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Frequência de *Escherichia coli* enteropatogênicas (EPEC) resistentes em carcaças de bovinos de um frigorífico no Brasil

Frequency of antibiotic resistant enteropathogenic *Escherichia coli* (EPEC) in bovine carcasses at a slaughterhouse in Brazil

Frecuencia de *Escherichia coli* enteropatógena (EPEC) resistente en canales bovinas de un matadero en Brasil

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Resumo

Escherichia coli (*E. coli*) ocupa o segundo lugar entre os microrganismos envolvidos em surtos de doenças transmitidas por alimentos no Brasil e está entre os quatro mais frequentes em todo o mundo. Devido à sua importância, o objetivo deste trabalho foi verificar a qualidade microbiológica pela presença de *E. coli* potencialmente patogênica em carcaças de bovinos. 365 cepas de *E. coli* foram isoladas por suabe de arrasto de 154 carcaças de bovinos, abatidos no município de Mineiros - GO, Brasil. A frequência de *E. coli* nas amostras coletadas foi de 81% (125/154). Destas cepas, 16 tinham o gene *eae* e nenhuma apresentava os genes *stx1* ou *stx2*, portanto, foram classificadas como EPEC. Assim, a frequência de EPEC nas carcaças foi de 9,7% (15/154). As cepas foram classificadas como parte dos grupos A ou B1. Quanto à resistência antimicrobiana, as cepas demonstraram maiores porcentagens de resistência à cefalotina com 82% (41/50), seguida por gentamicina e amicacina com 26% (13/50) cada. Nenhuma das cepas produziu a enzima beta-lactamase de espectro estendido, mas três EPEC foram classificadas como resistentes a vários medicamentos. Os resultados demonstram a presença de EPEC multirresistentes em carcaças de bovinos abatidas na cidade de Mineiros, Brasil.

Palavras-chave: Bovinos de corte; Resistência antimicrobiana; STEC; Segurança alimentar.

Abstract

Escherichia coli (*E. coli*) comes in second place among microorganisms involved in outbreaks of foodborne diseases in Brazil and is among the 4 most prominent worldwide. Due to its importance, the purpose of this work is to verify microbiological quality by the presence of *E. coli* in bovine carcasses. A total of 365 *E. coli* were isolated by swabs of 154 carcasses of cattle, slaughtered in the municipality of Mineiros – GO, Brazil. The frequency of *E. coli* in the samples collected was 81% (125/154). Of these *E. coli*, 16 had the gene *eae*, and none

presented the genes *stx1* or *stx2*, so were therefore classified as EPEC. Thus, the frequency of EPEC in the carcasses was 9.7% (15/154). The strains were classified as part of the A or B1 groups. As for antimicrobial resistance, the antibiotics with the highest percentages of resistance were Cephalothin with 82% (41/50), followed by Gentamicin and Amikacin with 26% (13/50) each. None of the samples showed any production of the extended-spectrum beta-lactamase enzyme, but three EPECs were classified as multi-drug resistant. The results demonstrate the presence of multi-resistant EPEC in bovine carcasses slaughtered in Mineiros city, Brazil.

Keywords: Beef cattle; Antimicrobial resistance; STEC; Food protection.

Resumen

Escherichia coli (*E. coli*) ocupa el segundo lugar entre los microorganismos involucrados en brotes de enfermedades transmitidas por alimentos en Brasil y se encuentra entre los 4 más prominentes a nivel mundial. Debido a su importancia, el propósito de este trabajo fue verificar la calidad microbiológica por la presencia de *E. coli* en canales bovinas. Se aisló un total de 365 *E. coli* con hisopos de 154 canales de ganado, sacrificados en el municipio de Mineiros - GO, Brasil. La frecuencia de *E. coli* en las muestras recolectadas fue del 81% (125/154). De estos *E. coli*, 16 tenían el gen *eae*, y ninguno presentaba los genes *stx1* o *stx2*, por lo que se clasificaron como EPEC. Así, la frecuencia de EPEC en los canales fue del 9,7% (15/154). Las cepas se clasificaron como parte de los grupos A o B1. En cuanto a la resistencia a los antimicrobianos, las cepas mostraron mayores porcentajes de resistencia a la cefalotina con 82% (41/50), seguidas de gentamicina y amikacina con 26% (13/50) cada una. Ninguna de las cepas produjo la enzima beta-lactamasa de espectro extendido, pero tres EPEC se clasificaron como resistentes a varios fármacos. Los resultados demuestran la presencia de EPEC multirresistente en canales de bovinos sacrificados en la ciudad de Mineiros, Brasil.

