Occurrence and Molecular Characteristics of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriales Recovered From Chicken, Chicken Meat, and Human Infections in Sao Paulo State, Brazil

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Specialty section: This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 12 November 2020
Accepted: 29 March 2021
Published: 22 June 2021

Citation: Cardozo MV, Liakopoulos A, Brouwer M, Kant A, Pizauro LJL, Borzi MM, Mevius D and de Ávila FA (2021) Occurrence and Molecular Characteristics of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriales Recovered From Chicken, Chicken Meat, and Human Infections in Sao Paulo State, Brazil. Front. Microbiol. 12:628738. doi: 10.3389/fmicb.2021.628738

This study aimed to investigate the phylogenetic diversity and epidemiology of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli and Klebsiella pneumoniae from chicken, chicken meat, and human clinical isolates in Sao Paolo, Brazil, and characterize their respective ESBL-encoding plasmids. Three hundred samples from chicken cloaca, chicken meat, and clinical isolates were phenotypically and genotypically assessed for ESBL resistance. Isolates were identified by MALDI TOF-MS and further characterized by MLST analysis and phylogenetic grouping. ESBL genes were characterized and their location was determined by I-Ceu-I-PFGE and Southern blot, conjugation, transformation, and PCR-based replicon typing experiments. Thirty-seven ESBL-producing isolates (28 E. coli and 9 K. pneumoniae) that were positive for the blaCTX-M-1 or blaCTX-M-2 gene groups were obtained. Two isolates were negative in the transformation assay, and the chromosomal location of the genes was deduced by Southern blot. The blaCTX-M genes identified were carried on plasmid replicon-types X1, HI2, N, FII-variants, I1 and R. The E. coli isolates belonged to nine sequence types, while the K. pneumoniae isolates belonged to four sequence types. The E. coli isolates belonged to phylotype classification groups A, B1, D, and F. This study demonstrated that isolates from cloacal swabs, chicken meat, and human feces had genetic diversity, with a high frequency of blaCTX-M-15 among chickens, chicken meat, and human feces. Thus, this reinforces the hypothesis that chickens, as well as their by-products, could be an important source of transmission for ESBL-producing pathogens to humans in South America.

Keywords: antibiotic resistance, plasmids, extended spectrum beta lactamases (ESBLs), poultry, food chain
INTRODUCTION

Enterobacteriales carrying extended-spectrum β-lactamase (ESBLs) genes with resistance to third- and fourth-generation cephalosporins have been detected widely in livestock (Wu et al., 2018). The role of chicken meat as a potential source of multidrug-resistant bacteria that carries ESBL genes have been demonstrated in several countries including China (Wu et al., 2018), Canada (Ghosh et al., 2019), The Netherlands (Liakopoulos et al., 2016), Senegal (Vounba et al., 2019), and Brazil (Casella et al., 2017).

Brazil draws attention as the world’s largest chicken meat and derivatives exporter (USDA, 2019). Therefore, European countries have demonstrated concerns regarding Brazilian imported chicken meat due to its possible role in transmitting antibiotic-resistant strains from food to humans (Liakopoulos et al., 2016). Although it has been shown that human-to-human transmission in the open community has a greater impact on the environment (Mughini-Gras et al., 2019).

Several studies in Brazil have shown the presence of ESBL-encoding isolates in animals, chicken meat, and humans, but none of them has demonstrated the presence and the relationship of CTX-M-producing Enterobacteriaceae in the food chain, comprising of broilers, chicken meat, and the consumers. Therefore, we aimed to investigate the origin, phylogenetic diversity, and epidemiology of ESBL-producing Escherichia coli and Klebsiella pneumoniae isolates from chickens and chicken meat, and their relationship with those causing clinical symptoms in humans in Sao Paulo, Brazil, as well as characterize their respective ESBL-encoding genes and plasmid replicon types.

MATERIALS AND METHODS

Bacterial Sampling, Identification, and Phentypic Characterization

A total of 300 samples, from the northwest region of the Sao Paulo, from two farms, two clinics and two slaughterhouses within a 80 km radius, were obtained between February and October of 2014 in Sao Paulo, Brazil, consisting of: (1) 100 cloacal swabs of clinically healthy chickens originated from two different farms in the same state [50 from Farm 1 (F1) and 50 from Farm 2 (F2)], (2) 100 chicken meat samples at a local retail (1 g each) [50 from Supermarket 1 (S1), and 50 from Supermarket 2 (S2)], and (3) 100 samples of human feces collected from an equal number of patients with gastrointestinal disease without prior antibiotic treatment of two different local hospitals [50 from Hospital 1 (H1), and 50 from Hospital 2 (H2)]. After collection, samples were transferred to a selective pre-enrichment broth (Luria–Bertani broth supplemented with 1 mg/L cefotaxime) and incubated overnight at 37°C. Subsequently, they were cultured on selective MacConkey agar plates supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany), and incubated for 24 h at 37°C. Thereafter, five morphologically different colonies per sample were tested for ESBL production using a combination disk test as previously described (Liakopoulos et al., 2016). The species of the recovered ESBL-producing isolates was determined by MALDI-TOF mass spectrometry (MALDI Biotyper, Bruker, Germany).

