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# A role for the *Clostridium perfringens* β2 toxin in bovine enterotoxaemia?

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## Abstract

Non-enterotoxigenic type A *Clostridium perfringens* are associated with bovine enterotoxaemia, but the oc toxin is not regarded as responsible for the production of typical lesions of necrotic and haemorrhagic enteritis. The purpose of this study was to investigate the putative role of the more recently described β2 toxin. Seven hundred and fourteen non-enterotoxigenic type A *C. perfringens* isolated from 133 calves with lesions of enterotoxaemia and high clostridial cell counts (study population) and 386 isolated from a control population of 87 calves were tested by a colony hybridisation assay for the β2 toxin. Two hundred and eighteen (31 %) *C. perfringens* isolated from 83 calves (62%) of the study population and 113 (29%) *C. perfringens* isolated from 51 calves (59%) of the control population tested positive with the β2 probe. Pure and mixed cultures of four *C. perfringens* (one α + β2 +, one α + enterotoxin + and two α+) were tested in the ligated loop assay in one calf. Macroscopic haemorrhages of the intestinal wall, necrosis and haemorrhages of the intestinal content, and microscopic lesions of necrosis and polymorphonuclear and mononuclear cell infiltration of the intestinal villi were more pronounced in loops inoculated with the oc and β2-toxigenic *C. perfringens* isolate. These results suggest in vivo synergistic role of the oc and β2 toxins in the production of necrotic and haemorrhagic lesions of the small intestine in cases of bovine enterotoxaemia. However, isolation of β2-toxigenic *C. perfringens* does not confirm the clinical diagnosis of bovine enterotoxaemia and a clostridial cell counts must still be performed.

**Keywords:** *Clostridium perfringens*; Toxins; Cattle-bacteria; Enterotoxaemia

## 1. Introduction

*Clostridium perfringens* is a Gram positive sporulated anaerobe responsible for necrotic and haemorrhagic enteritis and enterotoxaemia in humans and in different animal species. This bacterial species can produce up to 17 different toxins, five of which (the α, β, ε, t toxins and the enterotoxin) are responsible for the tissue lesions and the death of the hosts (Daube, 1992; Songer, 1996; Petit et al., 1999).

Bovine enterotoxaemia is characterised by a high case fatality rate, sudden deaths, lesions of necrotic and haemorrhagic enteritis of the small intestine and, most often, an absence of other clinical signs. Bovine enterotoxaemia occurs most frequently in beef cattle and is referred to as "Belgian Blue calf enterotoxaemia", although other breeds can suffer of this syndrome (Manteca and Daube, 1994; Manteca et al., 2000). The infectious aetiology of bovine enterotoxaemia has not been formally identified yet, but *C. perfringens* is often regarded as responsible (Popoff, 1989; Manteca and Daube, 1994; Songer, 1996). A recent survey of more than 70 typical cases in Belgium confirms toxin type A *C. perfringens* as putative aetiology (Daube et al., 1996; Manteca et al., 2001). However, if the α toxin is highly pathogenic for all species the reproduction of typical necrotic and haemorrhagic lesions has been performed only in chickens (Daube, 1992; Songer, 1996; Petit et al., 1999).

Another toxin of *C. perfringens* was described a few years ago, and named β2 (Gibert et al., 1997). β2 toxin is produced by *C. perfringens* isolated form piglets with haemorrhagic enteritis and from other animal species with, sometimes haemorrhagic, enteritis or diarrhoea (Herholz et al., 1999; Klaasen et al., 1999; Garmory et al., 2000; Gkiourtzidis et al., 2001). Because of the similarity of the lesions observed in calves with enterotoxaemia

(Manteca et al., 2000) and of those associated with the β2 toxin-producing *C. perfringens* isolates in piglets, the following study was done: (i) the collection of *C. perfringens* isolated from the bovine enterotoxaemia cases was re-examined by colony hybridisation for the presence of β2 toxin-encoding genes; (ii) β2-positive and β2-negative *C. perfringens* isolates were compared by ribotyping; and (iii) β2-positive and β2-negative *C. perfringens* isolates were compared in a ligated intestinal loop assay in one calf for the production of intestinal lesions.

