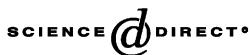




ELSEVIER

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)



Veterinary Microbiology 94 (2003) 121–129

**veterinary  
microbiology**

[www.elsevier.com/locate/vetmic](http://www.elsevier.com/locate/vetmic)

## Prevalence of *cpb2*, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype

Dawn M. Bueschel, B. Helen Jost, Stephen J. Billington,  
Hien T. Trinh, J. Glenn Songer\*

*Department of Veterinary Science and Microbiology, The University of Arizona,  
1117 East Lowell Street, Tucson, AZ 85721, USA*

Received 25 November 2002; received in revised form 6 March 2003; accepted 6 March 2003

### Abstract

Beta2 toxin, encoded by the *cpb2* gene, has been implicated in the pathogenesis of porcine, equine and bovine enteritis by type A *Clostridium perfringens*. By incorporating primers to *cpb2* into a multiplex genotyping PCR, we screened 3270 field isolates of *C. perfringens*. Of these, 37.2% were PCR positive for the *cpb2* gene. The majority of isolates from cases of porcine enteritis were positive for *cpb2* (>85%), and this was even more true for *C. perfringens* isolated from cases of porcine neonatal enteritis (91.8%). In contrast, isolates from normal pigs only contained *cpb2* in 11.1% of cases. The correlation between enteritis in other animal species and the presence of *cpb2* was not so strong. *cpb2* was found in 21.4% of *C. perfringens* isolates from cattle with enteritis, and in 47.3% of isolates from calves with enteritis or abomastitis. The prevalence of *cpb2* varied with genotype, with type A isolates being positive for this gene in 35.1% of cases. Furthermore, enterotoxigenic type D or type E strains almost always carried *cpb2*. We cloned a 6xHIS-tagged beta2 (HIS-beta2) and used this protein to raise antiserum against beta2. Culture supernatants from 68 *cpb2*-positive and 13 *cpb2*-negative strains were tested for the presence of beta2 by Western blotting. In *cpb2*-positive isolates of porcine origin, beta2 was almost always detected (96.9%). However, in *cpb2*-positive isolates from other animal species, only 50.0% expressed beta2 protein. The high rate of *cpb2*-positivity among strains from neonatal pigs with enteritis and the high correlation of genotype with phenotype, supports the contention that beta2 toxin plays a role in the pathogenesis of these infections. However, it may be important to consider the use of an additional method for the detection of beta2 toxin in non-porcine *cpb2*-positive isolates when making claims about the role of beta2 in enteritis in non-porcine species. © 2003 Elsevier Science B.V. All rights reserved.

**Keywords:** *Clostridium perfringens*; Beta2 toxin; Enteric disease; Pig-bacteria; Genotype; Phenotype

\* Corresponding author. Tel.: +1-520-621-2962; fax: +1-520-621-6366.

E-mail address: [gsonger@u.arizona.edu](mailto:gsonger@u.arizona.edu) (J. Glenn Songer).

## 1. Introduction

*Clostridium perfringens* is perhaps the most important clostridial enteric pathogen of domestic animals (reviewed in Songer (1996)). The species is divided into five types on the basis of production of four major toxins, alpha, beta, epsilon and iota, and each type is associated with specific enteric infections of various animal species (McDonel, 1986).

Initially thought to be a proteolytic breakdown product of beta toxin, the beta2 protein was purified from a *C. perfringens* type C strain isolated from a pig with typical neonatal, hemorrhagic, necrotic enteritis (Gibert et al., 1997). The 28 kDa beta2 protein was lethal for mice, and toxic for CHO and I407 cells (Gibert et al., 1997), hence its designation as a toxin. The structure of this toxin is so far unique, as the deduced amino acid sequence has no notable similarity with beta toxin or any other known toxin. The *cpb2* gene is plasmid-borne, at least in some strains (Gibert et al., 1997; Shimizu et al., 2002), which suggests the potential for mobility, and the subsequent transfer of *cpb2* among strains of *C. perfringens*.

