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BRIEF REPORT

Virulence genes of *Escherichia coli* in diarrheic and healthy calves

Ana Umpiérrez[a,*], Déborah Ernst[a], Magalí Fernández[a], Martín Oliver[a], María L. Casaux[b], Rubén D. Caffarena[b,c], Carlos Schild[b], Federico Giannitti[b], Martín Fraga[b], Pablo Zunino[a]

[a] *Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay*
[b] *Plataforma de Investigación en Salud Animal, Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA La Estanzuela, Colonia, Uruguay*
[c] *Departamento de Patología y Clínica de Rumiantes y Suinos, Facultad de Veterinaria, Universidad Nacional de La República, Montevideo, Uruguay*

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Abstract *Escherichia coli* ETEC, EPEC, NTEC and STEC/EHEC pathotypes are often isolated from bovine feces. The objective of this study was to detect 21 *E. coli* virulence genes in feces from 252 dairy calves in Uruguay (149 with neonatal diarrhea – NCD – and 103 asymptomatic). Genes *iucD*, *f17A*, *afa8E*, *papC*, *clpG* and *f17G(II)* were the most prevalent (81.3%; 48.4%; 37.3%; 35.7%; 34.1%; 31.3%, respectively). Genes *eae*, *stx1*and *stx2* were poorly represented; 13/252 animals harbored one or a combination of these genes. The prevalence of the *cnf* gene was 4.4%, while that of *cdt-IV* and *cdt-III* genes was 24.2% and 12.7% respectively. This study reports updated data about the virulence profiles of *E. coli* in dairy calves in Uruguay. A large number of adhesins and toxin genes were detected. Our results demonstrate that *E. coli* from bovine feces has diarrheagenic and extraintestinal profiles although other NCD risks factors may contribute to the disease outcome.

* Corresponding author.
E-mail addresses: aumpierrez@iibce.edu.uy, anitaump@gmail.com (A. Umpiérrez).

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PALABRAS CLAVE
Genes de adhesión;
NTEC;
DNT;
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Zoonosis

Genes de virulencia de *Escherichia coli* en terneros con diarrea neonatal y asintomáticos

Resumen Los patotipos de *Escherichia coli* ETEC, EPEC, NTEC y STEC/EHEC son frecuentemente aislados de heces bovinas. El objetivo del presente estudio fue detectar 21 genes de virulencia de *E. coli* en las heces de 252 terneros de leche en Uruguay, 149 de ellos con síntomas de diarrea neonatal (DNT) y 103 asintomáticos. Los genes *iucD*, *f17A*, *afa8E*, *papC*, *clpG* y *f17G(II)* fueron los más prevalentes (81,3; 48,4; 37,3; 35,7; 34,1 y 31,3%, respectivamente). Los genes *eae*, *stx1* y *stx2* estuvieron poco representados: 13/252 animales presentaron uno o una combinación de dichos genes. La prevalencia del gen *cnf* fue del 4,4%, mientras que la de los genes *cdt-IV* y *cdt-III* fue del 24,2 y 12,7%, respectivamente. Este trabajo aporta datos actualizados sobre el perfil de virulencia de *E. coli* en terneros en Uruguay. Fueron detectados un alto número de genes de adherencia y de toxinas. Se demuestra que los aislamientos de *E. coli* recuperados de heces de terneros presentan perfiles diarreogénicos y extraintestinales, aunque otros factores de riesgo de DNT podrán contribuir al desarrollo de la enfermedad.

Escherichia coli is a widely distributed gram-negative bacterium, and is the most numerous facultative anaerobe inhabiting the intestine of warm-blooded animals[2]. In this niche, these bacteria coexist with other microorganisms assembling the commensal gut microbiota. However, some *E. coli* variants have acquired specific attributes that allow them to infect the immunocompetent host and cause disease[4].

