Typing of *Clostridium perfringens* by *in vitro* amplification of toxin genes

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G. DAUBE, B. CHINA, P. SIMON, K. HVALA AND J. MAINIL. 1994. The strains of *Clostridium perfringens* are classified according to major toxins produced. Classically, this determination involves the seroneutralization of their lethal effect in mice. However, this method requires specific antiserum and a large number of mice. In this work, a new typing method was developed based on the amplification of toxin genes by polymerase chain reaction (PCR). By combination of several pairs of primers, the toxinoype of a *Cl. perfringens* strain was determined by looking at the pattern of bands on an agarose gel electrophoresis. This mixture contained primers amplifying simultaneously a part of α-toxin, β-toxin, ε-toxin and enterotoxin genes. In order to distinguish between toxinoype A and E, the τ-toxin gene fragment must be amplified in a separate PCR reaction. Moreover, with the primers combination, in most cases, a PCR product corresponding to the α-toxin gene was obtained from direct enrichments of animal intestinal contents.

**INTRODUCTION**

*Clostridium perfringens* is an anaerobe responsible for a wide range of diseases in animals and humans (Niilo 1986). The virulence of the organism is associated with the production of several toxins (exotoxins and enterotoxin). Among them, four are called the major lethal toxins (α, β, ε and τ). Intravenous injection of these toxins to mice is lethal. Among them, α-toxin is associated with gas gangrene in humans and with necrotic enteritis and enterotoxaemia in animals. It is a 43 kDa protein harbouring phospholipase C and sphingomyelinase activities. This toxin is involved in the increase of vascular permeability (Sugahawa 1977), cytotoxicity (Sato et al. 1989), intravascular haemolysis and clot formation (Freer 1988). α-Toxin stimulates the production of thromboxane A2 by endothelial cells (Fuji and Sakurai 1989). The β-toxin plays a major role in necrotic enteritis in animals and humans. In humans, the disease has been termed pigbel (Lawrence and Cook 1980). This 40 kDa protein induces a haemorrhagic necrosis in guinea pig intestinal loops (Lawrence and Cook 1980) and is cytotoxic for CHO cells (Jolivet-Reynaud et al. 1985). The ε-toxin is secreted as an inactive protoxin which is converted by trypsin to fully active toxin (Bhown and Habbeb 1977). This toxin is involved in enterotoxaemia in economically important livestock (McDonel 1986). The τ-toxin is a binary toxin consisting of two independent polypeptides: Ia, which is an ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin into the cell. The τ-toxin has been implicated in calf and lamb enterotoxaemias (Hathey 1990). The enterotoxin is a 320 amino acid protein proteolytically activated. The enterotoxin is known to be involved in food poisoning causing diarrhoea and abdominal cramps, sometimes with vomiting and fever (Stringer et al. 1982). It is generally accepted that enterotoxin production is associated with sporulation (Duncan et al. 1973).

The major lethal toxins constitute the basis of the frequently used classification of *Cl. perfringens* into five toxinoypes. Bacteria from toxinoype A produce α-toxin; those from toxinoype B produce α-, β- and ε-toxins; those from toxinoype C secrete α- and β-toxins; those from toxinoype D produce α- and ε-toxins and finally bacteria from toxinoype E secrete α- and τ-toxins. Classically, this classification was performed by two different methods: the seroneutralization of lethality by intravenous injection in mice and the seroneutralization of the dermonecrotic effect in guinea pigs (Sterne and Batty 1975). These methods, however, require a large amount of active toxin, specific
neutralizing antiserum for each toxin and a lot of laboratory animals. Taking into account the recently published sequences of the major toxin genes (Titball et al. 1989; Hunter et al. 1992, 1993; Perelle et al. 1993) and of the enterotoxin gene (Van Damme-Jongsten et al. 1989), a new in vitro method was developed, based on DNA amplification by polymerase chain reaction (PCR), to determine the Cl. perfringens toxigenotypes.

