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Detection of Diarrheagenic Escherichia coli in Bovine Meat in the Northern Region of Paraná State, Brazil

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HIGHLIGHTS

- Ground bovine meat samples were evaluated for the presence of diarrheagenic *E. coli* (DEC)
- The following DEC pathotypes were characterized: EAEC, STEC and aEPEC
- The samples were classified in phylogenetic groups: A, B1 and E
- The DEC strains showed a wide variety of serotypes

Abstract: Ground bovine meat is commonly consumed by the population of Brazil. However, it constitutes an excellent medium for the multiplication of microorganisms due to available nutrients and handling practices prior to consumption. Here, we examined 100 samples of ground beef for the presence of diarrheagenic *Escherichia coli* (DEC)

pathotypes by PCR, and characterized isolates by analyzing their adherence to HEp-2 cells, serotype, antimicrobial susceptibility, and phylogeny. Enteroaggregative *E. coli* was detected in five (5%) meat samples, Shiga toxin-producing *E. coli* in three (3%), and atypical enteropathogenic *E. coli* in two (2%). According to the phylogeny, six isolates (60%) were classified in group A, two (20%) in group B1, and two (20%) in group E. The detected serotypes were O3:H2, O93:H9, O93:H46, O105ab:H7, O152:H8, O156:H10, and O175:H7. The antimicrobial susceptibility testing showed that one sample (10%) was resistant to ampicillin, two (20%) to sulfamethoxazole-trimethoprim, and two (20%) to cephalothin. Based on these results, bovine ground meat for human consumption can serve as a reservoir of DEC, which emphasizes the importance of appropriate hygienic-sanitary conditions during handling at every stage from slaughter to table.

Keywords: Diarrheagenic *Escherichia coli*; ground beef; gastroenteritis; serotyping.

INTRODUCTION

In terms of bovine meat production, Brazil stands out as the world's second largest producer and number one exporter. Large amounts of beef are consumed by Brazilians, as approximately 80% of the beef produced in Brazil is destined for the domestic market [1].

Beef is rich in nutrients and easily accessible as a foodstuff to the majority of the population. However, its handling during food preparation contributes to its potential for contamination by pathogenic bacteria, such as *Escherichia coli*, and transmission of these pathogens to humans [2].

Although *E. coli* is part of the normal human intestinal microbiota, and these resident strains provide health benefits to the host, other strains of this species are pathogenic and can cause health problems, such as extra intestinal infections, caused by extraintestinal pathogenic *E. coli* (ExPEC), and gastroenteritis, which is caused by diarrheagenic *E. coli* (DEC) [3]. There are eight known DEC pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga producing-toxin *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), adherent invasive *E. coli* (AIEC) and enteroaggregative Shiga producing-toxin *E. coli* (STEAEC) [4].

EPEC is further subdivided into typical (tEPEC) and atypical (aEPEC); tEPEC contains the LEE region and an EPEC adherence factor (EAF) plasmid; aEPEC lacks the EAF plasmid and Shiga toxins (Stx1 and/or Stx2) [5]. For many decades, tEPEC was responsible for most cases of acute diarrhea occurring in children, especially during the first year of life. However, in recent years, the incidence of aEPEC has increased compared to that of tEPEC in both developed and developing countries [6,7].

STEC is an important foodborne enteropathogen, and ruminants, especially cattle and sheep, are its main reservoirs, and STEC infection can lead to severe diseases, such as hemolytic uremic syndrome (HUS) [8,9]. According to Gerber *et al*. [10], more than 83% of HUS cases in children occur following STEC infection. In Argentina, HUS is endemic, and approximately 400 new cases are reported annually in the nephrology units of hospitals in this country [11].

EAEC is strongly associated with persistent diarrhea, which can lead to malnutrition, growth problems, and cognitive development. This pathotype is also associated with traveler's diarrhea and outbreaks of diarrhea associated with the ingestion of contaminated food and water [12-14].

In this study, the virulence genes of DEC were investigated in *E. coli* isolates from samples of commercial bovine meat obtained in the city of Londrina, Brazil to assess the distribution and frequency of DEC.

