FULL ARTICLE

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Antimicrobial resistance of Shiga toxin-producing *Escherichia coli* isolated from sheep

Escherichia coli *produtora de toxina Shiga resistentes a antimicrobianos isoladas de ovinos*

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ABSTRACT

The presence of Shiga toxin-producing *Escherichia coli* (STEC) and resistance to beta-lactams in healthy sheep represents a potential public health risk. This study aimed to characterize STEC isolates in sheep feces for toxin production and resistance to beta-lactam antibiotics. In the present study, among the 40 isolates, we found a predominance of subtype Stx1 (22/40), followed by subtype Stx1 + Stx2 (11/40), while the less prevalent group was Stx2 (7/40). Also, we found phenotypical resistance to beta-lactam antibiotics in 50% (20/40) of the strains analyzed, forming two groups, one with resistant isolates and the other with non-resistant isolates. The cytotoxicity of the isolates did not vary among the groups. In addition to having this characteristic, some multiresistant isolates produced significant amounts of toxins. This leads to the conclusion that the mechanisms of antimicrobial resistance via beta-lactamases are present in sheep STEC and that the cytotoxicity of those isolates is variable regarding such resistance.

Keywords: STEC. Antibiotic. Beta-lactamases. Zoonoses. Toxins.

RESUMO

A presença de *Escherichia coli* produtora de toxina Shiga (STEC) e resistente a beta-lactâmicos em ovinos saudáveis, representa um risco potencial para a saúde pública. O objetivo deste estudo foi caracterizar isolados STEC presentes em fezes de ovinos, quanto a produção de toxina, bem como a resistência aos antibióticos beta-lactâmicos. No presente estudo, dentre os quarenta isolados, foi observada a predominância do subtipo *Stx1* (22/40), seguido do subtipo *Stx1+ Stx2* (11/40), enquanto o grupo menos prevalente foi *Stx2* (7/40). A resistência fenotípica aos antibióticos beta-lactâmicos foi observada em 50% (20/40) das cepas analisadas, formando dois grupos, um com isolados resistentes e outro de isolados não resistentes. A citotoxicidade dos isolados não variou entre os grupos. Alguns isolados multirresistentes, além de possuírem essa característica, produziram quantidades significativas de toxinas. Isto conclui, que os mecanismos de resistencia antimicrobiana, por meio de beta-lactamases estão presentes em STEC de ovinos, e que a citotoxicidade desses isolados é variável com relação a esta resistência.

Palavras-chave: STEC. Antibiótico. Beta-lactamases. Zoonoses. Toxinas.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic pathogens that may cause severe diarrheagenic diseases in humans, and other ruminant animals, such as sheep, are their natural reservoirs (Liu et al., 2022). Natural isolates of STEC can produce Stx1, Stx2, or both toxins (Melton-Celsa, 2014). The STEC producers of extended-spectrum β-lactam (ESBL) represent a constant public health threat worldwide, leading to severe infections and raising important therapeutic questions because beta-lactam antibiotics are frequently used in clinical routine (Puii et al., 2019). Therefore, the production of the Shiga toxin by STEC is an important virulence factor, as well as the resistance to beta-lactam antibiotics. However, the correlation between the output of the Shiga toxin and the resistance to beta-lactam in STEC is little studied. This study aimed to describe STEC isolates present in sheep feces for toxin production and resistance to beta-lactam antibiotics.

Materials and Methods

Samples and identification. Stella et al. (2017) identified the isolates used in this study. *E. coli* strains were isolated from rectal swabs of 23 healthy sheep southwest of Goiás, Brazil, and plated on MacConkey agar. Colonies were biochemically identified as belonging to *E. coli* based on lactose fermentation, indole production, Voges–Proskauer assay, citrate utilization, absence of urease, and hydrogen sulfide production.

