Papers

Cattle enterotoxaemia and *Clostridium perfringens*: description, diagnosis and prophylaxis

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Cattle enterotoxaemia is one of numerous pathologies caused by *Clostridium perfringens*. These anaerobic Gram-positive bacteria are naturally present in the intestinal flora of mammals, but their uncontrolled multiplication under certain conditions results in the overproduction of toxins in the intestinal tract. Major clinical signs are induced by the systemic spread of these toxins in the blood and tissues. Enterotoxaemia may be acute or peracute, and sudden death is often reported in rapidly growing, apparently healthy cattle. Enterotoxaemia can be prevented only with better understanding of its risk factors and pathogenesis. This paper provides an up-to-date overview of knowledge concerning the aetiology of the syndrome, its epidemiological context, pathogenesis, clinical signs and lesions, the diagnostic procedures and prophylactic tools, with specific attention to field aspects that are directly relevant to practitioners and clinical researchers.

THE word 'enterotoxaemia' denotes a toxaemia of intestinal origin that occurs when toxins produced in the intestines are absorbed into the bloodstream. Enterotoxaemia can be caused by various microorganisms, but the term is most frequently used in relation to the absorption of toxins produced by species of the genus *Clostridium* (Manteca and Daube 1994, Daube and others 1996a, Popoff 1998).

The genus *Clostridium* comprises more than 100 species, several of which are pathogenic, producing exotoxins that act locally or systemically on the host's cells and tissues. Although several species of *Clostridium* can cause enterotoxaemia in different hosts, the syndrome has come to be associated with the species *Clostridium perfringens* in ruminants (Popoff 1998).

Sheep enterotoxaemia caused by *C perfringens* is well documented. Different strains of *C perfringens* are responsible for several clinical syndromes, including lamb dysentery, pulpy kidney and struck (enterotoxaemia in adult sheep that causes sudden death) (Buxton and others 1978, Kadra and others 1999, Gkiourtzidis and others 2001, Soler-Jover and others 2004, Uzal and others 2004). Prevention is straightforward, through vaccination of ewes during pregnancy and of the lambs (Clarkson and others 1985, Green and others 1987, Odendaal and others 1988, 1989, de la Rosa and others 1997, Uzal and Kelly 1998, Uzal and others 1999, Rosskopf-Streicher and others 2004). The situation is less clear in cattle.

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This article reviews aspects of the aetiology, epidemiology and pathogenesis of cattle enterotoxaemia, with special reference to beef cattle. Its intention is to improve the accuracy of field and routine laboratory diagnosis, and to improve prophylaxis through management practices and vaccination.

C perfringens
Description

C perfringens is a Gram-positive, anaerobic, oxygen-tolerant, rod-shaped bacterium, successively named *Bacillus aerogenes capsulatus* (Welch and Nuttall 1892, Lucey and Hutchins 2004), *Bacterium welchii* and *Clostridium welchii* in honour of William Henry Welch, who first described it in 1892. The species name *perfringens* ('breaking through' in Latin), proposed by Veillon and Zuber (1898) (*Bacillus perfringens*), replaced *welchii* in the fifth edition of Bergey's Manual of Determinative Bacteriology (Bergey and others 1939). *C perfringens* is probably the best-known and most widespread anaerobic pathogen throughout the world. It is a normal component of the intestinal flora of healthy warm-blooded animals and human beings (Popoff 1989, Rood and Cole 1991, Songer 1996).

Like all bacterial species, *C perfringens* can be subdivided into strains according to the results of different typing methods. Although subdivision by serotyping was proposed in the past, division into strains according to the combinations of toxins produced (or toxinotypes) is still the most widespread and routinely useful method today. Genotyping is generally used only in PCR analysis of toxin genotype (Daube and others 1994, Meer and Songer 1997, Kanakaraj and others 1998, Baums and others 2004).

C perfringens can produce up to 30 potential toxins, and strains are traditionally classified into five categories (A, B, C, D and E) according to the combination of the four major toxins (α, β, ι and ϵ) they produce (Hatheway 1990, Songer 1996, Petit and others 1999) (Table 1). These five types can be further subdivided according to the production of two additional toxins: the enterotoxin (encoded by the *cpe* gene) and the β2 toxin (encoded by the *cbp2* gene) and numerous so-called minor toxins (Niilo 1987, Daube and others 1996a, b, Ghazi and others 1997, Gibert and others 1997, O'Brien and Melville 2004) (Table 1).

Associated pathologies

Type A strains cause most pathologies associated with *C perfringens* in human beings: gas gangrene (type A, non-enterotoxigenic), sporadic or antibiotic-associated diarrhoea (type A, ±enterotoxigenic, ±*cpb2*) and food poisoning (type A or D, enterotoxigenic) (Vogel 1995, Meer and Songer 1997, Sparks and others 2001, Carney and others 2002, Fisher and others 2005, Vaishnavi and others 2005). Necrotising enteritis (type C) is also seen in human beings (Kreft and others 2000).

In animals, the five toxinotypes cause numerous forms of enteritis and enterotoxaemia (Popoff 1989, Songer 1996, Greene and Jang 2003, Marks and Kather 2003, Coetzer and Tustin 2005, Radostits and others 2007a) (Table 2).

Type A strains are associated with diarrhoea, dysentery and enterotoxaemia in ruminants, pigs, horses and poultry (Manteca and Daube 1994, Songer 1996, Daube and others 1996b, Glock and DeGroot 1998, Herholz and others 1999, Manteca and others 2001, Bacciarini and others 2003, Bueschel and others 2003, Songer and Uzal 2005, Radostits and others 2007b). The role of the α toxin in enteritis and enterotoxaemia awaits full experimental confirmation: the α toxin has not yet been shown to be present in the bloodstream of mammals in non-experimental infections, experimental trials to reproduce enterotoxaemia in mammals using the α toxin have failed, and although clinical signs of necrotic enteritis can be reproduced in poultry (Fukata and others 1988), more recent work with mutant strains has questioned the supposed role of the α toxin (Keyburn and others 2006) and implicated the more recently discovered NetB toxin in this pathology (Keyburn and others 2008).

