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A Preliminary Molecular Typing by PCR Assays of *Clostridium perfringens* and *Clostridium difficile* Isolates from Dogs

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ABSTRACT

Clostridium perfringens and *C. difficile* have been associated with acute and chronic large and small bowel diarrhoea, and acute haemorrhagic diarrhoeal syndrome in dogs. The objective of this study was to investigate by toxin gene profile and PCR-ribotyping the molecular characteristics of 14 *C. perfringens* and 10 *C. difficile* isolates from 95 canine faeces (n = 36, diarrhoeic and n = 59, non-diarrhoeic). Concerning *C. perfringens*, 13 strains (92.9%) were type A, of which 3 (23.1%) also possessed the beta 2 toxin (CPB2)-encoding gene. One isolate (7.1%) was type D and possessed CPB2 gene. On the whole, 4 of the 14 strains (28.6%) tested *cpb*2-positive. Six *C. difficile* isolates (60.0%) demonstrated *tcdA*+/*tcdB*+ and *cdtA*+/*cdtB*+ genotype and tested positive for, *in vitro*, toxin production by EIA. Eight distinct ribotypes were observed. In conclusion, the PCR assays may provide useful and reliable tools for *C. perfringens* and *C. difficile* molecular typing in routine veterinary diagnostics.

Keywords: *Clostridium perfringens*; *Clostridium difficile*; Molecular Typing; Dogs; Toxigenic/Non-Toxigenic

1. Introduction

Clostridium perfringens and *C. difficile* are important enteropathogenic agents in veterinary medicine [1].

C. perfringens is one of the most widespread pathogen, inhabiting the gastrointestinal tract of human beings and animals as well as terrestrial and marine environments [2]. It has been associated with outbreaks of acute, often severe diarrhoea in humans, horses, dogs and cats. The elaboration of four major toxins, alpha (α), beta (β), iota (ι), and epsilon (ε), is the basis for typing the microorganism into five toxigenic phenotypes (A, B, C, D and E). The different toxinotypes cause different forms of enteritis and enterotoxaemia in various hosts [3-5]. Each type may also express a subset of at least 15 other established toxins, including *C. perfringens* enterotoxin (CPE), a well-characterized virulence factor whose production is co-regulated with sporulation [6,7]. Virtually all strains isolated from dogs are type A, with only one published report documenting a type C infection in five cases of canine peracute lethal hemorrhagic enteritis [2]. Although several studies have shown an association between the immunodetection of CPE in faecal specimens and canine diarrhoea, the pathogenesis of *C. perfringens*-associated diarrhoea in the dog is not fully understood, because CPE is also detected in up to 14% of non-diarrhoeic dogs. Isolation of non-enterotoxigenic type A strains from a diarrhoeic specimen does not preclude an involvement of such strains in disease, because there is a plethora of other virulence factors not yet evaluated. One of these is the recently characterized *C. perfringens* β2 toxin, which has been associated with both necrotic enteritis in piglets and equine typhlocolitis [3,8].

C. difficile is the major cause of antibiotic-associated pseudomembranous colitis in human patients. It has also been associated with diarrhoea and enterocolitis in foals and adults horses, as well as diarrhoea in dogs [6].

Three toxins produced by *C. difficile* have been described: toxin A (TcdA, enterotoxin), toxin B (TcdB, cytotoxin), and an adenosine diphosphate (ADP)-ribosyltransferase (binary toxin, CDT). Diseases associated with *C. difficile* have primarily been attributed to the activity of TcdA and TcdB, and strains have historically been thought to produce both toxins (toxigenic isolates) or neither (non-toxigenic). There are increasing reports of variant strains isolated from human clinical cases of *C. difficile*-associated infection (CDI) that produce only TcdA or TcdB, however [2].

Current diagnosis of *C. difficile*-associated diarrhoea is

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primarily based on detection of TcdA and/or TcdB in faecal specimens by EIA. Isolation of the microorganism alone is not sufficient for diagnosis, due to the presence of non-toxigenic strains. Toxigenic *C. difficile* has been isolated from dogs with chronic diarrhoea, and reports have documented a carriage rate of *C. difficile* ranging from 0% - 40% in diarrhoeic and non-diarrhoeic dogs [2, 9]. Toxigenic *C. difficile* can be isolated from up to 94% of neonate dogs in the absence of clinical signs of disease [2]. Clinical signs that have been associated with canine *C. difficile* infection range from asymptomatic carriage to a potentially fatal acute hemorrhagic diarrhoeal syndrome.

