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Short communication

Detection of *bla*$_{CTX-M}$-type genes in complex class 1 integrons carried by *Enterobacteriaceae* isolated from retail chicken meat in Brazil

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ABSTRACT

CTX-M-type extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* have been increasingly identified in humans and animals, and their potential transmission by contaminated food has been highlighted. In this study, we report for the first time the isolation of multidrug-resistant (MDR) *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* strains harboring *bla*$_{CTXM-2}$ or *bla*$_{CTXM-8}$ gene variants in chicken meat sold in markets in southeast Brazil. In this regard, the genetic environment of the *bla*$_{CTX-M-2}$ gene is composed of a complex class 1 integron and an IS*CR1*-associated sequence with *dfr* and/or *aadA* gene cassettes located within the variable region. In summary, chicken meat may be a reservoir of MDR *Enterobacteriaceae* harboring *bla*$_{CTX-M}$-type genes, which is a public health concern.

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1. Introduction

Healthcare-associated and community-acquired infections caused by extended-spectrum β-lactamases (ESBLs)-producing bacteria have been increasingly reported worldwide (Pitout and Laupland, 2008). Although the predominant ESBL families have been TEM, SHV and CTX-M (Bush and Jacoby, 2010), in the last years, the incidence of the CTX-M-type has risen dramatically (Wellington et al., 2013). In fact, currently the CTX-M-2, CTX-M-8 and CTX-M-15 variants are widely disseminated all over the world within *Enterobacteriaceae* from humans and animals, and their potential transmission by contaminated food, mainly chicken meat, has been highlighted (Egervärn et al., 2014; Kawamura et al., 2014; Kiiru et al., 2012). These enzymes are encoded by *bla*$_{CTX-M}$-type gene associated with mobile genetic elements, including insertion sequences IS*Ecp1* type and IS*CR1*, most carried on transferable plasmids (Zhao and Hu, 2013). In Brazil, although the production of the CTX-M enzymes has become the most prevalent mechanism of acquired resistance to broad-spectrum cephalosporins in gram-negative bacteria from clinical samples, poultry, food-producing animals, hospital wastewater and urban rivers (Aizawa et al., 2014; Carvalho-Assef et al., 2014; Ferreira et al., 2014; Peirano et al., 2011; Silva et al., 2013; Tollentino et al., 2011), there are no data available regarding the detection of CTX-M-producing bacteria in chicken meat intended for sale on the Brazilian market, which is curious, since the presence of CTX-M-2- and CTX-M-8-producing *E. coli* in raw chicken and broiler meat imported into European countries from South America countries, including Brazil, has been previously reported (Dhanji et al., 2010; Egervärn et al., 2014; Warren et al., 2008). So, the aim of this study was to investigate the presence of ESBL-producing bacteria and the genetic background of genes encoding ESBLs in retail chicken meat from markets, in southeastern Brazil.

2. Materials and methods

2.1. Sampling

We investigated the presence of ESBL-producing bacteria in four pork, three beef, and eight chicken meat samples, collected between March 2011 and July 2013 from seven traditional marketplaces in the

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state of São Paulo, south-eastern Brazil. The samples were immediately transported to the laboratory for microbiological analysis. For bacterial isolation, sub samples of 200 g were dispensed on sterile plastic bags (Whirl-Pak, Nasco, WI, USA) containing 200 mL of Tetrathionate broth plus 5% of Brilliant-Green broth, and incubated at 37 °C to 24 h. After, 1 mL of this diluent was submitted to serial dilution on Lactose broth and inoculated on MacConkey agar (Oxoid, Hampshire, England) and incubated at 37 °C for 24 h.