Type B and C strains cause most cases of dysentery and enterotoxaemia with necrotic or haemorrhagic enteritis, and type D strains cause the pulpy kidney enterotoxaemic syndrome in sheep, and perhaps in calves in which no necrotic or haemorrhagic intestinal lesions are present (Griner and Bracken 1953, Niilo 1986, Leary and Titball 1997, Popoff 1998, Radostits and others 2007c, d). The major β and ε toxins have been shown to have a role in these syndromes, and prevention by vaccination with toxoids is effective, at least in small ruminants (Uzal and Kelly 1998, Springer and Selbitz 1999, Uzal and others 2002, Rosskopf-Streicher and others 2004, Fernandez-Miyakawa and others 2007).

The role and importance of *C perfringens* type E (ι toxin) is still unclear, but type E strains appear to cause enteritis in sheep and goats in the Middle East and in calves in North America (Billington and others 1998, Bueschel and others 2003, Sipos and others 2003, Songer and Miskimmins 2004, Greco and others 2005, Radostits and others 2007c).

Specific toxins

The enterotoxin (*cpe* gene) is responsible for enteritis, diarrhoea and food poisoning in human beings (Schalch and others 1997, 1999, Lin and Labbe 2003, Miyamoto and others 2004, 2006, Fisher and others 2005) and could also be involved in cases of diarrhoea in animals (Tschirdewahn and others 1991, Kokai-Kun and McClane 1997, Bueschel and others 1998, 2003, Netherwood and others 1998).

The β2 toxin (*cpb2* gene) described in the late 1990s (Gibert and others 1997) has been associated with enteritis in piglets (Klaasen and others 1999, Bueschel and others 2003), typhlocolitis in horses (Herholz and others 1999, Bacciarini and others 2003) and diarrhoea in dogs (Thiede and others 2001). However, its role remains to be determined in human beings and in some animal species in which β2-positive strains have been isolated. The role of the β2 toxin will be further discussed in this paper in the specific context of cattle enterotoxaemia (see 'Strain typing' section).

The last *C perfringens* toxin discovered is NetB. It has been identified as a definitive virulence factor of the poultry necrotic enteritis syndrome (Keyburn and others 2008). This toxin is found in type A strains from poultry with or without necrotic enteritis and was also detected in one strain from a cow with liver abscesses (Martin and Smyth 2009).

Cattle enterotoxaemia

Cattle enterotoxaemia is an acute or peracute syndrome with a case fatality rate close to 100 per cent, associated with an uncontrolled multiplication of *C perfringens* in the small intestine with an overproduction of toxins (Manteca 2004). These toxins act both locally and systemically and may cause death within a few minutes to a few hours (Barker and others 1993, Manteca and others 2001). Recent cattle enterotoxaemia literature is summarised in Table 3.

TABLE 1: *Clostridium perfringens* conventional toxinotypes					
Toxin	Type A	Type B	Type C	Type D	Type E
α	X	X	X	X	X
β		X	X		
ε		X		X	
ι					X
Enterotoxin	(x)				(x)
β2	(x)	(x)	(x)	(x)	(x)

X Classic
(x) Potential

History

The first case of cattle enterotoxaemia was reported in Australia in young calves grazing on lush spring grass (Rose and Edgar 1936). Several cases were reported in the following years, mainly in suckling calves of beef cattle breeds (Griner and Bracken 1953, Niilo and others 1974, Manteca and Daube 1994, Manteca and others 2000). Unlike in sheep, enterotoxaemia in cattle has remained a poorly defined syndrome. Today, clostridial enterotoxaemia in cattle is characterised by sudden death, most often with lesions of haemorrhagic and segmental enteritis at postmortem examination and high colony counts of *C perfringens* in the small intestine (Daube and others 1996a, Songer 1996, Manteca and others 2001). Field cases of enterotoxaemia remain difficult to confirm, and the differential diagnosis of sudden death needs to be considered.

Moreover, the role and identity of the strains was uncertain until recent developments in molecular biology techniques (Manteca and Daube 1994, Songer 1996, Manteca and others 2001, Sawires and Songer 2005, 2006).

Most of the description of cattle enterotoxaemia hereafter is based on specific studies carried out on beef cattle breeds in Belgium (Daube and others 1996a, Manteca and others 2000, 2001, Manteca 2004), complemented with published data from other beef cattle breeds in other countries (Griner and Bracken 1953, Fleming 1985, Efuntoye and Adetosoye 2003). At the end of the 20th century a pathology closely related to enterotoxaemia was described in North America: the haemorrhagic bowel syndrome (HBS) or jejunal haemorrhage syndrome (JHS) (Dennison and others 2002, 2005, Abutarbush and others 2004, Abutarbush and Radostits 2005, Ceci and others 2006). This syndrome, affecting mainly high-producing dairy cattle, is so closely related that it will be discussed commonly with 'beef cattle' enterotoxaemia in this paper. Both syndromes (enterotoxaemia and HBS) share some common features: intensive rearing conditions, sudden onset, haemorrhagic enteritis (jejunitis), high *C perfringens* counts, identical *C perfringens* toxinotypes (mainly A) and stressful breeding conditions. The differences between enterotoxaemia and HBS may be linked to a differential evolution of *C perfringens* strains on both sides of Atlantic, as suggested by Johansson and others (2006). However, some cases have been described in Europe (Miyamoto and others 2006).

