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Original Article

Molecular and phenotypic characterization of *Escherichia coli* from calves in an important meat-producing region in Brazil

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Abstract

Introduction: *Escherichia coli* strains that lead to enteritis are considered an important cause of diarrhea in calves. For correct identification, these microorganisms must be differentiated from non-pathogenic members of the intestinal microbiota. The aim of the present work was to characterize *E. coli* isolates in calves regarding the presence of virulence genes that cause enteritis and evaluate the sensitivity of the isolates to different antimicrobials.

Methodology: One hundred forty-nine samples from beef cattle and 27 samples from dairy cattle were evaluated. All samples were submitted to microbiological identification and the disk diffusion antibiogram test. The polymerase chain reaction method was used to detect virulence genes.

Results: A hundred seventy-six samples were biochemically identified as *E. coli* and antibiograms were determined. The samples were then submitted to PCR; 35 were positive for the *eae* gene (19.88%), 135 (76.70%) for the *stx*1 gene, 62 (35.22%) for the *stx*2 gene, 159 (90.34%) for the *sta* gene and 35 (19.88%) for the *ltII* gene. No samples were positive for the *cnf* gene. Based on these results, the *E. coli* isolates were classified into pathotypes: enteropathogenic (n = 3), enterohemorrhagic (n = 32), Shiga toxin-producing (n = 122) and enterotoxigenic (n = 163). The antimicrobial sensitivity tests revealed that 77.2% of the isolates were resistant to three or more pharmacological groups, characterizing these isolates as multidrug resistant.

Conclusions: Enterotoxigenic *E. coli* was the predominant pathotype. Moreover, the prevalence of multidrug-resistant isolates was very high, accounting for the vast majority of isolates.

Key words: diarrhea; cattle; colibacillosis; virulence genes.

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Introduction

Cattle farming is one of the most representative features of Brazilian agribusiness on the world stage. Brazil has the largest commercial herd in the world (213.52 million heads) and is the second largest producer of beef, behind only the United States [1]. In 2017, the central western region of the country had 74.1 million heads, corresponding to 34.5% of the national total [1].

Enteritis is considered one of the main causes of economic losses in the livestock industry, especially in the first four weeks of life of the animals [2]. *Escherichia coli* is a bacterium that causes intestinal and extra-intestinal infections in bovine neonates. Strains of *E. coli*, which are commonly isolated from feces, are most often commensal and cause no diseases in the host [3]. However, other strains are grouped into pathotypes based on their pathogenic mechanisms and are frequently associated with diseases and harm in animals [4]. Strains such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC) and necrotoxigenic *E. coli* (NTEC) have been identified in calves. Other isolates, such as enteroinvasive *E. coli* (EIEC), diffuse-adherence *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) have not yet been confirmed as causes of bovine enteritis [3,5].

EPEC is characterized by the presence of the intimin (*eae*) gene, which promotes attachment to and the effacement of intestinal epithelial cells. The presence of one or more heat-stable (*st*I and *st*II) and labile (*lt*I and *lt*II) toxin genes characterizes ETEC strains. STEC and EHEC are characterized by the presence of two toxin genes – *stx*1 and *stx*2. The intimin gene (*eae*) is also an important virulence factor for the characterization of EHEC [5].

To confirm *E. coli*-associated enteritis, it is necessary to identify the pathotypes that cause this disease. Several studies have identified virulence factors in isolates obtained from calf feces with and without clinical signs of diarrhea [3,5] using the polymerase chain reaction (PCR) method, which is both fast and sensitive [6].

As colibacillosis is an important cause of economic losses, detailed studies are needed on virulence factors produced by strains of *E. coli* in farmed animals. Indeed, little is known regarding whether these strains are pathogenic, mainly due to the fact that the diagnostic method is expensive and requires a qualified professional. Therefore, the aim of the present work was to characterize *E. coli* isolates from calves regarding the presence of virulence genes that cause enteritis and determine the profile of antimicrobial resistance in an important beef cattle production region in Brazil.

Methodology

This study received approval from the Animal Ethics Committee of Universidade Federal de Mato Grosso do Sul (UFMS) (certificate number: 1.002/2018).

One hundred seventy-six stool samples were collected from calves aged one to 60 days and divided into two categories: up to 30 days and 31 to 60 days of age. Feces were analyzed for macroscopic visual appearance and the presence of blood streaks and classified as diarrheal or non-diarrheal according to parameters defined by Walker *et al.* [7]. An epidemiological questionnaire was also administered to collect clinical and epidemiological data (species, origin, age, treatments used, etc.) from each animal.

