Evaluation of two PCR-based procedures for typing *Clostridium perfringens*

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ABSTRACT

Two polymerase chain reaction (PCR)-based procedures for typing *Clostridium perfringens,* which affects most domestic animals, were compared and evaluated for efficiency as substitute to the guinea-pig intradermal test routinely used in our laboratory, namely a multiplex PCR and a protocol based on the individual amplification of gene sequences specific for each toxin. Reference isolates of *C. perfringens* types A, B, C and D as well as cultures from clinical specimens were tested. The sensitivity and specificity of the PCR was confirmed on reference isolates. There was similarity in results on 43 of the 46 samples typed by all 3 methods. Clear results were obtained by PCR on 5 clinical samples that showed either equivocal or weak skin reactions in guinea-pigs. The multiplex PCR protocol, in combination with the evaluation of bacterial growth, is a better alternative to *in vivo* toxin typing, since *C. perfringens* can only be incriminated as cause of a disease when it is present in large numbers in the intestine.

Key words: *Clostridium perfringens,* polymerase chain reaction, toxin typing.

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INTRODUCTION

Clostridium perfringens, a widely distributed microorganism found in soil and the alimentary tract of animals, is associated with a wide variety of diseases affecting most domestic animal species and humans (Table 1). The microorganism is classified into 5 types (A, B, C, D and E) according to the production of 4 major lethal toxins (Table 2). Types A, B, C and D occur most commonly in domestic animals[4,6].

Confirmation of the diagnosis of a disease caused by *C. perfringens* relies on the assessment of clinical signs and *post mortem* findings in combination with the isolation, determination of pre-formed toxins in intestinal contents and identification and typing of the organism[1,4,6]. The organism can be visualised microscopically on smears of affected tissues and intestinal contents as a Gram-positive stout rod. Bacteriological identification is conducted by anaerobic culturing of affected tissues and intestinal contents on blood agar or on selective agar media. The toxins are identified by means of the toxin-neutralisation test in the skin of guinea-pigs or neutralisation tests in mice[7].

These conventional methods are time-consuming, expensive and require the use of live animals. Apart from ethical considerations, the guinea-pig intradermal test (GPI) lacks precision, is technically difficult to perform and also requires experience to analyse and interpret.

Since the elucidation of genes encoding for the 4 major toxins of *C. perfringens,* many PCR-based detection methods have been developed and used for genotyping of *C. perfringens*[2,3,8]. Two approaches are commonly used: PCR based on individual reactions for each toxin gene and multiplex PCR for the simultaneous amplification of the major toxin genes in 1 tube[3]. The 1st method, although time-consuming and costly, seems to be more suited to detecting the organism directly from clinical or environmental samples, whereas the multiplex procedure produces better results on cultured material [2,3,8].

To assess whether the PCR-based genotyping of *C. perfringens* could be a better substitute to conventional *in vivo* methods used in our laboratory, a multiplex PCR assay (MP) and a PCR based on the individual detection of each toxin gene (IP) were evaluated and compared to results obtained with the conventional methods on isolates and overnight cultures from clinical specimens.

MATERIALS AND METHODS

C. perfringens reference isolates were obtained from the National Collection of Type Cultures (NCTC), Colindale, London, and were used together with field isolates prepared at the Onderstepoort Veterinary Institute (OVI) from cases submitted to the Bacteriology Department. They corresponded to *C. perfringens* types A, B, C and D.

As control material, isolates of *Clostridium botulinum, Clostridium septicum, Clostridium tetani, Clostridium sordellii* and *Clostridium chauvoei,* all from the OVI, were used to determine the specificity of the assays.

In addition, clinical samples submitted for routine diagnosis of *C. perfringens* were also used.

Isolates were cultivated on blood tryptose agar (BTA) or in brain-heart infusion broth (BHI) (Biolab Diagnostics), and incubated anaerobically overnight at 37 °C. Field specimens were cultivated on BTA plates under the same conditions.

