Veterinary Microbiology 195 (2016) 115–122

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Enteropathogen infections in canine puppies: (Co-)occurrence, clinical relevance and risk factors

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ARTICLE INFO

Article history:
Received 4 May 2016
Received in revised form 14 September 2016
Accepted 16 September 2016

Keywords:
Enteropathogens
Dogs
Diarrhoea
Clinical score
Co-infection
Determinants of infection
Breeding
Gastro-enteritis

ABSTRACT

Laboratory confirmation of the causative agent(s) of diarrhoea in puppies may allow for appropriate treatment. The presence of potential pathogens however, does not prove a causal relationship with diarrhoea. The aim of this study was to identify specific enteropathogens in ≤12 month old puppies with and without acute diarrhoea and to assess their associations with clinical signs, putative risk factors and pathogen co-occurrence. Faecal samples from puppies with (n = 113) and without (n = 56) acute diarrhoea were collected and screened for Canine Parvovirus (CPV), Canine Coronavirus (CCoV), *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens*,*Clostridium difficile*, β-hemolytic *Eschericha coli* (hEC), *Giardia* spp., *Toxocara* spp., *Cystoisospora* spp., and *Cyniclomyces guttulatus*. One or more pathogens were detected in 86.5% of diarrhoeic puppies and in 77.8% of asymptomatic puppies. Significant positive associations were found between CPV and CCoV, CPV and *Cystoisospora* spp., *Toxocara* spp. and hEC, *Giardia* spp. and *C. guttulatus*. Only CPV and CCoV were significantly associated with diarrhoea, hEC with a subset of puppies that had diarrhoea and severe clinical signs. CPV was more prevalent in puppies under 3 months of age. Puppies from high-volume dog breeders were significantly at increased risk for CPV (OR 4.20), CCoV (OR 4.50) and *Cystoisospora* spp. (OR 3.60). CCoV occurred significantly more often in winter (OR 3.35), and CPV in winter (OR 3.78) and spring (OR 4.72) as compared to summer.

We conclude that routine screening for CPV, CCoV and hEC is recommended in puppies with acute diarrhoea, especially if they are under 3 months of age and originate from high-volume dog breeders. Routine screening for other pathogens may lead to less conclusive results.

1. Introduction

Canine puppies under one year of age are highly susceptible to gastrointestinal infections. Acute diarrhoea is one of the most common clinical manifestations, potentially leading to severe dehydration and death (Hubbard et al., 2007). Several putative pathogens are associated with acute gastro-enteritis. Canine parvovirus (CPV) and canine coronavirus (CCoV) are considered the most common viral enteric pathogens in dogs worldwide (Decaro et al., 2011; Decaro and Buonavoglia, 2012). *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens*, and β-hemolytic *Escherichia coli* can also be associated with diarrhoea in dogs, but faecal presence of these bacteria in dogs with diarrhoea varies between studies (Marks et al., 2011). *Giardia* spp., *Toxocara* spp. and *Cystoisospora* spp. are parasitic enteropathogens and are often present in puppies, their claimed role as a causative agent however varies per study (Claerebout et al., 2009; Dupont et al., 2013; Grellet et al., 2014). *Clostridium difficile* is associated with severe diarrhoea in humans and animals but little is known about its pathogenicity in canine puppies (Koene et al., 2012; Wetterwik et al., 2013). The role of *Cyniclomyces guttulatus* in causing chronic diarrhoea in dogs was indecisive (Mandigers et al., 2014). Several studies have reported the simultaneous presence of multiple canine enteropathogens in dogs with diarrhoea, but studies targeting specifically puppies are rare, usually target puppies in kennels and/or are limited with respect to the number of

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http://dx.doi.org/10.1016/j.vetmic.2016.09.006

pathogens that was screened for (Cave et al., 2002; Sokolow et al., 2005; Yesilbag et al., 2007; Stavisky et al., 2011; Tupler et al., 2012; Dupont et al., 2013; Gizzi et al., 2014). The clinical relevance of the co-occurrence of multiple pathogens in faecal samples of dogs remains unclear, as this may simply mirror common exposure, but pathogens can also interact with one another in determining or aggravating disease. It is therefore important to screen for multiple pathogens in faeces of both healthy and diarrhoeic puppies to gain more insight into the role co-infections might play. Putative risk factors for acute infectious diarrhoea in puppies include breed, gender, vaccination history, age, season, breeder-origin, and/or stay in kennel (Houston et al., 1996; Stavisky et al., 2011; Ling et al., 2012; Saevik et al., 2012; Dupont et al., 2013; Bagshaw et al., 2014; Grellet et al., 2014). However these risk factors for infection with specific enteropathogens have mainly been reported in dogs housed in shelters or breeding facilities. The importance of some of these risk factors might be different for privately owned puppies kept under different living conditions.

The aim of this study was to assess possible associations between clinical signs and the presence of potential enteropathogens in canine puppies as well as the association between previously published risk factors and the occurrence of these pathogens. Pairwise co-occurrence of different enteropathogens in these puppies was also investigated.