The samples came from four regions in the state of Mato Grosso do Sul, Brazil: five ranches in the municipality of Campo Grande (20º26'34" S and 54º38'47" W) (39 samples), one in Sonora (17º34'37 "S and 54º45'28" W) (25 samples), one in Rio Verde do Mato Grosso (18º55'05" S and 54º50'39" W) (15 samples), one in Rochedo (19°56'55" S and 54°52'49" W) (11 samples), two in Água Clara (20º26'53" S and 52º52'41" W) (45 samples), one in Chapadão do Sul (18º47'39" S and 52º37'22" W) (15 samples), one in Rio Brilhante (21º48'07" S and 54º32'47" W) (15 samples) and one in Miranda (20º14'26" S and 56º22'42" W) (11 samples). Most properties produced beef cattle (149 samples) and some produced dairy cattle (27 samples). A total of 66.5% of the samples were from crossbred animals and 33.5% were from the Nellore breed.

Samples were collected in plastic bags (Wyda, São Paulo, Brazil) directly from the rectal ampoule and maintained refrigerated (4 °C) or on a swab containing Stuart medium (Absorve, São Paulo, Brazil) for up to 48 hours. The samples were sent to the Bacteriology Lab of the UFMS School of Veterinary Medicine and Animal Science.

Processing of the samples was based on Procop *et al*. [8]. The samples were sown on plates containing MacConkey agar (HiMedia®, Mumbai, India) and incubated at 37 ºC ± 1 ºC for 18 to 24 hours. After initial growth, samples from the cultures were separated according to colony and Gram staining.

A battery of specific biochemical tests for enterobacteria was performed using the following media (HiMedia®, Mumbai, India): triple iron sugar (TSI) agar, sulfide-indole-motility (SIM), Simmons citrate, phenylalanine, lysine iron, urea broth, methyl red (MR) and Voges-Proskauer (VP). Samples exhibiting biochemical characteristics compatible with *Escherichia coli* were submitted to antibiograms. The strain American Type Culture (ATCC) 25922 was used as control, which is recognized as a control strain by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility tests.

Analyses were performed using standard disc diffusion method and interpreted according to the CLSI guidelines [9], employing the following antibiotics: florfenicol (30 µg), tetracycline (30 µg), gentamicin (10 µg), oxacillin (1 µg), trimethoprim/sulfamethoxazole (25 µg), penicillin (10 IU), norfloxacin (10 µg), cephalexin (30 µg), enrofloxacin (5 µg), cephalothin (30 µg), amoxicillin (10 µg) and amoxicillin (20 µg) + clavulanic acid (10 µg).

DNA extraction was performed for the molecular analysis. Isolates from the MacConkey agar were sown in brain heart infusion (BHI) broth. After 24 hours, sedimentation was performed by centrifugation at 10,000 x g for five minutes. DNA extraction was performed from the sediment following the protocol described by Araujo *et al*. [10]. DNA purity analysis and quantification were performed in a NanoDrop® OneC Microvolume UV-Vis spectrophotometer.

PCR reactions for each gene were performed in a final volume of 25 µL containing 2.5 µL of 10x buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 1.25 U of Taq DNA polymerase (5 U/uL), 10 pmol of each primer (100 ng/uL) and 2 µL of DNA (average DNA concentration: 1,113,656 ng/uL). The amplification conditions included initial denaturation at 94 °C for five minutes, 35 cycles of denaturation at 94 °C for 1.5 minutes,

annealing at 50/52 or 56 °C for 1.5 minutes and extension at 72 °C for 1.5 minutes. A final extension step was performed at 72 °C for ten minutes. ATCC reference strains and donations from other institutions were used as the positive control. Milli-Q® water was used as the negative control. The primers are described in Table 1.

The amplified products were analyzed after electrophoresis on 2% agarose gel stained with GelRed®. The images were recorded with the aid of a photo-documentation system. *E. coli* isolates were classified into pathotypes as described in Table 2.

Statistical analysis involved the evaluation of associations between pathotypes and epidemiological characteristics (race, sex, color and appearance of feces) through analysis of variance with the aid of the SAS® program. Binomial data were transformed into percentages using the Glimmix procedure. Descriptive statistics were performed for the antibiograms and regions evaluated.

Results

Among the 176 animals, 149 (84.65%) had feces ranging from pasty to liquid with or without the presence of blood and were characterized as diarrheal. Twenty-seven samples (15.34%) did not have diarrheal characteristics. Bacterial colonies were isolated from all stool samples and were biochemically identified as *Escherichia coli* (glucose +, lactose +, H2S -, motility +, Indol +, citrate -, lysine iron +, presence of gas, urea -, red methyl + and Voges-Proskauer -).

