CLOSTRIDIUM PERFRINGENS TYPE A, ENTEROTOXIN, TOXIN GENOTYPES AND RIBOTYPES IN DOGS WITH EPISODIC DIARRHEA

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Abstract

During six episodes of diarrhea over the last 51 months, we monitored the feces from two dogs for a variety of diagnostic markers indicative of *C. perfringens*. A third dog living with the two affected animals was also studied. Symptoms, usually consistent within an episode, ranged from a mild, watery diarrhea to a mucoid and bloody diarrhea. Occasionally the diarrhea resolved spontaneously and, even within an episode, it was sometimes sporadic. We used quantitative, anaerobic culture on pre-reduced blood agar, with and without prior ethanol shock for the selection of spores. An EIA was used to detect *C. perfringens* enterotoxin (CpE, encoded by the *cpe* gene) in feces and broth cultures. We used PCR to identify, genotype and ribotype *C. perfringens* isolates. It was often necessary to work on multiple isolates from a single sample, representing presumptive isolates with different hemolytic patterns and colony types. This was because individual samples often contained several distinct strains, some potentially enteropathogenic (i.e. *cpe+*), some clearly not (*cpe-*). Five of the six episodes of diarrhea were associated with the presence of fecal CpE and strains of *C. perfringens* Type A carrying *cpe* and able to express the gene *in vitro* coupled with the absence of other known pathogens. Only diarrheic stools were positive for CpE. No formed stool was positive in the EIA. CpE was only present when *cpe+* isolates were seen. The reverse was not the case and in several instances *cpe+* isolates were recovered from dogs without fecal CpE and without symptoms. Although sometimes completely absent during health, *C. perfringens* spore and total counts were elevated during CpE diarrhea and only slightly less so in some normal stools, suggesting that culture, and the microscopic scrutiny of Gram stained smears for clostridia and spores may be unreliable features on which to base a diagnosis. Furthermore, PCR for *cpe* may not provide any greater diagnostic utility since *cpe+* isolates were common in both health and disease. The EIA was therefore the only assay, by itself, reliable for the diagnosis of CpE diarrhea in dogs. Treatment with metronidazole (25 mg/kg twice daily) rapidly led to the loss of symptoms and on most occasions the parallel elimination of both CpE and *cpe+ C. perfringens* from the diarrheic dogs.

Background

The literature on the role of *Clostridium perfringens* Type A and *C. perfringens* Enterotoxin (CpE) in diarrhea of dogs is confusing. The bacterium has been found during diarrhea and in health, especially in older animals (Benno *et al*., 1992). Different strains produce different combinations of toxins. Some toxins may contribute to symptoms in overlapping ways, while others, notably the more recently identified beta2, netB and tpeL toxins, await proven roles in diarrhea.

C. perfringens Enterotoxin (CpE) has a proven and direct role in diarrhea (Sarker *et al*., 1999). Even so, the story here is just as confusing. Not all *C. perfringens* carry *cpe*, the gene for CpE, while not all *cpe+* strains readily express CpE *in vitro*. Although dogs are susceptible (Bartlett *et al*., 1972), CpE has been detected in the stool of non-diarrheic dogs (van der Steeg *et al*., 1997). Despite these contradictions, both sporadic and recurrent diarrheas, often characterized by the presence of blood or mucus, have been linked to *C. perfringens* (Carman & Lewis, 1983; Kruth *et al*., 1989; Marks *et al*., 1999; Sasaki *et al*., 1999; Weese *et al*., 2001). However these studies, especially the earlier one, did not benefit from readily available and dependable immunoassays. Carman and Lewis (1983), for example, credited alpha toxin producing Type A isolates with causing diarrhea without considering any role for CpE.

Aims

To monitor *C. perfringens* counts, ribotypes and genotypes and CpE in 2 dogs with recurrent diarrhea and 3rd dog from the same household but without signs and to correlate these with symptoms and treatment.

Materials and methods

Feces : Feces were promptly collected by the dogs' owners and chilled to about 4 C for shipping to TechLab where the samples were stored below -20 C until testing.

Bacterial isolates: Following serial 10-fold dilution of the samples, we used anaerobic culture on pre-reduced sheep blood agar, with and without prior ethanol shock for the enumeration of spores and of total *C. perfringens* respectively. The inoculated plates were incubated anaerobically at 37 C for 24 h. Individual colonies were subcultured and stored in chopped meat broth until required. Isolates were confirmed as *C. perfringens* using PCR and species specific 16s rDNA PCR primers (Tonooka *et al*., 2005).