Palabras clave: Ganado vacuno; Resistencia a los antimicrobianos; STEC; Protección de alimentos.

1. Introduction

Cattle are a reservoir for potentially pathogenic *E. coli*. Bacteria that can represent a significant threat to public health, hence, it is crucial to monitor the prevalence of the genetic determinants of virulence and antimicrobial resistance among the *E. coli* population (Bok et al. 2015). *E. coli* is an important indicator of the industrial quality of food production, being

part of a group of bacteria that can cause infections in the gastrointestinal tract of humans and as the main indicator of fecal contamination during food processing. Pissetti et al. (2016) mention that contamination of the carcasses with intestinal microbiota can occur during slaughter, mainly during the skinning and evisceration processes. *E. coli* populations can also be used as an indicator for evaluating the prevalence of antimicrobial resistance. In addition, commensals *E. coli* may harbor resistance genes available for strains that have virulence factors and are, therefore, of great importance for public health considering the risk of introducing these bacteria into the food chain. EPEC was the first group identified and associated with cases of diarrhea in infants, with adherence being its main pathogenicity factor. The progression of the disease in humans is directly related to the ability of *E. coli* to adhere to the intestinal epithelium, which is crucial for its intestinal colonization.

High rates of resistance to antibiotics have been found, showing a worldwide trend toward the emergence of multiresistant bacteria. The increased resistance may be associated with host systemic stresses and may be an indicator of intestinal microbiota ecosystem disorders, as well as causing additional effects on the gut microbiota, such as the stimulation of gene transfer between bacteria and the reduction of immune responses in peripheral organs (Looft & Allen, 2012). The Extended-spectrum β-lactamase enzymes (ESBL) produced by bacteria are important because they are capable of inactivating widely-used antimicrobials in the treatment of infections caused by enterobacteria, such as penicillins, cephalosporins, and monobactams. Inadequate use of antibiotics can select multiresistant isolates, causing outbreaks and increasing the incidence of mortality (Nagai et al., 2016).

In many instances, carcass surfaces have been contaminated with enteric bacteria during evisceration in slaughterhouses. Therefore, the microbial quality of carcass surfaces should be correlated with the hygiene practices of abattoirs (Wheatley, Giotis and McKevitt, 2010). The objective of this research is to isolate and identify the strains of *E. coli* present in bovine carcasses, in addition to evaluating the frequency of the STEC and EPEC pathotypes in the samples, and to determine the susceptibility of the strains isolated against different antimicrobials.

2. Materials and Methods

Research is carried out to bring more light, understanding and knowledge to society as recommended by Pereira et al. (2018). In that study we used *E. coli* that was obtained from swab samples from bovine carcasses, in a slaughterhouse in Mineiros city,

Goiás State, Brazil, between February and June 2017. The slaughtered cattle came from the southwest region of Goiás state, from different farms and raised on pasture. One swab per carcass was used and three collection points were determined in each carcass: flank, round, and sirloin. The collected area was approximately 100cm² at each point, totaling an area of 300cm² per carcass (Matos et al., 2013). Swabs were wetted in Butterfield's solution containing 0.1% of Tween 20 (Elder et al., 2000) and later placed in nutrient broth and packaged in a microbiological stove.