Pathogenic *E. coli* comprises a vast number of virulent variants associated with human and animal illnesses. Such is the diversity of virulence factors, together with the ability of bacteria to horizontally transfer several virulence-related genes through plasmids, phages and transposons, that nowadays at least 9 different pathotypes or virulent variants have been described[4,6]. Enterotoxigenic *E. coli* (ETEC) is characterized by the expression of fimbrial and fibrillar adhesins and by the expression of heat-stable (ST) and heat-labile (LT) toxins[6]. ETEC F5 (formerly K99) and F17 fimbriae and ST and LT toxins have been significantly associated with neonatal calf diarrhea (NCD)[5]. Indeed, ETEC has been considered one of the primary causative agents of NCD[5]. Enteropathogenic *E. coli* (EPEC) belongs to the family of pathogens that produce attaching and effacing (AE) lesions when intimately attach to intestinal epithelial cells[4]. The ability to efface microvilli and to form pedestal-like structures in the intestine is encoded by the pathogenicity island called the locus of enterocyte effacement (LEE)[4]. Another pathotype that belongs to the AE family is the group known as enterohemorrhagic (EHEC)/Shiga toxin-producing *E. coli* (STEC). While all the strains belonging to this group are characterized by the expression of Shiga toxin 1 and 2 (individually or simultaneously), some of them produce AE lesions to the intestinal epithelial cells[4]. EHEC/STEC is a well-documented zoonotic pathogen, responsible for important outbreaks around the world[11]. Bovines are asymptomatic carriers of STEC, responsible for their spread[6]. Finally, necrotoxigenic *E. coli* (NTEC) is characterized by the expression of different virulence factors, including fimbrial and afimbrial adhesins, siderophores, and toxins (cytotoxic necrotizing factor, CNF and cytolethal distending toxin, CDT). So far, NTEC expresses two types of CNF. NTEC1 is frequently isolated from diarrhea in domestic animals and ruminants whereas NTEC2 is mostly associated with septicemia in ruminants[6].

Dairy farms are distributed across Uruguay, and the production of milk and dairy products is one of its most important agricultural activities. Pathogenic *E. coli* associated with NCD in the country has been previously characterized by our group. In that work, several *E. coli* virulence genes were detected in animals with signs of NCD and in healthy ones, F17 and CS31A adhesin genes being the most prevalent in both groups of calves[13].

The aim of this study was to detect the presence of 21 virulence genes, distinctive of the most relevant *E. coli* pathotypes in bovines: ETEC, EPEC, EHEC/STEC and NTEC, in feces from dairy calves with signs of diarrhea and asymptomatic ones throughout Uruguay. These analyses increased the panel of assessed *E. coli* virulence genes providing an update of the pathogenic profile of *E. coli* associated with NCD in dairy farms in Uruguay.

Feces of 252 (149 diarrheic and 103 healthy) calves younger than 35 days-old were processed between 2016 and 2018. Samples were collected throughout the Uruguayan territory by veterinarians and shipped chilled to the laboratory. All samples were plated onto selective MacConkey agar plates (OXOID) within 12 h following collection. After 24 h of incubation at 37 °C, at least 10 lactose positive colonies of each animal were selected and biochemically identified[8]. Molecular characterization included the evaluation of 21 *E. coli* virulence genes using conventional and multiplex PCR and previously described primers[7,9,15] (supplementary Table). PCR analyses were performed in pools of DNA, at a final concentration of 50 ng/μL. Each pool consisted of equal quantities of genomic DNA of *E. coli* isolates (a maximum of 10 isolates from each animal, see above) of every evaluated animal.

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Odds ratio (OR) was performed to measure the association between the presence of the evaluated genes, the occurrence of symptoms, and the geographical origins of the isolates, considering statistical significance when *p*-values were lower than 0.05.

All the evaluated *E. coli* virulence genes (VGs) were detected in this study. At least 1 animal presented one of the evaluated genes, and some showed more than one gene at the same time (a maximum of 7 VG was detected simultaneously in a pool of DNA). Additionally, all evaluated genes were detected in healthy calves and in calves with signs of NCD, except for Shiga toxin 2 gene (*stx2*) and heat-labile toxin gene (*eltA*), which were detected only in diarrheic animals (Table 1). No association between any VGs and animal signs was detected.