MATERIALS AND METHODS

Bacterial strains

Five well-toxinotypes reference strains of Cl. perfringens were used. These strains included: toxigenotype A strain ATCC 13124 (Tso and Siebel 1989), enterotoxigenic toxigenotype A strain NCTC 8239 (Van Damme-Jongsten et al. 1989), toxigenotype B strain ATCC 3626 (received from M. Popoff, Paris, France), toxigenotype C strain CWC 236 (received from M. Popoff, Paris, France), toxigenotype D strain CN 3978 (Wellcome Laboratories) and toxigenotype E strain NCIB 10748 (Perelle et al. 1993). Ten Cl. perfringens strains were isolated from animals or human and were labelled as unknown strains. These strains included: strain 460280-8 isolated from human, strain 88B21MF isolated from goat, strains 45017MF3, 45402C01 and 45497CF isolated from cattle, strains 945P, CP48 and 42718MF1 isolated from sheep, strain 92E1897RA isolated from deer and strain CWC 243 isolated from swine.

Isolation of total DNA

From pure culture. Clastoclitum perfringens was grown overnight in 5 ml volumes of Brain Heart Infusion (BHI; Gibco) supplemented with 1% (w/v) sodium thiosulphate under anaerobic conditions (MK3 anaerobic work station, Don Whitley Scientific, UK). For the extraction of DNA, the method described by Van Damme-Jongsten et al. (1989) was adapted for small volumes. Briefly, 1-5 ml of culture were centrifuged for 5 min in a minifuge (Eppendorf, model 54154). The pellet was resuspended in 1-2 ml of TES (Tris HCl 50 mmol l⁻¹ pH 8, 5 mmol l⁻¹ EDTA, 50 mmol l⁻¹ NaCl) and centrifuged for 10 min. The pellet was resuspended in TES containing 25% (w/v) sucrose and 2 mg ml⁻¹ lysozyme. The suspension was incubated for 20 min at 37°C. Sixty μl of 0-25 mol l⁻¹ EDTA, pH 8, 300 μl of TES containing 1% SDS and 30 μl diethylpyrocarbonate were then added. The suspension was incubated for 10 min at 60°C and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The DNA was then precipitated by 2-5 volumes of ethanol.

From intestinal contents. An ileal port of intestine (usually involved in this pathology) was extracted from cattle, sheep or goats which had died of enterotoxaemia. A sample of the intestinal contents was withdrawn in a sterile way. This sample was used to search for the presence of Cl. perfringens. The sample was diluted and plated on anaerobic blood agar base (Gibco BRL, Paisley, Scotland) supplemented with 8% of bovine blood and 0-05% of cyslocerin (Oxoid, Basingstoke, UK) and incubated overnight at 37°C in anaerobiosis. For amplification from the intestinal contents, 1 g of intestinal contents was placed in 9 ml of BH-thioglycolate supplemented with 0-05% (w/v) cyslocerin (Oxoid). The culture was incubated overnight at 37°C in anaerobic conditions. The DNA was isolated as described above.

DNA amplification and restriction

Primers derived from the published sequences with the Oligo software (National Biosciences, Plymouth, USA) are listed in Table 1.

Table 1 Characteristics of the primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Size of PCR product (bp)</th>
<th>Position*</th>
<th>Annealing temperature (°C)†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>α1: TGCTAATGTTACTGCGTGGATAG</td>
<td>247</td>
<td>1437</td>
<td>51-7</td>
<td>Titball et al. 1989</td>
</tr>
<tr>
<td></td>
<td>α2: ATAATCCAATCATCGCAACTGATG</td>
<td>1683</td>
<td>320</td>
<td>46-9</td>
<td>Hunter et al. 1993</td>
</tr>
<tr>
<td>β</td>
<td>β1: AGGAGGTTTTTTTTATGAG</td>
<td>1025</td>
<td>1345</td>
<td>46-9</td>
<td>Hunter et al. 1992</td>
</tr>
<tr>
<td></td>
<td>β2: TCCTATAGCTGTAGAGTTGGT</td>
<td>206</td>
<td>580</td>
<td>46-2</td>
<td>Perelle et al. 1993</td>
</tr>
<tr>
<td>ε</td>
<td>ε1: ATTTAAATTGCAATCATGGACATTGGG</td>
<td>298</td>
<td>1514</td>
<td>46-2</td>
<td>Van Damme-Jongsten et al. 1989</td>
</tr>
<tr>
<td></td>
<td>ε2: ATTTGGATATCAGTGAGTGATTTGGT</td>
<td>1812</td>
<td>254</td>
<td>46-2</td>
<td></td>
</tr>
</tbody>
</table>

* With respect to the published sequence.
† As calculated by the software Oligo (National Biosciences, Plymouth, USA).
PC: Polymerase chain reaction.

