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Original Article

Escherichia coli in Chicken Carcasses in Southern Brazil: Absence of Shigatoxigenic (STEC) and Isolation of Atypical Enteropathogenic (aEPEC)

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■Keywords

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ABSTRACT

The aim of this study was to investigate the presence of shiga toxin-producing *Escherichia coli* (STEC) and atypical enteropathogenic *Escherichia coli* (aEPEC) in frozen chicken carcasses sold at stores in southern Brazil. Typical *E. coli* colonies were enumerated in 246 chicken carcasses, and the presence of *stx1*, *stx2*, *eae* genes was investigated in their rinse liquid and in *E. coli* strains isolated from those carcasses. Strains of *E. coli* were also investigated for the presence of *bfp* gene. A median of 0.6 cfu.g^{-1}(ranging from <0.1 to 242.7 cfu.g^{-1}) of typical *E. coli* colonies was found in the carcasses. Shiga toxin-encoding genes (*stx1* and *stx2*) were not detected, indicating that the chicken carcasses were negative for STEC. The intimin protein gene (*eae*) was detected in *E.coli* isolated from 4.88% of the carcasses; all tested strains were negative for the *bfp* gene and were classified as aEPEC. Twenty-two aEPEC strains were tested for resistance to ten antimicrobials and subjected to macrorestriction (PFGE). All the tested aEPEC strains were fully susceptible to cephalosporins, ciprofloxacin and colistin. Resistance to sulfonamide (65%), ampicillin (55%), tetracycline (50%) and gentamicin (45%) were the most frequent. The PFGE profile demonstrated a low level of similarity among the resistant strains, indicating that they were epidemiologically unrelated. The results indicate that aEPEC strains can contaminate chicken meat, and their association with strains implicated in human diarrhea needs to be further investigated.

INTRODUCTION

According to estimates of the World Health Organization, almost one in ten people falls ill and 420,000 die every year from eating contaminated food (WHO, 2015). Among the pathogens involved in foodborne disease, Shiga toxin-producing *Escherichia coli* (STEC)was responsible for more than 1 million illnesses, resulting in more than 100 deaths (WHO, 2015). Regarding STEC, the severity of the disease in humans contributes to its relevance, and infections have been associated with clinical illness ranging from mild non bloody diarrhea to hemolytic uremic syndrome, which often includes acute kidney failure. A high proportion of patients are hospitalized, some develop end-stage renal disease, and some die (Baker *et al.*, 2016).

STEC comprises a large, highly diverse group of strains, which have the production of Shiga toxin (Stx) encoded by the genes *stx1* or *stx2* in common. Moreover, pathogenesis includes common steps, including entry into the human gut, attachment to the intestinal epithelial cells usually via an intimin protein encoded by the *eae* gene, and the production of toxin. Stx and the ability to adhere to intestinal epithelial cells are regarded as major STEC virulence traits (FAO/WHO, 2018).

A range of foods should be considered when managing the risk of foodborne STEC infection. Overall, beef was identified as the most frequent food category attributed in outbreaks reported in the African, American, European and Eastern Mediterranean regions. Although chicken does not figure among the most frequent category involved in outbreaks, it was estimated that 0.30% (CI95% 0.29-0.33) of outbreaks in the Americas is attributed to chicken meat (FAO/WHO, 2018). In Brazil, STEC was reported to be present in vacuum-packaged fresh beef samples collected from retailers (Castro *et al.*, 2019), while in other types of meats STEC has only scarcely been investigated.

The atypical enteropathogenic *E. coli* (aEPEC) is also involved in human gastroenteritis and has been reported as one of the most prevalent causes of diarrhea affecting children and immunocompromised patients in Brazil (Dias *et al.*, 2016). Some strains of this pathotype were found colonizing the gut of animals, including poultry (Alonso *et al.*, 2016). The role of meat products as vehicles of aEPEC has been suggested, but its importance as a foodborne pathogen is not yet clear. Moreover, aEPEC strains isolated from animal and food were identified carrying resistance genes against multiple antimicrobial classes, such as aminoglycosides, tetracycline and β-lactams (Comery *et al*., 2013). In China, multidrug resistance was detected in 47.9% of aEPEC strains isolated from human patients and animals; however, strains originated from diarrheal patients showed a significantly higher frequency of MDR strains (Xu *et al*., 2018). Thus, more information about the emergence and spread of aEPEC resistant to antimicrobials among animal and food is still needed.

