Detecting toxin genes of *Clostridium perfringens* isolated from diarrhea piglets using multiplex PCR

Dung H. M. Nguyen[1], Quynh T. X. Luong[1], Phuong T. Hoang[2], Duong T. T. Do[1], Thoai K. Tran[1], & Phat X. Dinh[1*]

[1]Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Vinh An Science and Technology Company Limited, Ho Chi Minh City, Vietnam

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***Corresponding author**

Dinh Xuan Phat
Email: dinhxuanphat@hcmuaf.edu.vn

ABSTRACT

Clostridium perfringens is currently classified into five types (A, B, C, D, E) based on the different toxins produced. Type A and C are known as the causative agent of enteritis and enterotoxemia in newborn and young piglets with severe intestinal lesions including edema, hemorrhage and necrosis. A multiplex PCR (mPCR) was developed in order to quickly and early determine the presence of genotypes of *C. perfringens* based on their genes of *cpa*, *cpb*, *cpb2* and *cpe* encoding alpha toxin, beta toxin, beta2 toxin and enterotoxin with predicted products of 324 bp, 196 bp, 107 bp and 257 bp respectively. The detection limit of the mPCR assay was 1×10^3 copies/reaction for each gene. Sequencing of mPCR products performed with clinical samples collected from *C. perfringens* suspected pigs showed that the mPCR test functioned specifically. In conclusion, the developed mPCR test successfully detected the presence of genes *cpa*, *cpb*, *cpb2* and *cpe* in the examined samples. Analysis of the bacteria isolated from field samples of diarrheal piglets collected in this study indicated that *C. perfringens* carrying gene *cpa* counted for 96.66% and 3.33% was identified as *C. perfringens* carrying genes *cpa* and *cpb* concurrently. Gene *cpe* was not found in this study, while gene *cpb2* was detected coincidently in 73.33% of the samples with *cpa* gene. The results indicate that the prevalence of these four toxin genes is *cpa*, *cpb2*, *cpb* and *cpe* in decending order.

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1. Introduction

Diarrhea neonatal piglets is one of the most causes of economic losses in the swine industry. Among the common infectious agents, *Clostridium perfringens* (*C. perfringens*) plays a key role in enteric diseases not only in domestic animals but also in humans. *C. perfringens* is a Gram-positive, anaerobic, rod-shaped bacterium. It is known to produce various toxins including alpha (α), beta (β), epsilon (ε), and iota (ι). These toxins play important roles in the pathogenesis of the disease and are used to classify *C. perfringens* into five biotypes, designated A-E. These five types can be subdivided according to the production of two additional toxins: the enterotoxin (encoded by the *cpe* gene) and the β_2 toxin (encoded by *cpb2* gene) and described in Table 1. Type A and C strains cause diarrhea, dysentery and enterotoxaemia in pigs (Lebrun et al., 2010; Markey et al., 2013).

Conventional isolation on agar media usually

Table 1. *Clostridium perfringens* conventional toxinotypes (Leburn et al., 2010; Mcclane et al., 2006)

Genes	Toxin	Type A	Type B	Type C	Type D	Type E
cpa	α	X	X	X	X	X
cpb	β		X	X		
etx	ε		X		X	
iap/ibp	ι					X
cpe	Enterotoxin (X)					(X)
cpb2	β_2	(X)	(X)	(X)	(X)	(X)
Host		Pigs, humans, lambs, dogs, chickens	Lambs (under 3 weeks old), neonatal calves, foals	Piglets, lambs, calves, foals, adult sheep, chickens	Sheep (all ages, except neonates), (goats, calves)	Calves, rabbits

X Classic; (X) Potential.

takes longer time in routine diagnostic process. In this study, a multiplex PCR (mPCR) protocol was developed to determine the presence of toxin genes coding for alpha toxin (*cpa*), beta toxin (*cpb*), enterotoxin (*cpe*) and beta2 toxin (cpb2) of *C. perfringens* isolates.

2. Materials and Methods

2.1. Control and clinical samples

Positive control: DNA fragments of *cpb* gene (beta toxin) and *cpe* gene (enterotoxin) were synthesized by IDT (Integrated DNA Technologies - USA); and *C. perfringens* reference strains contained *cpa* gene (alpha toxin) and *cpb2* gene (β_2 toxin) were supplied by Sanphar Vietnam laboratory (belonging to Erber group, Austria). The presence of *cpa* and *cpb2* in this positive control sample was confirmed by sequencing. The resultant sequences of *cpa* and *cpb2* has 97-100% identity to the Genbank Id MH213493.1 and MG720638.1, respectively.