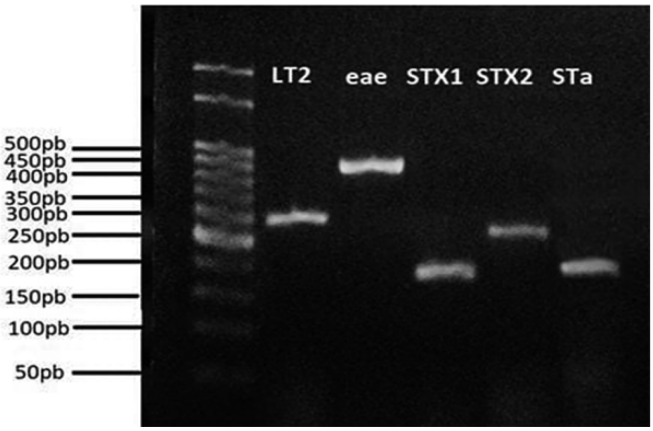
After the bacteriological analyses, DNA from the 176 samples was analyzed using the PCR method (Figure 1) to detect seven virulence genes and the pathotypes were classified, as shown in Table 3.

Table 2. Classification of Escherichia coli pathotypes according to virulence gene (Costa *et al*. [37]).

Pathogenic categories (8)	Characteristic virulence genes
EPEC	*eae* (intimina) with absence of *stx*
ETEC	elt (*ltII*) and / or this *sta* (enterotoxins)
EHEC	*stx*1 or *stx*2 and *eae* (intimine)
STEC	*stx*1 and / or *stx*2

ETEC was the most frequent pathotype on all the ranches (92.61%), followed by STEC (63.63%) and EHEC (18.18%). The exception was the ranch in the municipality of Sonora, which had no samples compatible with the EHEC pathotype. As the co-occurrence of pathotypes was found on some properties, the values are greater than 100%. The EPEC pathotype was the least frequent (1.13%), having been found on only two properties (ranches located in Água Clara and Rio Verde de Mato Grosso – each with one positive sample). A single isolate did not present any

Figure 1. Electrophoresis in 2% agarose gel with 50-base pair (pb) molecular marker.



Tab1e 1. Primer oligonucleotides used for PCR to amplify virulence factors in isolates of *Escherichia coli* from calves in Mato Grosso do Sul, Brazil, according to Costa *et al*. [37].

Virulence factors (genes)	Initiator	Initiator (5' – 3')	Product (pb)	Annealing temperature
Thermostable toxin (est)	ST-1 ST-2	CTGTATTGTCTTTTTCACCT GCACCCGGTACAAGCAGGAT	182	56 °C
Temolabic toxin (elt)	LT-1 LT-2	AGATATAATGATGGATATGTATC TAACCCTCGAAATAAATCTC	300	52 °C
Intimine (eae)	EAE-1 EAE-2	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCTCTGC	454	50 °C
Shiga toxin 1 (stx1)	STX-1[a] STX-1B	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	56 °C
Shiga toxin 2 (stx2)	STX-2[a] STX-2B	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	56 °C
Cytotoxic necrotizers (Cnf)	Cnf 1	GAACTTATTAAGGATAGT CATTATTTATAACGCTG	543	56 °C
	Cnf 2	AAT CTA ATT AAA GAG AAC CATTATTTATAAGCGTG	543	56 °C

pathotype; this isolate came from an animal with diarrhea. In some cases, more than one pathotype was detected per isolate, the most frequent of which was ETEC + STEC (61.93%), followed by ETEC + EHEC (15.34%) and ETEC + EPEC (1.13%).

The statistical analysis revealed a significant association between the STEC pathotype and the Nellore breed ($p = 0.0065$). No significant associations were found between the presence of the pathotype and age of the animal, sex of the animal, appearance of feces or color of feces.

The antibiograms revealed that 3.40% of the isolates were resistant to one a group of antimicrobials, whereas 77.2% were resistant to three or more groups, characterizing these isolates as multidrug resistant [11]. The results of the susceptibility analysis of the 176 isolates of *Escherichia coli* from calf feces with and without diarrhea are shown in Figure 2.