## 2. Materials and methods

### *2.1. Bacterial strains*

Seven hundred and fourteen *C. perfringens* isolated from 133 cases of enterotoxaemia (study population) and 386 isolated from a control population of 87 Belgian Blue calves were tested in a colony hybridisation assay with a gene probe for the β2 toxin. According to the numbers of *C. perfringens* tested per calf (2-10), nine classes were defined in each population. Most isolates originated from the small intestine (Manteca et al., 2001), but additional isolates from extra-intestinal organs were studied. All belonged to the toxin type A and one was also positive for the enterotoxin (Daube et al., 1996; Manteca et al., 2001). Four *C. perfringens,* one urease- and toxin-negative *C. sordellii,* one *C. bifermentans* (Table 1), all isolated from typical cases of enterotoxaemia (Manteca et al., 2001), and the urease- and toxin-positive *C. sordellii* reference strain C582 (Pasteur Institute Collection, Paris, France) were tested in the ligated loop assay in one calf.

***Table 1:*** *Description of the Clostridia tested in the ligated intestinal loop assay*

| Strain | Laboratory identification | Pathotype[a] | ELISA assays for toxin | | |
|---|---|---|---|---|---|
| | | | α | β | ε |
| *C. perfringens* | Cp1 | α+β2+θ+μ+ | ++ | - | - |
| *C. perfringens* | Cp2 | α+Ent+θ+μ+ | + | - | - |
| *C. perfringens* | Cp3 | α+μ+ | - | - | - |
| *C. perfringens* | Cp4 | α+θ+μ+ | ++ | - | - |
| *C. sordellii* | Csl | HT-LT- | NR[b] | NR | NR |
| *C. bifermentans* | Cb | HT-LT- | NR | NR | NR |
| *C. sordellii* (C582) | Cs2 | HT+LT+ | NR | NR | NR |

[a]HT: haemorrhagic toxin of *C. sordellii;* LT: lethal toxin of *C. sordellii.* [b]Not relevant.

### *2.2. β2 gene probe and colony hybridisation assay*

The β2 toxin probe was a 600 bp DNA fragment derived by a *Hind*III and *EcoRI* restriction of the recombinant plasmid pMRP109 carrying the gene coding for the β2 toxin from a porcine isolate of *C. perfringens* (Gibert et al., 1997), kindly provided by Dr. M. Popoff from the Pasteur Institute, Paris. The probe was radioactively labelled using a random primed hexanucleotide labelling kit (Boehringer Mannheim). The colony hybridisation assay was performed as previously described (Daube et al., 1996).

### *2.3. 16S rRNA probe and ribotyping*

The 16S rRNA probe was derived from the pBA2 recombinant plasmid carrying the genes coding for the rRNA of *Bacillus subtilis* (Iglesias et al., 1983). Two DNA fragments of ca. 4.0 and 1.2 kb were generated by digestion of pBA2 with *EcoRI* restriction endonuclease. The larger DNA fragment, corresponding to the vector plasmid and to the 3' end of the 16S rRNA-encoding gene, was autoligated (plasmid pCUR2). The 16S rRNA probe corresponds to the 1.1 kb insert of pCUR2 and was recovered after digestion of pCUR2 with *Hind*III + *Eco*RI restriction endonucleases. The 16S rRNA probe was radioactively labelled using the random primed hexanucleotide labelling kit described.

Total DNA of 13 β2-positive and 34 β2-negative *C. perfringens* isolated from 12 different calves with enterotoxaemia was extracted according to an adaptation in small volumes of the procedure described by Van Damme-Jongsten et al. (1989) and digested by *Hind*III restriction endonuclease. The restriction fragments were separated by electro-phoresis in agarose gel (0.6 %) in Tris-acetate-EDTA (TAE) buffer 1 x, for 16 h at 2 V/cm,

and subsequently transferred onto nylon membranes (Hybond-N+, Amersham) by southern blotting (Maniatis et al., 1982). The filters were treated according to the instructions of the manufacturer and hybridised with the 16S rRNA probe using the same conditions as for the colony hybridisation assay (Daube et al., 1996). The hybridisation profiles were compared using Boolean analysis.

### *2.4. ELISA assays*

The sandwich ELISA assays for the α, β and ε toxins were performed as previously described (Ginter et al., 1994, 1996). Briefly, 96-well microtiter plates were coated with monoclonal antibody to the α toxin (DY2F5), to the β toxin (DAN1, received from the Statens Veterinaere Serumlaboratorium, Copenhagen, Denmark), or to the ε toxin (CK6G4). Toxin fixation was revealed using peroxidase-conjugated specific polyclonal immune sera produced in rabbits.

