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Year: 2013

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DOI: <https://doi.org/10.1089/fpd.2012.1468>

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ZORA URL: <https://doi.org/10.5167/uzh-81823>

Journal Article

Published Version

Originally published at:

Drummond, N; Stephan, Roger; Haughton, P; Murphy, B; Fanning, S (2013). Further characterization of three *Yersinia enterocolitica* strains with a nalidixic acid-resistant phenotype isolated from humans with diarrhea. *Foodborne Pathogens and Disease*, 10(8):744-746.

DOI: <https://doi.org/10.1089/fpd.2012.1468>

## Further Characterization of Three *Yersinia enterocolitica* Strains with a Nalidixic Acid–Resistant Phenotype Isolated from Humans with Diarrhea

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### Abstract

Antimicrobial-resistant bacteria pose a threat to public health. Three *Yersinia enterocolitica* strains cultured from patients presenting with diarrhea and resistant to nalidixic acid were studied. Target gene mutations in *gyrA* alone were identified as part of the genetic basis for this phenotype. Efflux activity was also noted, since the presence of the efflux pump inhibitor, phenylalanine-arginine- $\beta$ -naphthylamide, increased susceptibility to nalidixic acid.

### Introduction

**Y**ERSINIOSIS, A FOODBORNE DISEASE, is characterized by symptoms including diarrhea, fever, abdominal pain, and vomiting (Drummond *et al.*, 2012). Fluoroquinolones (FQ) are used for the treatment of this infection, particularly in immunocompromised individuals (Capilla *et al.*, 2004). In Gram-negative bacteria, three mechanisms of FQ resistance have been described including target gene mutation(s); reduction in drug accumulation; and plasmid-mediated quinolone resistance (PMQR). DNA gyrase and DNA topoisomerase IV are tetrameric enzymes and are the targets for quinolone drugs. Resistance has been attributed to chromosomal mutations in the corresponding subunit-encoding genes (*gyrA*, *gyrB* for DNA gyrase and/or *parC* and *parE* for topoisomerase IV) (Ruiz, 2003). Within these loci, the quinolone-resistance-determining region (QRDR) is a mutational hot spot (Fàbrega *et al.*, 2010). Several mutations have already been identified in *gyrA* in *Yersinia enterocolitica*, the most common of which lead to amino acid substitutions at Ser-83 and Asp-87, conferring resistance to nalidixic acid (Sánchez-Céspedes *et al.*, 2003; Capilla *et al.*, 2004; Sihvonen *et al.*, 2011).

Compared to other members of the Enterobacteriaceae, quinolone resistance in *Y. enterocolitica* is not commonly encountered. In this study, chromosomal- and plasmid-mediated resistance mechanisms were investigated in three nalidixic acid–resistant *Y. enterocolitica* recovered from humans in Switzerland. The data presented extend our understanding of this resistance type in these foodborne pathogens.

### Materials and Methods

#### Bacterial strains and culture conditions

Three *Y. enterocolitica* strains (Table 1), isolated between 2006 and 2010 from humans in Switzerland, along with *Y. enterocolitica* 8081 and a reference-strain *Y. enterocolitica* ATCC™9610, were included in this study. All bacteria were subcultured and maintained as described previously (Murphy *et al.*, 2010).

#### Antimicrobial susceptibility testing

Susceptibility testing was performed by disc diffusion, using a panel of antimicrobial agents including the following: amoxicillin-clavulanic acid 30 (10+20)  $\mu$ g, ampicillin 10  $\mu$ g, cefoxitin 30  $\mu$ g, ceftazidime 30  $\mu$ g, cefpodoxime 10  $\mu$ g, cefuroxime 30  $\mu$ g, cephalothin 30  $\mu$ g, ciprofloxacin 5  $\mu$ g, gentamicin 10  $\mu$ g, kanamycin 30  $\mu$ g, nalidixic acid 30  $\mu$ g, streptomycin 10  $\mu$ g, tetracycline 30  $\mu$ g, and trimethoprim/sulfamethoxazole 25 (1.25+23.75)  $\mu$ g.

Susceptibility or resistance was interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines and minimum inhibitory concentrations (MIC) for nalidixic acid and ciprofloxacin were determined by E-test on Mueller-Hinton agar following the manufacturer's instructions (AB-Biodisk, Solna, Sweden). E-tests were also performed in the presence of the efflux pump inhibitor phenylalanine-arginine- $\beta$ -naphthylamide, which was added to Mueller-Hinton agar plates at a concentration of 20 mg/L. *Escherichia coli* ATCC™25922 was used for quality-control purposes.

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TABLE 1. BACTERIAL ISOLATES, THEIR CORRESPONDING BIO/SEROTYPES AND MINIMUM INHIBITORY CONCENTRATIONS (MICs) FOR NALIDIXIC ACID AND CIPROFLOXACIN IN THE PRESENCE AND ABSENCE OF THE EFFLUX PUMP INHIBITOR, PHENYLALANINE-ARGININE- $\beta$ -NAPHTHYLAMIDE (PA $\beta$ N)

Bacterial isolate	Bio/serotype	MIC (mg/L)			
		NA	NA + PA $\beta$ N	CIP	CIP + PA $\beta$ N
YE 8081	1B	>256	32 (8)	0.25	0.19 (1)
506-06	4:O3	>256	48 (5)	0.25	0.19 (1)
871-07	1A	>256	32 (8)	1	0.38 (3)
10-2307	4:O3	2	0.125 (16)	0.03	0.02 (1.5)
ATCC™ 9610	1:O8	0.75	0.19 (4)	0.004	0.004

Values represent mean results from three independent determinations. Numbers in parenthesis represent the fold-reduction in MIC in the presence of the efflux pump inhibitor.

