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Original Article

Detection and Antimicrobial Resistance Profile of Enteropathogenic (EPEC) and Shigatoxigenic Escherichia coli (STEC) in Conventional and Organic Broiler Chickens

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ABSTRACT

Enteropatogenic *Escherichia coli* (EPEC) and shigatoxigenic E. coli (STEC), are generally poultry and poultry product isolate and can cause serious human infections. Many strains may become resistant to various antimicrobials, which can hinder the treatment of bacterial diseases. Organic farming seeks to avoid the selection and frequency of antimicrobial-resistant bacteria. This study aims to verify the resistance of EPEC and STEC from organic and conventional (industrial) broiler isolates to antimicrobials. All isolates were submitted to disk diffusion test with tetracycline, gentamicin, enrofloxacin, ceftriaxone and amoxicillin + clavulanate (TET, GEN, ENO, CTX, AMC) and PCR to detect specific virulence genes for EPEC and STEC. A total of 297 *E. coli* strains were isolated, 213 from conventional. In organic broiler, 84 strains were isolated. The strains from the conventional broiler isolates were resistant to five antimicrobials tested: TET 48.82% (104/213), ENO 28.17% (60/213), CTX 15.49% (33/213), GEN 14.55% (31/213), and AMC 7.04% (15/213), and 9.86% (21/213) were considered multidrug-resistant. Organic chicken strains were resistant to four of the antimicrobials tested: TET 35.7% (30/84), ENO 9.5% (8/84), CTX 2.4% (2/84), GEN 4.8% (4/84). Of the strains from the organic broiler chicken isolates, only 1.2% (1/84) was considered multidrug-resistant. No EPEC and STEC were found in the organic chicken samples. The multidrug resistance was characterized in 9.52% (2/21) of the EPEC and 4.76% (1/21) of the STEC. The study demonstrated the absence of EPEC and STEC strains in organic broilers and carcasses and a lower frequency of multiresistant strains compared to conventional breeding.

INTRODUCTION

Escherichia coli is a commensal bacterium that inhabits the intestinal tract of several animal species. Nonetheless, it can acquire virulence attributes that when combined may cause intestinal or extraintestinal clinical syndromes in susceptible individuals (Pakbin *et al.*, 2021). The strain group that causes intestinal diseases in humans, called diarrheagenic, comprises the Enteropathogenic Escherichia coli (EPEC), Enterohemorrhagic Escherichia coli (EHEC), Shigatoxigenic Escherichia coli (STEC), Enteroinvasive Escherichia coli (EIEC), Adherent-invasive Escherichia coli (AIEC), Enterotoxigenic Escherichia coli (ETEC), Diffusely adherent Escherichia coli (DAEC), and Enteroaggregative Escherichia coli (EAEC) pathotypes (Mare *et al.*, 2021).

EPEC and STEC pathotypes are usually poultry and poultry product isolates (Dutta *et al.* 2011; Alonso *et al.*, 2012; Doregiraee *et al.* 2016), and are of public health relevance for they can cause enteric diseases in children and adults (Ifeanyi *et al.*, 2016; Fierz *et al.*, 2017; Torres, 2017). Thus, they should be considered in the transmission chain to humans. EPEC strains cause attaching-effacing lesions (A/E lesions) in

the intestinal epithelium. This lesion is characterized by the destruction of microvilli, intimate bacterial adherence to the intestinal epithelium, and formation and aggregation of actin and other cytoskeleton components at attachment sites of the bacteria. This injury is mediated by intimin, a protein on the surface of the bacterial cell wall and encoded by the Epithelium attaching-effacing gene (*eae* gene) (Gomes, Trabulsi, 2008).

The production of Shiga toxin (STX) is the main virulence factor of STEC pathotype strains, leading to direct endothelial damage, with increased production of pro-inflammatory cytokines, thus increasing the risk of thrombosis with injury in multiple organs, mainly kidneys. Intestinal infections generated by these strains may cause Hemolytic Uremic Syndrome (HUS), which is characterized by high morbidity and mortality with neurologic sequelae and chronic hypertension (Derad *et al.*, 2016). Two distinct groups of STX toxin are described, STX1 and STX2, encoded respectively by stx_1 and stx_2 genes (Gobius *et al.*, 2003).