2. Materials & methods

2.1. Study design and sample collection

From September 2009 to September 2011 60 small animal- or combined small and large animal veterinary practices in the Netherlands were invited to participate in this study, either via direct contact or via general announcements on symposia and via the internet. They were asked to submit faecal samples from puppies ≤1 year of age with acute diarrhoea (lasting ≤10 days). Diarrhoea was defined as stools that were too loose, and/or expelled too frequently. For each diarrhoeic puppy, the veterinarian was also requested to submit a faecal sample from another puppy under the age of 1 year, without diarrhoea in the three weeks preceding sampling. The samples were either taken directly from the rectum or from already expelled faeces. In case of spontaneous defecation, only the part that did not have contact with the environment was sampled. The samples were taken either by the veterinarian or by the owner, a training leaflet was provided with instructions for proper sampling. All owners were informed and consented with their dogs participating in this study. Sampling was performed by convenience. A maximum of 8 cases and 8 controls were collected per practice. Practices that did not return samples were reminded by e-mail once and they were considered non-participants if they did not reply since. In case of multiple puppies under lactation, a spontaneous sample counted as one dog, as the origin of the faecal sample could not be traced individually.

Each faecal sample was collected in two 15 ml containers (Sarstedt 80.734) and in an amies-charcoal medium swab (Sarstedt 80.1362) to be transported to the laboratory, either by post or by courier. The veterinarian was also asked to complete a questionnaire about the age, breed, gender, breeding circumstances, clinical signs, vaccine history and faecal characteristics of the sampled puppy.

The laboratory results were reported to the practitioner. All practices were contacted by telephone after participation, to check whether the participating puppies were still alive or had deceased.

2.2. Sample processing

Upon arrival at the Utrecht University Veterinary Microbiologic Diagnostic Centre (VMDC), the faecal samples were directly processed for routine bacterial culture and examined for the presence of parasite eggs and (oo)cysts via faecal flotation. A part of the sample was diluted 1:5 with Dulbecco's Phosphate Buffered Saline w/o Ca2+ Mg2+ (Lonza BE17-512F), centrifuged and the supernatant was stored at −80 °C for subsequent qPCR analysis for detection of CPV and CCoV. The remaining faecal sample was stored at −20 °C for later culturing of *C. difficile*. If the amount of faeces was too small to perform all analyses priority was given to the viral analyses, and subsequently the bacterial and parasitological analyses were performed. *C. difficile* testing had the lowest priority.

2.3. Bacterial culture

2.3.1. Escherichia coli

The classic three-way streak plate method was used for isolation and identification of discrete colonies in a mixed bacterial faecal flora (Cappuccino and Sherman, 2001). Each sample was inoculated in two sheep blood agar plates (Biotrading, Mijdrecht, the Netherlands K004P090KP) and MacConkey agar plates (Biotrading K039P090KP) and incubated both in aerobic and anaerobic (the blood agar plates) conditions for 24 h at 37 °C. If the culture resulted in multiple oxidase-negative and lactose-positive β-hemolytic colonies that were present in the third streak these colonies were tested biochemically (TSI (triple sugar iron)/ureum/ODC(ornithine de-carboxylase/trypton)) to confirm *E. coli* presence. Only β-hemolysin producing *E. coli* that were present in abundance in a mixed faecal culture was considered relevant in this study.

2.3.2. Salmonella *spp.*

Samples were directly cultured on *Salmonella* selective BGA (brilliant green agar) plates (biotrading K008P090KP), and 1 g of faeces was placed into 10 ml of selenite enrichment broth (biotrading K052F300HL) for 24 h to enhance detection of *Salmonella* spp. The BGA plates and broth were aerobically incubated for 24 h at 37 °C. Typical red colonies were confirmed using TSI, ureum, LDC (lysine de-carboxylase)/trypton and agglutinating sera (group level). When the directly inoculated BGA plates were negative, the selenite broth was inoculated on BGA plates and the above described procedure was repeated. Positive samples were sent to the National Reference Laboratory for Salmonella at the Dutch National Institute for Public Health and the Environment (RIVM) for serotyping according to the Kauffmann-White scheme.

2.3.3. Campylobacter *spp.*

Detection of *Campylobacter* spp. was performed by culture from the Amies-charcoal swab on *Campylobacter* selective plates (CCDA (charcoal cefoperazone deoxycholate agar) CM0739 Oxoid) as described by Koene et al. (Koene et al., 2009). The CCDA plates were incubated both at 37 °C and at 42 °C for 48 h under micro-aerobic conditions. Suspected clear small oxidase-positive colonies were confirmed using Gram staining and stored in −80° C for later confirmation. Bruker's MALDI TOF MS system, following the manufacturers procedure, was used to confirm positive colonies. Suspected colonies that could not be confirmed using MALDI TOF were confirmed using a *Campylobacter* genus specific real-time PCR as described by de Boer et al. (Boer de et al., 2013).

2.3.4. C. perfringens

The classic three-way streak plate method was used for isolation and identification of discrete colonies as described in the procedure for isolation of *E. coli* (Cappuccino and Sherman, 2001) The anaerobic plates were evaluated for presence of multiple characteristic colonies with double hemolysis suggestive of *C. perfringens* on strict anaerobic growth. These colonies were subject to gram staining and evaluated for presence of typical subterminal spores and further confirmed using the reverse CAMP reaction with *Streptococcus agalactiae*.