A total of 154 samples (154 carcasses) were collected, which were obtained after the processing, before entering the cold room. The isolates were obtained by inoculating the swabs on MacConkey agar, after incubation for 24 hours at 37°C were picked from 4 to 5 colonies, totaling 690 strains, that were identified biochemically as *E. coli*, by lactose fermentation tests, sucrose and glucose, indole production, methyl red and Voges-Proskauer reactions, citrate utilization, urease production, and sulfuric gas production. To detect the presence of genes *stx1*, *stx2*, and *eae*, we used the following: multiplex PCR in a final volume of 25µl, containing 2.5µl of DNA extracted by thermal lysis; 12.5µl Promega Mix (Promega Corporation, USA); 1.5µl of primer forward (0.5µl of each); 1.5µl of primer reverse (0.5µl of each) (Biotechnology Synthesis); and 7µl of ultrapure water (Stx1f: CAGTTAATGTGGTGGCGAAGG, Stx1r: CACCAGACAATGTAACCGCTG, 348 bp; Stx2f: ATCCTATTCCCGGGAGTTTACG, Stx2r: GCGTCATCGTATACACAGGAGC, 584 bp; eaef: TCAATGCAGTTCCGTTATCAGTT, eaer: GTAAAGTCCGTTACCCCAACCTG, 482 bp). Samples were amplified in 35 cycles, each cycle consisting of 1:30 min. at 94°C for denaturation, 1:30 min. at 60°C for annealing, and 1:30 min. at 72°C for extension (Vidal et al., 2005). For positive control of these genes, the strain O157: H7 (*stx1*$^+$, *stx2*$^+$, *eae*$^+$) was used. EPEC isolates were also tested by PCR in a final volume of 25µl, made up of 12.5µl Promega Mix (Promega Corporation, USA), 0.5µl of primer forward and 0.5µl of primer reverse (Biotechnology Synthesis), and 11.5µl of ultrapure millq water, to identify the presence of the adhesion genes *ToxB* (f: ATACCTACCTGCTCTGGATTGA, r: TTCTTACCTGATCTGATGCAGC, 602bp) and *efa1* (88AT: AAGGTGTTACAGAGATTA, 88TN:TGAGGCGGCAGGATAGTT; 88T14: GAGACTGCCAGAGAAAG, 88T9:GGTATTGTTGCATGTTCAG, 479 bp) (Tarr et al. 2002; Nicholls, Grant and Robins-Browne 2000). Samples were amplified in 35 cycles, each cycle consisting of 30s at 92°C for denaturation, 30s at 55°C for annealing, 45s at 72°C for extension, for the *ToxB* gene, and 1 min. at 94°C for denaturation, 1 min. at 51 ° C for annealing, 1 min. at 72 ° C for extension, for the *efa1* gene. For positive control of these

genes, the strain O157: H7 (*efa1*$^{+}$, *ToxB*$^{+}$) was used. All EPEC isolates were analyzed by multiplex PCR and classified into different phylogenetic groups (Clermont et al., 2013). For positive control of these genes the strain O157: H7 (*stx1, stx2, eae, efa1, ToxB;* source: Veterinary microbiology laboratory of the regional jataí of the Federal University of Goiás state) was used. *E. coli* MG1655 was used as a negative control.

EPEC strains were also tested for motility, Rappaport-Vassiliadis semi-solid medium (MSRV) was used, while, for hemolysis, sheep blood agar medium was used. For the antimicrobial resistance profile, the disk diffusion technique was used.

The antimicrobials tested were: ampicillin 10µg, imipenem 10µg, and meropenem 10µg (beta-lactam, carbapenem); 30 µg amikacin and 10 µg gentamicin (aminoglycosides); tetracycline 30 µg (tetracyclines); sulfazotrim 1.25/23.75µg (sulfonamides + trimethoprim); ciprofloxacin 5µg (quinolones); cephalothin 30µg (cephalosporin 1st generation); cefoxitin 30µg (cephalosporin 2nd generation); and cefepime 30µg (4th generation cephalosporin). For the detection of strains of extended-spectrum beta-lactamases (ESBL), a disc-approximation technique was used with the following antibiotics: 30 µg ceftriaxone, 30 µg cefotaxime, 30 µg ceftazidime, 30 µg aztreonam, and 20/10 µg amoxicillin-clavulanate (CLSI 2018).