Bacterial DNA: DNA was extracted from 48 h brain-heart infusion broth sub cultures using the QIAGEN DNA Mini Kit. Collected DNA was stored at -20ºC until ready for analysis.

PCR: See table 1 below.

EIA for *C. perfringens* Enterotoxin (CpE): Substituting dog feces for human but otherwise following the package insert, we used the *C. perfringens* Enterotoxin TEST (TechLab, Inc., Blacksburg, VA), a research-use-only, polyclonal antibody based EIA developed for use with human feces. All isolates were tested for their *in vitro* production of CpE after 48 h anaerobic growth in brain-heart infusion broth. We made no attempt to enhance expression by inducing sporulation. Any positives would thus indicate a more permissive regulation of CpE expression than is suggested by reports describing stringent *in vitro* regulation.

***C. perfringens* ribotyping:** We amplified the 16S to 24S rDNA inter-generic sequence using a forward primer (5' GGG TCA GCG ATT GGG GTG AAG T 3') specific for *C. perfringens* and a reverse primer (5' GCG CCC TTT GTA GCT TGA CC 3'), specific for *Clostridium botulinum* and the following cycling conditions: 94 ºC for 1 min, 56ºC for 1 min, 72ºC for 2 min for 35 cycles before 10 min at 72 ºC. Isolates were allocated to one of 5 ribotypes (R1 to R5) based on the patterns of amplicons produced (Figure 1).

Table 1. PCR for genotyping and ribotyping

16S rDNA (ribotyping)	Tonooka, T., Sakata, S., Kitahara, M., Hanai, M., Ishizeki, S., Takada, M., Sakamoto, M., Benno, Y., 2005. Detection and quantification of four species of the genus *Clostridium* in infant feces. Microbiol. Immunol. 49, 987-992.
cpa, cpb, etx, ia, cpe, cpb2	http://microvet.arizona.edu/research/ClostridiumWeb/multiplexprocedure.pdf
pfo	Deguchi A, Miyamoto K, Kuwahara T, Miki Y, Kaneko I, Li J, McClane BA, Akimoto S. 2009. Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. PLoS One. 19;4(5):e5598
pCPF5603-like *cpe*+ and pCPF4969-like *cpe*+ plasmid	Miyamoto, K., Wen, Q., McClane, B.A., 2004. Multiplex PCR genotyping assay that distinguishes between isolates of *Clostridium perfringens* type A carrying a chromosomal enterotoxin gene (*cpe*) locus, a plasmid *cpe* locus with an IS*1470*-like sequence, or a plasmid *cpe* locus with an IS*1151* sequence. J. Clin. Microbiol. 42, 1552-1558.

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Dedication: This work is dedicated to Zeus, Lucas and Sprout (Dogs Z, L and S), a man and a woman's best friends.

Results

Table 2. Course of illness and laboratory findings in two dogs with recurrent episodes of diarrhea and one dog without

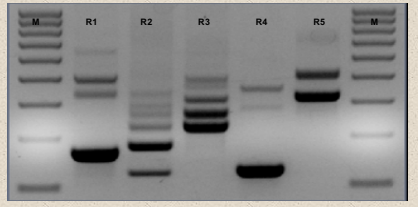
		Episode 1 11/7 to 11/28/05	Episode 2 2/14/06	Episode3 11/16-11/30/06	Non episode 2/5/07	Episode 4 7/17-7/27/07	Episode 5 (not a CpE associated diarrhea) 9/12-10/1/08	Non-episode 2/5-2/22/09
Diarrheic dogs	Dog(s)	Z, L	Z, L	Z, L		L	Z, L	
	Signs	Watery, bloody diarrhea	Watery diarrhea	Mucoid, bloody diarrhea		Mild watery diarrhea	Watery, mucoid diarrhea	
	Treatment	Loperamide	None	Metronidazole 250 mg bid for 7 d or 500 mg bid x 10 d		None	Metronidazole 500 mg bid x 10 d	
	C. perfringens enterotoxin test	+	Not done	+		+	+	
	C. perfringens total count/g	Not done	Not done	$10^{5.2}$ to $10^{6.8}$		$10^{6.1}$	None to $10^{3.1}$	
	C. perfringens spore count/g	Not done	Not done	Not done or none to $10^{7.0}$		$10^{6.1}$	$10^{5.1}$ to $10^{7.3}$	
	cpe $^{+}$ strains	R1G1	R1G1	R1G2, R2G2		R1G2	None	
	cpe $^{-}$ strains	R1G3, R1G4, R1G5	None	R1G3, R1G4, R2G4, R1G6, R4G7, R1G8		R1G3	R1G3, R1G6, R5G4	
Non-diarrheic dogs	Dog(s)	S	S	S	Z, L, S	S, Z	None	Z, L
	Signs	None	None	None	None	None		None
	Treatment	None	None	None	None	None		None
	C. perfringens enterotoxin test	Not done	Not done	-	-	-		-
	C. perfringens total count/g	Not done	Not done	$10^{4.5}$	None to $10^{3.0}$	None to $10^{6.2}$		None to $10^{5.2}$
	C. perfringens spore count/g	Not done	Not done	$10^{4.6}$	None to $10^{2.7}$	None to $10^{4.7}$		None to $10^{4.4}$
	cpe $^{+}$ strains	Not done	Not done	R1G2	R1G2	R1G2		None
	cpe $^{-}$ strains	Not done	Not done	R1G3, R1G6	R1G3	None		R1G6

