Original Article

Prevalence of *Escherichia coli* adhesion-related genes in neonatal calf diarrhea in Uruguay

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Abstract
Introduction: Neonatal calf diarrhea (NCD), one of the most important diseases of neonatal dairy and beef calves in Uruguay, has become relevant in association with intensive systems. This disease generates substantial economic losses every year worldwide as a result of increased morbidity and mortality. *Escherichia coli*, one of the pathogens associated with NCD, can express several fimbrial and afimbrial adhesins. The objective of this study was to assess the presence of *clpG*, *f5*, *f17A*, *f17G(II)*, and *f17G(I)* genes that encode three important adhesins expressed in diarrheagenic *E. coli*: F5, F17 and CS31A, isolated from feces of calves in Uruguay.
Methodology: Feces of 86 (70 diarrheic and 16 healthy) calves, from 15 animal facilities in Uruguay, were collected between 2012 and 2013. Biochemical and molecular identification were performed to finally obtain 298 *E. coli* isolates. Partial amplification of adhesion-related genes was performed by polymerase chain reaction.
Results: The most prevalent gene was *f17A* (31.2%), followed by *f17G(II)*, *clpG*, *f17G(I)* and *f5* (25.8%, 17.5%, 3.7% and 0.7%, respectively). All genes were present in diarrheic and healthy animals except *f5* and *f17G(I)*; these genes were present only in affected calves, although in low numbers.
Conclusions: This is the first report of the presence of F5, F17, and CS31A genes in *E. coli* strains from NCD cases in Uruguay. Prevalence values of the genes, except *f5*, were in accordance with regional findings. It is expected that further characterization of locally transmitted strains will contribute to control a problem of regional and international magnitude.

Introduction

Neonatal calf diarrhea (NCD), an important infectious disease that affects dairy and beef calves, is considered a worldwide challenge for bovine producers and industry [1]. Nowadays, there is a remarkable interest to promote production efficiency by improving productive intensive systems. However, these systems have disadvantages such as animal stress and inadequate sanitary conditions, among other factors, that favor the rapid spread of diseases caused by microorganisms [2-4]. Therefore, NCD generates substantial economic losses every year all over the world as a result of increased morbidity and mortality rates [2].

NCD has a complex etiopathogenesis, and *Escherichia coli*, together with rotavirus, coronavirus, *Cryptosporidium parvum* and *Clostridium perfringens*, are the pathogens most commonly associated with this disease [5].

E. coli colonizes the gastrointestinal tract of humans and animals and can coexist as part of the gut microbiota, except in immunocompromised patients or when the epithelial barrier is somehow altered [6]. Nonetheless, there are different highly adapted *E. coli* pathotypes that can settle in different niches and cause disease in healthy individuals and animals [6]. Adhesion to host cells is a crucial step for the establishment of the infection and is achieved through fimbrial and afimbrial adhesins and/or outer membrane proteins such as intimin [6-8], among other factors. Diverse fimbrial and afimbrial adhesins can be expressed in intestinal and extraintestinal *E. coli* strains causing disease in domestic and farm animals [9].

One of the main fimbrial antigens present in bovine diarrheagenic *E. coli* is the plasmid-encoded F5 (formely K99) fimbria [10,11], which mediates adherence to the ileum and establishes first steps of colonization in the bovine intestine [12]. F5 strains are

usually isolated form calves between one and five days of age with diarrhea, and it has been reported that this observation is due to the decline of the F5 receptors' expression in the bovine epithelia within days [13,14].

CS31A is an afimbrial adhesin of *E. coli*, immunologically related to the F4 fimbria (formerly K88), and also plasmid encoded. The CS31A antigen was first recognized as a capsule-like surface protein around the bacteria [15], and it has been extensively studied in septicemic and enterotoxigenic *E. coli* strains [15-18]. CS31A is usually expressed with other fimbrial antigens [16,19]. Particularly, it has been reported that F17 fimbria and CS31A are highly associated [19,20].

The F17-related fimbriae family comprises four types of fimbria: F17a, F17b, F17c, and F111 fimbria, which differ in the amino acid sequences of the structural subunit (F17A) and the adhesin minor subunit F17G, which is composed of the subfamilies of adhesins I and II [9]. This fimbriae family is encoded in the chromosome of *E. coli* strains isolated from diarrheagenic and septicemic cows and lambs from different outbreaks and also from humans suffering urinary tract infections [9,16,19,21,22].

The general aim of this study was to assess for the first time the presence of *clpG*, *f5*, *f17A*, *f17G(II)*, and *f17G(I)* genes that encode three crucial adhesins of *E. coli* associated with NCD, isolated from feces of healthy and ill calves throughout Uruguay.

