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Prevalence of Enterotoxigenic *Escherichia Coli* Pathotypes and Virotypes Isolated from Piglets Suffering from Post-Weaning Diarrhea in Belgium and the Netherlands

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Abstract

Post-weaning diarrhea (PWD) in pigs is a worldwide economically important disease characterized by reduced pig performance. Although relevant, recent data on prevalence of virulence genes and pathotypes in Belgium and The Netherlands are relatively scarce. The present study investigated the prevalence of fimbrial and toxin genes of E. coli using PCR in 539 PWD-affected farms in Belgium and The Netherlands. A total of 1404 samples were collected at early onset of PWD and submitted for diagnostic examination. Following standard bacteriological isolation of E. coli, PCR analysis was performed to detect genes encoding for fimbriae (F4, F5, F6, F18, F41) and toxins (LT, STa, STb, Stx2e). The prevalence of fimbriae and toxins among E. coli isolated from PWD-affected piglets was: F4 (43.1 %), F18 (39.0 %), F41 (3.8 %), F5 (1.4 %), F6 (1.2 %), STb (54.1 %), STa (49.7 %), LT (28.3 %) and Stx2e (5.0 %). Isolates carrying both fimbrial and toxin genes were detected in 50.5 % of the cases (709 out of 1404), with 94.9% classified as enterotoxigenic E. coli (ETEC). This study confirms that ETEC is frequently isolated in PWD-affected farms with F4- and F18-ETEC pathotypes involved in 34.7 and 27.8 % of the cases, respectively.

Keywords: *Escherichia coli; Prevalence; ETEC; Post; Weaning diarrhea*

Abbreviations: ETEC: Enterotoxigenic Escherichia coli; STEC: Shiga toxin-producing Escherichia coli; LT: Heat-labile toxin; STa: Heat-stable toxin a; STb: Heat-stabile toxin b; TSA: Tryptose soy agar; PWD: Post-weaning diarrhea; AIDA: Adhesion involved in diffuse adherence; EAST1: enteroaggregative E. coli heat stable enterotoxin

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Introduction

Post-weaning diarrhea (PWD) in pigs is a worldwide economically important disease (Fairbrother et al., 2005), characterized by increased mortality, weight loss, retarded growth, increased treatment costs, higher use of antibiotics and batch-to-batch variation (Hoa et al., 2013; Lyutskanov, 2011; Svensmark et al. 1989a; Svensmark et al., 1989b; Tubbs et al., 1993; USDA, 2002; Zhang et al., 2007). Enterotoxigenic E. coli (ETEC) is regarded the most important cause of PWD. The ETEC pathotype is typically characterized by the presence of fimbrial adhesins, which mediate attachment to porcine intestinal enterocytes, and enterotoxins, which disrupt fluid homeostasis in the small intestine. This results in mild to severe diarrhea within a few days post-weaning, associated with clinical signs of dehydration, loss of body condition (= disappearance of muscle volume) and mortality (Fairbrother et al., 2012). The adhesive fimbriae most commonly occurring in ETEC from pigs with PWD are F4 (K88) and F18 (Fairbrother et al., 2012). Other fimbriae such as F5 (K99), F6 (987P) and F41 rarely occur in E. coli isolates from PWD (Fairbrother et al., 2012; Chen et al., 2004; Frydendahl, 2002; Luppi et al., 2016; Vu-Khac et al., 2006). The main enterotoxins associated with porcine ETEC are heat-labile toxin (LT), heat-stable toxin a (STa) and heat-stable toxin b (STb). In some cases, both enterotoxins and a Shiga toxin (Stx2e) are produced by the pathogenic stains (Fairbrother et al., 2012). Classification of these strains is rather challenging, although some authors classify them as ETEC rather than Shiga toxin-producing E. coli (STEC) (Fairbrother et al., 2012).

