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Risk assessment of fluoroquinolones from poultry muscle consumption: Comparing healthy adult and pre-school populations

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ABSTRACT

Antibiotics, especially fluoroquinolones (FQs), have been largely used in animal husbandry namely poultry production. Therefore, this study aimed to identify, quantify and estimate the daily intake for adult and 3-year-old populations of the FQs norfloxacin (NOR), ciprofloxacin (CIP) and enrofloxacin (ENR) through poultry muscle consumption.

The results showed detection frequencies of 78% and 62% in supermarket and school canteen samples, respectively. Of the 182 analysed samples, 4 did not comply with ENR maximum residue level (MRL), and 9 were contaminated with NOR, not allowed as a veterinary medicine of food-producing animals.

The highest estimated daily intake value was obtained for the 3-year-old population regarding the sum of ENR and CIP ($0.46\,\mu g\,kg^{-1}\ day^{-1}$); value substantially lower than the established acceptable daily intake ($2.0\,\mu g\,kg^{-1}\ day^{-1}$). Although the low risk found, the high detection frequencies support the apprehension of the different international organizations, towards the emergence of human bacterial resistances to FQs originated from poultry production.

1. Introduction

The increase of human population has raised the necessity to breed a large number of food-producing animals and, consequently, has led to a higher usage of antibiotics in veterinary medicine.

In 2006, in the European Union (EU), the use of antibiotics as growth promoters was forbidden in food-producing animals (European Union, 2003). Nevertheless, the use of large amounts of antibiotics, especially in poultry production, has raised several safety questions regarding their use, toxic effects (musculoskeletal complications and arthrotoxicity), allergenic potential and, more alarming, the development of resistant strains of bacteria (Committee on Infectious Diseases, 2006; EFSA, 2013, 2015; FAO/WHO/OIE, 2007; JIACRA, 2017; Moniri and Dastehgoli, 2005).

Fluoroquinolones (FQs), widely use in human medicine, are one of the antibiotics group most frequently administered in veterinary medicine (EMEA, 2007; Leal et al., 2012). Enrofloxacin (ENR) is only used in veterinary medicine, and one of its major metabolites, ciprofloxacin (CIP), is an important antibiotic commonly administered to human and pets but banned from food-producing animals (Jong et al., 2012; Salehzadeh et al., 2007; The European Commission, 2010). Although ENR has been banned from the United States of America, where growth promoters are still allowed, it is extensively used in the EU (EMEA, 2007). In muscle, fat and skin, the maximum residue level (MRL) for ENR is $100\,\mu g\,kg^{-1}$, being the marker residue the sum of ENR and CIP (EMA, 2002; The European Commission, 2010). Norfloxacin (NOR), used in human medicine, is not allowed as a veterinary medicine in food-producing animals. Nonetheless, previous studies have confirmed the use of this FQ in several poultry productions from different countries (Al-Mustafa and Al-Ghamdi, 2000; Marni et al., 2011; Omotoso and Omojola, 2015; Pena et al., 2010).

The association of poultry meat to a healthy and hypocaloric diet as led to a higher demand and consumption over the last few years. In Portugal, the intake of poultry meat is of 37.5 kg $inhab^{-1}$ per year (EMA, ESFA, & ECDC, 2015; INE, 2015). This high consumption rate, promotes large scale productions, with the rearing of great number of animals in small spaces, leading to the increased use of antibiotics, namely FQs. In 2004, 3.6 tonnes of FQs were used in veterinary medicine in Portugal, being the European member-state with the highest rate of FQ consumption per kg of meat produced (EMEA, 2007). Since then, this value has been increasing, with 5.6 and 8.4 tonnes of ENR sold, alone, in 2010 and 2011, respectively (Almeida et al., 2014). In

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2012, analysing the ratio between the amount of FQs administered and kg of poultry produced, Spain was the EU country with the highest ratio (67.30 mg of FQs per kg of poultry produced), followed by Portugal (46.23 mg of FQs per kg of poultry produced) and the United Kingdom (22.12 mg of FQs per kg of poultry produced) (EFSA, 2015). Additionally, in Portugal the swine and cattle production has been decreasing, meaning that the increase in FQs consumption is related to the increase of poultry production (EMA et al., 2015).

The use of antibiotics in food-producing animals is one of the main causes of the emergence of bacterial resistances (Hooper, 2001; Panzenhagen et al., 2016). Furthermore, FQs are regarded as critically important antimicrobials for human medicine due to the lack of therapeutic alternatives and escalating emergence of bacterial of resistances, namely from non-human sources (Murphy et al., 2017; WHO, 2011). Many studies have been published regarding resistant strains of *Campylobacter spp*, *Salmonella spp* and *Escherichia coli* to FQs, which are positively correlated with the use of FQs in poultry production (EFSA, 2013; Griggs and Johnson, 2005; Hooper, 2001; Humphrey and Jørgensen, 2005; JIACRA, 2017). When these strains infect humans, the duration of the illness, time in hospital and death rates is increased (Barza and Travers, 2002; WHO, 2003).

According to the report presented by the European Food Safety (EFSA) for 2011, Portugal tops the list of countries with the highest incidence to CIP resistance of *Salmonella,* present in chickens, with a rate of 90.2% (EFSA, 2013). These results are similar to those obtained for 2013, where all strains of *Campylobacter coli* in chickens were resistant to nalidixic acid and CIP. These also presented high resistance values of *Campylobacter jejuni* to CIP (53.0%) and to nalidixic acid (50.3%) (EFSA, 2015).

