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Original Article

Escherichia coli O26 and O113:H21 on Carcasses and Beef from a Slaughterhouse Located in Mato Grosso, Brazil

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Abstract

Shiga toxin–producing *Escherichia coli* (STEC) is a group of emerging pathogens that can cause human diseases, including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). Monitoring slaughtering stages and checking contamination points are crucial for the production of safe food. In this context, the aim of this study was to verify contamination by STEC strains, to determine the contamination points and evaluate the resistance profile to 12 antimicrobials used in both veterinary and human medicine. A total of 80 samples were obtained from eight collection points (pen floor, rectum, hide, carcass swabs and esophagus, diaphragm, masseter, and retail beef tissue samples). The isolates were collected by dilution plating on MacConkey agar with sorbitol, cefixime, and tellurite and analyzed by multiplex polymerase chain reaction for virulence genes. Serotyping of non-O157 was performed, and testing for 12 antibiotics by disk diffusion was carried out. A total of 18 STEC strains were isolated, presenting different virulence profiles. Contamination by STEC was observed in the rectum (5/18), carcass surface (5/18), hide (3/18), diaphragm (2/18), retail beef (2/18), and masseter muscle (1/18). Pen floor swabs and esophagus tissues showed no STEC contamination. Moreover, three strains were identified as O26 and three as O113:H21 strains, which have been linked to HUS and HC outbreak cases in Brazil. All STEC isolates were susceptible to all evaluated antimicrobials, except streptomycin. The presence of STEC strains is a direct risk to the consumer, especially when isolated from retail beef, and contamination can occur during different slaughter stages. However, antimicrobial resistance profiles did not identify multidrug-resistant strains, limiting potential antimicrobial resistance transmission to other pathogens.

Keywords: STEC, antimicrobial resistance, foodborne disease, food safety

Introduction

Beef represents one of the main animal protein sources consumed worldwide, with Brazil as the second largest exporter and the third major producer (USDA, 2017). The Brazilian state of Mato Grosso is the main national producer, responsible for ~19.3% of the beef produced in the country (BRASIL, 2016). Considering the high potential for beef production and export, microbiological controls are required to ensure pathogen-free production. Currently, large consumer markets require the monitoring of specific bacterial species involved in meat contamination. For example, the United States and the European Union require Shiga toxin–producing *Escherichia coli* (STEC) monitoring, which is classified as pathogenic in beef products (USDA, 2011; ISO, 2012). However, for the efficient control of this pathogen during slaughtering, the entire process must be monitored regarding contamination and possible pathogen transmission to retail beef (Buncic *et al.*, 2014; Santos *et al.*, 2017).

E. coli strains reside in the mammal gastrointestinal tract, and most strain serotypes show a symbiotic relationship with hosts (Fouhy *et al.*, 2012). However, *E. coli* comprises six pathogenic groups, with the STEC group of main importance to public health due to its association to severe cases of

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foodborne disease (Clements *et al.*, 2012). The main characteristic of strains belonging to this group is the presence of two immunologically distinct toxins, Stx1 and Stx2, which affect protein transduction in host cells (Castro *et al.*, 2017). The STEC group can be divided into two subgroups: O157, comprising O157: H7, implicated in significant outbreaks, and the non-O157 subgroup, comprising serogroups O26, O45, O103, O111, O121, and O145, most commonly implicated in disease outbreaks (Scallan *et al.*, 2011).

Currently, the STEC prevalence rate in food products in Brazil ranges from 1.9% to 9.5% (Bergamini *et al.*, 2007; Freitas Filho *et al.*, 2014; Cardoso *et al.*, 2017), and cases of hemolytic uremic syndrome (HUS) have been reported in the country for both the O113:H21 and O26 strains (Guth *et al.*, 2002; Bando *et al.*, 2017). In addition to pathogen detection during slaughter, another widely studied factor is STEC resistant to antimicrobials, mainly due the increase in strains displaying multidrug resistance (Ayaz *et al.*, 2015; Amézquita-López *et al.*, 2016). Therefore, the aim of this study was to evaluate the presence of STEC strains during fresh beef processing and verify their antimicrobial resistance profile.