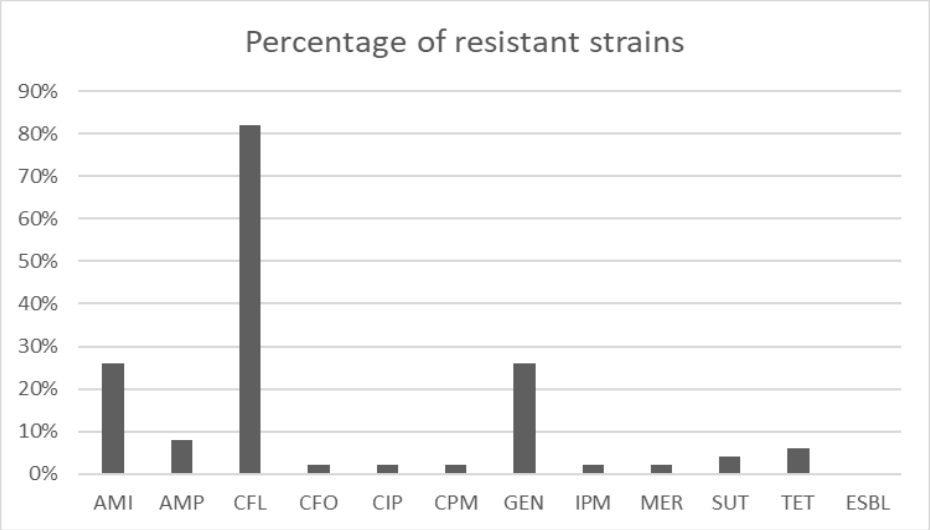
3. Results

In the present study, of the total of 154 samples (154 carcasses), we obtained 690 isolates, of which 365 strains were identified as *E. coli*. The frequency of *E. coli* in samples collected was 81% (125/154). Of the 365 *E. coli* strains, 4.38% (16/365) had the gene *eae* and 0% (0/365) the *stx1* or *stx2* genes, therefore, 16 strains were identified as EPEC, and the frequency of positive samples for STEC was zero.

Of the 154 carcasses collected, 15 (9.74%) presented EPEC strains. EPECs were also tested for the presence of the *ToxB* and *efa1* adhesion genes; no strain was positive for these genes.

The EPEC isolates were also submitted to multiplex PCR to determine their phylogenetic relationships. Most isolates belonged to phylogroup B1 (12/16). Regarding the motility test, all were positive, whereas for the hemolysis test only 6/16 (37.5%) had a positive result.

Figure 1. *Escherichia coli* resistance phenotypes, isolated from bovine carcasses, obtained in a slaughterhouse of Mineiros city, GO, Brazil. Veterinary Microbiology Laboratory UFG – Jataí.



*AMI: amikacin; AMP: ampicillin; CFL: cephalothin; CFO: cefoxitin; CIP: ciprofloxacin; CPM: cefepime; GEN: gentamicin; IPM: imipenem; MER: meropenem; SUT: sulfazotrim; TET: tetracycline; ESBL: Extended-spectrum beta-lactamase-producing *E. coli*.
Source: Authors.

It is important to note in Figure 1 that the strains were shown to be more resistant to the antimicrobials cefalotina, amicacina, and gentamicina.

No ESBL-producing strain was detected. All EPEC strains showed motility and 6 presented hemolysis. It was also observed that three EPEC strains showed multiple antimicrobial resistance, which makes the study of this pathogen even more relevant because multiresistant strains can cause serious problems for public health (Table 1).

Table 1. Group and phenotypes of EPEC, isolated from carcasses of cattle obtained in a slaughterhouse of Mineiros city, GO, Brazil. Veterinary Microbiology Laboratory UFG – Jataí.

