Clostridium perfringens in suckling piglets with diarrhoea and its PCR typing and prevalence in the Czech Republic in 2001–2003

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ABSTRACT: Out of the total of 153 farms under investigation that had been experiencing diarrhoea in suckling piglets, the presence of *Clostridium perfringens* was detected on 60 farms (39.2%). PCR typing of isolates prepared by anaerobic cultivation was carried out by multiplex PCR. *C. perfringens* type A (37.9%) and type C (1.3%) were detected on 58 and 2 farms, respectively. The *cpb2* gene was found in 79.3% strains of *C. perfringens* type A and in 100% strains of type C. An evaluation of the prevalence of positive identification of *C. perfringens* with the *cpb2* gene shows that the microorganism was diagnosed on large farms with the basic herd of over 700 or 1 000 sows significantly more frequently. None of the isolates was positive as to the specific sequences of the enterotoxin-coding gene.

Keywords: *Clostridium perfringens*; multiplex PCR; β2-toxin; pig; enteritis

Clostridium perfringens is an anaerobic G+ spore-forming bacterium that has been classified into 5 types based on the criterion of main toxin production. The clinical disease in pigs is caused by types A and C. *C. perfringens* type A produces the main α-toxin and is a part of the normal enteric microflora, but causes enteritis manifested by diarrhoea under conditions that have not been identified completely yet. *C. perfringens* type C produces two main toxins – α-toxin (*cpa*-lecithinase) and β-toxin (*cpb*-lethal necrotizing toxin), and a minor toxin (δ-toxin). *C. perfringens* type C is the agent causing fatal necrotic enteritis in piglets and its β-toxin is a highly trypsin-sensitive, single-chain polypeptide, whose molecular weight is 40 kDa. Some types of *C. perfringens* can produce some other toxins – enterotoxin (*cpe*) or β2-toxin (*cpb*2). The latter was discovered in piglets suffering from haemorrhagic enteritis in 1997. The toxin has a cytological effect on cell line 1407 (lysis-inducing activity) and is lethal to mice (Gibert et al., 1997). The gene coding *cpb2*-toxin production has been detected in *C. perfringens* strains isolated from many animal species (Garmory et al., 2000), e.g. horses (Herholz et al., 1999), elephants (Bacciarini et al., 2001), cattle (Manteca et al., 2001), sheep (Gkiourtzidis et al., 2001), and dogs (Thiede et al., 2001). The *cpb2* gene is being associated mainly with type A, but has also been identified in association with type C in piglets and in association with type E in calves (Garmory et al., 2000).

An opinion that the expression of enteric lesions requires a synergic action of α-toxin and β2-toxin (Manteca et al., 2002) has been voiced. Some epidemiological studies have reported a significantly more frequent isolation of *cpb*2-positive strains from piglets suffering from diarrhoea than from healthy animals (Klaasen et al., 1999; Garmory et

al., 2000). The association of *C. perfringens* enterotoxin with enteritis in piglets is not yet clear either. Our trial focused on *cpb2* and enterotoxin gene prevalence in *C. perfringens* strains isolated from ill suckling piglets from herds in the Czech Republic.

MATERIAL AND METHODS

Animals and representation of farms. Samples were taken from 153 different production farms in the Czech Republic in the period from 2001 to 2003 (Table 1). All samples were taken from piglets with apparent diarrhoea that were not treated with antibiotics. On each farm samples were taken from 3–10 clinically ill piglets. Individual farms differed in farming technologies and in the level of hygienic measures. Another factor distinguishing the farms was the scope of veterinary preventive and medical care.

Sample preparation. Rectal swabs were used for inoculation into transport medium AMIES W/CH and for subsequent bacteriological examination. Primary cultivation was done on blood agar (7% ovine blood) at 37°C under anaerobic conditions (atmosphere of 80.6:19.4, H_2:CO_2). Strains with typical double zone haemolysis were selected.

Suspensions of bacterial cells in distilled water (10^6 to 10^8 CFU/ml) were prepared in 2 ml polypropylene vials, incubated for 15 min at 100°C in a heat block and centrifuged for 10 min at 10 000 g at room temperature. Clear samples of supernatant were directly added to PCR mixture.

