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Detection, cloning, and sequencing of the enterotoxin gene of *Clostridium perfringens* type C isolated from goat

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Abstract: *Clostridium perfringens* enterotoxin (CPE) is an important virulence factor involved in *C. perfringens* food poisoning and other gastrointestinal illnesses. In our experiment a *Clostridium perfringens* enterotoxin gene (cpe) positive type C strain was obtained by multiplex PCR from 34 *C. perfringens* isolates from goat in Guizhou Province, China. Then a pair of specific primers was designed and synthesized according to the published cpe gene, and the complete cpe gene was amplified from the genomic DNA of the cpe-positive *C. perfringens* isolate. The PCR product was cloned into pMD18-T vectors and transformed into competent cell DH5a. The recombinant plasmid was identified by PCR and restriction enzyme then sequenced and analyzed. The results showed that the cloned gene was 960 bp in length; the gene coded 319 amino acids, which shared 99.4~99.8% sequence identity and 99.1~-99.7% amino acid sequence identity with reference strains in GenBank. These results provide a basis for further research on the structure, properties, and biological activities of *Clostridium perfringens* enterotoxin. It also set the groundwork for further investigation of the mechanism of disease caused by *C. perfringens* enterotoxin.

Key words: *Clostridium perfringens*, cpe, multiplex PCR, cloning, sequencing analysis

Introduction

Clostridium perfringens is an endospore-forming, gram-positive bacterium that ranks among the most important of the anaerobic pathogens affecting humans and domestic animals (1,2). The bacterium produces at least 15 different toxins (3) that are thought to play a role in pathogenesis, although in most cases their activity is poorly understood. Each individual *C. perfringens* isolate does not produce the entire toxin arsenal; this provides a pathogenic versatility that allows the bacterium to cause a spectrum of enteric and histotoxic diseases (4). The organism is grouped into 5 types (A, B, C, D, and E) on the basis of the production of 4 major toxins: alpha-, beta-, epsilon-, and iota-toxins (5). Its primary habitat is the bowels of warm-blooded animal species. It is also found in soil where animals congregate and where manure has been used to boost agricultural fertility (6).

C. perfringens enterotoxin (CPE) is a single polypeptide of 35 kDa with a unique amino acid sequence (3), which has been implicated as a virulence factor in several human gastrointestinal illnesses. These CPE-associated illnesses include *C. perfringens* type A food poisoning—which is the second most commonly reported foodborne disease

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in the United States—as well as non-foodborne diarrheas such as antibiotic-associated diarrhea and sporadic diarrhea (7). Only a small fraction (~1% to 5%) of *C. perfringens* isolates carry the enterotoxin gene (*cpe*), and these mainly belong to type A; expression of CPE by type C and D *C. perfringens* has also been described (8,9).

In this study, a *cpe*-positive *C. perfringens* type C strain was obtained using multiplex PCR from 34 *C. perfringens* isolates from goat in Guizhou Province, China. Next, the *cpe* gene was cloned and sequence analyzed, which provides a basis for further research on the structure, properties, and biological activities of CPE. It also set the groundwork for further investigation of the mechanism of disease caused by *C. perfringens* enterotoxin.

Materials and methods

Materials

C. perfringens type A strain CVCC38, *C. perfringens* type B strain CVCC54, and *C. perfringens* type E strain CVCC90 were purchased from the China Institute of Veterinary Drug Control. Both *C. perfringens* type C strain C59 and *C. perfringens* type D strain C60 were obtained from the Animal Husbandry and Veterinary Research Institute of Guizhou. The *C. perfringens* type A strain NCTC64609 carrying the *cpe* gene was obtained from the Guangzhou Center for Disease Control and Prevention. A total of 34 *C. perfringens* isolates from goat and *E. coli* DH5a were kept in our laboratory. We purchased pMD18-T vector, *Taq* DNA polymerase, restriction endonuclease, and 200 bp DNA markers from TaKaRa Company. A DNA fragment recovery kit, SDS, IPTG, and RNase A were purchased from Promega Company. Other reagents, if not described, were of analytic purity.