Materials and Methods

Sample collection

A total of 10 animals were followed from reception at the slaughterhouse lairage to retail beef cutting and wrapping. The samples were collected from eight different locations along the slaughter chain, totaling 80 analyzed samples. The samplings were carried out weekly between May 2015 and July 2015, in a slaughterhouse with an average slaughter capacity of 500 animals per day, under the Brazilian federal inspection system and located in the state of Mato Grosso, Brazil.

The pen floor, anal, and hide swabs (from the gluteobicep muscle region) were obtained with the use of 3M Swab Samplers® kits, inoculated in buffered peptone water (BPW). For esophagus, diaphragm, and masseter samples, tissue fragments (200 g) were collected and packed in sterile plastic bags. The 3M Sponge-Stick kit was used to obtain the swabs from the carcasses, with a sterilized field boundary bracket (10 cm by 10 cm or 100 cm^2) from each sampled neck, thorax, abdomen, and hindquarters. The retail beef tissue fragments (100 g) were collected and packed in sterile plastic bags. All samples were refrigerated (1–7°C) and transported to the laboratory for immediate analysis.

Culture enrichment and isolation

The samples were analyzed according to the U.S. Food and Drug Administration guidelines (USDA, 2002), with modifications during the pre-enrichment step. Briefly, a 1 mL aliquot of BPW from the swab samples was inoculated into 9 mL modified tryptone soy broth (mTSB). For tissue fragments, 25 g of each sample was inoculated into 225 mL of mTSB and incubated at 41.5°C. Both samples were homogenized in a stomacher (Marconi®) before plating, streaked in agar MacConkey cefixime telluride sorbitol (CT-SMAC), and incubated at 37°C for 24 h. Five typical colonies were striated on trypticase agar with yeast extract (TSA-YE) and incubated at 37°C for 24 h. A colony was inoculated on methylene Levine agar (L-EMB) and subjected to biochemical tests for indole, methyl red, Voges-Proskauer, and citrate (IMViC) and β-glucuronidase (coli-complete—Biocontrol®). The obtained isolates were then stored in a brain heart infusion (BHI) broth containing 10% glycerol at −80°C.

Polymerase chain reaction assay for STEC and major non-O157 strains

A 1 mL aliquot of each BHI-stored isolate with glycerol was reactivated on nutrient agar and the colonies were subjected to DNA extraction and purification using the DNeasy® Blood & Tissue kit (Qiagen®). DNA quantification was performed using the Qubit 2.0 kit (Invitrogen®). Subsequently, two polymerase chain reactions (PCRs) were carried out, based on the primers described by the FDA USDA (2011). In the first reaction, a duplex-PCR was used for the detection of *stx1* and *stx2* genes. In the second reaction, a triplex-PCR was used to amplify the *ehxA* (enterohemolysin), *+93 uidA* (β-glucuronidase mutation characteristic for *E. coli* Enterohemorrhagic (EHEC), and *y-eaeA* (adhesion) genes (Table 1). The *E. coli* O157:H7 strain (ATCC 43895) was used as the positive control, and sterile ultrapure milli-Q water was used as the negative control.

For the m-PCR of the major non-O157 strains, the isolates were analyzed regarding the six major serogroups, known as the ''big six.'' Two m-PCR assays with the primers described by Bai *et al.* (2012) were carried out, designed for the following genes: *wzxO26*, *wzxO45*, *wzxO103*, *wzxO111*, *wbqEO121*, and *wzxO145* (Table 1). The amplicons were visualized on a photodocumentator (MiniBis-Pro DNT; Bio-Imaging Systems®).

Serotyping by agglutination

Serotype determinations of strains negative for the ''big six'' were carried out by the method described by Guinée *et al.* (1981), employing all available O (O1 to O181) and H (H1 to H56) antisera. Serotyping was carried out at the Center of Bacteriology and Nucleus of Enteric Diseases and Infections at the Special Pathogens Reference Laboratory at the Adolf Lutz Institute.

