Letters to the Editor

Nalidixic Acid-Resistant Strains of *Salmonella* Showing Decreased Susceptibility to Fluoroquinolones in the Midwestern Region of the Republic of Ireland Due to Mutations in the *gyrA* Gene[▽]

Salmonellosis is a significant public health problem worldwide. It has been implicated in a number of food-borne outbreaks in the Republic of Ireland; its prevention, surveillance, and control have become a public health priority. To date, over 2,000 serotypes of *Salmonella* have been identified (6). The European Food Safety Authority (EFSA) has called for the monitoring of both nalidixic acid and ciprofloxacin resistance levels in this bacterium at both country- and European Union-wide levels (4).

Salmonella infection is associated primarily with gastroenteritis. This infection poses a serious health risk to populations in the community such as the elderly, the young, and the immunocompromised, where hospitalization may be required. In such cases, effective antimicrobial treatment is essential and the fluoroquinolones are an important class of antibiotic in the treatment of salmonellosis. Little published information on the fluoroquinolone antimicrobial susceptibility of *Salmonella* in the Republic of Ireland is available. Previous reports indicated the potential emergence of fluoroquinolone resistance in Irish *Salmonella* isolates (6, 7).

The CLSI ciprofloxacin resistance breakpoint stands at ≥ 4 mg/liter, a concentration that has been questioned in recent years. Many commentators believe a revision of the ciprofloxacin resistance breakpoint is needed, as treatment failures have occurred with both nontyphoidal and typhoidal salmonellae, where the ciprofloxacin MIC is much less than 4 mg/liter (1, 3, 12, 13, 14). A breakpoint of ≥ 0.125 mg/liter has been recommended (1).

We now provide an update on the occurrence of reduced susceptibility to nalidixic acid and the monitoring of susceptibility to ciprofloxacin and other fluoroquinolones in *Salmonella* isolates from humans and food animals in the midwestern region of the Republic of Ireland between 2007 and 2010.

Our study examined a total of 143 *Salmonella* isolates from human ($n = 16$), pet food ($n = 30$), and veterinary ($n = 97$) sources in the midwestern region of the Republic of Ireland. The human isolates were from the Mid West Regional Hospital; the veterinary isolates were from the Regional Veterinary Laboratory in Knockalisheen, County Limerick; and the pet food isolates were isolated from pig ear pet food treats manufactured and sold in the midwestern region.

In our laboratory, these *Salmonella* isolates were tested against nalidixic acid (30 μ g). Any nalidixic acid-resistant isolates were then tested against ciprofloxacin (5 μ g), ofloxacin (5 μ g), pefloxacin (5 μ g), norfloxacin (10 μ g), and enrofloxacin (5 μ g) (Oxoid, Hampshire, United Kingdom) in accordance with the CLSI guidelines for antimicrobial disc susceptibility testing (2). The ciprofloxacin MIC was then determined for nalidixic acid-resistant/intermediate isolates using Etests (Biodisk, Solna, Sweden) on Mueller-Hinton agar (Oxoid, Hampshire, United Kingdom). The CLSI breakpoints for the quinolones for *Enterobacteriaceae* are outlined in Table 1.

The *gyrA* PCR was performed using primers P1 and P2 as described by Griggs et al., and sequencing of the products was carried out (7). Accession numbers are listed in Table 2. *Esch-*

erichia coli ATCC 25922 was included on each test occasion, and all results were within the recommended limits, indicating the validity of our test procedures.

Nalidixic acid resistance was observed in 13.3% (19/143) of the isolates, and intermediate resistance was observed in 2.8% (4/143) of the *Salmonella* isolates; these isolates are listed in Table 2. The ciprofloxacin MICs obtained in this experiment showed reduced susceptibility, with the nalidixic acid-resistant isolates showing ciprofloxacin MICs of 0.125 to 1 mg/liter. The isolates with intermediate nalidixic acid resistance showed no reduced susceptibility to ciprofloxacin, with MIC values of 0.008 to 0.015 mg/liter. The nalidixic acid-resistant isolates also showed reduced susceptibility to the other fluoroquinolones in the disk diffusion tests (Table 2). For example, in *S. Typhimurium* nalidixic acid-susceptible strains, a 10- μ g norfloxacin disk showed a zone diameter of 29 mm. In contrast, nalidixic acid-resistant isolate *S. Typhimurium* DT1 SC53 showed a zone diameter of 19 mm, suggesting reduced susceptibility to fluoroquinolones. Similar differences in zone diameter can also be seen for the other *Salmonella* isolates tested in this study (Table 2).