Several workers have noted an association of *cpb2*-positive strains of *C. perfringens* type A and the occurrence of enteric disease in domestic animals, particularly piglets (Klaasen et al., 1999; Garmory et al., 2000), horses (Herholz et al., 1999), dogs (Thiede et al., 2001), and an African elephant with ulcerative enteritis (Bacciarni et al., 2001). The prevalence of *cpb2* in isolates from piglets with enteritis, and the lack of *cpb2* in isolates from normal piglets, revealed a strong epidemiologic association, if not a cause-and-effect relationship (Garmory et al., 2000). A similar association has been made in the case of horses with typical or atypical typhlocolitis (Herholz et al., 1999). Furthermore, data from some veterinary diagnostic laboratories suggest an association between *cpb2* and the occurrence of enteritis in calves and adult cattle (Carol Maddox, personal communication; Robert Ellis, personal communication), and a recent study implicated beta2 toxin in neonatal, bovine enterotoxemia (Manteca et al., 2002).

A previous comparison of genotype, as determined by multiplex PCR detection of the four major toxin genes and the enterotoxin gene, and phenotype revealed relatively few silent toxin genes (Meer and Songer, 1997). The only such genes documented previously are the *cpe* genes in strains of type E. All type E strains ( $n = 37$ ) in our collection are PCR positive for the *cpe* gene. However, those strains examined do not produce enterotoxin, because of the accumulation of multiple mutations, both within the *cpe* coding region and upstream promoter sequences (Billington et al., 1998). Such information is not available with regard to *cpb2*.

We report here the prevalence of *cpb2* and the correlation with beta2 expression in strains of the five genotypes of *C. perfringens* field isolates collected over a period of 9 years.

## 2. Materials and methods

### 2.1. Bacteria and growth conditions

*C. perfringens* strain 690D was a type A strain isolated from a case of porcine enteritis. Most of the *C. perfringens* isolates used in this study were received through the Clostridial

Enteric Disease Unit, University of Arizona. The other isolates used were from veterinary diagnostic laboratories or personal collections. The 3270 *C. perfringens* strains were grown on brain heart infusion (BHI, Difco) agar plates, supplemented with 5% bovine blood, at 37 °C in an atmosphere of 50:50 H<sub>2</sub>:CO<sub>2</sub>, or in BHI broth supplemented with 0.5% yeast extract, and 0.05% cysteine at 37 °C. *Escherichia coli* DH5 $\alpha$  strains (Gibco-BRL) were grown at 37 °C on Luria–Bertani (LB, Difco) agar or in LB broth with shaking, supplemented with 100  $\mu$ g/ml ampicillin, as appropriate.

## 2.2. Preparation of concentrated culture supernatant fluid

Concentrated culture supernatant fluid (CSF) was prepared from liquid cultures of *C. perfringens* grown for 24–48 h. Cells were removed by centrifugation at 5000  $\times$  g and the CSF was concentrated 20-fold by chloroform extraction and methanol precipitation (Wessel and Flugge, 1984).

## 2.3. DNA techniques

Genomic DNA from *C. perfringens* was isolated by the method of Pospiech and Neumann (1995). *E. coli* plasmid DNA extraction, transformation, DNA restriction, ligation, and agarose gel electrophoresis were performed essentially as described (Ausubel et al., 1994). DNA sequencing was performed on both strands, using primers designed to the sequence of pTrcHis B (Invitrogen). Sequencing reactions were performed by the genomic analysis technology core DNA sequencing facility at The University of Arizona, using a 377 DNA sequencer (Applied Biosystems).

## 2.4. PCR assay

A multiplex PCR assay, which amplifies *cpa*, *cpb*, *cpe*, *etx* and *ibp*, was used to type all the isolates in this study (Meer and Songer, 1997). PCR amplification of *cpb2* was performed by the addition of 0.36  $\mu$ M of each *cpb2* primer (5'-AGATTTTAAATATGATCCTAACC-3' and 5'-CAATACCCTTCACCAAATACTC-3'), to the multiplex PCR assay, which resulted in amplification of a 567 bp product in positive isolates (Garmory et al., 2000). PCR products were identified by UV transillumination, following electrophoresis through a 1.5% agarose gel containing 1.5  $\mu$ g/ml ethidium bromide.