Chicken is the most consumed meat by the Brazilian population; moreover, this country is one of the largest broiler meat producers and exporters (ABPA, 2018). In this scenario of scale production and high consumption, the assessment of hazards in products available to consumers is relevant for public health. Since there are still few studies in Brazil investigating STEC in meat products and, to the best of our knowledge, no report on aEPEC in chicken meat, the aim of this study was to assess the presence of STEC and aEPEC in frozen chicken carcasses sold in food stores.

MATERIAL AND METHODS

Study design

The sample size was calculated considering an expected STEC prevalence of 1.5% (Alonso *et al.*, 2012), assuming a 5% relative error and a 95% confidence interval. In this context, it was determined that a minimum of 224 samples were needed to ensure the detection of at least one STEC-positive carcass. To ensure representativeness, samples were collected at eight sales outlets in a medium-sized city (approximately 50,000 inhabitants) of the state of Santa Catarina. Each outlet was visited 10 times with a 7 to 15-day interval to obtain samples of different production batches. At each sampling event, one unit of product was collected per category and commercial brand available for sale. Only products packaged at the slaughterhouse were sampled to avoid any risk of cross contamination from handling at the retail stores. A total of 246 samples were collected, and they were distributed among the following categories: whole chicken (n=139, 57%); breeder (n=39, 16%); griller (n=37, 15%); marinade chicken (n=26, 11%); and backyard chicken (n=5; 2%). The chicken samples were distributed among 13 different commercial brands. From these, 227 (92.3%) originated from 16 slaughterhouses under the Federal Inspection Service located in seven Brazilian states. The remaining 19 samples (7.7%) came from a slaughterhouse under the State Inspection Service located in Santa Catarina. The collected samples belonged to 83 different slaughter batches processed between February 2017 and February 2018.

Sample processing

After defrosting, the carcasses were individually transferred to sterile plastic bags, weighed and rinsed with 400 mL of 1% buffered peptone water (BPW, Merck Millipore, Darmstadt, Germany), according to ISO 17604 (amendment 1:2009) and ISO/TS 13136:2012 (E).The rinse liquid from each carcass was drained into a sterile container and was considered the zero-hour (H0) sample.

To enumerate generic *E. coli*, aliquots of 1 mL from H0 were plated on Chromocult®Coliform agar (Merck Millipore, Darmstadt, Germany) and incubated at 36°C (± 1°C) for 48 hours. The violet blue colonies typical of *E. coli* were counted. The number of *E. coli* per gram of carcass was calculated by dividing the number of typical colonies by the carcass weight divided by 400.

Detection of *stx1*, *stx2* and *eae* genes in the carcass rinsing liquid

The detection of STEC on carcasses followed the methodology described in ISO/TS 13136:2012 (E). For this, an aliquot of 1 mL of H0 was transferred to 9 mL of BPW 1%, followed by incubation for 24 h at 36°C (± 1°C), resulting in the day one sample (H1). Total DNA

extraction from 1 mL aliquots of H1 was performed with the PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. After extraction, the DNA concentration was verified in the Quantus™ fluorometer quantifier (Promega, Madison, Wisconsin, USA), and the total DNA concentration was standardized between 10 and 25 ng/µL. The detection of *stx1*, *stx2* and *eae* genes was performed by multiplex-PCR as previously described (Souza *et al.*, 2013) using the primers depicted in Table 1.Each reaction was established to a final volume of 25 µL and included 20 pmol/µL of each primer, 10x PCR Rxn Buffer (Invitrogen), 50 mM $MgCl_2$, 10 mM dNTP's mix, 5 UTaq Polymerase (Invitrogen, Carlsbad, California, USA) and 2.5 µL DNA. Gene amplification was performed on a Veriti® 96-Well thermal cycler (Applied Biosystems, Foster City, California, USA) under the following conditions: 1 min at 95°C; followed by 35 cycles of 50 sec 95°C, 30 sec 62°C and 30 sec 72°C; and 10 min at 72°C. The amplicons were submitted to 2% agarose gel electrophoresis with Blue Green loading dye (LGC Biotecnologia, Cotia, São Paulo, Brazil). The samples were visualized on a transilluminator, and the fragments were compared to a 100 bp GelPilot® DNA molecular weight marker (Qiagen, Hilden, Germany). For all reactions, sterile ultrapure water was used as the negative control, and the *E. coli* strain CDC2010C-3114 O11:H8 was used as a positive control.

