Toxin genotyping of *Clostridium perfringens* strains using a polymerase chain reaction protocol

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Summary

A polymerase chain reaction protocol consisting of a multiplex to identify the *cpa*, *cpb1*, *cpetx*, *cpi* genes and a duplex to identify the *cpe* and *cpb2* genes encoding for α, β1, ε, ι, enterotoxin and β2 toxins, respectively, was applied to DNA extracted from two collections of *Clostridium perfringens* strains. The first collection involved 19 isolates from rabbits. The second collection of 41 isolates came from routine necropsies. The *cpa* gene alone, or in association with the *cpb2* gene, was detected in all DNA samples examined. The *cpa* gene, together with *cpb2* gene, were detected in seven of the rabbit *C. perfringens* strains (36.8%) and in nine isolates from necropsies (21.9%). The *cpa* gene was found in 63.2% of rabbit strains and 76.9% of strains from other animal species. In rabbits, the pathological lesions associated with *C. perfringens* detection were predominantly forms of non-inflammatory enteropathies. In other species, *C. perfringens* was mainly associated with congestive-haemorrhagic enteropathy, but also with fatal traumatic lesions, degenerative diseases and organs with post-mortem autolysis. No clear correlation was observed between detection of β2 toxin gene and species-specific pathological features.

Keywords

Animal, *Clostridium perfringens*, Clostridial toxin, Genotyping, Italy, PCR, Polymerase chain reaction, Rabbit.

Introduction

Clostridium perfringens strains are classified into five toxinotypes (A, B, C, D and E) on the basis of the production of four major toxins (α, β1, ε and ι), and on their fatal effect by intra-peritoneal injection and specific seroprotection with neutralising antibodies in mouse lethality tests (12).

C. perfringens type A is the most common toxinotype in the environment; it is ubiquitous and is responsible for gangrene in humans, haemorrhagic and necrotising enterotoxaemia in ruminants, abomasitis in calves, necrotic enteritis in poultry and diffuse enteritis in all mammals. It is also associated with food poisoning in humans (9). The enteropathogenicity of *C. perfringens* type A is mediated primarily by α toxin encoded by the *cpa* (*plc*), a gene located on a variable region of the chromosome near the origin of replication. Type A is also involved in enteritis in humans, after food poisoning, mediated by a toxin produced during sporulation, called enterotoxin, which is encoded by the *cpe* gene located in the chromosome. The same gene located in the plasmid is associated with non-foodborne enteritis in humans and animals (12).

C. perfringens types B, C, D and E are associated with dysentery in the young of many animal species, haemorrhagic enterotoxaemia (struck) in sheep, pulpy kidney disease in sheep and sudden death with dysentery in calves and lambs,

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respectively (9). Haemorrhagic necrosis of the intestinal mucosa and the oedematous parenchymal lesions, which characterise the pathogenesis of *C. perfringens* classified into toxinotypes B, C and D, are mediated by β1 and ε toxins encoded by genes *cpb1* and *etx*, located in plasmids. The β1 and ε toxins, in combination, are associated with toxinotype B, while, when individually expressed, they are associated with toxinotypes C and D respectively. The ι toxin, encoded by the *cpi* (*iap*) gene located in the plasmid, is responsible for the disruption of cell membranes and actin cytoskeleton and is associated with toxinotype E. Finally, the α toxin is always produced while enterotoxin can be produced by all these toxinotypes (12). A novel toxin, called β2 toxin, and its encoding plasmid gene *cpb2*, were recently described by Gibert *et al*. (6). It was so named to differentiate it from the β1 toxin expressed by a reference type B strain and a type C field strain isolated from a piglet with necrotising enterocolitis. More recently, several studies have shown the extensive distribution of β2-toxigenic *C. perfringens* isolated from diseased ruminants, carnivores, poultry and fish, and also in healthy animals (14). Following the recent widespread of epizootic rabbit enteropathy (ERE), the role of *C. perfringens* in rabbit enteropathies has been investigated by various authors (11, 15). Despite the fact that the aetiology of ERE still remains unknown, these studies speculate on the presence of the *cpa*, *cpa* + *cpb2* and *cpe* genotypes of *C. perfringens* in the spontaneous and experimental forms of ERE.

The polymerase chain reaction (PCR) employed in the genotyping of *C. perfringens* strains has been developed and commonly accepted as a practical, reliable method to provide a more accurate and comprehensive characterisation of *C. perfringens* pathovars than the toxinotyping (12).

The aim of this study was to contribute to knowledge of the genotypes of *C. perfringens* present in Italy in wild and domestic animals, with special regard to the rabbit, and to describe the main pathological profiles associated with the isolation of *C. perfringens*.

Materials and methods

Clostridium perfringens strain collections

Two *C. perfringens* strain collections were made between 2007 and 2008. They originated from an ad hoc study of rabbit enteric pathologies (13) and from necropsies that involved various animal species. The first collection consisted of 19 isolates from the caecal content of diseased rabbits while the second was made up of 41 isolates from the liver, kidney and intestinal content of farmed animals, pets and wildlife.