ETEC genes were detected in low numbers. Genes encoding for heat-stable (*sta*) and heat-labile (*eltA*) toxins were present in 10 animals (1 animal presented both toxin genes simultaneously), and only 7 *sta*+ animals were also *f5*+. F41 fimbriae were found in a limited number of animals (n = 7), where 5 of them were also *f5*+. Among all adhesins and adhesion-related genes, *f17A*, *afa8E*, *papC*, *clpG* and *f17G(II)* were the most abundant (48.4%, 37.3%, 35.7%, 34.1% and 31.3%, respectively). The gene that codifies for the F17A structural subunit was one of the most represented genes in this study, after the *iucD* gene of the aerobactin operon, whose prevalence was 81.3% (Table 1). On the other hand, the less prevalent adhesion-related genes were *saa* (STEC autoagglutinating adhesin), *sfaD-E* (mannose-resistant S fimbriae) and *f17G(I)* detected only in 15, 13 and 8 animals, respectively (prevalence 6.0%, 5.2% and 3.2%).

Genes associated with the EHEC/STEC group were also poorly represented. Seven of the 252 animals were *stx1*+/*eae*+ (6 calves with signs of NCD and 1 healthy animal), 2 animals with NCD signs were *stx2*+/*eae*+, 1 animal with signs of NCD was *stx1*+/*stx2*+/*eae*+, 2 animals with NCD signs were *stx1*+ and 1 animal with signs of NCD was *stx2*+. *E. coli* hemolysin gene *ehxA*, which can be used as an epidemiological marker for EHEC/STEC strains, was identified in 12 isolates (prevalence 4.8%). Seven of these 12 animals were *eae*+/*stx1*+/*ehxA*+, 3 were *eae*+/*ehxA*+, 1 was *eae*+/*stx2*+/*ehxA*+ and 1 was *eae*+/*stx1*+/*stx2*+/*ehxA*+. Likewise, the EPEC distinctive characteristic *eae*+ was detected in 7 (3 animals with NCD signs and 4 healthy) animals (Table 1).

As mentioned above, *iucD* was the most prevalent gene in the collection. This aerobactin gene is commonly present in extraintestinal *E. coli* strains, including NTEC. CNF was present in 11 animals (some animals were *cnf2*+ and *cnf1/2*+ simultaneously) and CDT was detected in animals with signs of NCD and in healthy ones, the gene variant *cdt-IV* being more prevalent than the *cdt-III* variant (24.2% and 12.7%, respectively) (Table 1).

Approximately 1700 homolog gene clusters form the core genome of *E. coli*, while the composition of the pangenome of this organism is about 16 400 gene clusters[3]. Variability in genome sizes is the consequence of the remarkable plasticity of the genetic material of this bacterium. The efflux of the flexible gene pool due to transposons, integrons, bacteriophages, plasmids, insertion elements and pathogenicity islands together with mutations, rearrangements, deletions and duplication events result in new combinations of genes and accelerate the emergence of virulent strains. When this combination of VGs persists, the pathotypes emerge[4].

F17 (*f17A* and *f17G(II)*) fimbriae and CS31A (*clpG*) afimbrial adhesin, essential in the first steps of attachment to the intestinal epithelium, were the most prevalent encompassing all adhesin and adhesion-related genes evaluated here. Similar results were observed in a smaller collection of *E. coli* in calf feces in our country in 2016[13]. On both occasions, *f17A*, *f17G(II)* and *clpG* were present in a high prevalence in both groups of animals (with and without signs of NCD). Meta-analyses performed by Kolenda et al.[5] statistically demonstrated that F17 is more frequent in diarrheic than in healthy animals; still, a high prevalence of this adhesin in healthy calves is observed. Two assumptions are raised regarding these observations, (i) F17 is not expressed even though the PCR method detects its presence, or (ii) this fimbria requires the presence of other virulence factors to participate in the etiology of NCD[5]. On the other hand, prevalence of F5 (*f5*) and F41 (*f41*), which are highly associated with NCD and are the most investigated ETEC fimbriae, was low (<2% each gene). Similar results were previously reported in Uruguay and regionally[12,13]. Furthermore, our results are consistent with the observation of several authors who showed a significant decline of *f5* over time, mainly attributed to the fact that available vaccines against NCD include this antigen[5]. Concomitantly with the level of *f5* and *f41* genes, heat-stable and heat-labile ETEC toxins genes were detected in low numbers.

