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Research Article

Incidence of Shiga-Toxin-Producing *Escherichia coli* (STEC) in Diarrheic Calves and its Profile of Sensitivity to Antimicrobials and to the Extract of *Eugenia uniflora L.*

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ABSTRACT

The present work had the goal of evaluating the presence of the Shiga-Toxin-Producing bacterium *Escherichia coli* (STEC) in diarrheic calves, its resistance profile against the antimicrobials usually employed in clinical practice, and evaluating the antimicrobial activity of the plant extract of the leaves of *Eugenia uniflora* L. against the isolates. The samples were obtained from animals belonging to dairy farms in the South of Minas Gerais and submitted to microbial isolation and identification. Molecular assays were performed, by the search for genes *stx1* and *stx2*, for STEC identification. The isolates were subjected to tests of antimicrobial sensitivity to the drugs usually employed in clinical treatment, using the disk diffusion method. The Minimum Inhibitory Concentration (MIC) of the antimicrobials gentamicin and sulfamethoxazole+trimethoprim was performed by the Etest technique. The sensitivity of the plant extract was investigated by the methods of diffusion in agar and broth microdilution, and the MIC and the Minimal Bactericidal Concentration (MBC) were determined. From the isolates analyzed, 17% presented the searched genes isolated (10%*stx1* and 1%*stx2*) and in combination (6%*stx1*+*stx2*). These isolates also presented multiresistance to the antimicrobials tested. The hydroalcoholic extract of the *E. uniflora* leaves presented antimicrobial activity against all isolates analyzed, with a MIC of 12.5 mg/mL and bacteriostatic activity. The data obtained contribute to the knowledge of STEC incidence associated to bovine diarrhea and point to the need of monitoring and controlling these multiresistant enteropathogens. Although Eugenia uniflora L. shows a potential antimicrobial action *in vitro*, other preclinical and/or clinical studies must be developed in order to ensure its therapeutic application.

Keywords: Bovine diarrhea; *Eugenia uniflora* L; *Escherichia coli*; PCR; Antimicrobial sensitivity; STEC

INTRODUCTION

Brazil has been presenting a gradual increase in the world milk production rates. This rise demands that the livestock products produced present the highest excellence in quality, mainly aiming at exporting. Therefore, each step of milk processing is monitored, as well as the diseases that might attack the animals, impairing the quality of the end product [1]. Among these diseases is neonatal diarrhea, one of the main diseases that attack the calves in their first weeks of life, compromising dairy production because of the costs with treatment or impairments caused by animal loss. Among the infectious agents frequently associated to this pathology is *Escherichia coli*, an opportunistic Gram-negative bacillus, present in the normal microbiota of the

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intestinal tract of humans and animals [2]. This microorganism is classified into different pathotypes according to its pathogenicity mechanisms, among which are the Shiga-Toxin Producers (STEC), which have major implications in the veterinary field. The domestic ruminants, mainly the bovines, are known reservoirs of STEC, occasionally suffering from diarrhea [3].

Since it is a microorganism related to cases of resistance to multiple drugs, some pathogenic strains, such as STEC, have been generating a major concern in the fields of public health and animal production, given their potential for dissemination. This promotes, therefore, inefficiency in the treatments of infections, as well as an increase in treatment costs. Cases of microbial resistance have been recurrent, because of the indiscriminate use of antimicrobial agents, which trigger the mechanism of selection of resistant strains. The antimicrobials frequently used in veterinary therapy have a wide spectrum of action, which favors the emergence of resistance. Therefore, it becomes necessary to execute studies that evaluate the resistance profile of pathogenic bacteria and search for new strategies to fight them [4,5].

Aiming at reversing this picture, medicinal plants have been greatly contributing to the search for new active principles with antimicrobial action. Given the wide existing ecological biodiversity, especially in Brazil, medicinal plants foster explorations and scientific research, and can be an interesting strategy for the control of resistant microorganisms. In this context, the present work had the purpose of evaluating the occurrence of Shiga-Toxin-Producing *E. coli* (STEC) in samples originated from diarrheic calves, as well as the resistance profile of this microorganism against the antimicrobials routinely used in veterinary therapy, besides investigating the antimicrobial action of the hydro-alcoholic extract of the leaves of Eugenia uniflora L.

