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Occurrence of *Clostridium perfringens* β2-toxin amongst animals, determined using genotyping and subtyping PCR assays

H. S. GARMORY[1]*, N. CHANTER[2], N. P. FRENCH[3], D. BUESCHEL[4], J. G. SONGER[4] AND R. W. TITBALL[1]

[1] *Department of Biomedical Sciences, Defence Evaluation and Research Agency, CBD Porton Down, Salisbury, Wiltshire SP4 0JQ*
[2] *Centre for Preventative Medicine, Animal Health Trust, P.O. Box 5, Newmarket, Suffolk CB8 7DW*
[3] *Department of Veterinary Clinical Science and Animal Husbandry, University of Liverpool, Leahurst, Neston, S. Wirral L64 7TE*
[4] *Department of Veterinary Science and Microbiology, University of Arizona, Tuscon, Arizona 85721, USA*

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SUMMARY

Clostridium perfringens isolates are currently classified into one of five biotypes on the basis of the differential production of α-, β-, ε- and ι-toxins. Different biotypes are associated with different diseases of man and animals. In this study a multiple PCR assay was developed to detect the genes encoding these toxins. In addition, detection of the genes encoding the *C. perfringens* enterotoxin and β2-toxin allowed subtyping of the bacteria. *C. perfringens* isolates taken from a variety of animals, including foals, piglets or lambs, were genotyped using this assay. Most of the isolates were found to be genotype A and the gene encoding β-toxin was present in 50% of the isolates genotyped. A significant association between *C. perfringens* possessing the β2-toxin gene and diarrhoea in piglets was identified, suggesting that β2-toxin may play a key role in the pathogenesis of the disease.

INTRODUCTION

Clostridium perfringens is an important cause of enteric disease in both humans and domestic animals [1–5]. The bacteria produce several toxins which play key roles in the pathogenesis of disease and are classified into five biotypes, designated A–E, on the basis of the differential production of alpha- (α-), beta- (β-), epsilon- (ε-) and iota- (ι-) toxins. The α-toxin is produced by all types and, in addition, β-toxin is produced by type B and C strains, ε-toxin is produced by type B and D strains, and ι-toxin is produced by type E strains. Different biotypes of *C. perfringens* are associated with different diseases. For example, type C strains have been reported to cause enterotoxaemia and necrotic enteritis in sheep, lambs, calves, piglets and fowl whilst type D strains are thought to cause lamb dysentery and pulpy kidney disease in sheep and lambs [1–4]. Type A strains have been associated with gas gangrene and food poisoning in humans [4, 5].

In addition to the so-called 'major' toxins, there are other toxins produced by some strains of *C. perfringens* which may play a role in disease. For example, the enterotoxin is important in type A food poisoning [6] and is thought to be important in some animal diseases [1]. A novel toxin produced by *C. perfringens*, termed beta2- (β2-) toxin, has recently been identified and its encoding gene characterized [7]. The β2-toxin

* Author for correspondence.

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sequence has no significant amino acid sequence homology with *C. perfringens* β-toxin or any other known toxin. However, β2-toxin does have similar biological activity to β-toxin, as both are cytotoxic for intestinal cells and lethal for mice [7]. Thus β2-toxin may play a role in disease. Preliminary studies suggested that β2-toxin-producing strains of *C. perfringens* are associated with necrotic enteritis in piglets [7] and enterocolitis and typhlocolitis in horses [7, 8]. Since enterotoxin and β2-toxin may play roles in disease, it is important to consider them in any typing system.

The identification of *C. perfringens* biotypes has traditionally been carried out using an *in vivo* toxin neutralization test in mice [9]. The difficulties and ethical unacceptability of carrying out such tests has limited the availability of suitable diagnostic tests for disease in humans and animals. Whilst antibodies against the toxins may be used to identify strains, it is known that toxin production is regulated in at least some strains [10]. Therefore toxins produced by some strains *in vivo* might not be detected when these strains are cultured *in vitro*. A genetic test for the presence of toxin-encoding genes should be more robust, reliable and useful than antibody-based methods and would save the unnecessary use of animals.