Pathological profiles

The pathological features of diseased rabbits were classified as inflammatory (entero-typhlitis) or non-inflammatory (liquid content, caecal impaction, mucoid content) entero-pathies, while the pathological profiles of the other animal species were grouped, on the basis of the principal diagnostic findings, into enteropathy (phlogistic or degenerative lesions) and other pathologies (traumatic, haemorrhagic or congestive syndrome, degen-erative disease or post-mortem autolysis).

Microbiology

Clostridium perfringens detection and identification

About 0.5 g or ml of inoculum from target organs or intestinal contents was suspended in 2 ml of meat broth. Of this suspension, 1 ml was inoculated into thioglycollate broth (BioMérieux SA, Marcy l'Étoile) and incubated at 37°C ±1°C for 24 h. Approximately 0.1 ml from each tube was inoculated by loop onto blood agar (BioMérieux SA, Marcy l'Étoile) and the plates were incubated in anaerobic conditions at 37 ±1°C for 24 h. *C. perfringens*-suspect colonies were sub-cultured onto blood agar in anaerobic conditions and the pure cultures were verified with Gram stain, catalase and oxidase tests. Biochemical identifications were performed using a commercial kit (API 20A™, BioMérieux SA, Marcy l'Étoile). The isolates were suspended in microbanks and stored at –80°C.

DNA analysis

Five colonies of each *C. perfringens* strain were used for DNA extraction with UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California) in accordance with the instructions of the manufacturer.

The extracted DNAs were selected on the basis of the molecular confirmation of *C. perfringens* with 16S rDNA-based PCR as described by Wang *et al.* (16) and subsequently tested for *cpa*, *cpb1*, *cpb2*, *cpetx*, *cpi* and *cpe* genes with PCR as described by Baums *et al*. (3) and Yoo *et al*. (17) and performed by Drigo *et al*. (5), in our in-house laboratory conditions. Briefly, a multiplex PCR was used to detect *cpa*, *cpb1*, *cpetx* and *cpi* genes and a duplex PCR was used to detect *cpb2* and *cpe* genes. The multiplex PCR was performed on a 25 µl mixture containing 2.5 mM $MgCl_2$, 250 µM each deoxyribonucleotide triphosphate (dNTP), 0.05 U/µl of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Carlsbad, California) and 0.1 µM of the primers listed in Table I. The duplex PCR was performed on a 25 µl mixture containing 1.5 mM $MgCl_2$, 250 µM of each dNTP, 0.05 U/µl of AmpliTaq Gold® DNA polymerase and 0.1 µM of the primers listed in Table II. The thermal cycling was performed as follows: initial denaturation for 2 min 30 sec at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C in the multiplex and 54°C in the duplex PCR, 1 min 20 sec at 72°C and a final step of 2 min at 72°C. The PCR products were separated by electrophoresis on 1.5% agarose gel (Eppendorf Italia, Milan) with 1X Sybr® safe gel stain (Invitrogen ltd, Paisley). Gels were viewed under a UV transilluminator and the image was captured using Chemilmager™ 5500, version 3.4 (Alpha Innotech Corporation, San Leandro, California).

The enterotoxin-toxinogenic ATCC 27324 type E, NCTC 3180 type C, NCTC 8346 type D and a β2-toxinogenic type A field strains were used as reference strains of *C. perfringens*.

Results

Rabbit strain collection

The rabbit *C. perfringens* strain collection contained genotypes expressing the *cpa* gene alone and others expressing *cpa* + *cpb2* genes.

Table I
Multiplex polymerase chain reaction: target toxin genes, nucleotide sequences and length of amplification products

Gene	Nucleotide sequence (5´-3´)	Length of amplicon (bp)	Reference
*cpa*_for	AGT CTA CGC TTG GGA TGG AA	900	Baums *et al.* (3)
*cpa*_rev	TTT CCT GGG TTG TCC ATT TC		
*cpb1*_for	TCC TTT CTT GAG GGA GGA TAA A	611	Baums *et al.* (3)
*cpb1*_rev	TGA ACC TCC TAT TTT GTA TCC CA		
*cpetx*_for	ACT GCA ACT ACT ACT CAT ACT GTG	541	Yoo *et al.* (17)
*cpetx*_rev	CTG GTG CCT TAA TAG AAA GAC TCC		
*cpi*_for	AAA CGC ATT AAA GCT CAC ACC	293	Baums *et al.* (3)
*cpi*_rev	CTG CAT AAC CTG GAA TGG CT		

Table II
Duplex polymerase chain reaction: target toxin genes, nucleotide sequences and length of amplification products

Gene	Nucleotide sequence (5´-3´)	Length of amplicon (bp)	Reference
*cpe*_for	TGG GAA CTT CGA TAC AAG CA	396	Baums *et al.* (3)
*cpe*_rev	TTA ACT CAT CTC CCA TAA CTG CAC		
*cpb2*_for	CAA GCA ATT GGG GGA GTT TA	200	Baums *et al.* (3)
*cpb2*_rev	GCA GAA TCA GGA TTT TGA CCA		

Table III shows the distribution of the rabbit *C. perfringens* strains by pathological profile and toxin genotyping results. Seventeen out of nineteen strains were associated with non-inflammatory enteropathies. Neither of the two genotypes was found to predominate in the various pathological profiles.