Negative control: viruses and bacteria potentially found in intestinal or fecal samples including *Salmonella* spp., *E. coli* (ATCC 25922), obtained from Sanphar's laboratory. *Salmonella* spp. was isolated from the field and identified by culture method as well as biochemical reaction; colonies of *Streptococcus suis* and a sample containing DNA of PCV2 virus confirmed by sequencing were obtained from the laboratory of Animal Molecular Pathogenesis and the Gene Technology laboratory respectively at the Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam.

Clinical samples: Thirty isolates of *C. perfringens* were selected from different samples of anal swabs or feces taken from piglets (< 25 days of age) having the symptoms or lesions of: 1/ sudden death or dying shortly after bloody diarrhea; 2/ diarrhea; 3/ diarrhea with blood or necrotic patches of tissues;4/ Dead piglets usually have bulging stomach and/or intestines; 5/ Haemorrhagic and/or necrotic intestinal mucosa.

2.2. Isolation of total DNA

Clostridium perfringens isolates were collected from clinical samples (feces and swab samples from *C. perfringens* - suspected pigs with the symptoms described above) using blood agar medium (Cat#M975A, Himedia) in anaerobic condition and these colonies were determined as *C. perfringens* by morphology. After 24 to 48 hours of culture at 37^0C, these colonies appeared with round, smooth and glossy shapes, covered by a double hemolysis, complete hemolysis inner zone and partial hemolysis outer zone. Suspected colonies were further confirmed by biochemical reactions on gelatin medium to test sugar fermentation, nitrate to nitrite transfer and negative catalase test (Markey et al., 2013). Then, TPGY (Tryptone Peptone Glucose Yeast extract) (Cat#M969, Himedia) broth was used as an enrichment broth for obtaining a high rate of bacterial biomass. Thus, cells from 50 mL of overnight cultures of TPGY broth were harvested by cen-

trifugation at 13,000 rpm for 10 min at 4^0C. The cells were washed in 5 mL of 1X PBS pH 7.0 (Cat#10010023, Gibco), centrifuged and resuspended in 1 mL of the same buffer. Twenty microliters of the solution mixture with 300 μL TEN buffer (20mM Tris-HCl, 5mM EDTA, 140 mM NaCl, pH 8.0) and 30 μL lysozyme (10 ng/μL) (Cat#90082, Thermo Fisher Scientific). The solution was incubated at 37^0C for 15 min. After incubation of the mixture with 30 μL of SDS 20% solution at 37^0C for 15 min, the bacterial DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution (Cat#P1037, Sigma; Cat#25666, Merck). The tubes were kept inverted then still in 5 min and centrifugation at 13,000 rpm for 10 min. The upper aqueous layer was recovered for DNA precipitation with 900 μL ethanol 100% at -20^0C overnight. The DNA was pelleted, washed with 70% ethanol, allowed to dry and dissolved in 40 μL TE, pH 8.0. Extracted DNA was stored at -20^0C until being used. Two microliters were used in each mPCR reaction.

2.3. Primer design

Primer pairs CPA (encoding alpha toxin), and CPB (encoding beta toxin) were adopted from Meer and Songer (Meer et al., 1997). Besides, CPE (encoding enterotoxin) and CPB2 (encoding β_2 toxin) primers were designed by Primer3plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) using the sequence data of cpe gene and cpb2 *gene* obtained from NCBI (Table 2), and validated by NCBI BLAST, OligoAnalyzer 1.0.2. The annealing temperature and the size of the amplified product were adjusted to become appropriate to be combined with the two adopted primer pairs in a new mPCR. Primers were synthesized by IDT (Integrated DNA Technologies - USA).

