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Short communication

# High diversity of genes and plasmids encoding resistance to third-generation cephalosporins and quinolones in clinical *Escherichia coli* from commercial poultry flocks in Italy

Giulia Niero[a], Valeria Bortolaia[b,1], Michele Vanni[c], Luigi Intorre[c], Luca Guardabassi[b,2], Alessandra Piccirillo[a,*,2]

[a] Department of Comparative Biomedicine and Food Science, University of Padua, 35020 Legnaro, PD, Italy
[b] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark
[c] Department of Veterinary Sciences, University of Pisa, 56124, San Piero a Grado, PI, Italy

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ABSTRACT

The aim was to investigate occurrence and diversity of plasmid-mediated resistance to third-generation cephalosporins (3GC) and quinolones in clinical *Escherichia coli* from 200 industrial poultry farms across Italy. *E. coli* was isolated from colibacillosis lesions in turkeys ($n$ = 109), broilers ($n$ = 98) and layers ($n$ = 22) between 2008 and 2012. 3GC-resistant isolates were screened for extended-spectrum and AmpC β-lactamase (ESBL/AmpC), while all isolates were tested for plasmid-mediated quinolone resistance (PMQR) genes. ESBL/AmpC- and PMQR-positive isolates were typed by pulsed-field gel electrophoresis and antimicrobial susceptibility testing, and their plasmids were characterised by replicon typing, multilocus sequence typing, restriction fragment length polymorphism and conjugation. EBSL/AmpC genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}12}$ and $bla_{CMY\text{-}2}$) were detected in 7%, 9% and 4% of isolates from turkeys, broilers and layers, respectively. We identified seven ESBL/AmpC-encoding plasmid types, usually conjugative (78%), with a marked prevalence of IncI1/pST3 plasmids carrying $bla_{CTX\text{-}M\text{-}1}$. PMQR occurred less frequently among isolates from turkeys (0.9%) compared to those from broilers (5%) and layers (4%). The PMQR genes *qnrS*, *qnrB19* and *oqxA/B* were located on three plasmid types and two non-typeable plasmids, mostly (85%) conjugative. ESBL/AmpC- and PMQR-positive isolates were genetically unrelated and 64% of them were additionally resistant to aminoglycosides, sulfonamides and tetracyclines. Our data show that 3GC- and quinolone-resistant clinical *E. coli* in Italian poultry production represent a highly diverse population often resistant to most antimicrobials available for poultry. These findings underline the crucial need to develop new strategies for prevention and control of colibacillosis.

## 1. Introduction

Colibacillosis is an avian disease caused by *Escherichia coli* and characterised by high morbidity, high mortality, reduced productivity and carcass condemnation. Antimicrobial treatment of infected flocks is crucial to prevent major economic losses to the poultry industry (Nolan et al., 2013). Thus, the spread of antimicrobial resistance in clinical *E. coli* from colibacillosis has important economic and animal health implications. Moreover, there is an increasing public health concern about zoonotic transmission of resistance to critically important antimicrobial classes such as third-generation cephalosporins (3GC) and fluoroquinolones (EFSA & ECDC, 2016). Both types of resistance can be mediated by conjugative plasmids that are transferrable across animal and human *E. coli* lineages (Carattoli, 2013).

Previous studies have evidenced high geographical variability in the occurrence of plasmid-mediated resistance to 3GC and fluoroquinolones in clinical *E. coli*, mainly broiler isolates (Briñas et al., 2005; Cerquetti et al., 2009; Yuan et al., 2009; Ahmed et al., 2013; Qabajah et al., 2014; Yang et al., 2014; Meguenni et al., 2015; Solà-Ginés et al., 2015; Awad et al., 2016; da Silva et al., 2017). Such variability likely reflects local antimicrobial usage practices as well as methodological differences between the studies. The same studies have also shown geographical differences in the distribution of ESBL/AmpC and plasmid-mediated quinolone resistance (PMQR) genes.

