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Assessing the molecular basis of transferable quinolone resistance in *Escherichia coli* and *Salmonella* spp. from food-producing animals and food products

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ABSTRACT

Enterobacteriaceae resistant to quinolones frequently arise in animals, being easily disseminated through the food-chain. The aim of this study was to investigate the presence of plasmid-mediated quinolone resistance (PMQR) determinants in *Salmonella* spp. ($n = 183$) and *Escherichia coli* ($n = 180$) isolates, collected from food-producing animals and food products among swine, poultry, rabbits and cattle. All isolates were subjected to antimicrobial susceptibility testing and molecular screening of PMQR determinants. β-Lactamase-encoding genes, and the quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes were also investigated in PMQR-positive isolates. Plasmid characterization was performed by conjugation, followed by replicon-typing. Genetic relatedness of PMQR-positive *E. coli* was examined by Multilocus Sequence Typing, while *Salmonella* was previously serotyped. The association of mobile genetic elements and PMQR was investigated through PCR mapping assays. Overall, 4.1% (15/363) isolates harbored *qnrB2* ($n = 3$), *qnrB19* ($n = 3$), and *qnrS1* ($n = 9$) genes. All but one isolate presented one to four mutations in QRDR of *gyrA* or *parC* genes, which is consistent with the range of MIC values detected (0.19–64 mg/L) for ciprofloxacin; 60% (9/15) of *qnr*-harboring isolates were non-susceptible to β-lactam antibiotics which was justified by the presence of β-lactamases from TEM (TEM-1, $n = 8$; TEM-135, $n = 1$) and SHV (SHV-108, $n = 1$) families. Analysis of mobile genetic elements revealed that *qnr* genes were detected nearby relevant genetic elements like *intI1*, *ISEcl2*, *IS26* and *ISCR1* and enclosed in diverse Inc. type plasmids. This study illustrated the existence of Qnr-producing *E. coli* and *Salmonella* from food-producing animals, associated to specific mobile elements that might mediate their transference between species and among distinct settings.

1. Introduction

Fluoroquinolones are a class of antimicrobials which are effectively used in the treatment of infections in both humans and animals, being also used as prophylactic agents in food-producing animals (EMA, 2012).

Bacterial resistance to fluoroquinolones has emerged quickly and has conventionally been attributed to chro-

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mosomally encoded mechanisms that allow the alteration of quinolone targets: DNA gyrase and topoisomerase IV (Eaves et al., 2004). However, the discovery of plasmid-borne determinants has increased the genetic background on the mechanisms of quinolone resistance. Currently, there are four main plasmid-mediated quinolone resistance (PMQR) mechanisms: the determinant Qnr, which includes genes such as *qnrA*, *qnrB*, *qnrS*, with several variants each, and *qnrC* and *qnrD*, that increase resistance to both nalidixic acid and fluoroquinolones (Tran and Jacoby, 2002; Hata et al., 2005; Jacoby et al., 2006; Wang et al., 2009; Cavaco et al., 2009); the cr variant of the common aminoglycoside acetyltransferase Aac(6′)-Ib, which is capable of acetylate and reduce the activity of certain fluoroquinolones (Robicsek et al., 2006); the QepA determinant, an efflux pump that confers decreased susceptibility to hydrophilic fluoroquinolones; and the multi-resistance (MR) efflux pump OqxAB that is also able to confer resistance to nalidixic acid and ciprofloxacin, among other antimicrobial agents (Poirel et al., 2012).

These PMQR mechanisms are frequently associated to transference events, which can be facilitated by their location on mobile genetic elements, such as transposons, insertion sequences, and integrons gene cassettes, among others, leading to the establishment of MR (Chen et al., 2009; García-Fernández et al., 2009).

Bacteria resistant to fluoroquinolones can arise and emerge in animals, being easily transferred to humans through the food-chain, which can ultimately lead to the development of infectious diseases (Poirel et al., 2012).

The lack of knowledge regarding the spread of PMQR determinants in Portugal has led us to investigate their presence among *Salmonella* spp. and *Escherichia coli* isolated from food-producing animals and food products, and to evaluate whether they could be disseminated between different settings by mobile elements.