Cells from BHI culture of *C. perfringens* were harvested by centrifugation of 1 mℓ broth at 5000 × g for 10 min. One colony from isolate cultures on BTA plate was resuspended in 1 mℓ of high-performance liquid chromatography (HPLC)-grade water and centrifuged at 5000 × g for 10 minutes. The pellets were resuspended in 200 µℓ of HPLC-grade water. The suspensions were boiled for 20 min, centrifuged (5000 × g for 5 min) and snapped cool on ice. Ten µℓ of the supernatant was used as template in the PCR assay. Mixed colonies on BTA plates containing cultures from clinical samples were harvested by washing the plates with 500 µℓ of HPLC-grade water. 200 µℓ of the suspended mixed culture was boiled, centrifuged and cooled in the same manner as for the isolates. Ten µℓ was also used as templates in the PCR assay.

Primers specific to alpha-, beta- and epsilon-toxin genes were used in both PCR protocols, as iota toxin is only produced by the rare E type[4,6].

The primers previously used by Uzal *et al.*[8] for the alpha-toxin gene (5'-TGG CTAATGTTACTGCCGTTGATAG-3' and

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Table 1: **Diseases associated with *Clostridium perfringens* in humans and animals.**

C. perfringens type	Description of disease	Animal affected
	Wound infection	
A	Gas gangrene, myonecrosis	Humans and animals
A	False blackleg	Cattle
A	Gangrenous mastitis	Cattle
A	Post-abortion septicaemia	Humans
	Enterotoxaemia	
A	Haemorrhagic/necrotic enteritis	Lambs, sheep
A	Yellow lamb disease	Lambs, calves
A	Enterotoxaemia	Lambs, calves, piglets, water buffalo, deer, elk
A	Sudden death	Cattle
A	Equine intestinal clostridiosis	Horses
A	Enterotoxin food poisoning	Humans and animals
B	Lamb dysentery	Lambs
B	Haemorrhagic enteritis, dysentery	Foals, calves
C	Struck	Sheep
C	Haemorrhagic necrotic enteritis	Lambs, calves, piglets, foals
C	Necrotic enteritis	Humans
D	Pulpy kidney disease (enterotoxaemia, overeating)	Sheep, goats
D	Enterotoxaemia	Cattle

5'-ATAATCCCAATCATCCCAACT ATG-3'), the beta-toxin gene (5'-AGGAG GTTTTTTTATGAAG-3' and 5'-TCTAAA TAGCTGTTACTTTGT-3') and epsilon-toxin gene (5'-TACTCATACTGTG GGAACTTCGATACAAGC-3' and 5'-CT CATCTCCCATAACTGCACTATAAT TTCC-3') were selected for the IP protocol. Ten µℓ of the templates from isolates were used with each set of primers (each to a final concentration of 0.8 µM), 250 µM of each dNTP, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100 and 1 unit of *Taq* polymerase (Takara Biomedicals) in a 50 µℓ reaction volume.

Amplification was conducted under the following conditions: 2 min at 93 °C followed by 30 cycles of 30 sec at 93 °C, 30 sec at 48 °C and 30 sec at 72 °C for mixes with primers specific for the alpha- and epsilon-toxin genes. Reaction mixes containing primers for the beta gene were denatured for 2 min at 93 °C, followed by 30 cycles of 45 sec at 93 °C, 45 sec at 50 °C and 45 sec at 72 °C.

Primer sets for the alpha (5'-TACTC ATACTGTGGGAACTTCGATACAAGC-3' and 5'-CTCATCTCCCATAACTGCACT ATAATTTCC-3'), beta (5'-GCGAATAT GCTGAATCATCTA-3' and 5'-GCAGGA ACATTAGTATATCTTC-3') and epsilon (5'-GCGGTGATATCCATCTATTC-3' and 5'-CCACTTACTTGTCCTACTAAC-3') toxin genes were used together in a 50 µℓ reaction volume for the MP protocol, under the conditions described by Meer *et al.*[3].

All amplicons were visualised under UV illumination after being subjected to electrophoresis for 20 min in a 1.5 % agarose gel (Roche Molecular Biochemicals) with 0.5 µg/mℓ of ethidium bromide.

Template DNA was extracted from *C. botulinum, C. septicum, C.tetani, C. sordelli* and *C. chauvoei* using the same protocol as for *C. perfringens.* The extracted DNA was used in PCR under the same conditions as for *C. perfringens* specimens to determine the specificity.

The GPI test was carried out according to the standard protocol[7].