Serious outbreaks caused by EHEC/STEC strains have been reported around the world, most of the time associated with contaminated raw meat or vegetables[11]. It is well known that infections caused by these pathogens are serious zoonoses since ruminants, particularly bovines, are the main reservoirs of these strains. In Uruguay, the incidence of hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC), two important diseases associated with EHEC/STEC infection, is low. It is estimated in 4 out of 100 000 children under 5 years old, whereas in Argentina, one of the countries of major incidence of HUS in the world is 12 out of 100 000 children[1,10]. In this work, only 13 animals harbored one of the distinctive genes *stx1* and *stx2* alone, or in combination with *eae*. This low prevalence was previously reported by our group and could corroborate the low incidences of EHEC/STEC diseases detected in Uruguay[14].

NTEC comprises a pathotype that shares many properties of typical extraintestinal *E. coli*, including the presence and expression of iron-sequestering systems, various fimbrial and afimbrial adhesins and resistance to the bactericidal action of complement[4]. Moreover, NTEC is defined by the presence and expression of CNF and CDT toxins and it can be detected in human and animal infections as well as in healthy individuals[6]. In this work, CNF and CDT toxins genes were detected in animals with or without signs of NCD, *cdt-IV* and *cdt-III* being more prevalent than *cnf2* and *cnf1/2*. In addition to the toxins, NTEC strains express factors associated with invasion, which are essential to cause septicemia and internal organ infections[6]. The *iucD* gene is a component of the aerobactin operon, and was the most prevalent gene in our collection, mostly detected simultaneously with adhesins (mainly *f17A*, *clpG*, *afa8E* and *papC*) and NTEC toxin genes. All these results are in accordance with previous

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Table 1 Presence of virulence genes in healthy calves and calves with signs of NCD.

	Diarrheic animals (n = 149)	Healthy animals (n = 103)	Total of positive animals	Total prevalence (n = 252)	Prevalence in diarrheic animals	Prevalence in healthy animals
clpG	53	33	86	34.1%	35.6%	32.0%
f5	6	1	7	2.8%	4.0%	1.0%
f17A	75	47	122	48.4%	50.3%	45.6%
f17G(II)	54	25	79	31.3%	36.2%	24.3%
f17G(I)	2	6	8	3.2%	1.3%	5.8%
f41	5	2	7	2.8%	3.4%	1.9%
eae	12	5	17	6.7%	8.1%	4.9%
stx1	9	2	11	4.4%	6.0%	1.9%
stx2	3	0	3	1.2%	2.0%	0%
ehxA	9	3	12	4.8%	6.0%	2.9%
saa	8	7	15	6.0%	5.4%	6.8%
eltA	1	0	1	0.4%	0.7%	0%
sta	8	2	10	4.0%	5.4%	1.9%
cnf2	2	6	8	3.2%	1.3%	5.8%
cnf1/2	2	7	9	3.6%	1.3%	6.8%
cdt-III	15	17	32	12.7%	10.1%	16.5%
cdt-IV	34	27	61	24.2%	22.8%	26.2%
iucD	118	87	205	81.3%	79.2%	84.5%
afa8E	58	36	94	37.3%	38.9%	35.0%
papC	57	33	90	35.7%	38.3%	32.0%
sfaD-E	9	4	13	5.2%	6.0%	3.9%

works that demonstrate the co-existence of diarrheagenic and extraintestinal *E. coli* VGs in each calf[4,6].

In summary, this study reports updated data about the virulence profile of *E. coli* in dairy calves in Uruguay, mainly diarrheagenic but also extraintestinal *E. coli* associated-genes. A large number of adhesins and toxin genes were observed, although no relationships between them and animal symptoms were noted. Therefore, other NCD risks factors such as co-infections, poor nutrition, inadequate colostrum consumption and animal hygiene may probably contribute to the disease outcome. Furthermore, the presence of EHEC/STEC genes exposes the occurrence of potentially zoonotic *E. coli* isolates in calf feces.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2020.04.004

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