## 2.5. Cloning and purification of a recombinant, 6xHIS-tagged beta2

The *cpb2* gene, lacking the coding region for the signal sequence, was amplified from *C. perfringens* strain 690D genomic DNA by PCR with a 5' primer containing a *Bam*HI site (5'-AATGAAAGCGGATCCAAAAGAAATCG-3') and a 3' primer containing an *Eco*RI site (5'-GTCACCTCAGAATTCTTTCTATGCAC-3') (restriction sites are underlined in the primer sequence). These primers amplified a 0.74 kb product from bases 91–798 of the *cpb2* gene (GenBank accession L77965). The PCR fragment was digested with *Bam*HI–*Eco*RI and cloned into *Bam*HI–*Eco*RI digested pTrcHis B (Invitrogen), to generate pJGS197. pJGS197, encoded 6xHIS-tagged beta2 (HIS-beta2), a 268 amino acid protein comprising

235 amino acids of the mature beta2, with an N-terminal extension of 33 amino acids encoded by pTrcHis B, including a 6xHIS sequence. DNA sequencing of the insert portion of pJGS197 was performed to ensure no mutations had been introduced during PCR.

Cultures for preparation of HIS-beta2 were grown to an OD<sub>600</sub> of 0.6, prior to induction with 2.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were harvested by centrifugation at 5000 × *g* and the cell pellet was resuspended in 20 mM Tris-HCl, 100 mM NaCl, pH 8.0. The cells were disrupted by two passages through a French pressure cell (Aminco) at 138 MPa and the insoluble material was removed by centrifugation at 12,000 × *g*. HIS-beta2 was purified from the soluble fraction using TALON metal affinity resin (Clontech), as per the manufacturer's instructions, and the protein was eluted from the resin with 50 mM imidazole, 20 mM Tris-HCl, 100 mM NaCl, pH 8.0. Total protein concentration was determined using Bradford protein assay reagent (Bio-Rad).

### 2.6. Preparation of goat antiserum to HIS-beta2

A male goat was immunized with 250 μg HIS-beta2 in Ribi adjuvant system (RAS) (Corexa) intramuscularly in the hindleg at two sites. A similar booster immunization of 250 μg HIS-beta2 in RAS was administered on days 14, 28 and 42. Blood was collected on day 64 and serum was harvested from the clotted blood by centrifugation at 400 × *g*.

### 2.7. SDS-PAGE and Western blotting

*E. coli* whole cells, purified HIS-beta2 or *C. perfringens* concentrated CSF were mixed 1:1 with SDS-sample buffer (0.2 M Tris-HCl, pH 6.8, 2.5% SDS, 10% β-ME, 20% glycerol, 0.013% bromophenol blue) and boiled for 10 min prior to electrophoresis in a 12% (w/v) SDS-polyacrylamide gel, essentially as described (Ausubel et al., 1994). Proteins were transferred to nitrocellulose and Western blots were immunostained as previously described (Ausubel et al., 1994) using goat anti-HIS-beta2 and rabbit anti-goat IgG(H+L)-peroxidase conjugate (KPL) as the primary and secondary antibodies, respectively.

## 3. Results and discussion

### 3.1. Prevalence of *cpb2*

A total of 3270 *C. perfringens* isolates were screened using a modification of the standard multiplex PCR used for genotyping this organism (Meer and Songer, 1997), by incorporation of *cpb2*-specific primers (Garmory et al., 2000). The vast majority of these isolates were from known disease cases from domestic animals (87.8%), and were predominantly type A (92.4%). Of the total, 37.2% were PCR positive for the *cpb2* gene.

*cpb2* was found in *C. perfringens* strains from all species examined, but the prevalence varied substantially. Pigs with enteritis were most likely to yield *cpb2*-positive isolates (85.8%). This correlation was particularly high among isolates from neonatal pigs with enteritis or diarrhea (91.8%). The majority of isolates in this group were type A (Table 1), suggesting that *C. perfringens* type A is a major cause of clostridial enteric disease in neonatal pigs.

