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VIRULENCE FACTORS OF *ESCHERICHIA COLI* ISOLATED FROM CALVES WITH DIARRHEA IN BRAZIL

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ABSTRACT

Two hundred and five *Escherichia coli* strains isolated from calves with diarrhea from mid-western Brazil were screened for the presence of virulence factors associated with bovine colibacillosis. One hundred and two (49.8%) of the *E. coli* strains produced toxins: Shiga toxins 1 (9.7%) and 2 (6.3%), α-hemolysin (9.7%), enterohemolysin (6.8%), Cytotoxic Necrotizing Factors type 1 (0.5%), and type 2 (4.4%), enterotoxins LT-II (8.3%) and STa (3.9%). No strain produced enterotoxin LT-I. Fimbrial adhesins F5 and F17 were produced by 7.3% and 4.8% of the strains, respectivly, and none expressed F41. Seven strains (3.4%) possessed the gene *eae* and belonged to serotypes O26:H-; O111:H- and O118:H16. These results suggest that calves in Brazil may be an important source of pathogenic *E. coli* for animals and humans.

Key words: calves, diarrhea, *E. coli*, virulence factors.

INTRODUCTION

Escherichia coli is an important pathogen in bovine neonates and may cause intestinal and extraintestinal infections. Bovine *E. coli* strains can produce Shiga-like toxins (Stx), heat-labile (LT) or heat-stable (ST) enterotoxins, cytotoxic necrotizing factors (CNF1 and CNF2) and hemolysins (α-Hly and E-Hly) (23). The Shiga toxin produced by *E. coli* strains (STEC) is similar to Shiga-toxin produced by *Shigella dysenteriae* type 1. *E. coli* producing Stx-1 and/or Stx-2 is a cause of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in humans. Most cases of HC and HUS are caused by ingestion of foods and drinks contaminated with faeces from cattle, specially ground beef, undercooked hamburgers, salami or other foods like raw milk or home made cheese from raw milk. Less frequent modes of transmission of the infection are cattle-to-person or person-to-person direct contact (34), however, asymptomatic STEC infection in household contacts represents a potential source of infection via person-to-person transmission (33).

Epidemiological investigations have shown that cattle frequently excrete strains of STEC in their faeces and this may represent a source of infection (12). Another type of Hly, enterohemolysin (E-Hly), which is different from Hly produced by ETEC strains of porcine origin and by *E. coli* strains which cause extraintestinal infections in humans, has been described in STEC strains (31).

The principal feature of infections caused by enteropathogenic *E. coli* (EPEC) is the attaching-and-effacing (A/E) histopathology observed in intestinal biopsies from patients or infected animals (37). The intimate adherence of enteropathogenic *E. coli* to epithelial cells is mediated by a 94-97 KDa outer membrane protein known as intimin, and the gene encoding intimin (*eae*, for *E. coli* attaching and effacing) was first reported by Jerse *et al* (30).

The name enterohemorhagic *E. coli* (EHEC) has been applied to strains that characterized by their ability to produce Shiga-like toxins and to induce attaching and effacing lesions (26).

Enterotoxin-producing *E. coli* (ETEC) have been identified as the causative agent of several important diarrheal diseases in animals and humans. These bacteria may produce thermolabile (LT-I and LT-II) and thermostable (STa and STb) enterotoxins (21). CNF-producing *E. coli*, known as necrotizing *E. coli*

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(NTEC), have been isolated from animals with enteritis (19) and from humans with extraintestinal infections (14). NTEC can produce two types of CNF (CNF1 or CNF2) that can be distinguished by the morphological alterations induced in HeLa cells, by cross-neutralization assays, by the specific necrotizing activity of CNF2 in mouse footpads, and by the presence of other virulence factors associated with NTEC (20). CNF1 is produced by α-hemolysin-positive *E. coli* strains that cause extraintestinal infections in humans (8).

E. coli strains can also produce a variety of adhesins that promote the attachment of bacteria to cell surface receptors and to components of the extracellular matrix. Although the strains which cause infections in humans are well characterized, there is limited information on those which cause diseases in animals (3). The fimbrial adhesin F5 (K99) plays a role in the colonization of enterotoxigenic *E. coli* in epithelial cells of the small intestine of calves (1) and occasionally piglets (35). Other fimbriae (F41 and F17) have also been identified in enterotoxigenic *E. coli* isolated from calves (36,39).

In this study, we investigated the prevalence of virulence factors associated with the pathogenicity of *E. coli* isolated from calves with diarrhea, using phenotypic and genotypic (Polymerase Chain Reaction - PCR) assays.