Table 3. *Clostridium perfringens* PCR toxin genotypes

Genotype	Possible cause of diarrhea in episode	Toxin and toxin-related PCR assays				
		cpa (alpha toxin)	*pfo* (theta toxin)	*cpe* (enterotoxin)	pCPF5603-like *cpe*+ plasmid	pCPF4969-like *cpe*+ plasmid
G1	1,2	+	-	+	+	-
G2	3,4	+	-	+	-	+
G3	None	+	-	-	-	-
G4	None	+	-	-	-	-
G5	None	+	+	-	-	-
G6	None	+	+	-	-	-
G7	None	+	+	-	-	-
G8	None	-	-	-	-	-

Only Type A isolates were recovered. No isolate amplified with primers for *cpb* (beta toxin gene), *etx* (epsilon toxin gene), *iA* and *iB* (iota A and iota B components of iota toxin). CpE was made, *in vitro*, by one or more isolates of each *cpe+* genotype.

Figure 1. *Clostridium perfringens* PCR ribotypes: Profiles of Riboptypes R1 to R5



(M = 100 bp ladder, 200 bp to 1 kbp)

Discussion

Over a period of 45 months two dogs had 5 episodes of diarrhea associated with fecal *C. perfringens* Enterotoxin. No other known pathogens were identified at any time.

Only diarrheic stools carrying spores of *cpe+ C. perfringens* were EIA positive; no formed stool was ever EIA positive. Confusingly however, the same ribotypes and genotypes of *C. perfringens* present during diarrhea were often abundant in formed stools. Thus, the PCR detection of *C. perfringens* with or without amplification of *cpe* is insufficient to diagnose *C. perfringens* Enterotoxin diarrhea. It is only the detection of CpE - the protein product of gene expression - that offers a definitive diagnosis.

Samples from the 6th episode (episode 5 chronologically) though diarrheic, were EIA negative and carried only *cpe-* strains of *C. perfringens*.

A third dog in the "family" never had diarrhea though on occasions did carry *cpe+ C. perfringens*.

Because individual fecal samples may carry two or even three different isolates, only genotyping of several distinct isolates, including those whose colonies have one or no zones of hemolysis, was sufficient for the identification of likely enteropathogenic isolates.

Counts of both total *C. perfringens* and of spores tended to be higher during diarrhea and lower to absent during health. Treatment with metronidazole very quickly reversed symptoms, lowered bacterial counts and lead to a negative EIA result.

After the resolution of treated and untreated diarrheas, the diversity among genotypes (but not necessarily ribotypes) increased. We speculate that this may reflect a loss of colonization resistance to newly colonizing strains after antibiotic therapy coupled with possible curing of plasmids in the presence of metronidazole, an antibiotic interacting with DNA. Thus, during the 14 days of episode 3 two sets of strains (the potentially enteropathogenic R1G1 and R2G2, and the cpe- strains R1G3, R1G4, R2G4, R1G6, R4G7, R1G8) were recovered. The likely pathogenic (since they were both recovered during other episodes of diarrhea) R1G1 and R1G2 are both *cpe+* isolates but with the gene on different plasmids (pCPF4969-like and pCPF5603-like respectively). After metronidazole treatment, the non-toxigenic R1G3 emerged. Were R1G2 to lose its pCPF4969-like *cpe+* plasmid, it would retain its ribotype but assume the non-toxigenic, genotype R1G3.