ε2 : CTTGTAAGGAGCATTATGAGTAA
The PCR reactions were performed in a DNA Thermal Cycler (Perkin Elmer, Norwalk, USA). The following conditions were applied: 1 unit of DNA polymerase from *Thermus aquaticus* (Taq, Beckman, Palo Alto, USA) was added to 50 μL buffer (10 mmol L⁻¹ Tris–HCl, pH 8.5, 50 mmol L⁻¹ KCl, 3 mmol L⁻¹ MgCl₂, 0.01% gelatin) containing 200 μmol L⁻¹ deoxynucleotides triphosphate (Pharmacia), 4 μmol L⁻¹ primers and 1 μmol L⁻¹ template DNA. The following procedure was used for all experiments: 5 min at 95°C followed by 45 cycles consisting of 30 s at 94°C, 30 s at 50°C and 30 s at 70°C. Ten μL of PCR product were then analysed by electrophoresis in 3% agarose gel.

The restriction enzymes (*Eco*RV, *Hind*III, *Spe*I, *Alu*I and *Kpn*I) were purchased from Gibco BRL and used following the manufacturer's recommendations.

**RESULTS**

**Control of primers specificity**

Five primers representative of each toxinoype were selected. After growth at 37°C in BHI-thioglycolate, DNA was isolated and a PCR reaction performed with the primer pairs defined in Table 1. Figure 1 shows the results obtained. A specific combination of primers permitted amplification of a single fragment from a toxin gene. With primers α1 and α2, derived from the sequence of the α-toxin gene, a 247 bp fragment from DNA of the toxinoype A strain ATCC 13124 was amplified. In the same way, toxinoype A *Clostridium perfringens* strain NCTC 8239 contained the α-toxin and the enterotoxin genes. With primers ent1 and ent2 derived from the sequence of the enterotoxin gene, a 163 bp fragment was amplified. From toxinoype B strain ATCC 3626, a 1025 bp PCR product was amplified with primers β1 and β2 derived from the β-toxin gene sequence. From toxinoype D strain CN 3978, a 206 bp PCR product was amplified with primers ε1 and ε2 derived from the sequence of the ε-toxin gene. Finally, primers ′ε1 and ′ε2, derived from the sequence of the ε-toxin gene, were used to amplify a 298 bp fragment from DNA of the toxinoype E *Clostridium perfringens* strain NCIB 10748. In each case, one single band of the expected size was obtained if the published sequence is considered. To confirm that the amplified product arose from the expected sequence, a restriction experiment was performed. Restriction fragments of the expected size for each PCR product were obtained (Fig. 1). The selected primers were thus useful to specifically amplify fragments of each toxin gene of *Clostridium perfringens*.
Fig. 2 Gel electrophoresis analysis of polymerase chain reaction (PCR) products obtained on Clostridium perfringens pure culture with a mixture of primers. (a) PCR reaction performed on reference strains with the primers x1, x2, β1, β2, α1, α2, ent1 and ent2. (1) Clostridium perfringens ATCC 13124 (toxotype A); (2) Clostridium perfringens NCTC 8239 (toxotype A, enterotoxigenic); (3) Clostridium perfringens ATCC 3626 (toxotype B); (4) Clostridium perfringens CWC 236 (toxotype C); (5) Clostridium perfringens CN 3978 (toxotype D); (6) Clostridium perfringens NCIB 10748 (toxotype E); M, molecular weight scale (123 bp DNA ladder from Gibco BRL). The less intense bands of small size are the primer oligomers. (b) PCR reaction performed on wild-type strains with primers x1, x2, β1, β2, α1, α2, ent1 and ent2. (1) Clostridium perfringens 46280-8; (2) Clostridium perfringens 88B21MF; (3) Clostridium perfringens 45017MF3; (4) Clostridium perfringens 945P; (5) Clostridium perfringens CP48; (6) Clostridium perfringens 45402C01; (7) Clostridium perfringens CWC 243; (8) Clostridium perfringens 92E1897RA; (9) Clostridium perfringens 45497C1; (10) Clostridium perfringens 42718MF1. The less intense bands of small size are the primer oligomers.

A PCR amplification product was obtained for: α-toxin; α-toxin and enterotoxin; α-, β- and ε-toxins; α- and β-toxins; α- and ε-toxins; and α-toxin, respectively (Fig. 2a). It was concluded that the amplification procedure correlates with the methods previously used for Clostridium perfringens classification.