MATERIAL AND METHODS

Sampling

The fecal samples were collected from newborn calves belonging to four rural properties, specialized in milk production, located in places near the city of Alfenas, South of the State of Minas Gerais, in the period from July 2014 to March 2015. The main breeds found were Dutch, Jersey, Gir and half-breed among them. A total of 100 samples from diarrheic animals were obtained, collected directly from the animal's rectum, using sterile swab and transported in PBS solution (100 mM of NaCl and 100 mM of NaH_2PO_4). Right after collection, the samples were taken to the Laboratory of Biology and Physiology of Microorganisms of the University José do Rosário Vellano (UNIFENAS), where the analyses were performed [6-8].

Microbial isolation and identification

After collection, the samples were diluted (1:10) and 50 µL aliquots were plated in dishes containing Hicrome *E. coli* agar (*HiCrome E. coli Agar*, M1295-500G, Himedia, Mumbai, India) and incubated at 35°C for 24 h. Subsequently, 10 indicative colonies (greenish coloration) were selected, randomly and subcultured in tubes containing BHI agar (*Brain Heart Infusion Agar*, M211-500G, Himedia, Mumbai, India) at 37°C for 24 h to obtain pure cultures. The selected isolates were submitted to identification by the aspect after Gram coloration (Gram-negative bacilli) and biochemical tests (Indole, Methyl Red (MR), Voges-Proskauer (VP), Citrate, Urease, and Hydrogen Sulfide). The isolates identified were stored in tubes containing BHI broth with the addition of 10% of glycerol at -70°C [9].

Obtaining the total genome

The bacterial cultures were developed in BHI broth (*Brain Heart Infusion Medium*, M211-500G, Himedia, Mumbai, India) at 37°C for 24 h. After growth, the cells were centrifuged at 10000 rpm for 5 min, and the supernatant was discarded. To the cell pellet obtained, 1 mL of PBS (100 mM of NaCl and 100 mM of NaH_2PO_4) was added, submitted to vortex shaking for 30 seconds and centrifugation at 15000 rpm for 2 min at 4°C. Subsequently, the supernatant was discarded and 500 µL of sterile purified water were added to the pellet, following vortex shaking, and it was placed in boiling water bath for 10 min. Right after, the cell material was submitted to the centrifugation process at the same conditions as previously. The supernatant obtained (~300 µL) was transferred to a microtube of 2 mL and stored at -20°C [10].

Primers and gene amplification conditions

Based on the DNA sequences of genes *stx1* and *stx2*, the following oligonucleotides were used to amplify the virulence factors for the STEC pathotype: primers *stx1*F (5'–AGAGCGATGTTACGGTTTG–3') and *stx1*R (5'–TTGCCCCCAGAGTGGATG–3'), and primers *stx2*F (5'–TGGGTTTTTCTTCGGTATC–3') and *stx2*R (5'–GACATTCTGGTTGACTCTCTT–3'), which amplify a fragment of 388 bp and 807 bp, respectively. A total of 5 µL of DNA samples was added to 45 µL of the PCR mixture consisting of 200 µM DNTPs (1 µL) (for each deoxynucleoside triphosphate – dATP, dCTP, dGTP and dTTP) (Promega Corporation, Madison WI, USA), 10 pmol of each primer (0.8 µL) (Integrated DNA Technologies, Inc., Iowa, USA), 25 mM $MgCl_2$ (6 µL) (Promega Corporation, Madison WI, USA), 5× Green GoTaq® Flexi Buffer (10 µL) (Promega Corporation, Madison WI, USA), 5U µL-1 GoTaq® Flexi DNA polymerase (0.25 µL) (Promega Corporation, Madison WI, USA), and the volume (50 µL) was completed with sterile purified water. The amplifications of the specific genes were conducted in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA, mod. 9902) with the cycles: initial denaturation at 94°C for 2 minutes, followed by 25 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds), and a final extension at 72°C for 7 minutes [11].

