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Beef Carcass Contamination by Shiga Toxin–Producing *Escherichia coli* Strains in an Abattoir in Brazil: Characterization and Resistance to Antimicrobial Drugs

Everlon Cid Rigobelo,[1] Edilene Santo,[2] and Jose Moacir Marin[3]

Abstract

A survey was performed to estimate the frequency of *Escherichia coli* and Shiga toxin–producing *E. coli* (STEC) in carcasses obtained from an abattoir in Brazil between February 2006 and June 2007. A total of 216 beef carcasses were sampled at three stages of the slaughter process—preevisceration, postevisceration, and postprocessing—during the rain and dry seasons, respectively. Of the carcasses sampled, 58% were preevisceration *E. coli* positive, 38% were postevisceration positive, and 32% postprocessing positive. At the postprocessing stage, the isolation of *E. coli* was twice as high in the rain season. *E. coli* was isolated from 85 carcasses of which only 3 (1.4%) were positive for *stx*-encoding genes. No *E. coli* O157 serogroup isolates were detected. No antimicrobial resistance was found in nine of the isolates (10% of the total). The most frequent resistances were seen against cephalothin (78%), streptomycin (38%), nalidixic acid (36%), and tetracycline (30%). Multidrug resistance (MDR) to three or more antimicrobial agents was determined in 28 (33%) *E. coli* isolates. The presence of STEC and MDR strains among the isolates in the beef carcasses emphasizes the importance of proper handling to prevent carcass contamination.

Introduction

Escherichia coli form part of the bacterial population of cattle's gastrointestinal tract. During beef carcass processing, the presence of *E. coli* is an indicator of fecal contamination. Levels of *E. coli* associated with cattle carcasses may increase or decrease during processing according to the extent of such contamination of the living cattle, efficiency of evisceration, and hygienic practices in the abattoir (Bell, 1997). Increased consumer's concern about beef safety started in 1983 (Riley *et al.*, 1983) and continued to rise in recent years due to the large number of reported outbreaks and sporadic cases of human infections with Shiga toxin–producing *E. coli* (STEC) (Hussein and Bollinger, 2005). STEC strains most frequently associated with diseases in the United States and Europe are of the O157:H7 serotype (Nataro and Kaper, 1998; Caprioli *et al.*, 2005). However, several other serotypes (O26, O103, O111, O113, and O121) are also commonly found in association with severe disease outbreaks; in some countries they are isolated more often from clinical cases than O157 (Bettelheim, 2007).

Cattle are considered primary reservoirs of both O157 and non-O157 STEC bacteria (Bettelheim, 2000), and frequently carry STEC without showing pathological symptoms (Blanco *et al.*, 1997). The complete list of bacterial virulence determinants required for STEC's pathological

[1]Faculdade de Zootecnia de Dracena, Universidade Estadual Paulista, Ribeirão Preto, Brazil.
[2]Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal, Universidade Estadual Paulista, Ribeirão Preto, Brazil.
[3]Departamento de Morfologia, Estomatologia e Fisiologia, FORP, Universidade de São Paulo-Campus, Ribeirão Preto, Brazil.

effects is not known, although *stx* appears to be a key factor in pathogenesis (Acheson, 2000). Other virulence factors such as intimin (*eae*) and hemolysin (*hly*A) are thought to enhance pathogenicity, but are not required for strains to produce severe disease, including hemolytic uremic syndrome (HUS) (Acheson, 2000; Caprioli *et al.*, 2005). STEC occurrence in feces of healthy or diarrheic cattle in Brazil has been reported with a high prevalence of strains and a great diversity of serotypes (Irino *et al.*, 2005; Rigobelo *et al.*, 2006; Aidar-Ugrinovich *et al.*, 2007; Farah *et al.*, 2007).