The disease is currently controlled using antimicrobials, although the emergence of antimicrobial resistance in E. coli strains isolated from cases of PWD urges the need for alternative control measures (Abraham et al., 2014; Abraham et al., 2015; Boyen et al., 2010; Jahanbakhsh et al., 2016; Luppi et al., 2013). Besides antimicrobials, zinc oxide (ZnO) has been demonstrated to prevent and heal PWD (Poulson, 1995). Therefore, ZnO has been admitted in the prevention and control of PWD at levels up to 3,000 parts per million (ppm) through the feed for a maximum of 14 days post-weaning in most European countries. However, Committee for Veterinary Medicinal Products (CVMP) has recently decided that the use of ZnO in post-weaning diets should be phased out the latest by 2022 throughout the EU (EMA, 2017). Thus, other preventive strategies have to be explored, such as an E. coli vaccination against PWD, which is specifically targeted against F4- and F18-ETEC (Fairbrother et al., 2005; Fairbrother et al., 2017; Nadeau et al., 2017).

In the light of these recent evolutions, it is prerequisite to investigate the occurrence and distribution of fimbriae and virulence factors among E. coli isolates from cases of PWD in order to determine the predominant pathotypes and virotypes present in post-weaning facilities suffering from PWD. A simple and easy method to determine the presence of ETEC fimbrial adhesins is slide agglutination, which is particularly useful for identification of F4 (Luppi et al., 2016). For other fimbrial adhesins, such as F5, F6 and F41, its reliability is lower due to their variable expression in vitro (Fairbrother et al., 2012; Mullaney et al., 1991). Nowadays, genotypic analysis is routinely used to investigate the E. coli virotype associated with intestinal infection. In most veterinary diagnostic laboratories, multiplex PCR analysis is applied to detect both genes encoding for fimbrial adhesins (F4, F5, F6, F18 and F41) and toxins (LT, STa, STb and Stx2e) (Luppi et al., 2016). In Poland, Denmark and Slovakia, differences in prevalence of the main fimbrial types in E. coli isolated from PWD cases have been reported (Osek, 1999; Frydendahl, 2002; Vu-Khac et al., 2006). Recently, prevalence of ETEC virulence factors was reported from several countries in Western Europe (Luppi et al., 2016), indicating that both F4 and F18 were the most prominent fimbriae in PWD. However, in this study, isolation and characterization of all samples was not performed in one laboratory and only a limited portion (< 20%) of the samples originated from Belgium and The Netherlands.

This study reports on the prevalence of enterotoxigenic E. coli pathotypes and virotypes isolated from piglets suffering from post-weaning diarrhea in Belgium and The Netherlands.

Materials and Methods

Sampling of pig farms with cases of PWD

A total of 539 pig farms with acute cases of PWD were sampled as part of the diagnostic support to the E. coli vaccine (Coliprotec® F4/F18; Elanco, Greenfield (IA), USA) between January 2015 and December 2016. Farms were located in Flanders, Belgium (n=262) and The Netherlands (n=277). Sampled herds had a history of PWD occurring from 3 days until 3 weeks post-weaning, as confirmed by the typical clinical signs: watery diarrhea, dehydration, decreased feed intake, general depression, increased mortality and increased use of antimicrobial therapy.

Diagnostic samples were obtained from 1403 three- to five-week old piglets with typical signs of diarrhea through rectal sampling within 48h after the occurrence of the first clinical symptoms of

PWD and before administration of specific antimicrobial treatment. All rectal samples were collected using a sterile transport swab containg Amies transport medium with charcoal (Copan Italia S.p.a., Bescia, Italy). The use of ZnO in these farms was not recorded. A detailed description of number of samples, number of samples positive for E. coli and type of samples by country and in total is presented in Table 1.

Country	Farms	Samples		
		n	Positive samples (% / number)	Type
Belgium	262	528	49.6 (262)	Rectal swab
The Netherlands	277	876	31.6 (277)	Rectal swab
Total	539	1404	38.4 (539)	

Table 1: *Number of farms, samples, sample results positive for E. coli (percentage / number) and sample type investigated in this study by country of origin.*

Isolation and characterization of *Escherichia coli* isolates

Samples were sent to one central veterinary diagnostic laboratory (Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Emilia Romagna, Italy) for further processing using standard procedures for isolation and characterization of intestinal E. coli (Luppi et al., 2013). Briefly, samples were plated on selective media and on tryptose soy agar (TSA) medium supplemented with 5% of defibrinated ovine blood and incubated aerobically overnight at 37°C. The semi-quantitative evaluation of pathogenic E. coli grown in culture, was considered as diagnostic criteria. Haemolytic activity was evaluated and single coliform colonies were further characterized by biochemical methods.

