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

Yuan TANG1,2,3,*, Ming WEN2, Bin LIAN1, Jianzhong CHENG1, Kaigong WANG2, Bijun ZHOU2
1Institute of Geochemistry, Chinese Academy of Science, Guiyang 550002 - CHINA
2College of Animal Science, Guizhou University, Guiyang 550025 - CHINA
3Graduate University of Chinese Academy of Sciences, Beijing 100049 - CHINA

Received: 08.01.2011

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 DH5α. 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

* E-mail: dh2468@163.com
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 MgCl₂, 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>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’→3’)</th>
<th>Product size (bp)</th>
<th>Primer concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa</td>
<td>GCTAATTGTTACTGCGGTGTTGA CTCCTGATACATCGTGTAAG</td>
<td>324</td>
<td>0.50</td>
</tr>
<tr>
<td>cpe</td>
<td>GAGAGATGGTTGGATATTAGGG GACCAGCAGTGGTTAGATA</td>
<td>233</td>
<td>0.40</td>
</tr>
</tbody>
</table>
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 BamHI restriction site) and downstream primer of 5’–GGGGAATTCTTAAAATTTTTGAAATAATATTGA-3 (including the underlined EcoRI 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 MgCl₂, 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 EcoRI and BamHI. 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.](image-url)
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 CaCl₂ 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 EcoRI and BamHI 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.

Discussion

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.4</td>
<td>99.7</td>
<td>99.4</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>99.6</td>
<td>99.7</td>
<td>99.4</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>99.8</td>
<td>99.8</td>
<td>99.7</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>99.7</td>
<td>99.7</td>
<td>99.9</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.7</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>99.8</td>
<td>99.8</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>99.8</td>
<td>99.8</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>99.8</td>
<td>99.8</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>99.8</td>
<td>99.8</td>
<td>100</td>
<td>99.9</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>99.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>99.4</td>
<td>99.4</td>
<td>99.6</td>
<td>99.5</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
<td></td>
</tr>
</tbody>
</table>

Amino acid identity (%)
Detection, cloning, and sequencing of the enterotoxin gene of *Clostridium perfringens* type C isolated from goat

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

This work was supported by the Guizhou Science and Technology Department, Key Project for Agriculture (grant no. QKHNY20093069).

References


158