Antimicrobial resistance

Antimicrobial susceptibility was assessed using the disk diffusion technique described by the Clinical and Laboratory Standards Institute (CLSI, 2017). Each isolate was tested for 12 different antibiotics (Oxoid®): ampicillin (10 μg), imipenem (10 μg), tetracycline (30 μg), gentamicin (10 μg), nitrofurantoin (300 μg), ciprofloxacin (30 μg), cefoxitin (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), sulfamethoxazole-trimethoprim (30 μg), ceftazidime (30 μg), and nalidixic acid (10 μg). Each colony was inoculated onto Müeller-Hinton broth 2 (MH; Himedia, India) and incubated between 2 and 4 h. The inoculum was then compared to the standard McFarland scale to 0.5 concentrations, followed by streaking on Müeller-Hinton 2 agar (MH; Himedia). The disks containing the antibiotics were then included and the plates were incubated at 37°C for 18 h. Finally, inhibition zones were measured (CLSI, 2017).

Pulsed-field gel electrophoresis

The pulsed-field gel electrophoresis (PFGE) technique was applied to discriminate between the O26 and O113:H21

Table 1. List of Primers, Their Sequences, the Amplicon Size, and the Reference Used in This Study

Genes	*Primer*	*Primer sequence (5′-3′)*	*Amplicon (bp)*	*References*
stx1	LP30	CAGTTAATGTGGTGGCGAAGG	348	USDA (2011)
	LP31	CACCAGACAATGTAACCGCTG		
stx$_2$	LP43	ATCCTATTCCCGGGAGTTTACG	584	USDA (2011)
	LP44	GCGTCATCGTATACACAGGAGC		
+93 uidA	PT-2	GCGAAAACTGTGGAATTGGG	252	USDA (2011)
	PT-3	TGATGCTCCATCACTTCCTG		
y-eaeA	AE22	ATTACCATCCACACAGACGGT	397	USDA (2011)
	AE20-2	ACAGCGTGGTTGGATCAACC		
ehxA	MFS1Fb	GTTTATTCTGGGGCAGGCTC	166	USDA (2011)
	MFS1R	CTTCACGTCACCATACATAT		
wzxO45	O45-F	GGGCTGTCCAGACAGTTCAT	890	Bai *et al.* (2012)
	O45-R	TGTACTGCACCAATGCACCT		
wzxO103	O103F2	GCAGAAAATCAAGGTGATTACG	740	Bai *et al.* (2012)
	O103R2	GGTTAAAGCCATGCTCAACG		
wbqEO121 *wbqFO121*	O121-F2	TCAGCAGAGTGGAACTAATTTTGT	587	Bai *et al.* (2012)
	O121-R2	TGAGCACTAGATGAAAAGTATGGCT		
wzxO145	O145F5	TCAAGTGTTGGATTAAGAGGGATT	523	Bai *et al.* (2012)
	O145R5	CACTCGCGGACACAGTACC		
wzxO26	O26F4	AGGGTGCGAATGCCATATT	417	Bai *et al.* (2012)
	O26R4	GACATAATGACATACCACGAGCA		
wzxO111	O111F2	TGCATCTTCATTATCACACCAC	230	Bai *et al.* (2012)
	O111R2	ACCGCAAATGCGATAATAACA		

strains. The analyses were performed according to the CDC PulseNet protocol (Ribot *et al.*, 2006), on a CHEF-DR III (Bio-Rad®) equipment. The X*bal* enzyme was used for digestion and the PFGE analyses and comparisons were performed using the GelJ software package (Heras *et al.*, 2015). A similarity analysis was performed using the Dice coefficient. Electrophoresis conditions were an initial switch time of 6.76 s, a final switch time of 35.38 s, and a run time of 18 h. The CDC *Salmonella* ser. Branderup isolate H9812 was used as the reference strain. Images of the PFGE gels were taken with the DOC PRINT II (Vilber Lourmat®).