A simple and rapid method is needed to differentiate toxigenic and non-toxigenic strains of *C. perfringens* and *C. difficile* in animals. In this regard, the objective of the current study was to investigate the molecular characteristics of various strains of *C. perfringens* and *C. difficile* isolates from diarrhoeic and non-diarrhoeic dogs, through the use of toxin gene profiling and PCR-ribotyping.

2. Materials and Methods

2.1. Samples

Ninety-five faecal samples were collected over an 8 month period (July 2006-March 2007) from diarrhoeic (n = 36) and non-diarrhoeic (n = 59) dogs. Thirty-eight were shelter dogs (diarrhoeic n = 3, non-diarrhoeic n = 35), 47 were privately-owned dogs (diarrhoeic n = 26, non-diarrhoeic n = 21) belonging to students or staff of the Veterinary Medicine Faculty of Parma (Italy), and another 10 dogs were patients at the Faculty Veterinary Hospital (diarrhoeic n = 7, non-diarrhoeic n = 3). Assays were performed on specimens collected within 3 hours after natural voiding. After analysis, samples were immediately stored at −20˚C.

2.2. Faecal Culture

All faecal samples were cultured onto pre-reduced Schaedler agar plates (Oxoid, Basingstoke, Hampshire, England), and at the same time inoculated into cooked meat broth (Oxoid, England). Samples were also streaked onto pre-reduced selective medium containing cycloserine-cefoxitin-fructose agar (CCFA) for *C. difficile* isolation. Plates were incubated anaerobically at 37˚C for 48 - 72 hours. After 3 days of incubation into cooked meat broth, the samples were subjected to heat shock for spore selection and then cultured onto Schaedler agar and/or CCFA. *Clostridium* identification was confirmed through the Rapid ID32A (bioMérieux SA, Marcy-l'Etoile, France).

2.3. Reference Strains

C. perfringens ATCC 12917 *cpa*+/*cpe*+ was utilized as positive control for duplex and multiplex PCRs. *C. perfringens* NCTC 8346, ATCC 373, and ATCC 27324 were used as *cpa*+/*etx*+, *cpa*+/*cpb*+/*cpb*2+ and *cpa*+/*iap*+/*cpe*+/*cpb*2+ controls, respectively, for multiplex PCR. *C. difficile* VPI 10463 and 51377 were used as *C. difficile tcdA*+/*tcdB*+ and *cdtA*+/*cdtB*+ controls, respectively. A strain characterized as PCR ribotype 078 was utilized to compare the PCR-ribotyping banding patterns.

2.4. Rapid Immunoassays

For rapid, *in vivo*, detection of TcdA/B in faecal samples, a commercial microplate EIA was performed according to manufacturer instructions (ProSpecT *Clostridium difficile* Toxin A/B, Remel, Lenexa, Kansas, USA). The, *in vitro*, toxin production by *C. difficile* was detected by two distinct immunological tests (ProSpecT *Clostridium difficile* Toxin A/B, Remel, USA, and *C. diff* Quik Chek Complete™, TechLab, Princeton, USA) on isolates following 3 and 5 days of anaerobic growth into cooked meat broth. *C. difficile* VPI 10463 was used as TcdA+/TcdB+ positive control.

2.5. Extraction of *C. perfringens* and *C. difficile* DNA

For each *C. perfringens* or *C. difficile* strain, a 100 µl suspension of cells in sterile water was vortexed, incubated at 100˚C for 5 and 10 min., respectively, and centrifuged at 12,000 g (Microliter Centrifuge, Hermle Z 233 M-2, Delchimica Scientific Glassware s.r.l.) for 2 min. Five µl of this preparation were used as the DNA template for all PCR assays. All PCRs were performed with a Techne TC-32 thermal cycler (Barloworld Scientific Ltd, Milano, Italy).

2.6. Duplex PCR for the *C. perfringens* Phospholipase C (PLC) and CPE Encoding Genes

All *C. perfringens* isolates and the ATCC 12917 reference strain were PCR-screened for the presence of PLC and CPE-encoding genes as previously described by Fach and Popoff [10]. Amplified products were subjected to 1.5% agarose gel electrophoresis (120 V, 1 h) and visualized by ethidium bromide staining and ultraviolet light exposure.

