Persistence of fluoroquinolones and of ciprofloxacin resistant *Enterobacteriacae* in soil after poultry manure application

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Abstract
This study aimed to evaluate the persistence (i) of two fluoroquinolones, enrofloxacin (ENR) and ciprofloxacin (CIP), and (ii) of CIP-resistant *Enterobacteriaceae* in soil after poultry manure application. The amounts of ENR and CIP in soil did not significantly change over the 48 day-experimental period. CIP ($\leq$ 30 µg kg^{-1}) was detected in weaker concentrations than ENR (20 to 250 µg kg^{-1} of soil). The concentration of *Enterobacteriaceae* (10^2-10^3 CFU g^{-1}) did not significantly decrease until Day 36. A total of 145 isolates were identified as belonging to *E. coli* and to 6 genera. The ciprofloxacin MIC of *E. coli* reached 32 mg L^{-1} whereas the other strains of *Enterobacteriaceae* had a MIC $\leq$ 0.25 mg L^{-1}. The 71 strains of *E. coli* were classified in 12 ERIC-PCR genotypes. One genotype, corresponding to a resistant strain, was detected until Day 89. This study showed that enrofloxacin and CIP-resistant *E. coli* persisted at least 48 and 89 days, respectively, in soil. However, their presence did not increase the MIC of *Enterobacteriaceae* originating from soil.

Introduction
Poultry manure which is generally applied to soil may constitute an environmental risk because of the possible dissemination of pharmaceutical residues and bacteria resistant to antibiotics. Thus, fluoroquinolones (Fqs) which represent one of the most widely used classes of antibiotics in veterinary medicine [1] may be transferred to soil through poultry manure application. Among Fqs, enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP) are highly effective broad-spectrum antimicrobials used to treat various veterinary infections, especially in the poultry industry. The prevalence of ENR in poultry manure ranges between 25% and 38% [2, 3] and the concentration of this antibiotic in soil impacted by manure application ranges between 17.4 and 370 µg kg^{-1} [2-4]. Because of their hydrophobicity, Fqs strongly adsorb to soil [1] and may thus persist in agricultural land after spreading manure. The aim of this study was to evaluate (i) the rate of degradation of ENR and CIP and (ii) the persistence of CIP-resistant *Enterobacteriaceae* in soil after poultry manure application.

Material and Methods
Poultry manure and manure application
One day-old chicks (around 4000) were separated into 2 groups of equal size. One group (control) was not treated. At 27 days of age, the chickens of the 2nd group (*ie.* treated group) were administered enrofloxacin (Baytril®, 10% oral solution) for 5 days at a dose of 10 mg/ kg body weight. The day of the departure of the animals (7 days after the end of the ENR treatment), manures (mixture of faeces and wood shavings) were transferred to an experimental field and applied to soil after 24 h of storage. The soil was a cambisol with a 2 % total organic carbon content. Manure application was performed on wheat crop at the end of the tillering stage. Manures were manually spread at a dose of 800 $g.m^{-2}$ on a field separated into two plots designated "soil T" (receiving manure from treated chickens) and "soil C" (receiving manure from control chickens). The two plots were subdivided into 6 equal subplots (6-m wide and 12-m long). Soil samples were taken from the 0-5 cm surface layer over a 3 month-period (from February to April). The concentrations of Fqs and of *Enterobacteriaceae* were measured 7 days before spreading, at Day 0 (after the manure application), and at Days 5, 12, 19, 36

and 48. A supplementary sample was taken on Day 89 for bacterial counts. Each sample was a composite of 4 soil 100 cm^3 cores randomly collected on each plot.

Chemical and microbial analyses

Fluoroquinolones extraction and analysis by LC-MS-MS were performed as described previously by Moraru *et al.* [5].