Multiplex PCR for detecting cpe-positive C. perfringens isolates

Template DNA was obtained from cultures of the 6 standard strains of *C. perfringens* and 34 *C. perfringens* isolates. Cells were grown for 18 to 20 h at 37 °C in TGY (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine). Then 1 mL of culture was centrifuged at 8000 r/min for 5 min, the cell pellet was washed twice with sterile saline and resuspended in 200 μL of sterile distilled water, and then placed in a boiling water bath for 15 to 20 min. After centrifugation, 10 μL of supernatant fluid was used as the template for multiplex PCR. The primers of the *C. perfringens* alpha toxin gene (*cpa*), *C. perfringens* beta toxin gene (*cpb*), *C. perfringens* epsilon toxin gene (*etx*), *C. perfringens* iota toxin gene (*iA*), and *C. perfringens* enterotoxin gene (*cpe*) were designed and synthesized (Table 1), with slight modification, based on previous studies (10-14). The 50 μL volume included 5.0 μL of 10 × PCR buffer, 10.0 μL of template DNA, appropriate concentrations of each primer (Table 1), 1.5 mmol/L $MgCl_2$, 0.3 mmol/L dNTP, and 1 U of *Taq* DNA polymerase. All amplification reactions consisted of an initial denaturation at 94 °C for 5 min prior to 25 cycles of 94 °C denaturation for 1 min, 55 °C

Table 1. The primers used to amplify 5 toxin genes of *C. perfringens*.

Gene	Primer sequences (5'→3')	Product size (bp)	Primer concentration (μM)
cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	0.50
cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	0.36
etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655	0.46
iA	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	0.52
cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233	0.40

annealing for 1 min, and 72 °C extension for 1 min, followed by a final 72 °C extension for 10 min. The PCR products were then analyzed by electrophoresis on 2.0% agarose gel.

PCR amplification of cpe gene

Template DNA was obtained from cultures of *cpe*-positive *C. perfringens* isolates. The genomic DNA was extracted using the previously described methods. From the available sequence 1 pair of *cpe* gene primers was designed (15-17). Primer sequences included upstream primer of AGGATCCATGCTTAGTAACAATTTAAATCC-3' (including the underlined *Bam*HIrestriction site) and downstream primer of 5'–GGGGAATTCTTAAAATTTTTGAAATAA TATTGA-3 (including the underlined *Eco*RI restriction site). Each PCR contained 5.0 μL of 10 × PCR buffer, 10.0 μL of template DNA, 0.4 μmol/L concentrations of each primer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, and 0.5 U of *Taq* DNA polymerase. Sterile distilled water was added to a 50 μL reaction system. The PCR reaction conditions were as follows: 94 °C for 5 min, 1 circle; 94 °C for 30 s, 40 °C for 30 s, 72 °C for 80 s, 5 circles; 94 °C for 30 s, 50 °C for 30 s, 72 °C for 80 s, 25 circles; and 72 °C for 10 min, 1 circle. PCR products were detected by 1.0% agarose gel electrophoresis.

Cloning and sequencing of cpe gene

The PCR product of the *cpe* gene was electrophoresed on 1.0% agarose gel. Fragments (~960 bp) were extracted from the gel using the DNA fragment recovery kit. The extracted fragments were then ligated into the pMD18-T cloning vector, overnight at 4 °C. Those recombinant plasmids were transformed into competent *E. coli* DH5a and selected on LB agar plates containing X-gal/IPTG and ampicillin (Amp). The positive plasmids were identified by PCR and sequential digestion with *Eco*RI and *Bam*HI. The correct recombinants were sequenced by TaKaRa Company.