Epidemiology
Target population

Cattle enterotoxaemia affects mainly suckling and veal calves in good to excellent body condition up to four months of age living in farms with high-quality management (Fleming 1985, Manteca and Daube 1994, Manteca and others 2000). Although beef cattle breeds (Belgian blue, Blonde d'Aquitaine, Charolais, Hereford) are more frequently affected, the rearing and breeding conditions have a greater effect than breed on whether an animal is at risk from enterotoxaemia. Therefore, cattle enterotoxaemia is tightly linked to intensive rearing conditions, both in beef and in veal production (Griner and Bracken 1953, Songer 1996, Popoff 1998, Manteca and others 2000). In Belgian blue cattle in Belgium, losses due to enterotoxaemia cause up to 10,000 deaths (4 to 5 per cent of the total annual registered mortalities) of suckling calves per year (Manteca and others 2000). These intensive breeding conditions seem to be present in the context of HBS too (Abutarbush and Radostits 2005, Berghaus and others 2005), with high-producing dairy cows most commonly affected (Dennison and others 2002, Berghaus and others 2005).

TABLE 2: Relationship between *Clostridium perfringens* toxinotypes and pathologies

Toxinotype	Animal species affected	Syndrome
A (α)	Cattle, lambs	Enterotoxaemia
	Fowl, piglets	Necrotic enteritis
B (α, β, ε)	Neonatal calves, foals	Haemorrhagic enteritis
	Adult sheep	Haemorrhagic enterotoxaemia
	Newborn lambs	Dysentery
C (α, β)	Neonatal pigs, lambs, calves, goats, foals	Necrotic or haemorrhagic enterotoxaemia
	Adult sheep	Acute enterotoxaemia (struck)
D (α, ε)	Lambs (as pulpy kidney disease), calves	Enterotoxaemia
	Adult cattle	Enterotoxaemia
	Fowl	Necrotic enteritis

Triggering factors

Cattle enterotoxaemia is a non-contagious sporadic disease, even if several fatal cases may be observed in a same herd (Shirley 1958, Sudaric and Nadazdin 1983, Katitch 1987, Popoff 1998, Schotte and others 2004). The disease is often associated with stressful conditions observed 24 to 36 hours before sudden death (Manteca and Daube 1994, Manteca and others 2000). Stress may be caused by an abrupt change in diet (for example, introducing high carbohydrate and protein substrates such as heavy grain, or grazing on rich pasture without a transition phase) or by a water supply defect. Stressful environmental conditions such as animals being regrouped or transported, hierarchical troubles or rough handling during dehorning or dipping should

also be considered. Medical treatment (antibiotherapy, antiparasitic prophylaxis) and some physiological states such as calving, lactation peak and oestrus in the dam are also considered as triggering factors of enterotoxaemia.

In a recent study on the risk factors associated with HBS in dairy cattle, the conclusion was that management practices implemented to achieve high milk production may increase the risk of HBS development. The increased consumption of a high-energy diet is the most plausible common pathway for all of the risk factors (Berghaus and others 2005).

Clinical data
Clinical signs

Cattle enterotoxaemia is an acute or peracute syndrome classically described by the farmer or the veterinarian as 'sudden death' or 'found dead'. Very few animals show premonitory clinical signs such as abdominal pain or nervous disorders. In Europe, there is no associated diarrhoea and only rarely tympanism (Fleming 1985, Manteca and Daube 1994). This is in contrast with the observations made in the USA in high-producing dairy cows or beef cattle suffering from HBS (Clarkson and others 1985, Dennison and others 2002, 2005, Abutarbush and others 2004, Abutarbush and Radostits 2005, Ceci and others 2006, Muskens and others 2007). If the animal does not die suddenly, some agonic signs may be observed (respiratory distress, lateral recumbency and convulsions before coma).

Postmortem lesions

Rapid putrefaction of the abdomen with gas production and a putrid smell is characteristic of clostridial infection. The most typical local

TABLE 3: Publications on cattle enterotoxaemia since 1980 (previous references were listed in Manteca and others 1994) showing the pathology, toxinotype, type of animal and country reported

Year	Authors	Pathology	Toxinotype	β2*	Animals	Country
1985	Fleming	Enterotoxaemia	A, B, C, D		Calves	USA
	Sakurai	Necrotic enteritis	A		Dairy cows	Japan
	Sato and others	Necrotic enteritis	C		Beef cattle	Japan
1987	Katitch	Enteritis	A, C		Cattle	France
	Niilo	Enterotoxaemia	C		Calves	Canada
	Worrall and others	Enterotoxaemia	A		Buffaloes	Indonesia
1989	Popoff	Enterotoxaemia	B, C, D, E		Calves and adult cattle	France
1990	Hatheway	Enterotoxaemia	A, E		Calves and adult cattle	USA
1993	Barker and others	Enteric infection	C, D		Feedlot cattle	USA
1994	Manteca and others	Enterotoxaemia	A		Cattle	Belgium
1996	Daube and others	Enterotoxaemia	A		Beef cattle	Belgium
	Songer	Enteritis, enterotoxaemia	A, B, C, D, E		Calves and adult cattle	USA
1998	Billington and others	Enteritis	E		Calves	USA
	Glock and Degroot	Sudden death	A, C, D		Feedlot cattle	USA
1999	Petit and others	Enteritis, enterotoxaemia	A, B, C ,D, E	Yes	Calves	France
2000	Garmory and others	Diarrhoea	A, E	Yes	Calves	USA
	Manteca and others	Enterotoxaemia	A	Yes	Cattle	Belgium
2001	Manteca and others	Enterotoxaemia	A	Yes	Calves	Belgium
2002	Dennison and others	Haemorrhagic bowel syndrome	A	Yes	Dairy cattle	USA
	Manteca and others	Enterotoxaemia	A	Yes	Calves	Belgium
2003	Bueschel and others	Sudden death, enterotoxaemia, enteritis	A, C, EE	Yes	Calves and adult cattle	USA
	Efuntoye and Adetosoye	Diarrhoea	A, C, D		Cattle	Nigeria
	Manteca	Enterotoxaemia	A	Yes	Cattle	Belgium
	Philippeau and others	Enterotoxaemia	Not specified		Cattle	France
	Sipos and others	Enteritis, enterotoxaemia	A, E		Domestic and exotic ruminants	Austria
2004	Abutarbush and others	Jejunal haemorrhage syndrome	A		Beef cows	Canada
	Manteca and others	Enterotoxaemia	A	Yes	Cattle	Belgium
	Schotte and others	Enterotoxaemia	A		Calves and adult cattle	Germany
	Songer†	Enteritis, enterotoxaemia	A, B, C, D, E		Calves and adult cattle	USA
	Songer and Miskimmins	Enteritis	E		Calves	USA
2005	Abutarbush and Radostits	Jejunal haemorrhage syndrome	A		Dairy and beef cattle	Canada
	Dennison and others	Haemorrhagic bowel syndrome	A	Yes	Dairy cattle	USA
	Jost and others	Enteric diseases	A, C, E	Yes	Cattle	USA
2006	Johansson and others	Enteritis	A	Yes	Roe deer	Sweden
	Rooney and others	Haemorrhagic enteritis	Not specified		Cattle	USA
	Sawires and Songer	Enteritis, sudden death	A, C, E		Cattle	USA
2007	Ceci and others	Haemorrhagic bowel syndrome	A	Yes	Dairy cattle	Italy
	Lebrun and others	Enterotoxaemia	A	Yes	Cattle	Belgium
	Muskens and others	Jejunal haemorrhage syndrome	A suspected		Dairy cows	Netherlands