Although antimicrobial therapy is an important tool for infection treatment, antimicrobial resistance may become a major problem in veterinary medicine as a consequence of the intensive use or misuse of antimicrobial drugs (Monroe and Polk, 2000). Susceptibility patterns of indicator bacteria obtained from healthy animals have been suggested as good predictors of resistance situation in a bacterial population as a whole (Van den Bogaard and Stobberingh, 2000). During the processing of carcasses, fecal contamination or transfer of bacteria from the animal's hide to the carcass can promote transmission of pathogenic *E. coli* to food supplies (Bell, 1997; Barkocy-Gallagher *et al.*, 2001). Antimicrobial drug resistance data of fecal *E. coli* strains from animals were difficult to find in the literature from Brazil, most of them showing high levels of resistance against several antimicrobial agents from commensal *E. coli* isolated from diarrheic calves (Rigobelo *et al.*, 2006) as well as from STEC strains isolated from meat (Rodolpho and Marin, 2007).

The aim of the present study was to determine the incidence of *E. coli* on beef carcass at three stages of the slaughter process, during the rain and dry seasons; the survey also included assessment of the prevalence of virulence genes and antimicrobial drug resistance in the isolates obtained at a chosen abattoir in Brazil.

Materials and Methods

Carcass samples

Two hundred and sixteen samples from bovine carcasses of pasture-raised cattle were collected between February 2006 and June 2007, at a small abattoir in São Paulo State (Dracena city), in southwestern Brazil. The abattoir had a slaughtering capacity of 100 cows per day; after antemortem inspection, healthy cows selected for slaughter rested in the bairage for a day prior to slaughter. Food was withdrawn, but water was given. Carcass sampling was performed according to the abattoir processing plan and permission. Sampling of the feedlot cattle was done on five different occasions, three in the dry and two in the rainy seasons, respectively, during three stages of the carcass handling process, namely, preevisceration, postevisceration, and postprocessing. Preevisceration samples were taken immediately after complete hide removal; postevisceration samples after splitting and trimming; postprocessing samples were taken after washing carcasses hanging in the cooler. Due to an abattoir ruling of its processing plan, all samples were taken from one carcass at only one stage of the process; it was therefore not possible to take samples of the same carcass at different stages of the processing. Each sample was obtained using a Specie-Sponge (3M-Brazil) moistened with sterile 0.1% peptone water (Basingstoke, Oxoid, UK) in a bag. Sponges were wrung out as much as possible within the bag, withdrawn, and used to swab the rump of each carcass, near the anus, over an area of 10×30 cm, delineated by a sterile metal template placed on the same half of each carcass. Each sponge was immersed in a stomacher bag with 25 mL of sterile-modified tryptone soy broth (Oxoid) supplemented with 2% novobiocin (Sigma, St. Louis, MO) (mTSB) and mixed by handling for 2 min. All samples were then taken to the laboratory in an ice-cooled bag and kept for 12 h at room temperature.

Bacterial isolates

One hundred microliters of each sample was streaked on a MacConkey agar plate (Oxoid), and incubated at 37°C for 24 h. Colonies showing *E. coli* characteristics were submitted to Gram-staining and identified by standard biochemical tests as oxidase negative, indole positive, Simon's citrate negative, urease negative, and hydrogen sulfide negative (Koneman *et al.*, 1997). The isolates were serotyped for the O serotype O157 using the O157 Latex Agglutination test kit (Oxoid). Negative isolates were

considered non-O157 strains. *E. coli* EDL 933 strain was used as a positive control for O157 serogroup. All isolates were confirmed as being *E. coli* by their biochemical analysis and submitted to PCR for the detection of *stx*, *eae*, and *ehly* genes. From each MacConkey agar plate, a loopful from a confluent bacterial growth was collected and analyzed. From each plate positive for *E. coli*, reisolation for individual colonies was done and the isolated colonies were used for polymerase chain reaction (PCR) and susceptibility testing.

PCR screening of samples

Bacterial strains grown overnight in nutrient broth (Sigma) at 37°C were pelleted by centrifugation at 12,000 *g* for 1 min, resuspended in 200 μL of sterile distilled water, and lysed by boiling for 10 min. Lysates were centrifuged as described above, and 150 μL of the supernatants was used as DNA template for the PCR (Wani *et al.*, 2003). A total of 85 *E. coli* isolates were subjected to PCR; *stx*1, *stx*2, and *eae* genes were detected using the primers and PCR conditions described by China *et al.* (1996). Control reference strains were *E. coli* EDL 933 (O157:H7, *stx*1, *stx*2, *eae*) and *E. coli* K12 (negative control).

