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High-virulence CMY-2- and CTX-M-2-producing avian pathogenic *Escherichia coli* strains isolated from commercial turkeys

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ABSTRACT

This study reports the high-virulence phylogenetic backgrounds of CMY-2- and CTX-M-2-producing avian pathogenic *Escherichia coli* strains isolated from turkeys sent to slaughter and condemned by airsacculitis in Brazil. Among 300 air sac samples, seven *E. coli* strains produced plasmid-mediated CMY-2-type AmpC, of which three carried also the $bla_{CTX\text{-}M\text{-}2}$ Extended Spectrum Beta-Lactamase encoding gene. Interestingly, the transfer of the $bla_{CMY\text{-}2}$ gene was positive for three *E. coli* strains, being associated with the presence of IncI1 plasmids. The complete sequence of the representative pJB10 plasmid revealed that the $bla_{CMY\text{-}2}$ gene was within a transposon-like element in the classical genetic environment consisting of *tnpA*-$bla_{CMY\text{-}2}$-*blc*-*sugE* structure. This plasmid with 94-kb belonged to the *sequence type* (ST) 12 among IncI1 plasmids, which has been associated with the worldwide spread of $bla_{CMY\text{-}2}$ among *Salmonella enterica* and *E. coli*. Furthermore, to the best of our knowledge, this is the first complete sequence of a CMY-2-encoding plasmid derived from an *Escherichia coli* isolated from food-producing animals in Latin America.

1. Introduction

Escherichia coli plays a key role in the spread of plasmid-mediated AmpC and extended-spectrum beta-lactamase (ESBL)-coding genes, which is a global challenge for human and veterinary medicine (Philippon et al., 2002; Woodford et al., 2011). Recently, food-producing animals have been described as an important reservoir for CTX-M- and CMY-2-producing pathogens with zoonotic potential (Trott, 2013).

Clinical presentation of *E. coli* infection in poultry are airsacculitis, perihepatitis, pericarditis, salpingitis, omphalitis, cellulitis, swollen head syndrome, and sepsis. The subpathotype of extraintestinal pathogenic *Escherihica coli* (ExPEC) that infects poultry is known as avian pathogenic *E. coli* (APEC) and is considered a heterogeneous group of pathogens with a broad range of virulence characteristics. Recently, several studies have shown that some APEC clones are very similar to extraintestinal pathotypes that affect humans: for this reason, some authors pointed to poultry as a reservoir for human ExPEC, suggesting a risk to public health (Cunha et al., 2014).

Here we describe for the first time the occurrence of high-virulence *E. coli* producing CMY-2 and CTX-M-2 enzymes in commercial turkeys in Brazil. In these strains, the $bla_{CMY\text{-}2}$ gene was associated with a transposon-like element located in a 94 kb IncI1 plasmid.

2. Material and methods

In 2012, a total of 300 turkeys condemned by airsacculitis were screened for the presence of Avian Pathogenic *Escherichia coli* (APEC). Initially, a single swab was collected from each air sac at the slaughterhouse and cultured in Brain Heart Infusion broth for 18 hours. Subsequently, a total of 227 *E. coli* strains were isolated in MacConkey agar plates and screened for ESBL and/or AmpC production. The antimicrobial susceptibility for ceftiofur, cefoxitin, cotrimoxazole, nalidixic acid, enrofloxacin, norfloxacin, streptomycin, amikacin, gentamicin, florfenicol and tetracycline was evaluated by disc diffusion (CLSI, 2014). Afterwards, ESBL/AmpC-like coding genes were identified for cephalosporin-resistant isolates by PCR using the primers $bla_{CTX\text{-}M}$ (F: 5′-TTTGCGATGTGCAGTACCAGTAA-3′; R: 5′-CGATATCGTTGGTGGTGCCATA-3); $bla_{CTX\text{-}M\text{-}1\text{-}group}$ (5′-GACGATGTCACTGGCTGAGC-3′; R: 5′-AGCCGCCGA CGCTAATACA-3′); $bla_{CTX\text{-}M\text{-}2\text{-}group}$ (F: 5′ GCGACCTGGTTAACTACAATCC-3′; R: 5′ CGGTAGTATTGCCCTTAAGCC-3′); and $bla_{CMY\text{-}2\text{-}group}$ (F: 5′ AACACACTG ATTGCGTCTGAC-3′; R: 5′ CTGGGCCTCATCGTCAGTTA-3′) (Lewis et al., 2007; Pérez-Pérez and Hanson, 2002). The minimal inhibitory concentrations (MICs) were determined by agar and/or micro dilution using