2.7. Multiplex PCR for the *C. perfringens* Toxins Encoding Genes

All *C. perfringens* isolates, along with the four reference strains, were PCR-subjected for the detection of α (*cpa*), β (*cpb*), ε (*etx*), CPE (*cpe*), ι (*iap*), and $\beta 2$ (*cpb*2) toxin encoding genes, as described by Baums *et al.* [3]. The reaction products were subjected to agarose gel electro-

phoresis as mentioned above.

2.8. Duplex PCRs for the *C. difficile* TcdA/B and Binary Toxin Encoding Genes

All *C. difficile* isolates and the reference strains were PCR-screened for the presence of (a) TcdA/B-encoding genes (624-bp *tcdA* and 412-bp *tcdB* gene fragments), as previously described by Spigaglia and Mastrantonio [11], and (b) binary toxin genes (375-bp *cdtA* and 510-bp *cdtB* gene fragments), as described by Stubbs *et al.* [12]. The reaction products were subjected to agarose gel electrophoresis as above.

2.9. *C. difficile* PCR-Ribotyping

PCR-ribotyping was conducted with the primer pair *RtFR*1/*RtFR*2, as described by Bidet *et al.* [13,14]. The amplified products were analyzed by 3% gel electrophoresis (85 V, 5 h) and visualized as above.

3. Results

Sixty-two faecal samples were positive for *Clostridium* spp. presence (62/95 samples, 65.3%, confidence interval 95%: 55.3 to 74.3). Eighty-nine *Clostridium* spp. were isolated from the 62 positive faecal specimens. Frequently, more than one species of clostridia was observed in the same faecal sample. The completed results were published in a precedent work [15].

Overall, 14 dogs were positive for *C. perfringens* (14/95: 14.7%; I.C. 95.0%: 8.6 to 23.0). The isolation rate from diarrhoeic dogs (6/36: 16.7%) was similar to the rate from healthy dogs (8/59: 13.6%). The difference was statistically not significant at 95% level (P = 0.679, Upton's Chi-square test). In one dog, affected by megaesophagus and treated with antibiotics for enteritis, *C. difficile* was also isolated [15].

None of the 14 strains were CPE-positive (*plc*+/*cpe*–) by duplex PCR. This result was confirmed by multiplex PCR assay (*cpa*+/*cpe*–). In particular, 13 isolates (13/14: 92.9%) were type A (*cpa*+), of which 3 (3/13: 23.1%) possessed the CPB2 toxin-encoding gene. Finally, 1 strain (1/14: 7.1%) was type D (*cpa*+/*etx*+) and possessed CPB2 gene (**Figure 1**). On the whole, 4 of the 14 strains (28.6%) tested *cpb*2-positive. Three of them (75.0%) were from diarrhoeic dogs, and 1 (25.0%) was from non-diarrhoeic dog. This difference was statistically not significant at 95% level (P = 0.486, Fisher's Exact test).

Six type A strains (3 *cpa*+, and 3 *cpa*+/*cpb*2+) were isolated from faecal samples of dogs with enteritis. The other 7 type A isolates and the type D strain were from canine non-diarrhoeic faeces.

Eight of 10 (80%) *C. difficile* culture-positive samples belonged to diarrhoeic dogs, 5 of which with enteritis after antibiotic therapy and 3 not treated with antibiotics since at least 6 months. The majority of *C. difficile* isolates (6/10, 60.0%) were toxigenic (*tcdA*+/*tcdB*+) and possessed *cdtA* and *cdtB* genes. All faeces tested EIA-negative. On the contrary, all PCR-positive strains were positive for, *in vitro*, toxin production when tested by both immunological tests. The isolation rates of *C. difficile* from diarrhoeic dogs (8/36, 22.2%) and non-diarrhoeic dogs (2/59, 3.4%) were statistically different (P = 0.006, Fisher's Exact Test).