Enterobacteriaceae were enumerated on violet red bile glucose (VRBG) agar (OXOID, France) supplemented or not with CIP (1 and 8 mg L^{-1} CIP) as described in Moraru *et al.* [5]. After incubation for 24 h at 37°C, purple-red colonies identified as *Enterobacteriaceae* were enumerated. A total of 145 colonies were randomly selected for further phenotypic and genotypic analyses. Colonies were streaked on TBX agar (OXOID, France) and incubated at 44ºC for 24 h. Characteristic colonies (β-glucuronidase positive) of *E. coli* were confirmed using API 20E biochemical strips (bioMerieux, France), whereas other colonies (β-glucuronidase negative) were identified by sequencing of their 16S rRNA gene as described in Cunault *et al.* [6]. Isolates were analyzed by molecular typing using ERIC PCR technique [7].

Ciprofloxacin MIC (minimum inhibitory concentration) values of isolates were determined according to EUCAST methodology. Isolates were classified as susceptible (MIC ≤ 0.5 mg L^{-1}) or resistant (MIC > 1mg L^{-1}) according to EUCAST clinical breakpoints for ciprofloxacin (http://www.eucast.org/mic_distributions/).

Results

Persistence of Fluoroquinolones in soil

Average concentrations of fluoroquinolones in manure of treated chickens before land application were 13 mg. kg^{-1} for ENR and 1.6 mg. kg^{-1} for CIP (data not shown). ENR, the concentrations of which ranged between 14 and 518 µg. $kg^{-1}$was still quantified in soil on Day 48 (Figure 1), suggesting a stability of the Fqs in the topsoil during the two months following the manure application. These results are in accordance with the persistence of ENR of several months reported by other authors [2, 4, 8]. CIP was quantified, as expected, in much smaller quantities (≤ 30 µg kg^{-1} of soil) than ENR. However, the highest values of CIP were observed for the highest levels of ENR which may suggest a similar persistence of both Fqs in the topsoil.

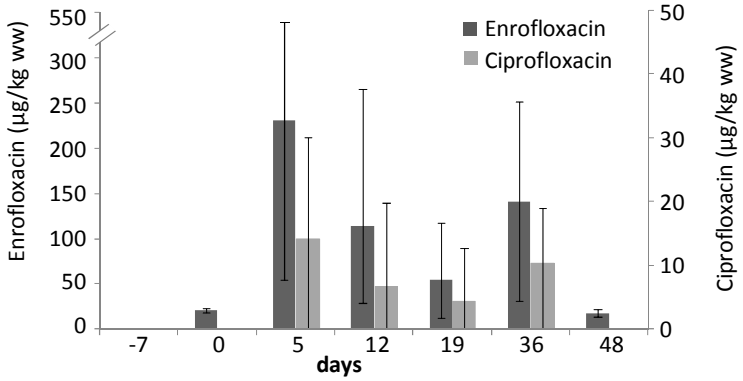

Figure 1. Mean concentrations of enrofloxacin and ciprofloxacin in the soil before and after application of manure of chickens treated with fluoroquinolones. Bars indicate minimum and maximum values.

It is noteworthy that most of the levels of ENR observed after poultry manure application were above the soil concentration trigger value of 100 µg kg^{-1} recommended for pharmaceutical residues by the European Commission [9].

Persistence of Enterobacteriaceae in soil

The concentrations of *Enterobacteriaceae* in both treated and control manures ranged between 1 10^5 and 4 10^5 CFU g^{-1}. They ranged between 10^1 and 10^2 CFU g^{-1} in soil before spreading. No significant decline of *Enterobacteriaceae* was observed until Day 36. Then, their levels significantly decreased (table 1). The decline of *Enterobacteriaceae* after Day 36 may be due to the rise in temperature over the period of experiment (February–May) as the average temperature increased by 11°C between Day

36 and Day 89. Indeed, temperature is one of the major environmental factors that impact the persistence of *E. coli* in manured soil [10, 11]. It is noteworthy that strains of *Enterobacteriaceae* present in soil T at Day 48 and Day 89 were identified as *E. coli*. The persistence of *E. coli* in this study after manure application is consistent with that reported by Unc and Goss [10].