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Sensititre -ESBL Confirmatory MIC plate (TREK Diagnostic Systems-Thermo Scientific) and/or agar dilution method (CLSI, 2014). The control quality *Escherichia coli* ATCC® 35,218 and *Escherichia coli* ATCC® 25,922 strains were used in all susceptibility tests according to CLSI (2014) guidelines. The genetic context of the CMY-2-encoding gene was determined by PCR (Cejas et al., 2014). Next, plasmids were extracted and transformed into chemically competent TOP10 *E. coli* recipient cells (Invitrogen™) according to the protocol previously described (Inoue et al., 1990). The transformant *E. coli* TOP10 strains were selected in Mueller-Hinton agar plates supplemented with 4 μg/mL cefoxitin, and further analyzed by PCR-based replicon typing (Carattoli et al., 2005). The acquisition of the bla_{CMY-2} gene was confirmed by PCR and MIC determination for the transformant strains. The representative plasmid pJB10 extracted from 10.4.1-T transformant strain was completely sequenced using MiSeq Illumina platform (Illumina, San Diego, CA, USA) generating 198.867 paired-end reads (300 bp read length) with a total coverage ~500X and *de novo* assembly was performed using Spades software v3.9.0 and Geneious 8.0.5 (Biomatters Ltd., Auckland, New Zealand) for further analysis, sizing and characterization of bla_{CMY-2} genetic context and pMLST (http://pubmlst.org/plasmid/). The complete sequence of the pJB10 plasmid was deposited in GenBank under accession number KX452392. APEC isolates were further characterized by phylogenetic grouping (Clermont et al., 2000) and virulence factors (*papC*, *tsh*, *iroN*, *iucD*, *irp-2*, *iss*, *cvi/cva*, *neuS*, *ibeA*, *vat*, *astA*, *aggR*) (Cunha et al., 2014). Finally, the genetic relatedness of isolates was evaluated by enterobacterial repetitive intergenic consensus (ERIC)-PCR (Syrmis et al., 2004).

3. Results and discussion

In this study, the identification of CMY-2 pAmpC in avian pathogenic *Escherichia coli* from turkeys condemned by airsacculitis in Brazil is reported for the first time. Among 227 *Escherichia coli* strains screened for plasmid-mediated AmpC and ESBL production, eight (3.52%) were resistant to cephalosporins, remaining susceptible to amikacin, gentamicin, piperacillin/tazobactam and carbapenems (Table 1). In fact, the higher MIC of cefoxitin (MIC ≥64) and ceftiofur (MIC ≥256) were associated with the presence of CMY-2- and CTX-M-2-encoding genes (Table 2), respectively. The bla_{CMY-2} gene was successfully transferred from three donors to the *Escherichia coli* Top10 recipient strain, being associated with IncI1 plasmids. The complete sequence of the representative plasmid pJB10 confirmed that the bla_{CMY-2} gene was within a transposon-like structure consisting of *tnpA*-bla_{CMY-2}-*blc*-*sugE*. Furthermore, the pJB10 plasmid contained 94 kilobases and belonged to the ST12 among IncI1 incompatibility group, which has been widely contributed to the spread of bla_{CMY-2} among *Enterobacteriaceae*.

For a long time, description of pAmpC among members of the *Enterobacteriaceae* family was restricted to inpatients, especially among *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* strains, which was later the first carrier of a plasmid-mediated bla_{AmpC} gene cassette, named MIR-1 (Papanicolaou et al., 1990; Philippon et al., 2002.). The increasing prevalence of pAmpC in hospitals and their emergence in the community, pets, environment and food production have identified these beta-lactamases as a global threat, which appears to be much more worrisome than initially thought (Mataseje et al., 2010; Guo et al., 2014, Pavez et al., 2008). In this regard, the CMY-2 variant is the most prevalent and worldwide-distributed pAmpC enzyme and favors carbapenem-resistance in porin-deficient strains (Mammeri et al., 2010).

In Brazil, after the bla_{CMY-2} had been first identified among *Salmonella* Heidelberg strains (Peirano et al., 2006), CMY-2-producing *E. coli* strains with carbapenem-resistant profiles were isolated from blood cultures from a 46-year-old man that died as a result of sepsis (Pavez et al., 2008). More recently, Botelho et al. (2015) recovered CMY-2-producing *E. coli* from frozen chicken carcasses at retail markets, two strains of which carried both CMY-2 and CTX-M-like determinants. Similarly, three CMY-2-positive strains from turkeys produced CTX-M-2 ESBL, which was also identified in the strain identified in this study as 6.22.1. Although CTX-M-2-producing *E. coli* had not been detected to date from Brazilian turkeys, its dissemination in chicken production and related products is well documented, indicating that bacteria present at farm and slaughterhouse can persist in final product (Warren et al., 2008). A European surveillance study a few years ago found the alarming presence of CTX-M-2-positive *E. coli* in chicken meat imported from Brazil, whose recurrence has been confirmed (Warren et al., 2008; Botelho et al., 2015). As a result, turkeys now constitute a new reservoir of CTX-M-2 producers.

