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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 antisera 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 toxinotype 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 toxinotype A and E, the ι-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 ι). Intravenous injection of these toxins to mice is lethal. The 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 (Fujii 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.* 1986). 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 ι-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 ι-toxin has been implicated in calf and lamb enterotoxaemias (Hatheway 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 toxinotypes. Bacteria from toxinotype A produce α-toxin; those from toxinotype B produce α-, β- and ε-toxins: those from toxinotype C secrete α- and β-toxins; those from toxinotype D produce α- and ε-toxins and finally bacteria from toxinotype E secrete α- and ι-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

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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* toxinotypes.

MATERIALS AND METHODS

Bacterial strains

Five well-toxinotyped reference strains of *Cl. perfringens* were used. These strains included: toxinotype A strain ATCC 13124 (Tso and Siebel 1989), enterotoxigenic toxinotype A strain NCTC 8239 (Van Damme-Jongsten *et al.* 1989), toxinotype B strain ATCC 3626 (received from M. Popoff, Paris, France), toxinotype C strain CWC 236 (received from M. Popoff, Paris, France), toxinotype D strain CN 3978 (Wellcome Laboratories) and toxinotype 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 45497C1 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. *Clostridium perfringens* was grown overnight in 5 ml volumes of Brain Heart Infusion (BHI; Gibco) supplemented with 1% (w/v) sodium thioglycolate 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 portion 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 cycloserine (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 BHI-thioglycolate supplemented with 0·05% (w/v) cycloserine (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^{R} software (National Biosciences, Plymouth, USA) are listed in Table 1.

Table 1 Characteristics of the primers used in this study

Gene	Primer sequences	Size of PCR product (bp)	Position*	Annealing temperature (°C)†	Reference
α	α1: TGCTAATGTTACTGCCGTTGATAG	247	1437	51·7	Titball *et al.* 1989
	α2: ATAATCCCAATCATCCCAACTATG		1683		
β	β1: AGGAGGTTTTTTTATGAAG	1025	320	46·9	Hunter *et al.* 1993
	β2: TCTAAATAGCTGTTACTTTGT		1345		
ε	ε1: ATTAAAATCACAATCATTCACTTG	206	580	46·2	Hunter *et al.* 1992
	[redacted]		786		
ι a	ι1: TTTTAACTAGTTCATTTCCTAGTTA	298	1514	46·2	Perelle *et al.* 1993
	ι2: TTTTTGTATTCTTTTTCTCTAGGATT		1812		
Enterotoxin	ent1: ATGTAATAGATAAAGGAGATGGTT	163	254	46·2	Van Damme-
	ent2: ATAAATTCAGAAGTAAATCCAACT		421		Jongsten *et al.* 1989

* With respect to the published sequence.

† As calculated by the software Oligo^{R} (National Biosciences, Plymouth, USA).

PCR, Polymerase chain reaction.