2. Materials and methods

2.1. Bacterial isolates

A total of 363 unduplicated *Salmonella* spp. ($n = 183$) and *E. coli* ($n = 180$) isolates were collected, during the years of 2009 and 2010, in a single food safety laboratory covering four mainland Portuguese regions (North, Center, Lisbon and Tagus Valley and South), and sent to the National Reference Laboratory of Antimicrobial Resistances (NRL-AR) at the National Institute of Health (NIH) in Lisbon, Portugal without any previous selection criteria. Among all isolates, 91 were collected from consumable food products which included meat ($n = 62$), processed meat ($n = 22$) and eggs ($n = 7$) from three distinct animal origins: pigs ($n = 51$), poultry ($n = 31$), and cattle ($n = 9$), being all *Salmonella* spp. isolates. The remaining 272 isolates (92 *Salmonella* spp. and 180 *E. coli* isolates), from poultry ($n = 255$), pigs ($n = 13$), rabbits ($n = 3$), and cattle ($n = 1$), were recovered from routine samples for bacteriological diagnosis: fecal ($n = 58$) and environmental samples ($n = 4$), collected using sterile boots/sock swabs, placed in sterile bags and transported to the laboratory; macerate of organs from food-producing animals ($n = 191$); and embryonated eggs ($n = 19$).

2.2. Antimicrobial susceptibility testing

For all isolates, antimicrobial susceptibility testing was performed by standard disk diffusion method, and interpreted according to the Antibiogram Committee of the French Society of Microbiology (Bonnet et al., 2012), using 28 distinct antibiotics, alone or in association with β-lactamase inhibitors: nalidixic acid, norfloxacin, pefloxacin, ciprofloxacin, flumequine, marbofloxacin, enrofloxacin, amoxicillin, amoxicillin plus clavulanic acid, piperacillin plus tazobactam, cephalotine, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, ceftiofur, cefepime, cefoxitine, aztreonam, imipinem, meropenem, tetracycline, kanamycin, gentamicin, chrolamphenicol, trimethoprim plus sulfamethoxazole, trimethoprim and nitrofurantoin. Minimum inhibitory concentrations (MICs) of nalidixic acid and fluoroquinolones were determined by Etest (bioMérieux, Marcy l'Etoile, France) for PMQR-producing isolates and their respective transconjugants, and interpreted according to the manufacturer's instructions. Isolates were considered MR if they presented non-susceptibility to three or more structurally unrelated antibiotics. Strains ATCC 25922, and CQURA270 CTX-M-15-producing *E. coli*, were used as controls for both antimicrobial susceptibility testing methods.

2.3. Molecular characterization of resistance

All isolates were investigated for the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac*(6′)-*Ib-cr*, and *qepA* genes, through PCR amplification using specific primers, as previously reported (Cavaco et al., 2009; Park et al., 2006; Wang et al., 2009, 2003). In all PMQR-producing isolates, the quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes, and class 1 and 2 integrons, were screened using primers and conditions previously reported (Ahmed et al., 2005; Bass et al., 1999; Everett et al., 1996; Leverstein-Van Hall et al., 2002; Lévesque et al., 1995; Mammeri et al., 2005; Sorlozano et al., 2007). Whenever PMQR-producing isolates were non-susceptible to penicillins, they were screened for bla_{TEM}, bla_{SHV} and bla_{OXA} genes (Manageiro et al., 2012). Positive controls were used in all PCR reactions. PCR products were purified with ExoSAP IT (USB Corporation, Cleveland, OH), and further sequenced directly, on both strands, using the automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK).

The inferred amino acid sequences of QRDR-encoding genes were compared with the corresponding regions of *E. coli* K-12 (GenBank accession no. AP012306) and reference strain LT2 (GenBank accession no. AE006468) for *E. coli* and *Salmonella* isolates, respectively.

2.4. Transfer of resistance

Transferability of the PMQR determinants was performed by broth mating-out assays using recipient strains *E. coli* C600 Rif^R, Str^R and *E. coli* J53 $NaN3^R$. Resistant *E. coli*

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