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Diversity of plasmids harboring bla_{CMY-2} in multidrug-resistant *Escherichia coli* isolated from poultry in Brazil

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ABSTRACT

Multidrug-resistance (MDR) has been increasingly reported in Gram-negative bacteria from the intestinal microbiota, environment and food-producing animals. Resistance plasmids able to harbor different transposable elements are capable to mobilize antimicrobial resistance genes and transfer to other bacterial hosts. Plasmids carrying bla_{CMY} are frequently associated with MDR. The present study assessed the presence of plasmid-encoded *ampC* genes (bla_{cmy}, bla_{mox}, bla_{fox}, bla_{lat}, bla_{act}, bla_{mir}, bla_{dha}, bla_{mor}) in commensal *E. coli* isolated from apparently healthy broiler chickens. Furthermore, we characterized the plasmids and identified those harboring the resistance genes. We isolated 144/200 (72%) of *E. coli* isolates with resistance to cefotaxime and the resistance gene identified was bla_{CMY-2}. The pulsed-field gel electrophoresis (PFGE) analysis showed high diversity of the genetic profiles. The phylogenetic groups A, B1, B2, and D were identified among *E. coli* isolates and group D was the most prevalent. The PCR-based replicon typing (PBRT) analysis identified four distinct plasmid incompatibility groups (Inc) in MDR isolates. Moreover, plasmids harboring bla_{CMY-2}, ranged in size from 50 kb to 150 kb and 51/144 (35%) belonged to IncK, 21/144 (14.5%) to IncB/O, 8/144 (5.5%) to IncA/C, 1/144 (0.5%) to IncI, while 63/144 (44.5%) were not typeable by PBRT. Overall, a high prevalence of bla_{CMY-2} genes was found in a diverse population of commensal MDR *E. coli* from poultry in Brazil, harbored into different plasmids.

1. Introduction

The use of antibiotics for prevention or treatment of gastrointestinal infections and as growth promoters in food-producing animals results in selective pressure for commensal microbiota and pathogens in the gut environment. Commensal *Escherichia coli* have shown a high capability to acquire and carry genes and mobile genetic elements (MGE) involved in antimicrobial resistance. Moreover, commensal *E. coli* also have the ability to harbor resistance genes and disseminate to other bacteria (da Costa et al., 2013).

Overexpression of intrinsic chromosomal *ampC* gene and high levels of AmpC protein may confer resistance to penicillin, third generation cephalosporins, β-lactamase inhibitor associated with β-lactams and cephamycins (Pfeifer et al., 2010). In *E. coli*, increased expression of the intrinsic *ampC* gene depends on mutations of promoter genes (Pfeifer et al., 2010). However,the extended spectrum resistance to cephalosporins generally occurs due to extended spectrum β-lactamase (ESBL) production or acquisition of plasmid-borne *ampC* β-lactamase (pAmpC) genes (Pfeifer et al., 2010). pAmpC have been isolated in *E. coli* and *Salmonella* from food-producing animals in many countries, becoming well adapted to these bacterial reservoirs (Jacoby, 2009; Liebana et al., 2013). In Brazil, bla_{CMY-2} gene is rarely identified in human clinical isolates (Rocha et al., 2015), and was never reported in live food-producing animals, only in retail poultry meat (Botelho et al., 2015).

The survival of *E. coli* during antimicrobial therapy can occur by the complex interaction of different mechanisms that confer resistance to different classes of antibiotics at the same time. This mechanism include drug efflux pump, enzymatic degradation of the antibiotic (*e.g.* β-lactamases) or protection of antimicrobial target protein (type II DNA topoisomerases) from quinolones, by *qnr* genes proteins (Rodriguez-Martinez et al., 2011; Szmolka and Nagy, 2013).

Resistance genes involved with enzymatic inactivation are frequently associated with MGE (Ferreira et al., 2016). Thus, the transferability capacity is higher, which facilitates the dissemination of resistance among *E. coli* and even other *Enterobacteriaceae* (Liebana et al., 2013).