We report here the adaptation and testing of a multiplex polymerase chain reaction (PCR) assay for the simultaneous identification of the *C. perfringens* α-, β-, ε-, ι- and β2-toxins and enterotoxin. In addition we have used individual PCR assays for these toxin-encoding genes to genotype *C. perfringens* isolated from a variety of healthy or diseased animals including piglets, foals and lambs, in order to identify any association between the possession of toxin encoding genes and disease.

MATERIALS AND METHODS

Bacterial reference strains and isolates

C. perfringens reference strains were obtained from the National Collection of Type Cultures or the Public Health Laboratory Service Anaerobe Reference Unit (listed in the text). *C. perfringens* isolates tested in the study were obtained from foals, lambs and piglets with diarrhoea. Control isolates were from foals that were not in contact with diarrhoea cases, lambs that were in the same group as the diarrhoea cases, and piglets. Isolates were also obtained from an alpaca, an elk, a swan, a llama, a horse and calves with diarrhoea.

C. perfringens isolates were cultivated on brain heart infusion agar (Oxoid Ltd., UK) in modified TPYG broth (50 g/l trypticase peptone, 5 g/l yeast extract, 5 g/l peptone, 5 g/l glucose, 0·5 g/l cysteine HCl, 1 g/l sodium thioglycollate, and distilled water to 1 l), and in cooked meat medium (Oxoid) at 37 °C under anaerobic conditions.

Isolation of total DNA

Cells from overnight 100 ml cultures of modified TPYG broth were harvested by centrifugation at 10000 ***g*** for 10 min at 4 °C. The cells were washed in 10 ml of 10 × TE buffer pH 7·5, centrifuged and resuspended in 2 ml of the same buffer. Lysozyme (10 μl of 100 mg/ml solution in 0·25 M Tris–HCl, pH 8·0) was added and the cells incubated for 1 h at 37 °C with agitation. After addition of 200 μl of 10% (w/v) sodium dodecyl sulphate, the samples were incubated for 10 min at 60 °C, followed by addition of 20 μl of 0·5 M EDTA and 5 μl of 20 mg/ml proteinase K, and incubation for 1 h at 37 °C. 440 μl of 5 M sodium perchlorate was added and the samples were incubated for 30 min at 65 °C. The cell lysate was extracted with 6 ml of chloroform:isoamyl alcohol (24:1) with agitation for 30 min, followed by centrifugation at 11000 ***g*** for 15 min at 4 °C to separate the phases. The upper aqueous layer was recovered and the DNA precipitated with just over one volume of isopropanol at −20 °C overnight. The DNA was pelleted, washed with 70% ethanol, allowed to dry, and dissolved in 200 μl–1 ml TE, pH 8·0.

Multiplex PCR

The multiplex PCR reactions each contained 50 ng *C. perfringens* template DNA, 62·5 pmol each *cpa* primer, 45 pmol each *cpb* primer, 55 pmol each *etx* primer, 70 pmol each *iA* primer, 45 pmol each *cpe* primer (*cpa*, *cpb*, *iA*, and *cpe* primer sequences as described by Meer and colleagues [6]), 50 pmol each *cpb2* primer (5′-AGATTTTAAATATGATCCTAACC-3′ and 5′-CAATACCCTTCACCAAATACTC-3′), dNTPs to a final concentration of 0·1 mM, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8·3, 2 mM $MgCl_2$), 0·1% Triton X-100, 2 units of *Taq* DNA polymerase (Roche) and water to 50 μl DNA was denatured for 2 min at 95 °C and amplified for 35 cycles (1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C for denaturation,

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annealing and extension phases, respectively), and followed by an additional period of extension for 10 min at 72 °C (model 9600 GeneAmp PCR System; Perkin–Elmer). PCR products were separated by electrophoresis in a 2% (w/v) agarose gel with 0·5 μg of ethidium bromide/ml. Twenty μl PCR products were subjected to electrophoresis for 45–60 min at 80 V. Amplified bands were visualized and photographed under UV illumination.