Detection of virulence genes in *E. coli* isolates

In parallel to the detection of genes by PCR, aliquots of the H1 broth after incubation for 24 h at 36°C (± 1°C) were transferred to CHROMagar™ (Becton Dickinson, Heidelberg, Germany) for the isolation of *E. coli*. Typical colonies originating from H1 broth that were positive for at least one of the tested genes by PCR (*stx1*, *stx2* and *eae*) were confirmed as positive for the aforementioned genes by the multiplex-PCR protocol described in the section 2.3. For this, 50 *E. coli* colonies from each plate were individually suspended in 90 µL of sterile 0.9% NaCl solution, and DNA was extracted by boiling (Ahmed & Dablool, 2017). From each DNA extraction, 1µL was collected and pooled into five DNA pools (representing ten colonies/pool). Moreover, the same procedure was conducted for typical *E. coli* colonies isolated from H0 corresponding to the same positive carcass samples. *E. coli* isolates from all pools that were positive for at least one virulence gene (*stx1*, *stx2* and *eae*) were tested individually by multiplexPCR (as described in 2.3).

E. coli strains positive for the *eae* gene but negative for the *stx* gene were further investigated for the presence of the *bfp* gene, which codifies for the bundle-forming pilus (Hu & Torres, 2016). Each reaction was established in a final volume of 20 µL and included 10 µL of GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA), 7 µL of sterile ultrapure water, 0.5 µL of each primer (20 pmol/µL)(Table 1) and 2.0 µL of DNA (10 ng/µL). Gene amplification was performed on a Veriti™ 96-Well thermal cycler (Applied Biosystems, Foster City, California, USA) under the following conditions: 1 min at 95 °C; followed by 29 cycles of 1 min at 94 °C,1 min at 60 °C and 1 min at 72 °C; and 5 min at 72 °C. The amplicons were visualized as described above.

The strain *E. coli* ATCC 25922 was used as a negative control; the strains *E. coli* CDC2010C-3114 O11:H8 and *E. coli* E2348/69 were used as positive controls for the amplification of the genes *stx* and *eae* or the gene *bfp*, respectively.

All typical *E. coli* isolates that had at least one virulence gene detected were confirmed by routine biochemical tests as previously described (Markey *et al.*, 2013).

Antimicrobial resistance (AMR) testing

An antimicrobial test against nine antimicrobials by the disk-diffusion method was performed and interpreted according to Matuschek *et al.* (2014) and EUCAST (2019). The following Oxoid™ antimicrobial susceptibility disks (Thermo Fisher Scientific, Waltham,

Table 1 – Primers used for screening genes encoding Shiga toxin (stx), intimin protein (eae) and bundle-forming pili (bfp).

Gene	Primer	DNA sequence (5'-3')	Amplicon (bp)	Reference
stx1	stxA1 598	AGT CGT ACG GGG ATG CAG ATA AAT	417	Bellin, Pulz, Matussek, Hempen & Gunzer (2001)
	stxA1 1015	CCG GAC ACA TAG AAG GAA ACT CAT		
stx2	stx2F	GGC ACT GTC TGA AAC TGC CC	255	Bellin, Pulz, Matussek, Hempen & Gunzer (2001)
	stx2R	TCG CCA GTT ATC TGA CAT TCT G		
eae	eaeF	ACT GGA CTT CTT ATT RCC GTT CTA TG	189	Hardegen, Messler, Henrich, Pfeffer, Würthner & MacKenzie (2010)
	eaeR	CCT AAA CGG GTA TTA TCA CCA GA		
bfp	bfpF	AATGGTGCTTGCGCTTGCTGC	326	Gunzburg *et al.* (1995)
	bfrR	GCCGCTTTATCCAACCTGGTA		

Massachusetts, USA) were tested: ampicillin (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), sulfonamide (300 µg) and tetracycline (30 µg). Furthermore, the minimum inhibitory concentration (MIC) of colistin (Sigma-Aldrich, St. Louis, Missouri, USA) was also determined (EUCAST, 2019). *E. coli* ATCC® 25922 strains was used for quality control purposes.