Table 1  
Prevalence of *cpb2* genotype in *C. perfringens* field isolates

Origin	Number	% Positive
Porcine (all ages, all conditions)	1132	75.8
Piglet (all conditions)	364	83.8
Piglet (enteritis, diarrhea)	256	91.8
Type A	220	90.9
Type C	36	97.2
Porcine (enteritis, diarrhea)	381	85.8
Porcine (normal)	9	11.1
Avian (all conditions)	133	33.8
Bovine (all conditions)	1537	12.8
Bovine (enteritis, enterotoxemia, sudden death)	336	21.4
Bovine calf	93	47.3
Type A	51	11.8
Type C	5	40.0
Type EE	37	97.3
Canine	140	37.9
Camelid (alpaca, llama)	43	18.6
Caprine	34	11.7
Cervid (caribou, deer, elk)	39	17.9
Equine	86	20.9
Feline (domestic, lynx, puma, tiger)	13	36.4
Other mammals (bear, beaver, bison, dolphin, ferret, kangaroo, mink, mouse, panda, seal, tapir)	23	17.4
Ovine	52	19.2
Rabbit	10	10.0
Environment (animal feed, human food, soil)	28	3.6

Moreover, the presence of *cpb2* in both type A and type C isolates (90.9 and 97.2%, respectively) strengthens the case for a direct association of beta2 toxin with enteric disease of neonatal pigs. This strong correlation with porcine enteric disease was highlighted by the fact that only one of the nine isolates from normal pigs was *cpb2*-positive (Table 1). Therefore, the high rate of occurrence of *cpb2*-positivity among strains from pigs with enteritis is consistent with the contention of other workers, that beta2 plays a role in pathogenesis of these infections (Gibert et al., 1997; Klaasen et al., 1999; Garmory et al., 2000).

*cpb2*-positive strains were more likely to be found in cattle with enteritis (21.4%) and in calves with enteritis or abomasitis (47.3%), than in the general population of bovine disease-associated isolates (12.8%). However, these percentages, even at their highest, were substantially less than that in piglets or adult pigs (Table 1). The high level of positivity among calf disease isolates was mainly due to type EE isolates (type E isolates carrying the *cpe* gene), a common cause of sudden death in calves. There was a near absolute correlation of *cpb2* in these strains (97.3%), while only 11.8% of type A isolates from calves contain *cpb2*. The presence of *cpb2* in *C. perfringens* isolates from other species ranged from ~12% in goats, ~20% in camelids, cervids, sheep, and horses, to ~35% in dogs and birds. Isolates from the environment, such as animal feed, human food and soil, showed only a very low occurrence of *cpb2* (Table 1).

Table 2  
Prevalence of *cpb2* by *C. perfringens* genotype

Genotype	% Positive
A ( <i>n</i> = 3021)	35.1
B ( <i>n</i> = 3)	66.6
C ( <i>n</i> = 178)	64.0
D ( <i>n</i> = 26)	0
DE ( <i>n</i> = 5)	100
EE <sup>a</sup> ( <i>n</i> = 37)	97.3

<sup>a</sup> The isolates are PCR positive for *cpe*, but do not express CPE (Billington et al., 1998).

*cpb2*-positivity varied with the *C. perfringens* type. *cpb2* was present in 35.1% of type A isolates, while approximately two-thirds of type B and C isolates were *cpb2*-positive (Table 2), although only a small number of type B isolates (*n* = 3) were tested. No *cpb2*-positive strains were found among 26 type D isolates, although 100% of *cpe*-carrying type D (DE) isolates (*n* = 5) contained *cpb2*. Furthermore, 36 of the 37 type EE isolates (97.3%) were *cpb2*-positive (Table 2). *cpe* is likely plasmid-borne in enterotoxigenic isolates, and taken together, these data suggest a possible genetic linkage between *cpe* and *cpb2*.

### 3.2. Cloning and expression of HIS-beta2

To facilitate the purification of recombinant beta2 from *E. coli*, the beta2-coding region, lacking the sequence for the putative signal peptide was cloned into pTrcHis B. SDS-PAGE and Coomassie brilliant blue staining of IPTG-induced cultures of DH5 $\alpha$ (pJGS197) indicated the presence of an over-expressed protein of approximately 31.2 kDa, compared to similarly induced cultures of DH5 $\alpha$ (pTrcHis B) (Fig. 1A). HIS-beta2 was purified from DH5 $\alpha$ (pJGS197) to >95% homogeneity using TALON resin (Fig. 1A), and the size of this protein corresponded to that of the predicted molecular mass of HIS-beta2. HIS-beta2 routinely purified as a doublet, which may indicate some processing of the protein in *E. coli*

