Veterinary Microbiology 280 (2023) 109691

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Clostridial diarrheas in piglets: A review

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ARTICLE INFO

Keywords:
Clostridioides difficile
Clostridium perfringens type A
Clostridium perfringens type C
Enteric infection
Necrotic enteritis
Pig

ABSTRACT

Clostridium perfringens type C and *Clostridioides difficile* are the main enteric clostridial pathogens of swine and are both responsible for neonatal diarrhea in this species. The role of *Clostridum perfringes* type A is under discussion. History, clinical signs, gross lesions and histological findings are the basis for a presumptive diagnosis of *C. perfringens* type C or *C. difficile* infection. Confirmation is based upon detection of beta toxin of *C. perfringens* type C or toxin A/B of *C. difficile*, respectively, in intestinal contents or feces. Isolation of *C. perfringens* type C and/or *C. difficile* is highly suggestive of infection by these microorganisms but it is not enough to confirm a diagnosis as they may be found in the intestine of some healthy individuals. Diagnosis of *C. perfringens* type A-associated diarrhea is more challenging because the diagnostic criteria have not been well defined and the specific role of alpha toxin (encoded by all strains of this microorganism) and beta 2 toxin (produced by some type A strains) is not clear. The goal of this paper is to describe the main clostridial enteric diseases of piglets, including etiology, epidemiology, pathogenesis, clinical signs, pathology and diagnosis.

1. Introduction

Clostridia are gram-positive, spore-forming bacilli that vary from strictly anaerobic to partially tolerant to oxygen (Rood, 2016). Several species of the *Clostridium* genus are responsible for enteric disease in multiple animal species, including humans. The incidence of these diseases remains high despite the availability of vaccines and other immunoprophylactic products against some of these microorganisms. *Clostridium perfringens* type C and *Clostridioides difficile* (formerly *Clostridium difficile*) are considered the most relevant enteric pathogenic clostridia of neonatal swine (Songer and Uzal, 2005; Diab, 2016; Diab et al., 2016). The involvement of *C. perfringes* type A in diarrhea of piglets has been suggested, but it has not been completely demonstrated (Klaasen et al., 1999; Garmory et al., 2000; Waters et al., 2003; Chan et al., 2013; Dors et al., 2016; Uzal et al., 2016). The role of other clostridia in neonatal diarrhea of piglets remains undetermined. This review describes the main clostridial enteric diseases of piglets, including etiology, epidemiology, pathogenesis, clinical signs, pathology and diagnosis.

1.1. *Clostridium perfringens*

The spore-forming, gram-positive, anaerobic bacillus, *C. perfringens*, is relatively aero tolerant (McClane B.A. et al., 2013; Rood et al., 2018). This microorganism is a fast grower, with a generation time of < 10 min (McClane B.A. et al., 2013), a trait that allows *C. perfringens* to quickly reach critical numbers in tissues and body fluids. *C. perfringens* has gliding motility mediated by type IV pili but lacks flagella. The pili are also involved in other virulence-related functions such as biofilm formation and adherence (Melville and Craig, 2013).

Although *C. perfringens* is a very important pathogen of animals and humans, this microorganism can be found ubiquitously in soil, decaying vegetation, and gastrointestinal (GI) content and feces of normal humans and other animals (McClane B.A. et al., 2013). *C. perfringens* produces at least 20 exotoxins, which are critical for its virulence; six of those toxins (referred to as major typing toxins) are used to classify this microorganism in 7 toxinotypes (A through G; Table 1) (Rood et al., 2018). Specific toxinotypes are associated with different diseases, but the role of some types in GI disease has not been demonstrated and/or it is under discussion. All *C. perfringens* types, with the exception of some type F strains carrying a chromosomal enterotoxin gene (*cpe*), carry their

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https://doi.org/10.1016/j.vetmic.2023.109691
Received 6 November 2022; Received in revised form 12 February 2023; Accepted 15 February 2023
Available online 23 February 2023

Table 1
Toxinotypes of *Clostridium perfringens*.

Toxinotype	Toxin produced					
	CPA	CPB	ETX	ITX	CPE	NetB
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	±	-
D	+	-	+	-	±	-
E	+	-	-	+	±	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

major toxin genes in plasmids (Rood et al., 2018). (Table 2).

1.2. *Clostridium perfringens type C*

Infection by *C. perfringens* type C in pigs occurs worldwide and is economically important (Songer and Uzal, 2005; Diab, 2016). Koch's postulates have been fulfilled for *C. perfringens* type C as the causative agent of GI infection in several animal species (Köhler et al., 1979; Johannsen et al., 1986; Sayeed et al., 2008; Garcia et al., 2012; Schumacher et al., 2013). The disease is characterized by necrotic enteritis (NE), which occurs most frequently in neonatal animals, including piglets, and is often fatal (Songer and Uzal, 2005).

Etiology and epidemiology. Two main typing toxin genes are carried by *C. perfringens* type C strains: *cpa,* which encodes alpha toxin (CPA), and *cpb,* which encodes beta toxin (CPB) (Rood et al., 2018), the latter being the main virulence factor involved in NE of pigs and other species (Posthaus et al., 2020). Some strains can also carry the *cpe* gene encoding enterotoxin (CPE), and other toxins not used for typing, including perfringolysin (PFO), beta 2-toxin (CPB2), and the large clostridial toxin TpeL (Rood et al., 2018). The role of these toxins in type C NE seems to be negligible.

Healthy animals, including sows, may variably carry small numbers of *C. perfringens* type C in the intestine. Neonatal piglets exposed to sow feces are colonized by these bacteria, which probably proliferate rapidly in the gut given the absence of a well-established intestinal microbiota (Songer, 1996; Songer and Uzal, 2005). *C. perfringens* type C spores are resistant to adverse environmental factors, including ultraviolet light, several disinfectants and heat. Transmission among piglets is also possible (Songer and Uzal, 2005).

Table 2
Samples, submission conditions and tests for the diagnosis of *Clostridium perfringens* enteric infections in pigs.