Table 1. Concentrations of *Enterobacteriaceae* in soil C and T

Days of sampling	*Enterobacteriaceae* (CFU g^{-1})	
	control mean (sd)	treated mean (sd)
-7	5.10^1 ($3.2\ 10^1$)	$1.0\ 10^2$ ($2.2\ 10^2$)
0	$9.6\ 10^1$ ($7.4\ 10^1$)	$2.2\ 10^3$ ($1.1\ 10^3$)
5	$2.2\ 10^2$ ($2.3\ 10^2$)	$3.1\ 10^2$ ($2.3\ 10^2$)
12	$7.6\ 10^2$ ($5.5\ 10^2$)	$4.3\ 10^2$ ($4.6\ 10^2$)
19	$4.1\ 10^3$ ($3.0\ 10^3$)	$8.6\ 10^2$ ($7.8\ 10^2$)
36	$1.6\ 10^3$ ($4.0\ 10^2$)	$3.5\ 10^2$ ($1.9\ 10^2$)
48	-[a]	3 (3)
89	-	10 (5)

[a] Not detected

The 145 strains isolated from chicken manure and soil were identified as belonging to *E. coli* and to 6 genera (table 2). Strains of *E. coli* isolated from soil T exhibited high-levels of resistance to CIP (with MIC values up to 32 mg L^{-1}). However, except a strain of *Yersinia massiliensis* isolated from soil T at Day 19 which had a MIC value of 0.25 mg L^{-1}, the strains of *Enterobacter, Rhanella, Buttauxiella, Raoutella and Serratia* exhibited low MICs ranging from 0.004 to 0.064 mg L^{-1}.

Table 2. Maximal ciprofloxacin MIC (mg L^{-1}) of *Enterobacteriaceae* isolated from manure and soil

Matrix	Day of sampling	*E. coli*		*Enterobacter*		*Rhanella*		*Buttauxiella*		*Raoutella*		*Serratia*		*Yersinia*	
		C[a]	T[b]	C	T	C	T	C	T	C	T	C	T	C	T
manure	-1	1	32	0.016	-	-	-	-	-	-	-	-	-	-	-
soil	-7	-[c]	-	0.004	-	-	0.032	0.032	-	-	0.016	-	0.004	-	-
	0	-	32	0.004	-	0.004	-	-	-	-	-	-	-	-	-
	19	-	1	0.008	0.008	-	0.064	-	-	-	-	-	0.032	-	0.25
	36	-	2	0.016	0.008	-	-	0.016	-	-	-	-	-		-
	48	-	8	-	-	-	-	-	-	-	-	-	-	-	-
	89	-	8	-	-	-	-	-	-	-	-	-	0.032	-	-

[a] soil spread with manure of control chickens; [b] soil spread with manure of treated chickens; [c] not identified on VRBG agar

Mechanisms involved in resistance to Fqs are mutations in chromosomal genes encoding for DNA gyrase and topoisomerase IV or plasmid mediated quinolone resistance (PMQR) which are horizontally transferable among bacteria [12]. Although PMQR has been identified among *Enterobacteriaceae* [13] and in *E. coli* chicken isolates [12], no attempt was made in this study to detect such PMQR genes in the resistant isolates. However, despite the persistence of Fqs in soils and the presence of Fqs-resistant *E. coli* in manure, there was no evidence of transfer of Fqs resistance from manure to soil *Enterobacteriaceae* throughout the experiment.

According to the results of ERIC-PCR, 30 fingerprint profiles were identified among 145 isolates of *Enterobacteriaceae.* The 71 strains of *E. coli* were classified in 12 ERIC genotypes which were divided into 8 unique profiles (found only once) and 4 multiple profiles (A, G, H and L) (table 3). The profiles identified in manure of control chickens were susceptible or intermediate whereas the 3 profiles identified in manure of treated animals were resistant (MIC $\geq$ 8 mg L^{-1}). Among the 7 profiles found in manure, only one profile (G), present both in manure of control and treated animals, was still detected in soil after spreading.

Table 3. Distribution of fingerprint profiles of strains of *E. coli* isolated on VRBG agar in manure from control (C) and treated (T) chickens and in soil T according to the MIC.