Analysis of toxin genes from unknown strains

Under the conditions described above, the primers mixture was used to determine the toxotype of 10 strains of unknown toxotype isolated from animals or humans (Fig. 2b). These experiments were performed on pure cultures of the Clostridium perfringens strains. A clear pattern was observed for each strain allowing the attribution of a toxotype. The results are shown in Table 2. This method required the isolation of the strain from crude material, the extraction of DNA and finally the PCR reaction. It was interesting to perform the PCR reaction directly on crude material such as intestinal contents. For this purpose BHI-thioglycollate was inoculated with 1 g of animal intestinal contents iso-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Toxotype*</th>
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<tbody>
<tr>
<td>460280-8</td>
<td>Human</td>
<td>A or E, enterotoxigenic</td>
</tr>
<tr>
<td>88B21MF</td>
<td>Goat</td>
<td>D</td>
</tr>
<tr>
<td>45017MF3</td>
<td>Cattle</td>
<td>A or E, enterotoxigenic</td>
</tr>
<tr>
<td>945P</td>
<td>Sheep</td>
<td>D</td>
</tr>
<tr>
<td>CP 48</td>
<td>Sheep</td>
<td>D</td>
</tr>
<tr>
<td>45402C01</td>
<td>Cattle</td>
<td>A or E, enterotoxigenic</td>
</tr>
<tr>
<td>CWC 243</td>
<td>Swine</td>
<td>C</td>
</tr>
<tr>
<td>92E1897RA</td>
<td>Deer</td>
<td>D</td>
</tr>
<tr>
<td>45497C1</td>
<td>Cattle</td>
<td>A or E</td>
</tr>
<tr>
<td>42718MF1</td>
<td>Sheep</td>
<td>D</td>
</tr>
</tbody>
</table>

* Toxotyping was done by polymerase chain reaction amplification using primers x1, x2, β1, β2, α1, α2, ent1 and ent2.
Fig. 3 Gel electrophoresis analysis of polymerase chain reaction (PCR) products obtained from intestinal contents with the mixture of primers. A PCR reaction was performed using primers α1, α2, β1, β2, x1, x2, ent1 and ent2 on intestinal contents from cattle (1–3, 5, 6, 9), sheep (4, 8) and goat (7). The products were by agarose gel electrophoresis. M, molecular weight scale (123 bp DNA ladder Gibco BRL). The less intense bands of small size are the primer oligomers.

l related from animals which had died of enterotoxaemia, containing Cl. perfringens. After incubation overnight at 37°C, the DNA was extracted from the culture and a PCR reaction performed with the mixture of primers. In each case except for one, an amplification was observed that corresponded to the α-toxin gene product (Fig. 3).

DISCUSSION

The pathogenicity of Cl. perfringens is associated with the production of major lethal toxins and enterotoxin. Each strain of Cl. perfringens produces its own set of toxins. Each set of toxins is related to a particular pathology. Classically, the toxinoityping was realized with in vivo tests. The development of molecular biology tools allowed an easier in vitro test. From the published sequences specific primers were derived for each toxin gene. With these primers a PCR toxinoityping method was developed. By this method a specific PCR product was amplified for each toxin gene using individual pairs of primers. Moreover, a combination of primers was used to amplify several toxin genes from the same strain. After electrophoresis, a pattern of bands corresponding to a specific toxinoitype was obtained. However, reproducible results with i-toxin primers could not be obtained. Thus it is impossible by this method to discriminate between Cl. perfringens of toxinoitypes A and E. However, toxinoitype E is rarely isolated in Europe. Type E was not isolated from 2569 wild type strains isolated in the laboratory (results not shown). Therefore, to discriminate between toxinoitype A and toxinoitype E, a two-step method is recommended. After PCR reaction with the mixture of primers, a PCR amplification may be done with only primers i.

In a further step, Cl. perfringens was characterized directly from intestinal contents. Nevertheless, the use of primer mixture directly on enrichments of intestinal contents is questionable. Indeed, the toxinoitype A is dominant in normal flora and this amplification could result in the preferential amplification of α-toxin gene which could mask the other amplifications. Hence, the multiple primers method should be limited to studying pure cultures of Cl. perfringens, while the crude intestinal extract should be analysed using the pair of primers corresponding to the gene sought. Nevertheless, the determination of the toxinoitype by PCR provides a sensitive, rapid (3 d) and reproducible method of characterizing Cl. perfringens strains. As the reagents can be kept stable for a long time, toxinoityping kits, including the primers, polymerase and reference DNA as a control, could be useful.

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REFERENCES