* Some β2-positive strains were detected in these studies
† Personal communication
EE Type E and enterotoxin positive

lesion is acute necrohaemorrhagic jejunoileitis, with a thickened haemorrhagic and necrotic intestinal wall and abundant liquid haemorrhagic contents (Manteca and others 2001). The affected intestinal loops may extend from only 10 cm to the entire length of the small intestine. In some cases, the lesions can involve more than one intestinal segment, with successive uninjured and injured loops providing a picture of segmentary enteritis (Worrall and others 1987). However, in a few peracute cases, the intestinal wall is only congested. There is also gas accumulation and a reactive secondary satellite adenitis of the mesenteric lymph nodes (Manteca and Daube 1994, Glock and DeGroot 1998). Similar lesions were described in the past in North American Hereford calves from high-quality dairy cows (Griner and Bracken 1953). The postmortem lesions of HBS cases (type A) differ to some extent, since blood clots sticking to the intestinal wall and sometimes fibrinous peritonitis are described (Dennison and others 2002, Abutarbush and Radostits 2005).

Microscopic lesions

Under the microscope, the intestinal lesions (haemorrhages, cell necrosis) extend from the tip of the villi to the base of the crypts. There is progressive erosion of the villi reaching the conjunctive axis and causing haemorrhages. White blood cell infiltration is present with massive margination of neutrophils and accumulation of lymphocytes in small blood vessels in the underlying mucosa (Arbuckle 1972, Sakurai 1985, Worrall and others 1987, Manteca and others 2001, 2002). In ortho- and parasympathetic nodes of the muscular and mucosal layers of the gut, hydropic degeneration is observed (Manteca and others 2000, Manteca 2004).

Internal organs can present lesions typical of toxaemia. The liver parenchyma can present centrilobular vacuolar degeneration or necrosis and the serous membranes may show petechiae. The kidneys may be congested with parenchymatous degeneration of convoluted tubules and sometimes haemorrhagic areas lining the cortex and medulla (Worrall and others 1987).

The local and systemic microscopic lesions of bovine enterotoxaemia are reminiscent of cases of necrotic enteritis in poultry caused by type A of *C perfringens* (Vissiennon and others 1996). In cattle, clusters of bacteria similar in appearance to *C perfringens* can be observed sticking to the intestinal cells. These bacteria remain localised in the necrotic areas (Manteca and others 2001) and do not cross the lamina propria or spread deeply through the intestinal wall.

Bacteriological findings
Clostridial population

In the past, bacteriological data were scarce, since diagnosis was usually limited to the growth of the bacteria in the intestinal contents on blood agar plates in anaerobic conditions followed by identification and typing of one or two colonies of the predominant colony type, most often *C perfringens* (Manteca and Daube 1994). More recently, quantitative bacteriological analysis of the intestinal contents of typical enterotoxaemia cases, and comparison with other cases of 'sudden death' for which the diagnosis of enterotoxaemia could be excluded, confirmed the fact that bovine enterotoxaemia is characterised by a drift of the commensal flora of the small intestine and by uncontrolled overgrowth of *C perfringens* at the location of the necrohaemorrhagic lesions (Popoff 1998, Manteca and others 2001). *C perfringens* is normally present at a level of 10^2 to 10^5 cfu/ml of intestinal contents but can reach counts higher than 10^6 to 10^8 cfu/ml of intestinal contents in cases of enterotoxaemia (Manteca and others 2001, Philippeau and others 2003).

In cattle, it is questionable whether the overgrowth involves the whole *C perfringens* population or is limited to one toxinotype (Manteca and others 2001, Manteca 2004) or even to one clone as described in poultry (Engström and others 2003, Nauerby and others 2003, Gholamiandekhordi and others 2006, Rooney and others 2006). Avian strains isolated from clinical outbreaks of necrotic enteritis (mainly *netB*-positive) present an interesting property. They are able to inhibit the growth of other *C perfringens* strains more efficiently than strains isolated from healthy chickens (Timbermont and others 2009). This intraspecies interstrain growth inhibition could be an advantage in the competition for the supply of essential amino acids, allowing exponential multiplication of those strains.

Strain typing

In Europe, type A non-enterotoxigenic *C perfringens* is mainly isolated from enterotoxaemic cattle (Daube and others 1994, 1996b, Garmory and others 2000, Manteca and others 2001, Johansson and others 2006). HBS, encountered in feedlot and dairy cattle in the USA, is also associated with type A non-enterotoxigenic *C perfringens* strains (Dennison and others 2002, 2005, Abutarbush and Radostits 2005, Ceci and others 2006).

