Fluoroquinolone degradation ratio in poultry litter fertilized soils and persistence of biological impacts below analytical limits of detection – a field study

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Introduction

Fluoroquinolones (FQs) are widely prescribed antibiotics in human and veterinary medicine. Among FQs, enrofloxacin (ENR) is massively administered in Brazilian poultry production, being metabolized in ciprofloxacin (CIP), an antibiotic commonly prescribed for human infections [1]. One of the main sources of FQs to agricultural environment result from application of poultry litter as soil fertilizer. This class of antibiotics exhibit in soils strong sorption, low mobility, high persistence and, therefore, long-term biological impact in soil microbiota [1, 2]. In this context, the continuous load of antibiotics can alter the structure of the soil microbiota, affecting biogeochemical cycles, besides the degradation and fate of agricultural contaminants [3]. In addition, bacterial strains resistant to antibiotics are commonly described in agricultural environments [4]. Even after the complete degradation of antibiotics, the maintenance of bacterial strains or even antibiotic resistance genes (ARGs) in the environment, can be a threat to human health through complex pathways of exposure. The aim of this research was to determine ENR and CIP contamination in short to long-term poultry litter fertilized soils and the presence of quinolone resistance genes. From the results, it was possible to observe a degradation pattern between ENR and CIP related to the time of land use.

Materials and methods

Poultry litter fertilized soils (Argisoil) were collected in São José do Vale do Rio Preto, main chicken production center in Rio de Janeiro state, southeastern Brazil. Soil samples (A horizon) were collected according to the time of poultry litter fertilization: 1) short-term (ST soil - 15 samples) with < 1 year (10 months) of fertilization; 2) medium-term (MT soil - 10 samples) with 1.5 year; 3) long-term 1 (LT 1 soil – 15 samples) with 10 years; 4) long-term 2 (LT 2 - 8 samples) with 15 years; 5) long-term 3 (LT 3 - 7 samples) with 30 years. ENR and CIP were extracted in lyophilized and sieved (< 1mm) soil samples according to Turiel et al. [5]. Briefly, 1 g of sample was extracted with 8 mL of MgNO3 50% (w/v) with 4% NH4OH solution. Extracts were cleaned-up with ultrasonic bath (30 min.), centrifugation (10 min with 3,400 rpm) and filtration with Millipore™ syringe filter 40 µm (Darmstadt, Germany) [1]. FQs were determined using a HPLC system (CBM-20A) equipped with a quaternary pump (LC-10ATVP) and a fluorescence detector (RF-10AXL) from Shimadzu Corp., Japan. Columns used were: guard C18, 10 x 4 mm, 5 µm and analytical C18, 250 x 4.6 mm, 5 µm, both Kromasil® (Sweden). Isocratic elution was used with flow rate of 1.0 mL min^{-1}. The mobile phase was composed of 0.02 M *o*-H_3PO_4:ACN (80:20) [2]. The fluorescence wavelengths were set at 280 and 450 nm for excitation and emission, respectively. Quantification was made by matrix-matched calibration curve with correlation coefficients ≥ 0.99. Recoveries and coefficient of variation (CV) were: 77.2% (2.9) to ENR and 77.5% (2.6) to CIP. The limits of detection (LOD) and quantification (LOQ) for ENR were 51.5 and 116 µg kg^{-1}, respectively. While for CIP were 68.1 and 189 µg kg^{-1}, respectively. The CIP ratio (CIP_R), degradation ratio between ENR and CIP, was calculated following the formula: $CIP_R = CIP/(CIP+ENR)$. For data analysis, measured values <LOQ were considered equal to Df * LOQ, where Df is the detection frequency in each sample set, as described by Das et al. [6]. The DNA was extracted from 4

soil samples (control, poultry house soil, ST soil and LT 1 soil) in triplicate using DNeasy PowerSoil Kit (QIAGEN, Inc., USA). The DNA extracted was used for polymerase chain reaction (PCR) amplification of quinolone resistance genes *qnrA*, *qnrB* and *qnrS*. PCR conditions were as previously described [7]. PCR products were analyzed by 1.5% agarose gel electrophoresis followed by staining with ethidium bromide to confirm their sizes.