Macrorestriction profile

Selected strains (n=20) were subjected to pulsed-field gel electrophoresis (PFGE) analysis following the procedures of Pulse-Net (https://www.cdc.gov/pulsenet/pathogens/pfge.html). Isolates were digested with XbaI (Promega),and electrophoresis was performed in a 1% agarose gel using 0.5X Tris-borate-EDTA buffer on a CHEF DR-II system (Bio-Rad Laboratories, Hercules, California, USA) at 6 V/cm for 21 h at 14ºC with an initial switch time of 6.76 s and a final switch time of 35.38 s. The *Salmonella* BraenderupH9812 strain was used as a marker. After PFGE, the gel was stained with ethidium bromide (2 µg/mL), photographed under UV transillumination, and the image digitalization was processed by an L-Pix Touch System (Loccus Biotecnologia). PFGE-banding patterns were compared using the Gel-Compare II software package (Applied Maths, Kortrijk, Belgium). Similarities between profiles were calculated using the Dice coefficient with 1.7% tolerance. The patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA), and dendrograms were constructed.

Statistical analyses

The frequency of strains positive for the tested genes (*stx1*, *stx2, eae* and *bfp*) and the antimicrobial resistance were analyzed by descriptive statistics. From the enumerations of typical colonies of Total Coliforms and *E. coli* on the carcasses, the median, minimum and maximum were calculated using R software (R Core Team, 2018).

RESULTS

Among the 246 chicken samples evaluated for *E. coli* enumeration, no typical colonies were detected in 26.83%. Among the positive samples, the typical *E.coli* colony median counts were 0.6 cfu.g^{-1}(Table 2). The food category breeder presented the highest *E. coli* median counts.

The detection of virulence genes in H1 broth from the 246 chicken samples was negative for the *stx1* and *stx2* genes. Thus, no STEC-positive chicken samples were detected. However, the H1 broth from 25 chicken samples (10.16%) were positive for the *eae* gene; among those samples, the presence of virulence genes in pools of *E. coli* typical colonies obtained from H0 and H1 was investigated.

Among the 159 ten-colony pools tested for the presence of *stx1* and *stx2* genes, all were negative. This result corroborated the absence of *stx1* and *stx2* amplifications, which has been performed directly from the H1 broth. Though, in 40 (25.15%) of the 159 pools, the *eae* gene was detected, and 400 typical *E. coli* colonies obtained from these pools were individually tested for *eae.* Among them, 149 (37.25%) were *eae*+ and were phenotypically confirmed as *E. coli.* All the149 confirmed *E. coli eae*+ strains were negative for the *bfp* gene and were thus identified as atypical enteropathogenic *E. coli* (aEPEC) strains. Therefore, among the 246 investigated chicken carcass samples, in 12 (4.88%) at least one aEPEC strain was detected. Positive samples belonged to whole chicken (n=9/139; 6.47%) and griller (n=3/37; 8.10%) categories.

Twenty two aEPEC strains from chicken were tested for AMR. Two strains were susceptible to all tested antimicrobials, while twenty strains were susceptible to ceftazidime, cefoxitin, ciprofloxacin and colistin. Moreover, resistance to sulfonamide (65%), ampicillin (55%), tetracycline (50%), gentamicin (45%),

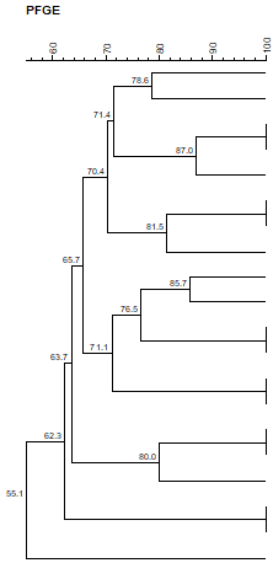
Table 2 – Enumeration of *Escherichia coli* in chicken samples collected at the retail level.

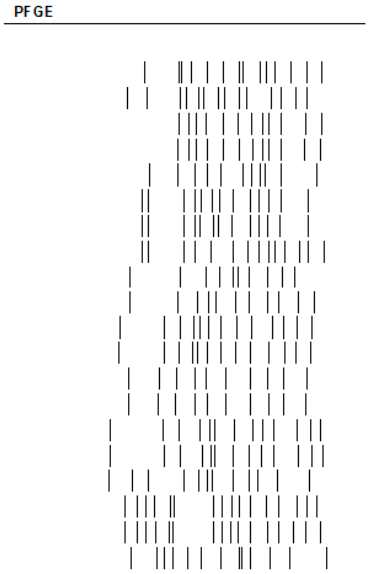
Food category	Number of samples	Not detected (%)	Positive		
			(%)	Median (cfu.g^{-1})	Maximum (cfu.g^{-1})
Whole chicken	139	19.43	80.57	0.8	36.8
Breeder	39	7.70	92.30	3.3	242.7
Griller	37	56.76	43.24	0.1	17.3
Marinade chicken	26	46.16	53.84	0.1	2.3
Backyard chicken	5	60.0	40.0	0.1	0.8
Total	246	26.83	73.17	0.6	242.7

cfu= colony forming unit.