2.4. Single PCR (sPCR) optimization

All primers were initially tested using gradient single PCRs according to the product specifications and protocols. The sPCR was performed in a 30 µl reaction mixture containing 1 μL DNA template, 0.33 μM each primer, 15 μL DreamTaq master mix 2X (Cat#K1081, Thermo Fisher Scientific), and nuclease-free water to adjust the final volume to 30 μL (Cat#R0582, Thermo Fisher Scientific). Nuclease-free water was also used as a negative control for all PCRs. The PCR was

Table 2. Primer sequences and estimated product sizes

Genes	Primers	Primer sequences (5' – 3')	Product size (bp)	Reference
cpa	CPA	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324	Meer & Songer (1997)
cpb	CPB	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATCTTC	196	Meer & Songer (1997)
cpe	CPE	F: ACAACTGCTGGTCCAAATGA R: GCAGCAGCTAAATCAAGGAT	257	Present study
cpb2	CPB2	F: TGCAACTTCAGGTTCAAGAGA R: CAGGGTTTTGACCATACACCA	107	Present study

carried out for pre-denaturation at 95^0C for 5 minutes, 35 cycles consisting of denaturation for 30 seconds at 95^0C, annealing at a temperature range for the gradient PCR: 53^0C, 55^0C, 57^0C, 59^0C, 61^0C for 30 seconds, extension for 70 seconds at 72^0C and a final extension of 72^0C for 10 minutes (model TC-512 GeneAmp PCR System; England). Ten microliters of amplified products were then analyzed by electrophoresis in a 2% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) with Midori Green Advance DNA stain (Cat#AG10, Nippon) using 1 kb Plus DNA ladder (Cat#10787018, Invitrogen) as the molecular weight markers to indicate the sizes of the amplification products.

2.5. Multiplex PCR (mPCR)

After several rounds of optimization, four ratios of each primer were investigated. Finally, a primer mix including the four primer pairs was generated with a ratio of CPA:CPB:CPE:CPB2 to be 0.67 μM: 0.33 μM: 0.67 μM: 1.0 μM respectively. The annealing temperature of mPCR was 57^0C to detect equal signal for each PCR product. The final mPCR mix included 15 µl of DreamTaq 2X primer concentration is used as mentioned above; 4 μL DNA template mix; and nuclease-free water to adjust the final volume to 30 μL. The mPCR conditions were similar to those described for sPCRs. Gel electrophoresis was extended to 70 minutes at 60V for better separation of the amplicons. After that, DNA fragments were recovered from low melting agarose using phenol-chloroform method and sequenced by University of Medicine and Pharmacy, Ho Chi Minh city, Vietnam. The sequences of the products were aligned with the target genes.

2.6. Specificity and sensitivity of multiplex PCR

In order to confirm the specificity of the mPCR conditions, genomic DNA of *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 were used as negative controls in the mPCR reactions as described above. Regarding the sensitivity, synthesized DNA fragments of *cpb* gene and *cpe* gene; and the purified PCR product of *cpa*, *cpb2* gene were used. These templates were diluted ten-fold serially in nuclease-free water and used for sensitivity test in the mPCR to estimate its limit of detection.

3. Results and Discussion

3.1. Multiplex PCR

In sPCRs, gel electrophoresis analysis confirmed the exact product size as predicted for each gene, including *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp. The results also indicated that 4 pairs of primers worked well in the annealing temperature range of 55^0C - 61^0C, and the 57^0C was chosen for mPCR. In addition, after the optimization of the mPCR, the products were clearly visible and easily distinguishable from each other, and sequencing of the four mPCR products showed that the mPCR functioned accurately (Figure 1).

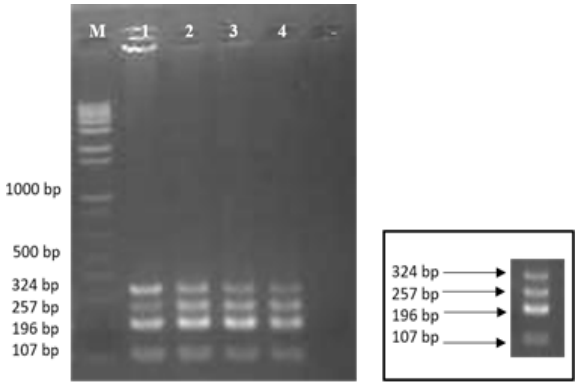

Figure 1. Results of the annealing temperature survey of multiplex PCR detecting four toxin genes of *C. perfringens*. *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp. M: 1 kb Plus ladder; (1) - (4): annealing temperature of 55^0C, 57^0C, 59^0C, 61^0C, respectively; (-) negative control with pure water.