PCR on colonies

For PCR analysis of *C. perfringens*, single colonies obtained after overnight cultivation on brain heart infusion agar were inoculated into 50 μl nuclease-free water and boiled for 5 min to lyse the cells. After centrifugation at 13000 ***g*** for 10 s at room temperature, the supernatant fluid was removed and 20 μl was used in the PCR reaction. Fifty pmol of each primer used in a primer pair to amplify a single *C. perfringens* toxin gene was added. All other PCR reaction components remain as described above.

Verification of PCR product

Verification of the *cpb2* PCR product was done by DNA sequencing of purified PCR product. The PCR product from amplification of *cpb2* (567 bp) was excised from an agarose gel and purified using the QIAEX II gel extraction kit (Qiagen). DNA sequencing reactions containing the purified PCR product and the PCR primers were performed using an automated Taq polymerase cycle sequencing protocol with fluorescently labelled dideoxy nucleotides (Applied Biosystems). The reaction products were analysed using an automated nucleotide sequencer (Applied Biosystems model 373A).

Specificity of multiplex PCR

50 ng total genomic DNA of *C. absonum*, *C. bifermentans*, *C. botulinum*, *C. histolyticum*, *C. novyi*, *C. septicum* or *C. sordellii* were used in the PCR reactions as described above.

Statistical methods

The selection of controls was different for lambs compared to foals and piglets. In the study of diarrhoea and faecal soiling in lambs, cases were individually matched with a randomly selected control lamb from the same group at the same point in time [11]. Both culture-positive (for *C. perfringens*) and culture-negative lambs were included in the analysis and a McNemar test for matched-pair data [12] was used to test for an association between the presence of β2-toxin and disease. The samples from foals and piglets were all *C. perfringens* culture-positive and were not from a matched study including culture-negative animals. A two-tailed Fisher's exact test [12] was used to test for an association between the presence of β2-toxin and disease in culture-positive animals.

RESULTS

Multiplex PCR of *C. perfringens*

A multiplex PCR assay to detect the genes for *C. perfringens* α-toxin (*cpa*), β-toxin (*cpb*), ε-toxin (*cpe*), ι-toxin (*iA*), and enterotoxin (*cpe*), was recently reported [13]. PCR products of 324, 196, 655, 446, and 233 bp, respectively, were obtained. When we used this assay it failed to determine correctly the genotype of all *C. perfringens* strains (e.g. *cpa* gene of isolate 97–6). Therefore the assay was modified by using a PCR buffer of pH 8·3 rather than pH 9·0, which allowed the detection of all strains. For the detection of the gene encoding *C. perfringens* β2-toxin (*cpb2*), PCR primers were designed from the reported nucleotide sequence of the gene [7]. This PCR primer pair amplified a gene fragment which was of a size easily distinguishable from the other gene fragments produced in the multiplex PCR (567 bp). The modified multiplex PCR assay was used to genotype correctly 20 *C. perfringens* reference strains and to detect the presence of the enterotoxin and β2-toxin genes in these strains.

The multiplex PCR assay was tested using DNA from *C. absonum*, *C. bifermentans*, *C. botulinum*, *C. histolyticum*, *C. novyi*, *C. septicum* or *C. sordellii*. None of the reactions produced a 324 bp *cpa* fragment, which was uniquely associated with *C. perfringens*. However, amplification products of approx. 580 bp were generated with DNA of *C. bifermentans* PHLSARU 2825 or *C. novyi* NCTC 9692. These products were amplified using primers for *cpb* and *cpe*, rather than with primers for amplification from one of the six *C. perfringens* toxin genes.