Polymerase chain reaction (PCR). A multiplex polymerase chain reaction assay for simultaneous

Table 1. Numbers of investigated farms and the percentages within the categories

Category	No. of sows in basic herd	No. of farms in CR*	No. of farms investigated	% of farms investigated
1	up to 100	5 032	28	0.6
2	101–200	787	44	5.6
3	201–400	374	34	9.1
4	401–700	94	16	17.0
5	701–1 000	31	11	35.5
6	1 001 and more	24	20	83.3
Total		6 342	153	

*Data for 2003 were provided by the State Veterinary Administration of the Czech Republic

Table 2. Oligonucleotide sequences used in the multiplex PCR system

Toxin gene	Primer	Oligonucleotide sequence	Fragment length (bp)	Source
alpha	cpa-f	5´-TTG ATG GAA CAG GAA CTC ATG C-3´	743	new sequences
	cpa-r	5´-TCC AAC TGA TGG ATC ATT ACC CT-3’		
beta	cpb-f	5´-GCG AAT ATG CTG AAT CAT CTA-3´	196	Meer and Songer, 1997 Gkiourtzidis et al., 2001
	cpb-r	5´-GCA GGA ACA TTA GTA TAT ATC TTC-3´		
beta2	cpb2-f	5´-GAA AGG TAA TGG AGA ATT ATC TTA ATG C-3´	567	Meer and Songer, 1997 Gkiourtzidis et al., 2001
	cpb2-r	5´-GCA GAA TCA GGA TTT TGA CCA TAT ACC-3´		
enterotoxin	cpe-f	5´-GGA GAT GGT TGG ATA TTA GG-3´	233	Meer and Songer, 1997 Gkiourtzidis et al., 2001
	cpe-r	5´-GGA CCA GCA GTT GTA GAT A-3´		
16S-rDNA	UNB-f	5´-AAC TGG AGG AAG GTG GGG AT-3´	370	new sequences
	UNB-r	5´-AAG AGG TGA TCC AAC CGC A-3´		

Table 3. Results of testing strains of *C. perfringens* for *cpa* by PCR and egg yolk agar

Strain	Internal strain source	Lecithinase activity (PCR)	Lecithinase activity (egg yolk agar)
19/79	ST1	+	+
2391	ST1	+	+
2388	ST1	+	+
2387	ST1	+	+
314	ANT	+	+
646	ANT	+	+
2390	ST1	+	+
49	ANT	+	+
2386	ST1	+	+
2389	ST1	+	+
312	ANT	+	+

detection of *cpa*, *cpb, cpb2* and *cpe* genes was constructed using specific oligonucleotide primer pairs described previously (Augustynowicz et al., 2000; Garmory et al., 2000; Gkiourtzidis et al., 2001; Yoo et al., 1997). As an internal reaction control the primers specific to bacterial 16S rDNA were newly added to the multiplex PCR reaction (Table 2). A new primer pair for detection of *cpa* gene was designed by ClustalW software using genomic sequences of *C. perfringens* (GeneBank, Accession numbers X17300.1, AB040737.1).

The new primer pair for detection of *cpa* gene was proved on 11 *C. perfringens* strains from National Reference Laboratories for Anaerobic Bacterials, Institute of Public Health, Ostrava, Czech Republic (Table 3), which were examined for lecithinase activity (α-toxin) by means of egg yolk agar. Agar was made by mixing 1 ml of stabilised egg yolk emulsion (SR0047, Oxoid) that was added directly to 10 ml of blood agar base. The tested strains of *C. perfringens* were inoculated and incubated for up to 5 days at 37°C.