* Corresponding author at: Department of Comparative Biomedicine and Food Science, University of Padua, viale dell'Università, 16, 35020 Legnaro (PD), Italy.
*E-mail address:* alessandra.piccirillo@unipd.it (A. Piccirillo).
[1] Present address: Research Group for Genomic Epidemiology, Technical University of Denmark, National Food Institute, DK-2800 Kgs. Lyngby, Denmark.
[2] These authors contributed equally as senior authors.

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Taken together, these studies indicate the need to study the epidemiology of ESBL/AmpC and PMQR genes locally to gather information relevant for both veterinary therapy and public health. The aim of this study was to investigate occurrence and diversity of plasmid-mediated resistance to 3GC and quinolones in clinical *E. coli* from broilers, layers and turkeys from industrial poultry farms in Italy.

## 2. Materials and methods

### 2.1. Bacterial isolates

Bacterial isolates were obtained from the private diagnostic laboratories of the 200 poultry farms involved in the study. The farms were located in northern Italy which is the most densely populated poultry area (DPPA) in the country and were representative of the main poultry production in this country. Swab samples from lesions consistent with colisepticemia (*e.g.* pericarditis, perihepatitis or airsacculitis) were collected from diseased birds between 2008 and 2012. The strain collection consisted of 229 clinical *E. coli* from individual birds, of which 109 were from 89 turkey farms, 98 were from 91 broiler farms, and 22 were from 20 layer hen farms. The majority of isolates derived from flocks raised at different farms. In few cases, isolates were collected from different flocks raised at the same farm at different times (2 isolates/10 farms and 3/3 for turkeys, and 2/3 for broilers) and from the same flocks sampled at different times (2 isolates/5 flocks for turkeys, 2/2 and 3/1 for broilers, and 4/2 for layers). All the isolates were stored in 20% glycerol at -80 °C prior to phenotypic and genotypic characterization.

### 2.2. Detection and identification of ESBL/AmpC genes

All isolates were screened for 3GC resistance by disk diffusion using cefpodoxime (10 µg), ceftazidime (30 µg) and cefotaxime (30 µg) (CLSI, 2013). All disks were purchased from DID (Italy). Isolates resistant to at least one 3GC were subjected to PCR for $bla_{TEM}$, $bla_{CTX-M}$, $bla_{SHV}$ and $bla_{CMY-2}$ using primers and conditions as previously described (Dierikx et al., 2012). $bla_{CTX-M}$-positive isolates were further tested with the group- and variant-specific primers for $bla_{CTX-M-1}$, $bla_{CTX-M-2}$ (Dierikx et al., 2012), $bla_{CTX-M-8}$ (Eller et al., 2013), $bla_{CTX-M-9}$ (Bouallègue-Godet et al., 2005), $bla_{CTX-M-14/17}$ (Dierikx et al., 2012) and $bla_{CTX-M-25}$ (Chmelnitsky et al., 2005). Amplicons were sequenced (Macrogen Europe, The Netherlands). Sequence data were analysed using CLC Main Workbench 6.8.4 (CLC Bio, Denmark). Nucleotide sequences and derived amino acid sequences were compared with publicly available sequences (www.ncbi.nlm.nih.gov/ and www.lahey.org/studies/webt.html).

### 2.3. Detection and identification of PMQR genes

All isolates were screened by PCR with primers and conditions used in a previous study (Dotto et al., 2014) for *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxA*, *oqxB* and those suggested by Kim et al. (2009) for *aac(6′)-Ib-cr*. Amplicons were sequenced and analysed as mentioned above.

### 2.4. Plasmid characterisation

Plasmid DNA was isolated from isolates positive for ESBL/AmpC and PMQR genes by the alkaline extraction method (Birnboim and Doly, 1979) and transformed into electrocompetent Genehog *E. coli* (Invitrogen, Denmark). Transformants were selected on Brain Heart Infusion agar (Oxoid, Denmark) supplemented with 1 µg/mL of cefotaxime (CTX) for selection of ESBL/AmpC-positive transformants or 0.06 µg/mL of ciprofloxacin (CIP) for selection of PMQR-positive transformants. The presence of the relevant ESBL/AmpC and PMQR genes was confirmed by PCR using the primers described above. S1 nuclease-digested genomic DNA from the transformants was used to determine plasmid number and size by pulsed field gel electrophoresis (PFGE) (Barton et al., 1995).

