The prevalence of *Clostridium perfringens* enterotoxin in dogs with acute diarrhea in southwestern Iceland.

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Summary

The objective of this study was to assess the prevalence of *Clostridium perfringens* enterotoxin (CPE) in dogs with acute diarrhea in southwestern Iceland and to find a possible association between the presence of CPE and endospore counts on fecal smears. Fecal specimens from 30 diarrheic dogs were evaluated by the use of enzyme-linked immunosorbent assay (ELISA) for the detection of CPE. Fourteen of these 30 dogs were CPE positive (46.64%). No association was found between the presence of CPE and endospore counts on smears.

Introduction

Clostridium perfringens is a gram-positive, anaerobic, spore-forming bacillus. The organism may be one of the most widespread pathogenic bacteria, inhabiting the gastrointestinal tract of human beings and animals as well as terrestrial and marine environments.[1] Strains of *C. perfringens* are classified into 5 types (A-E) based on the production of 4 major toxins (alpha, beta, epsilon, and iota).[1,2] *C. perfringens* type A is consistently recovered both from the intestinal tracts of animals and from the environment, while others (types B,C,D and E) are less common in the intestinal tracts of animals.[2] A variety of other toxins can be produced. Among these is *C. perfringens* enterotoxin (CPE). The production of CPE is most commonly associated with type A strains but can occur with all types. The *C. perfringens* strains that produce CPE carry the *cpe* gene which encodes for CPE.[1,2] The two bacterial phenotypes are designated CPE+ and CPE-. Almost all CPE- strains are capable of sporulation.[3] The enterotoxin is

an intestinally active polypeptide and is synthesized during sporulation of *C. perfringens* vegetative cells. CPE accumulates in the cytoplasm of the sporulating cell, and is liberated into the intestinal tract together with the endospores when the vegetative cell lysis.[4]

The transcriptional factors that activate the sporulation genes also up-regulate the *cpe* gene. Concentrations of CPE are therefore maximal during sporulation.[3]

Pathogenesis

Little information exist on the pathogenesis of *C.perfringens* entero-toxicosis in dogs. Most of our knowledge is derived from study of the disease in humans.[2] Humans acquire *C.perfringens* type A food poisoning when they ingest food contaminated with large numbers of vegetative cells of a CPE-producing *C.perfringens* type A strain. Many of those ingested bacteria perish when exposed to the acidity of the stomach, but some survivors pass on to the small intestine, where they multiply and commit to sporulation.[5] After being released from the vegetative cell the CPE binds to a specific receptor on cell membranes, where it forms a complex with membrane proteins after insertion into the plasma membrane. This causes alteration in the membrane permeability and thereby outpouring of water, sodium, and chloride, inhibition of glucose uptake, and decreased energy metabolism. Its action on the intestinal mucosa, mainly in the jejunum and ileum, is rapid, with profuse diarrhea and associated clinical signs appearing in 5-30 min.[2]

C. perfringens usually forms a part of the normal gastrointestinal flora in clinically normal dogs.[6] It has been readily cultured from more than 80%

of diarrheic and nondiarrheic dogs.[7,8] A disruption of the normal microflora of the gastrointestinal tract can lead to an increase in the concentration of *C. perfringens* followed by sporulation and release of enterotoxin by the enterotoxigenic strains. This might be due to dietary changes, stress, antibiotica administration or coinfection with another intestinal pathogen.[1] A study showed that dogs fed high protein diet had an increase in the fecal counts of *C. perfringens* enhancing enterotoxin production by this clostridial species.[9] CPE has been verified in feces from dogs with acute hemorrhagic gastroenteritis syndrome and parvovirus enteritis.[10]

Clinical signs

Most dogs with *C.perfringens* associated diarrhea exhibit signs of large-bowel diarrhea.[1,3,11] Some might have signs of small bowel diarrhea or a diffuse disease (small and large bowel).[7,12] The clinical signs may be acute or chronic.[9,11] In both the acute and chronic phase watery diarrhea, with blood or mucus are commonly observed. [1,2,9,11,13] Other signs are vomiting, tenesmus, anorexia and depression.[1,2,9,11,13] Fever or evidence of systemic involvement is uncommon.[11,13] Acute signs resolve within 3-7 days.[9,11,13] Most cases are self limiting but sometimes they need to be treated.[9] Chronic signs may have persisted for weeks to years in some dogs, with intermittent episodes as frequently as once every several weeks.[11]