MATERIALS AND METHODS

Bacterial strains

Two hundred and five *E. coli* strains isolated from 139 fecal samples of 139 diarrheic neonatal calves, with ages up to 60 days, provided by the Centro Nacional de Pesquisa de Gado de Corte, EMBRAPA, Campo Grande, MS. Brazil, were screened for the presence of virulence factors. Standard bacteriological methods were used to isolate and identify the *E. coli* strains. The reference *E. coli* strains used as positive and negative controls were H30 (O26:H11 Stx1$^+$), J2 (O157:H- Stx-2$^+$ *eae*$^+$), H10407 (LT-I$^+$ and STa$^+$), PCD (LT-II$^+$), 40MR48 (CNF1$^+$), B26a (CNF2$^+$), C3888 (E-Hly$^+$), B41M (O101:K-:F41$^+$), F82 (O101: K-:F5(K99)$^+$), ATT25 (F17-a$^+$) and K12C600 (nontoxigenic strain). All strains were stored in trypticase soy agar (TSA) (BBL, Cockeysville, USA) at room temperature.

Toxin production

For the production of Stx, heat-labile enterotoxin LT and heat-stable STa, bacteria were cultured in BHI (Difco, Detroit, USA) and incubated at 37ºC with shaking (150 rpm). After 18 hours, the cultures were centrifuged at 10.000 x *g* at 4ºC for 15 min. The supernatants were filtered through 0.22 μm membrane filters (Millipore Corp., Bedford, USA) and stored at -20ºC. CNF production by *E. coli* strains was assessed in the presence of mytomycin C (1 μg/mL) as described by Blanco *et al.* (7). The culture supernatants of all strains were tested in duplicate for detection of STx, CNF and LT toxins on Vero (African Green Monkey Kidney) cells supplied by Fort Dodge Laboratories, Inc. (Campinas, Brazil). Cytotoxic activity was detected as reported by Yano *et al.* (46). STa was detected by intragastric inoculation of *E. coli* culture supernatant in the suckling mouse assay as described by Dean *et al.* (16).

Hemolysin production

For the detection of α-Hly *E. coli* strains were plated on sheep blood-agar and incubated at 37ºC for 18 h. E-Hly was detected on Beutin basic medium (5) containing $CaCl_2$ (Merck) and washed sheep erythrocytes after incubation at 37ºC for 18 h.

Phenotypic detection of fimbriae

E. coli strains were cultured overnight at 37ºC on Minca medium (26). The detection of F5, F17 and F41 fimbriae was done by the slide agglutination test using specific antiserum produced in our laboratory using reference strains. Each antiserum was absorbed with its homologous strain grown at 16ºC before use.

Detection of virulence factors by PCR

Total bacterial DNA were prepared as described by Blanco *et al.* (13). The base sequences, annealing temperatures and predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. The analysis of the PCR products was performed in 2% horizontal agarose gel eletrophoresis stained with ethidium bromide under UV light.

Serogrouping and Serotyping

Serogrouping of the STEC strains associated with the *eae* gene was done using a microplate technique described by Guinée *et al.* (24) and modified by Blanco *et al.* (8), we used a kit containing all the 173 antisera, purchased from the Laboratório de Referência en *Escherichia coli* (LREC) from the Universidade de Santiago de Compostela in Lugo, Spain. The serotyping assays were kindly performed by Jesús Blanco, in the LREC.

RESULTS

Production of toxins

Two hundred and five strains of *E. coli* isolated from 139 calves with diarrhea were assayed for the production of Stx, CNF, LT and STa toxins. The toxins detected were: Stx-1 20 (9.75%), Stx-2 13 (6.34%), CNF1 1 (0.5%), CNF2 9 (4.4%), LT-II 17 (8.3%), and STa 8 (3.9%). No strains produced LT-I. Some strains produced more than one toxin (Table 2).

Hemolysin production

Of the 205 *E. coli* strains, 20 (9.8%) were α-Hly$^+$ and 14 (6.8%) were E-Hly$^+$ (Table 2). Two (1%) of α-Hly$^+$ strains were also CNF-producing strains. There was, a high association

Table 1. PCR primers used in this study.