Multidrug resistance was more frequent in the ETEC pathotype (71.02%), followed by the STEC (50%) and EHEC (12.5%) pathotypes. The ATCC strain had the following profile: sensitivity to amoxicillin + clavulanic acid, enrofloxacin, cephalothin, norfloxacin, trimethoprim/sulfamethoxazole and tetracycline. Resistance to amoxicillin, florfenicol, gentamicin, cephalexin, oxacillin and penicillin was also observed.

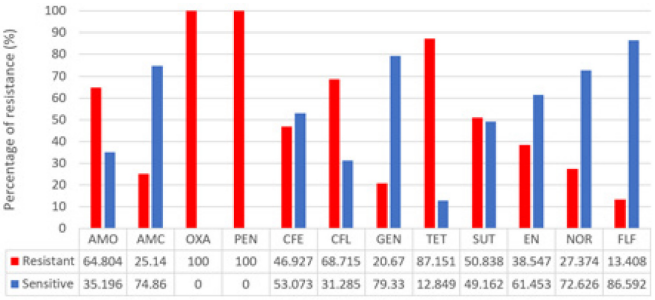
Discussion

ETEC is the most frequent pathotype in cattle [12,13]. In the present study, this pathotype was found in 92.61% of the isolates, with 90.34% of the isolates positive for *sta*, 15.34% for *sta* + and only 2.27% for *lt*II. Analyzing other types of samples, such as isolates from food, Hirish [14] described similar results, also reporting that strains that only produce thermostable enterotoxins are the most common, followed by those that secrete both thermostable and thermolabile enterotoxins and those that secrete only thermolabile enterotoxins. In the present study, the *sta* gene was more frequent than the prevalence reported by Rigobelo *et al*. [15]. Regarding the *lt*II gene, Salvadori *et al*. [16] found an 8.3% rate in *E. coli* isolates, which is lower than the rate reported here.

Table 3. Classification into pathotypes of *E. coli* isolates obtained in present study regarding virulence profile.

Virulence genes	Number of isolates	Patotype
eae + *lt*II	2	EPEC + ETEC
*lt*II	1	ETEC
sta	20	ETEC
ltII + *sta*	5	ETEC
eae + *stx*1 + *sta*	7	EHEC + ETEC
eae+*stx1*+*sta*+*ltII*	3	EHEC + ETEC
eae+*stx1*+*stx2*+*sta*+*ltII*	4	EHEC + ETEC
eae+ *stx2*+ *sta*	1	EHEC + ETEC
eae+ *sta*+ *stx1*+ *tx2*	12	EHEC+ ETEC
stx1 + *stx2*	3	STEC
stx1	5	STEC
stx2	3	STEC
stx1 + *sta*	56	STEC + ETEC
stx2 + *sta*	1	STEC + ETEC
sta+ *stx1* +*stx2*	34	STEC + ETEC
stx1+ *ltII*+ *sta*	9	STEC + ETEC
stx2+*ltII*+*sta*	2	STEC + ETEC
stx1 +*stx2*+ *sta*+*ltII*	7	STEC + ETEC

Figure 2. Results of evaluation of antimicrobial sensitivity. Antibiotics: FLF: florfenicol (30 µg): TET: tetracycline (30 µg): GEN: gentamicin (10 µg): OXA: oxacillin (1 µg): SUT: trimethoprim/sulfamethoxazole (25 µg): PEN: penicillin (10 IU): NOR: norfloxacin (10 µg): CFE: cephalexin (30 µg): EN: enrofloxacin (5 µg): CFL: cephalothin (30 µg): AMO: amoxicillin (10 µg); AMC: amoxicillin + clavulanic acid (30 µg).



The animals in the present study were up to 60 days of age and the ETEC pathotype was found more frequently in those with up to one week of life [17]. However, no significant association between age and this pathotype was found. Some researchers have found this pathotype in calves more than a week old [6]. The reason for the restriction of ETEC infection to calves in the first week of age is not fully understood. One explanation is that receptors for fimbriae are more expressed in immature cells of the intestinal villi. Thus, postnatal intestinal maturation limits ETEC infection to calves less than one week of age [17].

ETEC was identified in 138 (96.29%) of the fecal samples from beef cattle and 26 (92.61%) of the samples from dairy cattle. Therefore, the intake of both beef and milk may from animals carrying this pathotype poses a risk to the human population, especially when these foods are consumed without proper processing. ETEC is a major cause of enteritis among travelers and children under five years of age [18]. In 2010, a Global Burden of Disease study estimated an annual occurrence of 157,000 deaths due to ETEC; 9% of all deaths were attributable to enteritis and approximately

1% of all deaths involved children 28 days to five years of age, with many cases related to food poisoning [19].