DNA extraction and characterization of virulence factors by PCR

DNA samples were prepared from one up to three haemolytic and/or non haemolytic E. coli colonies and used to perform a multiplex PCR for the detection of fimbrial and toxin genes, including those encoding for F4 (K88), F5 (K99), F6 (987P), F18, F41, LT, STa, STb and Stx2e. The test could not discriminate between F4ab, F4ac and F4ad. The methodology for identification of these virulence genes has previously been described in detail (Fairbrother et al., 2005).

When E. coli isolates with the same combination of virulence factors (virotype) were detected in the same herd, they were considered duplicates of an E. coli prototype strain, which was considered for the prevalence calculations. The E. coli strains carrying a combination of both fimbrial and toxin genes were classified into pathotypes. Isolates encoding at least one of the investigated enterotoxins together with Stx2e and F18 fimbriae were classified as ETEC as previously proposed (Fairbrother et al., 2012). Isolates carry genes for adhesive fimbriae (F18) and Stx2e were classified as STEC.

Results

A total of 1404 DNA samples belonging to the same number of E. coli isolates and representing all of the 539 PWD-affected pig farms included in this study were analysed by PCR. These isolates were classified as being positive or negative for the presence of one or more virulence genes (F4, F5, F5, F18, F41, LT, STa, STb and Stx2e). Following these criteria and excluding isolates which were considered duplicates within herds, the prevalence of virulence genes was calculated. Sixty-two per cent (870 out of 1404) of E. coli isolates carried at least one virulence factor, while 38.0 % (534 out of 1404) resulted negative for all of the virulence factors currently investigated. Fimbrial genes were identified in 58.4 % (820 out of 1404) and toxin genes in 50.3 % (706 out of 1404) of the isolates. In 11,5% (161 out of 1404) of the E. coli isolates, a single virulence gene was detected.

The prevalence of fimbrial and toxin genes in non-duplicate E. coli isolates from PWD-affected pig farms in Belgium and The Netherlands is shown in Table 2. Overall, the adhesive fimbriae most commonly detected was F4 (43.1%), followed by F18 (39.0%). Nine isolates possessed genes for two types of fimbriae. The combination of F4-F18 was detected in 4 isolates, whereas other combinations were F18-F41 (n=2), F6-F18 (n=2) and F4-F6 (n=1), with or without toxin genes. The most prevalent toxin was STb (54.1%), followed by STa (49.7%) and LT (28.3%).

Based on the presence of genes for both fimbriae and toxins, 71.7% (389 out of 542) of the E. coli isolates were classified into pathotypes (Table 3). ETEC was the most prevalent pathotype with 94.9 % of the isolates (369 out of 389). The ETEC virotypes most commonly detected were (i) F18 STa STb (18.5%); (ii) F4 STa STb LT (15.2 %); (iii) F4 STa STb (14.9%); (iv) F4 STb LT (8.7%) and (v) F4 LT (8.0 %). Both F4 and F18 were mostly detected in combination with toxin genes encoding for STa and STb, and only in third instance with LT. Among the ETEC, 8 isolates encoded for Stx2e in addition to enterotoxins and F18 fimbriae. Twenty isolates (5.1%) were classified as STEC, only encoding for the combination of Stx2e with F18.