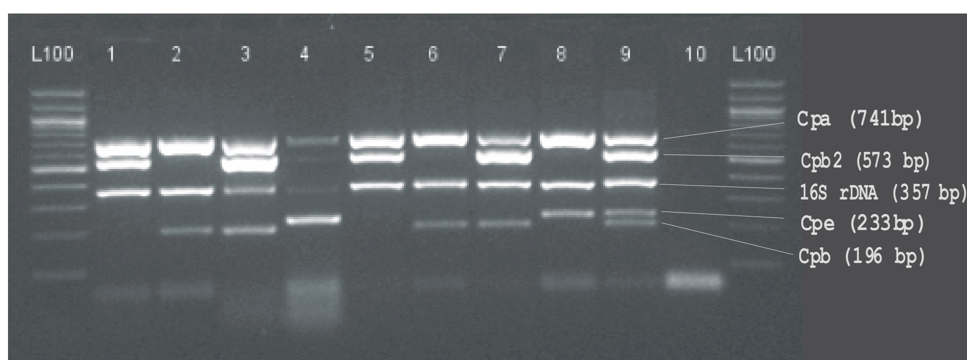

Figure 1. Specific amplification of *C. perfringens* toxin genes by multiplex PCR. Line 1 to 4: isolation of DNA by Qiagene. Line 5 to 8: supernatants of cell suspensions (see Sample preparation). Line **1**: *C. perfringens cpa-* and *cpb2*-positive strain (UN2814); line **2**: *cpa-* and *cpb*-positive strain (CNCTC 5460); line **3**: *cpa-*, *cpb2-*, and *cpb*-positive strain (UN2814 and CNCTC 5460); line **4**: *cpa-* and *cpe*-positive strain (UN2814 and 2389/ST/2001), the reaction was partially inhibited by the isolated DNA sample; line **5**: *cpa-* and *cpb2*-positive strain (UN2814); line **6**: *cpa*-and *cpb*-positive strain (CNCTC 5460); line **7**: *cpa-*, *cpb2-*, and *cpb*-positive strain (UN2814 and CNCTC 5460); line **8**: *cpa-* and *cpe*-positive strain (2389/ST/2001); line **9**: positive PCR control from isolated DNA; line **10**: negative PCR control; **L100**: 100 bp DNA weight marker (New England Biolabs, USA)