Much more concerning is the association of resistant-phenotypes with high invasive potential. In fact, some putative virulence genes are implicated in the pathogenicity of APEC, although a high heterogeneity in the virulence traits exists in strains isolated from sick and healthy birds (Dziva and Stevens, 2008). In this context, airsacculitis is the most common injury associated with colibacillosis in commercial birds. This leads to severe economic losses for the worldwide poultry industry because the air sac disease is often followed by the systemic spread of APEC, resulting in perihepatitis, pericarditis and sepsis.

Cunha et al. (2014) investigated the virulence profiles of APEC isolated from turkeys with airsacculitis and showed a predominance of B2 phylogroup, with virulence genes associated with iron uptake

Table 1
Minimal Inhibitory Concentration of CMY-2- and CTX-M-2-producing avian pathogenic *Escherichia coli* isolated from turkey production, *E. coli* TOP10 (transformed) and *E. coli* TOP10.

Strains	Minimal inhibitory concentration (mg/L)*																
	TAZ	FAZ	FEP	FOX	CEP	POD	FOT	AXO	CFT	IMI	MERO	GEN	AMP	CIP	P/TZ	T/C	F/C
3.15.1	16	≥16	≤1	≥64	≥16	≥32	8	32	16	≤0.5	≤1	≤4	≥16	2	≤4/4	8/4	8/4
TOP10 3.15.1-T**	16	≥16	≤1	≥64	≥16	≥32	8	64	16	≤0.5	≤1	≤4	≥16	≤1	≤4/4	8/4	8/4
3.2.1	8	≥16	≤1	64	≥16	≥32	8	8	32	≤0.5	≤1	≤4	≥16	≥4	≤4/4	8/4	4/4
3.23.1	32	≥16	≤1	≥64	≥16	≥32	16	32	32	≤0.5	≤1	≤4	≥16	2	8/4	32/4	8/4
3.38.1	16	≥16	≤1	64	≥16	≥32	8	8	32	≤0.5	≤1	≤4	≥16	≤1	≤4/4	4/4	8/4
4.23.1	≤0.25	≥16	≤1	64	≥16	2	≤0.25	≤1	1	≤0.5	≤1	≤4	≥16	≤1	≤4/4	1/4	0.5/4
6.22.1	4	≥16	≥32	≤4	≥16	≥32	≥64	≥128	≥256	≤0.5	≤1	≤4	≥16	≤1	≤4/4	0.25/4	≤0.12/4
10.4.1	16	≥16	≤1	≥64	≥16	≥32	16	16	8	≤0.5	≤1	≤4	≥16	2	≤4/4	8/4	8/4
TOP10 10.4.1-T	16	≥16	2	≥64	≥16	≥32	16	16	8	≤0.5	≤1	≤4	≥16	≤1	≤4/4	8/4	8/4
10.10.1	16	≥16	≤1	≥64	≥16	≥32	16	16	8	≤0.5	≤1	≤4	≥16	2	≤4/4	8/4	8/4
TOP10 10.10.1-T	16	≥16	≤1	≥64	≥16	≥32	16	16	8	≤0.5	≤1	≤4	≥16	≤1	≤4/4	8/4	8/4
***E. coli* TOP10**	0.5	≤8	≤1	≤4	≤8	1	≤0.25	≤1	0.5	≤0.5	≤1	≤4	≤8	≤1	≤4/4	0.5/4	≤0.12/4

* Faz = Cefazolin; TAZ = Ceftazidime; FEP = Cefepime; FOX = Cefoxitin; CEP = Cephalothin; POD = Cefpodoxime; FOT = Cefotaxime; AXO = Ceftriaxone; CFT = ceftiofur; IMI = Imipenem; MERO = Meropenem; GEN = Gentamicin; AMP = Ampicillin; CIP = Ciprofloxacin; P/TZ = Piperacillin/tazobactam; T/C = Ceftazidime/clavulanic acid; F/C = Cefotaxime/clavulanic acid.

** TOP10 3.15.1-T, TOP10 10.4.1-T and TOP10 10.10.1-T, transformed *E. coli* TOP10.