A Retrospective Study of the Prevalence of Virulence Factors of *Escherichia Coli* Causing Postweaning Diarrhoea in Spain.

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Research Article

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Abstract

Background: Postweaning diarrhoea is one of the most prevalent disease which occurs after weaning. The aim of the study reports the prevalence of virulence factors of *Escherichia coli* from clinical cases of postweaning diarrhoea on Spanish pig farms based on PCR directly from pooling of faeces samples. A total of 328 pig farms with acute cases of postweaning diarrhoea were sampled, between January 2018 and December 2020. These farms were all located in Spain. Animals were selected based on clinical signs (diarrhoea, dehydration, and increased mortality). A total of 984 four- to eight-week-old pigs within the first 24 hours of the acute phase of the disease were sampled and three rectal swabs were collected from three different pigs on each farm.

Results: A total of 328 farms with PWD cases were analyzed between 2018 and 2020. The classification was based on the presence or absence of the different *Escherichia coli*'s virulence factors. Only 1.2% (4 out of 328) of farms were negative for all the *Escherichia coli*'s virulence genes. Adhesin involved in diffuse adherence was the most prevalent adhesion factor (68.6%) followed by F18 (67.7%) and F4 (53.4%).

Conclusions: the present study shows the high prevalence *Escherichia coli* in postweaning diarrhoea cases. There is a high degree of variability in the *Escherichia coli*'s virulence factors within country as well as differences between countries. Further Investigation is needed to fight against postweaning diarrhoea since the use of zinc oxide will be withdrawn from veterinary medicinal products on 26 June 2022 [26,27] and the responsible use of antibiotics is being regulated very closely to be used only when is necessary.

Background

Post-weaning diarrhoea (PWD) caused by *Escherichia coli* (*E. coli*) remains a major cause of economic losses for the pig industry due to mortality, morbidity, decreased growth rate and cost of medication [7].

Nowadays, it is one of the most important diseases in the nursery period that can cause diarrhoea affecting up to 20% piglets and/or mortality up to 3% [13]. PWD usually occurs a few days after weaning causing dehydration, loss of body conditions and mortality [6]. However, in case of Spain, there has been a shift in the dynamic of infection, and clinical signs are mainly observed between the second and the third weeks after weaning.

There are different *E. coli* pathotypes, and enterotoxigenic *E. coli* (ETEC) is considered one of the most prevalent pathotypes [9, 3, 23]. It is characterized by the presence of several virulence factors (adhesion factors and enterotoxins). Adhesion factors are responsible for the attachment to porcine enterocytes and enterotoxins cause hypersecretion of electrolytes and water into the small intestine resulting in mild to severe diarrhoea.

Generally, the virulence factor carried by *E. coli* can differ over time [20, 10]. There are several fimbriae and, the most commonly found in PWD caused by ETEC are F4 (K88) and F18 (F107, 2134P, 8813) [8, 22]. Other adhesion factors can also be present such as the adhesion involved in diffuse adherence (AIDA-1). Regarding the enterotoxins, the heat-labile toxin (LT), heat-stable toxin a (STa) and heat-stable toxin b (STb) are very common in cases of PWD [12]. The enteroaggregative heat-stable enterotoxin 1 (EAST-1) can be found in cases of *E. coli* isolates both in human and animals with diarrhoea [10, 20, 24].

In relation to diagnosis, there are different approaches for PWD cases. A useful diagnostic tool is based on culturing the samples and performing a multiple polymerase chain reaction (PCR) from a single colony for the detection of genes encoding for toxins and adhesion factors. Another diagnostic tool carries out a multiple PCR directly in samples only.

The study started as a necessity to carry out a rigorous diagnosis in order to characterize the *E. coli*'s virulence factors and find the most appropriate solution in each PWD case.

The aim of this study is to report the prevalence of the *E. coli*'s virulence factors from clinical cases of PWD on pig farms in Spain based on PCR directly from pooling of faeces samples.

Methods

Sampling of PWD

A total of 328 pig farms with acute cases of PWD were sampled between January 2018 and December 2020. These farms were in Spain.

The main treatment of PWD was based on effective antibiotics against *E. coli* and the use of zinc oxide in the second feeding program, which was administered from two weeks after weaning to the end of the nursery period.

Animals were selected based on PWD clinical signs (diarrhoea, dehydration, and increased mortality). A total of 984 four- to eight-week-old pigs within the first 24 hours of the acute phase of the disease were sampled and three rectal swabs were collected from three different pigs on each farm. Swabs with fecal matter were inserted into Amies transport medium in airtight screw cap plastic vials and were submitted to the laboratory (Exopol; Zaragoza, Spain) for *E. coli* diagnosis.

DNA extraction

A multiplex PCR was carried out directly from 3 pooling of fecal samples contained in rectal swabs. PCR was performed to detect adhesion factor and toxin genes, including F4 (K88) and F18 fimbriae, adhesin involved in diffuse adherence (AIDA-1), heat-stable and heat-labile enterotoxins (STa, STb, LT), shigatoxin (STx2e), enteroaggregative heat-stable toxin (EAST-1) and, *E. coli* attaching and effacing (eae).