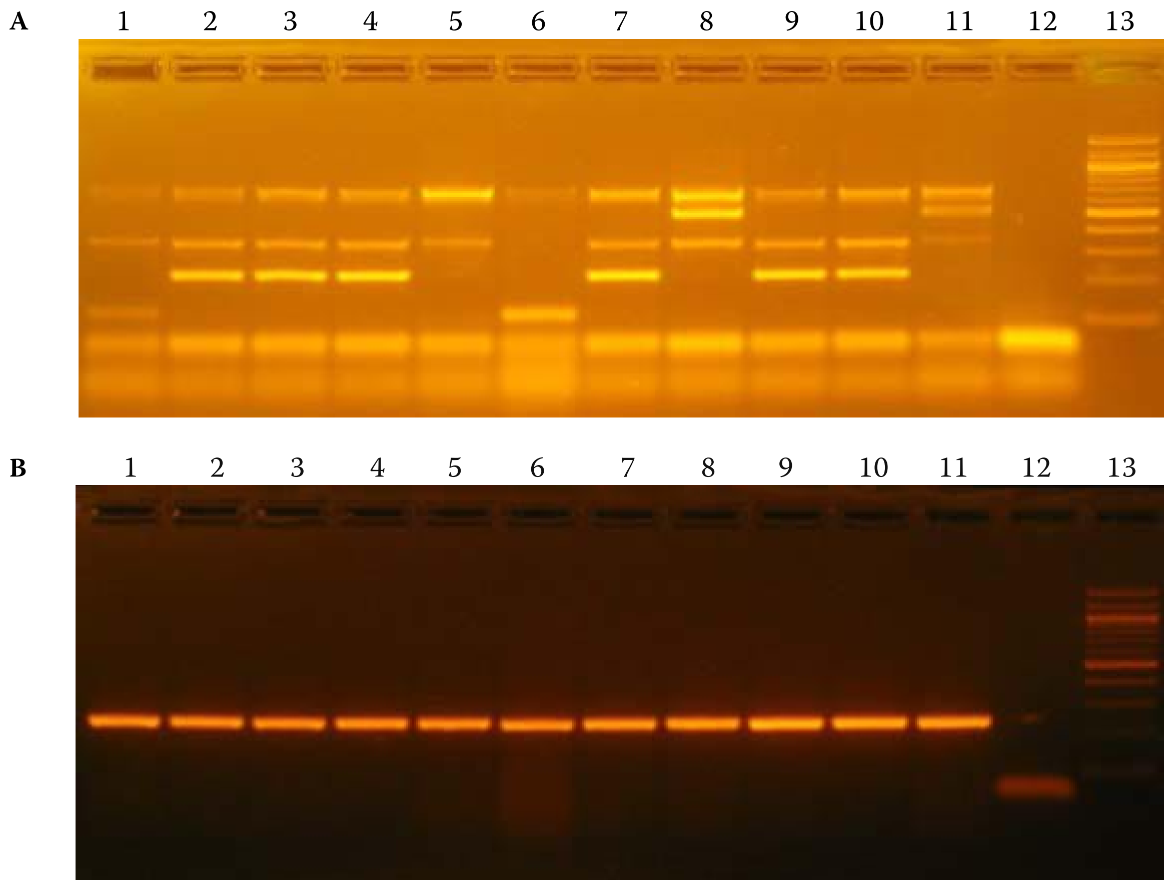

Figure 2. PCR detection of *cpa* toxin gene using previously published *cpa* primers and our newly designed *cpa* primers. **A**: novel multiplex PCR, **B**: PCR on previously published *cpa* primers. Line 1–11: bacterial strains characterized by egg yolk emulsion; Line 12: negative control; Line 13: 100 bp DNA weight marker (New England Biolabs, USA)

The reproducibility of the method was tested on strains of *C. perfringens* of defined genotypes (strain *cpa*, strain *cpa/cpb*, strain *cpb2* and strain *cpe*, and their mixture (Table 4)). These samples were isolated according to the standard protocol for the isolation of genomic DNA (Qiagene, USA) and by a "suspension-boiling" method. The two isolation methods were compared using our multiplex PCR reaction (Figure 1).

Each PCR reaction (total volume 40 µl) contained 2U DynaZyme polymerase (Finzyme, Finland), 4 µl of supplied 10× reaction buffer, 187.5mM dNTP (Roche), 2% glycerol, additional $MgCl_2$ to final concentration 2.5mM, 5 µg/ml of red cresol (Sigma), 4 µl of sample, primer pairs *cpa*, *cpb*, *cpb2*, *cpe*, and 16S rDNA (Table 2) in the concentration of 50 pmol, 5 pmol, 40 pmol, 5 pmol, and 2.5 pmol per reaction, respectively. The reaction was performed in a PTC200 thermocycler (MJ Research) at the following conditions: initial denaturation at 96°C for 2 min; 45 cycles of 96°C for 10 s; 56°C for 10 s; 72°C for 60 s; final elongation at 72°C for 2 min. The amplification products were separated on 3% ethidium bromide-stained agarose gels at 5 V/cm, visualised by UV illumination at 312 nm and photographed by CCD camera (Figure 1).

RESULTS

The new primer pair for detection of *cpa* gene was proved on 11 bacterial strains (Table 3). All 11 strains demonstrate lecithinase activity (presence of α-toxin) by production of zones of opacity around their colonies on the egg yolk agar. The identical results were obtained comparing new

Table 4. Registered strains used for the evaluation of the method

C. perfringens	No. of registered strain	Strain source
Toxin α	CAMP 5744 T	Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, CZ
Toxin α, β	CNCTC 5460	Czech National Collection of Type Cultures, National Institute of Public Health, Prague, CZ
Toxin α, β2	UN2814	National Collection of Type Cultures, Central Public Health Laboratory, London, UK
Toxin α, enterotoxin	2389/ST/2001	National Reference Laboratories for Anaerobic Bacterials, Institute of Public Health, Ostrava, CZ

Table 5. The distribution of the farms where *C. perfringens* and *cpb2* gene were identified

No. of sows in the basic herd	No. of farms investigated in Czech Republic	No. of farms with *C. perfringens* identified	% of positive farms
up to 100	28	10	35.7*§
101–200	44	13	29.5
201–400	34	12	35.3
401–700	16	5	31.3
701–1 000	11	10	90.9
1 001 and more	20	10	50*

*including farms with *C. perfringens* type C positive isolation
§including two farms with only *cpa* gene identification

primers with a previously published primer pair (Yoo et al., 1997) (Figure 2).