During the last decade, special concern has arisen from foods intended to be consumed by children. This population subtype (risk group) presents greater susceptibility to food contaminants, particularly due to the higher protein intake per kg of body weight when compared to the overall population (Nacano, 2012). Most of the published studies focused only on developing an analytical methodology. Only a few applied the developed methodology to real poultry samples and, as far as we know, no previous exposure study was performed on children.

Therefore, the aim of this study was to perform an updated picture of the FQs NOR, CIP and ENR contamination in poultry meat in the Portuguese supermarkets and in school canteens, contributing to establish contamination patterns (Chen et al., 2009). Additionally, we also aimed to calculate the estimated daily intake (EDI) in the adults and children risk group and compare the values found with the acceptable daily intake (ADI), performing the risk assessment of FQs in poultry meat.

2. Materials and methods

2.1. Sampling

The sampling campaign was performed in mainland Portugal, from 2013 to 2015. A total of 182 samples, 164 chicken and 18 turkey samples were collected. The turkey samples, although in relatively small number, were included as a preliminary study. Sampling was performed in mainland Portuguese supermarkets and in pre-school and primary school canteens in the Coimbra district, center of Portugal. Regarding supermarkets, 108 samples, 96 chicken and 12 turkey samples, all with Portuguese origin were collected. The samples from school canteens, 74, presented several origins: Spain (40 chicken samples), United Kingdom (10 chicken samples) and Portugal (18 chicken and 6 turkey samples).

2.2. Standards, chemicals and materials

All FQs were analytical standards with purity degree above 98.0%. NOR pharmaceutical standard was purchased from Sigma-Adrich Co. (St. Louis, USA) while CIP and ENR were acquired from Fluka, Sigma-Adrich Co. (St. Louis, USA). Individual stock solutions were prepared in sulphuric acid (0.005 M) at 1 mg mL^{-1} and stored at 5 °C. An intermediate standard solution of all antibiotics, with a concentration of 200 μg mL^{-1}, and working solutions prepared at concentration ranging from 0.0075 to 0.25 μg mL^{-1}, were also prepared in sulphuric acid.

Methanol and acetonitrile of HPLC grade and sulphuric acid (90–91%) were acquired from Carlo Erba (Milan, Italy). Phosphoric acid (85%) was purchased from Merck (Darmstadt, Germany) and tetrabutylammonium hydroxide (TBA) was acquired from Fluka, Sigma-Adrich Co. (St. Louis, USA). Ultrapure Milli Q water was daily obtained through a Millipore (Molsheim, France) equipment.

Polyamide membranes 0.45 and 0.2 μm were acquired from Whatman Schleicher and Schuell (Kent, USA) and from Whatman (Dassel, Germany), respectively. Oasis HLB cartridges (200 mg, 6 mL), from Waters Corporation (Milford, Massachusetts, USA), were used for solid phase extraction (SPE).

2.3. Sample pre-treatment and analysis

A portion of 100 g of muscle tissue was removed from each sample, properly labelled, and frozen at −20 °C until analysis. The analytical procedure was based on a previously reported methodology for the identification and quantification of FQs in poultry muscle samples (Pena et al., 2010).

Briefly, after defrosting and reaching room temperature, 1 g of muscle tissue sample was added of 7 ml of phosphoric acid (0.5 M). The sample was then stirred in a vortex for 1 min and sonicated for 15 min. After this process it was allowed to stand for 10 min, protected from light. Afterwards, centrifugation was performed for 10 min, at 5 °C and 13,000 g, the supernatant was removed, and the extraction procedure was repeated.

Extraction and clean-up was performed by SPE, through an Oasis HLB cartridge, previously conditioned with 2 mL methanol followed by 2 mL Milli-Q water. The extract (14 mL) was then applied, at a flow of 2 mL min^{-1}, and the cartridge was washed with 3 mL Milli-Q water. FQs were then eluted with 2 mL methanol. Finally, the eluent was evaporated until dryness, at 45 °C, under a gentle stream of nitrogen and the residue redissolved in 1000 μL of sulphuric acid (0.005 M).

Analysis of antibiotics was performed using a HPLC system consisting of a 805 manometric Module Gilson, with a loop of 20 μL from Gilson (Villiers le Bel, France) and a fluorimetric detector from Jasco (Tokyo, Japan) FP-2020 Plus. The system used a Chromolith® Performance RP-18e (100 × 4.6 mm) analytical column and a guard column (10 × 4.6 mm) of the same characteristics, both from Merck (Darmstadt, Germany).

Chromatography separation was achieved with a mobile phase composed by phosphoric acid solution (0.025 M), adjusted to pH 3.0 with TBA, methanol and acetonitrile (885:90:25). The flow rate was set at 1.5 mL min^{-1}.

2.4. Statistical analysis

Complete statistical analysis was performed using GraphPad Prism (6.01, GraphPad Software, Inc., San Diego, USA). To test whether the datasets were of Gaussian distribution, D'Agostino–Pearson normality test was used. Since most of the datasets were not normally distributed, with non-homogeneous variances, nonparametric tests were applied. For the comparison between each and the total of FQs in chicken and/or turkeys, in the Portuguese supermarkets and school canteens, and different production countries, Kruskal–Wallis test with Dunn's post-test were used. The statistical significance level was set to $p < 0.05$ (Magdeburg et al., 2012).