Sample	Condition	Test	Diagnostic significance
Small and large intestine	10% buffered formalin	Histopathology	Strongly suggestive[a]
Smears of small intestinal mucosa	Air-dried, room temperature	Gram stain	Suggestive[b]
Small intestinal content	Refrigerated or frozen (removed from intestinal loop)	Toxin detection, ELISA	CPB Confirmatory[a] CPA compatible[c]
Small intestinal swab or intestinal content	Refrigerated or frozen (removed from intestinal loop)	Anaerobic culture followed by typing (PCR)	Confirmatory[d] Compatible[e]
Formalin-fixed Paraffin-embedded intestine	Room Temperature	PCR typing	Compatible[f]

[a] Type C enteritis
[b] Type C and A enteritis, if many large Gram positive bacilli are observed
[c] Type A enteritis
[d] If type C and large numbers isolated
[e] If type A and large numbers isolated
[f] Type C (if *cpa* and *cpb* detected) or *C. perfringens* type A (if *cpa* detected)

C. perfringens type C-associated NE in piglets is most commonly seen in animals 1–3 days old, and it may occur as early as 12 h after birth. The disease is less common in older pigs (Jäggi et al., 2009). In non-vaccinated populations, the disease may manifest as an epizooty, with prevalence and lethality reaching 100% (Bergeland et al., 1966; Songer and Uzal, 2005; Posthaus et al., 2020). Occasionally, NE can re-occur in immunized herds (Wollschläger et al., 2009). Different factors responsible for this reoccurrence have been hypothesized, including failure of piglets to receive sufficient levels of antibodies *via* colostrum, trypsin inhibitors in the colostrum and deficiencies of trypsin secretion in piglets (Songer and Uzal, 2005; Diab, 2016).

Although type C-associated disease has been reported worldwide, documented information about its economic impact is lacking.

Pathogenesis. After ingesting the microorganism, there is overgrowth of *C. perfringens* type C in the intestine, facilitated by the still naïve intestinal microbiota of young piglets and the proliferative capabilities of *C. perfringens* (Posthaus et al., 2020). Vegetative forms produce and secrete CPB. Animal models of intestinal disease have indicated CPB as the critical virulence factor for type C strains to produce NE (Sayeed et al., 2008; Vidal et al., 2008; Uzal et al., 2009). CPB is a beta-barrel pore forming toxin of the α-hemolysin family (Popoff, 2014), characterized by its high sensitivity to trypsin and other proteases (Sakurai and Fujii, 1987). This sensitivity explains the occurrence of NE mainly in piglets, given the high level of trypsin-inhibitors present in the colostrum of the sow (Songer and Uzal, 2005). An intestinal loop model in rabbits has shown direct epithelial damage induced by CPB as early as 15 min after inoculation of those loops with purified CPB (Sayeed et al., 2008). Other *in vitro* studies, however, have not detected a direct effect on cultured porcine small intestinal epithelial cells when incubated with a recombinant CPB (Roos et al., 2015). In addition, a neonatal piglet jejunal model did not detect CPB binding to epithelial cells after 6 h of incubation with a type C strain (Schumacher et al., 2013). These observations suggest that the initial steps for CPB action in the intestine are probably more complicated than originally thought (Navarro et al., 2018; Posthaus et al., 2020). CPB does bind to porcine endothelial cells (Autheman et al., 2013; Schumacher et al., 2013) and exerts a cytotoxic effect on them (Gurtner et al., 2010; Autheman et al., 2013). Based on these results, it has been hypothesized that vascular endothelial damage in the lamina propria disrupts its permeability, leading to edema, hemorrhages, thrombi formation, ischemia and epithelial necrosis (Posthaus et al., 2020). Disruption of the intestinal mucosa integrity and permeability probably facilitates the absorption of CPB, and perhaps other toxins from the intestine, leading to toxemia and death (Songer and Uzal, 2005). Two putative receptors for CPB have been suggested in the last few years. In cultured monocyte cells, CPB binds to the $P2X_7$ receptor (Nagahama et al., 2015), and knockdown of $P2X_7$ reduced CPB binding and oligomer formation in those cells. Recently, the platelet endothelial cell adhesion molecule-1 (also known as PECAM-1 or CD31) was shown to be the receptor for CPB on endothelial cells (Bruggisser et al., 2020). It is possible that CPB receptors vary among cell types.

Clinical signs. Clinical presentation of NE can be peracute, acute, or chronic. The peracute and acute forms mainly affect piglets within the first 3 days of birth, and they are characterized by depression, abdominal pain, and hemorrhagic diarrhea. These presentations may last a few hours to up to 1 day after the initial exposure to *C. perfringens* type C (Songer and Uzal, 2005; Diab, 2016). Chronic presentation is usually seen in older piglets (> 3 day-old), and it is characterized by non-hemorrhagic diarrhea, dehydration, emaciation and reduced growth, persisting for up to 1 or 2 weeks (Songer and Uzal, 2005; Jäggi et al., 2009; Diab, 2016). Mortality in chronic presentations is usually seen during the second and third week of life (Posthaus et al., 2020).

Gross lesions. At necropsy, lesions are most frequently observed in the small intestine, particularly in the jejunum, and occasionally

extending to the colon (Figs. 1 and 2). Lesions restricted to the large intestine are much less common but may occur (Songer and Uzal, 2005). In most acute cases, lesions are characteristic, and consist of diffuse or segmental, fibrinonecrotizing to hemorrhagic enteritis, which can also be transmural and emphysematous (Figs. 1 and 2) (Songer and Uzal, 2005; Posthaus et al., 2020). Strands of fibrin may be found between intestinal loops. Mesenteric lymph nodes are red, and an excess of hemorrhagic fluid may be found in the pleural and peritoneal cavity (Diab, 2016).

Microscopic lesions. Histologically, the hallmark of *C. perfringens* type C-associated NE is hemorrhagic necrosis of the intestinal wall (Songer and Uzal, 2005; Diab, 2016). The luminal surface is usually covered by a pseudomembrane comprised of fibrin, degenerated and necrotic sloughed enterocytes, cell debris, and inflammatory cells including mainly neutrophils, but also macrophages, lymphocytes and plasma cells. Variable numbers of bacilli, very few of them with subterminal spores, are found singly or in clusters, free in the lumen, in the pseudomembrane, or demarcating the margin of the denuded mucosal surface. A few bacilli can also be seen in the crypts and invading the necrotic lamina propria. In the small intestine there is severe villus blunting and the necrotic epithelium and superficial lamina propria are homogenously acidophilic with scattered karyorrhectic debris and inflammatory cells composed of viable and degenerated neutrophils, and fewer lymphocytes, plasma cells and macrophages (Figs. 3 and 4). Necrosis can become deeper and affect all layers of the intestine (Songer and Uzal, 2005). Fibrin thrombi in the superficial arterioles and veins of the lamina propria and submucosa are frequently present (Köhler et al., 1979; Posthaus et al., 2020). There is diffuse edema throughout all layers of the affected intestinal segment, with severe congestion of subserosal vessels. All these intestinal changes are usually diffuse, although they can occur multifocally (Diab, 2016).(Fig. 5,6).

