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8 **Isolation of a clonal population of *Clostridium perfringens* type A from a**
9 **case of abomasal ulceration in a Belgian Blue calf**
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Summary

A case of abomasal ulceration in a 3 month old Belgian Blue calf is described. Histopathology showed a typical neutrophil demarcation line in the abomasal ulcers and intestinal mucosa, separating underlying healthy tissue from the superficial fibrinous necrotic material in which the bacteria resided. Pulsed Field Gel electrophoresis confirmed that a genetically clonal population of *Clostridium perfringens* type A was isolated from multiple ulcers and from the gut content of the animal.

SHORT PAPER

Abomasal ulceration and abomasitis are important problems in veal calves. High prevalence rates have been reported, indicating the economical importance of the disease (Wensing *et al.*, 1986; Welchman and Baust, 1987; Braun *et al.*, 1991; Marshall, 2009). Abomasal ulcers are classified into four types, depending on the severity of the lesions. Nonperforating abomasal ulcers either or not with severe blood loss are classified as type 1 and 2 ulcers, respectively, while perforating ulcers with local and diffuse peritonitis are classified as type 3 and 4 ulcers (Marshall, 2009). Clinically, abdominal pain and distension, bloat, diarrhea (containing blood in case of type 2 ulcers), lethargy, and sudden death can be observed. The underlying cause of abomasal ulceration is not yet clear, and different factors are believed to be associated with disease induction. Proposed predisposing factors include environmental and physical stress conditions, trichobezoars, mineral deficiencies, hyperacidity of the abomasum, and dietary factors, including overfeeding (Liley *et al.*, 1985; Wensing *et al.*, 1986; Mills *et al.*, 1990; Jelinski *et al.*, 1996; Marshall, 2009).

Some authors have tried to link infectious agents with abomasal ulcer formation in calves (Mills *et al.*, 1990; Jelinski *et al.*, 1995). *Clostridium perfringens* has often been mentioned as a potential pathogen involved in abomasal ulcer induction. This bacterium is widespread in

the environment and harbors a range of toxins and enzymes that cause different forms of tissue damage, depending on the host species (Songer, 1996; Gibert *et al.*, 1997). *Clostridium perfringens* has already been isolated from the abomasal content of calves with abomasal ulceration. When Roeder *et al.* (1987) cultured specimens of ruminal contents collected via stomach tube or at necropsy and abomasal contents collected at necropsy, *Clostridium perfringens* was isolated from all specimens. However in other reports no *Clostridium perfringens* bacteria were cultivated from the abomasum of affected animals (Mills *et al.*, 1990). The site of sampling (e.g. healthy or diseased tissue, content or scraping) could be one explanation, but also the lack of *Clostridium perfringens* bacteria in the abomasum. In a case-control study of Jelinski *et al.* (1995) involving 14 cases and 16 controls, *Clostridium perfringens* strains were isolated in about 75% of the animals in both populations. This indicates that *Clostridium perfringens* is most likely present in the abomasum, but it could be due to post-mortem multiplication or simply due to passage through the gastro-intestinal tract. This points to the importance of the sampling protocol. Own data show that sampling should be done in such a way that multiple necrotic areas at different locations are sampled and that only isolation of identical clones from different spots indicates the presence of a pathogenic strain, at least in chicken necrotic enteritis (Gholamiandehkordi *et al.*, 2006; Timbermont *et al.*, 2008). A few reports clearly indicate a role for *Clostridium perfringens* in abomasal ulceration. Firstly, Roeder *et al.* (1988) experimentally reproduced abomasal ulcers in neonatal calves after intraluminal inoculation of high numbers of *Clostridium perfringens*. Secondly, Songer *et al.* (2004) isolated huge numbers of *Clostridium perfringens* from a case of abomasitis, in which Gram-positive rods were associated with necrotic lesions in the gut tissue.

In the case of *C. perfringens* associated gastro-intestinal diseases in animals, it is speculated that a specific *Clostridium perfringens* strain is causative, when (1) the bacterium can be isolated from the diseased tissue in high numbers in nearly pure cultures, (2) the bacteria can be isolated at different spots in the diseased tissue, in clonal form, as confirmed by genetic

tools, and (3) the disease can be induced after inoculation in order to fulfill the postulates of Koch.

In order to show a possible etiological role of *Clostridium perfringens* in abomasal ulcer formation in calves, the first 2 points stated above were tested in a case of sudden death caused by abomasal ulceration. In February 2009 a 100 kg male Belgian Blue calf, aged 3 months, was presented at the Faculty of Veterinary Medicine with sudden onset of depression and sternal decubitus evolving into lateral decubitus and neurological symptoms. The calf had yellow pasty diarrhea with a trace of blood since one day. It was treated with lincomycin and spectinomycin by the local veterinarian and received electrolyte solutions. The local veterinarian gave one liter of polyionic perfusion. The animal was group housed (6 calves) on straw. The calf was fed an ad libitum 63% commercial calf concentrate (14 per cent rough protein) and 37% spelt combination. Fresh water and hay were provided. Mental state was severely depressed (stuporous) and the abdomen was bilaterally distended. Rectal temperature was 37°C and no faeces were present. Tachycardia (120 bpm) and tachypnea (60 bpm) with superficial breathing were present. The mucosae were pale, capillary refill time was increased and the extremities were cold. On simultaneous ballottement and auscultation fluid splashing sounds were audible in the right abdomen. Transcutaneous ultrasonography with a 2,5 mHz sector probe (Sonos 100; Hewlett Packard) showed a large amount of gas in the right abdomen. Moderately distended small intestines with no motility were seen in the loin region. The abomasum could not be properly evaluated because of reverberation artefacts, indicating the presence of gas in the intestinal tract. Blood gas analysis revealed a severe metabolic acidosis (pH= 6,661 (7.35-7.45), PCO₂= 46,1 mmHg (35.0-45.0), PO₂= 28,1 mmHg (30.0-50.0), Base Excess (BE)= out of range (-5 to 5), HCO₃⁻= out of range (20.0-30.0)) and moderate dehydration (PCV= 0,41 (0,25-0,35). White blood cell count was 21,1 x 10⁹/L (reference value 6,0-9,0 x 10⁹/L) with 77,9 per cent granulocytes. The animal died and a necropsy, including sampling for bacteriological analysis, was performed within 15 minutes after death, excluding post-mortem spread and extensive multiplication of the bacteria.