ESBL-Gene Typing

The genomic DNA was extracted by DNeasy Blood and Tissue Kit (QIAGEN, Germany). The presence of ESBL genes was assessed by microarray analysis using the Check-MDR CT-101 (Check-Points, Wageningen, Netherlands) and characterized by polymerase chain reaction (PCR) and sequence analysis as previously described (Dierikx et al., 2013). Sequence data were analyzed using Sequencher version 4.2 (Gene Codes Corporation, United States), and the sequences obtained were compared to ones deposited in GenBank.

Bacterial and Plasmid Typing

All E. coli and K. pneumoniae isolates were characterized by multilocus sequence typing (MLST) according to Achtman’s1 and Pasteur’s2 schemes, respectively. E. coli phylotyping was performed according to Clermont et al. (2013). After transformation and/or conjugation, plasmid characterization was performed by PCR-based replicon typing (PBRT) on transformants and/or transconjugants as previously described (Carattoli et al., 2005).

Genetic Support of the blaCTX-M Genes

The localization of ESBL genes on plasmids was assessed by transformation and/or conjugation experiments. For transformation experiments, the plasmids were extracted using QiaGen Plasmid Midi Kits (Qiagen, Netherlands) and electro-transformed in ElectroMaxTM H10BTM cells (Gibco Invitrogen, United States). Conjugation assays were performed in Luria–Bertani medium (LB-medium) using a rifampicin-resistant, indole-negative E. coli K12 strain as the recipient (Liakopoulos et al., 2016). Transformants were selected on MacConkey agar containing 1 mg/L cefotaxime, whereas transconjugants on MacConkey agar containing 1 mg/L cefotaxime and 100 mg/L rifampicin. The chromosomal location of the ESBL genes, when necessary, was confirmed by I-Ceu-I-PFGE followed by Southern blot hybridization, as previously described (Liakopoulos et al., 2016).

RESULTS AND DISCUSSION

From the MacConkey agar screening coupled with the combination disk test, we recovered 25 ESBL-producing isolates from 100 chicken cloacal samples (25%), seven isolates from 100 chicken meat samples (7%), and five isolates from 100 human fecal samples (5%). MALDI-TOF MS revealed

1http://mlst.ucc.ie/mlst/dbs/Ecoli
2http://bigd.db.pasteur.fr/klebsiella/
FIGURE 1 | Distribution of ESBL gene types (A), plasmid replicon types (B), and isolate sequence types (C) among the ESBL-producing isolates recovered from each of the reservoirs tested. Refer to Table 1 for the more detailed molecular characteristics of each isolate recovered.