RESULTS

Both PCR protocols yielded the expected amplification products when used on *C. perfringens* isolates (Figs 1, 2). For the MP, the products were of the following sizes: 324 bp for the alpha-toxin gene, 196 bp for the beta-toxin gene and 655 bp for the epsilon-toxin gene. For the IP assay, the product sizes were 247 bp, 1025 bp and 403 bp for the alpha-, beta- and epsilon-toxin genes, respectively. All reference isolates used in the evaluation yielded the expected amplicons.

Products of similar sizes to the reference isolates were obtained when the 2 PCR assays were used on cultures from clinical samples. The PCR results obtained were similar to those described earlier[3,8] and confirmed the good sensitivity and specificity of the PCR assays. PCR assays appear to be a suitable alternative typing method for *C. perfringens,* especially with regard to clinical samples.

Cultures from clinical samples were subjected to PCR after visual identification of *C. perfringens* colonies. They were also subjected to the GPI test. MP, IP and GPI tests results are summarised in Table 3. Except for 2 specimens with negative results, all samples yielded amplicons that could be characterised as corresponding to the alpha-, beta- or epsilon-toxin genes, allowing the isolates to be typed as types A, B, C or D.

No product resulted from DNA templates prepared from the other clostridial species, confirming the specificity of the PCR assays.

Table 2: **The major toxins of *Clostridium perfringens.***

C. perfringens type	Alpha toxin	Beta toxin	Epsilon toxin	Iota toxin
A	+	–	–	–
B	+	+	+	–
C	+	+	–	–
D	+	–	+	–
E	+	–	–	+

DISCUSSION

Except for the 3 clinical samples with equivocal results on the GPI test, there was similarity in results of the 43 samples typed by the GPI-, the IP- and the MP-based procedures. Samples 41 and 45 gave weak positive results in the GPI test, but specific for type D. They were clearly typed as D by PCR. All samples with equivocal results in the GPI test were

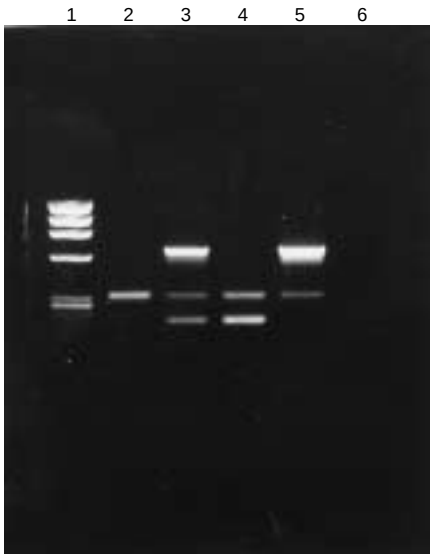

Fig. 1: **Agarose gel electrophoresis of the polymerase chain reaction** (PCR) **products (stained with ethidium bromide) obtained from the *Clostridium perfringens* multiplex PCR typing assay** (MP)**.** Lanes: **1**, molecular weight marker (ϕX174 DNA, Promega): 1353, 1078, 872, 603, 310, 281, 234, 194 and 118 bp; **2**, genotype A positive for α toxin gene; **3**, genotype B positive for α, β and ε toxin genes; **4**, genotype C positive for α and β toxin genes; **5**, genotype D positive for α and β toxin genes; **6**, negative control (no template DNA).

unequivocally typed by PCR. Samples 39 and 40 were negative on both tests. They originated from cases clinically suspected to be associated with *C. perfringens* infection, but never confirmed.

With the GPI test, 5 of 46 clinical samples (10.8 %) either showed equivocal results or showed weak skin reactions in guinea-pigs used for typing. The 5 samples yielded clear results with the PCR assays. *In vivo* typing methods for *C. perfringens* are known to some degree to lack precision and usually require an experienced diagnostician. This condition has limited the diagnostic capability of many laboratories, so that the PCR seems a better alternative typing method.