Primer	Sequence (5 -3)	Size of amplified product (base pairs)	Annealing temperature (°C)	Reference
eae	5 -GACCCGGCAACAAGCATAAGC -3 5 -CCACCTGCAGCAACAAGAGG-3	384	55	(39)
CNF1	5 -GAACTTATTAAGGATAGT -3 5 -CATTATTTATAACGCTG -3	543	45	(10)
CNF2	5 -AATCTAATTAAAGAGAAC -3 5 -CATTATTTATAACGCTG -3	543	44	(10)
LT-I	5 -TATCCTCTCTATATGCACAG -3 5 - CTGTAGTGGAAGCTGTTATA -3	480	48	(14)
LT-II	5 -AGATATAATGATGGATATGTATC -3 5 -TAACCCTCGAAATAAATCTC -3	300	48	(41)
STa	5 -TCCGTGAAACAACATGACGG -3 5 -ATAACATCCAGCACAGGCAG -3	244	60	(43)
Stx-1	5 -AGG TTGCAGCTCTCTTTCAATA -3 5 -TGCAAACAAATTATCCCCTGAG -3	364	57	(28)
Stx-2	5 -GGGCAGTTATTTTGCTGTGGA -3 5 -GTATCTGCCTGAAGCGTAA -3	386	59	(27)
F5(K99)	5 -TGGGACTACCAATGCTTCTG -3 5 -TATCCACCATTAGACGGAGC -3	450	60	(40)
F17	5 -GCAGAAAATTCAATTTATCCTTGG -3 5 -CTGATAAGCGATGGTGTAATTAAC -3	537	65	(3)
F41	5 -GAGGGACTTTCATCTTTTAG -3 5 -AGTCCATTCCATTTATCGGC -3	431	56	(21)

Table 2. Toxigenic and hemolytic *E. coli* strains isolated from calves with diarrhea.

Toxins	No. (%) of *E. coli* strains
CNF1	1(0.5)
CNF2	2(1.0)
LT-I	0
Stx-1	6(2.9)
Stx-2	4(1.9)
LT-II	14(6.8)
Sta	4(1.9)
α-Hly	18(8.7)
E-Hly	3(1.5)
CNF2 and Stx-1	1(0.5)
Stx-1 and Stx-2	2(1.0)
CNF2 and LT-II	1(0.5)
CNF2 and STa	2(1.0)
Stx-1 and LT-II	1(0.5)
CNF2 and LT-II and STa	1(0.5)
α-Hly and CNF2	2(1.0)
E-Hly and Stx-1	3(1.5)
E-Hly and Stx-2	6(2.9)
E-Hly and STa and Stx-2	1(0.5)

between E-Hly$^+$ and Stx$^+$, with 11 strains (5.3%) expressing both (Table 2). None of the strains producing Hly were associated with enterotoxins.

Production of fimbriae

Of the strains isolated, 15 (7.3%) were F5$^+$, 10 (4.9%) were F17$^+$ and none produced F41. The F5 and F17 positive strains were related to 7 (3.4%) and 5 (2.4%) toxigenic strains respectively, (Table 3).

Table 3- Fimbrial adhesins and *eae** producing *E. coli* strains isolated from calves with diarrhea and the associated toxins.

Fimbriae	No. (%) of strains
F5	6(2.9)
F17	5(2.4)
F41	0
F5 and Stx-2	2(1.0)
F5 and Sta	4(1.9)
F5 and α-Hly	1(0.5)
F5 and E-Hly	2(1.0)
F17 and LT-II	5(2.4)
Stx-1 and *eae*	6(2.9)
E-Hly and Stx-1 and *eae*	1(0.5)

* - *eae* detection by PCR assays only.

Genotypic assays

The results obtained in phenotypic assays were later confirmed by Polymerase Chain Reaction (PCR). Only the *eae* detection assays were made by PCR alone.

Analysis of strains producing Stx

Of 33 STEC, 7 (21.2%) showed the *eae* gene (Table 2), and belonged to serogroups O11 (1), O26 (1), O111 (1), O118 (2) and O153 (1); one strain was not typed. The strains of the serogroups O26, O11 and O118 were later assayed for determination of H (flagellar) antigen, and their serotypes were : O26:NM; O111:NM; O118:H14 and O118:H16.

DISCUSSION

Since colibacillosis is an important cause of economic loss on farms, detailed studies of the virulence factors produced by *E. coli* strains in farm animals are needed. Colibacillosis is common in Brazil, but the few studies reported so far have dealt with a restricted number of animals and regions, and represent only a small sample of the total brazilian bovine herd. For this study, we chose the State of Mato Grosso do Sul in mid-western Brazil which has the largest bovine herd in the country (27). From the 205 *E.coli* strains, we found 83 (40.05%) strains positive for at least one of the virulence factors involved in this study.