MATERIAL AND METHODS

Samples of ground beef

The study was carried out with 400 strains of *E. coli*, isolated from 100 samples of ground beef collected from 25 butchers and supermarkets in the city of Londrina, Paraná, Brazil, from January to November 2014. All meat samples were transported under isothermal conditions until the Laboratory of Bacteriology - State University of Londrina, where the bacteriological analyzes were carried out.

Isolation and identification of *E. coli*

From each ground beef sample, 25 g were weighed and placed in 225 mL of 0.1% peptone water (Difco, Detroit, USA) and homogenized for 20 minutes. Aliquots of 1 mL were inoculated into tubes containing 10 mL of Sodium Lauryl Sulfate Broth (Difco, Detroit, USA) and incubated at 35°C for 24 hours. The samples were then seeded on MacConkey agar (MC) (Difco, Detroit, USA) and incubated at 37°C for 18 hours. From each MC plate were selected from three to five presumptive colonies of *E. coli* and then identified biochemically through EPM, MILi and Simon's Citrate KIT (PROBAC - BRAZIL). Biochemically identified isolates such as *E. coli* were stored in infused heart and brain broth (BHI) (Difco, Detroit, USA) with 20% glycerol at -80°C.

Genotypic Characterization of DEC by PCR

All isolates were screened for the presence of virulence genes. Bacterial DNA was obtained by a boiling extraction method, and the supernatant was used in PCR performed on an Applied Biosystems® 2720 Thermal Cycler. All oligonucleotides used in this study are listed in Table 1.

The amplification reactions were performed in 25 µl reactions, containing 2 µl of bacterial lysate, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 20 pmol of each oligonucleotide primer, 1 U of Taq DNA polymerase (Invitrogen™), 1× reaction buffer, and ultrapure sterile water up to a final volume of 25 µl. The amplified products were separated by electrophoresis on a 1–2% agarose gel prepared in Tris-Borate EDTA (TBE) buffer. In each electrophoretic run, a molecular size marker (100 bp Ladder, Invitrogen™) was included to estimate the molecular size of the amplified fragments. The gels were stained with SYBER SAFE solution (Invitrogen™) and observed with ultraviolet light on a transilluminator (Vilbert Loumart™).

Adhesion, phylogeny, serotyping, and antimicrobial susceptibility assays were performed with all *E. coli* isolates that were positive for DEC virulence genes.

Several strains were used as positive controls in PCR, including EPEC 2348/69 (O127:H6), EHEC EDL 933 (O157:H7), EAEC 042 (O44:H18), EIEC FBC124-13 (O124:H-), and ETEC H10407 (O78:K80:H11). *E. coli* K-12 strain (HB 101) was also used as a negative control.

Table 1 - Primer sequence and size of products obtained used for the genes researched [15-22].