Table 3: **Comparison of *Clostridium perfringens* typing results using the multiplex PCR** (MP)**, the individual gene amplification PCRs** (IP) **and the guinea-pig intradermal test** (GPI) **on cultures from clinical specimens.**

Sample	MP-based typing	IP-based typing	GPI-based typing
1	A	A	A
2	A	A	A
3	A	A	A
4	A	A	A
5	A	A	A
6	A	A	A
7	A	A	A
8	D	D	D
9	D	D	D
10	A	A	A
11	A	A	A
12	D	D	D
13	A	A	A
14	A	A	A
15	A	A	A
16	A	A	A
17	A	A	A
18	A	A	A
19	D	D	D
20	D	D	D
21	A	A	A
22	A	A	A
23	A	A	A
24	A	A	A
25	A	A	A
26	D	D	Equivocal
27	A	A	A
28	D	D	D
29	A	A	A
30	A	A	A
31	A	A	A
32	A	A	A
33	A	A	A
34	D	D	D
35	D	D	D
36	D	D	D
37	B	B	B
38	D	D	D
39	Negative	Negative	Negative
40	Negative	Negative	Negative
41	D	D	Weak D
42	A	A	A
43	A	A	Equivocal
44	A	A	A
45	D	D	Weak D
46	A	A	Equivocal

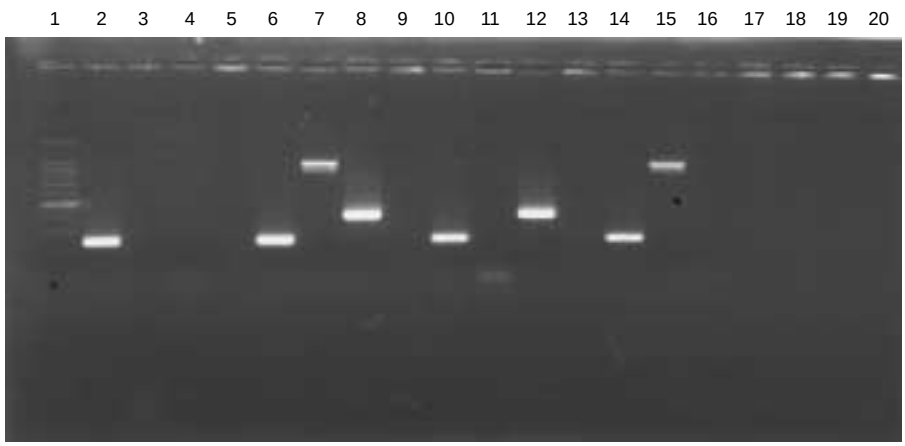

Fig. 2: **Agarose gel electrophoresis of the polymerase chain reaction** (PCR) **products (stained with ethidium bromide) obtained from the *Clostridium perfringens* individual gene amplification PCR typing assay** (IP)**.** Lanes: **1**, molecular weight marker (100bp DNA ladder, Promega): 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 bp; **2**, genotype A positive for the α toxin gene; **3**, genotype A negative for the β toxin gene; **4**, genotype A negative for the ε toxin gene; **6**, genotype B positive for α toxin gene; **7**, genotype B positive for β toxin gene; **8**, genotype B positive for ε toxin gene; **10**, genotype D positive for α toxin gene; **11**, genotype D negative for β toxin gene; **12**, genotype D positive for ε toxin gene; **14**, genotype C positive for α toxin gene; **15**, genotype C positive for β toxin gene; **16**, genotype C negative for ε toxin gene; **18**, **19** and **20**, negative control (*C. botulinum*), negative for α, β and ε toxin genes respectively.

Clinical specimens used in this evaluation were cultured before PCR amplification. No attempt was made to directly amplify DNA extracted from field specimens. *C. perfringens* forms part of the normal flora of the intestinal tract of animals and man. It can invade the parenchymatous organs shortly after death. *C. perfringens* is only considered to be significant if it is present in large numbers in the intestine, and if its isolation is correlated with the clinical signs and lesions seen at necropsy[5]. For bacterial evaluation, a heavy growth of *C. perfringens* is defined as a virtually pure growth obtained from both the initial inoculum and at least the 1st quadrant when streaked out using the normal method[1].

Performing the PCR assay after bacterial growth has been evaluated, therefore, becomes more appropriate in a diagnostic context. The use of multiplex PCR rather than single gene assays (more suited for direct detection of the organism in field samples) provides a better alternative to *in vivo* toxin typing. The combination of PCR with culture for identification of the organism allows a qualitative and quantitative evaluation of the specimen, and a more rapid and accurate typing of the *C. perfringens* type involved.

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