that these 37 isolates composed of nine *K. pneumoniae* and 28 *E. coli* strains. Interestingly, ESBL-producing *K. pneumoniae* is so far detected rarely among poultry (Zhuo et al., 2013; Daehre et al., 2018; Projahn et al., 2019). A micro-array showed that our isolates were positive for *bla*$_{CTX-M-1}$ or *bla*$_{CTX-M-2}$ group genes. Through sequencing, we have shown that all *bla*$_{CTX-M-1}$-group-harboring isolates encoded the *bla*$_{CTX-M-15}$ gene, and all *bla*$_{CTX-M-2}$-group encoded the *bla*$_{CTX-M-2}$ gene, with *bla*$_{CTX-M-15}$ being the most prevalent ESBL gene (86.4%; 32/37) among isolates of all sources (Figure 1A). Specifically, our data indicated that the *bla*$_{CTX-M-15}$ gene was present in 91.3% (23/25) of the chicken cloacal isolates, in 71.4% (5/7) of the chicken meat isolates, and in 83.3% (4/5) of the human feces isolates (Table 1). Despite the *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-8}$ genes being the most predominant ESBL genes so far in South America, the *bla*$_{CTX-M-15}$ gene has recently emerged in clinical isolates and is now detected as often as the *bla*$_{CTX-M-2}$ gene in humans, while it has only been reported sporadically from chickens in Brazil (Botelho et al., 2015; Ferreira et al., 2016; Casella et al., 2018). Either these cases reflect direct contamination through human handling or the potential emergence of the *bla*$_{CTX-M-15}$ gene in chickens on farms, they pose the risk for further spread of the *bla*$_{CTX-M-15}$ gene within the poultry production pyramid. We identified only five isolates encoding the *bla*$_{CTX-M-2}$ gene, in particularly two *E. coli* from cloaca, two from chicken meat and one from human feces, contrary to the previous studies documenting the high prevalence of the *bla*$_{CTX-M-2}$ gene among isolates recovered from chicken meat in Brazil (Casella et al., 2018). Overall, the detection of *E. coli* and *K. pneumoniae* isolates carrying *bla*$_{CTX-M}$ genes raises concerns about the broad dissemination of these antimicrobial resistance determinants in Brazil.
Transformation experiments revealed that the ESBL genes were plasmid-encoded in 35 of the 37 isolates with these plasmids belonging to diverse replicon-types (Figure 1B). In particular, plasmid replicon-types detected for the bla<sub>CTX-M-15</sub> gene were R, X1, FIIK, I1, N-FII(-FIIs), and R-FIIK, whereas for the bla<sub>CTX-M-2</sub> gene were R and H12(-P). In the two E. coli isolates that were negative for transformation and transmission pathways, it has been mostly reported to be chromosomally encoded (Casella et al., 2018). In our study, only a limited number of bla<sub>CTX-M-2</sub> genes were chromosomally encoded, suggesting that plasmids are important facilitators of their spread among the recovered isolates. Overall, our data highlight the contribution of plasmids on the epidemiology of ESBL-producing Enterobacteriales of poultry and human origin in Sao Paulo, Brazil.

Clermont’s classification demonstrated that our E. coli isolates belonged to A, B1, D, and F groups with one isolate being not classifiable. The B1 was the most prevalent with 64.7% (11/28) of cloacal isolates belonging to this group, followed by 17.6% (3/17) of D, 11.7% (2/17) of F, and 5.8% (1/17) of A. Similarly, B1 was the most prevalent group among the isolates that we recovered

### Supplementary Table 1

| Isolate | Origin | Reservoir | MALDI-TOF MS | PCR-ESBL | Sequencing | Plasmid | MLST | Phylogroup |
|---------|--------|-----------|--------------|----------|------------|---------|------|------------|
| 1       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncX1 | 47   | B1         |
| 2       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 3       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncX1 | 1125 | B1         |
| 4       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 345  | B1         |
| 5       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 345  | B1         |
| 6       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 345  | B1         |
| 7       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 345  | B1         |
| 8       | Cloacal swab | F1 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 345  | B1         |
| 9       | Cloacal swab | F1 | K. pneumonia | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncX1 | 307  | -          |
| 10      | Cloacal swab | F1 | K. pneumonia | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 15   | -          |
| 11      | Cloacal swab | F1 | K. pneumonia | CTX-M-1g | bla<sub>CTX-M-15</sub> | R     | 15   | -          |
| 12      | Cloacal swab | F2 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncN IncFII | 354 | F          |
| 13      | Cloacal swab | F2 | E. coli | CTX-M-1g | bla<sub>CTX-M-15</sub> | IncN IncFII | 354 | F          |
| 14      | Cloacal swab | F2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncN IncFII IncFIIIS | 349 | D          |
| 15      | Cloacal swab | F2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 16      | Cloacal swab | F2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 17      | Cloacal swab | F2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 1125 | B1         |
| 18      | Cloacal swab | F2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R     | 345  | D          |
| 19      | Cloacal swab | F2 | E. coli | CTX-M2-1g | bla<sub>CTX-M-2</sub> | IncH12 IncP | 47  | A          |
| 20      | Cloacal swab | F2 | E. coli | CTX-M2-1g | bla<sub>CTX-M-2</sub> | IncX1 | 3258 | D          |
| 21      | Cloacal swab | F2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R     | 15   | -          |
| 22      | Cloacal swab | F2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R     | 15   | -          |
| 23      | Cloacal swab | F2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R     | 485  | -          |
| 24      | Cloacal swab | F2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R     | 485  | -          |
| 25      | Cloacal swab | F2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | R IncFIIK | 273 | -          |
| 26      | Human feces | H1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 27      | Human feces | H1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 28      | Human feces | H1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 29      | Human feces | H1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncI1 | 38   | D          |
| 30      | Human feces | H2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 1125 | A          |
| 31      | Human feces | H2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | A          |
| 32      | Chicken meat | S1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 1125 | D          |
| 33      | Chicken meat | S1 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 34      | Chicken meat | S2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 345  | B1         |
| 35      | Chicken meat | S2 | E. coli | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncX1 | 1125 | B1         |
| 36      | Chicken meat | S2 | E. coli | CTX-M2-1g | bla<sub>CTX-M-2</sub> | IncH12 | 10  | Non-typable |
| 37      | Chicken meat | S2 | K. pneumonia | CTX-M1-1g | bla<sub>CTX-M-15</sub> | IncFIIK | 307 | -          |