Treatment

For the treatment of *C. perfringens* associated diarrhea, ampicillin, erythromycin, metronidazole and tylosin appear to be the most effective antibiotics, although it should be noted that some resistant strains do exist.[14] Dogs with chronic intermittent signs are more difficult to treat. They often respond to short-term antibiotic therapy but relapse following discontinuation of treatment, and require long-term antibiotic therapy. Tylosin has been used for years in the treatment of chronic diarrhea in dogs.[11,15] Some dogs also respond to dietary modification. Increasing the fiber content of the diet is reported to be beneficial. Soluble fiber sources decreases the colonic pH and thus inhibits sporulation or alters the bacterial microflora environment preventing *C.perfringens* proliferation and enterotoxin production.[11,16,17]

Diagnosis

Diagnosis of CPE-associated disease requires more than positive bacterial culture, since *C. perfringens* is commonly isolated from the feces of normal dogs.[2] Isolation of *C.perfringens* with fecal cultures have proven to be unreliable and of low diagnostic value.[3,7,8] Because sporulation is co regulated with enterotoxin production, fecal endospore enumeration has been suggested as a tool for diagnosing enterotoxigenic *C.perfringens* associated disease.[11] Most studies however show a poor association between endospore counts and the presence of CPE in diarrheic dogs.[3,7,8,12] Detection of CPE in fecal specimens is the most widely used diagnostic tool for *C. perfringens* in both human beings and animals. Two commercially

available immunoassays are currently used in veterinary diagnostic laboratories: a reverse passive latex agglutination assay (PET-RPLA; OXOID, Ogdensburg, NY) and an enzyme-linked immunoassay (ELISA, *C.perfringens* Enterotoxin Test; TECHLAB, Blacksburg, VA).[1] According to a human study comparing the two tests, it seems like the ELISA test is more specific than the RPLA, but equally as sensitive. RPLA showed some non-specific reactions, mostly false-positive reactions. The specificity and sensitivity for each of the test is not known.[18] The ELISA takes 3 hours to complete but the RPLA needs an overnight incubation.[3] The use of polymerase chain reaction (PCR) assays for the detection of genes (*cpe* gene) encoding specific toxins of *C. perfringens* has been reported in dogs.[8] Based on the result from a study by Marks et al,[8] the use of ELISA for detection of CPE in feces combined with PCR for detection of enterotoxigenic strains obtained via heat shock provides the strongest evidence for the presence of *C. perfringens* associated diarrhea in dogs.

In dogs with chronic intermittent signs the fecal samples should be tested during a clinical episode because there is usually no evidence of clostridial enterotoxin or spores during asymptomatic periods.[11,17]

The objective of this study was to assess the prevalence of CPE in dogs with acute diarrhea presented to veterinary clinics on the southwestern Iceland. This study also evaluated stained fecal smears for endospores. Based on earlier studies describing sensitivity, specificity, processing time for each test and the cost of the different tests, the ELISA test was chosen to detect CPE in feces from diarrheic dogs.

Materials and methods

In the period from the 1st of september to the 31st of december 2006, fecal samples from 30 diarrheic dogs were collected at 4 veterinary clinics in southwestern Iceland. Samples were collected from dogs with a primary complaint of diarrhea. The signalment, clinical signs, duration of diarrhea and recent antibiotica administration for each dog was noted. Samples were collected by veterinarians or were brought to the clinics by owners. The fecal samples were immediately brought into one container with 2 ml of 0.9% NaCl[12] and another container with 200 μL of diluent (TechLab, Blacksburg, Va). They where then kept refrigerated for 1-7 days until they were processed.

Exclusionscriteria: Dogs with history of a known chronic disease, f.ex. Inflammatory bowel disease (IBD), hepatitis etc.
Dogs with fever were tested for parvovirus and if they tested positive they where excluded. Dogs which had received antibiotics for the last 30 days were also excluded.[12]

Fecal endospore enumeration

One gram of fresh feces was added to 2 ml of sterile saline (0.9% NaCl) and vortexed. A 10 μL aliquot was spread on a 15x15 mm area of a glass slide and allowed to air dry. The smear was then gram-stained. From each dog, 10 random monolayered oil-immersion 100x fields were examined for *C.perfringens* endospores, which look like a safety pin.[12] One or more endospore in each field gave a positive result.(Appendix 1).