Antimicrobials are commonly used to treat various bacterial diseases, however, the use of these drugs in food-producing animals has an impact on the expression of resistance and selection of resistant bacteria. If resistant bacteria develop in a food-producing animal environment, they may be transferred to animals and their products and, thus, to consumers of animal products, such as poultry products. Hence, the growth of antimicrobial resistance from pathogenic bacteria in foods of animal origin, which can occur naturally, usually by genetic alterations (Wall *et al.*, 2016), has become a public health concern. To preserve the effectiveness of antimicrobials used to treat human diseases, several countries have restricted the use of various drugs in food animal production (ECDC, 2015). In Brazil, the use of several antimicrobials as performance-enhancing additives has been banned, but they continue to be used to treat diseases (BRASIL, 2003, 2004, 2005, 2009, 2016, and 2018).

Moreover, besides the legislation restrictions on antimicrobial use in food animal production, consumers concerned about the presence of antimicrobial residues in animal products have opted for organic products. Thus, there has been a worldwide increase in the consumption of organic products (Lima *et al.*, 2020). In addition, under organic systems, free-range broiler chickens should have access to outside areas at least for some time during the day (BRASIL, 2021). This system allows for fiber-rich grasses, regulating the intestinal microbiota, increasing the prevalence of beneficial bacteria, and thus reducing the population of pathogenic bacteria (Zheng *et al.*, 2021).

Hence, this study aimed to compare the frequency of EPEC and STEC E. coli, from conventional and organic broiler chicken isolates, and their antimicrobial resistance profile.

MATERIAL AND METHODS

Material collection

Samples were obtained from six flocks of conventional broiler chickens, bred in an industrial housing, with the use of antibiotics, and two flocks of organic broiler chickens from slaughterhouses inspected by the State Inspection Service (SIE), located in the eastern and southern regions of the state of Rio de Janeiro.

Forty broiler chickens per batch were randomly selected for cloacal material, in a total of five flocks of conventional broiler chickens and two organic broiler chickens at the reception area of each slaughterhouse. The material was collected using swabs that were placed in tubes containing Cary Blair medium (OXOID®). Also, from the same batch, 10 carcasses were randomly selected, removed from the conveyor belt after dripping, and individually packed in sterilized bags. All samples were transported under refrigeration in isothermal boxes.

The experimental protocol agreed with the Ethical Principles in Animal Experiments of the Brazilian Society of Laboratory Animal Science (SBCAL) and was approved by the Ethics Committee on Animal Use of the Federal Fluminense University (CEUA - UFF), under the no. 697.

Conventional bacteriological isolation

The swabs were placed in tubes containing 10 ml of 0.1% peptone saline solution (PSS), and 400 ml of 0.1% PSS were added to the bags containing the carcasses. After the carcasses were washed in PSS for one minute, 10mL of each wash was transferred to sterilized tubes. All samples were incubated at 37°C for 24h. Afterward, the samples were seeded on MacConkey agar (HIMEDIA®) and incubated under the same conditions. From each culture, three colonies with characteristics compatible with *E. coli* were submitted to biochemical characterization, using Triple Sugar Iron (TSI) agar (PRODIMOL®), Sulfide Indole Motility (SIM) (HIMEDIA®) medium, Methyl Red broth (VM), and VogesProskauer (VP) (MICRO MED®), and Citrate Agar (HIMEDIA®) (MacFaddin 2000).

PCR

All samples identified as *E. coli* were subjected to DNA extraction by thermal method (Andreatti Filho *et al.*, 2011) for the detection of *eae*, stx_1, stx_2, and *bfp* virulence genes for PCR using pairs of specific primers for each gene (Table 1).

For the amplification reaction of *eae* and *bpf* genes, each 100 ng of DNA extracted in the previous step was added; 1X 10X Buffer; 1.5mM MgCl2; 0.2mM dNTP; 0.4 µM of each primer (Table 1); 1U of Taq Polymerase, totaling a final volume of 25 µL.

In the reaction for the detection of stx_1 and stx_2 genes, 1X of 10X Buffer was added to each 100 ng of extracted DNA; 2mM MgCl2; 0.4mM dNTP; 0.4µM of each primer (Table 1); 1U of Taq Polymerase, totaling a final volume of 25 µL.

Amplification was conducted in a thermocycler (Programmable Thermal Controller PTC-100) under the following conditions: denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 45 seconds, 59°C for 45 seconds, 72°C for one minute, and a final extension at 72°C for 6 minutes (Dutta, 2011).