DNA electrophoresis

The products amplified by the PCR reaction were analyzed by the electrophoresis in agarose gel at 1.8% in 0.5x TBE (for 5X, 54.0 g Tris, 3.72 g EDTA [Na_2], 27.5 g Boric Acid, complete to

1000 mL). The electrophoresis was performed on a Pharmacia Biotech electrophoresis cube (max submarine unit HE 99 X), with Pharmacia Biotech power supply (EPS 300) at the conditions of 150 V for 1 h. The size of the DNA fragments was determined by a molecular marker of 100 bp (DNA ladder, Invitrogen) including in all amplifications a negative (sterile purified water) and a positive (positive strains for the enterotoxins tested) control. After the electrophoretic run, the gels were stained with ethidium bromide (1 µL/mL), visualized on a UV transilluminator (Major Science, USA), photographed and analyzed.

Tests of sensitivity to antimicrobials

The STEC isolates were submitted to susceptibility tests against antimicrobials. These isolates were inoculated in 3 mL of BHI agar (*Brain Heart Infusion Agar*, M211-500G, Himedia, Mumbai, India) and incubated at 35°C for 24 hours. Subsequently, an inoculum was adjusted to the turbidity of 0.5 at the McFarland scale with saline solution (150 mM of NaCl). Right after, the adjusted inoculum was plated with the use of sterile swabs in dishes containing Mueller-Hinton Agar (*Mueller-Hinton Agar*, M173-500G, Himedia, Mumbai, India) and, after approximately 3 minutes, the time necessary for drying the medium surface, the discs containing the antimicrobials were placed, as recommended by the Clinical and Laboratory Standards Institute [12]. The isolates were tested for the following antimicrobials, selected according to the therapeutic practice used in Veterinary Medicine: Ampicillin 10 µg, Cephalothin 30 µg, Streptomycin 10 µg, Gentamicin 10 µg, Ciprofloxacin 5 µg, Chloramphenicol 30 µg, Tetracycline 30 µg, Nitrofurantoin 300 µg, Sulfamethoxazole+Trimethoprim 25 µg and Nalidixic acid 30 µg (impregnated discs). The dishes were incubated at 35°C for 18 hours. The read was performed by the measurement of the inhibition halos, with the use of a caliper. The diameters, obtained in millimeters, were compared according to the CLSI guide.

At the Minimum Inhibitory Concentration (MIC), the Etest technique was employed (Epsilometer test, Biomérieux Ltda.), in which thin and inert strips containing the antimicrobials gentamicin and sulfamethoxazole+trimethoprim, calibrated with visual scale, indicating the drug concentration, were placed on the inoculum. Because of the high cost to perform this technique, the two antimicrobials mentioned were selected, encompassing a chemotherapeutic representative and an antibiotic, which are indicated in CLSI and are present in the antibiogram [12].

Thus, the isolates were inoculated in tubes containing BHI agar (*Brain Heart Infusion Medium*, M211-500G, Himedia, Mumbai, India) and incubated at 37°C for 24 hours. Subsequently, the turbidity of the isolates was adjusted to 0.5 in the *McFarland* scale with saline solution (150 mM of NaCl). Right after, the adjusted inocula were plated using sterile swabs in dishes containing Mueller-Hinton Agar (*Mueller-Hinton Agar*, M173-500G, Himedia, Mumbai, India) and after approximately 3 minutes, the time necessary for drying the surface of the medium, the strips containing the antimicrobials were placed. Then, they were incubated at 35°C for 18 hours. The elliptic growth inhibition halos were carefully inspected, using transmitted light for the detection of small colonies or sparse growth inside the inhibition zone, observing the ellipse intersection point. As quality control, the tests were performed using the standard strain *E. coli* ATCC 25922 following CLSI [12].

Tests of susceptibility to the hydroalcoholic extract of Eugenia uniflora leaves

The tests of susceptibility to the fluid extract of *Eugenia uniflora* leaves were performed by the method of microdilution in broth following CLSI guidelines (2012). The extract was commercially acquired and stored according to the manufacturer's recommendations (Aksy Comercial Ltda, São Paulo, SP, Brazil) (alcoholic strength: 57°, density: 0.920). The extract was concentrated by the evaporation of the solvent in an equipment of rotative evaporation (Rotary Evaporator, mod. 802, Fisatom Equipamentos Científicos Ltda, São Paulo, SP, Brazil), where 500 mL of the extract were subjected to water bath, under controlled temperature between 50°C-60°C, for solvent evaporation, and constant rotation and pressure of 40 rpm and 500 mmHg, respectively. The extract was maintained under the conditions mentioned until total solvent removal, evidenced by the interruption of alcohol release. After the end of the process, the extract was transferred to an amber bottle, properly identified, and submitted to the temperature of -20°C to evaluate the efficiency of the method [13].