Diagnosis. During necropsy, smears of affected intestinal mucosa and intestinal contents reveal abundant gram-positive bacilli, very few of them containing spores (Songer and Uzal, 2005). Even though *C. perfringens* may be more aerotolerant than other clostridia, culture requires an anaerobic environment (Posthaus et al., 2020). On blood agar plates, after 24 h of incubation, type-C colonies are around 3–5 mm in diameter and strains that produce PFO form a characteristic double zone of hemolysis; the inner zone of complete hemolysis is produced by PFO, and the outer zone of incomplete hemolysis is caused by CPA (Songer and Uzal, 2005). Isolation is ideally performed on selective growth media to avoid overgrowth of normal anaerobic bacterial microbiota. Selective media for isolation include tryptose-sulfite-cycloserine with egg yolk agar (TSC-EYA) and blood agar with polymyxin B (Møller and Ahrens, 1996; Kotsanas et al., 2010). Further identification can be achieved through biochemical panels, MALDI-TOF spectrometry or PCR detection of the *cpa* gene, which is present in all *C. perfringens* isolates (Albini et al., 2008; Rood et al., 2018; Posthaus et al., 2020). PCR detection of toxin-encoding genes (*cpa* and *cpb*) is used to type isolated strains (Albini et al., 2008). CPB in intestinal content or stool can be detected by ELISA (Posthaus et al., 2020).

A presumptive diagnosis of *C. perfringens* type C-mediated disease in piglets may be achieved by analyzing epidemiological data, clinical signs of disease, and characteristic gross and microscopic intestinal lesions. Other causes of enteritis must be ruled out. It is also important to consider that *C. perfringens* type C may colonize lesions initiated by other pathogens, including *Cytoisospora suis*, transmissible gastroenteritis virus, porcine epidemic diarrhea virus and/or rotavirus (Songer and

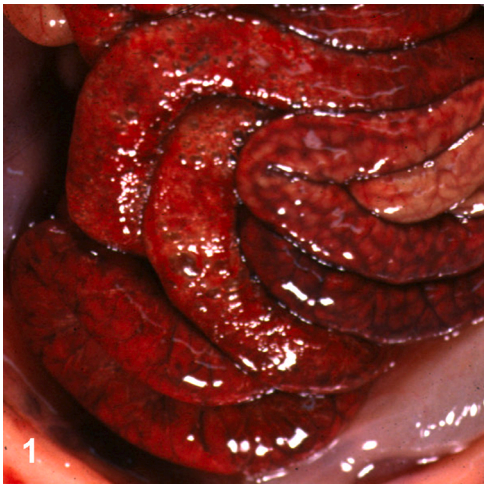

Fig. 1. Acute necrotic enteritis by *Clostridium perfringens* type C in a piglet (serosal view). Observe severe congestion and emphysema. Photo courtesy of Mark Anderson.

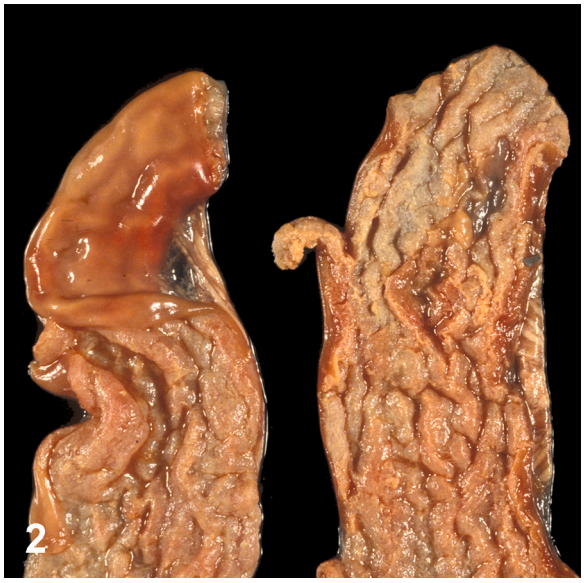

Fig. 2. Sub-acute necrotic enteritis by *Clostridium perfringens* type C in a piglet (mucosal view). Observe diffuse pseudomembrane covering the mucosa. Photo courtesy of Pat Blanchard.

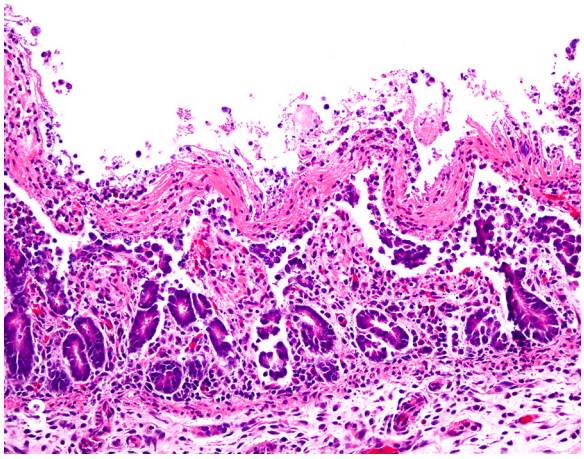

Fig. 3. Necrotic enteritis by *Clostridium perfringens* type C in a piglet. Observe mucosal necrosis and fibrino-necrotizing pseudomembrane. HE.

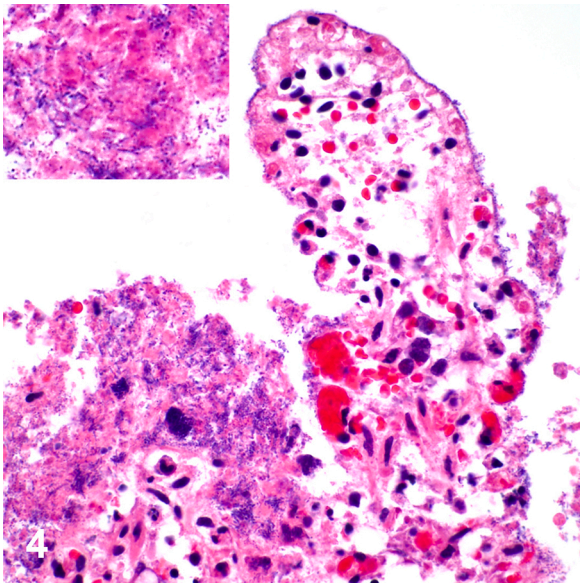

Fig. 4. Necrotic enteritis by *Clostridium perfringens* type C in a piglet. Observe loss of superficial epithelium and large number of rods in the lumen, mixed with cell debris and also lined the denudated villus. Inset: higher magnification of cluster of bacilli. HE.