Gene	Primer sequence (5´ - 3´)	Amplicon size (pb)
bfpA	(F) CAATGGTGCTTGCGCTTGGT	326
	(R) GCCGCTTTATCCAACCTGGT	
eae	(F) GACCCGGCACAAGCATAAGC	384
	(R) CCACCTGCAGCAACAAGAGG	
stx1	(F)ATAAATCGCCATTCGTTGACTAC	180
	(R)AGAACGCCCACTGAGATCATC	
stx2	(F) GGCACTGTCTGAAACTGCTCC	255
	(R) TCGCCAGTTATCTGACATTCTG	
stx2a	(F) GCGGTTTTATTTGCATTAGC	256
	(R) TCCCGTCAACCTTCACTGTA	
stx2b	(F) GGTAAAATTGAGTTCTCTAGTATA	175
	(R) CAGCAATCCTGAACCTGACG	
stx2c	(F) GCGGTTTTATTTGCATTAGT	124
	(R) AGTACTCTTTTCCGGCCACT	
stx2d	(F) CTTTATATACAACGGGTG	359
	(R) CTGAATTGTGACACAGATTAC	
hlyA	(F) GCATCATCAAGCGTACGTTCC	534
	(R)AATGAGCCAAGCTGGTTAAGCT	
aatA	(F) CTGGCGAAAGACTGTATCATC	630
	(R) AATGTATAGAAATCCGCTGTT	
aggR	(F) CTAATTGTACAATCGATGTA	308
	(R) ATGAAGTAATTCTTGAAT	
elt	(F) GGCGACAGATTATACCGTGC	450
	(R) CGGTCTCTATATTCCCTGTT	
est	(F) ATTTTTMTTTCTGTATTRTCTT	190
	(R) CACCCGGTACARGCAGGATT	
ipaH	(F) GTTCCTTGACCGCCTTTCCGATACCGTC	600
	(R) GCCGGTCAGCCACCCTCTGAGAGTAC	
arpa	(F) AACGCTATTCGCCAGCTTGC	400
	(R) TCTCCCCATACCGTACGCTA	
chuA	(F) ATGGTACCGGACGAACCAAC	288
	(R) TGCCGCCAGTACCAAAGACA	
yjaA	(F) CAAACGTGAAGTGTCAGGAG	211
	(R) AATGCGTTCCTCAACCTGTG	
TspE4.C2	(F) CACTATTCGTAAGGTCATCC	152
	(R) AGTTTATCGCTGCGGGTCGC	

Adherence Assay in HEp-2 cells

Diarrheagenic E. coli isolates were characterized by the pattern of adherence to HEp-2 cells as described by Rodrigues *et al*. [23]. The HEp-2 cells were grown in 24-well tissue culture microplates (BD Falcon, Bedford, MA, USA) with sterile round cover slips (13 mm diameter), containing 1 mL of Eagle's minimal essential medium (MEM, Invitrogen™) supplemented with 10% fetal bovine serum (Invitrogen™) and 1% antibiotic solution (penicillin 100,000 U and streptomycin 100 µg/mL, Sigma®). The mono layer of HEp-2 cells was cultured overnight at 37°C with CO2 at 5% to obtain at least 70% confluence. After this period, the culture medium was discarded, and the plates were washed 3 times with

sterilized saline phosphate buffer 0.05 M, pH 7.4 (PBS) and 1 mL of MEM and 2% SFB and 1% D-manose (Sigma®) were added to each well. To carry out the adhesion tests, the bacterial samples were inoculated in 3 mL triptone soya broth (TSB) (Difco, Detroit, USA) and incubated at 37°C for 18 hours. One 40 µL aliquot of the bacterial culture was added to each well. The plates were incubated for 3 hours at 37°C and after this period, washed five times with sterile PBS with the addition of 1 mL of MEM (2% SFB and 1% D-manose) and incubated for an additional 3 hours. Next, the plates were washed five times with PBS to remove the non-adhesive bacteria. The slides were fixed with absolute methanol, stained with May-Grunwald and Giemsa and observed under a light microscope using an oil immersion lens.

Phylogenetic Classification

The phylogenetic groups of the DEC isolates (A, B1, B2, C, D, E, and F) were determined by quadruplex PCR for four DNA markers (the genes *arpA*, *chuA*, and *yjaA* and the DNA fragment TSPE4.C2) as described by Clermont et al. [22].

Serotyping

The O and H antigens were determined by Dr. Armando Navarro of the National Autonomous University of Mexico, Mexico City, Mexico, using all available O (O1–O187) and H (H1–H56) antisera as described by Navarro *et al*. [24].

Antimicrobial Susceptibility Profile

The DEC strain were submitted to antimicrobial susceptibility testing using the disk diffusion technique on Müller-Hinton agar (Difco, Detroit, USA), as described by Bauer et al. [25], and according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) [26]. After depositing the antibiotics, the plates were incubated at 37°C for 18–24 hours. The diameters of the antibiotic sensitivity halos were recorded according to the recommendations of CLSI. The antimicrobial agents used were: nalidixic acid (NAL) 30 µg, amicacin (AMI) 30 µg, ampicillin (AMP) 10 µg, cephalotin (CFL) 30 µg, cefoxitin (CFO) 30 µg, ciprofloxacin (CIP) 5 µg, gentamycin (GEN) 10 µg, piperacillin-tazobactam (PPT) 100/10 µg, Ampicilina-sulbactam (20 µg), sulfamethoxazole-trimethoprim (SXT) 25µg and cefazolin (30 µg) CFZ (Oxoid, USA).