Next, the extract was submitted to the freeze-drying process. The extract obtained in the previous step was distributed into glass bottles, of the penicillin type, previously identified and weighed in analytical scale. The uncapped bottles were placed in the freeze dryer (Christ Freeze Dryer, mod. Beta 2-16), maintained for seven days for total water removal. Immediately after, the bottles were rapidly capped and sealed, so that moisture absorption would be avoided as much as possible. Subsequently, they were weighed on an analytical scale to determine the obtained mas [13]. To assess the activity of the extract of *E. uniflora* leaves, the minimum inhibitory concentration (MIC) was performed, using the method of microdilution in broth. Firstly, the isolates were cultivated in tubes containing BHI agar at 37°C for 24 hours. Isolate turbidity was adjusted to 0.5 of *McFarland* scale with sterile saline solution (150 mM NaCl), followed by a dilution at 1:10. The freeze-dried extracts were prepared in aqueous solvent at 50 mg/mL and sterilized by filtration (Millipore filter, 0.22 µm) and stored in 2 mL microtubes at -70°C.

The assay was conducted in sterile microdilution plates with multiple wells (96–well cell culture microplates), in which aliquots of 10 µL of the inoculate were added to the wells, containing 100 µL/well of Mueller Hinton broth (2X) (*Mueller-Hinton Broth*, M391-500G, Himedia, Mumbai, India) and of the extract of *E. uniflora* leaves (decreasing concentrations from 25 to 0.012 mg/mL). Then, the plates were incubated at 37°C for 24 hours. The read and interpretation of the results were performed following the CLSI guidelines (2012). The Minimum Bactericidal Concentration (MBC) was determined from the wells which, after 24 hours of incubation, did not present any

turbidity, in other words, higher concentrations than MIC. An aliquot of 10 µL was collected from these wells and inoculated on the surface of Petri dishes containing EMB agar (*Eosin Methylene Blue Agar*, M317-500G, Himedia, Mumbai, India), which were incubated at 37°C for 24 hours. Then, the lowest extract concentration that did not result in the characteristic bacterial growth was considered as MBC [12].

RESULTS

Isolation and identification

From the 100 samples analyzed 99 (99%) were positive regarding the presence of the bacterium *E. coli*. Considering that, from each sample, 10 indicative colonies were selected, 990 isolates were obtained in total.

Molecular identification of the virulence genes

For the total of isolates, in the molecular analysis, approximately 10% carried only gene *stx1*, 1% only carried gene *stx2* and 6% presented a combination of the searched genes (*stx1* and *stx2*). The results were important because of the detection of pathogens having the virulence genes characteristic of the development of the disease in the animals (Figure 1).

Tests of sensitivity to antimicrobials

Using the data obtained in the antibiogram reads, the isolates were classified according to their profile of sensitivity and/or resistance to the agents tested, using the parameters provided by CLSI (2013) (Table 1).

The following percentage values of resistance to the antimicrobial agents tested were verified: Gentamicin 61.2%, Streptomycin 100%, Tetracycline 97%, Nalidixic acid 75.7%, Chloramphenicol 32.3%, Ampicillin 82.3%, Ciprofloxacin 41.1%, Sulfamethoxazole+Trimethoprim 88.2%, Nitrofurantoin 76.4%, and Cephalothin 88.2%.

The determination of the MIC, for the isolates considered resistant in the antibiogram, was performed for the antimicrobials gentamicin and sulfamethoxazole+trimethoprim. For the first, values of 0.75 µg/mL (6.25%), 1.5 µg/mL (6.25%), 24 µg/mL (12.5%), 38 µg/mL (6.25%), 48 µg/mL (12.5%) and 64 µg/mL (18.75%) were found; the others (43.75%) did not form inhibition ellipse.

On the other hand, for sulfamethoxazole+trimethoprim none of the isolates presented the formation of inhibition ellipse, in other words, none of the concentrations present in the strip was capable of inhibiting bacterial growth.

