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Frequency of virulence genes of *Escherichia coli* among newborn piglets from an intensive pig farm in Argentina

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ABSTRACT

The enterotoxigenic and porcine enteropathogenic *Escherichia coli* (ETEC and PEPEC) strains are agents associated with swine neonatal diarrhea, causing economic losses in swine production. The main goal of this study was to identify virulence genes of ETEC, verotoxigenic (VTEC) and PEPEC in intestinal strains responsible for swine diseases, by molecular typing using PCR in newborn piglets from an intensive farm system. Two hundred and sixty seven rectal swabbings from 7-15 days- old Landrace x Large White crossbred piglets were taken, and 123 randomly selected samples, biochemically compatible with *E. coli*, were tested for *E. coli* virulence genes by PCR. A frequency (%) compatible with: 68 ETEC, 24 VTEC, and 8 EPEC were found. Of all *E. coli* strains studied, 19.51 % carried at least one virulence gene. These data showed conclusively that, in spite of the application of strict sanitary measures in the intensive farm, genes encoding virulence factors of intestinal pathogens compatible with ETEC are still detected; therefore these strains will probably keep circulating among animals.

Key words: virulence genes, *Escherichia coli*, piglets, intensive farm

RESUMEN

Frecuencia de genes de virulencia de *Escherichia coli* en lechones neonatos de un criadero intensivo de Argentina. El objetivo del trabajo fue identificar genes de virulencia de cepas intestinales de *Escherichia coli* de los grupos enterotoxigénico (ETEC), verotoxigénico (VTEC) y enteropatogénico porcino (PEPEC), responsables de patologías en cerdos, mediante tipificación molecular por PCR. Para ello se trabajó en un criadero intensivo, donde se tomaron 267 hisopados rectales de lechones cruza Landrace por Large White de 7-15 días de edad. Del total de aislamientos obtenidos se seleccionaron al azar 123 de ellos, bioquímicamente compatibles con *E. coli*, los que fueron analizados por PCR. La frecuencia de genes compatibles con ETEC, VTEC y PEPEC fue de 68 %, 24 % y 8 %, respectivamente. De las cepas de *E. coli* seleccionadas, el 19,51 % portaban al menos un gen codificante de un factor de virulencia. Estos hallazgos muestran de manera concluyente que la aplicación de estrictas medidas sanitarias en el criadero en estudio no logró evitar la presencia de genes que codifican factores de virulencia de patógenos intestinales compatibles con ETEC. Es probable que las cepas circulen entre los animales.

Palabras clave: genes de virulencia, *Escherichia coli*, lechones, criadero intensivo

Swine neonatal diarrhea is one of the major causes of death and economic losses in swine production. The enterotoxigenic (ETEC), verotoxigenic (VTEC) and porcine enteropathogenic (PEPEC) strains are one of the most representative pathogenic variants of *E. coli* (9, 11, 12). There are a number of virulence factors associated with ETEC strains, such as the production of heat-labile (LT) and heat-stable (ST) toxins with two variants (STa and STb) (2).

The mechanism of pathogenicity of ETEC is to

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first colonize the small intestine, followed by toxin production. This colonization is achieved under other virulence factors known as fimbriae**.** The main fimbriae are F4 (K88), F6 (P987) and F18. Each fimbriae interacts with specific glucidic residues from the mucus layer (5, 9). These virulence factors are not required for vegetative replication or bacterial commensalism, due to the fact that *E. coli* is one of the most successful commensal microorganisms which is related to a great diversity of hosts (2). However, such virulence factors provide the microorganism with greater ability to colonize intestinal surfaces in the host and cause disease through damage of cells and tissue (2, 5, 9).

The intensification of swine production has led producers to carry out breeding practices under strictly controlled sanitary conditions, such as temperature control, reduction of contact between animals and waste, improvement of effluent treatment, parturition control with human intervention, and use of autovaccines with local strains. However, all these practices are not enough to achieve ideal sanitary conditions, mainly due to the environmental survival capacity of *E. coli* strains.

Authors from different countries have described the recent situation of swine production regarding the prevalence of *E. coli* (2, 6, 10, 14, 15). In Argentina, as in other countries, enteric colibacillosis is a very common disease among newborn piglets (3). However, little is known about the distribution of strains carrying virulence factors in Argentina. The objective of this study was to identify genes encoding virulence factors causing intestinal disease in swine, in possible ETEC, VTEC and PEPEC strains by molecular typing using PCR in newborn piglets in an Argentine intensive farm system. The results of our study may offer valuable information about the presence of virulence factors compatible with ETEC, VTEC and PEPEC in a breeding facility.

Farm description. The crossbred pigs used in this study, Landrace x Large White, came from a single-source, a 2500 multi-site swine production system having high-health status, without clinical signs of diarrhea. The farm is located in Santa Eufemia - Cordoba Province, in the central region of Argentina. The piglets were housed in pens in a nursery site with plastic floor separated by metal partitions, allowing contact between animals and their mothers.