2.3.5. C. difficile

Samples were analyzed in batches for the presence of *C. difficile* using the method described by Koene et al. (Koene et al., 2012). In short, samples were 1:1 diluted with saline solution and subjected to heat-treatment (Julabo SW 22) at 60 °C for 60 min. After enrichment with BHI (brain heart infusion) broth (Oxoid CM1135) *C. difficile* selective supplement (Oxoid SR0096) and sodium taurocholate hydrate 97% (Alfa Aesar, A18346) was added to the samples which were then incubated anaerobically for 7 days. Subsequently the samples were inoculated on two different *C. difficile* selective agar plates: *C. difficile* selective medium (CDSM, Oxoid PB5054A) and Brazier's *C. difficile* selective medium (Brazier, Oxoid PB5191A). Plates were evaluated daily for 4 days and *C. difficile* like colonies were confirmed via Gram staining and inoculated on a blood agar plate. A glu-D PCR as described in Koene et al. (2012) with minor alterations was performed in order to confirm *C. difficile* (Koene et al., 2012). DNA was isolated by a boiling method rather than with a purification kit. The PCR mix used for this PCR reaction comprised 25 μl Hot Start Green Master Mix (including loading buffer), 0.5 μl Glu-D primer forward, 0.5 μl Glu-D primer reverse, 19 μl Nuclease-Free water and 5 μl isolated DNA, making a total volume of 50 μl, using primers as described in Koene et al. (2012). Promega (M5122) was used instead of Qiagen. The PCR program was adjusted to this master mix by shortening the first heating step of the protocol from 12 to 2 min at 94 °C (Koene et al., 2012).

2.3.6. Faecal flotation

Faecal samples were examined for the presence of helminth eggs and/or protozoan (oo)cysts and typical "spectacle case shaped" *C. guttulatus* yeast cells using the Centrifugal Sedimentation Flotation (CSF) method with saturated Zinc Sulphate (ZnSO4) as a flotation solution (sg.1.34 g/cm2) (Houwers and Blankenstein, 2001). The slides were evaluated at 100× magnification and identification was performed at 400× magnification.

2.3.7. qPCR for CCoV

RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). A TaqMan RT-PCR (lightCycler Roche 480) for the detection of CCoV was designed according to Decaro et al. (2004) using FeLV strain FL74 as an internal RNA control which was added to the sample before the RNA extraction (Decaro et al., 2004). The primers and probes used to detect CCoV (Invitrogen/Applied Biosystems) were as described by Decaro et al. (2004). The primers and probes used to detect FeLV (Invitrogen/Aplied Biosystems) were those described by Tandon et al. (2005). A Ct value of 39 was used as cut-off; >39 was considered negative.

2.3.8. qPCR for CPV

DNA was isolated using a boiling method as described by Decaro et al. (2005b). A TaqMan RT-PCR (lightCycler Roche 480) for the detection of CPV was designed according to Decaro et al. (2005b) using FHV as an internal DNA control which was added after the boiling method. The primers and probes used to detect CPV (Invitrogen and TIP Molbiol) were as described by Decaro et al. (2005b). The primers and probes used to detect the internal FHV control were as described in Helps et al. (2003).

2.3.9. Data analysis

Clinical signs were recorded via a questionnaire and scored in a weighed clinical score system (Table 1). In case more than 1 faecal consistency characteristic was mentioned in the questionnaire, the most severe characteristic was included in the clinical score system. The added scores resulted in 3 categories that were defined as follows: a total score of 1–3 resulted in category 1 (mild clinical signs), a total score of 4–6 resulted in category 2 (moderate clinical signs) and a total score of 7–9 resulted in category 3 (severe clinical signs). For each pathogen the association with single clinical signs and with the three clinical score categories was explored. Categorical variables were examined using Chi-square test or Fisher's exact test, as appropriate. For the continuous variable puppy's age, the non-parametric Mann-Whitney's or Kruskal-Wallis' tests were used, as this variable was not normally distributed (Kolmogorov-Smirnov's test, $p < 0.05$). Logistic regression was used to identify possible bivariate associations between different pathogens in the same samples, while adjusting for puppy's age.

Logistic regression was also used to sort out which pathogens were associated with acute diarrhoea and/or severe clinical signs. For these pathogens logistic regression was used to assess the association with previously published risk factors for gastro-intestinal infections in dogs, namely age, breed (purebred vs. mixed breed), gender, vaccination history (fully vaccinated vs. partially or not vaccinated), sampling season (Summer: June–August; Autumn: September–November; Winter: December–February; Spring: March–May), and whether the puppy originated from a high-volume dog breeder (Houston et al., 1996; Stavisky et al., 2011; Ling et al., 2012; Saevik et al., 2012; Dupont et al., 2013; Bagshaw et al., 2014; Grellet et al., 2014). Age was categorized in 2 groups: younger than 3 months and older than 3 months, as 3 months is the age at which maternal immunity is expected to have diminished to an insignificant level for the majority of puppies and at which the final puppy vaccines generally are administered in the Netherlands (Davis-Wurzler, 2014).

Statistical analysis of the risk factors was performed as described in Nijsse et al. (Nijsse et al., 2015). When appropriate,

Table 1
Weighed clinical score system used to semi-quantify severity of clinical disease in puppies with diarrhoea.

Clinical signs	Numerical score			
	0	1	2	3
Health status	Normal	Clinically ill	Hospital admission	N/A
Appetite	Normal	Decreased	N/A	N/A
Vomiting	No	Yes	N/A	N/A
Temperature	38.0–39.0 °C	39.0–40.0 °C	>40.0 °C	N/A
Faecal characteristics	Normal	Mushy	Watery without blood	Bloody diarrhoea

Score 1–3: category 1 = mild clinical signs, score 4–6: category 2 = moderate clinical signs, score 7–9: category 3 = severe clinical signs.
N/A = not applicable.

associations were expressed as odds ratios (OR), providing 95% confidence intervals (95%CI). The statistical software SPSS-22 was used and statistical significance was set to $p < 0.05$. Clustering of observations at the veterinary practice level was assessed using the Likelihood Ratio(LR) test.