PCR. DNA extraction from the isolates was performed by thermal lysis and stored in a freezer at -30 °C. The isolates were tested for the presence of genes stx1 and stx2 by PCR, as described by Vidal et al. (2005). Strains that carry one or both stx genes have been considered as belonging to the STEC pathotype. Strain EDL933 (O157:H7, stx1+, stx2+, eae+) was used as a positive control for the PCR assay. Conventional PCR was also performed to investigate CTX-M groups. For the five classes of CTX-M, the primers were: class 1, 5' AAA AAT CAC TGC GCC AGT TC and 5' AGC TTA TTC ATC GCC ACG TT (415 bp); class 2, 5' CGA CGC TAC CCC TGC TAT T and 5' CCA GCG TCA GAT TTT TCA GG (552 bp); class 9, 5' CAA AGA GAG TGC AAC GGA TG and 5' ATT GGA AAG CGT TCA TCA CC (205 bp); class 08 TCG CGT TAA GCG GAT GAT GC and 5' AAC CCA CGA TGT GGG TAG C (666 bp), and class 25, 5' GCA CGA TGA CAT TCG GG and 5' AAC CCA CGA TGT GGG TAG C (327 bp) (Woodford et al., 2006). As a positive control, strains belonging to the bacterial collection of the Laboratory of Veterinary Microbiology of the Federal University of Jataí were used. PCR reactions were assembled with a final volume of 25 μL, 12.5 μL of which were Promega Mix (Promega Corporation, USA), 0.5 μL of each primer, 5 μL of ultrapure water, and 2.5 μL of DNA.

Antimicrobial resistance. Antimicrobial resistance was assessed by disk-diffusion to the following beta-lactam antimicrobials: Penicillin [Amoxicillin (10 μg) and Ampicillin (10 μg); Cephalosporins of 1st generation [Cephalothin (30 μg)]; 2nd generation [Cefaclor (30 μg), Cefoxitin (30 μg)]; 3rd generation [Ceftriaxone (30 μg) Cefotaxime (30 μg), Ceftazidime (30 μg)]; and 4th generation [Cefepime (30 μg)]; Carbapenems [Meropenem (10 μg)]. The disk-diffusion method was used according to Clinical and Laboratory Standards Institute (2018). The *E. coli* strains were inoculated in Mueller Hinton broth (Difco) and incubated at 37 °C for 3 h. The colony suspension was then diluted in a saline solution until set to 0.5 McFarland standard turbidity (approximately 106 CFU/mL). After seeding the aliquots in Mueller Hinton agar, the antibiotics' disks were added. After incubating the plates at 37 °C for 16 to 20 h, the halos were measured.

Cytotoxicity assay. Cytotoxicity assay was performed as described by Rocha et al. (2012). Vero cells (1×105 cells/mL) were grown in 96-well plates in DMEM medium in the presence of 10% FBS for 24 h at 37 °C and 5% CO2. Bacteria cytotoxicity was assayed by incubating the Vero cells in the presence of a 1/10 dilution of the bacterial culture supernatant in DMEM with 2% FBS for 72 h at 37 °C and 5% CO2. The supernatant was obtained by growing the strains overnight in LB, followed by centrifugation at 1,690 g for 5 min. MTT (Sigma-Aldrich) was used to determine cell viability according to the manufacturer's instructions.

EDL933 and DH5-α (a commensal K12 strain) were used as positive and negative controls, respectively. Cytotoxicity of the isolates was normalized against the cytotoxic activity of strain EDL933. At least three independent assays were performed for each bacterial strain.

Statistical analysis. Analysis by Student's t-test was performed to compare the average cytotoxicity production between the group of bacteria resistant to beta-lactam antibiotics and the group of bacteria sensitive to beta-lactam antibiotics.

Results and Discussion

STEC can be found in several domestic and wild animals. However, ruminants are considered the main reservoirs, eliminating the bacteria via feces and contaminating humans directly (rural workers) or indirectly (contaminated foods) (Coura et al., 2014; Yang et al., 2017). STEC is considered an emergent pathogen and a challenge for public health as they have significant pathogenicity to humans and are routinely involved in outbreaks of foodborne diseases of animal origin (Caldorin et al., 2013; Liu et al., 2022). There are several *E. coli* strains pathogenic ones, among the pathogenic strains, STEC is considered an emergent pathogen and highlighted due to its pathogenicity to humans and for being routinely involved in foodborne disease outbreaks. Contagion may occur via the ingestion of raw or undercooked meat, non-pasteurized milk, and plants with some fecal contamination, setting off severe settings of bloody diarrhea, nausea, vomiting, and gastroenteritis. Thus, the presence of STEC in healthy sheep represents a potential risk to public health that cannot be neglected (Loiko et al., 2016). In this work, 40 STEC strains were characterized from healthy sheep and identified as described in the