Plasmids from transformants were typed by PCR-based replicon typing (PBRT) using a commercial kit (Diatheva, IT) and, if typeable, by plasmid multi-locus sequence typing (pMLST) (https://pubmlst.org/plasmid/). The same approach was used to analyse plasmid content of the clinical *E. coli* isolates that were used as donors for the transformants. Furthermore, plasmid DNA from transformants was typed by restriction fragment length polymorphism (RFLP). Plasmid DNA was isolated using PureLink® HiPure Plasmid Midiprep Kit (Invitrogen, Denmark) and digested (on separate digestions) with *Bgl*II and *Pst*I for IncI1-Iγ plasmids, *Ecor*V and *Pst*I for IncK plasmids, *Pst*I, *Sal*I and *Ecor*I for IncX1 plasmids. All restriction enzymes were purchased from ThermoScientific (Sweden). Restriction profiles were visualised on 0.8% agarose gels and band patterns were compared by visual inspection. Plasmids showing indistinguishable profiles were designated with the same capital letter (*e.g.* 'A') and a number was added to indicate closely related subtypes (*e.g.* A1, A2, A3) differing by one or two bands.

To detect co-transfer of resistance to antimicrobials other than β-lactams and/or quinolones, transformants were tested by disk diffusion for susceptibility to chloramphenicol (30 µg), florfenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), sulfamethoxazole-trimethoprim (25 µg), tetracycline (30 µg), streptomycin (10 µg) and sulfonamides (30 µg) according to CLSI guidelines (2013). Human CLSI breakpoints were used for compounds without established veterinary breakpoints (2013).

Plasmid conjugative transfer was tested by filter-mating experiments using a rifampicin-resistant, lactose-negative *E. coli* J62-2 strain as recipient. Transconjugants were selected on MacConkey agar (Merck, Denmark) supplemented with 2 µg/mL CTX and 25 µg/mL rifampicin (for selection of ESBL/AmpC-positive transconjugants) or 0.06 µg/mL CIP and 25 µg/mL rifampicin (for selection of PMQR-positive transconjugants). Transfer of the relevant genes was confirmed by PCR as detailed above.

### 2.5. Strain typing

ESBL/AmpC- and PMQR-positive *E. coli* were characterised by XbaI-PFGE as previously described (Ribot et al., 2006). *Salmonella enterica* serovar Braenderup H9812 was used as molecular size marker. PFGE profiles were analysed with GelCompar II version 6.6.11 (Applied Maths, Belgium) using the Dice similarity coefficient and clustered by the unweighted pair group method with arithmetic averages. Band optimisation and position tolerance were set at 1%. Isolates were considered related if the Dice similarity index was ≥ 80%.

The same isolates were also tested by disk diffusion according to the CLSI (2013) guidelines. The following disks (Oxoid, UK) were used: ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), florfenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), sulfamethoxazole-trimethoprim (25 µg), streptomycin (10 µg), sulfonamides (30 µg) and tetracycline (30 µg).

## 3. Results

### 3.1. Occurrence of ESBL/AmpC and PMQR genes

ESBL/AmpC genes were detected in 18 (8%) isolates (Table 1) with a prevalence of 7%, 9% and 4% among isolates from turkeys, broilers and layers, respectively. While $bla_{CTX-M-1}$ was found in 12 isolates from all types of poultry production, $bla_{CTX-M-14}$, $bla_{CTX-M-2}$, $bla_{SHV-12}$ and $bla_{CMY-2}$ were sporadically detected (Table 1). More than half ($n$ = 11) of the ESBL/AmpC-positive isolates additionally harboured $bla_{TEM-1b}$ (Table 1).

PMQR genes were identified in seven (3%) isolates with a prevalence of 0.9%, 5% and 4% among isolates from turkeys, broilers and layers, respectively (Table 1). *qnrS1* was detected in four and one