Macroscopical lesions included abomasal nonperforating type I ulcers containing hemorrhagic material (Figure 1). Several hundreds of small lesions were located on the edges of the abomasal folds and in the abomasal wall (Figure 1c). One large ulcer, about 4 cm in size, surrounded by fresh blood was also observed (Figure 1b). The small intestinal wall was moderately thickened and the mucosa was covered with mucous content. Liver and spleen were slightly swollen. Histologically, abomasal ulcers presented demarcated areas of coagulative mucosal necrosis and hemorrhage, sometimes extending into the submucosa. Moderate numbers of neutrophils and macrophages were infiltrating these lesions, demarcating small numbers of rod-shaped bacteria adherent to the surface of the necrotic mucosa. In the small intestinal wall, there was multifocal to coalescing coagulation necrosis of the epithelial cell layer and lamina propria at villous tips, resulting in blunting of villi, marked suppurative inflammation and fibrin accumulation. Crypts were dilated and often filled with necrotic debris, some crypts were completely necrotic. Numerous rod-shaped bacteria were observed attached to the necrotic debris (Figure 2). All layers were congested and the lamina propriae and submucosa were diffusely infiltrated by macrophages and neutrophils. The histological view of the small intestine almost completely matched the picture as seen in poultry necrotic enteritis (Gholamiandehkordi *et al.*, 2007). In the liver, there were randomly distributed foci of hepatocellular coagulative necrosis ranging in size from 500µm to 5000µm. Portal areas were congested and moderately infiltrated by mononuclear cells (Figure 3). In peri-arteriolar sheaths of the spleen, multiple pyknotic nuclei were seen. Histological diagnoses of abomasal ulceration and multifocal to coalescing fibrinonecrotic enteritis with intralesional rod-shaped bacteria, multifocal necrotizing hepatitis and splenitis were made.

Five (5) swabs were taken from different focal ulcers, 2 from the hemorrhagic ulcers at the edges of the abomasal folds, 2 from the large abomasal ulcer, and 2 from a hyperemic zone in the small intestine. Swabs were streaked (within 30 min after sampling) on Columbia agar (Oxoid, Basingstoke, UK) with 5% defibrinated sheep blood, and on similar plates containing

150 12 mg kanamycin sulphate and 30.000 U polymyxin B sulphate per l. Plates were incubated
151 during 24h at 37°C under anaerobic conditions. *C. perfringens* colonies were identified by
152 characteristic colony morphology, dual haemolysis and Gram staining. After incubation,
153 plates showed an overgrowth of *Clostridium perfringens* (ranging from a few hundreds to
154 uncountable numbers), even when no antibiotic selection was performed. Two (2) colonies
155 from each plate (one plate per swab) were randomly picked and frozen for further analysis.
156 The toxin type of the 22 *C. perfringens* isolates was determined by a multiplex PCR, as
157 described by Yoo *et al.* (1997), while the presence of the enterotoxin, the NetB and the beta2
158 toxin genes was detected with single PCR reactions (Herholz *et al.*, 1987; Meer and Songer,
159 1997; Keyburn *et al.*, 2008). All isolates were toxin type A, and negative for all toxin genes
160 except the alpha toxin. The isolates were further characterized by Pulsed Field Gel
161 Electrophoresis (PFGE) after overnight anaerobic culture, while being shaken at 37°C in
162 Luria-Bertani broth (LB). The *Sma*I PFGE patterns were determined for all 22 *C. perfringens*
163 isolates using previously described PFGE methods with some slight modifications
164 (Gholamiandehkordi *et al.*, 2006). The patterns were grouped in a dendrogram with
165 GelCompar II software (Applied Maths, St.-Martens-Latem, Belgium) by using the Dice
166 coefficient and the unweighted pair group method with an arithmetic averages clustering
167 algorithm. A similarity of more than 95% was considered as the same PFGE type. Twenty-
168 one (21) out of the 22 isolates had an identical (100% similarity) PFGE pattern, pointing to a
169 clonal population in the abomasal ulcers, and even in the gut of the animal. The latter could be
170 caused by passage of *C. perfringens* from the abomasum into the small intestine. Anyhow,
171 these bacteria seemed to cause necrotic lesions in the gut. It has indeed been shown that
172 hemorrhagic enteritis and enterotoxemia due to *Clostridium perfringens* occur frequently, also
173 in combination with abomasal ulcers (Songer and Miskimins 2005; Lebrun *et al.*, 2007).
174 In conclusion, a clonal population of *Clostridium perfringens* toxin type A strain was isolated
175 from a case of abomasal ulceration and fibrinonecrotic enteritis in a Belgian Blue calf. This

suggests, at least in this calf, an etiological role of *Clostridium perfringens* in the abomasal ulcer induction, although other additional factors may also be involved.

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