Among the MGE, plasmids have been described as the most efficient tool involved in the acquisition and dissemination of antimicrobial resistance genes in *Enterobacteriaceae*. The role of plasmids in the

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dissemination and maintenance of resistance genes among multidrug-resistant bacteria (MDR) has been increasingly demonstrated by different studies (Fernandez-Alarcon et al., 2011; Hiki et al., 2013; Li et al., 2007). These elements are well adapted to the respective bacterial hosts. Conveniently selected by the progeny due to the abusive use of antimicrobials, resistance plasmids interfere on the efficacy of therapies, hampering the control of MDR bacteria (Canton and Ruiz-Garbajosa, 2011; Carattoli, 2013; Livermore, 2012). Different families of plasmids have been identified, although epidemiological data shows a variable frequency of dissemination. Some families, such as IncI1, IncHI1, IncN and IncA/C have been associated to MDR pathogens. Their efficient conjugative system and broad host range, contribute to the dissemination in commensal and pathogenic bacteria (Carattoli, 2013; Liebana et al., 2013).

Thus, the present study assessed the presence of extended spectrum cephalosporin-resistant *E. coli*, pAmpC genes, determined the size and Inc. group of plasmids-carrying *ampC* genes and evaluated the population structure of pAmpC-producing *E. coli* in the commensal microbiota of apparently healthy broiler chickens.

2. Material and methods

2.1. Isolates

Two-hundred cloacal swabs were obtained from commercial broilers in two poultry farms from São Paulo State, Brazil, from 2011 to 2012. Cloacal swab samples were streaked on MacConkey (MC) agar containing cefotaxime (1 μg/mL) and on MC agar with ceftazidime (1 μg/mL), incubated at 37 °C for 24 h. One colony from each plate containing cefotaxime was selected to conduct the present study. The bacterial colonies were identified by classical biochemical methods and confirmed by API 20E system (bioMérieux, France).

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolates were determined by using the disk diffusion methods (CLSI, 2012), and the results were interpreted according to recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2013), document M100-S23. Fifteen antimicrobial agents were tested, including β-lactam antibiotics: amoxicillin-clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), aztreonam (ATM), ertapenem (ETP) and, non β-lactam antibiotics: nalidixic acid (NAL), ciprofloxacin (CIP), levofloxacin (LEV), tetracycline (TET), gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT) and chloramphenicol (CHL).

2.3. Pulsed-field gel electrophoresis (PFGE) and phylogenetic analysis

Genetic relationship among isolates was determined using analysis of *Xba*I-digested genomic DNA followed by PFGE, performed in CHEF DRIII System (Bio-Rad, USA), as previously described (CDC, 2004). Profiles were analyzed with the BioNumerics fingerprinting software v 5.0 (Applied Maths, Belgium). The normalized profiles were compared using the Dice similarity coefficient and the dendrogram was constructed with the unweighted-pair group method using arithmetic average linkage algorithm (UPGMA). The homology cutoff value of 85% was used to group the related isolates within the same PFGE-type.

The phylogenetic groups were assigned by PCR, according to previously described method (Clermont et al., 2000). Briefly, this method characterizes the phylogenetic groups (A, B1, B2, or D) of each *E. coli* isolate based on the presence of *chuA*, *yjaA* genes and TSPE4.C2 DNA fragment.

2.4. Detection of plasmid-mediated ampC genes

The investigation of bla_{cmy}, bla_{mox}, bla_{fox}, bla_{lat}, bla_{act}, bla_{mir}, bla_{dha}, bla_{mor}, genes was carried out by PCR (D'Andrea et al., 2006). Purified PCR amplicons (illustra™ GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare, USA) were directly sequenced using the ABI 3730 DNA Analyzer (Life Technologies-Applied Biosystems). The DNA sequences and translated amino acid sequences obtained were compared with reference sequences from the LAHEY home page (http://www.lahey.org/Studies/).