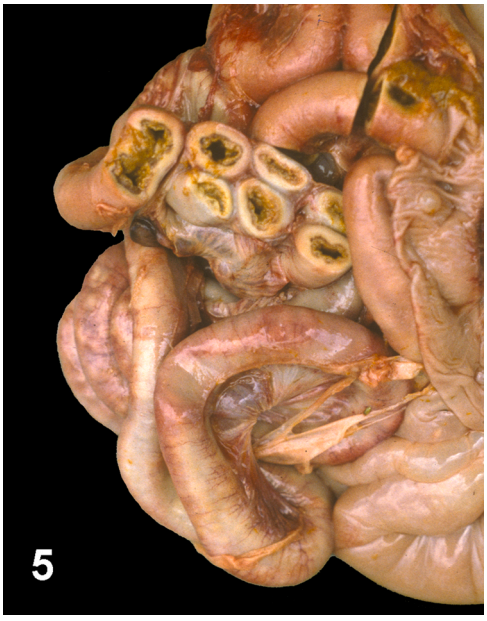

Fig. 5. Enteritis associated with *Clostridium perfringens* type A in a piglet. Notice a fibrinous pseudomembrane lining the intestinal mucosa. Photo courtesy of Carlos Perfumo.

Uzal, 2005). Therefore, cases with more than one etiology are possible. Isolation of *C. perfringens* type C from intestinal contents, followed by genotyping by PCR is highly suggestive of type C infection, although it is not confirmatory, as this microorganism may be found in the intestinal content of a small number of healthy pigs (Songer and Uzal, 2005).

Confirmatory diagnosis requires CPB detection in intestinal contents or feces by ELISA. Because CPB is highly sensitive to proteases, samples should be collected and processed as soon as possible or kept frozen until processed. Failure to detect CPB in intestinal contents does not necessarily preclude a diagnosis of *C. perfringens* type C-mediated disease, as this toxin is very labile and may break down before the samples are processed (Songer and Uzal, 2005; Macias-Rioseco et al., 2012).

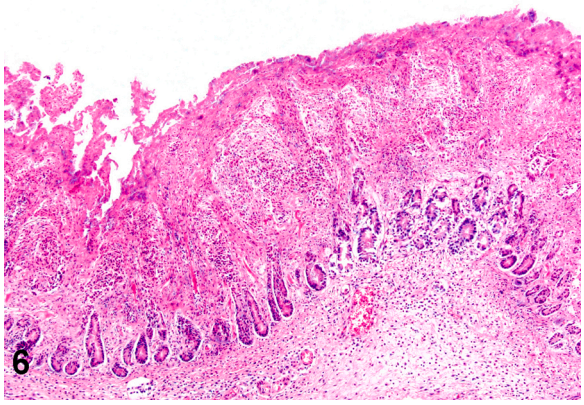

Fig. 6. Enteritis associated with *Clostridium perfringens* type A in a piglet. The mucosa is diffusely necrotic and covered by a fibrinous pseudo-membrane. HE.

Conversely, CPB detection is confirmatory.

Treatment and prophylaxis. Treatment of piglets with *C. perfringens* type C infection is usually unsuccessful because the disease is mostly acute and the lesions are irreversible once the diarrhea starts. During outbreaks, administration of type C antitoxin to the rest of the herd has proved to be of some help to prevent new cases of the disease (Uzal and Songer, 2019). Parenteral or oral antibiotics arealso preventive if given to piglets within 2 h of birth. Because *C. perfringens* type C enteritis tends to recur on infected premises, vaccination of pregnant sows with two doses of type *C. p*erfringens type C bacterins at 6 and 3 weeks, respectively, before parturition confers immunity to piglets at birth *via* colostrum. Sows so vaccinated are recommended to receive a booster of vaccine 3 weeks before each farrowing (Burrough, 2022).

1.3. Clostridium perfringens type A

C. perfringens type A is a normal component of the swine intestinal microbiota (Mansson and Smith, 1962; Uzal, 2016), although it is also considered by some authors as a cause of enteric disease in neonatal and, occasionally, weaned pigs (Ramisse et al., 1979; Nabuurs et al., 1983; Jestin et al., 1985; Chan et al., 2013; Dors et al., 2016). *C. perfringens* type A is largely the most ubiquitous toxinotype in the intestine of animals (Uzal, 2016), including piglets, which renders isolation of this type, or detection of its alpha toxin alone, of no diagnostic significance in diarrheic piglets (Yaeger et al., 2002; Chan et al., 2013).

Etiology. *C. perfringens* type A is similar to other types of *C. perfringens* in culture, but among the major typing toxins only encodes CPA (Table 1). In addition, most type A strains of porcine origin encode and produce CPB2, which has been suggested to play a role in enteritis of pigs (Bueschel et al., 2003; Waters et al., 2003). Information in this regard is, however, contradictory. While clinical signs have been reproduced by oral inoculation with pure cultures and culture supernatants of *C. perfringens* type A (Johannsen et al., 1993a; Wang et al., 2013), several other studies failed to demonstrate a pathogenic effect of this toxinotype in pigs (Farzan et al., 2013; Lee et al., 2014; Kongsted et al., 2018).

Whole genome sequencing (WGS) studies on *C. perfringens* strain JXJA17 isolated from diarrheic piglets revealed a close relation with the JGS1495, Cp-06, Cp-16, and FORC_003 strains, all of which have been isolated from different samples and belonged to different toxin-types. However, contrary to other strains, the *cpb2* gene of JXJA17 was located on a large plasmid with no co-localization of other toxin genes, antibiotic resistance genes or insertion sequences (Zeng et al., 2021). In another study, WGS of isolates from chickens and pigs revealed that

C. perfringens contained diverse antimicrobial resistance genes [*tetA*(P), *ant*(6)-Ib, *erm*(Q), *etc.*] and toxin genes (*cpb2*, *colA*, *cloSI*, *pfoA*, *etc.*) (Li et al., 2016). By comparative analysis, four of those *C. perfringens* isolates from three different pig farms possessed cpb2-carrying plasmid p1 with 100% nucleotide sequence identity, suggesting horizontal gene transfer among these microorganisms (Li et al., 2016). These studies highlight the importance and value of genome sequencing analyses from a pathogenic and epidemiologic perspective.

