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

Lorena Pérez Esteruelas (lorena.perez@elancoah.com)
Elanco International Inc: Elanco Animal Health Inc
https://orcid.org/0000-0002-6353-7724

Miguel Claver Mateos
Elanco International Inc: Elanco Animal Health Inc

Pedro José Sánchez Uribe
Elanco International Inc: Elanco Animal Health Inc

Research Article

Keywords: Escherichia coli, virulence factor, post-weaning diarrhoea, enterotoxin, adhesion factor

DOI: https://doi.org/10.21203/rs.3.rs-552758/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 Further Investigation is needed to fight against postweaning diarrhoea since the use of zinc oxide will be withdrawn from veterinary medicinal products on 26 June 2022 [26,27] and the responsible use of antibiotics is being regulated very closely to be used only when is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 of 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 enterotoxin (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          |
| eae    | 4          |
| STb    | 1          |
| EAST   | 7          |

| Number | Percentage |
|--------|------------|
| F4     | 0.3        |
| eae    | 1.2        |
| STb    | 0.3        |
| EAST   | 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 | eae  |
|----------------|--------|------|
| **F4/F18/AIDA (115/328)** | Sta 99.1 | STb 100 |
|                | LT 91.3 | Stx2e 26.1 |
|                | EAST-1 99.1 | eae 87.0 |

|                | Sta 57.1 |
|----------------|--------|
| **AIDA (21/328)** | 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 withdraw 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), Enteroaggregative 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

**Funding:** not applicable
**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.

**Acknowledgement:** We thank participation of farmers and veterinarians who took the samples on the farm level. We also thank the Exopol laboratory where samples were analyzed.

**References**

1. Berberov EM, Zhou Y, Francis DH, Scott MA, Kachman SD, Moxley RA. Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic *Escherichia coli* that produces multiple enterotoxins. Infect Immun. 2004; 72:3914–3924.

2. Blanco M, Lazo L, Blanco JE, Dahbi G, Mora A, López C, González EA, Blanco J. Serotypes, virulence genes, and PFGE patterns of enteropathogenic *Escherichia coli* isolated from Cuban pigs with diarrhea. Int Microbiol. 2006; 1:53–60.

3. Chen, X., Gao, S., Jiao, X., & Liu, X. F. Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. Veterinary Microbiology. 2004; 103, 13–20.

4. Choi C, Kwon D, Chae C. Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *coli* isolated from diarrheic piglets. J Vet Diagn Invest. 2001. 13:26–29.

5. Costa M.M., Drescher G., Maboni F., Weber S.S., Schrank A., Vainstein M.H. S. Schrank1, A.C. Vargas. Virulence factors, antimicrobial resistance, and plasmid content of *Escherichia coli* isolated in swine commercial farms. Arq. Bras. Med. Vet. Zootec., 2010; v.62, n.1, p.30-36.

6. Fairbrother JM, Gyles CL. Colibacillosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Disease of Swine. 10th ed. UK: Wiley-Blackwell. 2012; p. 723–

7. Fairbrother, J.M., Nadeau, É., and Gyles, C.L. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Animal Health Research Reviews 6. 2005; p. 17-39

8. Kim, Y. J., Kim, J. H., Hur, J., & Lee, J. H. Isolation of *Escherichia coli* from piglets in South Korea with diarrhea and characteristics of the virulence genes. Canadian Journal of Veterinary Research. 2010; 74, 59–64.

9. Li S., Wang L., Zhou Y., Miao Z. Prevalence and characterization of virulence genes in *Escherichia coli* isolated from piglets suffering post-weaning diarrhoea in Shandong Province, China. 2020.

10. Liu, W., Yuan, C., Meng, X., Du, Y., Gao, R., Tang, J., & Shi, D. Frequency of virulence factors in *Escherichia coli* isolated from suckling pigs with diarrhea in China. Veterinary Journal. 2014; 199, 286–289.

11. Luppi, A. Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. Porcine Health Management. 2017; 3, 16
12. Luppi A., Gibellini M., Gin T., Vangroenweghe F., Vandenborucke V., Bauerfeind R., Bonilauri P., Labarque G., Hidalgo A. Prevalence of virulence factors in enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea in Europe. Porcine Health Management. 2016.