The situation could nevertheless be different in some cases. Glock and Degroot (1998) identified types C and D as responsible for enterotoxaemia in feedlot cattle in the USA. Moreover, the presence of the *cpe* gene (with or without cpe expression) in type A strains or other types complicates the interpretation of the results. Bueschel and others (2003) demonstrated that 51 out of 91 bovine strains were of type A *cpe*-negative, 37 were of type EE (type E, *cpe*-positive) and the other five were of type C *cpe*-negative. But the *cpe*-positive strains seemed to be unable to produce the corresponding toxin (in vitro). Silent *cpe* gene carriage in type A *C perfringens* was also described in healthy and diarrhoeic human beings (Fisher and others 2005, Heikinheimo and others 2006). In a Nigerian study, six of the 11 type A cattle strains were *cpe*-positive and able to produce enterotoxin (Efuntoye and Adetosoye 2003). However, the origin of the strains was not clearly described in those studies; the information was limited to 'calves with enteritis or abomasitis' or 'diarrhoeic cattle'.

If type A represents the main toxinotype isolated in cattle (Yoo and others 1997), strains of this toxinotype present a high degree of genetic diversity (Ginter and others 1996, Manteca and others 2002, Waters and others 2003). Johansson and others (2006) observed that epidemiologically related isolates were of the same pulsed-field gel electrophoresis type, which could partially explain some differences linked to geography and breeding usage (Johansson and others 2006). In the same way, the association between a similar clinical condition and the toxinotype was also described by Rooney and others (2006). The most recent study on *C perfringens* clonal relationships was performed by multilocus sequence typing and allowed regrouping of strains in clusters (Jost and others 2006). Type A strains represent the majority of one cluster and are found in another of the three major clusters. This technique helps to identify host-species relationships with respect to these *C perfringens* isolates.

There is thus strong evidence that type A strains are the main strains involved in cattle enterotoxaemia. But, within type A, it is likely that toxins other than α toxin contribute to the pathophysiological process. In this context, some studies related to the β2 toxin provide new insights on the subject.

The β2 toxin

The β2 toxin is the fifth major toxin of *C perfringens*. The major toxins are lethal for mice after intravenous administration. The β2 toxin-producing strains (type C) were originally isolated from necrotic enteritis in young piglets (Gibert and others 1997). The β2 toxin has comparable biological activities to the first described β toxin (lethality to mice, haemorrhagic necrosis of intestinal wall in the guinea pig ligated loop model and cytotoxic properties) but the two toxins differ in molecular size and lack a significant amino acid identity (Gibert and others 1997).

Different variants of the β2 toxin have been described in animals and in human beings, with some being more toxic than others (Fisher and others 2005) and some encoding genes or alleles that are expressed more than others due to differences in the gene sequence promoting or impairing expression (Jost and others 2005). The variant present in the porcine strains associated with haemorrhagic diarrhoea (type C) seems to be toxic and highly expressed (Waters and others 2003). This good correlation between the presence of the gene and toxin expression has also been observed in horses with typhlocolitis (type A) (Bacciarini and others 2003). The situation is less clear in cattle. Bueschel and others (2003) reported that less than 50 per cent of *C perfringens* bovine strains (from enteritis and enterotoxaemia) produced the β2 toxin. In a study performed on ligated intestinal loops, Manteca and others (2002) suggested that α and β2 toxins act in a synergistic way to induce intestinal lesions in calves. Lebrun and others (2007) demonstrated the exclusive pres-

ence of a specific allelic variant, *cpb2*, and the highest expression of the corresponding toxin variant among isolates from enterotoxaemic calves compared with healthy animals.

These results strengthen the possible role of the β2 toxin in cattle enterotoxaemia, but further studies related to different variants are needed (van Asten and others 2010).

Pathogenesis
Bacteriology
Threshold of toxicity and possible toxin association
For many years the striking characteristics of cattle enterotoxaemia were the dramatic and sudden change in the intestinal flora at the location of the lesions of necrohaemorrhagic enteritis, characterised by the uncontrolled multiplication and overgrowth of *C perfringens*, with local overproduction of toxins (Barker and others 1993, Songer 1996, Popoff 1998). The following hypothesis can be formulated to explain the pathogenesis of cattle enterotoxaemia: a local overgrowth of some *C perfringens* clones associated with overproduction of highly expressed α toxin and a potent and highly expressed β2 toxin variant or another α-synergistic toxin and the absorption of, at least, the α toxin into the bloodstream (Barker and others 1993, Popoff 1998, Manteca and others 2001).

If the absorption of the α toxin is the most likely explanation of the sudden death of calves suffering from enterotoxaemia, although this remains to be proved, the hypothesis of a synergy between the α toxin and a β2 toxin variant was proposed to explain the lesions of necrohaemorrhagic jejunoileitis observed in cattle enterotoxaemia with toxinotype A strains. Moreover, the level of production of the α toxin may also play a role. Indeed, in a ligated intestinal loop assay in one calf, Manteca and others (2002) reproduced typical lesions of necrohaemorrhagic enteritis with one β2 toxin-positive strain, and not with any of the β2 toxin-negative strains. However, this β2 toxin-positive strain also had a higher level of production of the α toxin. The next experimental steps should therefore be: to demonstrate the actual production of the β2 toxin variant in vivo in the calf intestine; to compare the level of production of the α toxin between strains isolated from cases of enterotoxaemia and other calves, and between strains negative or positive for the different β2 toxin variants; to test different mutants and complement strains (those genetically modified) for the level of expression and production of the α and β2 toxins in the ligated intestinal loop assay; and to test the competition and inhibition between different strains of the same species of bacteria.

Some specific conditions
When *C perfringens* grows in high numbers, the bacteria seem to be able to attach or, at least, get close to the enterocytes. This protects the bacteria from certain immune system components (Arbuckle 1972, Niilo 1986, Popoff 1998). This situation creates a microspace where toxins are concentrated and partially protected against destruction, and also enhances the imbalance between production and elimination of toxins (by transit, proteases and antibodies) (Popoff 1998, Manteca and others 2001, Efuntoye and Adetosoye 2003).