Strain collection from necropsies

This collection also contained genotypes expressing the *cpa* gene alone or together with *cpb2* gene. Table IV shows the distribution of the two genotypes by pathological profile. *C. perfringens* was predominantly detected from animals with congestive-haemorrhagic enteropathy and, to a lesser extent, from cases of mortality associated with fatal traumatic lesions or degenerative diseases, as well as organs undergoing post-mortem autolysis. The genotype *cpa* + *cpb2* was found in 27.3% of dead animals with intestinal lesions and in 15.8% of those with other pathologies. This genotype was found in dogs (three cases with haemorrhagic enteropathy and three cases with other pathologies), cattle, sheep and goats (three cases with haemorrhagic enteropathy).

The main results of our study were as follows:

- only *C. perfringens* toxinotype A was found in 60 strains originating from rabbits (19 strains), cattle (6 strains), sheep and goats (7 strains), pigs (5 strains), poultry (5 strains), dogs (13 strains), wildlife (3 strains), cats and rats (1 strain each)
- the genotype with the *cpa* gene encoding the α toxin was found in approximately 73% of strains
- the genotype which had *cpa* and *cpb2* genes encoding for α and β2 toxins was found in approximately 27% of strains
- there was no clear correlation between genotype *cpb2* and species-specific pathological findings

Table III
Pathological profiles associated with the detection of *Clostridium perfringens* in rabbits and toxin genotyping results

Pathological profile	No.	Toxin genotype (n)	
		cpa	*cpa* + *cpb2*
Enterotyphlitis	2	1	1
Bloating, liquid content in the caecum without enteric phlogosis	8	5	3
Dilation of small intestine, caecal impaction, mucus content in the colon, absence of enteric phlogosis	9	6	3
Total	19	12	7

Table IV
Pathological profiles associated with the detection of *Clostridium perfringens* from necropsies and toxin genotyping results

Pathological profile	Animals involved (n)	No.	Toxin genotype (n)	
			cpa	*cpa* + *cpb2*
Phlogistic enteropathy				
Haemorrhagic syndrome	Cattle (4), dogs (7), poultry (2), sheep (2), goats, rats, pig	18	12	6
Congestive, necrotic lesion	Cattle, dogs, sheep, poultry	4	4	0
Other pathologies				
Traumatic death	Wildlife (2), dogs, sheep	4	3	1
Haemorrhagic, congestive syndrome	Dogs (2), pigs (2)	4	3	1
Degenerative disease or post-mortem autolysis	Dogs (2), goats (2), poultry (2), pigs (2), cats, wildlife, cattle	11	10	1
Total		41	32	9

- approximately 90% of *C. perfringens* strains isolated from rabbits with intestinal disorders were associated with forms of non-inflammatory enteropathies.

Discussion

The PCR protocol used in this study proved adequate for determining the genetic potential of *C. perfringens* strains to produce toxins. Pathological features should also be considered when determining the competence of *C. perfringens* isolates to cause fatal enterotoxaemia. About 46% of *C. perfringens* strains isolated from necropsy, were probably not the primary cause of fatal disease (fatal cases of traumatic, haemorrhagic syndrome and degenerative disease). Our results confirmed the wide distribution of toxino-type A in animals. The genotype expressing *cpa* gene alone was found in 73.3% of the strains examined (63.2% of rabbit origin and 78.1% from other species). Furthermore, 36.8% of rabbit strains and 21.9% of isolates from other species also expressed the *cpb2* gene. Similar epidemiological findings in rabbits (4) and other animal species have also been reported in recent studies conducted by authors in Italy (K. Forti and M. Cagiola, unpublished data).

The β2-toxinogenic *C. perfringens* type A strains revealed a synergic action of α and β2 toxins, resulting in an increase in necrotic and haemorrhagic intestinal lesions in bovine enterotoxaemia (10). In contrast, the evidence of *cpb2* and *cpa* genotypes in *C. perfringens* strains isolated from healthy animals emphasised the role of predisposing factors in the pathogenesis of animal clostridial enterotoxaemia, such as sudden change in diet or the presence of trypsin-inactivating factors in feed (14). The rapid multiplication of *C. perfringens* in the small intestine is itself a factor of the pathogenicity of the bacterium through the acquisition of extrachromosomal elements containing additional toxin genes (12). These considerations can assist discussion on the role of *C. perfringens* in the non-inflammatory rabbit enteropathies revealed by our study. The pathological features, correlated to about 90% of the *C. perfringens* rabbit strains examined, are indeed compatible to ERE. This non-inflammatory enteropathy of growing rabbits has been spreading in Europe since 1998 (7), requiring antibiotics that act against Gram-positive bacteria, such as Zn-bacitracin and avilamycin (1, 2). Although the aetiology of ERE remains unknown, a recent review by Licois and Marlier (8) suggested that an anaerobic bacterium that produced an unknown toxin that is active in the early phase of the infection, could be involved. *C. perfringens* appears to play a role in the high mortalities observed during spontaneous ERE.

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