### *2.5. Intestinal ligated loop assay*

One healthy 2-week-old black and white calf originating from a farm with no record of vaccination against enterotoxaemia, and negative for *Salmonella,* intestinal parasites, rotavirus and coronavirus using routine detection methods, was anaesthetised with Xylazine® (0.1 ml/kg) and Gutajal® (500 ml)-Thiopental® (2 g) (1 ml/kg) prior to abdominal surgery. Twelve ligated loops of ca. 15 cm were created in the small intestine with interloop regions of ca. 10 cm. Eleven loops received 1 ml of pure or mixed cultures of the clostridial isolates and one loop received 1 ml of sterile broth.

The inocula were prepared by growing the bacteria overnight at 37 °C in Brain Heart Infusion broth with 0.5% thioglycollate (Manteca et al., 2001) to a cell concentration of $10^8$ CFUs (colony forming units)/ml. An aliquot of each bacterial culture was transferred into dextrin-liver broths to a final concentration of ca. $10^7$ CFU/ml.

The calf was monitored overnight (body temperature, respiratory and heart rates) under anaesthesia and euthanised with Nembutal® (50 mg). Necropsy was performed immediately after euthanasia and each loop was sampled for bacterial isolation and counts, histopathology and ELISA assays for the α, β and ε toxins. The lungs, liver and kidneys were also sampled for histopathology. Intestinal tissue sections were scored (0: absence of lesions; 1: presence of weak lesions; 2: presence of heavy lesions) for the haemorrhagic and necrotic aspects of the intestinal content and wall and for the inflammatory response in the intestinal villi.

For bacterial analysis the content of each loop was inoculated onto Ana-Blood base agar plates, Ana-Blood base agar plates with polymyxin and kanamycin, and Ana-Blood base agar plates with cycloserin, using a Model D Spiral Systems plater (LED Techno), as previously described (Manteca et al., 2001).

### *2.6. Statistical analysis*

The percentage of calves with β2-positive *C. perfringens* amongst all calves (=Pβ2) and the percentage of β2-positive *C. perfringens* amongst all isolates (=Cβ2) were computed within each class (2-10 isolates tested) and within either population of calves (study and control populations). Chi-square analyses were used to test the hypothesis that Pβ2 and Cβ2 were higher in the study population than in the control population. The degree of homogeneity for Pβ2 and Cβ2 across the nine classes was tested before combining evidence from all classes. The significance of the weighted average percentages was then tested by referring the Cochran's chi-square to a chi-square with 1 degree of freedom (Fleiss, 1973).

## 3. Results

### *3.1. Colony hybridisation assay*

Of the 714 *C. perfringens* isolated from the study population, 218 (31%) tested positive with the β2 probe. Of the 386 *C. perfringens* isolated from the control population, 113 (29%) tested positive with the β2 probe. There was no relation between the hybridisation results and the site of isolation.

According to the colony hybridisation results, 83 calves (62%) from the study population and 51 calves (59%) from the control population harboured at least one isolate positive with the β2 probe. Amongst calves with β2-positive isolates, the average of study calves (Pβ2) was estimated at 57.46% (standard error: 4.27%) and was

significantly, although slightly, higher than for control calves ($0.05 < p < 0.01$).

As 2-10 *C. perfringens* were isolated from each calf, the proportion of positive colonies varied greatly (Table 2). The numbers of calves with high proportion of β2-positive colonies (Cβ2) tended to be higher in the study population (Table 2 and Fig. 1), but this was not statistically significant.

### *3.2. Ribotyping*

Twelve ribotype profiles were observed for the 47 *C. perfringens* which were typed (Table 3 and Fig. 2). The 13 β2-positive isolates were present in five of them and there was as much variability amongst the β2-positive as amongst the β2-negative *C. perfringens.* Ribotype profiles could be heterogeneous amongst the β2-positive or the β2-negative *C. perfringens* isolated from the same calf (Table 3).

### *3.3. Intestinal loop assays*

The pathotypes of the *C. perfringens* isolates were confirmed using the ELISA assays for toxins α, β and ε, prior to the inoculation of the intestinal loops: two isolates (Cp1 and Cp4) produced high levels and one (Cp2) classical level of α toxin, whereas one isolate (Cp3) tested negative (Table 1). The inocula of the 11 loops were pure or mixed cultures of the different *C. perfringens, C. sordellii* and *C. bifermentans* isolates (Tables 4 and 5).