Figure 2a is a result of the sensitivity testing of the optimized mPCR showing the four clear products. The mPCR could detect all four bands with equal signals when the template concentration present at 1 x 10^3 copies/reaction.

Specificity test of the mPCR was performed with unrelated DNA from virus and bacteria commonly found in the intestine and feces of pigs including *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 as the four negative controls. Results showed that no amplified products were seen. It means that four primer pairs do not cross-react with DNA from the investigated organisms, avoiding false-positive results (Figure 2b).

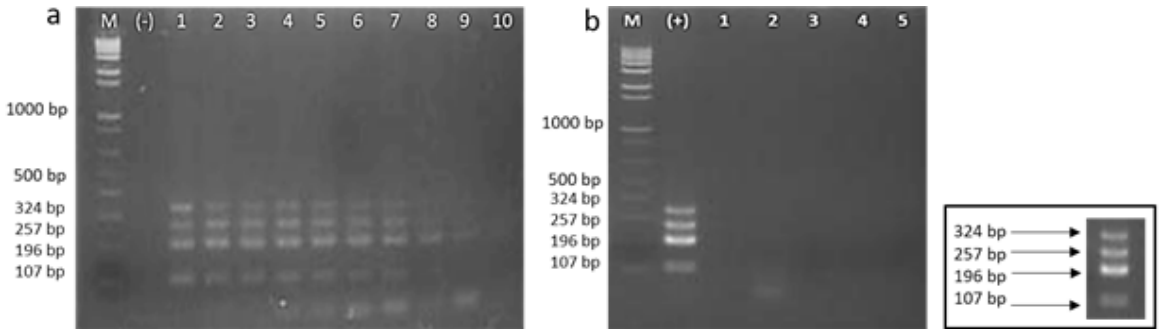

Figure 2. Multiplex PCR detecting four toxin genes of *C. perfringens*. *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp.
a. Sensitivity test. M: 1 kb Plus ladder; (-) negative control with pure water; (1) - (10): dilution starting from 1 x 10^9 to 1 x 10^0 DNA copies of each template.
b. Specificity test. (+): positive control; (1) - (4): negative controls (DNA of *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 respectively); (5) negative control with pure water.

Figure 3. Multiplex PCR test using clinical samples.
M: 1 kb Plus ladder, (+) positive control, (-) negative control with pure water, (1) - (14) clinical samples.

3.2. Detecting the presence of toxin genes from clinical samples

The mPCR was evaluated using 30 colonies isolated from clinical samples of different farms suspected to be *C. perfringens* based on biochemical test following instruction by Markey et al. (2013). The results are summarized in Table 3 while Figure 3 showed the agarose gel analysis for mPCR products of 14 out of 30 isolates examined.

All 30 isolates were shown to carry the cpa gene (100%), further confirming these isolates are *C. perfringens* even though this is not surprising, as gene *cpa* has been reported to be the dominant genes of *C. perfringens* in swine. Only one out of 30 samples (3.33%), in the well number 10 showed positive for both alpha (*cpa*) and beta toxin (*cpb*) gene together (Figure 3). Recently, Yadav et al. (2017) also reported the presence of only 3% *C. perfringens* carrying the cpa and *cpb* gene from diarrheal cases in swine in India. Additionally, 22/30 isolates (73.33%) positive for the cpa and cpb2 gene (encoding β_2 toxin) in the present study was similar to the detection rate (70% - 90.3%) from previous reports (Van Asten et al., 2010; Chan et al., 2012; Yadav et al., 2017). It has been shown that β_2 toxin may play a key role in enteric diseases of pigs, even though the issue is still controversial. On the other hand, none of the isolates tested in this examination was *cpe*-positive, this is in accordance with a previous study carried out in America with 89 samples (Kanakaj et al., 1998). In the present communication, according to the toxinotypes of Leburn

Table 3. Results of mPCR detecting four toxin genes of thirty *C. perfringens* isolates from diarrheal piglets