Country and number of isolates tested	Percentage (number) of positive E. coli isolates								
	Fimbriae					Toxins			
	F4	F5	F6	F18	F41	LT	STa	STb	Stx2e
Belgium (n=264)	45.5 (120)	1.1 (3)	1.1 (3)	33.3 (88)	3.8 (10)	20.1 (53)	42.0 (111)	44.3 (117)	6.1 (16)
The Netherlands (n=316)	41.1 (130)	1.6 (5)	1.3 (4)	43.7 (138)	3.8 (12)	35.1 (111)	56.0 (177)	62.3 (197)	4.1 (13)
Total (n=580)	43.1 (250)	1.4 (8)	1.2 (7)	39.0 (226)	3.8 (22)	28.3 (164)	49.7 (288)	54.1 (314)	5.0 (29)

Table 2: *Prevalence of examined genes for fimbriae and toxins among 580 isolates of E. coli recovered from pigs with PWD in Belgium and The Netherlands.*

Virulence factor combination	Number of positive E. coli isolates			
	Belgium	The Netherlands	Total	
	n	n	n	%
F18STaSTb	29	43	72	18.5%
F4STaSTbLT	20	39	59	15.2%
F4STaSTb	34	24	58	14.9%
F4STbLT	10	24	34	8.7%
F4LT	10	21	31	8.0%
F18STa	7	16	23	5.9%
F18Stx2e	15	5	20	5.1%
F18STb	5	11	16	4.1%
F18STaSTbLT	2	9	11	2.8%
F4STa	6	3	9	2.3%
F18STbLT	1	7	8	2.1%
F18LT	2	4	6	1.5%
F4STb	3	3	6	1.5%
F41STb	3	2	5	1.3%
F4STaLT	3	2	5	1.3%
F41STa	1	3	4	1.0%
F5STa	1	3	4	1.0%
F18STaSTbStx2e	0	3	3	0.8%
F6STaLT	2	1	3	0.8%
F18STaLTStx2e	0	2	2	0.5%
F5STaSTb	1	1	2	0.5%
F5STb	1	1	2	0.5%
F18STaLT	0	1	1	0.3%
F18STaSTbLTStx2e	0	1	1	0.3%
F18STaStx2e	0	1	1	0.3%
F18STbStx2e	1	0	1	0.3%
F4F18STaLT	0	1	1	0.3%
F6F18STbLT	0	1	1	0.3%

Table 3: *Distribution of encoded virulence factor combinations among 389 non duplicate E. coli isolates from cases of PWD in Belgium and The Netherlands classified as ETEC and STEC in decreasing order of prevalence.*

E. coli characterized as ETEC pathotype were haemolytic in 75.6% of all cases (294 out of 389), while the remaining 24.4 % were non haemolytic. All STEC isolates (F18 Stx2e) were haemolytic.

ETEC was detected in 66.8% of the 539 PWD-affected farms investigated in Belgium and The Netherlands. The rate of ETEC-positive farms was slightly higher (76.5%) in The Netherlands as compared to Belgium (56.5%). In both countries, F4-ETEC was the main pathotype with 34.7% of the examined farms. F18-ETEC was detected in 27.8% of the PWD-affected farms. In only one farm, an ETEC harbouring both F4 and F18 was detected. A detailed description of ETEC prevalence at farm level in both Belgium and The Netherlands is given in Table 4.

ETEC subtype	Percentage[a] (number) of affected pig farms in a given country		
	Belgium	The Netherlands	Total[b]
F4-ETEC	30.9 (81)	38.3 (106)	34.7 (187)
F18-ETEC	22.1 (58)	33.9 (94)	28.2 (152)
F4-F18-ETEC	-	0.4 (1)	0.2 (1)
F5-ETEC	1.1 (3)	1.8 (5)	1.5 (8)
F6-ETEC	0.8 (2)	0.4 (1)	0.6 (3)
F41-ETEC	1.5 (4)	1.8 (5)	1.7 (9)
All subtypes	56.4 (148)	76.5 (212)	66.8 (360)

[a]Percentage of positive farms over total farms investigated in a given country: Belgium (n=262), The Netherlands (n=277)
[b]Percentage of positive farms over all farms investigated (n=539)

Table 4: *Prevalence of ETEC fimbrial subtypes among 539 pig farms with cases of PWD examined in Belgium and The Netherlands.*