The calf was monitored overnight. During that time, the body temperature and the respiratory rate were normal. The heart rate was very high for 8 h (up to 160 beats/min) but was again normal (ca. 70 beats/min) during the following 10 h.

At necropsy weak lesions of congestion and infarcts were present in the control loop. The scores of the control loop were subtracted from the scores of the inoculated loops (Table 4). Macroscopic haemorrhagic lesions of the intestinal wall were present in loops 2, 5, 11, and 12. Dilatation by liquid accumulation was also observed in loop 6, and to some extent in loops 10 and 12.

Macroscopic lesions of the intestinal content, when present, were necrosis and, in some loops, haemorrhages (Table 4). Microscopic lesions of the intestinal villi consisted in necrosis and infiltration of polymorphonuclear and mononuclear cells (Table 4). The lesion scores were highest in loop 5 inoculated with the β2-positive *C. perfringens* isolate (Cp1) and in loop 11 inoculated with both Cpl and the *C. sordellii* reference strain (Cs2). High lesion scores were also observed in loop 2 inoculated with the toxin-negative *C. sordellii* (Cs1), in loop 3 inoculated with the *C. bifermentans* (Cb) and in loop 10 inoculated with both Cs1 and Cp2 (Table 4). In contrast loop 4 inoculated with Cs2 gave a low lesion score (Table 4).

The challenge clostridial species were recovered from all loops, with the exception of loop 4 from which endogenous *C. perfringens,* was recovered, as from the control loop 1 (Table 5).

A great variation was observed in the CFU per millilitre of intestinal content recovered from the loop inoculated with pure cultures. On the other hand, the variation was smaller for the loops inoculated with two strains. Enterococci and non-haemolytic *E. coli* were also isolated from all loops except loops 3 and 5.

The α toxin was detected in loops inoculated with any *C. perfringens* isolate positive in vitro (Cpl, Cp2, Cp4). The loops inoculated with sterile broth, *C. sordellii* (Csl and Cs2) or *C. bifermentans* strains and the *C. perfringens* isolate (Cp3) which was already negative in vitro gave negative results (Table 5). Moreover, all loops gave negative results with the ELISA for the β and ε toxins.

No macroscopic lesions were observed in internal organs. Histologically, the lungs and the kidneys were congested and evidence of centrolobular to panlobular hydropic to vacuolar degeneration was observed in the hepatic tissue.

***Fig. 1**. Comparison of the percentage of calves from both populations with 100% of β2-positive isolates.*

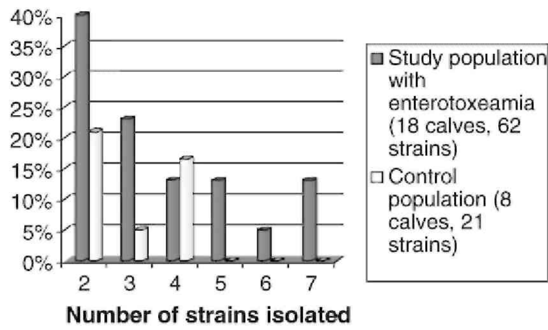


***Fig. 2**. Example of ribotype profiles of 11 α+ and one β2+ (42834I2) C. perfringens isolates (Table 3) from two calves after total DNA extraction and restriction by HindIII. From lane 1 to lane 12: 42942S3; 42942S2; 42942S1; 42942I4; 42942I3; 42942I2; 42942I1; 42834I3; 42834I42; 42834I1; 42834S3; 42834S2 (S: splenic isolate; I: intestinal isolate).*