2.2. Bacterial identification and antibiotic susceptibility testing

Bacterial identification and initial susceptibility testing and detection of ESBL producers were performed using the Vitek 2 system (bioMérieux, France). Identification of ESBL producers was based on the comparison of MIC values for ceftazidime, ceftriaxone and cefepime tested alone and in association with clavulanic acid, according to CLSI recommendation (CLSI, 2011). Additionally, antimicrobial susceptibility profiles were determined by the disc diffusion method using commercially available discs (Oxoid, UK). Interpretation was performed according to criteria of the Clinical and Laboratory Standard Institute (CLSI, 2008, 2011) or following EUCAST guidelines (EUCAST, 2011). *Escherichia coli* ATCC 25922 was used as reference strain.

2.3. Identification of ESBL genes and PCR mapping of class 1 integron

ESBL genes were screened by PCR as previously described (Tollentino et al., 2011). Sequencing was performed using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) with the ABI PRISM 3130 automated sequencer (Applied Biosystems, Foster City, CA). The sequences were annotated in DS Gene 2.0 software and compared with GenBank.

The architecture of the class 1 integron was assessed using the following primers: *intI1* gene, I5 (5′-ACCGCCAACTTTCAGCACAT-3′) and I3 (5′-GCGTTCGGTCAAGGTTCTGG-3′); gene cassettes region, 5′-CS (5′-GGCATCCAAGCAGCAAGC-3′) and 3′-CS (5′-AAGCAGACTTGACCTGAT-3′); *orf513*, ORFend (5′-CCGTTAAGCTCTTATGTGGG-3′); $bla_{CTX-M-2}$, blaI (5′-TTAATGATGACTCAGAGCATT-3′) and blaII (5′-GATACCTCGCTCCATTTATTGC-3′); *sul1* + IS*CR1*, F12F (5′-GTATTGCGCCGCTCTTAGAC-3′) and F12R (5′-AAACCAGCATGGTTGGCTAC-3′); and 3′-CS (*qacEΔ1*), qacEΔ1B (5′-CAAGCTTTTGCCCATGAAGC-3′) (Power et al., 2005).

3. Results

During the study, six broad-spectrum cephalosporin-resistant *Enterobacteriaceae* strains were recovered from four different retail chicken meat samples, purchased from distinct markets (Table 1). These antibiotic-resistant isolates identified as *Proteus mirabilis* ($n = 1$), *Citrobacter diversus* ($n = 1$), *Klebsiella pneumoniae* ($n = 1$) and *Escherichia coli* ($n = 3$) exhibited additional resistance to human and veterinary quinolones, aminoglycosides and/or trimethoprim-sulfamethoxazole and were positive for the production of ESBL. In fact, PCR screening for bla_{ESBL} genes showed that the six strains carried CTX-M-encoding genes. According to sequencing results, while most strains harbored the $bla_{CTX-M-2}$ gene, the presence of the $bla_{CTX-M-8}$ was only confirmed in one *E. coli* strain from market D (Table 1). On the other hand, the analysis of the genetic environment revealed that $bla_{CTX-M-2}$ genes in *P. mirabilis* S11, *K. pneumoniae* S105, and *E. coli* S137 and Lac60 strains comprised a complex class 1 integron and an IS*CR1*-associated sequence, with *dfr* and/or *aadA* gene cassettes located within the variable region of the class 1 integron (Fig. 1). Curiously, for *C. freundii* Ec32 and *E. coli* CF1 strains the genetic environment of the $bla_{CTX-M-2}$ and $bla_{CTX-M-8}$ gene variants could not be determined by the methodology used herein, since the presence of class 1 integron was not observed in either of them.