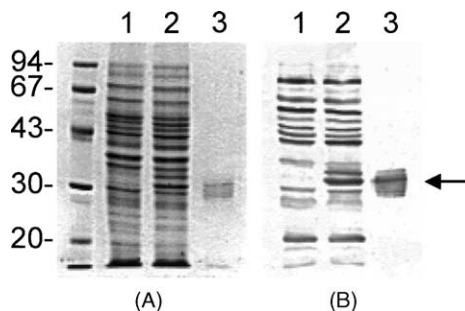


Fig. 1. Over-expression and purification of HIS-beta2. Whole cell lysates of IPTG-induced cultures of (1) DH5 $\alpha$ (pTrcHis B), (2) DH5 $\alpha$ (pJGS197), or (3) 100 ng purified HIS-beta2 were subjected to electrophoresis on 12% SDS-PAGE. Separated proteins were stained with Coomassie brilliant blue (A), or were transferred to nitrocellulose by Western blotting and immunostained with 1/100 dilution of HIS-beta2 antiserum (B). The positions of the molecular size standards are shown in kDa on the left. The arrow indicates the position of HIS-beta2.

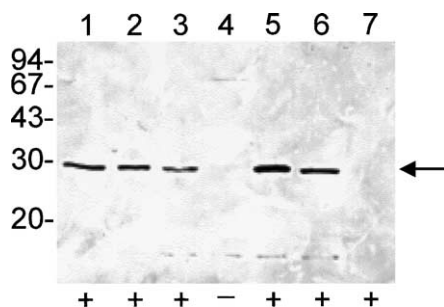


Fig. 2. Expression of beta2 in concentrated CSF of *C. perfringens* isolates. Concentrated CSF from strains (1) 690D (porcine type C), (2) porcine type A, (3) porcine type C, (4) porcine type A, (5) avian type A, (6) bovine type A, and (7) bovine type A were subjected to electrophoresis through a 12% SDS-PAGE. The separated proteins were transferred to nitrocellulose by Western blotting and immunostained with 1/100 dilution of HIS-beta2 antiserum. The positions of the molecular size standards are shown in kDa on the left. The + or – indicates the presence or absence, respectively, of *cpb2* in that strain. The arrow indicates the position of beta2.

(Fig. 1A). In Western blots, antiserum prepared against HIS-beta2 detected a protein of approximately 31.2 kDa in IPTG-induced cultures of DH5 $\alpha$ (pJGS197) and preparations of purified HIS-beta2, but not IPTG-induced cultures of DH5 $\alpha$ (pTrcHis B) (Fig. 1B).

### 3.3. Expression of beta2 by field isolates

Western blotting with antiserum against HIS-beta2 was used to detect expression of beta2 in concentrated CSF of 68 *cpb2*-positive and 13 *cpb2*-negative *C. perfringens* isolates. Strain 690D was used as a positive control, and a representative blot is shown in Fig. 2. With the exceptions of two equine and one cervid isolate, *C. perfringens* from all other species displayed a less than 100% correlation between genotype and beta2 toxin expression (Table 3). The bovine isolates are of particular interest, as they represent a substantial population which have silent genes. Bovine type A isolates only expressed beta2 toxin in 50% of cases, whereas expression was never detected in seven bovine type EE isolates (Table 3). Further genetic analysis is warranted to determine the nature of failure of these isolates to express beta2.

The high level of *cpb2*-positivity among strains from neonatal pigs with enteritis and the high correlation of genotype with phenotype (>96.9%) supports the contention that beta2 toxin may play a role in the pathogenesis of these infections (Gibert et al., 1997; Klaasen et al., 1999; Garmory et al., 2000). Of interest in this regard, is that the only *cpb2*-positive porcine isolate in which beta2 expression was not detected, was isolated from an apparently normal pig (Tables 1 and 3). However, in *cpb2*-positive *C. perfringens* isolated from non-porcine species, only 50% expressed beta2 protein. Therefore, care should be taken when interpreting the PCR results alone in the diagnosis of disease. It may also be important to consider the use of an additional method for the detection of beta2 toxin in non-porcine *cpb2*-positive isolates. Preferably, detection of beta2 should be performed directly from the tissue in enteritis cases where beta2 toxin may be expected to play a role.