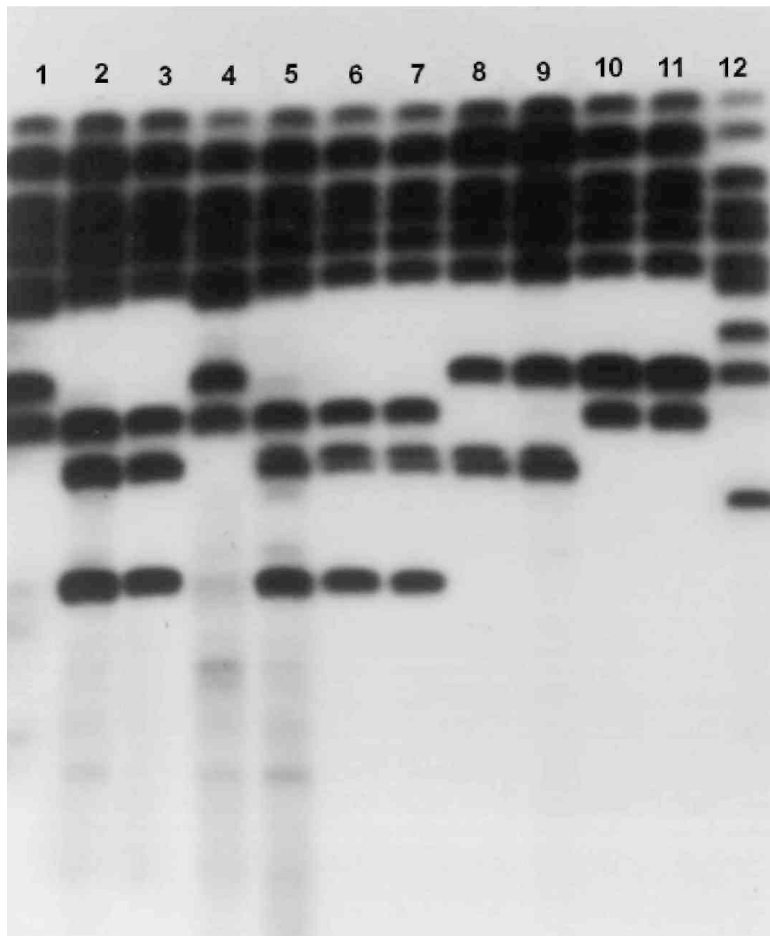

***Table 2**: Numbers of β2-positive colonies according to the number of colonies isolated from calves from both populations*

| Class[a] (number of calves) | Number of β2 + colonies per calf | | | | | | | | | | Total β2+ calves (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| *Population of calves without enterotoxaemia* | | | | | | | | | | | |
| 2(24) | 5[b] | 5 | | | | | | | | | 10 (42) |
| 3 (22) | 9 | 3 | 1 | | | | | | | | 13 (59) |
| 4(11) | 1 | 4 | - | 2 | | | | | | | 7(64) |
| 5(3) | 1 | - | - | 1 | - | | | | | | 2(67) |
| 6(7) | 1 | - | 3 | - | - | - | | | | | 4(57) |
| 7(4) | - | - | 1 | 1 | 1 | - | - | | | | 3(75) |
| 8(7) | 2 | 1 | 2 | - | - | - | - | - | | | 5(71) |
| 9(3) | 1 | - | - | - | - | - | - | - | - | | 1 (33) |
| 10(6) | 2 | - | - | - | 1 | 3 | - | - | - | - | 6 (100) |
| *Population of calves with enterotoxaemia* | | | | | | | | | | | |
| 2(15) | 2 | 6 | | | | | | | | | 8 (53) |
| 3(26) | 6 | 4 | 6 | | | | | | | | 16 (62) |
| 4(16) | 5 | 2 | 2 | 2 | | | | | | | 11 (69) |
| 5(15) | 2 | 1 | 1 | 2 | 2 | | | | | | 8 (53) |
| 6(21) | 7 | 1 | - | 2 | 2 | 1 | | | | | 13 (62) |
| 7(8) | 2 | - | - | 1 | 1 | 1 | 1 | | | | 6(75) |
| 8(11) | 4 | - | 1 | 1 | 2 | - | - | - | | | 8 (73) |
| 9(13) | 3 | 2 | 1 | - | - | - | 1 | 1 | - | | 8 (62) |
| 10(8) | - | 1 | - | 1 | 3 | - | - | - | - | - | 5 (63) |

[a] Number of colonies isolated per calf. [b]Number of calves.