Results and discussion

In short-term fertilized soils (ST soil) were measured 453 ± 529 µg kg^{-1} ENR and 184 ± 133 µg kg^{-1} CIP (Table 1). In this area a long persistence of ENR + CIP was observed, with concentrations between 130 and up to 540 µg kg^{-1} being measured eight months after the last poultry litter application. A high persistence of ENR was also reported by Karci and Balcıoğlu [8] that measured 50 µg kg^{-1} after seven months of application in Turkey's agricultural soils. In this same area, poultry litter applications for truck farming production (eg. cucumber, zucchini and peppers) were carried out periodically. After eight months, a new sampling campaign was carried out, being called medium-term fertilized soils (MT soil) this new set of samples. The concentrations found for ENR and CIP were 1,330 ± 1,718 µg kg^{-1} and 374 ± 237 µg kg^{-1}, respectively. In both sets of samples (ST soil and MT soil), higher ENR concentrations were observed compared to CIP.

Table 1. Fluoroquinolone concentration (µg kg^{-1}) in each sample set and basic statistics

Samples	FQs	Mean	SD[a]	Median	Range	CIP_R[b]
Short-term soil (n=15)	ENR	453	529	178	83.5 - 1,612	0.29
	CIP	184	133	106	189 - 555	
	ENR + CIP	637	651	284	189 - 2,167	
Medium-term soil (n=10)	ENR	1,330	1,718	893	330 - 6,138	0.22
	CIP	374	237	339	106 - 960	
	ENR + CIP	1,704	1,669	1,250	560 - 6,244	
Long-term soil 1 (n=15)	ENR	238	177	164	51.0 - 521	0.64
	CIP	415	417	315	83.2 – 1,371	
	ENR + CIP	653	421	505	134 – 1,563	
Long-term soil 2 (n=8)	ENR	164	220	51.0	51.0 - 686	0.63
	CIP	276	318	83.2	83.2 - 976	
	ENR + CIP	440	536	134	134 – 1,662	
Long-term soil 3 (n=7)	ENR	51.0	n.d.[c]	51.0	n.d.[c]	0.75
	CIP	151	178	83.2	83.2 - 555	
	ENR + CIP	202	178	134	134 - 606	

a: standard deviation; b: ciprofloxacin ratio; c: not determined

On the other hand, CIP concentrations predominated in long-term fertilized soils (LT1 to LT3). In LT soil 1, concentrations ranged from 238 ± 177 µg kg^{-1} and 415 ± 417 µg kg^{-1} for ENR and CIP, respectively. In LT soil 2, were measured 164 ± 220 µg kg^{-1} ENR and 276 ± 318 µg kg^{-1} CIP. While in LT soil 3, only CIP was measured in one sample

(555 µg kg^{-1}) and ENR concentration was estimated by the detection frequency multiplied by the LOQ [6]. The CIP_R is presented in Table 1 for each area, where a ratio > 0.5 indicates the CIP predominance. According to previous studies that measured ENR and CIP in poultry litter samples, a clear predominance of the parent compound (ENR) can be observed [1, 9]. Figure 1 describes the study area and the five poultry litter fertilized soil sample sets. As can be seen, short and medium-term fertilized soils presented an ENR predominance. However, despite the continuous agricultural activity in all areas, there is a shift in the degradation patterns of both antibiotics related to the time of land use. In long-term-fertilized soils (Fig. 1 LT1 to LT3), is clear a CIP predominance with a CIP_R between 0.64 and 0.75.