Methodology

Media and reagents

The isolation of *E. coli* strains from feces was done in MacConkey agar plates, whereas tryptonse soya broth (TSB) and tryptone soya agar (TSA) were employed for further routine cultures. All media were purchased from Oxoid (Basingstoke, UK). Routine cultivation was at 37°C for 24 hours. Strains were stored at -20°C and -80°C, using TSB medium supplemented with 15% glycerol.

Sample collection

Feces of 86 (70 diarrheic and 16 healthy) calves younger than 35 days of age from 15 animal facilities throughout Uruguay were collected by technicians and shipped chilled to the laboratory between 2012 and 2013 (Figure 1). The criteria for sampling included animals with and without symptoms of diarrhea as well as animal samples from intensive (n = 14) and extensive systems (n= 1). All samples were plated onto selective MacConkey agar plates within 12 hours following collection. After 24 hours of incubation at 37°C, at least

Figure 1. Map of Uruguay. Collection zones (provinces) throughout the country are identified in dark gray.
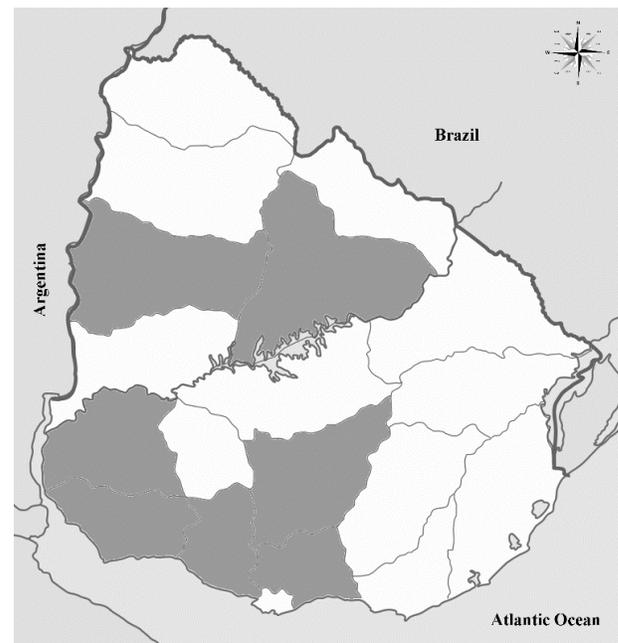
five lactose-positive colonies of each animal were selected.

Biochemical and molecular identification

For biochemical identification of each isolate, the following tests were performed: Gram stain and oxidase, catalase activity, citrate utilization, oxidative/fermentation glucose, indol, and Voges-Proskauer; growth at 45°C in EC (DIFCO; New Jersey, USA) medium was also investigated. All biochemical analyses were performed after 24 hours of incubation at 37°C, except for the citrate test (up to 72 hours of incubation at 37°C) and EC growth (24–48 hours at 45°C). Also, morphology of the colony and occurrence of swarming motility were taken into account in the isolate descriptions.

Further molecular identification was done by polymerase chain reaction (PCR) amplification of the 16S rDNA with 27F and 1492R universal primers. PCR was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) thermocycler, using 0.2 mM dNTPs, 5 μM of each primer, 2 μL of DNA, 3.0 mM of $MgCl_2$, 1X of buffer, and 1U Taq DNA polymerase (Invitrogen, Waltham, USA). Total DNA was extracted by boiling a single colony of each strain resuspended in distilled water followed by a spin-dry step to separate the cell debris from the DNA. Briefly, the cycling program comprised an initial activation step at 94°C for 3 minutes, 30 cycles of amplification (denaturation at 94°C for 1 minute, annealing for 1

minute at 50°C followed by an extension at 72°C for 90 seconds), a final extension step at 72°C for 10 minutes, and chilling at 4°C. PCR products were analyzed by electrophoresis in 0.8% agarose gels, stained with GelRed, and sequenced (MACROGEN, Seoul, Korea). Obtained sequences were aligned using the sequence alignment editor program BioEdit (version 7.0.5.3) and analyzed with BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ribosomal Database Project (http://rdp.cme.msu.edu/).