Moreover, the toxinogenic clostridia would be better adapted to obtain nutrients essential for their proliferation than non-toxinogenic strains (Jost and others 2006, Schotte and others 2004). Indeed, *C perfringens* strains possess few metabolic enzymes and are unable to produce some essential amino acids, which they must find in their environment. Enzymes and/or toxins therefore allow a more efficient and earlier food supply because they are associated with eukaryotic cells and extracellular matrix lesions that release nutrients not normally available to bacteria, and consequently multiplication is more effective. This metabolic situation may partially explain why toxinogenesis occurs very early in *C perfringens* growth phases (early in the logarithmic phase). This effect should be emphasised if an intraspecies interstrain growth inhibition was demonstrated between cattle strains, as has been shown in poultry strains (Timbermont and others 2009).

Paralytic ileus
Causes and predisposing factors
The causes of the flora drift are still not completely elucidated, although a poststress modification in the intestinal environment is strongly suspected (Worrall and others 1987, Huis in 't Veld 1991, Manteca and others 2000). This stress would lead to intestinal stasis (paralytic ileus) or, at least, to a decrease of the intestinal transit (Manteca and others 2001).

According to epidemiological studies (Braun and others 1993, Manteca and others 2000, 2001, Berghaus and others 2005), problems in feeding are the main origin of the stress-induced paralytic ileus and small intestinal flora drift. As in other species (sheep, poultry and pigs) drastic feeding modifications, overfeeding, or a protein- and easily fermentable starch-rich diet with few raw fibres (cellulose, hemicellulose and lignin), favour enterotoxaemia as well as other digestive problems (Niilo 1986, Riddell and Kong 1992, Annett and others 2002). These situations occur mainly in beef and veal production, in which the aim is to reach maximal average daily weight gain, but can also occur in high-producing dairy cattle near the lactation peak (Glock and DeGroot 1998, Dennison and others 2002, Abutarbush and Radostits 2005). A maximal risk level is also observed in suckling calves grazing on lush grass (Manteca and Daube 1994).

Some reports of human (Zoppi and others 1998) and pig (Okewole and others 1991) diseases showed that constipation may also lead to significant *C perfringens* overgrowth of the intestinal flora.

The degenerative lesions in the ortho- and parasympathetic nodes of muscular and mucosal layers of the gut may also be correlated with the existence of such a paralytic ileus (Manteca and others 2000, Manteca 2004).

Other causes favouring paralytic ileus are other infections, parasitism, injuries and/or hepatic insufficiency, which increase the absorption of the toxins (Philippeau and others 2003).

Consequences
The paralytic ileus cancels the beneficial flush effect of intestinal transit and therefore favours the multiplication and overgrowth of *C perfringens* in the small intestine, with consequent toxin(s) production in the location of the paralysed intestinal segments (Fleming 1985). The paralytic ileus would therefore explain the localised, sometimes segmentary, aspect of the intestinal lesions and the localised clostridial overgrowth.

The effects of the toxins themselves must be added, since some increase intestinal permeability, enhancing their own absorption and the absorption of other toxins.

Cause of death
Toxaemia with consequent multi-organ failure is the most probable cause of the sudden death. The vital parenchymas (for instance, in the lungs, liver or kidneys) present lesions of toxaemia (Radostits and others 2007b, c, d).

Diagnosis
Field criteria
Diagnosis of cattle enterotoxaemia requires an integrated approach. Sudden death without any premonitory signs and the presence of acute haemorrhagic jejunoileitis with haemorrhagic intestinal contents at postmortem examination are highly specific, although not exclusively of cattle enterotoxaemia in Europe. Information on breeding conditions and triggering factors previously described must be taken into account, with particular attention to any stress observed 12 to 36 hours before death.

Laboratory results
In the attempt to confirm the suspicion of enterotoxaemia in cases of sudden death, laboratory analysis is a necessity to make the distinction from other causes of sudden death (for example, septicaemia or the animal being struck by lightning). It must be emphasised that the results of the laboratory analysis come to support the field suspicion, based on the above reviewed criteria, and never the opposite.

Sampling
The ideal sample obtained at postmortem examination for bacterial analysis is a ligated intestinal loop in the location of the necrohaemorrhagic lesions. Meaningful samples for bacterial isolation come from freshly dead animals. *C perfringens* is a member of the normal microbiota and can rapidly invade and multiply in high numbers in the

intestine and in the internal tissues, taking part in the postmortem putrefaction process, but most importantly biasing the quantitative bacterial analysis. Ideally, intestinal samples should be taken less than three hours after death. After this delay, in euthanased healthy animals, clostridial (*C perfringens*) counts reach levels similar to those in enterotoxaemia cases (Philippeau and others 2003).

Unfortunately, since calves are most frequently 'found dead', the time of death is rarely determined and the delay between death and sampling is most often unknown. Therefore, in practice, postmortem examination must be performed on-site (or on-farm) as quickly as possible and the ligated intestinal loop should be kept at 4°C in appropriate anaerobic transport conditions (in an anaerobic jar) to reduce the multiplication of *C perfringens* for the next 24 hours (Songer and Miskimmins 2004, J. Songer, personal communication). If any sample must be kept for more than 24 hours before bacterial analysis, it should be frozen at –20°C, keeping in mind that freezing is lethal for a proportion of the bacteria present, including *C perfringens*.