A report by Gorman and Adley included 195 *S. enterica* subsp. *enterica* isolates from human, food, and veterinary sources in the midwestern region between 2000 and 2003. Nalidixic acid resistance was observed in 2.6% (5/195) of the isolates (6). The 13.2% of nalidixic acid-resistant isolates in our study represents a rise. This could be potentially due to the spread of resistant strains and the overuse of antibiotics in humans and previous usage in animals (9, 10, 11). No resistance or reduced susceptibility to ciprofloxacin was detected in any of the isolates in this study (6).

In a report by Kilmartin et al., isolates of *S. Enteritidis* PT1 from all over Ireland were tested for susceptibility to nalidixic acid and ciprofloxacin. Twenty-two out of 50 isolates were found to be nalidixic acid resistant. The ciprofloxacin MICs for these isolates were 0.25 to 0.5 mg/liter (8).

Mutations within the quinolone resistance-determining region (QRDR) of *gyrA* are believed to be one of the main mechanisms of quinolone resistance. *gyrA* is a subunit of the DNA gyrase enzyme. DNA gyrase is essential to the DNA replication process; quinolones act by inhibiting this process.

TABLE 1. Resistance to quinolones and equivalent MIC breakpoint for ciprofloxacin according to CLSI standards

Resistance ^a	Zone size (mm) ^b						CIP MIC breakpoint
	NAL	CIP	OFX	PEF	NOR	ENR	
R	≤ 13	≤ 15	≤ 12	NA	≤ 12	NA	≥ 4
I	14–18	16–20	13–15	NA	13–16	NA	
S	≥ 19	≥ 21	≥ 16	NA	≥ 17	NA	≤ 1

^a R, resistant (no zone diameter was observed); I, intermediate resistance; S, susceptible.

^b NAL, nalidixic acid; OFX, ofloxacin; PEF, pefloxacin; NOR, norfloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; NA not applicable.

TABLE 2. Summary of the mutation patterns in the QRDR of *gyrA* and antibiotic susceptibilities of various *Salmonella* isolates