We found 20 (9.75%) e 13 (6.34%) positive strains for Stx-1 and Stx-2 respectively (Table 2); investigations by Blanco *et al.* (10) found a rate of Stx-1 and Stx-2 producing strains in Spain similar to our data. Among those Stx^+ strains, 7 were positive for *eae* gene, belonging to the serogroups (O11, O26, O111, O118, O153); then determined the type of flagellar antigen of these strains and their serotypes are: O26:H-; O111:H-; O118:H14 and O118:H16; according to Mainil (34) and Nataro and Kaper (37), strains from these serotypes are EHEC strains. The most important animal species in terms of human infection by EHEC is cattle and high rates of colonization by Stx-positive *E. coli* have been found in bovine herds in many countries (37). Our findings suggests that cattle is an important reservoir of STEC and EHEC in Brazil. There is a previous report (2) of eae^+ strains isolated from bovines in Brazil, but these strains were not positive for any enterotoxin or cytotoxin.

Blanco *et al.* (9) reported that 62% of Stx-producing strains from cattle and humans produced E-Hly. However, we observed only 35.5% of $E\text{-}Hly^+$ strains associated with Stx synthesis. It is unclear whether the absence of hemolytic activity in some STEC strains corresponds to a characteristic phenotype, since the Stx and E-Hly phenotypes are not always stably inherited in certain *E. coli* strains (31).

One (0.5%) and nine (4.4%) of the culture supernatants of the 205 *E. coli* strains produced CNF1 and CNF2, respectively. These results are comparable to those recently reported by Orden *et al.* (38). We observed a low association between the production of CNF and a-Hly, in contrast to other reports (9). CNF2 and Stx production were associated in one of the strains (Table 2). The significance of this observation in relation to pathogenesis is unclear. However, even with the potential mobility of *cnf2*, the production of both CNF2 and Stx-1 (0.5%) by the same strain remains a rare event (39).

For the enterotoxins, we found eight (3.9%) STa positive strains. Such a low frequency may be comparable to French (0%) and Spanish (1.3%) data (18,6). No LT-I positive strain was found. Interestingly, we found 17 (8.3%) strains positive for LT-II, in contrast with the fact that there are few reports of the incidence of this toxin in human and animals (26). In 2002, Ugrinovich *et al.* reported, for the first time, the isolation of a $LT\text{-}II^+$ strain among 52 isolates in Brazil (45).

Fimbrial adhesin F5 plays a role in the colonization of bovine small intestine epithelial cells by ETEC (1). F5 and heat-stable toxin (ST)-producing ($F5^+$ and STa^+) strains occur frequently in calves in several countries (9). Our results (Table 3) were similar to those of Blanco *et al.* (6) in Spain, who detected $F5^+$ STa^+ strains in 1% of their cases. We also isolated 10 (4.8%) $F17^+$ strains, which is comparable to the frequency reported by Shimizu *et al.* (43) and Leite *et al.* (32). F5 and F17 were associated with other toxins (Table 3). None of the strains produced F41 fimbriae (17).

In conclusion, these results show that bovine *E. coli* produce several toxins and colonization factors (Tables 2 and 3), some of which may be involved in human diseases. More extensive studies involving a larger number of animals of different bovine races and types of farms (dairy or meat) are required in order to establish precisely the identity and prevalence of virulence factors associated with colibacillosis in Brazil. Such studies will provide important epidemiological data about this disease.

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RESUMO

Fatores de virulência das amostras de *Escherichia coli* isoladas de bezerros com diarréia no Brasil

Duzentas e cinco amostras de *Escherichia coli* isoladas de bezerros com diarréia da região centro oeste do Brazil foram examinados quanto a presença de fatores de virulência associados à colibacilose bovina. Cento e duas amostras (49,8%)

de *E. coli* produziram toxinas: toxina de Shiga do tipo 1 (9,7%) e 2 (6,3%), α-hemolisina (9,7%), enterohemolisina (6,8%), Fatores Citotóxicos Necrotisantes tipo 1 (0,5%) e 2 (4,4%), enterotoxinas LT-II (8,3%), e STa (3,9%). Nenhuma amostra produziu enterotoxina LT-I. Adesinas fimbriais F5 e F17 foram produzidas por 7,3% e 4,8% das cepas, respectivamente, e nenhuma expressou F41. Sete das amostras (3,4%) apresentaram o gene *eae* e pertenceram aos sorotipos O26:H-; O111:H- e O118:H16. Estes resultados sugerem que bezerros no Brasil podem ser uma importante fonte de *E. coli* patogênica para animais e humanos.

Palavras-chave: bezerros, diarréia, *E. coli*, fatores de virulência.

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