ε2 : CTTGTGAAGGGACATTATGAGTAA

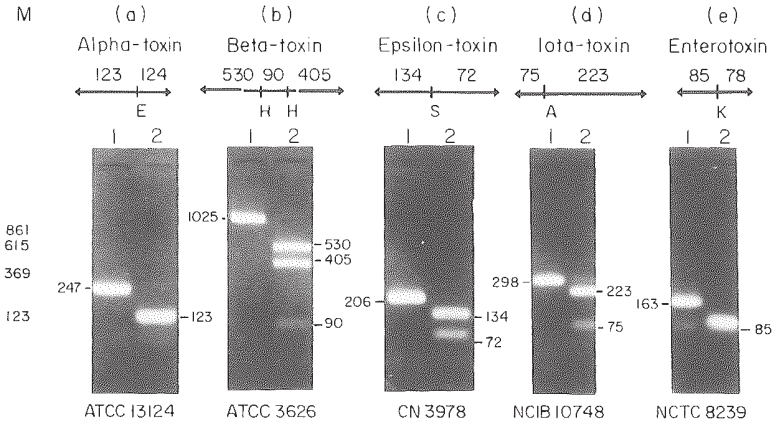

Fig. 1 Amplification of specific toxin gene using a single pair of primers. DNA of *Clostridium perfringens* was amplified with a single pair of primers. Half of the polymerase chain reaction product was analysed by agarose gel electrophoresis (1). The second half was digested by an appropriate restriction enzyme and then analysed by agarose gel electrophoresis (2). a, *Clostridium perfringens* ATCC 13124 DNA amplified with primers α1 and α2 (1) and restricted by (E) *Eco*RV (2); b, *Cl. perfringens* ATCC 3626 DNA amplified with primers β1 and β2 (1) and restricted by (H) *Hin*dIII (2); c, *Cl. perfringens* CN 3978 DNA amplified with primers ε1 and ε2 (1) and restricted by (S) *Spe*I (2); d, *Cl. perfringens* NCIB 10748 DNA amplified with primers ι1 and ι2 (1) and restricted by (A) *Alu*I (2); e, *Cl. perfringens* NCTC 8239 DNA amplified with primers ent1 and ent2 (1) and restricted by (K) *Kpn*I (2); M, molecular weight scale (123 bp ladder from Gibco BRL). The numbers indicate the size of DNA fragments

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} $MgCl_2$, 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, *Hin*dIII, *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 strains representative of each toxinotype 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 toxinotype A strain ATCC 13124 was amplified. In the same way, toxinotype A *Cl. 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 toxinotype B strain ATCC 3626, a 1025 bp PCR product was amplified with primers β1 and β2 derived from the β-toxin gene sequence. From toxinotype 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 ι1 and ι2, derived from the sequence of the ι-toxin gene, were used to amplify a 298 bp fragment from DNA of the toxinotype E *Cl. 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 *Cl. perfringens*.

To determine the experimental conditions for analysis of unknown strains, the toxinotype of reference strains was first tested with a mixture of primers. The different sizes of amplified fragments should provide a clear DNA pattern allowing determination of the toxinotype. For unknown reasons, good results were not obtained when ι-toxin primers were added (results not shown). Hence, a mix of the primers for α-, β-, ε- and enterotoxin genes was used. As expected, from the DNA of *Cl. perfringens* strains ATCC 13124, NCTC 8239, CWC 236, CN 3978 and NCIB 10748,