Strain	Group	Hemolysis	Resistance Phenotypes*
1	B1	-	CFL^{SDR}
2	B1	-	CFL^{SDR}
3	B1	+	AMP^{SDR}
4	A	-	$AMI\text{-}CFL\text{-}GEN^{SDR}$
5	A	-	CFL^{SDR}
6	B1	-	CFL^{SDR}
7	B1	+	$AMP\text{-}CFL\text{-}TET^{MDR}$
8	B1	+	CFL^{SDR}
9	B1	+	$AMI\text{-}CFL\text{-}GEN^{SDR}$
10	A	+	$CFL\text{-}TET^{SDR}$
11	B1	-	GEN^{SDR}
12	A	-	$AMI\text{-}CFL\text{-}GEN^{SDR}$
13	B1	+	CFL^{SDR}
14	B1	-	$AMP\text{-}CFL\text{-}CFO\text{-}CPM\text{-}IPM\text{-}MER\text{-}SUT\text{-}TET^{MDR}$
15	B1	-	AMI^{SDR}
16	B1	-	$AMI\text{-}CFL\text{-}TET^{MDR}$

*AMI: amikacin; AMP: ampicillin; CFL: cephalothin; CFO: cefoxitin; CPM: cefepime; GEN: gentamicin; IPM: imipenem; MER: meropenem; SUT: sulfazotrim; TET: tetracycline. SDR: Single Drug resistance, MDR: Multidrug resistance.

Therefore, it was observed, in Table 1, that strains 7, 14 and 16 were resistant to three or more different groups of antimicrobials, demonstrating a multiresistance phenotype.

4. Discussion

There is a correlation between the use of unhygienic slaughterhouse practices and the incidence of meat-borne disease outbreaks (Ali et al., 2010), which occurs probably because the nutrient composition of meat is attractive to a broad spectrum of microorganisms (De Filippis et al., 2013).

The main source of microbiological contamination of beef carcasses along the slaughter line is of fecal origin, therefore, *Escherichia coli* and Enterobacteriaceae seem to be the most suitable indicators for assessing the hygienic status of the slaughter process (Barco et al., 2005). The presence of *E. coli* in the samples indicates a contamination frequency of 81%, while the frequency of EPEC in bovine carcasses was 9.74% and STEC 0%. Not only in Brazil but in other parts of the world the presence of diarrheagenic *E. coli* has been demonstrated in beef (Matos et al., 2013; Tanih, Sekwadi and Ndip, 2015; Roman et al., 2013). The microbial surface contamination of blefe carcasses by EPEC probably constitutes

a significant risk to meat handlers and consumers, especially during the processing of and/or eating of contaminated meat or meat products.

Among diarrheagenic *E. coli*, EPECs are prominent because they can cause prolonged liquid diarrhea, with high mortality – especially in children – in regions of Latin America, Africa, and Asia especially (Gomez-Duarte, 2014). In the intestinal infection by EPEC, there are changes in the normal physiological activity of the enterocyte due to the increase of the secretion of electrolytes by the cells to extracellular space, to the increase of the permeability of the intra and intercellular junctions, and to the structural change in the form of the apical region of the enterocyte.

This loses its absorptive capacity and the solutes accumulate in the intestinal lumen, which leads to watery diarrhea (Farfán-García et al., 2016). Most of the EPEC strains belong to phylogroup B1, as already described by other studies (Bok et al., 2015; Unno et al., 2009; Carlos et al., 2010). This is a predominant group in the feces of beef cattle. It should be noted that the beef cattle diet, consisting of unprocessed, natural feed for ruminants (grass and straw) promotes the predominance of phylogroup B1 (Bok et al., 2015).

No STEC strains are identified in the samples, although ruminants are considered to be the main reservoirs of this pathotype, contrasting with studies by other authors that report significant frequencies (Carvalho et al., 2012; Ojo et al., 2010). This is probably due to the collection carried out in post-processing, after the carcass washing (and in this case probably the presence of EPEC would be even higher), or because of the low contamination of the carcasses by the bovine feces during the slaughter process, which shows that the establishment takes the necessary care during the processing of the carcasses with an efficient evisceration.

EPEC pathotypes cause an injury called attaching and effacing (A/E) lesion, which is characterized by an intimate adhesion (adhesin intimine) of the bacterium to the intestinal epithelium, with the destruction of microvilli, changes in the cytoskeleton, the formation of pedestal-like structures and an accumulation of polymerized actin just below the cell attachment (Mainil and Daube, 2005). Intimine adhesin, a 94 kDa protein from the external membrane of the bacterium, is encoded by the *eae* gene (Mainil, 2013).