4. Discussion

In this study, we report the occurrence of MDR *Enterobacteriaceae* in chicken meat intended for sale in the Brazilian market. Resistance to broad-spectrum cephalosporins was associated with CTX-M-2 and CTX-M-8 enzymes, which is a cause of clinical concern. In order to determine the genetic background of bla_{CTX-M} genes, different PCRs were conducted, using several primer combinations (Power et al., 2005). The results showed that $bla_{CTX-M-2}$ was part of a structure similar to the complex class 1 integrons InS21 and In116, except for the variable region (Di Conza et al., 2002; Power et al., 2005). This kind of structure has been very common in South America (Andrade et al., 2010; Power et al., 2005; Quiroga et al., 2013), whereas, it has been also found sporadically in Spain (Valverde et al., 2006). Additionally, in a recent study in Belgium, similar class 1 integron structures were identified in *Salmonella enterica* isolated from poultry (Doublet et al., 2014). Thus, this study report for the first time the presence of $bla_{CTX-M-2}$ genes harbored by complex class 1 integrons in *Enterobacteriaceae* strains recovered from retail chicken meat in Brazil. With regard to *P. mirabilis* strain S11, the genetic environment of $bla_{CTX-M-2}$ is composed of a complex class 1 integron and an IS*CR1*-associated sequence, with *dfrA1* and *aadA1* gene cassettes located within the variable region, which confer resistance to trimethoprim and streptomycin, respectively. This arrangement of gene cassettes was found in *E. coli* and *Salmonella* isolated from poultry in Korea and in Belgium (Dessie et al., 2013; Doublet et al., 2014) and from clinical isolates from animals in Ireland (Karczmarczyk et al., 2011). *K. pneumoniae* S105 presented only the *dfrA1* gene in the variable region, which has not been previously reported; and while *E. coli* S137 presented the *dfrA7* gene, a variant of *dfrA1* previously found in a *E. coli* from an unhealthy animal in Ireland (Karczmarczyk et al., 2011), *E. coli* Lac60 carried only the *aadA1* gene in the variable

Table 1
Characteristics of multidrug-resistant CTX-M-producing *Enterobacteriaceae* isolated from chicken meat in Brazilian markets.

			Resistance profile (Kirby-Bauer)[a]																	
Strain[b]	Date	Market	AMC	CTX	CRO	EFT	CAZ	FEP	FOX	ATM	NA	ENO	CIP	LEV	MXF	SXT	GEN	AK	EST	β-lactamases
Pm S11	03/14/2011	A	**R**	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	S	S	**R**	**R**	S	S	**R**	CTX-M-2
Cd Ec32	04/25/2011	B	**R**	**R**	**R**	**R**	S	S	**R**	S	S	S	S	S	S	S	S	S	S	CTX-M-2
Kp S105	08/23/2011	C	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S	S	CTX-M-2, SHV-11, TEM-1
Ec S137	08/23/2011	C	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	S	S	**R**	S	S	**R**	CTX-M-2, TEM-1
Ec Lac60	08/23/2011	C	**R**	**R**	**R**	**R**	S	**R**	S	**R**	**R**	**R**	**R**	S	**R**	S	S	S	S	CTX-M-2
Ec CF1	07/10/2013	D	**R**	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	**R**	I	**R**	S	**R**	S	S	CTX-M-8, TEM-1

[a] AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; EFT, ceftiofur; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; NA, nalidixic acid; ENO, enrofloxacin; CIP, ciprofloxacin; LEV, levofloxacin; MXF, moxifloxacin; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; AK, amikacin; EST, streptomicin. Resistance is indicated in bold. The CLSI breakpoints were used to interpret the susceptibility results (CLSI, 2008, 2011), except for moxifloxacin where the EUCAST criteria (resistant <17 mm) was applied (http://www.eucast.org/clinical_breakpoints/).

[b] *Pm*, *Proteus mirabilis*; *Cd*, *Citrobacter diversus*; *Kp*, *Klebsiella pneumoniae*; *Ec*, *Escherichia coli*.