N/A: Not applied as the ESBL gene for these isolates was confirmed to be encoded on the chromosome.
from human clinical samples (60%; 3/5) and chicken meat (50%; 3/6). Isolates assigned to the B1 group have been previously associated with mostly intestinal pathogenic *E. coli* with high virulent potential in animal models (Morchatti et al., 2018).

Multilocus sequence typing classification demonstrated that the 28 *E. coli* isolates belonged to nine sequence types (ST10, ST38, ST47, ST93, ST345, ST354, ST1125, and ST3258) with the ST345 and ST1125 being the most prevalent ones (46.4% and 17.8%, respectively) (Figure 1C). *K. pneumoniae* isolates were assigned to four sequence types (ST15, ST273, ST307, and ST485) with ST15 being the most prevalent (44.4%) one (Figure 1C). Of note, we observed some known epidemic clones among the *E. coli* (i.e., ST10 and ST38) and *K. pneumoniae* (i.e., ST15 and ST307) isolates (Woodford et al., 2011; Mathers et al., 2015; Navon-Venezia et al., 2017). *K. pneumoniae* ST15 isolates harboring *blaCTX-M−15* are emerging among patients with respiratory tract infections in China (An et al., 2012; Xu et al., 2019) and have been previously isolated from companion animals in Paris (Morchatti et al., 2018). As previously described, our data reveal clonal diversity among the recovered isolates and highlight Brazilian poultry meat as a reservoir of ExPEC lineages (i.e., ST10) (Casella et al., 2018).

As observed by Casella et al. (2018), we show genetic identity in the ESBL gene, plasmid type, isolate ST, and phylogroup suggesting clonal similarity in *K. pneumoniae* isolates between F1 and F2 (*blaCTX-M−15*, R, ST15) but also *E. coli* isolates among (1) F1, F2, H1, and S2 (*blaCTX-M−15*, IncX1, ST345/B1); (2) F1 and S1 (*blaCTX-M−15*, R, ST345/B1); and (3) F1, F2, and S2 (*blaCTX-M−15*, IncX1, ST1125/B1) (Table 1). In addition, we observed genetic identity in ESBL gene and plasmid type suggesting plasmid spread among (1) F1, F2, H1, H2, S1, and S2 (*blaCTX-M−15*, IncX1) and (2) F1, F2, and S1 (*blaCTX-M−15*, R) (Table 1). Overall, our data highlight a complex epidemiology of ESBL-producing Enterobacteriales driven by both clones and plasmids, as well as the potential transmission of these clones and plasmids along the poultry meat chain to humans and/or vice versa.

In conclusion, we demonstrated that despite their overall genetic diversity, isolates from cloacal swabs, chicken meat, and human feces present genetic similarities highlighting that Brazilian chickens, as well as their by-products, may be an important source of transmission for ESBL-producing pathogens to humans. In addition, we indicated the occurrence and high frequency of *E. coli* and *K. pneumoniae* isolates harboring the *blaCTX-M−15* gene from chicken and chicken meat products in South America for the first time.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author(s).

**AUTHOR CONTRIBUTIONS**

MC conceptualized and designed the study. AL and LP aided with data analysis, and manuscript preparation and revision. AK aided in data acquisition. MBo in data acquisition and manuscript preparation. All authors read, contributed to, and approved the final manuscript.

**FUNDING**

Financial support was obtained from the European Union’s Horizon 2020 Research and Innovation Program under grant agreement no. 773830 OneHealth EJP: ARDIG and also grants 2021/03188-7, 2015/10410-0, and 2013/18280-0 from São Paulo Research Foundation (FAPESP).

**ACKNOWLEDGMENTS**

We thanks the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for the scholarship, the Wageningen University and Research, and the São Paulo State University.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.628738/full#supplementary-material

**Supplementary Figure 1** | Chromosomal localization of the *blaCTX-M−2* gene by l-Ceu-l-PFGE and Southern blot hybridization. Columns 1 (Isolate 19) and 2 (Isolate 19), and 5 (Isolate 30) depict the hybridization results using intragenic *blaCTX-M−2* gene probe, whereas columns 3 (S. enterica ser. Braenderup strain H9812—marker), 4 (Isolate 19), and 5 (Isolate 30) depict the hybridization results using intragenic 16S rDNA probes.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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