2.5. Characterization of plasmid replicon typing and genomic localization

After PCR and DNA sequencing, isolates carrying pAmpC genes were selected for investigation and characterization of resistance plasmids. PCR-based replicon typing (PBRT) method was used as previously described (Carattoli et al., 2005) to search for major plasmid incompatibility (Inc) groups among field *E. coli* isolates. Plasmid DNA was digested with *S1* nuclease and analyzed on PFGE gels (*S1*-PFGE). Southern blot and hybridizations were performed as described previously (Sambrook et al., 1989) using specific probes to locate the plasmid carrying the resistance gene.

2.6. Conjugation experiments

Transferability of plasmids carrying *amp*C β-lactamase genes was determined by conjugation using as recipient strain the *E. coli* K12 C600, which is streptomycin resistant, lactose negative, and plasmid free. Transconjugants were selected on MacConkey agar containing 2 μg/mL of cefotaxime and 300 μg/mL of streptomycin. The presence of acquired *amp*C genes in the transconjugants was confirmed by PCR. Inc. groups of resistance plasmids from transconjugants were assigned using the PBRT method.

3. Results and discussion

Surveillance of antimicrobial resistance in commensal *Enterobacteriaceae* has a critical impact to evaluate the presence and the prevalence of MDR bacteria and resistance genes in the microbiota of food-producing animals (Szmolka and Nagy, 2013). The inappropriate use of antimicrobials in food-producing animals concerns the food safety authorities. Commensal bacteria found in gastrointestinal tract of farm animals may cause extraintestinal infections or serve as reservoirs for resistance genes that could potentially be transferred to pathogenic organisms. The concept of "farm-to-fork" involves the risk of dissemination of pathogens through the food chain (Liebana et al., 2013). Although there is little evidence reported up to now (Huijbers et al., 2014), this concept may also be applicable to commensal MDR bacteria considering the increasing prevalence found in livestock. MDR bacteria present in raw meat and even processed food may contaminate humans through handling and consumption of these products, offering risk to public health when colonizing the community or causing foodborne infections (Botelho et al., 2015; Landers et al., 2012).

In the present study, 144 *E. coli* isolates resistant to cefotaxime (CTX) were obtained from the culture of 200 different samples of cloacal swabs. Additionally, isolates resistant to CTX also showed resistance to other β-lactams tested, including 100% (144/144) resistance to AMC and 84% (121/144) to CAZ. Resistance to FOX was present in 90% (130/144) and to ATM was found in 55% (80/144) of the isolates. However, only 4% (6/144) of these isolates showed resistance to TZP and 100% were susceptible to FEP and ETP. Furthermore, 99% (143/144) of the isolates were also resistant to the non-beta-lactam antibiotics NAL and CIP, 97% (140/144) to LEV, 75% (108/144) to TET, 50% (73/144) to GEN, 35% (50/144) to SXT and 24% (34/144) to CHL (Table 1).

Thus, all 144 isolates were considered MDR, not-susceptible to at least one agent in three or more antimicrobial categories (Magiorakos

Table 1
Characteristics of the CMY-2 producing *E. coli* isolates from commercial broiler chickens from Brazil.

Replicon type carrying bla_{cmy-2}	Isolates (n)	Phylogenetic group (n)				Resistance β-lactams (n)						Resistance non β-lactams (n)							Clusters
		A	B1	B2	D	CTX	CAZ	AMC	FOX	ATM	TZP	NAL	CIP	LEV	GEN	TET	SXT	CHL	
K	51	20			31	51	39	51	40	26	1	51	51	49	28	33	8	4	A,B,C,F,H,I,K,M,N,O,P
B/O	21		6		15	21	21	21	21	20	2	21	21	21	8	4			F,H,I
A/C	8			8		8	8	8	8	4	2	8	8	8		8	6	8	A,D,E,F,J,S
I	1				1	1	1	1	1						1	1			R
NT	63	24		1	38	63	52	63	60	30	1	63	63	61	35	61	31	22	A,B,F,G,H,I,K,L,N,Q,U,T