3. Results

Sixty veterinary practices distributed throughout the Netherlands signed up for this study and received sampling materials and information; 49 of these practices submitted samples. In total 195 samples and related questionnaires were collected; 169 (86.6%) of these samples met the inclusion criteria, whereas 26 were discarded because the puppies had diarrhoea for more than 10 days. Of the 169 enrolled samples 113 (66.8%) originated from puppies with acute diarrhoea (cases) and 56 from asymptomatic puppies (controls). There was no evidence of clustering in the data (i.e. lack of independence of observations) at the veterinary practice level for any of the outcomes studied (LR test, $p > 0.05$). Median age of the puppies was 91 days (range: 29–333 days). Median age of the cases was 88 days (range: 30–314 days) and that of the controls was 99 days (range: 29–304 days). Of all enrolled puppies 53.3% were male, 42.6% were female, and 4.1% were undefined (litter samples). Cases and controls did not differ significantly from one another regarding age ($p = 0.330$) and gender ($p = 0.710$).

Pathogens were analyzed for their association with diarrhoea; results are presented in Table 2. Diarrhoea in puppies was shown to be significantly associated only with CPV (OR 3.03, 95%CI 1.09–8.41, $p = 0.027$) and CCoV (OR 3.80, 95%CI 1.67–8.99, $p = 0.001$) infection. Association between the presence of agents and the occurrence of diarrhoea may depend on the severity of the disease, and co-occurrence of pathogens might influence the clinical outcome. The co-occurrence of pathogens and the association between pathogens and specific clinical signs was therefore further analyzed.

3.1. Co-infection with multiple pathogens

A total of 96 and 45 samples from cases and controls respectively, were tested for the full panel of pathogens and were therefore used to compare the prevalence of multiple pathogens between cases and controls. In 13 (13.5%) diarrhoeic puppies no pathogen could be detected in the faeces, whereas most of the diarrhoeic puppies were infected with 1, 2, 3 or 4 pathogens (respectively 29.2%, 27.1%, 16.7% and 10.4%). In 3 puppies 5 pathogens were detected (3.1%). Of the asymptomatic puppies 22.2% did not test positive for any of the pathogens, and most of the puppies were colonized with 1, 2 or 3 pathogens (respectively 40.0%, 24.4%, and 13.4%). In none of the asymptomatic puppies more than 3 pathogens were found.

When combined in four groups (bacterial pathogens, viral pathogens, parasitic pathogens and yeast infection), only the presence of ≥ 1 viral pathogens was significantly associated (OR 3.10, 95%CI 1.49–6.42, $p = 0.020$) with the occurrence of diarrhoea, whereas bacteria, yeast or parasites were not detected significantly more often in diarrhoeic puppies (respectively OR 1.23, 95%CI 0.61–2.48, $p = 0.568$; OR 1.60, 95%CI 0.49–5.24, $p = 0.430$; OR 1.46, 95%CI 0.68–3.12, $p = 0.356$). *Salmonella* was left out from further analyses because there was only one positive sample, which was shown to be *Salmonella* Typhimurium.

Taking into account all faecal samples, significant positive associations were found between CCoV and CPV (OR 3.00, 95%CI 1.30–7.00, $p = 0.013$), *Toxocara* spp. and hEC (OR 18.40, 95%CI 3.20–105.90, $p = 0.001$) *and Giardia* spp. and *C. guttulatus* (OR 4.50, 95%CI 1.20–16.70, $p = 0.023$), correcting for puppies age. A borderline significant association was found between CPV and *Cystoisospora* spp. (OR 2.60, 95% CI 0.98–6.80 $p = 0.054$). In diarrhoeic puppies significant positive associations were found for CPV and CCoV (OR 3.40 95% CI 1.30–9.00 $p = 0.013$), *Toxocara* spp. and hEC (OR 13.10, 95% CI 1.60–105.90 $p = 0.016$) and CPV and *Cystoisospora* spp. (OR 3.50 95% CI 1.20–10.50, $p = 0.025$). The association between *Giardia* spp. and C. *guttulatus* (OR 4.20 95% CI 0.90–19.30 $p = 0.065$) was borderline significant in the diarrhoeic puppies.

3.2. Pathogens and clinical signs

In 40.0% of the diarrhoeic puppies bloody diarrhoea was reported, combined with either watery faeces (21.8%) or mushy faeces (18.2%). Soft or mushy faeces was found in 59.0% of the puppies, 62.0% had watery faeces and 26.4% showed both mushy and watery faeces. When more than one faecal consistency characteristic was reported, the most severe characteristic was used in de clinical score system. A combination of vomiting and diarrhoea was found in 51.0% of puppies, 51.0% had diarrhoea combined with general depression, while 56.0% of diarrhoeic puppies had reduced appetite and 16.0% presented with fever (>39 °C). In total 19.0% of diarrhoeic puppies were hospitalized for intensive treatment.

Applying the clinical score system as presented in Table 1 to grade the severity of clinical signs in 3 categories, the association between carriage of pathogens (not corrected for co-occurrence) and the severity of disease was analyzed and reported in Table 3.

