype 2, serovar E) developed isolated colonies within the inhibition zone. These colonies were purified, and maintenance of resistance was confirmed by serial incubations on medium without antibiotics. Using the disk diffusion method, CG100 was shown to be resistant to SAM and F and CECT 4604 to F (see Table S1 in the supplemental material). The MICs for OA, NAL, flumequine (UB), and ciprofloxacin (CIP) were determined by using the microplate assay according to the recommendations of the Clinical and Laboratory Standards Institute and the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (8, 12) and interpreted according to the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (13). The MICs for OA and NAL and for the fluoroquinolones UB and CIP exhibited by the mutants and their counterparts are shown in Table 2. The inhibition zone diameters correlated well with MICs (data not shown). Mutants FR1, FR2, FR3, and FR4 were resistant to NAL and sensitive to the remaining quinolones, although they showed higher resistances than their parental strains (Table 2). Thus, these four mutants showed increases of 32- to 128-fold for NAL MICs, 4- to 8-fold for UB MICs, and 16-fold for CIP MICs (Table 2). The fifth mutant, FR5, was resistant to the two tested quinolones and to UB, a narrow-spectrum fluoroquinolone. This mutant, although sensitive to CIP, multiplied its MIC for this drug by 128 with respect to the parental strain (Table 2).

For other gram-negative pathogens, quinolone resistance relies on spontaneous mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes that occur in a specific region of the protein known as the quinolone resistance-determining region (QRDR) (1, 11, 17, 24, 25, 26, 28). To test the hypothesis that mutations in these genes could also produce quinolone resistance in *V. vulnificus*, the QRDRs of these genes were sequenced in the naturally resistant strains and in the two sensitive strains that

had developed resistances by selective pressure in vitro. The genomic DNA was extracted (3), and the QRDRs of *gyrA*, *gyrB*, *parE*, and *parC* were amplified using the primers shown in Table 3, which were designed from the published genomes of biotype 1 strains YJ016 and CMCP6 (7, 22). PCR products of the predicted size were sequenced in an ABI 3730 sequencer (Applied Biosystems). Analysis of the QRDR sequences for *gyrA*, *gyrB*, *parC*, and *parE* of the mutants and the naturally resistant strains revealed that all naturally resistant strains, except one, shared a specific mutation at nucleotide position 248 with the laboratory-induced mutants (Table 2). This mutation gave rise to a change from serine to isoleucine at amino acid position 83. The exception was a mutation in the adjacent nucleotide that gave rise to a substitution of arginine for serine at the same amino acid position (Table 2). All the isolates that were resistant to the quinolone NAL had a unique mutation in the *gyrA* gene, irrespective of whether resistance was acquired naturally or in the laboratory (Table 2). This result strongly suggests that a point mutation in *gyrA* that gives rise to a change in nucleotide position 83 can confer resistance to NAL in *V. vulnificus* biotypes 1 and 2 and that this mutation could be produced by selective pressure under natural conditions. *gyrA* mutations consisting of a change from serine 83 to isoleucine have also been described in isolates of *Aeromonas* from water (17) and in diseased fish isolates of *Vibrio anguillarum* (26). Similarly, replacement of serine by arginine at amino acid position 83 in diseased fish isolates of *Yersinia ruckeri* (16) suggests that this mechanism of quinolone resistance is widespread among gram-negative pathogens. In all cases, these single mutations were also related to increased resistance to other quinolones (OA) and fluoroquinolones (UB and CIP) (Table 2), although the mutants remained sensitive according to the standards of the Clinical and Laboratory Standards Institute and the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (9, 13). A total of 50% of the naturally resistant strains, all of them of biotype 1, showed additional mutations that affected *parC* (a change in amino acid position 113) or *gyrB* (changes in amino acids at positions 425 and 438) (Table 2). These strains exhibited higher MICs for OA and fluoroquinolones (Table 2), although they were still sensitive to these drugs (9, 13). Finally, one isolate of biotype 2, serovar E, which was naturally resistant to quinolones and UB, showed a mutation in *parC* that gave rise to a

TABLE 3. Oligonucleotides used in this study

Primer	Sequence	Annealing temp (°C)	Size (bp)
GyrAF	GGCAACGACTGGAATAAACC	55.8	416
GyrAR	CAGCCATCAATCACTTCCGTC		
ParCF	CGCAAGTTCACCGAAGATGC	56.6	411
ParCR	GGCATCCGCAACTTCACG		
GyrBF	CGACTTCTGGTGACGATGCG	57.4	642
GyrBR	GACCGATACCACAACCTAGTG		
ParEF	GCCAGGTAAGTTGACCGATTG	56.8	512
ParER	CACCCAGACCTTTGAATCGTTG		

substitution of leucine for serine at amino acid position 85 (Table 2). This mutation was shared only with the laboratory-induced mutant, also a biotype 2, serovar E mutant, which was resistant to the fluoroquinolone UB. The same mutation in *parC* had been previously described in diseased fish isolates of *V. anguillarum* that were highly resistant to quinolones (28), but this had not been related to fluoroquinolone resistance in *Vibrio* spp. nor in other gram-negative bacteria. These results strongly suggest that resistance to fluoroquinolones in *V. vulnificus* is related to specific mutations in *gyrA* and *parC* and that mutations in different positions for *parC* or in *gyrB* could contribute to increased resistance to quinolones and fluoroquinolones. Our results also agree with previous studies confirming that the acquisition of higher quinolone resistance is more probable when arising from a *gyrA parC* double mutation than from a *gyrA gyrB* double mutation (29).