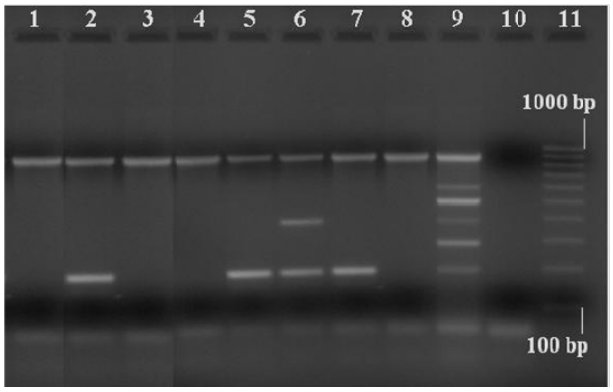

Figure 1. Detection of toxins encoding genes by multiplex PCR in *Clostridium perfringens* strains isolated from dogs. Lanes 1, 3, 4 and 8: type A strains (*cpa*+); lanes 2, 5 and 7: type A, *cpb*2+ strains; lane 6: type D, *cpb*2+ strain; lane 9: *C. perfringens* positive control (*cpa*+/*cpb*+/*cpe*+/*etx*+/*iap*+/*cpb*2+); lane 10: negative control ("0 DNA"); lane 11: molecular size markers (100 bp Molecular Ruler, Biorad, Italy).

The proportion of toxigenic isolates (5/8, 62.5%) in diarrhoeic dogs was similar to the proportion (1/2, 50.0%) in non-diarrhoeic dogs. Such difference was not significant (P = 0.667, Fisher's Exact Test).

Finally, the 10 *C. difficile* strains were subjected to ribotype analysis by comparing the primer-targeted amplicons of the intergenic spacer region localized between the 16S and the 23S rRNA genes. Eight ribotypes were noted (arbitrarily designated VETPR 1 - 8) (**Figure 2**). The observed ribotype distribution suggested wide diversity of *C. difficile* within the dog population. In particular, one ribotype (VETPR1) was predominant among the isolates, comprising 3/10 total strains (30.0%) (derived from 2 diarrhoeic and 1 non-diarrhoeic dogs) with a *tcdA*+/*tcdB*+ and *cdtA*+/*cdtB*+ genotype (**Table 1**). None of the observed ribotypes showed the ribotype 078.

4. Discussion

Detection of *C. perfringens* and *C. difficile* in canine faeces is important. It has been well documented that culture isolation of *C. perfringens* has not diagnostic value for canine *C. perfringens*-associated diarrhoea. Culture may be useful in procuring isolates for toxin neutralization tests and molecular techniques like PCR to

Table 1. *Clostridium difficile* PCR-ribotype prevalence versus toxin profile by PCR.

Ribotype	No. of isolates				Total
	tcdA+/tcdB+cdtA+/cdtB+		*tcdA–/tcdB–cdtA–/cdtB–*		
	Diarrhoeic	Non-diarrhoeic	Diarrhoeic	Non-diarrhoeic	
VETPR 1	2	1			**3**
VETPR 2	1				**1**
VETPR 3	1				**1**
VETPR 4	1				**1**
VETPR 5			1		**1**
VETPR 6			1		**1**
VETPR 7				1	**1**
VETPR 8			1		**1**
Total	**5**	**1**	**3**	**1**	**10**

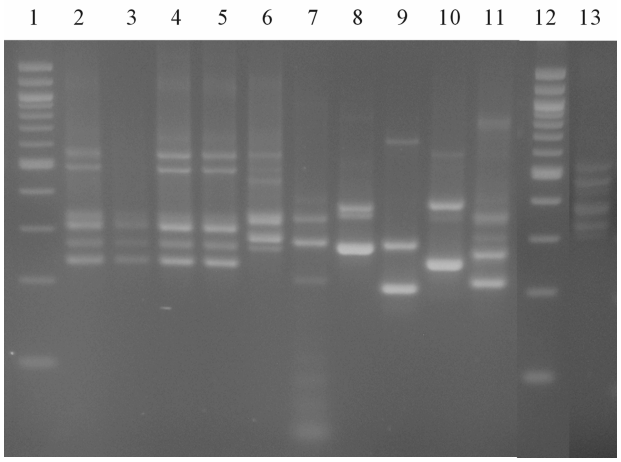

Figure 2. PCR-ribotyping of *Clostridium difficile* strains isolated from dogs. Lanes 1 and 12: molecular size markers (100 bp DNA Ladder, Celbio, Milano, Italy); lanes 2-11: *C. difficile* isolates. In particular, lanes 2, 4 and 5: ribotype VETPR 1; lane 3: VETPR 2; lane 6: VETPR 3; lane 7: VETPR 4; lane 8: VETPR 5; lane 9: VETPR 6; lane 10: VETPR 7; lane 11: VETPR 8; lane 13: ribotype 078.

detect specific toxin genes, or molecular typing of strains to establish clonality in suspected outbreaks. Two commercially available immunoassays are currently used in veterinary diagnostic laboratories for CPE. It is important to note that the performance of these assays have not been validated in the dog, and there are concerns about their sensitivities and specificities [2]. Moreover, they not detect the CPB2 or other toxins.