Epidemiology. Reported presumptive cases of type A disease occur during the first week of life, and as with type C infections, sows are considered to be the source of infection. However, since the diagnosis is complicated by the fact *C. perfringens* type A is part of the normal intestinal microbiota of pigs and a virulence marker for these strains has not been identified, differentiation between disease-causing strains and normal population is currently not possible.

This does not preclude, however, that under some circumstances, normal microbiota may cause disease. Because of this, the epidemiology of type A enteric infections in piglets is uncertain (Sangild et al., 2013; Dors et al., 2016). Because of the scarcity of documented cases of this condition, data on economic impact of this condition are not available.

Pathogenesis. The pathogenesis of *C. perfringens* type A infection in piglets is poorly understood. It is likely multifactorial (Dors et al., 2016). Spores of *C. perfringens* are likely to be responsible for keeping the organism in the environment, including, sometimes, in pig feed. It has been suggested that when some strains of *C. perfringens* type A multiply beyond control (probably 10^8-10^9 colony forming units per gram of intestinal content) they produce toxins that induce enteric disease. The toxins responsible for this disease have not been identified but CPA and CPB2 have been suggested. A diarrheic condition was reproduced with intragastric inoculation of *C. perfringens* type A in piglets (Johannsen et al., 1993a). However, no consistent changes were seen when ligated intestinal loops were inoculated with purified CPA in a different experiment (Johannsen et al., 1993b). Therefore, further experimental studies are needed to clarify the role of *C. perfringens* type A on the pathogenesis of piglet diarrhea.

An early study showed that more than 90% of *C. perfringens* type A strains isolated from cases of neonatal piglets with enteritis were positive for CPB2, and the *cpb2* gene was rarely silent (Bueschel et al., 2003). Based on these results, it was postulated that there was a strong association between CPB2 and cases of porcine diarrhea, and this toxin was proposed as a virulence factor of *C. perfringens* type A (Gibert et al., 1997; Herholz et al., 1999; Bueschel et al., 2003; Songer and Uzal, 2005). However, more recent information disputed this idea and showed that the prevalence of CPB2 positive strains is similar in the intestine of pigs with and without diarrhea (Chan et al., 2013; Kongsted et al., 2018).

Due to the lack of conclusive evidence of the role of *C. perfringens* type A in enteric disease, a multifactorial pathogenesis has been suggested to explain possible cases of diarrhea associated with *C. perfringens* type A (Dors et al., 2016).

Clinical signs and lesions. *C. perfringens* type A enteric infection has been associated with watery and mucoid, non-hemorrhagic diarrhea in suckling and feeder pigs (Uzal, 2016). Cases with no intestinal lesions have been described, although most cases seem to be characterized by mucosal necrosis with villus atrophy, and, occasionally, serositis. These lesions are usually most severe in the jejunum and ileum (Olubunmi and Taylor, 1985), although all parts of the intestine can be affected. Different to the usual transmural enteritis observed in cases of *C. perfringens* type C infection, type A cases are usually limited to the mucosa. Occasionally, pseudomembranous enteritis has been reported associated with *C. perfringens* type A in piglets (Sanz et al., 2007).

CPE-producing *C. perfringens* (formerly known as CPE positive *C. perfringens* type A, and now reclassified as *C. perfringens* type F; (Rood et al., 2018) strains have been isolated from 3-month-old pigs entering fattening units that presented watery, mucoid diarrhea (Jestin et al., 1985). These pigs, however, had minimal or no histological abnormalities. CPE inoculated into ligated ileal loops of axenic pigs induced fluid accumulation (Jestin et al., 1985). Based on this, it was suggested that CPE may be responsible for diarrhea in pigs.

Diagnosis. The main diagnostic challenge of type A enteritis is the difficulty establishing causality for strains and toxins that can be also found in healthy animals. Furthermore, neither conventional nor molecular Koch's postulates have been fulfilled for this disease and the presence of *C. perfringens* type A or its toxins is not enough for the diagnosis. It has been proposed that compatible clinical signs and lesions (non-hemorrhagic diarrhea of unexplained origin associated with necrotizing enteritis) and isolation of large numbers of *C. perfringens* type A from the small intestine are strongly suggestive of type A disease (Songer and Uzal, 2005; Wang et al., 2013). In bacterial cultures, *C. perfringens* type A is similar to other types of this microorganism. Type A strains can reach 10^8-10^9 CFU per gram of contents in pigs with diarrhea (Songer, 1996). Detection of CPA in intestinal content is supportive but not diagnostic, as this toxin can be found in a significant number of healthy animals. Although several ELISAs are available to detect CPA, no commercial tests are currently available for detection of CPB2 in body fluids. Combined infections of *C. perfringens* type A with other enteric pathogens are frequently described, thus a broad differential diagnostic panel is always indispensable (Mengel et al., 2012; Wang et al., 2013).

Therapy and prophylaxis. Although very little information is available about therapy and prophylaxis of *C. perfringens* type A infections in piglets, it is assumed that the general guidelines mentioned above for type C disease, may apply for type A.

1.4. *Clostridioides difficile*

During the last two decades, there has been increasing evidence of the role of *C. difficile* as a cause of typhlocolitis and diarrhea in neonatal piglets. Experimental reproduction of *C. difficile*-associated disease (CDAD) using pure isolates has been achieved (Arruda et al., 2013; Lizer et al., 2013). Furthermore, its zoonotic potential has been suggested, which has fueled research on the putative role of pigs in animal-human transmission aoodborne disease (Diab et al., 2016). Confirmatory evidence of this role is, however, lacking.

Etiology. *C. difficile* is a gram-positive, rod-shaped, anaerobic, spore forming bacterium that causes enteric disease in humans and several animal species, including horses, pigs, and various laboratory animals, and has zoonotic potential (Diab et al., 2016; Weese, 2020). In addition, *C. difficile* has been isolated from the gastrointestinal tract of multiple species in which its role in disease is not clear (Diab et al., 2016). In swine, it is considered an opportunistic pathogen that colonizes the intestinal tract at very early age (Grześkowiak, Ł., Zentek, J., Vahjen, W, 2016). The spores of *C. difficile* are very resistant in the environment, where they may prevail for long times, and are present in large numbers in feces and, therefore, in soil and fecal-contaminated products. The vegetative forms, which produce toxins, are strictly anaerobic and replicate in the intestines of hosts (Keel and Songer, 2006; Weese, 2020).