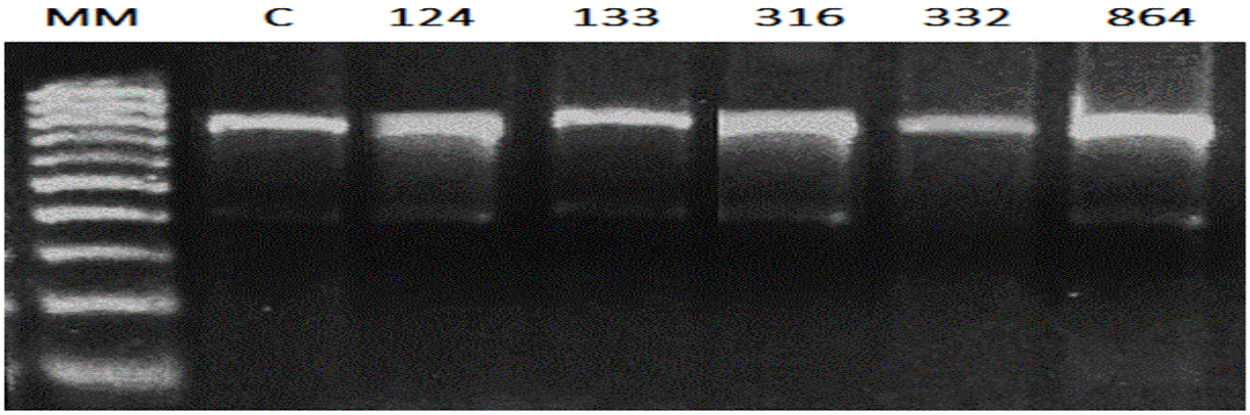

Figure 1: Amplicons of the *stx2* gene (807 bp) of *Escherichia coli* clinical isolates coming from fecal samples of diarrheic calves. MM: Molecular marker 100 bp DNA ladder (DNA ladder, Invitrogen); C: positive control (strain ECL6611, positive for the enterotoxins tested, obtained from the Reference Laboratory for *Escherichia coli*, Faculty of Veterinary Medicine of the University of Montreal); 124, 133, 316, 332 and 864 corresponds to E. coli isolates from the four farms analyzed.

Table 1: Profile of sensitivity to antimicrobials and to the extract of *E. uniflora* leaves of the clinic isolates of Shiga-Toxin-Producing *E. coli* (STEC) following CLSI guidelines (CLSI document M100-A23) [12].

Farms	Code of the isolates	Antimicrobials										Extract of E. uniflora leaves MIC Halo size (mm)				
		Gene	GEN	EST	TET	NAL	CLO	AMP	CIP	SUT	NIT	CFL	50 mg/ml	100 mg/ml	150 mg/ml	mg/ml
E. coli ATCCâ 25922			S	S	S	S	S	S	S	S	S	S				
A	39	stx2	S	R	S	S	S	I	S	S	R	I	8	9	10	12.5
A	108	stx1+stx2	R	R	R	R	R	R	R	R	R	R	8	9	10	12.5

A	116	stx1	R	R	R	R	S	R	I	R	R	R	8	9	10	12.5
A	118	stx1+stx2	R	R	R	R	I	R	R	S	R	R	8	9	10	12.5
A	124	stx1+stx2	S	R	R	I	S	R	S	R	S	I	8	9	10	12.5
A	125	stx1+stx2	S	R	R	S	S	R	S	R	S	R	8	9	10.1	12.5
A	133	stx1	R	R	R	R	R	R	I	R	R	R	7.9	9	10	12.5
B	197	stx1+stx2	R	R	R	R	S	R	I	R	R	R	7.9	9	10	12.5
B	204	stx1+stx2	S	R	R	S	I	R	S	R	R	R	8	9	10	12.5
B	213	stx1+st	S	R	R	R	S	S	S	S	R	S	8	9	10	12.5
B	284	stx1+stx2	S	R	R	R	R	R	R	R	R	I	8	9	10	12.5
B	292	x2 stx1+stx2	I	R	R	R	R	R	R	R	R	R	8	9	10.1	12.5
B	300	stx1+stx2	R	R	R	R	R	I	R	R	S	R	8	9	10	12.5
B	301	stx1+stx2	R	R	R	R	R	R	R	R	R	R	8	9	10	12.5
B	308	stx1	I	R	R	S	S	R	S	R	R	R	8	9	10	12.5
B	316	stx1	R	R	R	R	S	R	I	R	R	R	8	9	10	12.5
C	332	stx1	R	R	R	R	I	R	I	R	R	R	8	9	10.1	12.5
C	419	stx1+stx2	R	R	R	R	S	R	R	R	R	R	8	9	10.1	12.5
C	420	stx1+stx2	R	R	R	R	S	R	R	R	R	R	8	9	10	12.5
C	451	stx2	S	R	R	R	S	R	S	R	S	R	8	9	10	12.5
C	452	stx2	S	R	R	R	S	R	S	R	S	R	8	9	9.9	12.5
C	467	stx1	I	R	R	R	S	I	R	R	R	R	7.9	9	9.9	12.5
D	483	stx1+stx2	R	R	R	S	R	R	I	R	S	R	8	9	9.9	12.5