Materials and Methods Section

Primarily, STEC may produce the Shiga 1 (stx1) and Shiga 2 (stx2) toxins (Jajarmi et al., 2017). Several studies have shown that the frequency of STEC in sheep varies widely according to the methodology employed, sampling (feces, milk, meat), as well as the region studied (Ferhat et al., 2019; Furlan et al., 2019; Liu et al., 2022; Martins et al., 2015; Oporto et al., 2019; Otero et al., 2017; Zaheri et al., 2020). This study (Table 1) observed a predominance of subtype Stx1 (22/40) followed by subtype Stx1 + Stx2 (11/40), while the less prevalent group was Stx2 (7/40). Other authors state that, in animals, the main environmental force that molds the genetic structure of the intestine population of *E. coli* is the domestication status of the host (Escobar-Páramo et al., 2006) and socioeconomic factors, such as diet and hygiene (Skarżyńska et al., 2020), instead of geographic or genetic conditions of the host.

E. coli is one of the seven species the World Health Organization uses as a sentinel organism for antibiotic resistance. There has been a significant concern with the emergence and dissemination of ESBL-producing *E. coli* associated with ruminants (Liebana et al., 2013). Beta-lactams are antimicrobials that inhibit the synthesis of the bacterial cell wall and are characterized for containing the beta-lactam ring in their structure. Phenotypical resistance to those antibiotics was observed in 50% (20/40) of the strains analyzed, forming two groups, one with resistant isolates and the other with non-resistant isolates (Table 1). The cytotoxicity of the isolates did not vary between the groups, and Student's t-test showed there is no significant difference in the average production of cytotoxins between the group of isolates resistant to beta-lactam antimicrobials (M:73.3; n=20) when compared with the group of non-resistant isolates (NDR) (M:66.45; n=20).

Antimicrobial resistance in STEC isolates is routinely reported (Beceiro et al., 2013; Colello et al., 2015; Pereira et al., 2014). It may be considered an important virulence factor for the establishment of infections. Acquired resistance results from structural and biochemical alterations in the bacterial cell, determined by genetic chromosomal or extrachromosomal alterations (Santana et al., 2012). Therefore, the presence of resistant isolates in sheep feces also indicates the possibility of propagating mobile genetic elements to other bacteria in the intestinal tract, thus contributing to the selection of emerging pathogens (Beceiro et al., 2013). Identifying isolates carrying the CTX M genes and phenotypically resistant (6, 7, and 36) shows this possibility in the herd studied.

The indiscriminate use of antibiotics, such as cephalosporins, in veterinary medicine results even more in a new generation of multiresistant bacteria. In agricultural regions, the inefficient use of antibiotics in animal production may contribute to the continuous increase of antimicrobial resistance (Coura et al., 2014; Van Boeckel et al., 2015). In many cases, there may not be effective antibiotics for treating complicated infections, except for carbapenems, since the resistance mediated by enzymes capable of hydrolyzing all beta-lactams, including carbapenems, has grown worldwide (Yu et al., 2016).

As can be observed in Table 1, some multiresistant isolates (Magiorakos et al., 2012), such as strains 6, 14, and 22, in addition to having this characteristic, produce significant amounts of toxins, showing a combination of virulence factors. However, overall, as observed between

Table 1 – Phenotypic and genotypic profile of Shiga toxin production and antimicrobial resistance of two groups of STEC isolated from sheep in Jataí-GO, Brazil