Disk Diffusion Test

The disk diffusion test was performed for phenotypic evaluation of resistance to antimicrobials of the classes: tetracyclines (tetracycline – TET 30), aminoglycosides (gentamicin – GEN 10), quinolones (enrofloxacin – ENO 05), cephalosporins (ceftriaxone – CTX 05) and penicillins (amoxicillin + clavulanate – AMC 30) (CLSI, 2022). Multidrug-resistant strains were those with resistance to three or more classes of antimicrobials (Magiorakos *et al.*, 2012).

Table 1 – Sequence of Oligonucleotide primers and size of the PCR products obtained for the detection of virulence genes of Enteropathogenic and Shigatoxigenic *Escherichia coli* from broiler chickens.

Oligonucleotide primers	Sequence	Size of PCR products	Reference
eae-F	5′ GACCCGGCACAAGCA TAAGC 3′	384pb	Dutta *et al* 2011
eae-R	5′ CCACCTGCAGCAACAAGAGG 3′		
stx_1-F	5′ ATA AATCGCCATTCGTTGACTAC 3′	180pb	
stx_1-R	5′ AGAACGCCCACTGAGATCATC 3′		
stx_2-F	5′ GGCACTGTCTGAAACTGCTCC 3′	255pb	
stx_2-R	5′ TCGCCAGTTATCT GACATTCTG 3′		

Statistical analysis

Chi-square and Fisher's exact tests were used, when needed, with a significance level of 0.05 for the comparison of contamination sources.

RESULTS AND DISCUSSION

A total of 297 *E. coli* strains were isolated, 213 from conventional broiler chickens, of which 106 were cloaca isolates and 107 carcass isolates. Of the organic broiler chickens, 84 strains were isolated, 52 cloaca isolates, and 32 carcass isolates.

EPEC and STEC Characterization

Of the 213 strains isolated from conventional broiler chickens, 26.76% (57/213) were characterized as EPEC, since they harbor only the *eae* gene (Gomes; Trabulsi, 2008), and 16.43% (35/213) as STEC (Table 2), for they carry stx_1 and/or stx_2 genes (Gobius *et al.*, 2003). Moreover, the frequency of these diarrheagenic strains in poultry or carcasses was higher than that found in other studies. Hence, EPEC strains continue to be the most prevalent when compared to STEC strains (Dutta *et al.*, 2011; Alonso *et al.*, 2012; Samanta *et al.*, 2015; Badi *et al.*, 2018; Cerutti *et al.*, 2020; Shen *et al.*, 2022), which was corroborated by the same reports. Nonetheless, this differs from reports by Nagwa *et al.*, (2022), where 30% of STEC and 10% of EPEC were found.

In broiler chickens and carcasses from organic farming, no EPEC or STEC strains were detected. Access to pasture, one of the characteristics adopted by the organic system, may promote fiber intake, which regulates the intestinal microbiota, thus increasing the prevalence of beneficial bacteria, and reducing the population of pathogenic bacteria (Zheng *et al.*, 2021). Also, antimicrobials used in conventional farming can unbalance the intestinal microbiota. In the absence of these drugs, as seen in organic farming, the microbiota can be more balanced, enabling competitive exclusion of pathogenic bacteria that coexist in the same environment (Praxedes *et al.*, 2012).

Among the analyzed sources, the EPEC strains were more frequent in the cloacal material, detected in 52.63% (30/57) of the strains, and in the carcasses in 47.37% (27/57). Among the STEC strains, there was a higher frequency of strains in carcasses, detected in 54.29% (19/35) of the strains. In the cloacal samples,

Table 2 – Distribution of diarrheagenic *E. coli* strains of the Enteropathogenic (EPEC) and Shigatoxigenic (STEC) pathotypes isolated from cloacal material and conventional and organic chicken carcasses.

Pathotype	Conventional			Organic		
	Source		Total	Source		Total
	Cloaca	Carcasse		Cloaca	Carcasse	
EPEC	30 (52,63%)	27 (47,37%)	57 (100%)	0	0	0
STEC	16 (45,71%)	19 (54,29%)	35 (100%)	0	0	0
No EPEC/STEC	60 (49,59%)	61 (50,41%)	121 (100%)	52 (61,90%)	32 (38,10%)	84 (100%)

45.71% (16/35) of STEC strains were isolates. There was no statistical difference between the cloacal and carcass sources, still, the Fisher's test showed a significant difference (p=0.0099) between the frequencies of EPEC and STEC strains (Table 2). Also, in a study using the same sources, Alonso *et al.* (2012) detected a higher percentage of EPEC strains in cloacal samples (11.9% -102/859) compared to STEC strains in the same source (0.1% - 1/ 859). In carcasses, the same authors reported a frequency of 3.9% (18/457) of EPEC strains and 3.3% (15/457) of STEC strains. According to the same authors, contamination of carcasses may result from cross-contamination during slaughter from strains of the intestinal tract of poultry, and, therefore, adopting hygienic practices during slaughter so as to provide consumers with a safe product is paramount.