Finally, the evolutionary history for each protein was inferred from previously published DNA sequences of the whole genes from different *Vibrio* species after multiple sequence alignment with MEGA4 software (32) by applying the neighbor-joining method (30) with the Poisson correction (35). The distance tree for each whole protein showed a topology similar to the phylogenetic tree based on 16S rRNA analysis, with the two isolates of *V. vulnificus* forming a single group, closely related to *Vibrio parahaemolyticus*, *Vibrio cholerae*, *V. anguillarum*, and *Vibrio harveyi* (see Fig. S1A in the supplemental material). A second analysis was performed with the QRDR sequences of the different mutants and isolates of *V. vulnificus* (GenBank accession numbers FJ379836 to FJ379927) to infer the intraspecies relationships (see Fig. S1B in the supplemental material). This analysis showed that QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were highly homogeneous within *V. vulnificus*.

In summary, the zoonotic serovar of *V. vulnificus* can mutate spontaneously to gain quinolone resistance, under selective pressure in vitro, due to specific mutations in *gyrA* that involve a substitution of isoleucine for serine at amino acid position 83. This mutation appears in biotype 2, serovar E diseased-fish isolates and biotype 1 strains, mostly recovered from fish farms. An additional mutation in *parC*, resulting in a substitution of lysine for serine at amino acid position 85, seems to endow partial fluoroquinolone resistance on biotype 2, serovar E strains. This kind of double mutation is present in diseased-fish isolates of the zoonotic serovar but not in resistant biotype 1 isolates, which show different mutations in *gyrB* or in *parC* that increase their resistance levels but do not make the strains resistant to fluoroquinolones. Thus, antibiotics other than quinolones should be used at fish farms to prevent the emer-

TABLE 2. MICs for quinolones and fluoroquinolones and mutations in *gyrA*, *gyrB*, and *parC* detected in naturally and artificially induced resistant strains

Strain(s)	MIC (µg ml⁻¹) for indicated antibiotic[b]				Gene mutation[a]											
					gyrA				*gyrB*				*parC*			
	NAL	OA	UB	CIP	Position nt	aa	Codon change	aa change	Position nt	aa	Codon change	aa change	Position nt	aa	Codon change	aa change
CG100	0.5 (S)	0.125 (S)	0.0625 (S)	0.0078 (S)	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
FR1	16 (R)	1 (S)	0.25 (S)	0.125 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC
FR2	16 (R)	1 (S)	0.25 (S)	0.125 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC
CECT 4604	0.25 (S)	0.0625 (S)	0.0625 (S)	0.0078 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC
FR3	32 (R)	2 (S)	0.5 (S)	0.125 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC
FR4	32 (R)	2 (S)	0.5 (S)	0.125 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC
FR5	256 (R)	16 (R)	16 (R)	1 (S)	248	83	AGT→ATT	S→I	1156	386	GCA→ACA	A→T	254	85	TCA→TTA	S→L
CECT 4602	128 (R)	8 (R)	64 (R)	1 (S)	248	83	AGT→ATT	S→I	1236	412	CAG→CAC	Q→H	254	85	TCA→TTA	S→L
CECT 4603, CECT 4606, CECT 4608, PD-5, PD-12, JE	32 (R)	2 (S)	<1 (S)	<1 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	254	85	TCA→TTA	S→L
CECT 4862	64 (R)	2 (S)	2 (S)	<1 (S)	249	83	AGT→AGA	S→R	1274	425	GAG→GGG	E→G	338	113	GCA→GTA	A→V
A2, A4, A5, A6, A7, PD-1, PD-3	64-128 (R)	2 (S)	4 (S)	<1 (S)	249	83	AGT→AGA	S→R	1314	438	AAC→AAA	N→K	338	113	GCA→GTA	A→V
VI	128 (R)	4 (S)	4 (S)	<1 (S)	248	83	AGT→ATT	S→I	NC	NC	NC	NC	NC	NC	NC	NC

[a] Mutations in a nucleotide (nt) that gave rise to a codon change and to a change in amino acids (aa) are indicated. NC, no change detected.

[b] The resistance (R) or sensitivity (S) against the antibiotic determined according to the Clinical and Laboratory Standards Institute and the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (9, 13) is indicated in parentheses.

gence and spread of quinolone resistances, especially to CIP, a drug widely recommended for human vibriosis treatment.

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