Isolate	Genes (Toxin)			
	cpa (α)	*cpb* (β)	*cpe* (Entero-toxin)	*cpb2* (β_2)
1	(+)	(-)	(-)	(+)
2	(+)	(-)	(-)	(-)
3	(+)	(-)	(-)	(+)
4	(+)	(-)	(-)	(+)
5	(+)	(-)	(-)	(-)
6	(+)	(-)	(-)	(+)
7	(+)	(-)	(-)	(+)
8	(+)	(-)	(-)	(+)
9	(+)	(-)	(-)	(+)
10	(+)	(+)	(-)	(-)
11	(+)	(-)	(-)	(+)
12	(+)	(-)	(-)	(+)
13	(+)	(-)	(-)	(+)
14	(+)	(-)	(-)	(+)
15	(+)	(-)	(-)	(-)
16	(+)	(-)	(-)	(-)
17	(+)	(-)	(-)	(+)
18	(+)	(-)	(-)	(+)
19	(+)	(-)	(-)	(-)
20	(+)	(-)	(-)	(-)
21	(+)	(-)	(-)	(+)
22	(+)	(-)	(-)	(+)
23	(+)	(-)	(-)	(+)
24	(+)	(-)	(-)	(+)
25	(+)	(-)	(-)	(+)
26	(+)	(-)	(-)	(+)
27	(+)	(-)	(-)	(+)
28	(+)	(-)	(-)	(+)
29	(+)	(-)	(-)	(-)
30	(+)	(-)	(-)	(+)

(+):Positive;(-):Negative.

et al. (2010) and Mcclane et al. (2006) (Table 1), 96.66% of the isolates showing positive for *cpa* can be considered as *C. perfringens* type A; 3.33% isolates positive for both *cpa* and *cpb* can be considered as C. perfringens type C; 73.33% isolates showing positive for *cpa* and *cpb2* gene are *C. perfringens* type A carrying additional minor *cpb2* gene.

4. Conclusions

To summarize, the mPCR developed in this study enables the simultaneous detection of two major toxin genes (*cpa*, *cpb*) and two minor toxin genes (*cpe*, *cpb2*) of *C. perfringens*. The optimal annealing temperature was 57^0C/30 s. The ratio of primers CPA:CPB:CPE: CPB2 were 0.67 μM: 0.33 μM: 0.67 μM: 1.0 μM respectively. The mPCR was specific and the sensitivity was at 1 x 10^3 copies/template per reaction. Thirty colonies isolated from clinical samples were tested to determine the presence of these toxin genes. Results showed that in this set of samples, the detection rate of *cpa*, *cpb*, *cpb2* and *cpe* was 100%, 3.33%, 73.33% and 0% respectively. The results indicate that the prevalence of these four toxin genes is *cpa*, *cpb2*, *cpb* and *cpe* in decending order.

References

Chan, G., Farzan A., Soltes, G., Nicholson, V. M., Pei Y., Friendship, R., & Prescott, J. F. (2012). The epidemiology of *Clostridium perfringens* type A on Ontario swine farms, with special reference to cpb2-positive isolates. *BMC Veterinary Research*, 8(1), 156.

Kanakaraj, R., Harris, D. L., Songer, J. G.,& Bosworth, B., (1998). Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. *Veterinary microbiology* 63(1), 29-38.

Lebrun, M., Mainil, J. G., & Linden, A. (2010). Cattle enterotoxaemia and *Clostridium perfringens*: description, diagnosis and prophylaxis. *Veterinary Record* 167(1), 13-22.

Markey, B., Leonard, F., Archambault, M., Cullinane, A., & Maguire, D. (2013). *Clinical veterinary microbiology* (2nd ed.). Edinburgh, UK: Mosby Elsevier.

Mcclane, B. A., Uzal, F. A., Miyakawa, M. E. F., Lyerly, D., & Wilkins, T. (2006). *The prokaryotes* (3rd ed.). New York, USA: Springer.

Meer, R. R., & Songer, J. G. (1997). Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *American Journal of Veterinary Research* 58(7), 702-705.

Van Asten, A. J., Nikolaou, G. N., & Grone, A. (2010). The occurrence of *cpb2*-toxigenic *Clostridium perfringens* and the possible role of the β_2 toxin in enteric disease of domestic animals, wild animals and humans. *The veterinary journal* 183(2), 135-140.

Yadav, J. P., Das, S. C., Dhaka, P., Vijay, D., Kumar, M., Mukhopadhyay, A. K., Chowdhury G., Chauhan P., Singh R., Dhama K., Malik S., & Kumar A. (2017). Molecular characterization and antimicrobial resistance profile of *Clostridium perfringens* type A isolates from humans, animals, fish and their environment. *Anaerobe* 47, 120-124.