Regarding the STEC pathotype, the *stx*1 and *stx*2 genes were present in 68.18% of the isolates. This fact is cause for concern, as STEC is often isolated from healthy animals [20]. In the present study, 6.25% of the isolates were from calves with no clinical signs. According to Mainil [4], the lack of systemic signs is explained by the fact that ruminants do not have receptors for *stx* in the vascular endothelium. For the 93.75% of the isolates that presented the STEC pathotype in animals with diarrhea, the occurrence of symptoms may be attributed to co-occurrence with the ETEC pathotype. STEC colonization rates in cattle herds vary and can reach as high as 60%, but typical rates range from 10 to 25% [21]. However, even higher rates are reported in some studies. Analyzing feces from cattle belonging to dairy herds in the municipality of Jaboticabal in the state of São Paulo, Vicente *et al*. [22] found the *stx* gene in 72.16% of the samples.

The EPEC pathotype was found in only 1.13% of the positive isolates in the present study. This pathotype is an intestinal pathogen that causes acute, persistent enteritis in animals and humans [23]. In several studies conducted in different countries, such as in Brazil, Chile, Peru and Iran, EPEC was considered the main cause of endemic diarrhea in children under one year of age, accounting for 5 to 10% of cases of pediatric diarrhea [24]. Analyzing fecal samples from dairy cows in the state of São Paulo, Pereira *et al*. [25] found the *eae* gene in only 5.6%, confirming the low rate of this isolate, as reported by other authors [6,26].

None of the isolates was identified as NTEC, as the samples were negative for both *cnf*1 and *cnf*2. Similar results have been reported in other studies. Analyzing 630 isolates, Shahrani *et al*. [27] found *cnf* in 22 isolates (3.49%), demonstrating the low prevalence of this pathotype in herds. Regarding the only isolate for which no pathotype was found but the animal had diarrhea, this may have been due to other agents, such as viruses, protozoa and bacteria, which were not investigated in in the present study.

The spread of these pathotypes among domestic herds causes additional concerns related to the emergence of antimicrobial-resistant bacteria as a result of the widespread use of these agents for the treatment of infectious diseases in young animals [28, 29]. The most common resistance pattern was related to both oxacillin and penicillin (100%). Evaluating the resistance profile of STEC from calves with diarrhea, Shahrani *et al*. [27] also found 100% resistance to penicillin. According to Nepomuceno *et al*. [30], *E. coli* is naturally resistant to penicillin G. Therefore, high resistance is expected.

The third most common antimicrobial-resistance pattern was related to tetracycline (87.15%). This result is similar to findings described by Franco *et al*. [31] in a study involving pigs, who found a resistance rate of 70.6%, and Maciel *et al*. [32] in a study involving cattle, who reported a 63.3% resistance rate. The epidemiological questionnaire administered in the present study revealed that that all properties reported having used tetracycline at some point during management.

The antimicrobial to which bacteria were the most sensitive was florfenicol (86.5%). Differences in sensitivity and resistance occur due to factors related to the existing bacterial population and the indiscriminate use of antimicrobials [33]. Sato *et al*. [34] found a resistance rate of 95.2% in a study of isolates from piglets, whereas Reis [35] found a sensitivity rate of 100% for this drug.

There is evidence that the use of antimicrobials in veterinary medicine contributes to the occurrence of antimicrobial-resistant bacterial infections in humans, which underscores of the importance of adhering to the "One Health" concept [36].

Determining the prevalence of virulence genes in a population helps veterinarians perform the proper management of properties and determine whether the cause of enteritis is related to *Escherichia coli*, as these enteric conditions can be due to several factors. Determining prevalence can contribute to reducing the incidence of disease in humans. Another concern is related to multidrug-resistant strains, which corresponded to the vast majority of isolates analyzed in the present investigation. Thus, veterinarians should be warned to use antimicrobials with caution. Penicillin, oxacillin and tetracycline are not indicated for the properties analyzed in this study, as these antibiotics were the most widely employed and were also those to which the isolates had higher degrees of resistance. A recommended alternative would be the determination of antibiograms in cases of outbreaks or after the identification of an isolated strain to reduce economic losses resulting from the inappropriate use of these drugs.

Conclusions

Enterotoxigenic *E. coli* was the predominant pathotype in calves up to 60 days of age farmed in the state of Mato Grosso do Sul, Brazil (99.43%). The detection of pathotypes directly related to enteritis in humans is relevant to the epidemiology of these

infections. Moreover, the prevalence of multidrug resistant isolates was very high, accounting for the vast majority of isolates.

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