JMP 15.1.0. was used to represent the descriptive results.

Results

The classification was based on the presence or absence of the different *E. coli*'s virulence factors.

Only 1.2% (4 out of 328) of farms were negative for all the *E. coli*'s virulence factor genes.

The total prevalence of genes for adhesion factors and toxins of *E. coli* among 328 farms with PWD cases are shown in Table 1.

AIDA-1 gene was the most commonly found (68.6%) followed by F18 (67.7%) and F4 (53.4%). The most prevalent toxin was EAST-1 (98.1%) followed by STb (87.6%) and STa (79.3%). Enteropathogenic *E. coli* (EPEC) was present in 78.0% of isolates.

Table 1. Total prevalence of genes codifying for adhesion factors and toxins of *E.coli*

Virulence factor		Percentage (%)
Adhesion factor	F4	53.4
	F18	67.7
	AIDA-1	68.6
Toxins	Sta	79.3
	STb	87.6
	LT	68.4
	Stx2e	19.8
	EAST-1	98.1
	eae	78.0

In 3.9% of the 324 *E. coli*'s positive results, only a single virulence gene was detected.

Table 2. Number and percentage of results that showed only a single virulence gene

	Number	Percentage
F4	1	0.3
eae	4	1.2
STb	1	0.3
EAST	7	2.1

From the 328 diagnosed farms, adhesion factor genes were identified in 87.8% (288 out of 328). The most prevalent combination of adhesion factors above overall farms was F4, F18 and AIDA with a percentage of 35.1% (115 out of 328), followed by 17.4% (57 out of 328) of F18 and AIDA. The most associated enterotoxins depending on the existence of F4, F18 and/or AIDA adhesion factors are shown in table 3. Adhesive fimbria F4 was detected as a single *E. coli* virotype in 5.2% (17 out of 328) of isolates and it was more commonly associated with STb (82.4%) enterotoxin. Adhesive fimbria F18 was detected as a single *E. coli* virotype in 7.9% (26 out of 328) of isolates and was mainly in combination with genes encoding for STb (92.3%). AIDA was detected as a single adhesion factor in 6.4% (21 out of 328) of isolates and it was more commonly associated with EAST-1 (100%). The presence of genes encoding for F4, F18 and AIDA adhesion factors were mainly in combination with genes encoding for STb (100%), EAST-1 (99.1%) and STa (99.1%) enterotoxins. When F18 and AIDA adhesion factors were present, the most prevalent genes were encoding for EAST-1 (100%), STb (98.2%) and STa (93.9%). The presence of genes encoding F4 and AIDA adhesion factors were mainly in combination with genes encoding for EAST-1 (100%) and STb (91.7%). Genes encoding for F4 and F18 showed a combination with genes encoding for Stb (100%) STa (96.4%), and EAST-1 (96.4%).

Table 3. Distribution of toxins based on the adhesion factor genes.

ADHESION FACTOR	Percentage of toxin	
F4 (17/328)	Sta	58.8
	STb	82.4
	LT	70.6
	Stx2e	0
	EAST-1	82.4
	eae	76.5
F18 (26/328)	Sta	88.5
	STb	92.3
	LT	69.2
	Stx2e	23.1
	EAST-1	84.6
	eae	53.8
F4/F18 (28/328)	Sta	96.4
	STb	100
	LT	92.9
	Stx2e	14.3
	EAST-1	96.4
	eae	67.9
F4/AIDA (24/328)	Sta	70.8
	STb	91.7
	LT	70.8
	Stx2e	4.2
	EAST-1	100
	eae	75.0
F18/AIDA (57/328)	Sta	93.9
	STb	98.2
	LT	68.4
	Stx2e	33.3

	EAST-1	100
	eae	71.9
F4/F18/AIDA (115/328)	Sta	99.1
	STb	100
	LT	91.3
	Stx2e	26.1
	EAST-1	99.1
	eae	87.0
AIDA (21/328)	Sta	57.1
	STb	81.0
	LT	28.6
	Stx2e	4.8
	EAST-1	100
	eae	52.4

Respecting to *E. coli* virotypes, the most prevalent were F4, F18 and AIDA in combination with the STa, STb, LT and EAST (20.7%), followed by F4, F18 and AIDA in combination with STa, STb, LT, EAST and STx2e (6.4%); and F18 and AIDA adhesion factors in combination with STa, STb, LT, EAST toxins and intimin (5.2%).

Discussion

Distribution of adhesion factor genes:

There has been a shift in the dynamics of infection, and clinical signs are mainly observed between the second and the third week after weaning. It is assumed that the responsible use of antibiotics and changes in the management of zinc oxide play a key role. For example, colistin has been reduced by 97.18% from 2015 to 2018 [18].

The present study searched for F4, F18 and AIDA-1 adhesion factor genes. Results showed that the most prevalent adhesin gene was AIDA-1 (68.6%) followed by F18 (67.7%) and F4 (53.4%).