Expression of ehly

Expression of enterohemolysin was determined as described by Beutin *et al.* (1989). Plates were incubated at 37°C for 24 h and observed for hemolysis after 3 h (for expression of α-hemolysin) and 24 h (for *ehly*), respectively, and the genotype was confirmed by PCR using the primers hlyA1 and hlyA4 described by Schmidt *et al.* (1995). The reference strains used in this assay were *E. coli* U4-41 (positive control for α-hemolysin), *E. coli* 32511 (STEC O157:H7) (positive control for *ehly*), and *E. coli* K12 (negative control).

Susceptibility testing

Antimicrobial disk susceptibility tests were performed using the disk diffusion method, as recommend by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). From each *E. coli*–positive plate, one isolated colony was tested against 11 antimicrobial agents: ampicillin, amoxicillin/clavulanic acid, cephalothin, ceftriaxone, tetracycline, gentamicin, streptomycin, amikacin, cotrimoxazole, nalidixic acid, and ciprofloxacin. *E. coli* reference strains ATCC 25922 and ATCC 35218 were used for strain quality control.

Results

The distribution of positive carcass responses for *E. coli* corresponding to each sampling season is shown in Table 1. At the postprocessing stage, 32% (37/116) of the carcasses sampled were *E. coli* positive, showing that the isolation of *E. coli* was twice as high (44%, 25/56) in the rainy season when compared to the dry season (20%, 12/60). In the fourth collection, at the dry season, a reduction was detected in the *E. coli*–positive carcasses at the postevisceration stage (38%, 19/50) when compared with the preevisceration stage (55%, 29/50).

Among the 216 carcasses analyzed, only 3 (1.4%) (data not shown) were positive for *stx* genes in *E. coli* isolates when submitted to PCR analysis. One of the three was positive for the *stx*1, and the other two for *stx*1/*stx*2 genes;

Table 1. Distribution of *Escherichia coli* Isolates from Three Different Stages of Processing of 216 Beef Carcasses at an Abattoir During Two Different Climatic Seasons in Brazil, Between February 2006 and June 2007

Carcass Collection	*Season*	*Preevisceration*	*Posteviceration*	*Postprocessing*	*Number of positive carcass/total*
1°	Dry	NS	NS	12/60	12/60
2°	Rainy	NS	NS	3/16	3/16
3°	Rainy	NS	NS	22/40	22/40
4°	Dry	29/50	19/50	NS	48/100
					85/216

NS, not searched for.

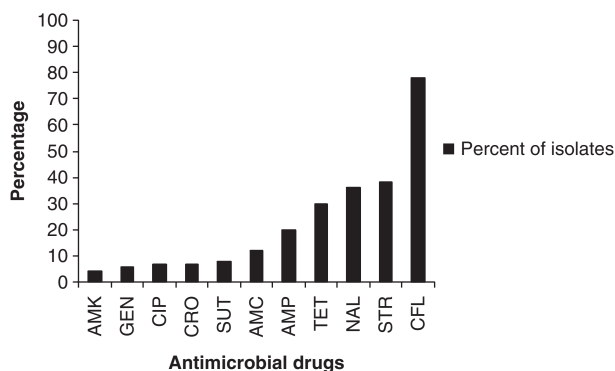

FIG. 1. Antimicrobial resistance patterns of 85 *Escherichia coli* strains of cattle from an abattoir in Brazil. AMC, amoxicillin/clavulanic acid; AMK, amikacin; AMP, ampicillin; CRO, ceftriaxone; CFL, cephalothin; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; SUT, cotrimoxazole.

all of the isolates were negative for genes *eae* and *ehly*.