Presence of fimbrial and non-fimbrial adhesins

For the evaluation of virulence factors in the whole *E. coli* collection, partial amplification of the *f5* gene of F5 fimbria, the structural gene *clpG* of CS31A capsule-like adhesin, the structural fimbrial gene *f17A*, and adhesins genes *f17G(I)* and *f17G(II)* of F17 fimbria was accomplished using specific primers (Table 1) and a 2720 Thermal Cycler thermocycler (Applied Biosystems). DNA was released as described before. Each reaction mix comprised 0.2 mM of each dNTPs, 0.32 μM of primers, 3.0 mM of MgCl$_2$, 1X of buffer, 2.5 ng/μL of DNA, and 1U Taq DNA polymerase (Invitrogen). The thermocycler program comprised an initial activation step at 94°C for 5 minutes, then 30 cycles of amplification (denaturation at 94°C for 2 minutes, annealing for 1 minute [at 58°C for *f5*, 61°C for *clpG*, 59°C for *f17A*, and 58°C for *f17G(I)* and *f17G(II)*] followed by an extension at 72°C for 1 minute), a final extension at 72°C for 10 minutes, and chilling to 4°C. PCR products were analyzed by electrophoresis in 1.2% agarose gels stained with GelRed and photographed under UV light. A 1 Kb Plus DNA ladder (Thermo Scientific, Waltham, USA) was used to determine the molecular size of the PCR products. After PCR amplifications, one representative PCR product of each gene was sequenced to confirm the presence of *clpG, f5, f17A, f17G(II)*, and *f17G(I)*.

Statistical analyses

Odds ratio (OR) was used to measure the association between the presence of the evaluated genes, the occurrence of symptoms, and geographical origins of the isolates.

Results

Construction of E. coli collection from feces of calves

After biochemical identification, a collection of 298 *E. coli* isolates was generated. Then, molecular identification by PCR amplification and sequencing of 16S rRNA gene confirmed previous biochemical identification results. In all cases, nucleotide sequences presented 100% similarity with published sequences for the *E. coli* 16S rRNA gene. Out of the 298 isolates, 237 corresponded to 70 animals with symptoms of diarrhea (mild to severe), whereas 61 isolates corresponded to 16 animals that did not have any symptoms of the disease at the moment of feces sampling.

Presence of F5, F17, and CS31A genes

A PCR product representative of each gene was sequenced, confirming the presence of *clpG, f5, f17A, f17G(II)*, and *f17G(I)* along the collection. Obtained nucleotide sequences presented over 98% of similarity with published sequences for each gene.

The presence of the different *E. coli* adhesion-related genes is summarized in Table 2. The structural subunit gene of CS31A adhesin *clpG* was present in 17.5% of the isolates, whereas 31.2% of the isolates were positive for the F17 structural fimbrial subunit gene *f17A*. Regarding F17 adhesin genes, 25.8% of the isolates were positive for *f17G(II)*, while only 3.7% were positive for *f17G(I)*. Furthermore, 22.8% of the isolates were positive for *f17A* and *f17G(II)* simultaneously, and 3.7% carried both *f17A* and *f17G(I)*

Table 1. Primers used in polymerase chain reaction for the determination of virulence genes in the *E. coli* sample collection

Primer	Sequence (5´--3´)	Gene position	Size (bp)	Reference
clpG-F	GGGCGCTCTCTCCTTCAAC	153-172	403	[23]
clpG-R	CGCCCTAATTGCTGGCGAC	537-556		
f5-F	TATTATCTTAGGTGGTATGG	21-40	314	[24]
f5-R	GGTATCCTTTAGCAGCAGAGTATTTC	311-334		
f17A-F	GCAGAAAATTCAATTTATCCTTGG	3-26	537	
f17A-R	CTGATAAGCGATGGTGTAATTAAC	517-540		
f17G(I)-F	CGGAGCTAATACTGCATCAACC	399-420	615	[20]
f17G(I)-R	TGTTGATATTCCGTTAACCGTAC	992-1014		
f17G(II)-F	CGTGGGAAATTATCTATCAACG	396-417	615	
f17G(II)-R	TGTTGATATTCCGTTAACCGTAC	992-1014		

Table 2. Prevalence of virulence genes in healthy and diarrheic animals

	Diarrheic animals (n = 237)	Healthy animals (n = 61)	Total isolates	Total prevalence	Prevalence (diarrheic)	Prevalence (healthy)
f17A	68	25	93	31.2%	28.7%	40.9%
f17G(II)	52	25	77	25.8%	21.9%	40.9%
f17G(I)	11	0	11	3.7%	4.6%	0%
f5	2	0	2	0.7%	0.8%	0%
clpG	41	11	52	17.5%	17.3%	18.0%
f17A-f17G(II)	43	25	68	22.8%	18.1%	40.9%
f17A-f17G(I)	11	0	11	3.7%	4.6%	0%
f17A-f17G(II)-clpG	9	6	15	5.0%	3.8%	9.8%
f17A-f17G(I)-clpG	1	0	1	0.3%	0.4%	0%