13. Lyutskanov M. Epidemiological characteristics of post-weaning diarrhoea associated with toxin-producing *Escherichia coli* in large intensive pig farms. Trakia Journal of Sciences. 2011; Vol. 9, No 3, 68-73.

14. Moxley RA, Francis DH. Significance of heatstable and heat-labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. Infect Immun. 2006; 74:3107–3114.

15. Ngeleka M, Pritchard J, Appleyard G, Middleton DM, Fairbrother JM. Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. 2003.

16. Osek J, Gallien P, Truszczyński M, Protz D. The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. Comp Immunol Microbiol Infect Dis. 1999; 22:163–74.

17. Pittman J.S. Enteritis in grower-finisher pigs caused by F18-positive *Escherichia coli*. Journal of Swine Health and Production. 2010; Volume 18, Number 2.

18. Plan Nacional frente a la Resistencia a los Antibióticos (PRAN) 2019-2021. http://www.resistenciaantibioticos.es/es/publicaciones/plan-nacional-frente-la-resistencia-los-antibioticos-pran-2019-2021. Accesed 18 July 2019.

19. Sánchez PJ, Hidalgo A, Núñez P, Pérez L. Prevalence of virulence factors in *Escherichia coli* isolated from pigs with post-weaning diarrhoea in Spain. Bacteriology and bacterial diseases. 2016; IPVS 2016.

20. Toledo A, Gomez D, Cruz C, Carreon R, Lopez, J, Giono S, & Castro AM. Prevalence of virulence genes in *Escherichia coli* strains isolated from piglets in the suckling and weaning period in Mexico. Journal of Medical Microbiology. 2012; 61, 148–156.

21. Vu-Khac H, Holoda E, Pilipcinec E, Blanco M, Blanco JE, Mora A, Dahbi G, López C, González EA, Blanco J. Serotypes, virulence genes, and PFGE profiles of *Escherichia coli* isolated from pigs with postweaning diarrhea in Slovakia. BMC Vet Res. 2006;2:10.

22. Vu-Khac H, Holoda E, Pilipcinec E, Blanco M, Blanco JE, Dahbi G. ... Blanco, J. Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhea in Slovakia. Veterinary Journal. 2007; 174, 176–187.

23. Wang XM, Jiang HX, Liao XP, Zhang, WJ, Zhu HQ, Zhang Y, & Liu YH. Prevalence of serotypes and virulence genes and antimicrobial susceptibility of pathogenic *Escherichia coli* isolates from swine. Scientia Agricultural Sinica. 2010; 43, 4109–4115.

24. Zhang W, Zhao M, Ruesch L, Omot A, Francis D. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. Vet Microbiol. 2007;123:145–52.
25. Renzhammer R, Loncaric I, Spergser J, Roch F, Pinior B, Schwarz L, Ladinig A, Unterweger C. Virulence genes from *Escherichia coli* isolates in Austrian swine stocks. A retrospective study (2016-2018). ESPHM 2021.

26. European Medicines Agency: Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally to food-producing species. Outcome of a referral procedure under Article 35 of Directive 2001/82/EC (EMEA/V/A/118) - 26 June 2017 EMA/394961/2017 Veterinary Medicines Division. https://www.ema.europa.eu/documents/referral/zinc-oxide-article-35-referral-questions-answers-veterinary-medicinal-products-containing-zinc-oxide_en.pdf. Accessed 26 June 2017. European Medicines Agency: Zinc oxide

27. Article-35 referral Annex I, II. https://www.ema.europa.eu/documents/referral/zinc-oxide-article-35-referral-annex-iii_en.pdf. Accessed 26 June 2017.

28. Vangroenweghe F, Fellows J, Bonnilauri P. Prevalence of virulence factors of *Escherichia coli* from piglets with postweaning diarrhoea in United Kingdom and The Republic of Ireland. ESPHM 2021.

29. Congreso de Producción Porcina en Resistencia. Available at: www.engormix.com/pig-industry/articles/recent-trends-virulence-antimicrobial-t39746.htm. Accessed 14 October 2016.
Figure 1

Associated E. coli’s virulence factors results.