As already reported, 16 strains carried the gene *eae*, and did not carry *stx* genes, therefore, they were classified as EPEC. In addition to intimin, other proteins are important for the adhesion and colonization of intestinal cells, such as for adherence 1 (*Efa1*) and toxin B (*ToxB*) (Bardiau, Szalo and Mainil, 2010). The *efa1* gene encodes an adhesion factor, being

an adhesin that was originally described in some EHEC strains, which contributes to the adhesion of EPEC to epithelial cells.

This is fundamental for intestinal colonization and is strongly related to the virulence and severity of disease (Vieira et al., 2010). While the *ToxB* gene contributes to cell adhesion through the production and/or secretion of type III proteins (Michelacci et al., 2014). In the present study, EPEC strains were tested for the *ToxB* and *efa1* adhesion genes, but none of the strains show a positive result for them. As already reported by other authors, the frequency of the presence of these genes in EPEC is so varied (Monaghan et al. 2013; Pradel et al. 2015).

In their paper on global trends in the use of antimicrobials in feed production animals, Boeckel et al., (2015) estimate that antimicrobial consumption will increase by 67% until 2030, and that in Brazil, Russia, India, China, and South Africa this increase will be 99%, with the aim of protecting animal health and increasing productivity. This will bring selection pressure to the bacteria, making them more resistant. In the present work, we can observe this trend, where 100% of the samples presented single or multiple resistance to antimicrobials. The strain EPEC 14 was the one with the most extended resistance phenotype, which was found for 8 antibiotics, from 4 different classes, representing a high risk to public health.

Antibiotic resistance remains a major challenge in human and animal health. Contamination of beef by bacteria resistant to antibiotics can be a major threat to public health because the genetic elements that determine the resistance are mobile. That is, they can be transferred to other bacteria potentially pathogenic to humans. These foods can be contaminated if they are not properly handled during slaughter and processing. In Brazil, a large proportion of the population depends on beef as a source of protein.

Studies have shown a great variability of antimicrobial resistance between strains isolated from bovine carcasses (Fontcuberta et al., 2016; Loiko et al., 2016; Murutu et al., 2016). In the present study, the most effective antimicrobials were cefoxitin, ciprofloxacin, cefepime, imipenem, and meropenem, with only 2% resistance between the bacteria. On the other hand, cephalothin, with 82% of resistance between strains, followed by amikacin and gentamicin, both with 26%, were the least effective.

The results obtained in the present work are important in terms of public health, showing that beef can carry multiresistant EPECs. This is in line with that described by Arias et al. (2012), who reported that in several studies antimicrobial resistance in humans may be related to the use of antimicrobials in animals, because the classes of drugs used in both cases are the same, thus, causing therapeutic failure due to the transfer of multiresistant bacteria through the food chain.

However, it is possible to reduce the bacteria count to levels considered hygienically acceptable, through practices such as pre-slaughter fasting, separation between clean and dirty areas in the slaughterhouse, and rigorous inspection and sanitization of the carcasses, so that a low number of multiresistant bacteria enter the food-processing chain and reach the consumer (Pissetti et al., 2016). Therefore, it is extremely important, that the slaughterhouses of bovines present in this region, take all possible measures of hygiene and sanitization in the slaughter, to avoid that the meat produced in the region transports EPEC multi-resistant.

5. Final Considerations

In this research, antibiotic multiresistant EPEC, belonging to group A and B1, were identified in bovine carcasses, showing that at some point in the production process there was contamination. This calls attention to problems related to public health, with a risk of transmission to humans.

The result obtained is extremely important for demonstrating the relevance of hygienic and sanitary conditions of food-producing establishments to avoid the possibility of contamination of carcasses from pathogenic microorganisms. And finally, it is important that more research is done in the main beef producing regions in Brazil, in order to know the real frequency of resistant EPEC strains, as well as if this resistance profile changes according to the geographic region.

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