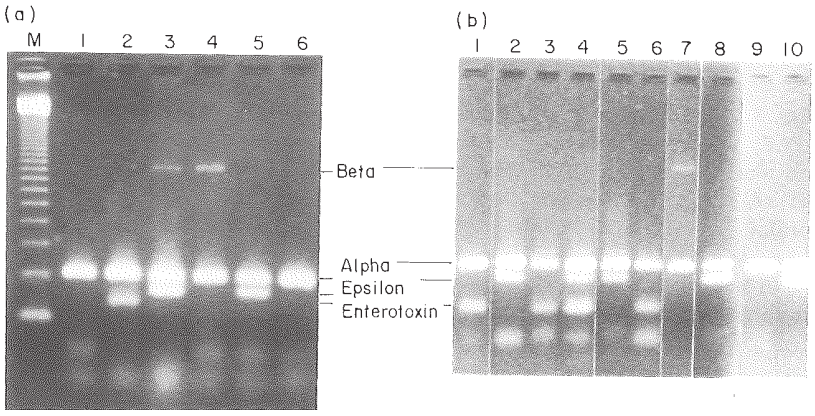

Fig. 2 Gel electrophoresis analysis of polymerase chain reaction (PCR) products obtained on *Clostridium perfringens* pure culture with a mixture of primers. (a) PCR reaction performed on reference strains with the primers α1, α2, β1, β2, ε1, ε2, ent1 and ent2. (1) *Clostridium perfringens* ATCC 13124 (toxinotype A); (2) *Cl. perfringens* NCTC 8239 (toxinotype A, enterotoxigenic); (3) *Cl. perfringens* ATCC 3626 (toxinotype B); (4) *Cl. perfringens* CWC 236 (toxinotype C); (5) *Cl. perfringens* CN 3978 (toxinotype D); (6) *Cl. perfringens* NCIB 10748 (toxinotype 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 α1, α2, β1, β2, ε1, ε2, ent1 and ent2. (1) *Clostridium perfringens* 46280-8; (2) *Cl. perfringens* 88B21MF; (3) *Cl. perfringens* 45017MF3; (4) *Cl. perfringens* 945P; (5) *Cl. perfringens* CP48; (6) *Cl. perfringens* 45402C01; (7) *Cl. perfringens* CWC 243; (8) *Cl. perfringens* 92E1897RA; (9) *Cl. perfringens* 45497C1; (10) *Cl. 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 *Cl. perfringens* classification.

Analysis of toxin genes from unknown strains

Under the conditions described above, the primers mixture was used to determine the toxinotype of 10 strains of unknown toxinotype isolated from animals or humans (Fig. 2b). These experiments were performed on pure cultures of the *Cl. perfringens* strains. A clear pattern was observed for each strain allowing the attribution of a toxinotype. 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-thioglycolate was inoculated with 1 g of animal intestinal contents iso-

Table 2 Toxinotyping of unknown *Clostridium perfringens* strains

Strain	Origin	Toxinotype*
460280-8	Human	A or E, enterotoxigenic
88B21MF	Goat	D
45017MF3	Cattle	A or E, enterotoxigenic
945P	Sheep	D, enterotoxigenic
CP 48	Sheep	D
45402C01	Cattle	A or E, enterotoxigenic
CWC 243	Swine	C
92E1897RA	Deer	D
45497C1	Cattle	A or E
42718MF1	Sheep	D

* Toxinotyping was done by polymerase chain reaction amplification using primers α1, α2, β1, β2, ε1, ε2, ent1 and ent2.

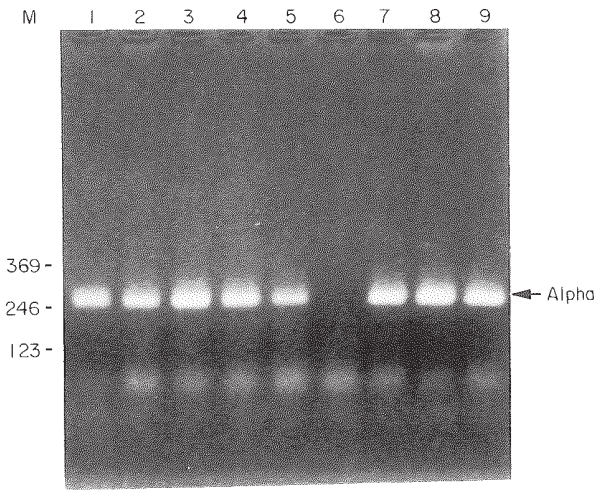

Fig. 3 Gel electrophoresis analysis of polymerase chain reaction (PCR) products obtained from intestinal contents with the mixture of primers. A PCR reaction was performed using primers $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\varepsilon 1$, $\varepsilon 2$, ent1 and ent2 on intestinal contents 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

lated 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 toxinotyping 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 toxinotyping 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 toxinotype was obtained. However, reproducible results with ι-toxin primers could not be obtained. Thus it is impossible by this method to discriminate between *Cl. perfringens* of toxinotypes A and E. However, toxinotype 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 toxinotype A and toxinotype E, a two-step method is recommended. After PCR reaction with the mixture of primers, a PCR amplification may be done with only primers ι.

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 toxinotype 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 toxinotype 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, toxinotyping kits, including the primers, polymerase and reference DNA as a control, could be useful.

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