RESULTS AND DISCUSSION

In this work, the presence of DEC in 400 *E. coli* isolates from 100 bovine ground beef samples was investigated.

In the search for DEC virulence genes, by the PCR, the following pathotypes were found: two (2%) aEPEC, three (3%) STEC, and five (5%) EAEC. The tEPEC, EHEC, ETEC, and EIEC pathotypes were not isolated from the meat samples. The prevalence of DEC isolates and their genotypic and phenotypic characteristics are shown in Table 2.

Table 2 - Genotypic and phenotypic characteristics of DEC isolated from bovine ground meat**.**

Sample origin	Genotypic profile	Resistance profile	Adhesion pattern	Phylogenetic group	Serotype	Pathotype
A	*aggR,*	AMP	AA	E	O93:H9	EAEC
B	*aggR,*	-	AA	E	O93:H9	EAEC
C	*aggR,*	-	AA	A	O3:H2	EAEC
D	*aggR,*	-	AA	A	O3:H2	EAEC
E	*aggR,*	SUT	AA	A	O3:H2	EAEC
F	*stx*2a,	SUT	ND	A	O152:H8	STEC
G	*stx*2*ND*	CFL	ND	A	O93:H46	STEC
H	*stx*2*ND*	-	ND	ND	O175:H7	STEC
I	*eae*	-	ALL	B1	O105ab:H7	aEPEC
J	*eae*	CFL	ALL	B1	O156:H10	aEPEC

[a.]Antimicrobials: AMP, ampicillin (10 µg); SUT, sulfamethoxazole-trimethoprim (25 µg); CFL, cephalothin (30 µg). b Adherence: AA, aggregative adhesion; ALL, localized-like adhesion. ND: not defined

Adherence is the first step to host bacterial colonization. The *in vitro* adherence assay in HEp-2 cells is used to verify the different adherence patterns that DEC presents [27]. In this study, EAEC strains exhibited characteristic aggregative adherence that defined this pathotype. The aEPEC strains exhibited localized-like adherence. Although this pathotype may present any adherence patterns described, the localized-like pattern is the most common [27]. The STEC strains exhibited an undefined adherence pattern, the most common pattern presented by this pathotype [27].

Several studies have shown that the number of tEPEC isolates from both food and fecal samples is increasing when compared to the number of aEPEC isolates. In Mexico, Estrada-Garcia *et al*. [28], studied fecal samples from children, and obtained 117 (out of 795) DEC isolates; 44.5% (52/117) were aEPEC, and 10% (12/117) were tEPEC. Mora *et al*. [7], isolated EPEC strains from 94 stool samples from children with diarrheal disease in Quito, Ecuador, and they found that aEPEC was more prevalent (89.36%) than tEPEC (10.64%).

In our study, STEC isolates only contained the *stx2* gene, which has variants that differ in their pathogenic potential. Studies have demonstrated a relationship between carriage of *stx2a*, *stx2c*, or *stx2d* and the development of both hemorrhagic colitis (HC) and HUS. In contrast, *stx2b* and *stx2e* showed little association with human diseases [29,30]. In the present study, an *stx2a* variant was found in one isolate, while the other two STEC isolates did not contain any of the tested *stx2* variants. In Brazil, Lascowski *et al*. [31], conducted a

search for DEC isolates in samples of water for human consumption and isolated 12 strains of STEC; five of which contained *stx1* and *stx2*, two contained *stx1*, and five contained *stx2*.

According to the serotyping, two EAEC isolates were serotype O93:H9 and three were O3:H2. Each of the three STEC isolates were different serotypes, i.e., O152:H8, O93:H46, and O175:H7, and the two aEPEC samples also were different serotypes (O105ab:H7 and O156:H10).