Antibiotic resistance patterns of the isolates ($n = 85$) are presented in Fig. 1. Isolates presenting intermediary resistance were classified as resistant. The most frequent resistances were to cephalothin (78%), streptomycin (38%), nalidixic acid (36%), and tetracycline (30%), and were less frequent to amikacin (4.0%) and gentamicin (6.0%). No antimicrobial resistance was determined in nine (10%) isolates. Multidrug resistance (MDR) to three or more antimicrobial agents was shown by 28 (33%) of the *E. coli* isolates, and the most common MDR pattern was to streptomycin, tetracycline, and cephalothin (Table 2).

Discussion

E. coli strains are part of the microbiota of the gastrointestinal tract of cattle raised for human meat consumption. Transfer of fecal material to the carcass at slaughtering leads to potential contamination of raw meat (Elder *et al.*, 2000). In the present study, the collection dates of each material are not exactly comparable because of the size of samples and the fact that all three points were not collected on the same sampling days. Based on the first, second, and third sample collection, we verified that the isolation of *E. coli* from the carcasses examined was twice as high in the rainy season when compared to the dry season, confirming other reports (Barkocy-Gallagher *et al.*, 2001; Varela-Hernandez *et al.*, 2007). In the fourth collection at the dry season, a reduction in the *E. coli* car-

Table 2. Resistance Patterns of 28 Multidrug-Resistant *Escherichia coli* Strains Isolated from Cattle Carcasses During Slaughtering

Number of strains	*AMP*[a]	*AMK*	*STR*	*GEN*	*TET*	*CRO*	*CFL*	*AMC*	*NAL*	*CIP*	*SUT*
6			+		+		+				
2	+		+		+		+		+		
2	+		+		+		+	+			
1							+		+		+
1			+				+		+		
1			+		+		+		+		
1			+			+	+				
1	+				+		+	+			
1		+	+		+		+				
1	+		+				+		+		
1							+	+	+		+
1	+						+	+			+
1					+		+		+		
1	+		+		+		+		+		
1	+		+			+	+	+	+		
1			+	+	+	+	+		+	+	
1	+		+		+		+	+	+		
1	+		+	+	+	+	+			+	
1		+	+	+	+	+			+	+	
1	+		+		+	+	+		+	+	+
1	+	+	+	+		+	+	+	+	+	

[a]Antimicrobial drugs.
AMP, ampicillin; AMK, amikacin; STR, streptomycin; GEN, gentamicin; TET, tetracycline; CRO, ceftriaxone; CFL, cephalothin; AMC, amoxicillin/clavulanic acid; NAL, nalidixic acid; CIP, ciprofloxacin; SUT, cotrimoxazole.

casses contamination was detected at the post-evisceration stage in agreement with the data reported by Elder *et al.* (2000) for an U.S. abattoir.

In the present study, a superficial contamination of the carcass by STEC strains was established, but at a low level (1.4%) that agrees with reports by other authors. Rogerie *et al.* (2001) reported a low (1.9%) postprocessing non-O157 STEC prevalence in carcasses sampled during the summer in processing plants in France. Similarly, the non-O157 STEC prevalence in carcasses processed in Hong Kong has been reported as being of 1.7% (Leung *et al.*, 2001). However, a different situation has been reported by carcasses processed in Mexico and the United States; Varela-Hernandez *et al.* (2007) and Arthur *et al.* (2002) reported a high level of contamination with non-O157 STEC, of 20.5% and 54.0%, respectively. Because a large number of variables (e.g., management practices, diets fed, animal factors, and methods of STEC detection) can influence STEC prevalence, comparisons among studies should be carefully evaluated.

Traditionally, Brazil is characterized as a beef cattle producer. Animals are fed mainly at pasture, considering they evolved as grazing herbivores. However, cereal grains ferment at a faster rate than fiber, and grain can be a valuable supplement for cattle production. Several studies have suggested continuous feeding of high grain diets that promotes the proliferation of *E. coli* population, lowers the pH of gut contents, and selects for acid-resistant STEC (Diez-Gonzales and Russell, 1999; Vanselow *et al.*, 2005), increasing the shedding of enterohemorrhagic *E. coli* (EHEC O157:H7). These conditions, in addition to the high animal density in feedlot (traditional in the United States), make it reasonable to assume that a selection of acid-resistant *E. coli* serotypes in grain-fed cattle differ from those isolated from grazing-fed animals, in number as well as in serotypes.