C. difficile is typed into different PCR ribotypes by amplifying a region of the rRNA. Ribotypes are subsequently classified into five clades. Additionally, it can also be divided into several toxinotypes depending upon various polymorphisms in the genes that encode for toxins A (TcdA) and B (TcdB), *tcdA* and *tcdB*, respectively (Rupnik and Janezic, 2016). The most commonly detected strain in swine in Europe, Asia, and America is ribotype 078 (in clade 5) / toxinotype V (Keel et al., 2007; Baker et al., 2010; Weese et al., 2010; Kim et al., 2018; Krutova et al., 2018; Andino-Molina et al., 2019; Zhang et al., 2019). No clear association between any of the ribotypes and disease has been described in swine. Ribotyping is not routinely performed in most veterinary diagnostic laboratories. Genomic diversity of clostridial species associated with swine production has been studied in the last few years. Particularly, the One Health perspective has aimed to assess the zoonotic potential of some clostridial pathogens, with particular emphasis on

C. difficile. For instance, a WGS analysis revealed a toxinogenic RT033 clone found in environmental and animal samples, positive for all *C. difficile* toxin genes (*tcdA*, *tcdB*, *cdtA/cdtB*) with a unique combination of genetic elements that may contribute to its host tropism and environmental dissemination and preservation (Alves et al., 2022). In another study using WGS, two human *C. difficile* isolates (RT046) were closely related to pig isolates in Sweden, suggesting a possible transmission (Werner et al., 2020). In addition, *C. difficile* RT014 has been characterized as well-established in both human and porcine populations in Australia; a core genome single nucleotide variant (SNV) analysis found 42% of human strains of this ribotype showed a clonal relationship with one or more porcine strains, suggesting a recent inter-host transmission (Knight et al., 2017). The same study also showed clustering of human and porcine strains indicative of very recent shared ancestry, based on phylogenies based on MLST (7 loci, STs 2, 13, and 49) and core orthologous genes (1260 loci). Furthermore, two *C. difficile* RT078 isolates detected in samples of rat intestinal content and feces in fattening pig farms showed a close genetic relationship with a virulent strain isolated from a human patient, suggesting a potential reservoir for humans and other animal species (Martín-Burriel et al., 2017).

Epidemiology. In most domestic animals and humans, CDAD occurs in several age groups and in association with the use of antibiotics. However, in pigs, the disease affects mostly neonatal, 1–7 days old piglets, and previous antibiotic administration is not considered a predisposing factor (Yaeger et al., 2002; Arruda et al., 2013; Schneeberg et al., 2013). Neonatal piglets become infected very shortly after birth and carry a high burden of *C. difficile* in the gastrointestinal tract. In fact, *C. difficile* may be isolated from the gastrointestinal tract of piglets as soon as 1 h postpartum, and positivity may reach up to the 100% of the litter at 32–48 h of age (Hopman et al., 2011a). The prevalence of infection in post-weaning and growing-finishing pigs decreases dramatically (Asai et al., 2013; Kim et al., 2018).

Transmission is mostly fecal-oral, and the source of infection for the piglets is fecal material from the sow. Airborne transmission may probably occur too (Weese et al., 2010; Hopman et al., 2011a). *C. difficile* may also be isolated from feces of clinically healthy pigs of several age groups, which likely contributes to further environmental contamination and transmission (Weese et al., 2010, 2011; Hopman et al., 2011b). Mortality and morbidity vary considerably in different reports. A range of 50–80% of affected litters with a mortality of 11–14% was recorded among three farms with CDAD in Australia (Squire et al., 2013). Weekly total losses among suckling piglets of a farm affected by CDAD ranged from 7% to 58%, and 90% of those losses were directly attributed to *C. difficile* by the farmer (Waters et al., 1998).

Recent attention has focused on zoonotic or foodborne sources. Toxigenic strains of *C. difficile* are common in pigs, cattle, poultry, dogs, and a variety of other mammals. Contamination of carcasses at slaughter is uncommon and foodborne transmission is unlikely (Warriner et al., 2017). Direct transmission from human carriers or from infected animals has not been confirmed as a direct cause of CDAD.

Ribotype 078 is one of the most frequently detected strains in cases of nosocomial and community acquired, multidrug resistant CDAD in humans. Pigs are thus considered a reservoir of this organism, and their role as possible source of foodborne infections has been proposed (Rupnik and Songer, 2010). Ribotype 078 has been isolated from carcasses in slaughterhouses and, remarkably, genetically-indistinguishable strains have been detected in both pigs and humans, including farm workers, in multiple countries (Debast et al., 2009; Harvey et al., 2011; Keessen et al., 2013; Rodriguez et al., 2013; Knetsch et al., 2014). Nevertheless, foodborne acquisition is still considered uncommon by some authors, and direct transmission from infected animals to humans has not been confirmed to date (Uzal and Songer, 2019).

Other ribotypes such as 013, 014, 019, 046, 062, 066 and 126 may be also encountered (Norén et al., 2014; Diab et al., 2016; Weese, 2020). Interestingly, ribotype 078 does not seem to occur in Australia, where a different one, 237, has been identified (Squire et al., 2013).

The earliest published report of natural infection with *C. difficile* in swine was that of two piglets diagnosed with enterocolitis in the 1980 s (Jones, Hunter, 1983). In the 1990 s, a large outbreak of *C. difficile* infection was reported in a pig Canadian farm, which had a weekly mortality rate of up to 58% in piglets (Waters et al., 1998). A 12 year surveillance study performed at the University of Iowa Veterinary Hospital showed a prevalence of *C. difficile* infection in pigs of 55 (Yaeger et al., 2002). The carrier rate of C. difficile in 1–2 week old piglets varies from 50% to nearly 100% in many pig operations throughout the world; these numbers decrease significantly in older pigs (Moono et al., 2016). Although the morbidity of piglets infected with *C, difficile* can be as high as 100%, the mortality of the disease is usually low, although it has been reported to be as high as 16% in severe outbreaks (Songer et al., 2007). Countries in Europe, North America and Europe have seen a recent rise in the incidence of *C. difficile* infection in piglets (Slimings, Riley, 2014).