Resistant Group (Strains)	Pathotype Definition Gene		Cytotoxicity assay ±SEM[a,b]	Antimicrobial resistance		Non Resistant Group (Strains)	Pathotype Definition Gene		Cytotoxicity assay ±SEM[a,b]	Antimicrobial resistance	
	Stx1	*Stx2*		CTX M genes	Antimicrobial resistant pattern*		*Stx1*	*Stx2*		CTX M genes	Antibiogram disk diffusion*
1	-	+	85 ± 22.5		CFL	2	-	+	53 ± 30.6		NDR
6	-	+	23 ± 23.0	• Group 9	CFL-AMP-CFC-CTX-CEF	3	-	+	0 ± 15.1		NDR
7	-	+	14 ± 18.9	• Group 9	CFL	4	-	+	35 ± 19.4		NDR
8	+	+	90 ± 14.7		CFL	5	-	+	22 ± 6.1	• Group 9	NDR
10	+	+	14 ± 12.7		CFL-CFC	9	+	+	65 ± 26.1		NDR
12	+	+	79 ± 19.9		CFL	11	+	+	72 ± 16.8		NDR
14	+	+	62 ± 5.6		CFL-CTX-CRO	13	+	+	43 ± 43.8		NDR
18	+	+	63 ± 20.8		CFL	15	+	+	65 ± 3.29		NDR
22	+	-	66 ± 25.2		CFL-AMP-CFC	16	+	+	71 ± 22.1		NDR
23	+	-	88 ± 14.4		CFL-CFC	17	+	+	88 ± 19.6		NDR
24	+	-	113 ± 14.4		CFL-CTX-CRO	19	+	-	77 ± 13.3		NDR
25	+	-	95 ± 28.2		AMO-AMP	20	+	-	65 ± 17.1		NDR
27	+	-	83 ± 25.4		AMP	21	+	-	72 ± 19.5		NDR
32	+	-	90 ± 11.3		AMO-AMP	26	+	-	100 ± 13.9		NDR
33	+	-	59 ± 22.0		CFL-CRO	28	+	-	87 ± 18.0	• Group 1	NDR
35	+	-	50 ± 19.7		CFL-AMP	29	+	-	79 ± 23.8		NDR
36	+	-	115 ± 7.8	• Group 1	CFL	30	+	-	83 ± 19.0		NDR
37	+	-	74 ± 8.7		CFL-CFC	31	+	-	96 ± 8.5		NDR
38	+	-	93 ± 21.7		AMP	34	+	-	79 ± 13.4		NDR
39	+	-	110 ± 14.2		AMP	40	+	-	77 ± 16.8	• Group 1	NDR

[a]Each value represents the mean ± S.E.M. (standard error of the mean) of three independent experiments. [b]Cytotoxicity against Vero cells relative to the cytotoxicity displayed by strain EDL933. *CFL (Cefalotina); AMP (Ampicilina); CFC (Cefaclor); CTX (Cefotaxima); CEF (Cefepime); CRO (Ceftriaxona); AMO (Amoxicilina).

the two STEC groups, resistant and non-resistant, there was no correlation between toxin production and phenotypical resistance since this resistance, as well as the possession of the CTX M genes, has been homogeneously distributed between the groups. Pereira et al. (2014), when correlating the presence of stx genes and antimicrobial resistance, also observed that resistance had been homogeneously distributed between the resistant and non-resistant groups.

Certain antimicrobials, such as quinolones, may induce the production of the Shiga toxin and, therefore, promote the appearance of acute disease symptoms in humans (Amézquita-López et al., 2018), through the lysis of cells causing the infection, thus releasing substantial amounts of toxin in the host organism. However, the association between resistance to beta-lactam and Shiga toxin production is little studied. Shiga toxin production is disseminated via phages, and the evolutionary interaction between phage and its bacterial host is dynamic, with phases of inhibition, selection, and evolution. Furthermore, the temperate phages may not transport antimicrobial resistance genes but play a more prominent role, interfering in the metabolic regulation that changes bacterial sensitivity to antibiotics (Holt et al., 2017).

The commensal microbiota, and particularly the intestinal microbiota, has been shown to have an essential role in the appearance of antimicrobial resistance, where mobile genetic elements play a leading role in facilitating the acquisition and dissemination of resistance genes. As observed in this work, *E. coli* has a diversified and flexible mobilome regarding antimicrobial resistance (Table 1). Thus, that may mean mobile genetic elements are more mobile in *E. coli* when other bacteria species are considered, whether because they are frequently mobilized in plasmids or due to a higher conjugation rate (Leekitcharoenphon et al., 2021).

Kong et al. (2019) reported the presence of antimicrobial resistance genes in ruminant manure, indicating the possibility of transformation in this environment. In addition to this critical environmental contamination, Oporto et al. (2019) reported that those resistance profiles are like human strains, indicating the possibility of a connection between animal production and human infections. Considering the multidrug-resistant and potentially pathogenic microorganisms identified in this work (Table 1), and, as highlighted by Van Den Brom et al. (2020), that STECs of sheep origin are important causative agents of foodborne diseases, strict hygiene practices must be applied throughout the sheep production process (Ferhat et al., 2019). Moreover, bacterial resistance to antimicrobials has become a global public health issue that involves different ecological circles, including animals and humans (Furlan et al., 2019). Therefore, resistance to beta-lactam in STEC is an issue of growing concern, supporting the need to monitor the use of those agents by the agricultural and livestock sectors.

Conclusions

This study concludes that the resistance mechanism by beta-lactamases is present among STEC isolates of sheep origin. The cytotoxicity did not vary among resistant and non-resistant STEC isolates, evidencing a high variability in the tested parameters.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

The authors declare no direct animal use in the experiment.

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