Sample collection. Two hundred and sixty seven samples were taken from randomly selected 7-15 day-old piglets by rectal swabbing. In order to guarantee a safe, correct and careful handling of the animals, we proceeded according to specifications of the Universidad Nacional de Rio Cuarto Ethics Committee (4). When samples were taken, animals were apparently healthy; they did not show any signs or symptoms of diarrhea. Samples were collected every season during a 2-years period (March 2008 - December 2010).

Table 1. Primers used in the amplification reaction of virulence genes

Virulence Factor gene	Primer	Sequency (5´ - 3´)	Amplicon size (pB)	Reference
General Verotoxin (VTg)	VTg -F VTg-R	GAGCGAAATAATTTATATGT CGAAATCCCCTCTGTATTTGCC	322	13
Heat-labile Toxin (LT)	LT-B LT-FN	CCGAATTCTGTTATATATGTC GGCGACAGATTATACCGTGC	696	1
Heat-stable Toxin type b (STb)	STb-F STb-R	ATCGCATTTCTTCTTGCATC GGGCGCCAAAGCATGCTCC	175	1
Heat-stable Toxin type a (STa)	STa-A STa-B	TCTTTCCCCTCTTTTAGTCAG ACAGGCAGGATTACAACAAAG	166	11
Intimin (Eae)	eae-MBR eae-V3F	TCCAGAATAATATTGTTATTACG CATTGATCAGGATTTTTCTGGT	510	Access GenBank: AF453441
Fimbria 18 (F18)	F18-FN F18-RN	GGGCTGACAGAGGAGGTGGGG CCCGGCGACAACTTCATCACCGG	411	14
Fimbria 4 (F4)	K88-A K88-B	GGTGATTTCAATGGTTCGGTC ATTGCTACGTTCAGCGGAGCCG	772	8
Fimbria 6 (F6)	P987 A P987 B	GCGCCCGCTGAAAACAACACCAGC GTACCGGCCGTAACTCCACCG	467	8

The swabs were transported in a Stuart's transport medium to the Bacteriology Laboratory of the Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto. The samples were cultured on Mac Conkey Agar. After growth, lactose positive colonies were biochemically characterized by using tests described for the *Enterobacteriaceae* family, according to Bergey's Manual (7).

DNA extraction. For DNA extraction, the confluent growth area of the plate was extracted and diluted in 300 µl of sterile bi-distilled water in Eppendorf tubes. After dilution, the sample was boiled for 3 min, and centrifuged at 11 000 x *g* at 4 °C for 2 min. The supernatant was used as a template for the PCR reaction.

PCR reaction. The primers listed in Table 1 were used for the reaction. Each of them codified different virulence factors of *E. coli* under the following conditions of reaction: amplification carried out with 7 µl of template and 6µl of reaction buffer (Green go Taq 5X) (pH = 8.5), which includes $MgCl_2$ (1.5 mM); then 0.6 µl of dNTP (10 mM), 0.5 µl for each primer (300 ng/µl), and 0.2 µl of Taq DNA polymerase (5 U/µl) added and taken to final volume with distilled sterile water (30 µl). PCR cycling conditions were: initial denaturalization at 94 °C for 2 min; 30 amplification cycles (denaturalization at 93 °C for 1.5 min, suitable annealing for 1 min, then extension at 72 °C for 1 min), and final extension for 7.5 min.

PCR products were electrophoresed in agarose gel at 2 %, with TAE buffer (tris-acetate-EDTA). A marker of 100-1000 bp molecular weight was used. Reference *E. coli* strains were run for each gene as a positive control, and distilled water was used as a negative control. Gel was stained with Ethidium Bromide and visualized under UV light.

Two hundred and sixty seven samples were processed. Of the *E. coli* isolates, 123 were randomly selected and analyzed by PCR. Figure 1 shows PCR products of the evaluated virulence genes: F4 (lane 2), VTg (lane 3), STb (lane 4), F18 (lane 5), LT (lane 6), eae (lane 8), F6 (lane 9) and STa (lane 10).

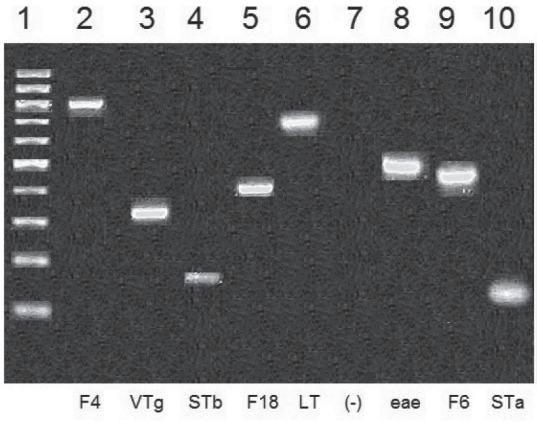

Figure 1. Agarose gel electrophoresis of PCR products. Lanes: 1, 100-bp DNA ladder; 2, *E. coli* F4$^+$ (772 bp); 3, *E. coli* VTg$^+$ (322 bp); 4, *E. coli* STb$^+$ (175 bp); 5, *E. coli* F18$^+$ (411 bp); 6, *E. coli* LT$^+$ (696 bp); 7, negative control (distilled water); 8, *E. coli eae*$^+$ (510 bp); 9, *E. coli* F6$^+$ (467 bp); 10, *E. coli* STa$^+$ (166 bp).