With respect to AIDA, a recent study carried on China showed AIDA-1 as the most prevalent adhesion factor gene (19.9%) [9]. Another study from Austria remarks the possible importance of this adhesion factor since it was detected in a high percentage of affected animals (99). This adhesin is related with ETEC strains isolated from weaned pigs with PWD, but its role is not totally clear [11]. In relation to F4 and

F18 fimbriae, another Spanish study carried out in 2015 found that F18 gene was the most prevalent in comparison to F4, F5, F6 and F41 [19]. Other countries such as Slovakia, Poland, Cuba, United Kingdom and The Republic of Ireland, concluded that F18 fimbria gene was the most prevalent [2, 16, 21, 28]. With respect to F4, it was the most commonly found fimbria gene in Belgium, The Netherlands, France and Italy [12]. Also in The United States, F4 fimbria gene was the most prevalent [24].

To find different combination of virulence factors is acceptable insofar as the diagnostic tool carries out a PCR directly in faeces samples. Then, it is common to find all the possible virulence factor's genes present in the respective samples.

Among the 324 *E. coli* positive farms, carrying virulence genes, four different combinations of adhesion factor genes were identified. The most common combination was F4, F18 and AIDA adhesion factors with a percentage of 35.5% (115 out of 324), followed by 17.6% (57 out of 324) of F18 and AIDA.

Other studies differ from the present study and find a lower prevalence of combined adhesion factor genes [12] because their diagnostic tools are based on culturing the fecal sample as first step. However, there are other studies based on previous culture that resulted in a total of 7 possible associations [9].

Distribution of enterotoxins genes:

The most prevalent enterotoxin gene has been EAST-1 (98.1%), followed by STb (87.6%) and STa (79.3%). Other studies are in accordance with the present study and showed EAST-1 as the most prevalent enterotoxin [9]. EAST-1 must be further investigated since in inoculating piglets with a strain of the EAST-1 pathotypes, isolated from diarrheic piglet, did not affect 72 hours after infection [15]. It was also showed that the detection of EAST-1 gene occurred both in diarrheic and non-diarrheic piglets suggesting that EAST-1 by itself, is probably insufficient to cause diarrhea in piglets. However, other studies suggested that EAST-1 maybe produces diarrhea in piglets [4].

STb enterotoxin gene was found as the main enterotoxin in cases of diarrhoea caused by ETEC [12]. In contrast, there are other studies, which showed a low percentage of Stb in comparison with other enterotoxin genes [9].

Distribution of the combination between adhesion factors and enterotoxin genes:

From the total, 10.4% (34/328) isolates did not show any adhesion factor gene and only presented one or more enterotoxin genes. It has been described that *E. coli* strains exist and are able to produce enterotoxins without the necessity of any adhesion factor to adhere the intestine [5, 8]. The present results are in accordance with other studies, which did not contain any adhesion factor gene [9, 10].

Adhesive fimbria F4 was more commonly associated with STb (82.4%) and EAST-1 (82.4%) enterotoxinS. Adhesive fimbria F18 was mainly in combination with genes encoding for STb (92.3%) and STa (88.5%). AIDA was more commonly associated with EAST-1 (100%) followed by STb (81.0%). The presence of

genes encoding for F4, F18 and AIDA adhesion factors were mainly in combination with genes encoding for Stb (100%), Sta (99.1%) and Stb (99.1%) enterotoxins.

Other studies showed AIDA-1, and EAST-1 as the most common association, and there was a significant relationship ($p > 0.05$) [9].

It has been shown that *E. coli* strains which produce multiple enterotoxins are able to cause a more severe diarrhea [1, 14].

Conclusions

The present study shows the high prevalence of *Escherichia coli*, which can cause PWD. There is a high degree of variability in the *E. coli* virulence factors within country as well as differences between countries.

Further investigation is needed to fight against postweaning diarrhoea caused by Enterotoxigenic *E. coli* since the use of zinc oxide will be withdrawn from veterinary medicinal products on 26 June 2022. (77,88) and the responsible use of antibiotics is being regulated very closely to be used only when is necessary. Some virotypes are more severe and show a higher resistance to certain antibiotics [29]. Then, an appropriate diagnosis should be carried out to detect the main pathogen implicated in every case of postweaning diarrhoea.

List Of Abbreviations

Postweaning diarrhoea (PWD), *Escherichia coli* (*E. coli*), Adhesin involved in diffuse adherence (AIDA), Polymerase chain reaction (PCR), Heat-labile toxin (LT), Heat-stable toxin a (STa), Heat-stable toxin b (STb), Intimin (eae), Enteraggregative heat-stable enterotoxin 1 (EAST-1), Shigatoxin (Stx2e), Fimbria F4 (F4), Fimbria F18 (F18)

Declarations

Ethics approval and consent to participate: not applicable

Consent for publication: not applicable

Availability of data and materials: The datasets used and analysed during the current study are available from the corresponding author on reasonable request

Competing interests: not applicable

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Author's contributions: LPE, MCM and PSU designed the protocol. LPE analysed the results. MCM critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Associated *E. coli*'s virulence factors results.