Table 3  
Correlation of *cpb2* genotype with beta2 expression

Origin	Genotype	Beta2 expression (%)
<i>cpb2</i> -positive		
Avian ( <i>n</i> = 5)	A	40
Bovine ( <i>n</i> = 10)	A	50.0
Bovine ( <i>n</i> = 7)	EE	0
Canine ( <i>n</i> = 2)	A	0
Caprine ( <i>n</i> = 1)	DE	0
Caribou ( <i>n</i> = 1)	A	100
Equine ( <i>n</i> = 1)	A	100
Equine ( <i>n</i> = 1)	AE <sup>a</sup>	100
Ovine ( <i>n</i> = 1)	B	100
Ovine ( <i>n</i> = 1)	DE	0
Porcine ( <i>n</i> = 32)	A	96.9
Porcine ( <i>n</i> = 6)	C	100
<i>cpb2</i> -negative		
Alpaca ( <i>n</i> = 1)	A	0
Avian ( <i>n</i> = 2)	A	0
Bovine ( <i>n</i> = 2)	A	0
Equine ( <i>n</i> = 1)	A	0
Ovine ( <i>n</i> = 1)	D	0
Porcine ( <i>n</i> = 6)	A	0

<sup>a</sup> Type AE refers to *C. perfringens* type A carrying the *cpe* gene.

#### 4. Conclusions

The use of PCR to determine the presence of genes requires phenotypic characterizations before conclusions should be drawn about the functionality of a particular toxin in disease. The finding that 50.0% of non-porcine, *cpb2*-positive *C. perfringens* isolates did not express beta2 protein highlights this fact. More work is required to elucidate the role of beta2 in enteritis in pigs and other animal species, and determine the genetic basis of lack of beta2 expression in vitro in some *C. perfringens* isolates.

#### References

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), 1994. *Current Protocols in Molecular Biology*. Greene Publishing Associates/Wiley, New York, NY.
- Bacciarni, L.N., Pagan, O., Frey, J., Grone, A., 2001. *Clostridium perfringens* beta2-toxin in an African elephant (*Loxodonta africana*) with ulcerative enteritis. *Vet. Rec.* 149, 618–620.
- Billington, S.J., Wieckowski, E.U., Sarker, M.R., Bueschel, D., Songer, J.G., McClane, B.A., 1998. *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. *Infect. Immun.* 66, 4531–4536.
- Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer, J.G., Titball, R.W., 2000. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol. Infect.* 124, 61–67.

- Gibert, M., Jolivet-Reynaud, C., Popoff, M.R., 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. *Gene* 203, 56–73.
- Herholz, C., Miserez, R., Nicolet, J., Frey, J., Popoff, M.R., Gibert, M., Gerber, H., Straub, R., 1999. Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. *J. Clin. Microbiol.* 37, 358–361.
- Klaasen, H.L.B.M., Molkenboer, M.J.C.H., Bakker, J., Miserez, R., Hani, H., Frey, J., Popoff, M.R., van den Bosch, J.F., 1999. Detection of the beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in The Netherlands and Switzerland. *FEMS Immunol. Med. Microbiol.* 24, 325–332.
- Manteca, C., Daube, G., Jauniaux, T., Linden, A., Pirson, V., Dettleux, J., Ginter, A., Coppe, P., Kaeckenbeeck, A., Mainil, J.G., 2002. The role of *Clostridium perfringens* beta2-toxin in bovine enterotoxemia? *Vet. Microbiol.* 86, 191–202.
- McDonel, J.L., 1986. Toxins of *Clostridium perfringens* type A, B, C, D, and E. In: Dorner, F., Drews, H. (Eds.), *Pharmacology of Bacterial Toxins*. Pergamon Press, Oxford, pp. 477–517.
- Meer, R.R., Songer, J.G., 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am. J. Vet. Res.* 58, 702–705.
- Pospiech, A., Neumann, B., 1995. A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet.* 11, 217–218.
- Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S., Hayashi, H., 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* 99, 996–1001.
- Songer, J.G., 1996. Clostridial enteric disease of domestic animals. *Clin. Microbiol. Rev.* 9, 216–234.
- Thiede, S., Goethe, R., Amtsberg, G., 2001. Prevalence of beta2-toxin gene in *Clostridium perfringens* type A from diarrhoeic dogs. *Vet. Rec.* 149, 273–274.
- Wessel, D., Flugge, U.I., 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138, 141–143.