D	484	stx1+stx2	R	R	R	R	R	R	I	R	R	R	8	9	10	12.5
D	500	stx1+stx2	S	R	R	R	R	R	R	R	R	R	8	9	10	12.5
D	516	stx1+stx2	R	R	R	S	R	R	S	R	R	R	8	9	9.9	12.5
D	628	stx1	R	R	R	R	R	R	R	R	R	R	8	9	10	12.5
D	644	stx1+stx2	I	R	R	S	S	R	S	R	R	R	8	9	10	12.5
D	833	stx1	S	R	R	R	S	R	I	R	R	R	7.9	9	10	12.5
D	849	stx1	R	R	R	S	I	R	S	R	R	R	8	9	10	12.5
D	864	stx1	S	R	R	R	S	S	R	R	S	R	8	9	10	12.5
D	896	stx1	R	R	R	R	I	R	R	R	R	R	8	9	9.9	12.5
D	1089	stx1	S	R	R	R	S	R	R	R	S	R	8	9	10	12.5
D	1179	stx1	R	R	R	R	S	I	I	S	R	R	8	9	10.1	12.5
Resistance (R)		n	18	34	33	25	11	28	14	30	26	30				
		%	61.2	100	97	75.7	32.3	82.3	41.1	88.2	76.4	88.2				
Intermediary (I)		n	4	0	0	1	5	4	9	0	0	3				
		%	11.7	0	0	2.9	14.7	11.7	26.4	0	0	8.8				
Sensitive (S)		n	12	0	1	8	18	2	11	4	8	1				
		%	12	0	1	8	18	2	11	4	8	1				
∑		n	12	0	1	8	18	2	11	4	8	1				
		%	100	100	100	100	100	100	100	100	100	100				

Note: Gentamicin 10 mg (GEN), Streptomycin 10 mg (EST), Tetracycline 30 mg (TET), Nalidixic Acid 30 mg (NAL), Chloramphenicol 30 mg (CLO), Ampicillin 10 mg (AMP), Ciprofloxacin 5 mg (CIP), Sulfamethoxazole+Trimethoprim 25 mg (SUT), Nitrofurantoin 300 mg (NIT), Cephalothin 30 mg (CFL). Minimum inhibitory concentration (MIC).

Tests of susceptibility to the hydroalcoholic extract of *Eugenia uniflora* leaves

In the screening assay, the formation of inhibition halos with diameters of 10 mm (150 mg/mL), 9 mm (100 mg/mL) and 8 mm (50 mg/mL) was observed in all STEC isolates, against the extract of the leaves of *E. uniflora*. Since this is a qualitative assay, the assays for the determination of the minimum inhibitory and bactericidal concentrations were performed, in order to assess the sensitivity profile of the isolates to the extract. In the assay for MIC determination, performed by the method of microdilution in broth, the bacterial growth was inhibited from the concentration of 12.5 mg/mL, for all isolates analyzed. In the assay for MBC determination, none of the concentrations evaluated was capable of causing bacterial death. Therefore, it is understood that the extract of *E. uniflora* leaves presents bacteriostatic action, that is, it is capable of inhibiting bacterial growth.

DISCUSSION

Bovines are known for being asymptomatic reservoirs of STEC, thus representing an important source of infection. Because of this fact, several studies have been conducted, in order to understand STEC pathogenesis mechanism and search for new ways to suppress future infections, such as the study performed by Moura et al. in which they molecularly characterized STEC strains derived from samples belonging to diarrheic calves, similar to one of the goals of the present study. The cited researchers identified percentages above 40% of positivity [14].