Significant differences between clinical score categories mild and severe were found for CPV ($p = 0.000$), CCoV ($p = 0.025$) and hEC ($p = 0.021$) (Table 3). For none of the other pathogens a significant association was found.

To further investigate these associations for those three pathogens, carriage in severely diseased puppies only (category 3 animals) was compared to the asymptomatic controls, thereby excluding puppies with mild or moderate signs. The occurrence of

Table 2
Prevalence of pathogens in diarrhoeic and asymptomatic puppies.

Pathogen	Prevalence in asymptomatic puppies	Prevalence in diarrhoeic puppies	Total prevalence	95% CI	OR (95% CI)	P-value
Salmonella	0% (0/56)	0.9% (1/112)	0.6% (1/168)	0.5–1.7%	0.66(0.60–0.74)	0.481
Campylobacter	37.5% (21/56)	41.1% (46/112)	39.9% (67/168)	32.5–47.3%	1.16(0.60–2.25)	0.656
C. perfringens	10.7% (6/56)	18.8% (21/112)	16.1% (27/168)	10.5–21.7%	1.92(0.73–5.10)	0.183
C. difficile	17.0% (8/47)	15.4% (16/104)	15.9% (24/151)	10.1–21.7%	0.89(0.35–2.24)	0.799
hEC	1.8% (1/56)	7.4% (8/108)	5.5% (9/164)	2.0–9.0%	4.40(0.54–36.10)	0.117
Toxocara	5.7% (3/53)	4.6% (5/109)	4.8% (8/165)	1.5–8.1%	0.85(0.20–3.69)	0.829
Cystoisospora	10.7% (6/56)	18.3% (20/109)	15.8% (26/165)	10.2–21.4%	1.87(0.71–4.97)	0.205
Giardia	7.1% (4/56)	9.2% (10/109)	8.5% (14/165)	4.2–12.8%	1.30(0.39–4.39)	0.660
Cyniclomyces	7.1% (4/56)	11.0% (12/109)	9.7% (16/165)	5.1–14.2%	1.60(0.49–5.24)	0.430
CPV	10.2% (5/49)	23.6% (26/110)	18.9% (31/164)	12.9–24.9%	3.03(1.09–8.41)	**0.027**
CCoV	14.8% (8/54)	40.2% (43/107)	31.7% (51/161)	24.5–38.9%	3.80(1.67–8.99)	**0.001**

The bold values denote statistically significant p values ($P < 0.05$).

Table 3
Association of pathogens with severity of disease using weighed clinical score system.

Pathogen	Clinical signs score categories				
	Cat 1: mild	*Cat 2: moderate*	*Cat 3: severe*	n	P-value
Campylobacter	43.9% (18/41)	40.0% (16/40)	38.1% (8/21)	102	0.900
C. perfringens	26.8% (11/41)	10.0% (4/40)	19.0% (4/21)	102	0.149
C. difficile	15.8% (6/38)	13.2% (5/38)	26.3% (5/19)	95	0.516
hEC	2.5% (1/40)[a]	2.6% (1/39)[a]	20.0% (4/20)[b]	99	**0.021**
Toxocara	2.4% (1/41)	2.6% (1/39)	5.3% (1/19)	99	0.796
Cystoisospora	22.0% (9/41)	10.3% (4/39)	26.3% (5/19)	99	0.225
Giardia	17.1% (7/41)	5.1% (2/39)	0.0% (0/19)	99	0.079
Cyniclomyces	14.6% (6/41)	10.3% (4/39)	5.3% (1/19)	99	0.708
CPV	14.6% (6/41)[a]	13.2% (5/38)[a]	61.9%(13/21)[b]	100	**0.000**
CCoV	22.5% (9/40)[a]	39.5% (15/38)[ab]	57.1% (12/21)[b]	99	**0.025**

P-value = overall p-value for the differences among the groups (chi-square test).
[a,b]Each superscript letter denotes a subset of clinical score categories in whose column proportions do not differ significantly from each other at the 0.05 level.
The bold values denote statistically significant p values ($P < 0.05$).

CPV (p = 0.000), CCoV (p = 0.000) and hEC (p = 0.0162) was significantly associated with severe clinical signs compared to the asymptomatic (control) puppies. For none of the other pathogens a significant association was found with severe clinical signs compared to the asymptomatic control puppies.

Associations with specific clinical signs were also investigated for these three pathogens.

CPV was significantly associated with bloody diarrhoea (OR 2.59 95%CI 1.06-6.34, p = 0.030), vomiting (OR 3.09 95% CI 1.21–7.90, p = 0.020), general malaise (OR 5.77 95% CI 1.97–16.86, p = 0.001), decreased appetite (OR 12.90 95% CI 2.86–58.13, p = 0.000) and hospital admission (OR 8.04 95% CI 2.78–23.22, p = 0.000).

CCoV was significantly associated with vomiting (OR 2.67 95%CI 1.19–5.96, p = 0.016), decreased appetite (OR 3.40 95% CI 1.43–8.06, p = 0.004) and hospitalization (OR 5.56 95%CI 1.93–16.07, p = 0.001).

hEC was significantly associated with hospitalization (OR 7.00 95%CI 1.43–34.31, p = 0.020), and borderline significantly associated with bloody diarrhoea (OR 4.92 95%CI 0.95–25.61, p = 0.060), vomiting (OR 6.77 95%CI 0.79–58.27, p = 0.060) and general malaise (OR 7.4495%CI 0.88–62.71, p = 0.060).