Results

A total of 18 isolates were positive for one or more virulence genes and classified as STEC. The detected virulence gene profiles were the following: *stx2*/*ehxA* (38.8%), *stx1*/*stx2*/*ehxA* (22.2%), *stx2* (16.6%), *stx1*/*stx2*/*ehxA*/*uidA* (5.6%), *stx2*/*uidA*/*eaeA* (5.6%), *stx2*/*uidA* (5.6%), and *stx1*/*eaeA*/*uidA* (5.6%). After virulence gene detection, the 18 STEC isolates were subjected to PCR analyses for the six main non-O157 serogroups involved in cases of food outbreaks and serotyping by serum agglutination. Of the total isolates, 3 (16.6%) were positive for the *wzyO26* gene, characterizing *E. coli* O26 strains (Table 2), with one strain isolated from a retail beef sample. Moreover, serum agglutination showed that three isolates belonged to O113:H21, the serotype is involved in HUS and hemorrhagic colitis (HC) cases worldwide.

Furthermore, different STEC virulence profiles were detected in the same samples in some animals. For example, animal 5 had two STEC strains isolated from the carcass swab. However, the S44 and S46 strains were different regarding serogroup determination. In addition, animal 9 was positive for two strains in the hide swab (S101 and S102), but with different virulence genes.

The PFGE profiles indicated that S94 and S95 strains were clones, both obtained from the same sample, while another O113:H21 strain (S97) showed 75% similarity with the clones. In addition, the virulence gene profile was different, with only the presence of the *stx2* gene detected. For the O26 serotype isolated from the same animal (S31 and S35), the PFGE profile showed difference in the strains, and both were different to S101 strain (Fig. 1). Moreover, S35 strain showed the most distant similarity profile and the presence of the *stx2* toxin gene.

All *E. coli* STEC strains were sensitive to the evaluated antimicrobials, except for streptomycin, in which resistance was detected in four isolates (two resistant and two intermediate). Moreover, all *E. coli* O26 were sensitive to the 12 evaluated antimicrobials. The S97 strain showed intermediate resistance to streptomycin (Table 2).

Discussion

Contamination with STEC is a health risk to the consumer due to association with HUS, HC, and purpura thrombocytopenia (Karpman *et al.*, 2017; Castro *et al.*, 2017). The data reported herein indicate a high STEC prevalence in the analyzed samples (22.5%). In addition, all isolates except one presented the *stx2* gene, which encodes the main toxin involved in the most severe disease cases (Fuller *et al.*, 2011).

As expected, most STEC contamination was detected during the first slaughter stages, mainly in the rectum (5/18), hide (3/18), and carcass surface (5/18). This may be due to the fact these areas are more exposed to fecal contamination, with a greater possibility of STEC presence during the first processing stages (Martin and Beutin, 2011).

However, no STEC isolates were detected in swabs obtained from the pen floor, probably due to the high microbial load present in feces, increasing competition before or during enrichment (Vimont *et al.*, 2006). No STEC were detected in

Table 2. Collection Points, Profile of Virulence Genes, Antimicrobial Resistance, and Serotype by Polymerase Chain Reaction and Agglutination by Serum

			Genes						
Strain	*Animal*	*Point*	Stx1	Stx2	y-eaeA	+93uidA	ehxA	*Antimicrobial resistance*	*Serotype*
S09	2	Masseter muscle	−	+	−	−	+	Streptomycin	O83:H19
S31	4	Rectal swab	−	+	−	−	+	—	O26:HNT
S35	4	Hide swab	−	+	−	−	+	—	O26:HNT
S37	4	Diaphragm muscle	−	+	−	−	+	—	O73:H45
S44	5	Carcass swab	+	+	−	−	+	Streptomycin	O8:H21
S46	5	Carcass swab	−	+	−	−	+	—	O79:H7
S72	6	Diaphragm muscle	+	+	−	+	+	—	O79:H7
S73	6	Carcass swab	−	+	−	−	+	—	O79:H7
S94	7	Retail beef	+	+	−	−	+	—	O113:H21
S95	7	Retail beef	+	+	−	−	+	—	O113:H21
S97	8	Carcass swab	−	+	−	−	−	Intermediary streptomycin	O113:H21
S99	8	Carcass swab	−	+	−	−	+	—	O22:H16
S101	9	Rectal swab	+	+	−	−	+	—	O26:HNT
S102	9	Rectal swab	−	+	−	+	+	—	O117:H7
S103	9	Hide swab	−	+	−	+	−	—	O21:H19
S117	10	Rectal swab	−	+	−	−	−	—	ONT:H10
S119	10	Rectal swab	−	+	−	−	−	Intermediary streptomycin	ONT:H10
S123	10	Hide swab	+	−	+	+	−	—	O132:H21