In another work developed in Turkey, Güler et al. identified the bacterium *E. coli* in samples of diarrheic calves. Among the isolates, carriers of genes *stx1* and *stx2* were found, alone or in concomitance [14]. Pereira et al. in the State of São Paulo, observed, also, strains characterized as STEC, with percentages of identification that corroborate with the present study, 11% for *stx1*, 12% for *stx2* and approximately 5% for *stx1* and *stx2* [15]. On the other hand, in the research developed by Carvalho et al. the presence of genes *stx1* and *stx2* was analyzed by PCR in bovines in the State of São Paulo, obtaining a confirmatory ascertainment in 42.3% of the cases [16]. Similarly, Costa et al. in the States of Rio de Janeiro and Rondônia, verified strains genetically characterized as STEC, 55.5% being carriers of the gene *stx* [17].

Assumpção et al. also investigated the occurrence of the virulence genes *stx1* and *stx2* in *E. coli* isolated from dairy cows. They obtained a total of 561 *E. coli* isolates, 90 (16%) of which were carriers of *stx1*, 97 (17.3%) of *stx2* and 37 (6.6%) of *stx1* and *stx2* [18]. On the other hand, in the study performed by Iweriebor et al. STEC strains were isolated from dairy farms of the Eastern Cape Province in South Africa, where 44% of the isolates presented the gene *stx1*, 45.3% the gene *stx2*, and 10.7% the genes *stx1* and *stx2*, differently from the present study, which found a higher incidence of the gene *stx1* (Figure 1) [19].

Noll et al. also found a high incidence of gene *stx2*, analyzing 576 fecal samples of feedlot cattle, and from these, 18.8% (108/576) were positive for STEC, of which 94.1% presented the gene *stx2*, and 64.4% the gene *stx1* [20]. Similarly, in the study performed by Bonardi et al. samples obtained from cattle in the North of Italy were analyzed regarding the presence of the genes *stx* [21]. The authors found 13.1% of samples positive for STEC, prevailing the presence of gene *stx2*. Andrade et al. found a high prevalence of gene *stx1* (82.8%) in isolates from calves with and without diarrhea in dairy farms in Minas Gerais, in comparison with gene *stx2* (4%) [22]. This corroborates the data obtained in the present work. Likewise, in a study performed between 2010 and 2011 in Iran, a prevalence of STEC isolates (84.61%) was found in fecal samples from calves with diarrhea. From the isolates characterized as STEC, it was found that 48.92% presented the gene *stx1*, 32.79% the gene *stx2*, and 18.27% the genes *stx1* and *stx2*, also evidencing a higher prevalence of gene *stx1* [23].

As the incidence of STEC strains has been high in animals with diarrhea, it becomes necessary to evaluate the profile of susceptibility to the antimicrobials, with the purpose of guaranteeing the therapeutic success. Therefore, cases of microbial resistance in STEC have been generating an increasing concern in veterinary therapy. Thus, several studies have evaluated the profile of susceptibility and resistance of STEC strains around the world, as performed by Shahrani et al. who evaluated the resistance profile of STEC strains from fecal samples of diarrheic calves, finding an elevated percentage of resistance to Penicillin (100%), Streptomycin (98.25%), Tetracycline (98.09%), Lincomycin (92.69%), Chloramphenicol (73.8%), Ampicillin (71.11%), Trimethoprim (62.22%), Enrofloxacin (61.42%) and Ciprofloxacin (60.31%) [23]. In the study performed by Aslam et al. strains of *E. coli* O157:H7, isolated from feedlot cattle from three commercial farms, presented resistance to tetracycline (69%) and sulfisoxazole (68%). Nevertheless, the resistance profile varied among the farms, being predominant in only two, by which the authors conclude that the management conditions can influence the incidence of resistant strains [24].