YE, *Yersinia enterocolitica*; NA, nalidixic acid; CIP, ciprofloxacin. Shaded blocks indicate resistant values.

#### Detection of virulence markers and the *Y. enterocolitica*-specific 16S rRNA gene by polymerase chain reaction (PCR)

Template DNA was prepared and three specific primer sets (Eurofins MWG Operon, Ebersberg, Germany) were used to amplify *ail* (Falcão *et al.*, 2004), *pYad* (Lantz *et al.*, 1998), and the *Y. enterocolitica*-specific chromosomal 16S rRNA genes (Murphy *et al.*, 2010). Amplification reactions were performed as described previously (Murphy *et al.*, 2010).

#### PCR amplification and DNA sequence analysis of QRDR and PMQR

QRDR containing regions of the target genes were amplified by PCR. Primer sequences and PCR conditions applied in 50- $\mu$ L final reaction mixtures are shown in Supplementary Table S1 (Supplementary Data are available online at [www.liebertpub.com/fpd](http://www.liebertpub.com/fpd)). DNA sequences were analyzed for mutations using DNASTar software (Madison, WI), BLAST (<http://blast.ncbi.nlm.nih.gov/>), and ClustalW (<http://www.ebi.ac.uk/clustalw>). Similarly, PMQR primers and amplification conditions are shown in Supplementary Table S2. All amplicons produced were separated by electrophoresis in a 1.5% agarose gel (SeaKem® LE Agarose, Lonza Wokingham, Ltd., UK) containing 0.1  $\mu$ g/mL EB (Sigma, Ireland) in 1 X Tris-boric acid-EDTA buffer (pH 8) (Sigma).

TABLE 2. AMINO ACID SUBSTITUTIONS IDENTIFIED WITHIN THE QUINOLONE-RESISTANCE-DETERMINING REGIONS OF DNA GYRASE

Bacterial isolate	GyrA
YE 8081	Asp87 $\rightarrow$ Tyr
506-06	Asp87 $\rightarrow$ Tyr
871-07	Ser83 $\rightarrow$ Ile
10-2307	none

YE, *Yersinia enterocolitica*.

## Results and Discussion

The three *Y. enterocolitica* strains were isolated from humans between 2006 and 2010, in Switzerland. Two of the isolates were bio/serotypes 4:O3 (506-06 and 10-2307) and are classified as pathogenic. The remaining isolate was of biotype 1A (871-07). Isolates 506-06 and 10-2307 harbored the large pVYe plasmid of 67-kb (data not shown) and were positive for the *ail* and *pYad* markers, features associated with pathogenic *Y. enterocolitica* bio/serotypes (Lantz *et al.*, 1998; Falcão *et al.*, 2004; Thisted Lambertz *et al.*, 2006). Isolate 871-07 did not contain this plasmid, consistent with the lack of *ail* and *pYad* markers, and the 1A biotype.

All three clinical isolates were resistant to nalidixic acid by disk diffusion with resistance profiles including 506-06: AmpKfNaSSxt; 871-07: AmcAmpKfNa; 10-2307: AmpKfNa. Two of the three clinical isolates (506-06 and 871-7, Table 1) were resistant to nalidixic acid with MIC > 256 mg/L. All three clinical isolates were susceptible to ciprofloxacin and none contained PMQR markers (data not shown).

The amino acid substitutions associated with nalidixic acid resistance are summarized in Table 2. A mutation in *gyrA* was identified in isolate 871-07 consistent with the substitution of Ser-83-Ile, and an Asp-87-Tyr substitution was identified in isolate 506-06, similar to the change in *Y. enterocolitica* 8081. Mutations in these loci contributed to a nalidixic acid-resistant phenotype, as determined by E-test (Table 1). Furthermore, no mutations in *gyrB*, *parC*, or *parE* were detected.

Measurement of MICs to nalidixic acid and ciprofloxacin in the presence of phenylalanine-arginine- $\beta$ -naphthylamide contributed to an increase in susceptibility with the exception of the reference strain, in respect of ciprofloxacin (Table 1). These findings suggest that efflux activity in this bacterium contributes to quinolone/FQ resistance, a feature that confirms earlier observations (Capilla *et al.*, 2004).

Compared to other Enterobacteriaceae, small numbers of *Y. enterocolitica* resistant to nalidixic acid have been reported to date (Sánchez-Céspedes *et al.*, 2003; Capilla *et al.*, 2004; Fàbrega *et al.*, 2010; Sihvonen *et al.*, 2011; Fredriksson-Ahomaa *et al.*, 2012). Although target gene mutations in *gyrA* are the primary mechanism of resistance to nalidixic acid, efflux pump activity, also contributes to support this phenotype. This finding supports data reported earlier (Capilla *et al.*, 2004). Importantly, all three clinical isolates remain susceptible to ciprofloxacin.

## Acknowledgments

The authors acknowledge the assistance of Dr. Karen Power in reviewing the manuscript.

## Disclosure Statement

No competing financial interests exist.

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