Follow-up data regarding survival was gathered in 156 puppies: 2 non-diarrhoeic and 11 diarrhoeic puppies died (cause unknown). No significant differences (p = 0.220) were found between the cases and controls regarding survival. Both CPV and CCoV were significantly associated with death (respectively OR 8.00 95%CI 2.40–26.66, p = 0.001 and OR 3.98 95%CI 1.23–12.92, p = 0.020).

Pathogen interaction in aggravating disease was explored for CPV, CCoV and hEC.

In total 43 diarrhoeic animals were CCoV positive, however not all faecal samples had a complete report of clinical signs therefore only 36 CCoV positive puppies could be categorized using the clinical score system. Fourteen (38.9%) CCoV positive puppies were also infected with CPV. Eleven of the puppies with a CCoV-CPV co-infection were severely diseased, 2 were moderately ill, and 1was mildly ill. There was a significant association between CCoV-CPV co-infection and severe clinical signs (p = 0.014).

Of the CCoV-positive but CPV- negative animals (n = 22, 61.1%) only one animal was severely ill, 13 were moderately ill, and 8 were mildly diseased. In these puppies the earlier significant association between CCoV infection and severe clinical signs disappears (p = 0.217).

Out of 24 CPV- positive puppies that were included in the clinical score system, 14 were double infected with CCoV (58.3%) and 10 had single infections (41.7%). Five of the single infected puppies were mildly ill, 3 were moderately ill, and 2 were severely ill. In these puppies the earlier significant association between CPV infection and severe clinical signs disappears (p = 0.802).

Of the 9 hEC positive samples, 8 originated from diarrhoeic puppies. Complete clinical data were available for 6 of these diarrhoeic puppies. Four hEC-positive puppies were CPV negative, and 2 animals showed co-infections with CPV. These two double

Table 4
Determinants of infection for 3 enteropathogens in canine puppies.

All puppies					
	Pathogen	Exposed pathogen positive puppies	Not exposed pathogen positive puppies	OR (95% CI)[a,b]	P-value
High-volume breeder					
	CPV	12/31	13/99	4.20 (1.70–10.60)	**P = 0.003**
	CCoV	18/29	26/98	4.50 (1.90–10.90)	**P = 0.001**
	hEC	3/32	5/101	1.90 (0.42–8.40)	P = 0.410
Season[c]					
Winter	CPV	12/48	19/116	3.78 (0.98–14.56)	**P = 0.054**
Autumn	CPV	6/45	25/119	1.74 (0.41–7.51)	P = 0.456
Spring	CPV	10/34	21/130	4.72 (1.17–19.00)	**P = 0.029**
Summer	CPV	3/37	28/127	nc.	nc.
Winter	CCoV	23/47	28/114	3.35 (1.30–8.90)	**P = 0.015**
Autumn	CCoV	14/44	37/117	1.63 (0.60–4.48)	P = 0.341
Spring	CCoV	6/34	55/127	0.75 (0.23–2.44)	P = 0.633
Summer	CCoV	8/36	53/125	nc	nc
Winter	hEC	2/49	7/115	1.53 (0.13–17.57)	P = 0.732
Autumn	hEC	3/45	6/119	2.57 (0.26–25.82)	P = 0.422
Spring	hEC	3/33	6/131	3.60 (0.36–36.43)	P = 0.278
Summer	hEC	1/36	8/128	nc.	nc.
Age (<3 months)					
	CPV	26/82	5/82	0.14 (0.05–0.39)	**P = 0.000**
	CCoV	30/82	21/79	0.64 (0.32–1.23)	P = 0.174
	hEC	7/86	2/78	0.30 (0.60–1.48)	P = 0.138

nc = not calculable.
The bold values denote statistically significant p values ($P < 0.05$).
[a] Adjusted for age, except for determinant age.
[b] Binary log regression; constant included in the model.
[c] summer was used as reference category.

infected puppies were also double infected with CCoV and showed severe clinical signs. The 4 hEC-positive and CPV-negative samples were also CCoV-negative. Of these puppies 1 showed mild clinical signs, 1 showed moderate signs, and 2 showed severe signs.

3.3. Risk factors

For CPV, CCoV and hEC, the pathogens associated with (severe) diarrhoea, a risk factor analysis was performed. Significant risk factors for infection with CPV, CCoV, and hEC are presented in Table 4. Not all questionnaires included full data about the risk factors, therefore n varies per risk factor.

Using summer as the reference category, puppies were significantly more likely to be infected with CPV in spring. Puppies were borderline significantly more likely to be CPV positive in winter. Puppies were also significantly more likely to be infected with CCoV in winter than in summer. No seasonal effects were found for hEC. CPV was significantly more likely to be detected in puppies of $\leq$3 months. For hEC and CCoV no significant age effect was found. Puppies originating from a high-volume breeder were significantly more likely to be infected with CPV and CCoV.

Both case and control puppies were equally distributed among the seasons ($p = 0.950$), and there was no significant difference in the mean age of puppies between the seasons ($p = 3.93$).

Breed and vaccination history, despite being previously described risk factors for acute diarrhoea, were not analyzed here since the puppies in this study were mainly purebred and most animals were partially or fully vaccinated making the mixed breed group and unvaccinated group too small for comparison in the analysis. For none of the pathogens gender was a significant determinant of infection.