Another study performed by Amézquita-López et al. investigated the microbial resistance profiles of STEC O157 and non-O157 strains, isolated from cattle, sheep and poultry [25]. All strains analyzed were susceptible to the Antimicrobials Ceftazidime, Ceftriaxone, Ciprofloxacin, Nalidixic acid and Sulfamethoxazole+Trimethoprim (Table 1). Nonetheless, they observed a predominant resistance to the Antimicrobials Ampicillin, Cephalothin, Chloramphenicol and Kanamycin, which corroborates the existing literature data and those found in the present work. Assumpção et al. analyzed STEC strains, derived from dairy cows, regarding the resistance against the Antimicrobials Ampicillin, Tetracycline, Trimethoprim, Erythromycin, Nalidixic acid, Streptomycin, Novobiocin, Lincomycin, Penicillin and Neomycin. In this study, the level of resistance to all antimicrobials evaluated was high, besides a multiresistance profile. The dissemination of resistant strains can lead to several implications for public health, since it can hamper the treatment.

STEC strains were isolated from fecal samples derived from dairy cattle, and evaluated in terms of their susceptibility profile to diverse antimicrobial agents. In this study, a high prevalence of multiresistance was found among the isolates analyzed, and resistance to Ampicillin (94.74%), Tetracycline (96.84%), Oxytetracycline (94.74%), Amoxicillin+Clavulanate (84.2%), Cephalothin (94.74%), Ceftazidime (32%), Norfloxacin (10.5%), Ciprofloxacin (12.6%), Enrofloxacin (7.4%), amikacin (6.3%), Chloramphenicol (89.5%), Kanamycin (5.3%), Streptomycin (84.2%), Gentamicin (8.4%) and Sulfamethoxazole+Trimethoprim (84.2%). The elevated incidence of bacterial resistance profiles, both in STEC and non-STEC strains, has been prompting the search for new antimicrobial agents that are efficient against these increasingly resistant strains. With this intention, several studies with medicinal plants have been performed, with the aim of obtaining new active principles with antimicrobial properties, especially against resistant strains.

Coutinho et al. evaluated the antimicrobial activity of *E. uniflora* ethanolic extract against *E. coli* strains, where they found a minimum inhibitory concentration ≥ 1.024 µg/mL [26]. Similarly, in the study performed by Costa et al. a MIC>1000 µg/mL was also recorded against E. coli [27]. Fiúza et al. investigated the antimicrobial activity of the crude ethanolic extract from *E. uniflora* and obtained a MIC of 17.5 mg/mL, a result relatively similar to that found in the present work [28]. Gonçalves et al. also obtained positive results of the action of the hydroalcoholic extract of *E. uniflora* against *E. coli* [29]. Also, in the study performed by Holetz et al. a moderate antimicrobial activity against *E. coli* was found, with a MIC of 500 µg/mL [30].

On the other hand, in the study performed by Auricchio et al. the hydroalcoholic extract of *E. uniflora* leaves did not demonstrate antimicrobial activity against *E. coli*, only in strains

of *Staphylococcus aureus*, *Salmonela cholerasuis* and *Pseudomonas aeruginosa* [31]. This result is similar to what was found in the work of Bezerra et al. in which the antimicrobial activity of *E. uniflora* hydroalcoholic extract was also investigated, and a significant activity was only observed against *S. aureus* and *P. aeruginosa* [32-34]. Contradictory data might occur, since, regarding medicinal plants, questions such as the origin of the plant (climate, altitude, soil composition), time of harvest (season of the year, plant maturity stage), and extractive processes might interfere in the presence and/or absence of certain active principles, as well as in their amount in the plant.

CONCLUSION

The present work contributes with the knowledge on STEC incidence in animals from dairy farms in the State of Minas Gerais. Furthermore, the work also contributes demonstrating the resistance profile of these strains, and the importance of knowing it for treatment success. The high multiresistance level found evidences the indiscriminate use of antimicrobials in veterinary therapy. This finding shows the need of adoption of measures that promote the reduction of the abusive use of antimicrobials, such as performing the antibiogram before the treatment. It is also pertinent to highlight the importance of searching for new antimicrobial agents, especially those derived from medicinal plants, which have been demonstrating positive effects on multiresistant strains. The antimicrobial activity found in the extract of *E. uniflora* leaves justifies its use in popular medicine. Therefore, medicinal plants represent an interesting alternative for the treatment of infectious diseases, mainly those caused by multiresistant microorganisms.

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HIGHLIGHTS

- High prevalence of multi-drug resistant isolated from diarrheic calves.
- Antimicrobial activity of the extract of leaves against isolates.
- Low incidence of the and genes in from diarrheic calves.

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