Pathogenesis. TcdA and TcdB are two large clostridial toxins that principally mediate the pathogenesis of CDAD (Songer et al., 2016). In addition, some strains of *C. difficile* produce a third, actin-specific binary ADP-ribosylating toxin (CDT), which is encoded by the *tcdC* gene. The role of CDT has not been clearly defined and it is still under discussion if it takes any part in the pathogenesis of the disease in piglets (Gerding et al., 2014; Songer et al., 2016). TcdA is an enterotoxin and TcdB is both an enterotoxin and a cytotoxin (Uzal et al., 2016). Over the years, there has been intense debate about the essential role of each one of these two toxins in disease development and, eventually, animal models demonstrated that both are able to induce lesions and cause disease (Kuehne et al., 2010). Most of the toxicogenic swine isolates carry both *tcdA* and *tcdB* genes, although there are rare strains that carry *tcdB*, only (Fry et al., 2012).

Ingested *C. difficile* spores germinate in the small intestine after exposure to bile salts (Crobach et al., 2018). Vegetative forms colonize the colon and cecum of piglets and, if toxins are produced in sufficient amount, mucosal damage and disease occur (Uzal and Songer, 2019). *C. difficile* toxins induce damage by a variety of mechanisms, including mitochondrial dysfunction, ATP depletion, production of reactive oxygen species, release of pro-inflammatory and chemotactic cytokines, mast cell degranulation, substance P secretion, and apoptosis (Voth and Ballard, 2005; Di Bella et al., 2016; Songer et al., 2016). Death often ensues as a consequence of toxemia (Songer et al., 2016).

Clinical signs. Clinical presentation may be variable and generally of mild severity (Diab et al., 2016). Non-hemorrhagic diarrhea, poor body condition, reduced weight gain, respiratory distress, abdominal distention, and scrotal edema can be seen in affected piglets (Waters et al., 1998; Songer and Uzal, 2005; Yaeger et al., 2007; Squire et al., 2013). In a few cases, sudden death ensues without prior diarrhea (Waters et al., 1998).

Gross lesions. Grossly, edema of the mesocolon is considered a hallmark of CDAD (Fig. 7), although this finding is not 100% specific and might be missing in some cases (Songer et al., 2000; Yaeger et al., 2002, 2007). Furthermore, other agents such as *E. coli*, porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus may also produce mesocolonic edema. Colon and cecum are often distended, contents are commonly soft to watery and yellowish (Fig. 8), and there may be patchy foci of ulceration/necrosis in the mucosa, although grossly unremarkable mucosal surfaces are not uncommon (Uzal et al., 2016).

Microscopic lesions. Histologically, there is usually marked edema in the submucosa and mesocolon (Fig. 10), with neutrophils in the colonic and cecal lamina propria, and epithelial hyperplasia and goblet cell loss in the crypts. Multiple foci of erosion and ulceration in the superficial epithelium are common (Waters et al., 1998; Songer et al., 2000; McElroy et al., 2015). A combination of degenerated neutrophils, mucus, fibrin, and debris may be expelled into the lumen through these

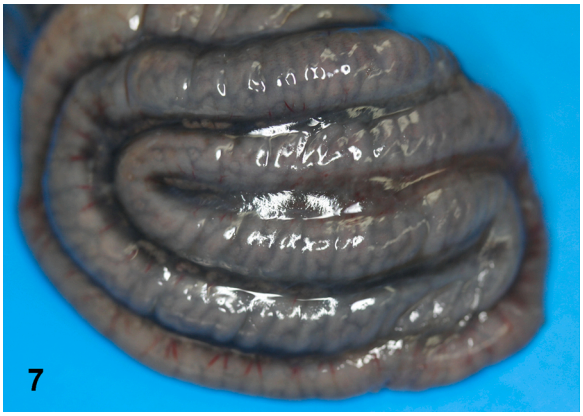

Fig. 7. Colitis by *Clostridioides difficile* in a piglet. Observe diffuse mesocolonic edema.

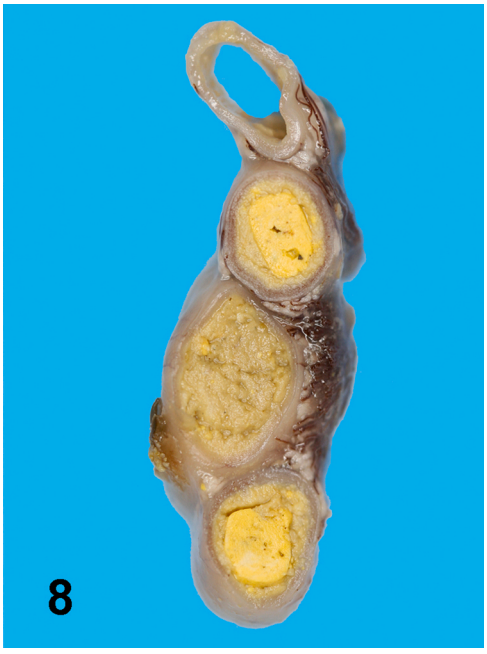

Fig. 8. Colitis by *Clostridioides difficile* in a piglet. Observe several distended colonic segments with yellowish content.

surface defects and are therefore consistent with the so-called "volcano" lesions described in other animal species and humans with CDAD (Fig. 9) (Uzal et al., 2016). Pseudo-membrane formation has also been described (Diab et al., 2016). Transmural necrosis of cecum and colon with serositis may occur in certain cases (Waters et al., 1998; Lizer et al., 2013). In a study, loss of goblet cells, neutrophilic infiltrates in the lamina propria, and multifocal erosions in the colon were significantly associated with detection of *C. difficile* toxins (Yaeger et al., 2007).

Diagnosis. CDAD can be presumptively diagnosed based on compatible clinical signs and lesions in the adequate age group. In particular, edema of the mesocolon and fibrinosuppurative typhlocolitis with "volcano" type ulcers in neonatal piglets should prompt further investigations into the possible role of *C. difficile* (Uzal and Songer, 2019). Detection of TcdA and/or TcdB in intestinal contents or feces confirms the diagnosis. Cell cytotoxicity assays, which detect the toxins by demonstrating their cytotoxic effects in cell culture, were considered by many years the gold standard (Delmée, 2001). However, commercial ELISAs are currently the most utilized method in most veterinary diagnostic laboratories (Yaeger et al., 2007; Grześkowiak et al., 2016; Uzal and Songer, 2019). TcdA concentration in feces are negatively affected by storage at 4 °C and freezing and thawing cycles, although storage at − 30 °C does not significantly alter them after at least 21 days. TcdB is more stable, and its concentrations seem to be unaltered after 21 days of storage at 4 °C, − 30 °C, or even after repetitive freezing and thawing cycles at − 30 °C (Grześkowiak et al., 2020).