Sequence analysis of cpe gene

Sequence comparison for sequencing results was carried out at the National Center for Biotechnology Information, USA (NCBI). The DNA sequence was translated into protein sequence, and homologous comparison was done with DNAMAN software. Phylogenetic trees were constructed by the bootstrap neighbor-joining method using clustalX1.83 and mega3 software. There were 8 reference strains, and their GenBank Accession Numbers were: AJ000766, M98037, Y16009, CP000312, AB236337, AF416450, X71844, and X81849. With the exceptions of AJ000766 and X81849 (type not shown in GenBank and related references), reference strains belonged to type A *C. perfringens* (2, 16, 17, 18, and GenBank description).

Nucleotide sequence accession number

The nucleotide sequence of the complete *cpe* gene from *cpe*-positive *C. perfringens* isolates has been submitted to GenBank (accession number FJ205886).

Results

Multiplex PCR for detecting cpe-positive C. perfringens isolates

A multiplex PCR method was used to detect *cpe*-positive *C. perfringens* from 34 isolates from Guizhou Province according to *cpa*, *cpb*, *etx*, *iA*, and *cpe*. To ensure the reliability of multiplex PCR results, control PCRs were run using template DNA prepared from 6 standard strains. The results showed that there was 1 *cpe*-positive *C. perfringens* type C isolate, which was named CP2 (Figure 1). There was 1 *cpe*-negative *C. perfringens* type C strain and 1 *cpe*-negative *C. perfringens* type D strain; the others were all *cpe*-negative *C. perfringens* type A strains (data not shown).

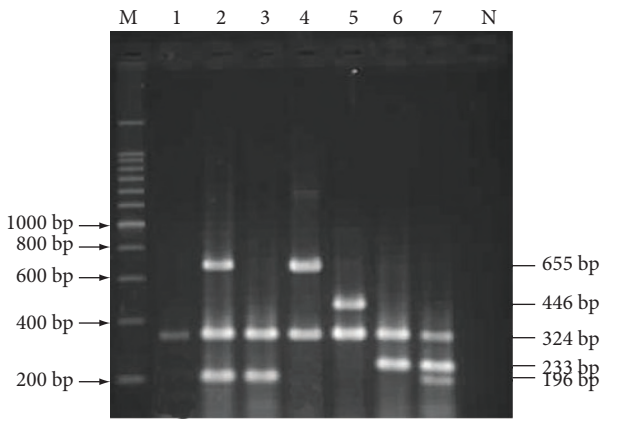

Figure 1. Multiplex PCR for detection of *cpe*-positive *C. perfringens*. M: 200 bp marker; 1: cvcc38; 2: cvcc54; 3: C59; 4: C60; 5: cvcc90; 6: NCTC64609; 7: CP2; N: blank control.

Cloning and identification of cpe gene

Primers were designed according to the open reading frame of the *cpe* gene, as previously described. A single band of about 960 bp (Figure 2) was obtained with the total DNA isolated from CP2 strains as templates through PCR amplification of the *cpe* gene. The PCR product was purified with the DNA fragment recovery kit and evaluated by agarose gel electrophoresis. The fragment was ligated to the cloning vector pMD18-T. The cloning vector containing the PCR product was introduced into competent *E. coli* DH5a cells by $CaCl_2$ transformation. Transformed *E. coli* was grown at 37 °C in medium containing X-gal/IPTG and ampicillin. The positive plasmids were identified by PCR and sequential digestion with *Eco*RI and *Bam*HI and *cpe* gene; about 960 bp was obtained (Figure 3).

Sequence, homology, and phylogenetic analysis of cpe gene

After sequencing by TaKaRa Company, the results (GenBank Accession Number FJ205886) showed that the open reading frame of the *cpe* gene was 960 bp long and encoded 319 amino acids. The *cpe* gene of CP2 was compared with 8 reference strains of *C. perfringens cpe* genes using the DNAMAN software. The nucleotide sequence homologies were: 99.6%, 99.8%, 99.7%, 99.8%, 99.8%, 99.8%, 99.8%, and 99.4%; amino acid sequence similarities were 99.4%, 99.7%, 99.4%, 99.7%, 99.7%, 99.7%, 99.7%, and 99.1% (Table 2). Using clustalX1.83 and mega3 software, a phylogenetic tree was constructed based on the nucleotide sequences of the *cpe* gene (Figure 4). The gene tree showed a close relationship between CP2 and the reference strains. However, the relationship between CP2 and X81849 was slightly more distant than the relationships among other strains.