***Table 3:*** *Ribotyping results of 34 β2-positive and 13 β2-negative C. perfringens isolated from 12 calves*[a]

| Calf | Isolate | Pathotype | Ribotype |
|---|---|---|---|
| 42709 | I1 | α+ | H |
| | I2 | α+β2+ | A |
| | SI | α+ | E |
| 42384 | I1 | α+ | A |
| 42834 | I1 | α+ | A |
| | I2 | α+β2+ | D |
| | I3 | α+ | D |
| | S2 | α+ | C |
| | S3 | α+ | A |
| 42942 | I1 | α+ | G |
| | I2 | α+ | G |
| | I3 | α+ | G |
| | I4 | α+ | A |
| | S1 | α+ | G |
| | S2 | α+ | G |
| | S3 | α+ | A |
| 42948 | I1 | α+β2+ | A |
| | I4 | α+β2+ | L |
| | I6 | α+β2+ | L |
| | I11 | α+ | K |
| | I12 | α+ | K |
| 90E287 | I1 | α+ | J |
| | I2 | α+ | J |
| | I3 | α+ | J |
| | I4 | α+ | C |
| | I5 | α+β2+ | B |
| | I6 | α+ | A |
| | I7 | α+ | I |
| | L1 | α+β2+ | C |
| | S1 | α+β2+ | C |
| 90L883 | I1 | α+ | I |
| | L1 | α+β2+ | G |
| | S1 | α+β2+ | G |
| 90L1448 | I1 | α+ | A |
| 90L1608 | I1 | α+ | G |
| | I2 | α+ | G |
| | I3 | α+ | A |
| | I4 | α+β2+ | A |
| | I5 | α+β2+ | A |
| | I6 | α+ | A |
| | I110 | α+ | B |
| | 111 | α+β2+ | A |
| | I12 | α+ | A |
| | I13 | α+ | A |
| G939 | I | α+ | A |
| G1239 | I | α+ | B |
| G1422 | I | α+ | F |

[a] I: isolation from the intestine; L: isolation from the liver; S: isolation from the spleen.

***Table 4:** Macroscopic and microscopic lesions of the 12 ligated intestinal loops*[a]

| Loop number | Inoculum strain | Pathotype | Intestinal content | | Histology of intestinal wall and villi | | | | Total score |
|---|---|---|---|---|---|---|---|---|---|
| | | | N | H | N | H | PMN | MN | |
| 1 | Broth | NR | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | Cs1 | HT-LT- | 2 | 0 | 1 | 1 | 1 | 0 | 4 |
| 3 | Cb | HT-LT- | 1 | 0 | 2 | 0 | 1 | 0 | 4 |
| 4 | Cs2 | HT+LT+ | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| 5 | Cp1 | α+β2+θ+μ+ | 2 | 0 | 2 | 1 | 1 | 0 | 5 |
| 6 | Cp2 | a+Ent+θ+μ+ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Cp3 | a+μ+ | 1 | 0 | 0 | 0 | 2 | 0 | 3 |
| 8 | Cp4 | a+θ+μ+ | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| 9 | Cs1/Cp1 | HT-LT-/a+β2+θ+μ+ | 1 | 0 | 0 | 0 | 1 | 0 | 2 |
| 10 | Cs1/Cp2 | HT-LT-/a+Ent+θ+μ+ | 1 | 1 | 1 | 0 | 0 | 1 | 4 |
| 11 | Cs2/Cp1 | HT+LT+/a+β2+θ+μ+ | 2 | 0 | 2 | 1 | 2 | 0 | 6 |
| 12 | Cs2/Cp2 | HT+LT+/a+Ent+θ+μ+ | 1 | 0 | 0 | 1 | 0 | 0 | 1 |

[a] N: necrosis; H: haemorrhages; PMN: polymorphonuclear infiltration; MN: mononuclear infiltration; NR: not relevant; HT: haemorrhagic toxin of *C. sordellii;* LT: lethal toxin of *C. sordellii;* Ent: enterotoxin of *C. perfringens;* 0: absence of lesions; 1: presence of weak lesions; 2: presence of clear lesions.

***Table 5** : Bacterial growth and α toxin ELISA results on the contents of the 12 ligated intestinal loops*[a]

| Loop number | Inoculum strain | Pathotype | Isolation | CFU/ml ($x10^9$) | α toxin ELISA |
|---|---|---|---|---|---|
| 1 | Broth | NR | *C. perfringens* | 0.56 | - |
| 2 | Cs1 | HT-LT- | *C. sordellii* | 1.7 | - |
| 3 | Cb | HT-LT- | *C. bifermentans* | 7.6 | - |
| 4 | Cs2 | HT+LT+ | *C. perfringens* | 2.3 | - |
| 5 | Cpl | α+β2+θ+μ+ | *C. perfringens* | 7.5 | + |
| 6 | Cp2 | α+Ent+θ+μ+ | *C. perfringens* | 11 | + |
| 7 | Cp3 | α+μ+ | *C. perfringens* | 0.45 | - |
| 8 | Cp4 | α+θ+μ+ | *C. perfringens* | 3.8 | + |
| 9 | Cs1/Cp1 | HT-LT-/α+β2+θ+μ+ | *C. sordellii/C. perfringens* | 0.15/0.57 | + |
| 10 | Cs1/Cp2 | HT-LT-/α+Ent+θ+μ+ | *C. sordellii/C. perfringens* | 0.44/0.46 | + |
| 11 | Cs2/Cp1 | HT+LT+/α+β2+θ+μ+ | *C. sordellii/C. perfringens* | 0.96/0.17 | + |
| 12 | Cs2/Cp2 | HT+LT+/α+Ent+θ+μ+ | *C. sordellii/C. perfringens* | 0.39/0.35 | ND |