Detection of enterotoxin

Fifty μL of liquid fecal specimens or 3 mm of more formed fecal specimens were transferred to 200 μL of diluent (Techlab, Blacksburg, Va) vortexed for 10 seconds and stored between 2°C and 8°C until the ELISA test (TechLab, Blacksburg, Va) was performed according to the manufacturer´s instructions. The ELISA test and the spectrophotometric reading, were performed at Keldur, The Pathological Institute in Reykjavík. Positive test result indicated the presence of *C.perfringens* enterotoxin. Interpretation of results:

1. Visual reading
    - negative = colorless
    - positive = any yellow color

2. Spectrophotometric Single Wavelength at 450 nm.
    - negative = $OD_{450} < 0.120$
    - positive = $OD_{450} \geq 0.120$

The results from 3 of the 4 first dogs were read visually because no yellow color was detected in the wells. After that the spectrophotometer was used on all samples to give quantitative results.

Statistical analysis

The Win Episcope 2.0 program was used to calculate the sample size. Estimating that a total of 10000 dogs living in Iceland and half of them in southwestern Iceland, then we have a total of 5000 dogs. According to Weese et al,[7] diarrhea is a presenting complaint in 2.2% of dogs examined at primary care clinics. That gives us a sample size of 110 dogs with diarrhea. Using the studies from Weese et al,[7] Marks et al,[8] and Kruth et al,[13] as measurements for expected prevalence, expecting 34.5 % prevalence from a population of 110 dogs with diarrhea, with 95% confidence, the sample size should consist of 49 dogs. The sample size in this study was 30 dogs. The prevalence of CPE was calculated using Wilsons procedure.[19]
To evaluate the association between the detection of CPE and endospore counts, the program Measures of association[20] was used.

Results

C. perfringens enterotoxin was detected in feces from 14 of 30 dogs with acute diarrhea.(App.1) According to Wilsons procedure[19] with a continuity correction and 95% confidence interval(CI) this gives a prevalence of 14/30, (46.64%), with 95% CI =[28.8-65.36].

The median age of the dogs in this study were 22.2 months. The median age for the dogs who were CPE positive were 22.07 months.

Eighteen dogs were males and 12 were females. Nine males (50 %) and 5 females (41.66%) were CPE positive.

The odds ratio between CPE and the findings of endospores on smears was OR=1,037 with 95% CI =[0.244-4.411], chi=0.102 and p=0,749. So a

statistically significant association between CPE and the findings of endospores on smears could not be demonstrated.

The clinical signs in the CPE positive dogs ranged from watery diarrhea, bloody diarrhea, diarrhea with mucus, vomiting, tenesmus, but no fever. Intermittent diarrhea was also registered.

The time from sample taking until processing the ELISA test varied from 1 hour up to 4-5 days.

Discussion

The findings in this study are in agreement with Marks et al,[8] Kruth et al,[13] Weese et al,[7] which found the prevalence of CPE in dogs with acute diarrhea to be 34.4%, 41.1% and 28% respectively. The ELISA test was used in all the studies. Marks et al,[3] using a RPLA assay, detected CPE in 26.8% of diarrheic dogs. Cave et al,[12] using both ELISA and RPLA described a prevalence of 14.3% and 45.2% respectively. However, the tests were used on two different groups so no comparison on the assays sensitivity or specificity could be made.[12]

Care must be taken when comparing results from different studies, because different toxin assays and sampling populations may be used. The sampling population in the present study were dogs presented to primary care veterinary clinics. Only the study by Weese et al,[7] used the same sampling population. The others,[3,8,12,13] used hospitalized dogs or dogs referred to referral centers for sampling population. These dogs may not be representative for the population of dogs presented to primary care clinics.[7]

In the animal hospitals there might be enviromental contamination with spores and lots of stressful events leading to the development of diarrhea.[11,13]

The poor association between CPE and endospore counts is consistent with results from other studies.[3,7,8,12] The endospores found in feces from dogs with a CPE negative ELISA test, could have been from sporulating CPE- strains. A CPE positive ELISA test and no endospores, might be due to excretion of endospores through the feces, because the 6 CPE positive and endospore negative dogs, had suffered from diarrhea a few days before the visit to the veterinarian.