genes. These results showed that the majority of F17-positive isolates carried the *f17G(II)* adhesin gene. In addition, 5% of the *f17A-f17G(II)* positive strains were also *clpG* positive, and 0.3% of the *f17A-f17G(I)* were also *clpG* positive. In this study, *f17A* and *f17G(II)* fimbrial genes were present in diarrheic animals with similar prevalences (28.7% and 21.9%, respectively), as well as in healthy animals (40.9% each). Also, the *clpG* gene was present in diarrheic and healthy animals, with very similar prevalence values (17.3% and 18%, respectively). Finally, PCR analyses showed that the *f5* gene was present in 0.7% (two isolates) of the *E. coli* samples. Moreover, *f5* was not present together within either of the other evaluated genes. Even *f5* and *f17G(I)* genes were found in small proportions within the samples, and both were present only in animals with symptoms of NCD.

No significant association between the presence of F17, CS31A, and F5 genes and NCD symptoms was observed. Moreover, it was not possible to associate the different genotypes with geographical origins, productive systems, or isolation dates.

Discussion

Although *E. coli* is part of the commensal gut microbiota, different strains are responsible for intestinal infections of humans and animals [8,25]. F5 and F17 fimbriae and CS31A adhesin are among the most relevant adhesins in *E. coli* infections in calves, lambs, pigs and dogs, and are essential in the first colonization stages [8,14,17,26]. Additionally, recent studies have demonstrated that F17 and F5 together with F41 are significantly associated with calf diarrhea [27]. Furthermore, previous work has established that the *E. coli* F17 family is associated with CS31A in bovine diarrhea and septicemia [16]. Also, an explicit association between the presence of these adhesins and the heat-stable enterotoxin (STa) and the enteroaggregative heat-stable toxin 1 (EAST1) has been observed [28].

In the present work, genotypic analyses of 298 isolates of *E. coli* allowed us to demonstrate the presence of *E. coli* fimbrial and non-fimbrial adhesin genes in the feces of calves. Prevalences of CS31A (17.5%) and F17 (31.2%) genes were in accordance with those from previous reports in France, New Zealand, Iran, and in countries of the region such as Argentina and Brazil, but the F5 gene was less prevalent (< 1%) in the present study [17,28-31]. These results are in accordance with a study by Kolenda *et al.*, who reported a significant F5 prevalence reduction over the last decades [28]. One explanation for this reduction could be the use in pregnant cows of vaccine formulations that include the F5 antigen [32].

Otherwise, F17 was the most prevalent virulence factor gene in our collection, followed by CS31A. However, only a small proportion of F17+ strains were also CS31A+, a lower prevalence compared to that in other studies [17,28]. Moreover, we revealed that *f17A* and *f17G(II)* were present together in a high proportion of the isolates compared to *f17A* and *f17G(I)*. This observation had been reported previously, and a prominent presence of F17 subfamily II adhesin compared to subfamily I adhesin in bovine *E. coli* isolates was previously detected [20,33]. Conversely, F17 and CS31A genes were also present in some animals without NCD symptoms. This observation could be explained by the occurrence of horizontal gene transfer, particularly in the case of CS31A genes, which are plasmid encoded [26]. In this study, we observed a relatively high F17 gene prevalence in healthy animals. Even though recent studies have observed an association between the presence of F17 and calf diarrhea, Kolenda *et al.* observed a high prevalence of this fimbria in healthy calves [27]. These authors propose that this fimbria could require the presence of

other virulence factors intervening in the etiology of diarrhea or that they are not functionally expressed [27].

It is important to highlight that although the presence and expression of *E. coli* virulence genes in locally transmitted strains is relevant for the development of the disease, there are other risk factors involved. Rotavirus, coronavirus, *Cryptosporidium parvum*, and *Clostridium perfringens* are also associated with NCD, particularly following the first week of birth [5]. Also, the status of the animals, management procedures, and environmental conditions are all together risk factors that would increase the incidence of NCD [2,4]. Hence, the presence of F5, F17, and CS31A genes alone, regardless of the management factors and the presence of other pathogens mentioned above, do not necessarily determine the occurrence of NCD.

Conclusions

This study reports the presence of the genes that encode important adhesion-related factors such F5, F17, and CS31A in *E. coli* strains from NCD cases for the first time in Uruguay. Prevalence of all evaluated adhesion-related genes, except *f5*, was in accordance with regional diverse surveys. *F17A* and *f17G(II)* were the most frequently detected genes within our collection.

Results obtained in this study will contribute to the knowledge and characterization of locally transmitted *E. coli* strains in our country and will provide information to manage a problem of regional and international magnitude.

Acknowledgements

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