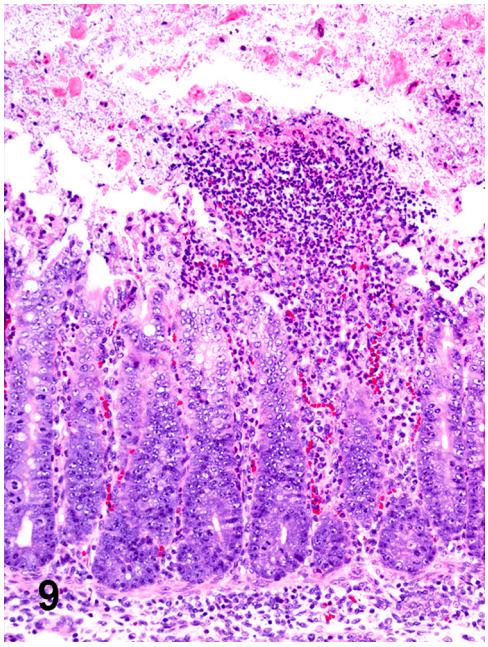

Fig. 9. Colitis by *Clostridioides difficile* in a piglet. Observe focal mucosal necrosis and many neutrophils being expelled through a mucosal ulcer (volcano lesion). HE.

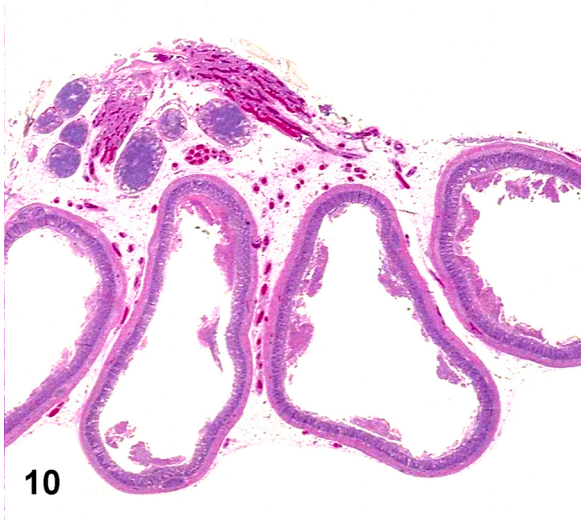

Fig. 10. Colitis by *Clostridioides difficile* in a piglet. Observe mesocolonic edema and multifocal pseudomembrane formation. HE.

Isolation of *C. difficile* supports, but does not confirm, a diagnosis of CDAD because it can be also isolated from healthy animals (Alvarez-Perez et al., 2009; Hopman et al., 2011a). Enriched media that contain cefoxitin, cycloserine, fructose and taurochocolate are needed to grow and permit sporulation of *C. difficile* (Uzal and Songer, 2019). Toxinotyping of the isolates, generally done by examining the presence of *tcdA*, *tcdB*, and *tcdC* genes *via* PCR, is recommended to rule out non-toxinogenic strains, which may be carried by swine (Fry et al., 2012; Knight et al., 2015). PCR typing of isolates is, however, unfrequently

done in veterinary diagnostic laboratories.

There are other gastrointestinal diseases that may mimic CDAD and cause diarrhea in piglets, including enterotoxigenic colibacillosis, coccidiosis, cryptosporidiosis, porcine epidemic diarrhea, transmissible gastroenteritis, rotaviral enteritis, and other clostridial diseases (see above) (Chan et al., 2013; Diab et al., 2016; Kongsted et al., 2018; Thomson and Friendship, 2019; Jung et al., 2020). Also, similarly to what was stated above for *C. perfringens* type C, CDAD may occur concurrently with one or more of those conditions in complex disease processes (Yaeger et al., 2007; Chan et al., 2013; Kongsted et al., 2018). Therefore, a comprehensive set of tests aiming to confirm or rule out these conditions should be always applied in cases of suspect CDAD.

Therapy and prophylaxis. The immunoprophylaxis of *C. difficile* diarrhea in piglets and other domestic animals has not been studied, but studies in laboratory anijmls and humans suggest that immunity is antitoxic. This is based on the fact that antibodies against TcdA and TcdB prevent toxin binding in mouse and hamster models, preventing intestinal lesions and clinical disease (Uzal and Songer, 2019). Likewise, information about treatment of piglets with *C. difficile* is lacking, but results of *in vitro* antimicrobial susceptibility testing suggest that tylosin may be effective in treatment of piglets (Uzal and Songer, 2019).

2. Discussion

The clostridial enteric infections presented here have a complex pathogenesis and epidemiology. Most importantly, the 3 clostridial agents involved in these conditions (*C. perfringens* types C and A, and *C. difficile*) can be found with different prevalence in the intestine of healthy pigs. Therefore, diagnosis of these conditions cannot be based solely on isolation of the clostridia involved. Several other factors should be thus taken into consideration to make a final diagnosis. Thus, factors associated with farm management (degree of intensification of the farm, housing of sows/piglets, hygiene and disinfection procedures, control of births and fostering, genetics, immune status of sows, pig stress [cut teeth / tail], the use of antibiotics in the sow or piglets), vaccination programs, clinical signs, lesions and exposure to other intestinal pathogens should be considered during the diagnostic workup.

For *C. perfringens* type C and *C. difficile*, detection of their main toxins in intestinal content and feces, coupled with clinical and pathologic findings, is diagnostic. However, the limited sensitivity of some of the ELISAs used for toxin detection can hamper the diagnostic. Microbiological isolation and molecular typification (PCR) can support, but do not confirm, the final diagnosis.

Diagnosis of type A enteritis, however, is seldom unequivocal as no diagnostic criteria have been published, no pathognomonic gross or microscopic lesions are described, and this toxinotype can be found in the intestine of healthy pigs with much higher frequency than *C. perfringens* type C or *C. difficile* (Manson and Smith, 1962; Uzal et al., 2016). Finally, although CPB2-producing *C. perfringens* type A has also been suggested to be linked to disease in several animal species, including pigs (Bueschel et al., 2003; Waters et al., 2003), most evidence implicating CPB2 in the pathogenesis of porcine infections is based on isolation on CPB2-positive *C. perfringens* from sick animals. However, these strains can be also isolated from healthy animals so no clear evidence of their role in virulence has been provided. The condition has not been reproduced experimentally with CPB2-producing strains, and the importance of including CPB2 detection (*in vivo* or *in vitro*) in the diagnostic definition of type A infections remains unknown.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109691.

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