Routine bacterial analysis

Examination of a Gram-stained direct smear of the intestinal content can help in detecting a large number of Gram-positive bacilli, but quantitative anaerobic growth on blood agar is the gold standard to confirm the field suspicion of enterotoxaemia due to *C perfringens* (Popoff 1998, Manteca and others 2001, Philippeau and others 2003). To generate anaerobic conditions, a jar with an oxygen-consuming system is enough, since *C perfringens* is oxygen-tolerant (Bergey and others 1939). After overnight growth, the typical fried egg-shaped colonies of *C perfringens* are surrounded by a double β-haemolysis zone (Bergey and others 1939, Manteca and others 2001). If necessary, a 2 per cent agar plate with phenylethanol limits the overgrowth of other bacteria (Songer and Miskimmins 2004). Some specific selective media allow the rapid growth and specific detection of *C perfringens* by means of chromogenic and/or fluorogenic substrates (Gubash and Ingham 1997, Adcock and Saint 2001).

As already mentioned, the number of *C perfringens* present in the region of the lesions is the most important bacterial criterion. This is one further reason to carry out bacterial analysis only as a support to the field suspicion. The first authors to advise systematic *C perfringens* enumeration were Sato and others (1985) and Sakurai (1985). The cut-off number of cfu, however, remains debatable and varies according to the authors (Popoff 1998, Manteca and others 2001, Philippeau and others 2003). In cattle, counts higher than 10^6 to 10^7 cfu/ml of intestinal contents are considered as a hallmark of *C perfringens* overgrowth and confirmatory of a field suspicion.

Toxin typing

Toxin typing can be performed by phenotypic or genotypic assays. Phenotypic assays such as the mouse seroneutralisation assay after intraperitoneal or intravenous injection of four- to six-hour culture supernatants (gold standard) or ELISAs aim to detect the production of the toxins (Hatheway 1990). Genotypic assays such as DNA colony hybridisation with gene probes and PCRs aim to detect the toxin-encoding genes (Daube and others 1996b, Kadra and others 1999, Johansson and others 2005).

However, toxin typing of the isolates of *C perfringens* is rarely applied routinely. The laboratories must be appropriately equipped to perform some tests and several colonies (up to 10) have to be typed to give an appropriate result. Moreover, in the case of cattle enterotoxaemia, the typing should include the β2 toxin, which can be routinely detected only by genetic methods. Genotyping results are sometimes difficult to interpret because of the existence of different gene variants and different levels of expression of the same variant.

Nevertheless, when applied, *C perfringens* toxin typing is performed mostly by PCR in specialised laboratories because PCR can detect any combination of toxin-encoding genes present in the isolates in a one-step procedure and can also be applied to clinical cases involving *C perfringens* in other diseases and/or animal species. Some PCR methods do not require the complicated, time-consuming and costly bacterial culture and DNA extraction phases but can by applied on (almost) raw samples, such as faeces or urine (Persson and Olsen 2005, van den Berg and others 2006, Zhang and others 2006). Unfortunately, there

also exist limits and biases such as the detection of silent or underexpressed genes or the loss of the gene of interest during in vitro growth (Rood and Cole 1991). Indeed, the genes coding for different toxins are (*cpb, etx, iap-ibp, cpb2*) or can be (*cpe*) located on mobile elements (plasmids or transposons) that can be lost. It is especially advisable to limit the number of handlings and subcultures in an aerobic atmosphere to reduce this risk (Johansson and others 2005), and the use of an anaerobic cabinet is therefore highly recommended.

Direct or in vivo toxin detection

Different tests based on toxin detection with specific antibodies can also be directly applied to biological samples, such as intestinal contents, faeces or serum. Commercial kits are available. They are rapid and easy to use in the diagnostic laboratory, for example, ELISA (Bio-X) (Ginter and others 1994) or slide latex agglutination kits (Gentaur; Oxoid) (McClane and Snyder 1987, Fach and Popoff 1997, Kadra and others 1999), or primarily by the practitioner on-site during the postmortem examination such as immunosticks (Bio-X). These tests are available for α, β and ε toxins, but not yet for the ι and β2 toxins.

When these tests are applied in the laboratory, delay and conditioning of the samples are also crucial factors, because toxins are rapidly degraded in the gut by proteases (Popoff 1998). In any case, a positive result (presence of the toxin) is meaningful, taking into account the general context and the other criteria, while a negative result is not interpretable.

Prophylaxis

Since cattle enterotoxaemia is a peracute syndrome with a case fatality rate close to 100 per cent, there is no treatment. Prevention measures are important and should be applied when some risk factors are present or when some animals die of enterotoxaemia and other animals are exposed to the same stressors.

Management

Good practice in animal management is crucial. The aim is to promote slow food transitions and diets rich enough in fibre to allow equilibrium of the intestinal flora. Careful attention should be given to avoid stressful situations related to feeding or the environment, such as a sudden change in diet or animal regrouping (Popoff 1998, Manteca and others 2000). These suggestions are based on field observations and should be adapted to each individual situation according to the case history and farm management.

Medical
Vaccination

There are several commercial vaccines are against clostridial enterotoxaemia in ruminants. All marketed vaccines in Europe contain at least one of *C perfringens* toxinotypes and most contain the β and ε toxoids. Several also contain the α toxoid and raw culture supernatants of *C perfringens*. In addition, several other clostridial species toxoids and/or bacteria are usually present in the vaccines: *Clostridium chauvoei, Clostridium novyi, Clostridium sordellii, Clostridium septicum* and *Clostridium tetani*. A few vaccines also include other bacteria such as *Mannheimia haemolytica* or enterotoxigenic *Escherichia coli*.