Isolate	Source	Inhibition zone diam (mm) ^a							CIP MIC (mg/liter)	Accession no.	Codon (amino acid) ^b at <i>gyrA</i> position:					
		NAL (30 µg)	CIP (5 µg)	OFX (5 µg)	PEF (5 µg)	NOR (10 µg)	ENR (5 µg)	82			83	84	85	86	87	88
<i>S. Typhimurium</i> NCTC74	NCTC ^c	24 (S)	32 (S)	28 (S)	27	29 (S)	32	0.008	FR716800	GAT (Asp)	TCC (Ser)	GCA (Ala)	GTG (Val)	TAT (Tyr)	GAC (Asp)	ACC (Thr)
<i>S. S. Derby</i> SV2	Veterinary (porcine)	15 (I)	29 (S)	23 (S)	21	23 (S)	25	0.015	FR716801							
<i>S. S. Dublin</i> SV29	Veterinary (bovine)	17 (I)	30 (S)	27 (S)	27	30 (S)	26	0.008	FR716802							
<i>S. Typhimurium</i> 15a SV99	Veterinary (bovine)	17 (I)	23 (S)	21 (S)	16	22 (S)	21	0.015	FR716803							
<i>S. S. Dublin</i> SV141	Veterinary (bovine)	16 (I)	30 (S)	27 (S)	27	30 (S)	26	0.008	FR716804							
<i>S. Typhimurium</i> DT104 ULPT23	Pig ear	9 (R)	22 (S)	24 (S)	19	22 (S)	26	0.25	FR716805		TTC (Phe)					
<i>S. S. Dublin</i> SV11	Veterinary (bovine)	6 (R)	24 (S)	21 (S)	18	22 (S)	22	0.25	FR716806		TTC (Phe)					
<i>S. Typhimurium</i> DT104 SV130	Veterinary (bovine)	10 (R)	24 (S)	21 (S)	18	23 (S)	21	0.25	FR716807		TTC (Phe)					
<i>S. S. Dublin</i> SV166	Veterinary (bovine)	9 (R)	22 (S)	22 (S)	17	24 (S)	26	0.125	FR716808						AAC (Asn)	
<i>S. S. Enteritidis</i> SC41	Clinical	8 (R)	25 (S)	23 (S)	19	23 (S)	22	0.125	FR716809						TAC (Tyr)	
<i>S. S. Typhimurium</i> DT1 SC53	Clinical	11 (R)	22 (S)	24 (S)	16	19 (S)	24	0.125	FR716810		CCC (Pro)					
<i>S. S. Typhimurium</i> DT203 SC54	Clinical	5 (R)	24 (S)	21 (S)	16	21 (S)	20	0.125	FR716811						GTC (Val)	
<i>S. S. Haifa</i> SC55	Clinical	9 (R)	27 (S)	21 (S)	15	23 (S)	23	0.25	FR716812		TAC (Tyr)					
<i>S. S. Blockley</i> SC56	Clinical	7 (R)	23 (S)	20 (S)	12	21 (S)	21	0.25	FR716813		TTC (Phe)					
<i>S. S. Virchow</i> SC57	Clinical	7 (R)	25 (S)	23 (S)	16	24 (S)	21	0.25	FR716814		TTC (Phe)					
<i>S. S. Enteritidis</i> SC58	Clinical	10 (R)	22 (S)	24 (S)	16	26 (S)	20	0.125	FR716815						TAC (Tyr)	
<i>S. S. Agona</i> SC59	Clinical	11 (R)	24 (S)	21 (S)	15	22 (S)	25	0.25	FR716816		TTC (Phe)				TAC (Tyr)	
<i>S. S. Enteritidis</i> PT146 SC60	Clinical	9 (R)	23 (S)	23 (S)	16	26 (S)	21	0.125	FR716817						TAC (Tyr)	
<i>S. S. Enteritidis</i> PT1 SC61	Clinical	5 (R)	26 (S)	24 (S)	13	22 (S)	23	0.125	FR716818						TAC (Tyr)	
<i>S. S. Heidelberg</i> SC62	Clinical	7 (R)	27 (S)	19 (S)	16	26 (S)	21	1	FR716819		TAC (Tyr)					
<i>S. S. Virchow</i> SC63	Clinical	7 (R)	29 (S)	24 (S)	16	23 (S)	22	0.125	FR716820						TAC (Tyr)	
<i>S. S. Typhi</i> SC64	Clinical	11 (R)	22 (S)	22 (S)	16	20 (S)	24	0.25	FR716821		TAC (Tyr)					
<i>S. S. Enteritidis</i> PT1 SC65	Clinical	9 (R)	29 (S)	23 (S)	17	24 (S)	26	0.125	FR716822						TAC (Tyr)	
<i>S. S. Enteritidis</i> PT6a SC66	Clinical	10 (R)	30 (S)	25 (S)	15	25 (S)	22	0.125	FR716823						AAC (Asn)	

^a NAL, nalidixic acid; OFX, ofloxacin; PEF, pefloxacin; NOR, norfloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; R, resistant (no zone diameter was observed); I, intermediate resistance; S, susceptible.
^b Boldface type indicates a mutation.
^c NCTC, National Collection of Type Cultures.

The nalidixic acid-resistant isolates all contained mutations in the QRDR of *gyrA*. The four intermediately nalidixic acid-resistant isolates had no mutations in the QRDR of *gyrA*. Five nalidixic acid-resistant isolates contained a mutation of serine 83 (Ser-83) to phenylalanine, four contained a mutation of Ser-83 to tyrosine, and another one contained a mutation of Ser-83 to proline. Six nalidixic acid-resistant isolates were found with the aspartic acid 87 (Asp-87)-to-tyrosine mutation, two had Asp-87 mutated to asparagine, and one had Asp-87 mutated to valine (Table 2). These mutations have all been reported previously. Giraud et al. suggested that a mutation at codon 83 conferred a higher resistance to quinolones than mutations at codon 87 (5). Results of our study mostly conform to this hypothesis; the strains with mutations at codon 83 had ciprofloxacin MICs of 0.25 to 1 mg/liter, except for the one isolate; *S. Typhimurium* DT1 SC53, which had a ciprofloxacin MIC of 0.125 mg/liter. All isolates that had mutations at codon 87 had a MIC of 0.125 mg/liter (Table 2). A similar sequence analysis was also carried out by Kilmartin et al. on the *gyrA* gene of their nalidixic acid-resistant isolates (8). They also found that isolates that had reduced susceptibility to ciprofloxacin had mutations at the same positions. Many similar studies from other countries indicate that a reduction in susceptibility to ciprofloxacin is linked with mutations of the *gyrA* gene (9, 11).