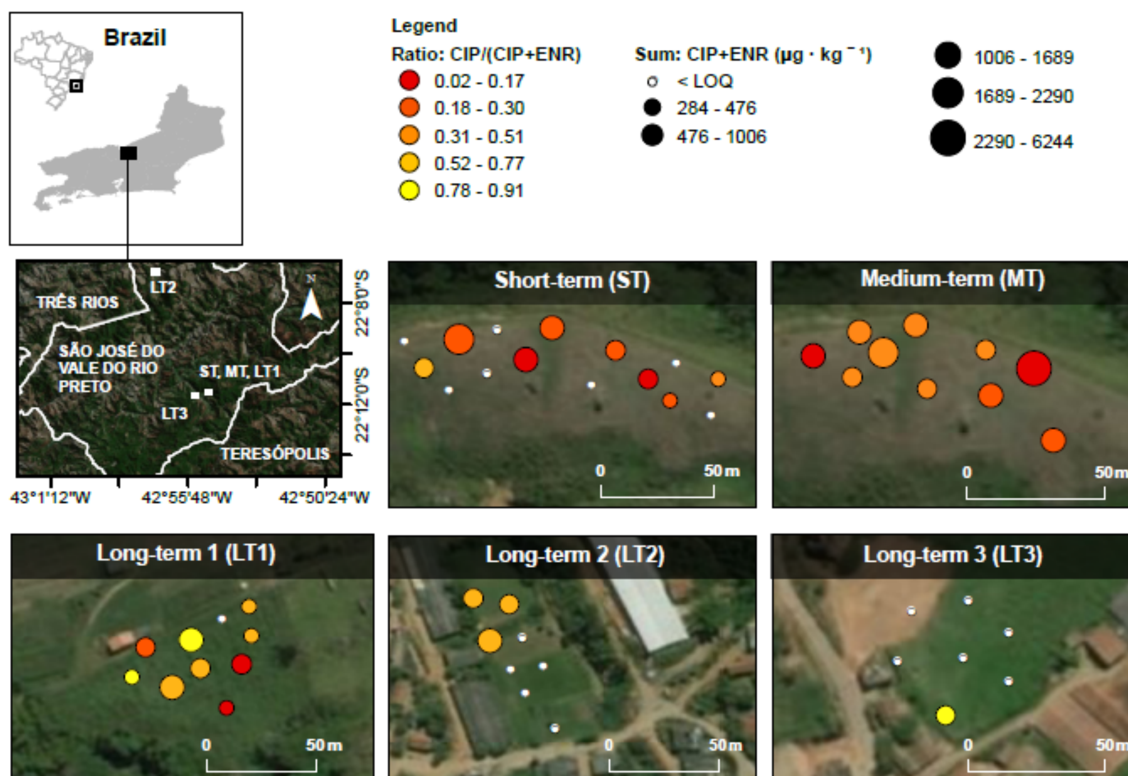

Figure 1 Study area and soil ENR and CIP concentrations along short to long-term fertilized soils

After reach soil, antibiotics can cause disturbances for local microbiota through its specific action mainly on the bacterial community. In this context, other groups may become dominant through the adaptive capacity of microbial community, such as fungi and other FQ-tolerant microorganisms [10, 11]. Previous studies cited, for example, a great degradation of ENR, CIP and norfloxacin by fungi commonly found in soil and in poultry litter [12, 13]. Therefore, it is expected that soils impacted for a long time with antibiotics have their microbiome altered and adapted to a faster and more efficient degradation. This adaptation due ENR impact over the years could explain the higher concentrations of CIP in LT soil areas. In addition to the impacts on the microbiota and its effects on soil functions, antibiotics in soil exert a selective

pressure and may lead to the transfer (vertical and horizontal) of ARGs [10]. According to the PCR results, *qnrS* gene was detected in a sample of LT1 soil, while *qnrA* and *qnrB* genes were not detected in soil samples. The *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* genes are associated with resistance mechanisms to low quinolones concentrations and therefore may provide high levels of resistance to this class of antibiotics [14]. Although these soils were highly impacted by FQs, the *qnr* genes were present in a low number in the samples evaluated. This absence is probably due to its occurrence below the limit of detection of the technique (PCR) used. In addition, the other genes involved in quinolone resistance (*qnrC* and *qnrD*) were not evaluated in this study. According to Huang et al. [14], was observed a significant increase of CIP-resistant bacteria in soil communities exposed to low CIP concentrations (40 and 400 µg kg^{-1}) compared to high CIP concentrations 4,000 µg kg^{-1}. The soil concentrations associated with a higher CIP-resistant bacteria communities, are in the same range of ENR 483 µg kg^{-1} and CIP 278 µg kg^{-1} found in the present study. The fact that FQs undergo strong sorption in soils, favors their diffusion and sorption in micro and nanopores reducing their biological contact and, therefore, their toxicity and biodegradability. However, the same phenomenon may increase its persistence through desorption at subinhibitory concentrations, implying a chronic selection pressure on soil microbiota. The increasing burden of human and veterinary antibiotics in the environment leads to the need for further investigations of fate, environmental persistence, and possible adverse effects on organisms, especially at sub-lethal doses. The results demonstrate the importance of a broader approach in the evaluation of environmental contamination, where not only was consider the presence of target compounds, but their biological impacts and changes in the environment.

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