4. Discussion

Infectious causes of diarrhoea can have devastating effects on canine health and can even cause death. Since young animals are most vulnerable to gastrointestinal infections we performed a case-control study in a population of dogs of one year or younger. Information about the association of potential enteropathogens with clinical signs, the co-occurrence of enteropathogens, and putative risk factors for enteropathogens can aid the veterinary practitioner to improve treatment and prevention. To our best knowledge this study with 11 putative enteropathogens reports the highest infection rates not only in diarrhoeic puppies (86.5%) but also in asymptomatic puppies (77.8%). Gizzi et al. (2014) studied dogs of all ages and report enteropathogens in 68.3% of diarrhoeic dogs and 30.2% of control dogs (Gizzi et al., 2014). Grellet et al. (2014) report enteropathogens in 77.1% of puppies regardless of clinical signs (Grellet et al., 2014). However the studies are difficult to compare due to differences in study design.

We found that CPV, CCoV and β-hemolytic *Eschericha coli* (hEC), were pathogens associated with (severe) clinical signs in canine puppies, whereas infection with *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens, Clostridium difficile, Giardia* spp., *Toxocara* spp., *Cystoisospora* spp., and *Cyniclomyces guttulatus* was not significantly associated with acute diarrhoea.

CPV is a serious threat to dogs (Schulz et al., 2008; Decaro et al., 2009; Decaro et al., 2011; Decaro and Buonavoglia, 2012). CPV prevalence varies between studies, depending on the inclusion criteria for participation. The prevalence of CPV found in our study (18.9%) fits within the range of other prevalences previously published (respectively 16% and 48.7%) (Schulz et al., 2008; Decaro et al., 2011).The most distinctive clinical signs associated with CPV observed here match the typical signs described in the literature. Bloody diarrhoea was reported in 40.0% of the puppies and one third of these puppies were CPV-positive. The occurrence of CPV infections in asymptomatic puppies can be explained by low-grade subclinical infection with field strains that can occur in puppies with a partial protective immune response, either via maternal immunity or acquired immunity, or shedding of vaccine virus in recently vaccinated puppies (Decaro et al., 2005a; Decaro et al., 2014; Grellet et al., 2014). The 31.7% prevalence of CCoV infections in this study was in line with that of earlier studies (Decaro et al., 2011; Stavisky et al., 2012). Genetic variability within canine enteric coronaviruses is due to naturally occurring recombination and mutation. Genotype CCoV-I and CCoV-II (with the subtypes CCoV-IIa and CCoV-IIb) have all been detected in dogs with diarrhoea (Stavisky et al., 2011; Decaro et al., 2013; Licitra et al., 2014). In our study no further genetic characterization was conducted. CCoV infection was associated significantly with severe clinical signs. Most of these puppies were however co-infected with CPV. Puppies that were co-infected with CCoV and CPV showed severe clinical signs, which was in concordance with previous reports of frequently occurring co-infections with these pathogens (Decaro et al., 2011; Castro et al., 2013). CPV and CCoV synergize in their pathogenicity by interacting at the gut wall level (Zicola et al., 2012). Also CPV induces suppression of the immune system which facilitates superinfection with other pathogens (Castro et al., 2013). CPV single infections have been shown to cause severe clinical signs (Decaro and Buonavoglia, 2012). Novel antigenic CPV variants (CPV-2a,CPV-2b,CPV-2c) are increasingly spread across the continent and have all been found in dogs with gastro-intestinal signs (Decaro and Buonavoglia, 2012). In this study antigenic typing of CPV was not conducted. In our study no association with severe clinical signs was found. However, since most of the CPV infected pups in our study were co-infected with CCoV, only a limited number of CPV single infected pups ($n = 10$) remained for statistical analysis. Shedding of CCoV in clinically healthy puppies was expected, this can be explained by prolonged shedding of this virus after previous subclinical or clinical infections (Pratelli et al., 2002). Although the presence of hEC was not significantly associated with diarrhoea, it was associated with severe clinical signs, regardless of coinfection with other pathogens. *E. coli* is part of the normal gut microbiome in dogs, making it difficult to differentiate between pathogenic and non-pathogenic strains based on culture identification alone. In our study a classic virulence factor: the production of β-hemolysin, was used to detect virulent *E. coli* strains. β-hemolysis is a phenotypic indicator that can easily be screened for in routine bacterial culture. Starčič et al. (2002) found that hemolysin production in *E. coli* strains from dogs with diarrhoea was frequently combined with cytonecrotising toxin 1 and P fimbriae production. This was suggested to account for the pathogenicity of these types of strains (Starčič et al., 2002). Using β-hemolysis as a criterium for pathogenicity, the significant association of hEC with severe clinical signs provides clues for the possible role of this bacterium in causing acute diarrhoea in puppies. Additional characterization of these isolates towards toxigenic, adhesive, and necrotizing properties was not performed. The presence of *Salmonella* spp. in puppies was rare, as was expected (Stavisky et al., 2011; Marks et al., 2011; Dupont et al., 2013). Although clinical relevance could not be established, the zoonotic potential of this bacterium justifies monitoring for this pathogen. *Campylobacter* spp. is a common enteropathogen in humans, and is found in dogs as well. The 39.9% *Campylobacter* spp. prevalence found here is rather high compared to those determined in other studies and is, considering the zoonotic potential of this pathogen, worth further investigation (Parsons et al., 2010; Marks et al., 2011; Stavisky et al., 2011). The faecal shedding of *C. difficile* also appears to be high in puppies and is certainly much higher than that reported in earlier studies, but was not associated with acute diarrhoea (Koene et al., 2012; Wetterwik et al., 2013). The samples