chloramphenicol (20%) and cefotaxime (5%) was found. Half of the strains were resistant to three or more antimicrobial classes and were considered multi-drug resistant (MDR) strains.

The macrorestriction of the strains subjected to the AMR test (Figure 1) demonstrated 100% similarity only between *E. coli* strains isolated from H0 and H1 from a same chicken carcass sample. All of these strains except one (#107) displayed a common AMR profile, indicating that they might be the same strains isolated before and after the incubation of the rinse solution. The remaining strains presented similarities lower than 90% and were considered unrelated.





Key	stx1	stx2	eae	bpf	Phenotypic resistance profile	MIC colistin
195.4	negative	negative	positive	negative	Sul	1
58.42	negative	negative	positive	negative	AmpCloGenSulTet	1
108.1	negative	negative	positive	negative	AmpGenSulTet	1
108.10	negative	negative	positive	negative	AmpGenSulTet	1
92.16	negative	negative	positive	negative	SulTet	1
25.1	negative	negative	positive	negative	Amp	1
25.10	negative	negative	positive	negative	Amp	1
4.1	negative	negative	positive	negative	susceptible to all antimicrobials	1
195.14	negative	negative	positive	negative	GenSulTet	1
58.1	negative	negative	positive	negative	AmpGenSul	1
88.19	negative	negative	positive	negative	GenSul	1
88.3	negative	negative	positive	negative	GenSul	1
83.1	negative	negative	positive	negative	AmpCloSulTet	1
83.16	negative	negative	positive	negative	AmpCloSulTet	1
107.3	negative	negative	positive	negative	SulTet	1
107.9	negative	negative	positive	negative	AmpCloCtxSul	1
201.15	negative	negative	positive	negative	susceptible to all antimicrobials	1
124.2	negative	negative	positive	negative	AmpGenSulTet	1
124.3	negative	negative	positive	negative	AmpGenSulTet	2
246.12	negative	negative	positive	negative	Gen	1

DISCUSSION

In the present study, shiga toxin-producing *E. coli* was not detected in frozen chicken carcasses originated from different slaughterhouses and sampled at retail points in a medium-sized city in Brazil. Cattle and sheep are considered to be the major reservoir of STEC, which can result in the contamination of meat and milk products (FAO/WHO, 2018). In Brazil, STEC was isolated from bovine carcasses at slaughter (Loiko *et al.*, 2016) and was later reported in 4.67% of vacuum-packaged fresh beef samples collected at retail stores (Castro *et al.*, 2019). Other reported sources of STEC include water samples (3.8% positive) collected from lettuce farming systems (Ceuppens *et al.*, 2014) and fecal samples (1.27% positive) of farmed Nile tilapia or wild fish (Cardozo *et al.*, 2018). In both cases, environmental contamination with livestock manure was suggested as the possible origin.

In poultry, the presence of STEC has been reported in cloacal swabs taken from broilers in Argentinian farms, giblets and chicken meat in several countries (Lukásová *et al.*, 2004; Chinen *et al.*, 2009; Alonso *et al.*, 2012; Bagheri *et al.*, 2014; Bai *et al.*, 2015). The frequency of STEC isolation from chicken meat in those studies was highly variable, ranging from 1.96% (Bagheri *et al.*, 2014) to 8.0% (Chinen *et al.*, 2009). The high frequencies reported in ground chicken and carcasses in studies conducted in Argentina (Chinen *et al.*, 2009; Alonso *et al.*, 2012) were attributed to hygiene failures in the production chain and processing and cross-contamination with beef at the retail level. Of the few studies conducted in Brazil in which STEC was investigated in chicken meat, no positive samples were detected in raw chicken legs and chicken cuts sampled at the retail level (Alvares, 2011; Ristori *et al.*, 2017). To the best of our knowledge, chicken carcasses have not yet been investigated; thus, we conducted a sampling with a detection power of 1.5% prevalence and performed a protocol that allowed the screening of STEC virulence genes after an enrichment step. Since all samples were negative, we can infer that STEC is absent in frozen chicken carcasses or that its prevalence is very low.