+93uidA, non-glucuronidase encoding gene; *eaeA*, intimin encoding gene; *ehxA*, enterohemolysin-encoding gene; HNT, H nontypeable; *stx1*, gene encoding Shiga toxin type 1; *stx2*, gene encoding Shiga toxin type 2.

animals 1 and 3. Moreover, the lack of a ''gold standard'' methodology with adequate STEC and EHEC strain recoveries (Enterohemorrhagic *E. coli*) and inhibition of accompanying microflora remains a challenge (Conrad *et al.*, 2014).

Furthermore, the absence of strains exhibiting beta-glucuronidase enzyme production was verified in 14 of the 18 strains isolated in this study. The heterogeneity of the STEC group has been reported since the first outbreak in 1999 with a sorbitol-positive strain (Ammon *et al.*, 1999). Some studies report the absence of beta-glucuronidase enzyme production (Yang *et al.*, 2004; Ogura *et al.*, 2007; Perera *et al.*, 2015). Due to rapid cell multiplication and gene transmission, mainly through plasmids, the heterogeneity of *E. coli* metabolic pathways becomes an extra challenge in the development of culture media specific to certain pathogenic groups.

In this study, STEC strains were likely not transferred during processing. Moreover, the presence of pathogenic O26 was detected in animals 4 and 9, and O113:H21 strains were detected in animals 7 and 8, both linked to certain foodborne illnesses cases (Hoshina *et al.*, 2001; CDC, 2015; Peron *et al.*, 2016).

The presence of *E. coli* O26 strains in central Brazil has been described previously by Salvadori *et al.* (2003) in calves with diarrhea. In addition, *E. coli* O26 was the first non-O157 STEC strain identified in HC and HUS cases in Brazil (Guth *et al.*, 2002). However, to date, this study is the first to report *E. coli* O26 contamination in retail beef produced in Brazil.

The *E. coli* O26 strains isolated herein were initially identified as enteropathogenic (EPEC), and *stx* genes were subsequently detected (Combes *et al.*, 2011). In addition, the presence of the enterohemolysin-encoding gene was verified in all *E. coli* O26 isolates, similar to reports by Lorenz *et al.* (2016), who demonstrated that *E. coli* O26 isolated from food frequently displayed the enterohemolysin gene without the presence of Intimin (*eaeA*). The enterohemolytic toxin can