Discussion

In the present study, the prevalence of fimbrial and toxin genes is described during the early phases of PWD under field conditions in Belgium and The Netherlands. From the different fimbrial adhesins detected, F4 was the type most commonly detected, immediately followed by F18 (43.1% and 39.0%, respectively). These results from Belgium and The Netherlands are accordance with previous studies in several European countries (Belgium, France, Italy, Germany and The Netherlands) (Luppi et al., 2016) Denmark (Frydendahl, 2002), Slovakia (Vu-Khac et al., 2004), Poland (Zajacova et al., 2012). Nevertheless, in Slavakia and Poland, F18-ETEC had a higher prevalence (Vu-Khac et al., 2006; Osek et al., 1999). In Australian pigs, F4 was the most common fimbrial adhesin associated with diarrhea during the post-weaning period (Smith et al., 2010), whereas in Cuban pigs F18-ETEC was apparently more prevalent in PWD-affected farms (Blanco et al., 2006). However, in a recent study (de la Fé Rodriguez et al., 2011), both F4 and F18 were found to be highly prevalent in Cuban piggeries. In the United States, 64.6% of E. coli isolates from diarrheal pigs were F4-positive (Zhang et al., 2007).

Although atypical for the post-weaning phase, some ETEC strains were detected with genes encoding for F5, F6 and F41, which are predominantly associated with diarrhea in younger suckling piglets. This observation is in accordance with previous studies (Kwon et al., 2002; Frydendahl, 2002; Chen et al., 2004; Vu-Khac et al., 2006; Luppi et al., 2016). In 54.1% of the cases, enterotoxin STb was prevalent. This enterotoxin is associated with severe fluid loss in the small intestine of the weaned piglet, consequently resulting in dehydration and depression of the PWD-affected piglets (Fairbrother et al., 2005; Fairbrother et al., 2012). Other studies (Frydendahl, 2002; Zhang et al., 2007; Luppi et al., 2016) also observed STb as the predominant enterotoxin in PWD-affected piglets.

In 71.7% of the isolates, the detected combination of fimbriae and toxins could be classified as a specific pathotype (Fairbrother et al., 2005). In a previous study in Western Europe, only 52.5% of the isolates could be recognized as a specific pathotype (Luppi et al., 2016). Both ETEC and STEC were identified among the E. coli isolates from PWD-affected farms in Belgium and The Netherlands, with the vast majority being classified as ETEC (94.9%), which is an identical percentage as compared to the previous study in Western Europe (Luppi et al., 2016). The low percentage of STEC seems logical as specific sampling instructions described that rectal sampling of individual piglets with diarrhea should be performed. These specific criteria predominantly exclude Shiga toxin-related edema disease from the clinical picture, since the disease is not necessarily related with clinical diarrhea. The recovery of 71.7% of isolates being classified as a specific pathotype is more successful as compared to the earlier study, resulting in 52.5% recovery rate (Luppi et al., 2016). This high recovery rate indicates that sampling has been conducted in the early stage of onset of PWD, before antibiotic treatment and thus increasing the likelihood of detecting the causal agent rather than other resident E. coli.

A total of nine ETEC isolates had a combination of two fimbrial adhesins, namely F4-F18, F4-F6, F6-F18 or F18-F41. ETEC pathotypes with combination of genes encoding for two adhesins has been described previously (Kwon et al., 2002; Frydendahl, 2002; Chen et al., 2004; Vu-Khac et al., 2004; Luppi et al., 2016) and could offer them a pathogenic advantage through multiple adhesive options (Nagy and Fekete, 1999).

In the present study, virotypes with a specific combination of fimbrial adhesine F4 and toxins STa STb were most prevalent (117 out of 389), followed by STa STb LT (59 out of 389) and STb LT (34 out of 389). These results are partly in line with previous results in Western Europe and Denmark, where F4 combined with the toxins STb LT and STa STb LT was detected as the most prevalent (Frydendahl, 2002; Luppi et al., 2016). In contrast to other regions where a dramatic increase in F4 STa was reported over time, F4 STa was only present in 2.3 % of our E. coli isolates (Noamani et al., 2003). A more detailed analysis of virotype prevalences in Belgium and The Netherlands demonstrated no major differences, except for F4 STa STb being more prevalent in Belgium, whereas F18 STa was more prevalent in The Netherlands. These data suggest geographical differences among the major virotypes of F4-ETEC detected in cases of PWD.

