Diagnostic Microbiology and Infectious Disease 88 (2017) 361–364

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

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

Joseane Cristina Ferreira [a], Rafael Antonio Casarin Penha Filho [b], Leonardo Neves Andrade [a], Angelo Berchieri Junior [b], Ana Lúcia Costa Darini [a,*]

[a] School of Pharmaceutical Sciences of Ribeirao Preto –University of Sao Paulo (USP), Ribeirão Preto, SP, 14040-903, Brazil
[b] School of Agricultural and Veterinary Sciences – São Paulo State University (UNESP), Jaboticabal, SP, 14884-900, Brazil

ARTICLE INFO

Article history:
Received 7 February 2017
Received in revised form 18 April 2017
Accepted 26 April 2017
Available online 3 May 2017

Keywords:
AmpC β-lactamases
PBRT
Enterobacteriaceae
Food-producing animals
Chicken

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

* Corresponding author. Tel.: +55-16-36024291; fax: +55-16-36024725.
E-mail address: aldarini@fcfrp.usp.br (A.L.C. Darini).

http://dx.doi.org/10.1016/j.diagmicrobio.2017.04.014
0732-8893/

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