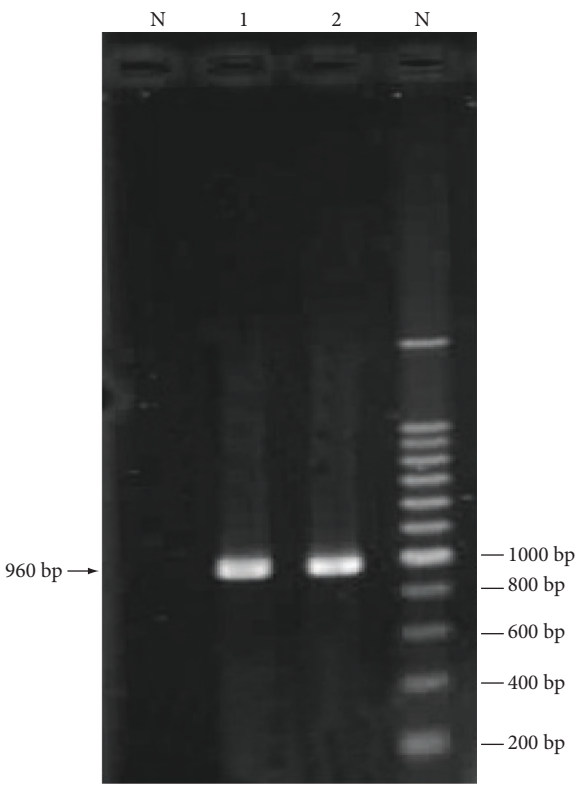

Figure 2. Identification of PCR-amplified *cpe*. N: blank control; 1: CP2 strain; 2: NCTC64609; M: 200 bp marker.

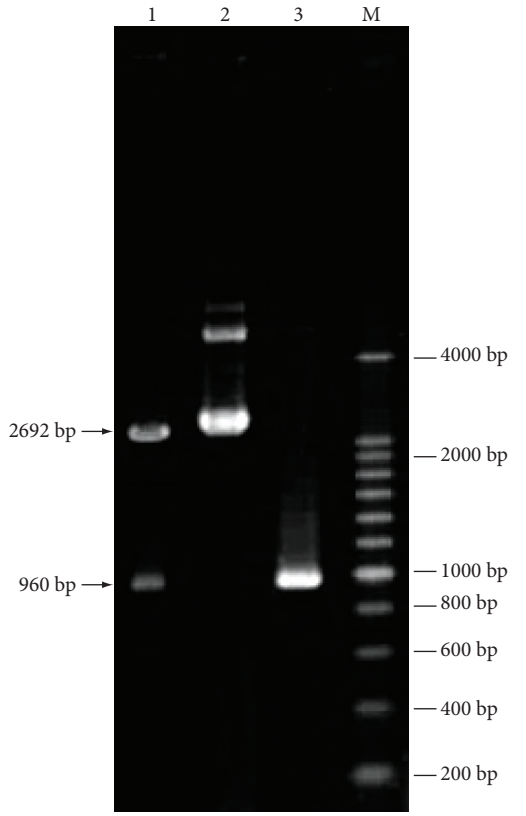

Figure 3. Identification of the recombinant plasmid. 1: Products from recombinant plasmid digested with *Eco*R and *Bam*H; 2: recombinant plasmid; 3: PCR products of the recombinant plasmid; M: 200 bp marker.

Discussion

C. perfringens is a gram-positive, rod-shaped, and spore-forming bacterium that produces several characteristic toxins. At the present time several

Table 2. Homologous comparison of CP2 with reference strains of *C. perfringens* enterotoxin gene.