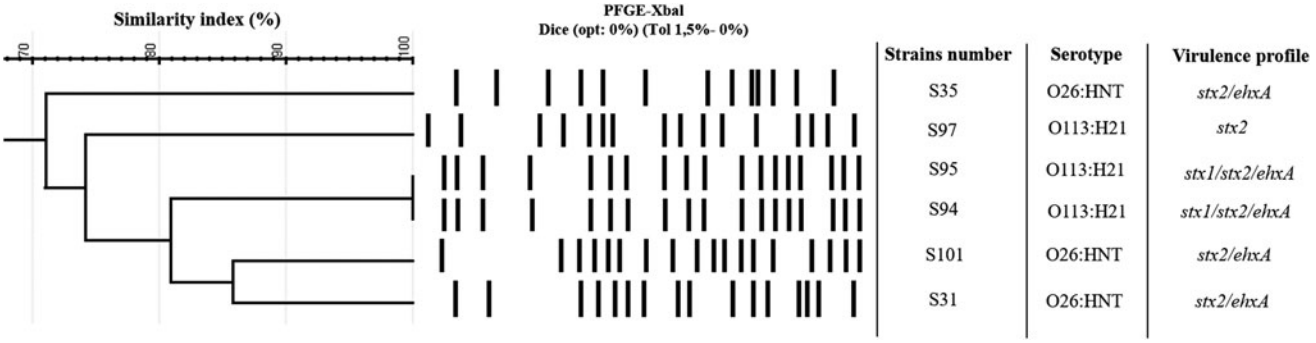

FIG. 1. PFGE profiles for O26 and 0113:H21. Dendrogram of XbaI PFGE for strains S35 (O26); S97 (O113:H21); S95 (O113:H21); S94 (O113:H21); S101 (O26); and S31 (O26) associated with bacterial virulence genes profile. PFGE, pulsed-field gel electrophoresis.

break down red blood cells, causing iron release and leading to HC (Fagan *et al.*, 1999).

E. coli O113:H21 strains have been linked to HUS in a Brazilian patient (Bando *et al.*, 2017). The presence of this strain during the beef processing represents a direct risk to the consumers. In addition, the high production and export of Brazilian beef increase the risk of disease cases or foodborne outbreaks.

The presence of the O group was observed in this study for O26 strains, although the H flagellum was nontypeable, perhaps being the cause of the phylogenetic distance observed between the isolates. Moreover, several studies have indicated genetic heterogenicity between strains with the O26 serotype, and differences in the analyzed virulence genes (Zhang *et al.*, 2007; Miko *et al.*, 2010; Sekse *et al.*, 2011).

Regarding antimicrobial resistance, all evaluated antimicrobials were effective in inhibiting the isolates, except for streptomycin, with only four isolates showing resistance to this agent. Streptomycin belongs to the aminoglycoside class and acts on bacteria through the inhibition of protein synthesis by binding to prokaryotic ribosomes (Mingeot-Leclercq *et al.*, 1999; Kester *et al.*, 2012). Bacterial resistance to streptomycin is primarily due to alteration of the binding site of the ribosome to the agent molecule (Murray, 1991).

In Brazil, the government instituted the National Program for the Prevention and Control of Antimicrobial Resistance in Agriculture in 2017, with the aim of preventing and monitoring antimicrobial resistance throughout the production chain (BRASIL, 2017). In addition, the use of antimicrobials as growth promoters has been controlled in the country since 1999 (BRASIL, 1999).

However, the use of antimicrobials in herds is common in clinical treatment, and the use of streptomycin is related to the treatment of diseases such as *Dermatophilosis* in the state of Mato Grosso (Bacha *et al.*, 2014). In addition, other cattle-related diseases are commonly treated with streptomycin, such as *Leptospira* (Martins and Lilenbaum, 2017).

Resistance to streptomycin has been reported by Park *et al.* (2015) as being prevalent in STEC from slaughterhouse beef and supermarkets. In a Turkish study, 11 STEC isolates from cattle were tested against several antimicrobial agents and all were resistant to streptomycin (Goncuoglu *et al.*, 2010). In Brazil, Cergole-Novella *et al.* (2006) reported that 75% of STEC isolated in the state of São Paulo from different origins exhibited resistance to streptomycin. However, in the study carried out by Cergole-Novella *et al.* (2006), resistance to tetracycline was most prevalent, while all of the STEC isolates in this study were sensitive to this antibiotic.

Conclusions

Contamination by Shiga toxin–producing bacteria can occur throughout the slaughter chain, with transference from hide to carcass and beef cuts through cross-contamination during the process. The presence of *E. coli* O26 and O113:H21 is a public health risk, due to association with cases of foodborne disease, including HC and HUS in Brazil. In addition, antimicrobial resistance for streptomycin detected in this study would only slightly increase the risk in treating bacterial infections, as, unlike other studies available in the literature, isolates were not resistant to multiple antimicrobials.

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Disclosure Statement

No competing financial interests exist.

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