Effectiveness of vaccination is better in small ruminants and pigs (Kelneric and others 1996, Songer 1996) than in cattle. Indeed, there are examples of breakdown in the field when clostridial vaccines are used in cattle (Glock and Degroot 1998). A first difficulty is the absence of an experimental model of the enterotoxaemia syndrome (Manteca and others 2001). Moreover, no current vaccines contain β2 or enterotoxin toxoids and α toxoid is present in only some vaccines. For α toxin, divergent opinions exist concerning its implication in clostridial enteritis/enterotoxaemia and its subsequent importance in vaccine composition. In healthy cattle, α toxin is present and there is a basic level of antibodies to α in serum, meaning that a part of produced toxin may be absorbed in the blood and processed by the immune system. The serological threshold to obtain clinical protection is empirically fixed because any experimental protection has not been determined in vivo. But after vaccination, it was demonstrated that seroconversion was positively correlated with the quantity of antigen present in the vaccine (Manteca and others 2004). In that study, the highest titres were

obtained in two- to four-month-old calves using a first injection at one month of age followed by a booster one month later. Further boosters were necessary twice a year. If the risk of infection is considered high at birth, the cows may be vaccinated at seven and eight months of gestation. In this case, calves from vaccinated dams should not be vaccinated before the fourth month of life because passive immunity interferes with active immunisation. For vaccines without α toxoid, a booster is recommended every year. Finally, it must be emphasised that the sporadic course of the disease in small-scale farms (one to two cases per year) limits the economic interest of vaccination against enterotoxaemia compared with vaccination against epidemic pathologies.

Improvements in the vaccines currently available are needed. At the present time, anticlostridial vaccines are raw culture supernatants. Cultures are inactivated and bacterial bodies are removed, but they contain a lot of uncharacterised and unstandardised elements such as minor toxins and metabolic waste. These elements may disturb the immune response to toxoids of interest and induce a local inflammatory response and allergic reactions (Stokka and others 1994, Uzal and others 1999). The production of vaccines from virulent strains also requires stringent safety conditions, costly detoxification and control steps. It may be possible to create targeted vaccine(s) composed of non-toxic peptides. That seems feasible, as all *C perfringens* toxins genes have been cloned and expressed as recombinant proteins (α: Bennett and others 1999, Schoepe and others 2006; β: Steinthorsdottir and others 1995; α–β: Bai and others 2006; β–β2: Xu and others 2005; ε: Goswami and others 1996, Oyston and others 1998; ι: Sirard and others 1997; enterotoxin: Belyi and Varfolomeeva 2003; β2, Gibert and others 1997, Lebrun and others 2007). Another way would be to select strains producing naturally protective immunogenic but non-toxic variants of the toxins of interest, such as the 1121A/19 strain of *C perfringens*, which produces a non-toxic variant of α toxin that induces a protective response against different toxic variants of α (Schoepe and others 2001).

Antibiotics

In some rare cases of an enzootic-like form of the syndrome, it is sometimes recommended to administer antibiotics and/or immune serum prophylactically to protect all the animals living in the same conditions as the first victims, considering they were all exposed to the same initiating stress (Fleming 1985, Daube and others 1992, 1996a, Popoff 1998). Antibiotics like penicillin G can prevent the multiplication of *C perfringens* and the production of new toxin(s) but have no effect on the toxins already produced. *C perfringens* normally has little resistance to antibiotics, but can acquire multiple resistances in the case of frequent antibiotic use on the farm. Some mechanisms of resistance transmission are well studied, such as the tetracycline resistance model (Daube and others 1992, Lyras and Rood 1996, 1997, Dubreuil and others 2003, Dubreuil and Neut 2004, Johansson and others 2004, Lucey and Hutchins 2004, Menozzi and others 2004).

Serotherapy

Immune serum contains antibodies that can neutralise the circulating toxins, but treatment has no effect on toxins already fixed on their target cells. Moreover, neither antimicrobial agents nor hyperimmune serum has much therapeutic value, because death occurs too quickly to allow effective therapeutic intervention. The lack of identification of the toxins involved in cattle enterotoxaemia, the cost of immune serum and the existence of a risk of anaphylaxis, since the sera are produced in horses, render this approach very uncommon.

Probiotics

In the context of an enzootic-like form with a common initiating stress, the administration of probiotics, in a preventive way, could help the gut flora to overcome the stress. Some of these probiotic organisms are also used as growth promoters in the context of intensive breeding such as in feedlots (Klaasen and others 1999, Reid and Friendship 2002, Beauchemin and others 2003, Ewaschuk and others 2004, Barbosa and others 2005, Timmerman and others 2005, Jouany 2006, Stein and others 2006, Kim and others 2007, Yasuda and others 2007).

Conclusions

Cattle enterotoxaemia affects predominantly young beef cattle, in an intensive breeding context such as the feedlot or veal industry, or high-producing dairy cattle in the case of HBS. For the veterinary clinician, the first diagnostic hypothesis is based on the case history (sudden death, stress) and the lesional aspect at postmortem examination (haemorrhagic jejunoileitis). These suspicions would be ideally confirmed by both bacterial anaerobic culture and *C perfringens* counting if samples can be processed in good conditions and rapidly. After bacterial identification, the toxin type(s) associated preferentially with cattle enterotoxaemia has to be determined. In cattle enterotoxaemia and HBS, type A is the dominant toxinotype.

There are difficulties in making an exhaustive review of cattle enterotoxaemia. In some studies the exact origin of *C perfringens* strains is not indicated but only associated with 'intestinal troubles, enteritis, diarrhoea' or just 'sudden death'; the citation 'suspected of clostridial disease' is also a generic term commonly used. The criteria to determine whether or not it is enterotoxaemia are not always clearly defined, for example when using methods such as *C perfringens* counting. Two other elements can be confusing. First, with evolution in the techniques used, some strains originally classified into one toxinotype by the mouse seroneutralisation assay have been reviewed and reclassified into another toxinotype. As a consequence, some older studies are difficult to interpret. Secondly, the classification of the toxins into 'major' and 'minor' toxins is somewhat debatable. Indeed, some minor toxins have been proved to play a more important role than initially expected, like θ toxin or perfringolysin in the pathogenesis of gas gangrene. New classification of the strains of *C perfringens* should also be a future major research domain.

In the field, clostridial enterotoxaemia has both cost and animal welfare implications for cattle breeders, who may lose calves of high value to the disease. While further studies are expected to provide better understanding of the pathogenicity of *C perfringens*, as well as progress in vaccination strategies, good breeding management and feeding practices remain the best means of preventing enterotoxaemia in cattle.

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