Strans	1	2	3	4	5	6	7	8	9
								Amino acid identity (%)	
1		99,4	99,7	99,4	99,7	99,7	99,7	99,7	99,1
2	99,6		99,7	99,4	99,7	99,7	99,7	99,7	99,1
3	99,8	99,8		99,7	100	100	100	100	99,4
4	99,7	99,7	99,9		99,7	99,7	99,7	99,7	99,1
5	99,8	99,8	100	99,9		100	100	100	99,4
6	99,8	99,8	100	99,9	100		100	100	99,4
7	99,8	99,8	100	99,9	100	100		100	99,4
8	99,8	99,8	100	99,9	100	100	100		
9	99,4	99,4	99,6	99,5	99,6	99,6	99,6	99,6	
	Nucleotide identity (%)								

1: CP2; 2: AJ000766; 3: M98037; 4: Y16009; 5: CP000312; 6: AB236337; 7: AF416450; 8: X71844; 9: X81849.

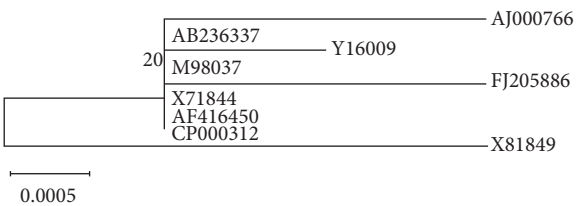

Figure 4. Phylogenetic tree of *cpe* nucleotide sequences of CP2 with reference strains from GenBank database. Neighbor joining trees; bootstrap values (1000 replicates) are shown at the nodes.

C. perfringens-associated toxin genes have been characterized (12). Among these, 4 (*cpa*, *cpb*, *etx*, and *iA*) have been proposed for classification of *C. perfringens* into 5 isotypes (A, B, C, D, and E) (19,20,21), each type carrying a different combination of the toxin genes. The *cpa* toxin is found in *C. perfringens* types A, B, C, D, and E, whereas the *cpb* toxin is found in types B and C. In addition, the *etx* toxin is found in types B and D. The *iA* toxin is found only in E. Enterotoxin, a single polypeptide of 35 kDa with a unique amino acid sequence, is reportedly produced by all 5 types of *C. perfringens* (4), although it is doubtful that type A plays a role in disease production. CPE causes the symptoms associated with *C. perfringens* type A food poisoning, which is among the most common human foodborne illnesses, and it also appears to be involved in other common human and veterinary gastrointestinal illnesses. It is generally accepted that CPE formation in *C. perfringens* is associated with sporulation, and it has been suggested that CPE is a structural protein of the spore coat (15).

Multiplex PCR is now routinely used to assign *C. perfringens* isolates to one of 5 types (A-E) based on whether an isolate carries genes encoding alpha-, beta-, epsilon-, or iota-toxin. Recent versions of the multiplex PCR toxin genotyping assay can also determine whether a *C. perfringens* isolate possesses the *cpe* gene (22). The use of multiplex PCR for detecting *cpe*-positive *C. perfringens* from 34 strains isolated from goat in Guizhou Province, China, is reported for the first time in this paper. A *cpe*-positive *C. perfringens* type C strain was obtained and named CP2. Next, the gene encoding CPE from CP2 strain was successfully cloned, and the nucleotide sequence was analyzed. The open reading frame of the *cpe* gene was 960 bp long and encoded 319 amino acids polypeptide, which shared 99.4%-99.8% nucleotide sequence identity and 99.1%-99.7% amino acid sequence identity with reference strains in GenBank. The phylogenetic tree based on the nucleotide

sequence shows a close relationship between the CP2 strain and the reference strains. Most of the reference strains belong to type A *C. perfringens*. The high identity and close relationship with type A *C. perfringens* strains reveal that the *cpe* gene is highly conserved. In recent years there have been many reports of *cpe* gene cloning, mainly *C. perfringens* type A isolates. In our study, the *cpe* gene of *C. perfringens* type C isolate was successfully cloned and sequence analyzed. This has set the groundwork for further investigation of the mechanism of disease caused by *C. perfringens* enterotoxin.

Acknowledgement

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