This multiplex PCR assay proved to be successful for genotyping *C. perfringens* from isolated DNA

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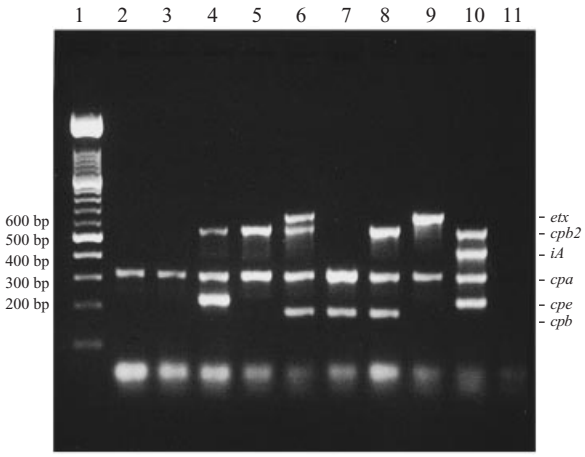

Fig. 1. Agarose gel electrophoresis of the PCR products obtained from the multiplex PCR assay for genotyping *Clostridium perfringens* and detection of the genes encoding enterotoxin and β2-toxin. PCR products represent amplification of *cpb* (196 bp), *cpe* (233 bp), *cpa* (324 bp), *iA* (446 bp), *cpb2* (567 bp) and *etx* (655 bp). Lanes: 1, DNA molecular weight markers; 2, *C. perfringens* NCTC 8237 (genotype A); 3, *C. perfringens* isolate 97–6 (genotype A); 4, *C. perfringens* PHLSARU 1289 (genotype $Ae^{\beta 2}$); 5, *C. perfringens* NCTC 528 (genotype $A^{\beta 2}$); 6, *C. perfringens* NCTC 4964 (genotype $B^{\beta 2}$); 7, *C. perfringens* NCTC 3227 (genotype C); 8, *C. perfringens* NCTC 10719 (genotype $C^{\beta 2}$); 9, *C. perfringens* NCTC 8503 (genotype D); 10, *C. perfringens* isolate 294 (genotype $Ee^{\beta 2}$); 11, negative control (no template DNA).

(Fig. 1), but PCR from bacterial colonies was most reliable when the six toxin genes were amplified in individual PCR reactions. The latter also ensured that amplification of PCR products as a result of cross-reactivity between primers in the multiplex PCR did not occur.

PCR genotyping

A total of 140 *C. perfringens* isolates were genotyped using multiple monoplex PCR assays. For 96% of the isolates a genotype was determined from colonies, but for five isolates no amplification products were found in the PCR.

Foals

A total of 24 *C. perfringens* isolates from foals with diarrhoea, and a further 10 control isolates from foals without diarrhoea, were genotyped. All isolates possessed the *cpa* gene, but not the *cpb*, *etx* or *iA* genes, indicating that they were all genotype A (Table 1). These results agreed with previous genotyping of these isolates (data not shown) [14].

Lambs

A total of 27 *C. perfringens* isolates from lambs with chronic diarrhoea and 12 control isolates from lambs without diarrhoea were genotyped. Twenty-four disease and 10 control isolates were genotype A, and 3 disease and 2 control isolates were genotype D, possessing the *cpa* and *etx* genes (Table 1). For some of the isolates, however, the genotyping results were different to those indicated previously using ELISA [11]. Eleven and six of the isolates genotyped here as A (*cpa* gene only) were types D (*cpa* and *etx*) and B (*cpa*, *cpb* and *etx*), respectively by ELISA. Spiking of these colony lysates with 25 pmol of plasmid DNA encoding the cloned *etx* gene resulted in amplification of the gene, suggesting that these reactions did not contain inhibitors of the PCR reaction. Conversely, three isolates genotyped as type D here were previously typed A by ELISA.

Piglets

A total of 33 *C. perfringens* isolates from piglets with diarrhoea and 7 control isolates from those without diarrhoea were genotyped (Table 1). Of the disease isolates, 29 were type A (possessing the *cpa* gene) and 4 were type C (possessing *cpa* and *cpb* genes). All of the control isolates were genotype A. These results were in agreement with previous genotyping (data not shown), except that two of the disease isolates genotyped A here were previously genotyped C.