This antimicrobial susceptibility report highlights the need for continuous monitoring of fluoroquinolone usage in animal and human medicine and for surveillance of antimicrobial resistance of *Salmonella* in accordance with the call of the EFSA.

REFERENCES

1. Aarestrup, F. M., C. Wiuff, K. Molbak, and E. J. Threlfall. 2003. Is it time to change fluoroquinolone breakpoints for *Salmonella* spp.? *Antimicrob. Agents Chemother.* **47**:827–829.
2. Clinical and Laboratory Standards Institute. 2008. Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement; CLSI document M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
3. Crump, J. A., T. J. Barrett, J. T. Nelson, and F. J. Angulo. 2003. Reevaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and for non-Typhi salmonellae. *Clin. Infect. Dis.* **37**:75–81.
4. European Food Safety Authority. 2010. Manual for reporting on zoonoses, zoonotic agents and antimicrobial resistance in the framework of Directive 2003/99/EC and of some other pathogenic microbiological agents for information derived from the reporting year 2009. EFSA J. **8**:1579. <http://www.efsa.europa.eu/en/scdocs/doc/1579.pdf>.
5. Giraud, E., A. Brisabois, J. L. Martel, and E. Chaslus-Dancla. 1999. Comparative studies of mutations in animal isolates and experimental in vitro- and in vivo-selected mutants of *Salmonella* spp. suggest a counterselection of highly fluoroquinolone-resistant strains in the field. *Antimicrob. Agents Chemother.* **43**:2131–2137.
6. Gorman, R., and C. C. Adley. 2003. Nalidixic acid-resistant strains of *Salmonella* showing decreased susceptibility to fluoroquinolones in the mid-west region of the Republic of Ireland. *J. Antimicrob. Chemother.* **51**:1047–1049.
7. Griggs, D. J., K. Gensberg, and L. J. Piddock. 1996. Mutations in *gyrA* gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals. *Antimicrob. Agents Chemother.* **40**:1009–1013.
8. Kilmartin, D., D. Morris, C. O'Hare, G. Corbett-Feeney, and M. Cormican. 2005. Clonal expansion may account for high levels of quinolone resistance in *Salmonella enterica* serovar Enteritidis. *Appl. Environ. Microbiol.* **71**:2587–2591.
9. Marimón, J. M., M. Gomariz, C. Zigorraga, G. Cilla, and E. Perez-Trallero. 2004. Increasing prevalence of quinolone resistance in human nontyphoid *Salmonella enterica* isolates obtained in Spain from 1981 to 2003. *Antimicrob. Agents Chemother.* **48**:3789–3793.
10. Matheson, N., et al. 2010. Ten years experience of *Salmonella* infections in Cambridge, UK. *J. Infect.* **60**:21–25.
11. Murray, A., J. E. Coia, H. Mather, and D. J. Brown. 2005. Ciprofloxacin resistance in non-typhoidal *Salmonella* serotypes in Scotland, 1993–2003. *J. Antimicrob. Chemother.* **56**:110–114.
12. Parry, C. M., and E. J. Threlfall. 2008. Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. *Curr. Opin. Infect. Dis.* **21**:531–538.
13. Parry, C. M., et al. 2010. Suitable disk antimicrobial susceptibility breakpoints defining *Salmonella enterica* serovar Typhi isolates with reduced susceptibility to fluoroquinolones. *Antimicrob. Agents Chemother.* **54**:5201–5208.
14. Vasallo, F. J., et al. 1998. Failure of ciprofloxacin therapy for invasive nontyphoidal salmonellosis. *Clin. Infect. Dis.* **26**:535–536.

Michael P. Ryan

Colm Dillon

Catherine C. Adley*

Microbiology Laboratory

Department of Chemical and Environmental Sciences

University of Limerick

Limerick, Ireland

*Phone: 353 61 202646

Fax: 353 61 202568

E-mail: Catherine.Adley@ul.ie

† Published ahead of print on 9 March 2011.