Despite the detected pathotypes and their frequencies, both are of great public health concern, for they are responsible for enteric diseases in humans (Blanco *et al.*, 2006; Rasko *et al.*, 2011).

Antimicrobial resistance

Strains isolated from conventional broiler chickens were resistant to the five antimicrobials tested, in the following frequency: TET 48.82% (104/213), ENO 28.17% (60/213), CTX 15.49% (33/213), GEN 14.55% (31/213), and AMC 7.04% (15/213). Strains from organic broiler chickens were resistant to four of the antimicrobials tested, in the following frequency: TET 35.7% (30/84), ENO 9.5% (8/84), CTX 2.4% (2/84), and GEN 4.8% (4/84). In the strains from organic broiler chickens, all strains were sensitive to AMC (Table 3). All samples had a statistically significant difference in the chi-square test (p<0.05). The ban on the use of antimicrobials during all stages of production may account for the lower number of resistant strains in organic broiler chickens (BRASIL, 2007). Wall *et al.* (2016) reported that the use of these drugs in food-producing animals can select resistant strains and increase their frequency in these animals. Macedo *et al.* (2013) described in their study a higher frequency of resistant bacteria in the carcasses of conventional broiler chickens when compared to those of organic broiler chickens. However, Millman *et al.* (2013) observed a greater number of drugs to which *E. coli* strains were resistant in samples from organic broiler chickens (13/14) compared to those from conventional broiler chickens (9/14), despite no significant difference.

Table 3 – Resistance Profile of *Escherichia coli* chicken isolates and cloacal material, and conventional and organic broiler chicken carcasses.

Antibiotics	Conventional	Organic
TET	104 [a] (48,82%)	30 [b] (35,7%)
ENO	60 [a] (28,17%)	8 [b](9,5%)
CTX	33 [a](15,49%)	2 [b](2,4%)
GEN	31 [a](14,55%)	4 [b](4,8%)
AMC	15 [a](7,04%)	0 [b]

TET = Tetracycline; ENO = Enrofloxacin; CTX = Ceftriaxone; GEN = Gentamicin; AMC = Amoxicillin + clavulanate. Different letters on the line indicate significant difference (p<0,05).

In the present study, *E. coli* strains, isolated from conventional and organic broilers, showed high percentages of resistance to tetracycline (35.02% - 134/297) and to enrofloxacin (22.89% - 68/297). Cardoso *et al.* (2015) found higher percentages of resistance to the same antimicrobials. However, this was already expected as these antibiotics are widely used in poultry production and can be easily purchased.

In the strains from the conventional broiler chickens, 9.86% (21/213) were resistant to three or more classes of antimicrobials, being considered multi-resistant. In the strains isolated from the organic broiler chickens, only 1.2% (1/84) were considered multidrug-resistant. Statistically, the chi-square test showed a significant difference (p<0.05) among the analyzed samples. Similar data, in which the occurrence of multidrug resistance is lower in organic creations compared to conventional ones, were also obtained by Vieira *et al.* (2022). Taking into account the legal restrictions on the

use of these drugs in organic farming, this result was already expected, for the absence of antimicrobials in this organic system ends up developing less selection pressure for resistant strains.

Antimicrobial multidrug resistance in diarrheagenic EPEC and STEC strains

Among the diarrheagenic strains isolated from conventional broiler chickens considered multidrug-resistant, 9.52% (2/21) were of the EPEC pathotype and 4.76% (1/21) of the STEC pathotype. Furthermore, Fisher's exact test showed no statistical difference among the samples (p>0.05). Infections caused by *E. coli* from EPEC and STEC pathotypes may require antimicrobial treatment in severe cases in humans (Wong *et al.*, 2012, Ifeanyi *et al*, 2016, Canizalez-Roman *et al.*, 2016). Yet, the presence of multidrug-resistant strains, such as those detected in this study, may be the cause of treatment failures when using these drugs.

CONCLUSION

This study allowed characterizing the presence of strains of the EPEC and STEC pathotypes only in cloacal samples and carcasses of conventional creation broilers, with the EPEC pathotype being the most frequent. In these strains, the multidrug resistance phenotype was also characterized, which may constitute an additional risk, as this condition can lead to failures in therapies against infections caused by these pathotypes.

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