Other animals

Twelve *C. perfringens* isolates from calves with diarrhoea and five *C. perfringens* isolates from other diseased animals (alpaca, horse, elk, llama, swan) were all genotype A (possessing the *cpa* gene), except one calf isolate which was genotype E (*cpa* and *iA* genes) (Table 1).

Associations of disease with genotype A

All disease and control foal isolates were genotype A, and there was no significant difference between the presence of genotype A in isolates from diseased and control piglets. Although only 39 lamb isolates were genotyped in this study, a further 126 isolates obtained from diseased (55 isolates) and control (71 isolates) lambs which were culture-negative for *C. perfringens* were included in statistical analyses. When culture-negative lambs were included in a matched analysis,

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Table 1. *Genotypes of* Clostridium perfringens *isolates*

C. perfringens isolates	No. of isolates screened	Genotype*												% β2-positive isolates
		A	$A^{\beta 2}$	Ae	$Ae^{\beta 2}$	B	C	$C^{\beta 2}$	D	$D^{\beta 2}$	$De^{\beta 2}$	E	$Ee^{\beta 2}$	
Foal disease	24	12	10	0	2	0	0	0	0	0	0	0	0	50
Foal control	10	5	5	0	0	0	0	0	0	0	0	0	0	50
Piglet disease	33	6	23	0	0	0	0	4	0	0	0	0	0	82
Piglet control	7	7	0	0	0	0	0	0	0	0	0	0	0	0
Lamb disease†	27	17	7	0	0	0	0	0	1	2	0	0	0	33
Lamb control†	12	4	6	0	0	0	0	0	0	0	2	0	0	67
Calf disease	12	9	2	0	0	0	0	0	0	0	0	0	1	25
Other disease	5	3	2	0	0	0	0	0	0	0	0	0	0	40

* A, B, C, D, E refers to genotype; e refers to presence of the gene encoding enterotoxin; $^{\beta 2}$ refers to presence of the gene encoding β2-toxin.

† Lamb isolates were obtained as part of a matched case control study.

there was a significant association between genotype A and diarrhoea (matched odds ratio = 2·30, 95% CI 1·11–5·05, $P = 0{\cdot}037$).

Prevalence of *cpe* in *C. perfringens* isolates

The gene encoding *C. perfringens* enterotoxin (*cpe*) was detected in 4% of isolates genotyped in this study. Two foals with diarrhoea and two lambs without diarrhoea were *cpe*-positive. None of the piglet isolates were *cpe*-positive. No significant associations between possession of the *cpe* gene and disease in these animals were identified.

Prevalence of *cpb2* in *C. perfringens* isolates

The gene encoding *C. perfringens* β2-toxin (*cpb2*) was present in 50% of the isolates genotyped. However, the prevalence of this gene varied between animal isolates (Table 1). Twenty-seven of the 33 isolates (82%) from piglets with diarrhoea were positive for the *cpb2* gene, compared with none of the piglet control isolates, strongly suggesting that this gene was significantly associated with diarrhoea in piglets ($P = 0{\cdot}00045$).

The prevalence of the *cpb2* gene in *C. perfringens* was similar for both diseased and control isolates from foals. The *cpb2* gene was more frequently found in control isolates than in case isolates from lambs, but this association was not significant. The inclusion of culture-negative lambs revealed a positive but non-significant association of *cpb2* and diarrhoea (matched odds ratio, including culture-negative cases and controls = 1·33, 95% CI 0·46–3·84, $P = 0{\cdot}79$).