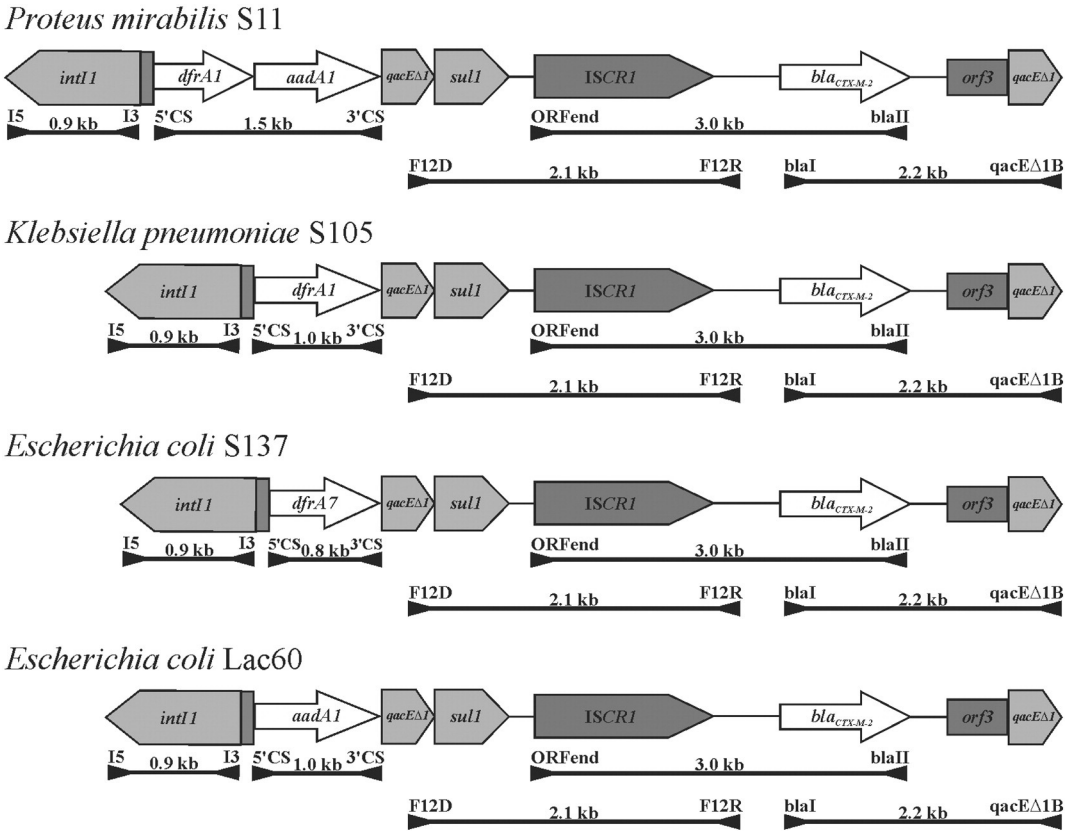

Fig. 1. Schematic representation of class 1 integrons carrying $bla_{CTX\text{-}M\text{-}2}$ genes in *Enterobacteriaceae* strains isolated from retail chicken meat. These integrons had approximate sizes of 7.7 kb (*P. mirabilis* S11), 7.2 kb (*K. pneumoniae* S105), 7 kb (*E. coli* S137) and 7.2 kb (*E. coli* Lac60). Fat arrows indicate the direction of transcription. Thin arrows indicate primers used to identify gene cassette arrays in integrons (Power et al., 2005).

region, such as In2::IS*CR1*::$bla_{CTX\text{-}M\text{-}2}$ integron previously reported in Argentina (Quiroga et al., 2013).

In summary, to the best of our knowledge, this is the first Brazilian study showing retail chicken meat as an important vehicle for the dissemination of ESBL-producing enterobacteria, which is a major commercial and public health issue, since Brazil is the third largest producer of chicken meat and the largest exporter of this product, with a high domestic consumption (Alves et al., 2012). So, surveillance of antimicrobial resistance in bacteria from food-producing animals and derived food products needs to be established as a priority. Moreover, strategies for the rational use of antimicrobial agents in food animals need to be taken urgently, in order to inhibit the release of MDR bacteria harboring $bla_{CTX\text{-}M}$-type genes in food.

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