Thirty-eight per cent of the E. coli isolates lacked any of the virulence factors investigated, whereas 11.5% of the isolates encoded for only one of the virulence factors. Therefore, nearly half of the E. coli isolates were considered non pathogenic. However, isolates carrying only a toxin without any known fimbriae and vice versa have been described previously (Vu-Khac et al., 2006; Nagy et al., 1990; Luppi et al., 2016). The role of these strains in the development of PWD need further investigation into other currently unknown adhesins and enterotoxins, which might play a role in PWD

pathogenesis. Some of these virulence factors such as AIDA (adhesin involved in diffuse adherence) and toxin EAST1 (enteroaggregative E. coli heat stable enterotoxin 1) have been recovered from piglets with diarrhea, although their specific role in PWD still has to be clarified (Zajacova et al., 2012; Ravi et al., 2007).

In the current study of PWD-affected farms, 360 of the farms investigated (66.8%) were positive for the presence of ETEC in samples for diarrheic piglets post-weaning. This result is higher as compared to the recent study in different countries in Western Europe, which reported 59.6% of positive samples. This might be due to different approach on sample analysis. In our study, all collected samples were analyzed in one diagnostic veterinary laboratory (IZSLER, Italy) using a uniform sample analysis procedure, whereas in the previous study, several different laboratories with slightly different procedures were enrolled for the study. Moreover, in the current study, veterinary practitioners were clearly briefed on specific sampling criteria and correct sampling procedures (timing, clinical signs), which might have impacted the recovery rate of E. coli isolates.

Overall, examination of ETEC subtypes associated with PWD revealed that F4-ETEC is the main fimbrial subtype in Belgium and The Netherlands, followed by F18-ETEC. In both countries, the prevalence of F4-ETEC was consistently higher as compared to F18-ETEC, although the difference was not as pronounced as in earlier studies (Luppi et al., 2016). It has been shown that specific ETEC pathotypes are recurrent and herd-specific. Therefore, reporting prevalence of pathotypes at farm level is of high clinical relevance (Fairbrother et al., 2005; Fairbrother et al., 2012; Luppi et al., 2016). Nevertheless, most studies only report the percentage of isolates recovered and not the proportion of clinical cases at farm level (Kwon et al., 2002; Osek, 1999; Vu-Khac et al., 2004; Zajacova et al., 2012; Zajacova et al., 2013).

However, taking into account serious changes in the preventive approach of PWD in the near future, with the limitation of preventive use of antimicrobials (EU/2019/06, 2018), the general pressure on reduction of antimicrobial use and the pending ban of ZnO use in the European Union (EMA, 2017), knowledge of herd-specific pathotypes will be increasingly important in order to assess other preventive options such as an E. coli vaccination. Currently, a live, non-pathogenic E. coli vaccine (Coliprotec® F4/F18; Elanco) is on the market in Europe for preventive protection of post-weaned piglets against both F4-ETEC and F18-ETEC (Nadeau et al., 2017). In order to successfully apply this alternative approach towards prevention of PWD, knowledge on the presence of F4-ETEC or F18-ETEC as the etiological agent of the PWD problems is prerequisite for vaccine implementation.

Conclusions

This study confirms that ETEC is the main pathotype involved in clinical cases of PWD in pig farms in Belgium and The Netherlands. The main fimbrial pathotype is F4-ETEC, immediately followed by F18-ETEC. This information is of practical relevance in relation to prevention of PWD, especially in the current climate of antibiotic reduction and the pending EU ban on the use of ZnO as a preventive measure towards PWD. One of the promising future preventive strategies is use of an E. coli vaccine, which is specifically focused at prevention of F4-ETEC and F18-ETEC. Therefore, correct sampling of diseased piglets, combined with a laboratory confirmation of the etiological agent and its associated virulence factors are required to archieve a correct early diagnosis and understand which herd-specific pathotypes are responsible at the onset of the PWD outbreak.

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