Out of the total of 153 farms under examination, the presence of *C. perfringens* was detected on 60 farms (39.2% of farms). The identification of the isolates was confirmed by demonstration of 100% presence of the specific sequence of *cpa* gene in the isolated strains.

C. perfringens type A was identified on 58 farms (37.9% of the farms under investigation) that had been experiencing diarrhoea in suckling piglets. *C. perfringens* type A with *cpb2* gene was found on 56 farms (36.6% of farms). The identification of the *cpb2* gene was positive in 79.3% out of 116 isolated strains of *C. perfringens*. The sequence specific to β-toxin was identified in strains from two different farms (1.3% of farms under investigation). These strains were also *cpb2*-positive. None of the isolates was positive in terms of specific sequences of the enterotoxin-coding gene. The distribution of the farms under investigation within the trial where *C. perfringens* and the *cpb2* gene were identified in terms of the number of sows in the basic herd is presented in Table 5.

DISCUSSION

Multiplex PCR identification of genes coding the production of toxins in *C. perfringens* species was done for several years. The multiplex PCR methods developed earlier were adapted using some new primers in this trial. A new primer pair for *cpa* toxin gene detection was designed and used in our multiplex PCR since the length of amplification products using previously published *cpa* primers (Yoo et al., 1997) was not suitable for our multiplex assay. The modified multiplex PCR allows for an accurate typing of *C. perfringens* pig isolates and for distinguishing between type A and type C. Apart from this, the method allows for subtyping *C. perfringens* based on the identification of genes for β2-toxin and enterotoxin production.

The 79.3% prevalence of *C. perfringens* type A strains with *cpb2* gene in piglets suffering from diarrhoea is in correlation with previous surveys – reporting 82% prevalence for piglets suffering from diarrhoea (Garmory et al., 2000) and as high as 91.8% prevalence in neonatal piglets suffering from diarrhoea or enteritis (Bueschel et al., 2003).

C. perfringens type C, the agent causing fatal haemorrhagic-necrotic enteritis in neonatal piglets, was diagnosed on 2 farms only. Both cases were identified in 2001; no later incidence was recorded.

None of the isolates was positive to specific sequences of the gene coding the enterotoxin production, which is consistent with a previously published study (van Damme-Jongsten et al., 1990).

Assessing the distribution of the farms under investigation, we may state that the group of farms selected by us includes a sufficient number of farms for each category (Table 1) even if the numbers are not representative of some categories. The farms included in the survey tend to rank among farms with over 100 sows in the basic herd, despite the fact that 79% of farms in the Czech Republic have less than 100 sows. The main reason for this situation is better cooperation with relatively large and large farms, whose more stabilised economic situation allows them to make more investments into laboratory diagnostics.

Our analysis of the prevalence of positive identification of *C. perfringens* with the *cpb2* gene on farms of different sizes shows that the type was diagnosed more frequently on large farms. On farms with over 700 sows in the basic herd, the prevalence of *C. perfringens* with the gene coding β2-toxin production was 90.9% and the prevalence was 50% on farms with basic herds of over 1 000 sows. The finding is in sharp contrast with the average prevalence of *C. perfringens* with the *cpb2* gene in other size categories of farms (33%).

CONCLUSIONS

Our findings allow us to say that *C. perfringens* type A and C was detected in piglets suffering from diarrhoea on farms in the Czech Republic. The study corroborated a high frequency of *cpb2* gene in *C. perfringens* type A isolates. A relationship between the identification of *C. perfringens* with the β2-toxin coding gene and the size of the farm was also detected. We could not prove the involvement of *C. perfringens* enterotoxin in the diarrhoea experienced by suckling piglets.

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