The low level of STEC strains detected as contaminants in the carcass, in this study, contrasts with the high number of these strains detected in feces of healthy or diarrheic cattle in Brazil (Irino *et al.*, 2005; Rigobelo *et al.*, 2006; Aidar-Ugrinovich *et al.*, 2007) what could suggest a efficient work during removal of the hide or the gastrointestinal tracts during slaughtering.

Absence or rarity of the *eae* gene observed in STEC isolates coincides with earlier reports in Brazil (Lira *et al.*, 2004; Irino *et al.*, 2005). Absence of serotype O157:H7 in STEC isolates is not unexpected; it is extremely rare (0.6%) in Brazilian cattle (Irino *et al.*, 2005), although a gold standard method as a immunomagnetic separation using beads coated with O157 antibodies was already used to select the STEC O157 strains in feces of cattle (Aidar-Ugrinovich *et al.*, 2007). Magnetic beads labeled with antibodies to alternative non-O157 serotypes are now available commercially, but other aspects of their isolation (e.g., their optimum enrichment media and enrichment temperature) are still in development (Drysdale *et al.*, 2004).

It is not clear to what extent non-O157 STEC bacteria detected in cattle feces or on beef carcasses are able to cause disease in humans. Gyles *et al.* (1998) proposed that all STEC bacteria could become pathogenic according to the presence or absence of favorable conditions; Bettelheim (2007) claims that non-O157 STEC's ability to cause diseases is, in general, underestimated.

For over 4 decades, it has been a common practice in farms to use antimicrobial agents for animal disease prevention and growth promotion. Pathogenic organisms are clearly the antimicrobial drug's target bacterial population on which selection pressure can be exerted. It is also important to consider that antimicrobial drugs may exert selection pressure on commensal bacteria (Catry *et al.*, 2003).

Levels of antimicrobial resistance in fecal commensal bacteria can reflect the selection pressure exerted by the use of antimicrobial agents in a certain environment (Van den Bogaard and Stobberingh, 1999). In the present study, high levels of resistance as well as MDR were detected among the isolates agreeing with other reports from Brazil (Lira *et al.*, 2004; Rigobelo *et al.*, 2006), all of them showing resistance predominantly to cephalothin, tetracycline, streptomycin, and less frequently to nalidixic acid. These findings agree with data from previous studies showing that resistance is common among strains isolated from food, animals, and humans (Sáenz *et al.*, 2001; Schroeder *et al.*, 2002).

The multiple antimicrobial-resistant phenotypes observed in this study (Table 2) may

have resulted from the spread of mobile genetic elements. For example, the observation that nearly 62% of ampicillin-resistant *E. coli* isolates were also resistant to streptomycin and tetracycline suggests that resistance genes for these drugs are linked on plasmids, agreeing with data previously reported by Schroeder *et al.* (2002) for generic *E. coli* and STEC strains. High levels of resistance to antimicrobial agents have also been reported for STEC strains isolated in India (Khan *et al.*, 2002), in Europe (Mora *et al.*, 2005), and in Palestine (Adwan and Adwan, 2004) with some strains also exhibiting MDR.

It is generally accepted that antimicrobial resistance in veterinary medicine could form a potential public health hazard. Indeed, the commensal gastrointestinal flora of healthy animals harbors a reservoir of resistance genes (Witte, 2000) that can colonize human flora through the food chain or by direct contact. Underlying resistance horizontal gene transfer to human pathogenic bacteria can result in treatment failures, which constitute a reason for concern (Van den Bogaard and Stobberingh, 2000; Catry *et al.*, 2003).

In conclusion, we report here a small (1.4%) level of STEC strains on beef carcasses during processing at an abattoir in Brazil. Analyzed *E. coli* isolates showed a high level of antimicrobial resistance as well as MDR, again causes a reason for concern.

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Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Jose Moacir Marin, Ph.D.
Departamento de Morfologia
Estomatologia e Fisiologia, FORP
Universidade de São Paulo-Campus
Ribeirão Preto 14040-904
Brazil

E-mail: jmmarin@forp.usp.br