Knowing that the real pathogenic mechanism of ETEC strains is the production of toxins and that adherence to the enterocyte is carried out through fimbriae, it is necessary to correlate toxin production with the presence of fimbriae-coding genes. The frequency of fimbriae and enterotoxins are shown in Table 2.

A remarkable circulation of ETEC-compatible genes with a cumulative frequency of 68 % is shown in Table 2, being the thermostable toxin b (STb) the prevailing virulence factor with a frequency of 28 %, followed

Table 2. Frequency of intestinal virulence genes for fimbriae and enterotoxins among positive strains

Genes encoding virulence factor	Frequency (%)	Compatible with	Cumulative frequency (%)
F4	8		
F18	4		
P987	16	ETEC	68
STb	28		
LT	8		
STb, F18	4		
Eae	8	PEPEC	8
STa, VTg	12		
Eae, VTg	8	VTEC	24
P987, VTg	4		

by fimbriae 6 (P987) with 16 % and thermolabile toxin (LT) with 8 %. As shown in Table 2, the frequency of isolates carrying genes coding for fimbriae 4 (F4) were 8 %, fimbriae 18 (F18) and the association between both thermostable toxin b and fimbriae 18 (STb, F18) were 4 %, respectively**.** Circulating genes compatible with PEPEC (*Eae*) 8 %, and VTEC (VTg) 24 %, were also found.

Relationships were observed between the presence of general verotoxin and fimbriae 6 (VTg, P987) with a frequency of 4 %. No association was found between the heat-stable toxins (both a and b) and the heat-labile toxin.

No seasonal differences were observed among the frequency of virulence genes studied.

There is no information about the prevalence or frequency of *E. coli* virulence genes causing intestinal disease in swine in Argentina. This study provides updated information about the presence and circulation of *E. coli* among newborn piglets in an intensive system.

No animals with clinical symptoms of diarrhea were detected. Genes coding for heat-stable a and b toxins, and fimbriae F4, F6 and F18 are consistent with the presence of ETEC, indicating a great circulation of this pathogen among animals (frequency = 68 %). These genes have high pathogenic potential and may trigger piglet diarrhea, under some conditions (2, 5, 9). Therefore, the absence of the disease may be related to the application of adequate environmental, hygienic and sanitary measures.

The gene coding for general verotoxin (VTg) is present in 24 % of the samples, showing clear evidence about its circulation. Since bacteria carry the VTg gene, the etiologic agent of Hemolitic Uremic Syndrome (HUS), they might represent a potential risk to public health, mainly to susceptible hosts. There is an increased risk of transmission to hosts through carriers, persons, who are infected because they work in close contact with animals. Several frequencies of isolates compatible with ETEC / VTEC and PEPEC strains have been found and described (24 % and 8 %, respectively). Among others, in Brazil, diarrheic piglets under 11 days presented 65.7 % of ETEC, and non-diarrheic ones presented 42.8 %. However, 25.7 % and 9.5 % of PEPEC strains were observed in animals with and without diarrhea, respectively analyzed by PCR (10). In Spain, strains of ETEC obtained through serotyping were found with the following percentages: 31.9 % of F6, 11.6 % of F5, 10.1 % of F41 and 8.7 % of F4 (6). In Slovakia, at least one virulence gene was found by PCR in 80 % of the isolates from diarrheic and non-diarrheic piglets, as well as 65 % of ETEC and 17 % of PEPEC (14). In Germany Wieler *et al.* (15) found by PCR and colony hybridization that 17.6 % (49/278) of the animals were infected with enterotoxigenic *E. coli* [ETEC: 10.1 % (28/278) ETEC-ST-Ia, and 8.6 % (24/278) ETEC-LT-I]. In China, Cheng *et al.* (2) found by PCR 67.92 % of ETEC strains in nursing piglets from different farms. As for Argentina, Cicuta *et al.* (3) found 24.5 % of ETEC STa positive and 2.9 % of LT; and 2.1 % of VTEC from 164 samples of 23 intensive farms in several regions of the country analyzed by PCR. The results of our study show a greater circulation of ETEC compatible genes than that observed in the studies above. Our results, however, are consistent with those obtained by Cicuta *et al.* (3), which were obtained from 123 samples from a pig farm in the central region of Argentina.

In conclusion, this study observed a frequency of 68 % in ETEC compatible genes, which may appear to be high, considering that the selected pig farm complies with all needed requirements and adequate sanitary conditions for intensive farming. The presence of 24 % of genes encoding for general verotoxin is considered to pose serious risk on Public Health. These kinds of studies have not been performed for a long time in our country. These results offer updated information about virulence genes of *Escherichia coli* responsible for intestinal disease among piglets in the central region of Argentina.

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