et al., 2012). Moreover, the PCR and DNA sequencing showed that all these isolates carried the resistance gene bla_{CMY-2}. *E. coli* carrying pAmpC genes are usually co-resistant to other commonly used antimicrobial agents (Liebana et al., 2013). The persistence and dissemination of pAmpC-producing isolates in food-producing animals may be aggravated by the prophilatic use of cephalosporins. Co-resistance phenotypes are also involved in the maintenance of resistance genes and plasmids in *E. coli*, (Gouvêa et al., 2015); thus, other antimicrobials frequently used, such as fluoroquinolones, may also play a role in the selection of MDR isolates in the animal environment (Canton and Ruiz-Garbajosa, 2011; Szmolka and Nagy, 2013).

The distribution of phylogenetic groups has been suggested to be related with health status or geographical region of each host species analyzed (Asai et al., 2011). The *E. coli* phylogenetic group D was the most prevalent, containing 60% (87/144) of the isolates, 30% (43/144) were assigned to the phylogenetic group A, 6% (8/144) of the isolates belonged to phylogenetic group B2 and 4% (6/144) belonged to phylogenetic group B1 (Table 1). Similar results were previously described on *E. coli* harboring bla_{CMY-2} isolated from retail chicken meat in Brazil (Botelho et al., 2015). Phylogenetic group D and B2 are associated virulent extra-intestinal *E. coli* (Clermont et al., 2000). Thus, MDR *E. coli* harboring bla_{CMY-2} from both these phylogenetic groups may have the capacity to cause extra-intestinal infections, representing higher risk, in such cases when treatment is difficult.

The clonal dissemination of antimicrobial resistance has been reported, highlighting the capability of resistant strains to prevail in different environments, especially communities or hospitals (Liebana et al., 2013). However, in the present work, among 144 *E. coli* isolates carrying bla_{CMY-2}, 75 different PFGE-types were found, classified within 21 clusters (Table 1). Thus, we show a non-clonal *E. coli* population carrying the same resistance gene or even the same resistance plasmid (Table 1). The combination of a rich microbiological environment and the selective pressure in current poultry production systems, caused by measures such as the use of antibiotics in the animal feed, might contribute for the successful establishment of a diverse population of MDR bacteria.

Among 144 CTX-resistant isolates, all harbored bla_{CMY-2}, a remarkable high rate of this gene in broilers. In Europe, bla_{CMY-2} is the most prevalent pAmpC gene (Liebana et al., 2013). However, in Brazil, presence of this gene is rarely reported in clinical isolates, thus the continuous surveillance is extremely important to prevent environmental dissemination or maintenance of this resistance gene in hospital bacteria (Campana et al., 2013; Rocha et al., 2015). Recently, this gene was reported in retail chicken meat in Brazil (Botelho et al., 2015; Mattiello et al., 2015). However, the present study reports for the first time the disseminated bla_{CMY-2} in commensal *E. coli* isolates from the microbiota of live healthy poultry in Brazil. Together, these results show an important concern to the public and animal health. The high prevalence of these MDR bacteria in the animal environment and retail meat may represent risk of dissemination to the human environment.

Resistance plasmids carrying bla_{CMY-2} belonged to four distinct Inc. groups corresponding to 51 (35%) from IncK, 21 (14.5%) from IncB/O, 8 (5.5%) from IncA/C and one (0.5%) from IncI, while 63 (44.5%) plasmids were non-typeable by PBRT (Table 1). As shown by the S1-PFGE, plasmids harboring bla_{CMY-2} ranged in size from 50 kb to 150 kb. However, the large size did not prevent the resistance plasmids to be conjugative. These were successfully transferred to *E. coli* K12 C600, conferring CTX resistance to this strain. The conjugative ability of resistance plasmids increases the risk of successful dissemination to other bacterial hosts. Thus, commensal *E. coli* carrying these MGE may be capable to transfer the resistance plasmids to pathogens in the microbiota, such as *Salmonella* (Winokur et al., 2001).