The high rate of occurrence of *cpb*2-positivity among strains isolated from animals with enteritis would give strength to the hypothesis that CPB2 plays a role in pathogenesis of the disease [8,16]. On the contrary, the detection of strains harbouring *cpb*2 in healthy animals is not necessary itself a risk, although β2-toxigenic *C. perfringens* can become an emerging health threat when associated to enteric dysbiosis or immunosuppression [17].

In this work, the frequency of *C. perfringens* isolation from healthy and diarrhoeic dogs was similar. By multiplex PCR, 13 out of the 14 *C. perfringens* strains belonged to type A. This is in accord with literature [2]. Only one isolate tested type D. None strain resulted *cpe*-positive, but a relatively high percentage of strains (4/14: 28.6%) were *cpb*2-positive. On the contrary, the type D isolate, positive for *cpb*2, came from a healthy dog.

We can not conclude that CPB2 is responsible for the enteritis in our strains because we didn't verify the β2 protein expression *in vitro*, although we found a high revelation percentage of *cpb*2-positive diarrhoeic dogs. It may be important to consider the use of an additional method for the detection of CPB2 in *cpb*2-positive isolates, such as neutralization test. Preferably, detection of CPB2 should be performed directly from the tissue in enteritis cases where CPB2 may be expected to play a role [8].

Concerning *C. difficile*, the role that this microorganism plays in dogs is not well defined, and only a few studies evaluating the presence of toxins in diarrhoeic and non-diarrhoeic animals have been done [2].

The laboratory diagnosis of *C. difficile*-associated diarrhoea in the dog is controversial. The apparently high prevalence of EIA-positive, culture-negative canine specimens obtained with some commercial assays, never validated in the dog, is questionable, and may represent the consequence of false-positive results [2].

The results of this study confirmed the low sensitivity of EIA when performed directly on faecal specimens. This low sensitivity is not surprising, since none of the commercial EIA kits currently available has been vali-

dated in the dog. In contrast, the sensitivity and specificity for TcdA/B detection were higher when EIA was performed directly on isolates rather than on faecal samples. However, these results should be interpreted with caution, as toxins production, *in vitro*, does not automatically imply that toxin is produced and secreted in the intestinal tract [9].

Our significatively higher isolation rates from diarrhoeic dogs compared to non-diarrhoeic are in disagreement with previous reports [9]. However, it is important to underline that 5 out of the 10 *C. difficile* strains were isolated from dogs with enteritis consequent to antibiotic therapy which could have caused an overgrowth of *C. difficile* in intestine, thus predisposing the animals to enteritis.

The majority of *C. difficile* strains (60.0%) were toxigenic on the basis of results of the duplex PCR assays for the identification of TcdA/B and binary toxin genes. The carriage rates of toxigenic isolates in diarrhoeic dogs (62.5%) was similar than those in non-diarrhoeic dogs (50.0%). These findings are in agreement with those reported in previous studies [9,18].

None of our ribotypes showed the ribotype 078 that has emerged as hypervirulent genotype and predominant strain in pigs and calves [19]. The comparison of our ribotypes and *tcd*-profiles with additional *C. difficile* isolates from other sources could be useful to determine whether certain ribotypes are associated with variant toxin profiles in dogs, other animals and/or humans.

In conclusion, ideally, the application of PCR assays on *C. perfringens* and *C. difficile* isolates for the detection of toxins genes, combined with EIA tests for the demonstration of toxins production (*in vivo* and *in vitro*), should be implemented for diagnosing canine disease.

The results of this study highlight that the PCR assays may provide a useful and reliable tool for *C. perfringens* and *C. difficile* genotyping in routine veterinary diagnostics. The genotype, in many cases, could provide the final piece of information needed to establish a diagnosis [20].

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