DISCUSSION

Multiplex PCR assays have recently been used to detect the genes encoding the *Clostridium perfringens* α-, β-, ϵ- and ι-toxins, and enterotoxin and β2-toxin [8, 13, 15, 16]. In this study, an existing multiplex PCR assay [13] has been adapted, for the detection of all six of these toxin genes. This assay correctly identified *C. perfringens* isolates as genotypes A–E, and further subtyped the bacteria on the basis of the enterotoxin or β2-toxin genes. This assay is an improvement on existing genotyping assays, since it may be used to detect all of the genes important in biotyping and subtyping *C. perfringens*, including atypical isolates such as isolate 97–6. Researchers have not previously reported cross-reactivity of other clostridia in *C. perfringens* multiplex PCR assays. Our assay was most reliable when used as multiple monoplex PCR assays, particularly with lysates of *C. perfringens* colonies. Our finding that multiplex PCR assay of *C. perfringens* from colony lysates was less reliable than from purified DNA is in keeping with others [17], although recently Herholz and colleagues and Kanakaraj and colleagues used colony lysates in their multiplex PCR assays [8, 15].

The multiplex PCR and multiple monoplex PCR assays were reliable for the genotyping of reference strains using either *C. perfringens* DNA or colony lysates. Only 5 of 140 isolates from animals could not be genotyped by PCR from colony lysates and required the use of extracted DNA [14, 15].

All *C. perfringens* isolates from foals were genotype A, a genotype which has previously been associated with diarrhoea in foals [14]. Netherwood and colleagues used PCR to analyse *C. perfringens* associated

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with foal diarrhoea on the basis of possession of the genes encoding α-, β-, ϵ- and ι-toxins as well as θ-(theta-) toxin, large and small sialidases, hyaluronidase and virulence regulation, but did not identify a marker for subtyping *C. perfringens* which was associated with disease [14]. Such a marker may yet be identified in association with diarrhoea in foals or lambs or other animals such as goats, calves and fowl.

Most isolates from lambs, piglets and calves were also genotype A, although genotypes D, C and E were also identified. *C. perfringens* type A has previously been associated with lamb enterotoxaemia and *C. perfringens* type D has been identified as the aetiological agent of pulpy kidney disease in lambs [1–4]. Recently, a significant association was identified between diarrhoea in 2- to 6-month-old lambs and the presence of *C. perfringens* type A in the faeces [11]. Similarly, type A isolates were associated with diarrhoea in the same lambs in this study.

However, the genotype of 20 of the lamb isolates was not the same as the biotype determined using ELISAs. For some isolates this might reflect the loss of plasmids known to encode the β-toxin and ϵ-toxin [18], while the gene may be silent in other isolates that did not produce detectable ϵ-toxin but possessed the *etx* gene. These isolates need to be further typed to confirm their biotype before associations with disease can be studied.

C. perfringens types A and C have previously been suggested to be the aetiological agents of necrotizing enterocolitis and necrotic enteritis (enterotoxaemia) in piglets, respectively [1–4], and that *C. perfringens* α-toxin and β-toxin may play key roles in these diseases. Here we showed a significant association between *C. perfringens* possessing the gene encoding β2-toxin (*cpb2*) and diarrhoea in piglets in *C. perfringens*-positive samples. This association is in agreement with preliminary epidemiological studies [7] and supports the view that β2-toxin may play a role in enteric disease of piglets.

C. perfringens is a frequent cause of acute enterotoxaemia in pre-weaned pigs and is reported to be increasing in prevalence [19]. Immunization of pre-weaned pigs with a *C. perfringens* antitoxin immediately after birth provides protection against disease, but this procedure is costly and requires extensive handling of newborns. Passive protection of piglets has also been achieved by immunization of pregnant gilts with a *C. perfringens* type C toxoid [20]. However, incomplete protection against disease has been seen in piglets immunized with type C strains, possibly reflecting the use of *cpb2*-negative strains in the preparation of vaccines.

Although associations between *cpb2*-positive *C. perfringens* and diarrhoea in foals and lambs were not found in this study, the finding that β2-toxin is associated with piglet enteric disease suggests that virulence determinants other than the major toxins may be important in disease.

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