Some studies confirm that CPE can be found in feces from normal, asymptomatic control dogs. Weese et al[7] and Kruth et al[13] used the ELISA test, and detected CPE in the feces from 5%, respectively 7%, of normal dogs. Marks et al,[3] used RPLA and found 14 of 53 (26.42%) control dogs with CPE in feces. Cave et al,[12] used both ELISA and RPLA on two different groups of control dogs, and detected CPE in feces from that 12% and 25% respectively. These findings underscore the high incidence of possible false positive results obtained with the RPLA assay.[7] CPE in feces from asymptomatic dogs may also be due to sporulation at a low rate, resulting in concentrations of enterotoxin too low for inducing clinical disease. Some degree of sporulation is constantly occurring regardless of clinical status both in humans and animals.[8]

When comparing the prevalence of CPE in diarrheic dogs and in healthy control dogs, toxigenic strains have been more likely demonstrated in diarrheic specimens compared to normal specimens. This provides further evidence that CPE is associated with diarrhea in dogs.[8]

The present study used the ELISA test for detecting CPE. Most of the studies that have been done on this subject in dogs have used the same

ELISA test. But there are some concerns about the tests sensitivity and specificity. The performance caracteristics for both the ELISA test and the RPLA test has not been analyzed in the dog. The concerns are mostly about the specificity of the RPLA and reports of potential false-positive results.[1] That is the reason for this study not choosing the RPLA test. To date the ELISA tests sensitivity and specificity in the dog have not been validated but nevertheless it seems like the ELISA test is a more reliable choice than the RPLA test.

From the results of this study and other similar studies it seems obvious that CPE plays a part in clostridia-associated diarrhea. Whether CPE is the primary cause of the diarrhea or is released secondarily in response to other conditions affecting the gastrointestinal tract, is still not clear. However, *Clostridium perfringens* and CPE associated diarrhea should be considered as a possible differential diagnosis of diarrhea in dogs, even though the role of these organism needs further clarification.

The weakness of this study may be the lack of healthy control dogs. However, the prevalence of CPE in diarrheic dogs was the goal with this study. The sample size in this study is also smaller than it should be due to short time and also because there were a few dogs that were excluded because they tested positive for parvovirus.

Conclusion

The present study demonstrated that CPE is relatively common in dogs with diarrhea in Iceland. CPE should be considered as a possible differential diagnosis in diarrheic dogs with acute, chronic or intermittent clinical symptoms like watery diarrhea with blood or mucus, vomiting, tenesmus, anorexia and depression but without fever.

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19. Vassars hjemmeside: http://faculty.vassar.edu/lowry/VassarStats.html

20. Microsoft Excel, 2by2.xls, Helle Stege

Appendix 1

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Results

Dog nr.	Age of dog/ Gender	ELISA (nm)	Endospore counts
1	1 year old/ male	Visual reading negative	7/10 fields
2	10 months old/female	0.891 **Positive**	8/10 fields
3	10 years old/male	Visual reading negative	10/10 fields
4	5 months old/female	Visual reading negative	2/10 fields
5	2 years old/female	0.152 **Positive**	Nothing
6	18 months old/ male	0.240 **Positive**	8/10 fields
7	18 months old/male	0.722 **Positive**	9/10 fields
8	3 years old/male	1.006 **Positive**	9/10 fields
9	8 years old/male	0.438 **Positive**	1/10 fields
10	5 months old/female	0.551 **Positive**	Nothing
11	5 years old/male	0.053 Negative	Nothing
12	6 months old/female	0.070 Negative	Nothing
13	2 months old/male	0.478 **Positive**	2/10 fields
14	3 months old/female	0.064 Negative	7/10 fields
15	6 months old/male	0.171 **Positive**	7/10 fields
16	2 months old/female	0.108 Negative	1/10 fields
17	1 year old/female	0.052 Negative	3/10 fields
18	4 months old/male	0.595 **Positive**	5/10 fields
19	4 months old/female	0.225 **Positive**	Nothing
20	2 years old/male	0.089 Negative	Nothing
21	2 years old/male	0.488 **Positive**	Nothing
22	3 months old/male	0.076 Negative	Nothing
23	15 months old/male	0.043 Negative	Nothing
24	30 months old/female	0.042 Negative	5/10
25	1 year old/male	0.084 Negative	Nothing
26	30 months old/ male	0.511 **Positive**	Nothing
27	3 year old/female	0.091 Negative	1/10
28	3 year old/female	0.127 **Positive**	Nothing
29	14 months old/male	0.093 Negative	Nothing
30	4 months old/male	0.076 Negative	10/10