The dissemination of pAmpC gene in both humans and animals has been associated to the IncF, IncI, IncN, IncA/C, IncL/M, and IncK plasmid families (Liebana et al., 2013). Plasmids co-harboring multidrug-resistance determinants are usually large (>50 kb), self-conjugative, and may encode resistance to all main antimicrobial classes used in therapies, including β-lactams, quinolones, fluoroquinolones and tetracyclines. Moreover, these plasmids are highly efficient in acquisition and transmission of most resistance genes (Carattoli, 2013).

The Inc. groups of plasmids identified in this study have been previously associated with CMY-2-producing *Enterobacteriaceae* worldwide (Hiki et al., 2013; Voets et al., 2013), but never described in isolates from Brazil. In 2010, IncI1 and IncK plasmids harboring bla_{CMY-2} were detected in live poultry and hospital patients in Netherlands (Dierikx et al., 2010). The same plasmid replicons were later described in isolates from retail poultry meat in the same country in 2013, suggesting the association between these isolates (Voets et al., 2013). In Japan, the presence of IncB/O carrying bla_{CMY-2} in *E. coli* from livestock animals was recently described for the first time (Hiki et al., 2013). IncA/C plasmid replicon has been associated with the spread of bla_{CMY-2} in *E. coli* and *Salmonella* spp. isolated from humans in the United States (Carattoli, 2009). Furthermore, plasmids harboring bla_{CMY-2} frequently harbor other resistance genes associated to different antimicrobial classes (Hiki et al., 2013), increasing the spectrum of resistance. In Australia, the replicons IncI1 and IncF were identified carrying bla_{CMY-2} in *E. coli*; however, IncI1 was predominant in this country, present in 96% of the isolates (Sidjabat et al., 2014; Tagg et al., 2015). According to our findings, IncK was the most frequent plasmid typeable by PBRT, carrying the gene bla_{CMY-2} in different *E. coli* PFGE-types. These results may suggest a higher conjugation and dissemination capacity of this replicon, and further studies may also identify this replicon in CTX resistant isolates from Brazil.

Plasmid-mediated AmpC in *E. coli* from retail chicken meat was found for the first time in 2015 in Brazil (Botelho et al., 2015). In Europe, Asia and United States, *E. coli* carrying bla_{CMY-2} has already been reported in food-producing animals, however the prevalence in the United States is high, in both food-producing animals and humans (Carattoli, 2008; Hiki et al., 2013; Winokur et al., 2001). In Brazil, there are no reports characterizing the plasmids Inc. groups carrying bla_{CMY-2} in human clinical isolates. In this study, only IncA/C plasmids carrying bla_{CMY-2} were identified in *E. coli* isolates from phylogenetic group B2, usually characterized as the most virulent in comparison with other groups. These findings brought new knowledge involving the dissemination of bla_{CMY-2} in live poultry from farms in Brazil. Although there is little evidence of dissemination through the food chain, farm workers in animal production facilities are exposed to higher risks of contamination by MDR *E. coli* of animal origin, which is another *via* of community colonization (da Costa et al., 2013).

E. coli are efficient hosts, capable to receive and disseminate resistance to antimicrobials, through resistance determinants, associated to MGE that can be horizontally transferred. Furthermore, these bacteria have high potential to harbor and become reservoirs of antimicrobial resistance genes (da Costa et al., 2013). Overall our results show a high rate (72%) of bla_{CMY-2} disseminated by different plasmid replicon types, in a diverse commensal *E. coli* population. These findings, taken together with other recent studies worldwide, demonstrate the disseminated resistance to third generation cephalosporins in apparently healthy food-producing animals, which may impact on reduced therapeutic options and concern the public health due to the environmental contamination by these MDR bacteria.

Conflict of interest statement

None to declare.

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