Serogroup O156, which was detected in one of our aEPEC isolates, is associated with both aEPEC and STEC strains [32]. Other authors also describe isolation of aEPEC serotype O105:H7, but they did not find the ab serogroup variant [33,34]. Then to our knowledge there have been no reports of aEPEC strain belonging to serotype O105ab:H7.

STEC O152:H8 has also been isolated from animal stool samples by other investigators. In Brazil, Farah *et al*. [35] reported the presence of STEC serotype O152:H8 isolates containing *stx2* genes in bovine feces. In Bangladesh, Johura et al. [36] analyzed 35 *E. coli* isolates from goats, sheep, cattle, chickens, and ducks found a STEC-ETEC hybrid strain belonging to serogroup O152:H8, indicating that such animals may be STEC reservoirs. Vernozy-Rozand *et al.* [37], detected STEC serogroup O175 in cheese samples, which also contained *stx2* gene.

In our study, EAEC was isolated from 5 out of 100 (5%) meat samples. In Japan, three outbreaks of EAEC have been reported to be caused by contaminated foods. The first one involved approximately 2697 high school students who consumed school meals that were contaminated with an EAEC isolate of the ONT:H10 serotype [38]. The second and third outbreaks involved high school students and adults who attended a party where they were infected with EAEC strains belonging to the O126:NM and O111:NM serogroups [39].

In 2011, in northern Germany, *E. coli* was the causal agent of a major outbreak associated with the consumption of contaminated food, which was responsible for the largest number of HUS cases (852) and deaths (50) recorded in a single *E. coli* outbreak. Genome sequencing of this strain showed that it was an O104:H4 serotype EAEC strain that acquired genes from a phage encoding *stx2* [40].

An interesting finding in our study was the isolation of three EAEC strains belonging to the O3:H2 serotype, which was the same serotype as the 17-2 EAEC prototype sample [41]. The O93 serogroup has been detected in STEC, Avium Pathogenic *E. coli* (APEC), and other DEC strains, thus showing the variety of serogroups and serotypes in the EAEC pathotype [42]. Is important finding of our study is that both STEC and EAEC pathotypes of serogroup O93 were found.

According to the phylogenetic typing, the isolates were classified into three phylogenetic groups, A, B1, and E. Group A contained six (60%) isolates, three EAEC and three STEC; Group B1 contained two aEPEC isolates; and group E contained two EAEC pathotype isolates. These results are consistent with those reported by other researchers, such as Salmani *et al*. [43] who also showed a high prevalence of group A (35%), followed by group B1 (26%), in DEC isolates from feces. In Osaka-Japan, Wang *et al*. [44] studied 333 food samples (meat, fruits, and vegetables) and detected DEC in 82 samples. In the phylogenetic typing, groups A and B1 were also predominant among these isolates.

Regarding antimicrobial resistance, one EAEC isolate (10%) was resistant to ampicillin, and two (20%) were resistant to sulfamethoxazole-trimethoprim. One STEC and aEPEC isolate each (10%) were resistant to cephalothin. These data are similar to those of other researchers. In a study of *E. coli* isolates from food, Canizalez-Roman *et al*. [45] found that 29% were resistant to ampicillin and 14% were resistant to sulfamethoxazole-trimethoprim. Wang *et al*. [44] showed that among 82 DEC strains isolated from food, tetracycline resistance was most common (49%), followed by resistance to nalidixic acid (28%), ampicillin (24%), sulfamethoxazole/trimethoprim (20%), and cephalothin (18%). None of the DEC isolates showed resistance to more than one antimicrobial, and five (50%) were sensitive to all tested antimicrobials.

CONCLUSION

Based on our results, we can conclude that bovine ground beef, which is widely consumed by the population, can be contaminated by DEC pathotypes, such as aEPEC, STEC, and EAEC, which may present a health risk for the population.

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Conflicts of Interest: The authors declare no conflict of interest.

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