Matrix	Day of sampling	Nb profiles (nb strains)	MIC (mg L^{-1})			
			<0.05	1	2	≥8
Manure (C)	-1	7 (18)	**A**[a], B, C, D, E, F (4)[b]	**G** (9)		
Manure (T)	-1	3 (18)				**G** (8), **H** (9), **A**
Soil (T)	0	1 (10)				**H** (10)
	19	1 (1)		I		
	36	3 (17)	J, K		**G** (15)	
	48	1 (2)			**L**	**L**
	89	1 (5)	**L**			**L** (4)

[a] Letters indicate the code of the profiles (profiles found in more than one sample are in bold); [b] number of strains if different from 1.

Our results are in agreement with those reported by Topp *et al.* [11] who also observed a shift in *E. coli* community fingerprinted by ERIC PCR between swine manure and manured soil. One profile (L), which corresponded to sensitive and resistant strains has been detected at Day 48 and at Day 89, suggesting the ability of this genetic profile to persist in soil.

Conclusion

These results showed that enrofloxacin persisted at least 6 weeks in manured soil without a significant decrease in concentration and that CIP-resistant *E. coli* can survive up to three months. However, Fqs and CIP-resistant *E. coli* brought by manure did not increase levels of CIP-resistance in *Enterobacteriaceae* originating from soil.

References

[1] Pico Y, Andreu V, 2007. Fluoroquinolones in soil-risks and challenges. Analytical and Bioanalytical Chemistry 387, 1287-1299

[2] Martinez-Carballo E, Gonzalez-Barreiro C, Scharf S, Gans O, 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. Environnemental Pollution 148, 570 - 579

[3] Pereira Leal RM, Figueira RF, Tornisielo VL, Regitano JB, 2012. Occurrence and sorption of fluoroquinolones in poultry litters and soils from São Paulo State, Brazil. Science of the total Environment 432, 344-349

[4] Karcı A, Balcıoğlu IA, 2009. Investigation of the tetracycline, sulfonamide and fluoroquinolone antimicrobial compounds in animal manure and agricultural soils in Turkey. Science of the total Environment. 407, 4652-4664

[5] Moraru R, Pourcher AM, Jadas-Hecart A, Kempf I, Ziebal C, Kervarrec M, Communal PY, Maresa M, Dabert P, 2012. Changes in concentrations of fluoroquinolones and of ciprofloxacin-resistant *Enterobacteriaceae* in chicken feces and manure stored in a heap. Journal of Environmental Quality 41, 754-763

[6] Cunault C, Pourcher AM, Burton CH, 2011. Using temperature-time criteria to control the effectiveness of continuous thermal sanitation of piggery effluent in terms of set microbial indicators. Journal of Applied Microbiology 111, 1492-504

[7] Burr MD, Josephson KL, Pepper IL, 1998. An evaluation of ERIC PCR and AP PCR fingerprinting for discriminating *Salmonella* serotypes. Letters in Applied Microbiology 27, 24-30

[8] Boxall ABA, Johnson P, Smith Edward J, Sinclair CJ, Stutt E, Len SL, 2006. Uptake of Veterinary Medicines from Soils into Plants. Journal of Agricultural and Food Chemistry 54, 2288-2297

[9] Golet EM, Xifra I, Siegrist H, Alder AC., Giger W, 2003. Environmental Exposure Assessment of Fluoroquinolone Antibacterial Agents from Sewage to Soil. Environmental Science and Technology 37, 3243–3249

[10] Unc A, Goss MJ, 2006. Culturable *Escherichia coli* in Soil Mixed with Two Types of Manure. Soil Science Society of America 70, 763-769

[11] Topp E, Welsh M, Tien YC, Dang A, Lazarovits G, Conn K, Zhu H, 2003. Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. FEMS microbiology ecology 44, 303-308

[12] Kuo HC, Chou CC, Tu C , Gong SR, Han CL, Liao JW, Chang SK, 2009. Characterization of plasmid-mediated quinolone resistance by the qnrS gene in *Escherichia coli* isolated from healthy chickens and pigs Veterinarni Medicina 54 , 473-482

[13] Nordmann P, Poirel L, 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. Journal of Antimicrobial Chemotherapy 56, 463-469