Screening detected the *eae* gene in chicken carcass rinse fluid samples, indicating that enteropathogenic *E. coli* (EPEC) could be present. EPEC pathogenesis is related to the ability to produce attaching and effacing lesions on the intestinal epithelium (*eae* + gene) without shiga toxin production (Trabulsi *et al.*, 2002). EPEC strains are further divided into typical (tEPEC) and atypical (aEPEC) strains by bundle-forming pilus (BFP)

presence in tEPEC or absence in aEPEC (Hu & Torres, 2016). Therefore, typical *E. coli* colonies originated from rinse fluid before and after the enrichment step were investigated for *eae* and *bfp* genes, and 12 (4.88%) of the chicken carcass samples were confirmed as aEPEC positive. Animals and humans are reservoir of aEPEC, whereas tEPEC generally is carried in the guts of humans (Hernandes et al., 2009); however, there are reports of tEPEC in captive wild birds in Brazil (Sanches et al., 2017). While tEPEC strains are recognized as a cause of severe diarrhea in children (Trabulsi *et al.*, 2002), the role of aEPEC as a human disease pathogen is less clear (Hernandes *et al.*, 2009). However, studies have strongly associated aEPEC with acute or persistent diarrhea in children (Gomes *et al.*, 2016). Although there is no confirmation of direct transmission from animals to humans, it has been suggested that some aEPEC strains may have zoonotic potential (Hu & Torres, 2016). Moreover, animal stools carrying aEPEC may contribute to the dissemination of these strains into the environment, and raw meat contaminated with the intestinal content of reservoirs may be a possible transmission vehicle of aEPEC in human infections (Alonso *et al.*, 2016; Gomes *et al.*, 2016; Xu *et al.*, 2016). However, the contribution of food as a vehicle of aEPEC in human disease is still unclear.

Atypical EPEC was detected in poultry cloacal samples (Alonso *et al.*, 2011; Bagheri *et al.*, 2014), indicating that chicken is a reservoir for these bacteria. In Argentina, aEPEC was detected in 3.9% of chicken carcasses sampled in retail shops (Alonso *et al.*, 2012), which is similar to the frequency reported in our study. The authors suggested that contamination during the evisceration process and the difficulty of removing bacteria with washing may cause the contamination of chicken carcasses, particularly the internal surface (Alonso *et al.*, 2011; Alonso *et al.*, 2012).In our study, we investigated the fluid that resulted from washing internal and external surfaces of the chicken carcasses, and the median value of *E. coli* was 0.6 cfu.g^{-1} (ranging from <0.1 to 242.7 cfu.g^{-1}). The low counts of generic *E.coli* indicate a low level of fecal contamination, possibly related to a suitable process of evisceration and efficient hygienic measures taken at slaughter.

Antimicrobial resistance in potential zoonotic pathogens has been a growing worldwide concern (WHO, 2017). Although human diarrheagenic *E. coli* infections are usually self-limiting, persistent diarrhea caused by aEPEC might require treatment with antimicrobial dugs (Dias *et al.*, 2016). Therefore, aEPEC strains isolated from each positive chicken carcass sample were tested for AMR. The results demonstrated the absence of resistant strains to antimicrobials classified as highest priority critically important for human medicine (WHO, 2017), such as ceftazidime, cefoxitin, ciprofloxacin and colistin. On the other hand, AMR frequency was higher for ampicillin and gentamicin than previously reported in aEPEC isolated from humans in Brazil (Dias *et al.*, 2016).The resistance frequency to all tested antimicrobials, however, was lower than those found in generic *E.coli* isolated from poultry production systems in Argentina (Dominguez *et al.*, 2018), China (Yassin *et al.*, 2017) and Brazil (Stella *et al.*, 2016). The aEPEC strains subjected to AMR testing showed low similarity on PFGE, indicating that there was no epidemiological relationship among the resistant or MDR strains.

This is the first report of aEPEC detection in chicken meat in Brazil, and the association of these strains with those implicated in human diarrhea needs to be further investigated. In the meantime, consumers should be encouraged to observe good hygiene when handling chicken meat and cooking it thoroughly to avoid the transmission of foodborne pathogens.

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