[a] NR: not relevant; HT: haemorrhagic toxin of *C. sordellii;* LT: lethal toxin of *C. sordellii;* Ent: enterotoxin of *C. perfringens;* ND: not done.

## 4. Discussion

The role of non-enterotoxigenic *C. perfringens* toxin type A in enteritis and enterotox-aemia in mammals is controversial (Daube, 1992; Songer, 1996; Petit et al., 1999). Epidemiological data favour a role for instance in horses and cattle (Griffiths et al., 1998; Netherwood et al., 1998; Manteca et al., 2001), and in vivo role of the α toxin has been proved in necrotic enteritis in poultry (Popoff, 1989; Daube, 1992; Songer, 1996). The identification of β2-toxigenic *C. perfringens* isolated from several animal species with, sometimes necrotic and haemorrhagic, enteritis and enterotoxaemia revived the discussion (Gibert et al., 1997; Herholz et al., 1999; Klaasen et al., 1999; Garmory et al., 2000; Gkiourtzidis et al., 2001).

Colony hybridisation confirmed the existence of β2-positive *C. perfringens* in cattle (Garmory et al., 2000), but brought no argument for its involvement in bovine enterotoxaemia since: (i) almost as many *C. perfringens* isolates and calves of both study and control populations are positive; and (ii) β2-positive *C. perfringens* are not isolated from 40% of the calves of the former population. The isolation of a β2-positive *C. perfringens* from the intestinal content thus does not confirm the clinical diagnosis of bovine enterotoxaemia, as the probability of this calf being enterotoxaemic is only 0.6. As previously stated and described (Manteca et al., 2001), a clostridial cell count must still be performed.

The results of the ligated loop assay in one calf bring new evidence for the importance of β2 toxin since necrotic and haemorrhagic lesions developed in two of three loops inoculated with a β2-positive *C. perfringens* strain, and not, or not as much, in the other loops, including those inoculated with toxin-positive and toxin-negative *C.*

*sordellii* (Table 4). However, this *C. perfringens* strain also produces high level of the α toxin and a synergistic action between these two toxins must also be considered.

Previous data suggested an anarchic multiplication of the *C. perfringens* population ($10^6$-$10^{10}$ CFU/ml of small intestinal content in the study population versus $10^3$-$10^7$ CFU/ ml in the control population) and invasion of the small intestine (Manteca et al., 2001). As a result of the multiplication of the *C. perfringens* population, there would be an increase of the production of the α and/or β2 toxins in the small intestine followed by their absorption through the gut wall and the production of necrotic and haemorrhagic lesions. This multiplication indeed affects many, if not all, α-positive and α/β2-positive clones of the *C. perfringens* population, as illustrated by the ribotyping results.

To prove an actual role of the β2 toxin in the bovine enterotoxaemia syndrome, in synergy with the α toxin or not, future experiments must: (i) compare the growth rates of the β2-negative and β2-positive strain; (ii) demonstrate, like for the α toxin, the actual production in vivo of the β2 toxin by ELISA or by immunocytochemistry; (iii) reproduce necrotic and haemorrhagic lesions of the bovine small intestinal wall and a sudden death syndrome with other *C. perfringens* isolates and their allelic mutants in the α and β2 toxin-encoding genes.

In summary our results suggest in vivo synergistic role of the β2 and α toxins of *C. perfringens* in the production of necrotic and haemorrhagic lesions of the small intestine in the bovine enterotoxaemia syndrome. On the other hand, any role for the enterotoxin is definitely ruled out. The enterotoxin is responsible for fluid accumulation in the loops, but not for necrotic and haemorrhagic lesions.

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