were not investigated for toxin presence given the low sensitivity of the assay (Chouicha and Marks, 2006). Isolates were tested for the presence of toxin A and B encoding genes and one isolate in the control group and one isolate in the diarrhoeic group was positive for toxin A and B encoding genes (data not shown). *C. perfringens* is part of the normal gut flora, therefore only shedding of large quantities of *C. perfringens* was considered positive in this study. In our study no significant association between the presence of *C. perfringens* and acute diarrhoea could be established. Regular deworming of puppies may have accounted for the relatively low prevalence and non-significant clinical impact of helminth infections and, depending on the product used, G*iardia* spp. or *Cystoisospora* spp. infections. The presence of *Cystoisospora* spp. in our puppies was not associated with diarrhoea, which was in concordance with the study of Dupont et al. (Dupont et al., 2013). *Cyniclomyces guttulatus* is prevalent in canine faeces, and its role as an opportunistic pathogen associated with chronic or reoccurring episodic diarrhoea has been described earlier (Mandigers et al., 2014). As no information about the role of this yeast in causing acute diarrhoea exists, this pathogen was incorporated in this study. In dogs with chronic diarrhoea a prevalence of 14% was reported earlier (Houwers and Blankenstein, 2001). A similar prevalence was found in our study but faecal presence of this yeast was not significantly associated with acute diarrhoea in puppies. In only 13.5% of the diarrheic puppies and 22.2% of asymptomatic puppies none of the pathogens searched for could be detected. Other causes for diarrhoea in these puppies could range from non-infectious causes (e.g. food hypersensitivity, food allergy, toxin ingestion etc.) to pathogens not included in our study (amongst others Canine Adenovirus, Norovirus, Astrovirus, Canine Distemper Virus, *Brachyspira* spp. *Cryptosporidium* spp.).

Co-occurrence of pathogens was expected in the puppies. Shedding of multiple pathogens was found in 57.3% of the diarrhoeic puppies and in 37.7% of the asymptomatic puppies. Mixed infections with several enteropathogens in puppies were reported earlier (Dupont et al., 2013; Grellet et al., 2014; Gizzi et al., 2014). However, differences in study populations, selected pathogens and detection methods can account for differences with our results. We have screened our samples with techniques that are commonly used as routine procedures in diagnostic laboratories.

Whereas our study used a population of client-owned puppies <1 year of age, Dupont et al. (2013) and Grellet et al. (2014) included puppies with gastrointestinal signs of unknown duration housed mainly in groups, which were thus more prone to dog-to-dog transmission, while Gizzi et al. (2014) included dogs of all ages suffering either from acute or chronic diarrhoea gathered from only one veterinary practice (Dupont et al., 2013; Gizzi et al., 2014; Grellet et al., 2014). In contrast to results of previous studies we found co-infections with multiple pathogens not only in the diseased but also in the healthy puppies emphasizing the need for developing a plan for first and second priority testing in infectious canine diarrhoea. Besides CPV and CCoV, hEC and *Toxocara* spp., CPV and *Cystoisospora* spp., and *Giardia* spp. and *C. guttulatus* were significantly more often found together. Puppies are considered more susceptible to *Toxocara* spp., *Giardia* spp. and *Cystoisospora* spp. compared to adult dogs. The co-occurrences found here are likely to reflect common risk exposures for the pathogens in question (Claerebout et al., 2009; Dupont et al., 2013).

High-volume dog breeders produce puppies of a variety of different breeds in large numbers each year that can be bred under hygienically poor and overcrowded conditions. The high volume dog breeders in this study were denoted by the participating clinicians for having poor hygienic conditions and overcrowding. In our study high-volume breeders are linked to a higher risk for CPV and CCoV further supporting the association between these pathogens and breeder-size found by Grellet et al. and Licitra et al. (Grellet et al., 2014; Licitra et al., 2014). Moreover puppies originating from high-volume breeders were significantly (OR 3.60, $p = 0.014$) more likely to carry *Cystoisospora* spp. especially along with CPV as mentioned before. Other studies have also found a higher prevalence of *Cystoisospora* spp. in puppies obtained from pet shops and large breeding facilities. (Claerebout et al., 2009; Dupont et al., 2013; Grellet et al., 2014). Young animals are more vulnerable to infectious diseases. Apart from this, the moment of first exposure to an infectious disease in puppies will vary, and both criteria will determine the age at which puppies are first infected. Most of the CPV infections were found in puppies < 3 months of age. In the Netherlands puppies are generally vaccinated at 6, 9 and 12 weeks against CPV and most of the puppies in our study were vaccinated accordingly. Only 4 diarrhoeic puppies older than 3 months were CPV-positive, possibly due to either incomplete vaccination or inadequate vaccine-response due to high levels of maternal immunity. CCoV infection was not significantly different in puppies under and above 3 months of age. The role of CCoV is not unequivocal between studies, Schulz et al. (2008) found no significant association between CCoV infection and diarrhoea, in contrast to Stavisky et al. (Schulz et al., 2008; Stavisky et al., 2011). The use of different study populations and viral detection methods makes it difficult to compare the results of these studies. However, our study unequivocally shows a significant association with diarrhoea in puppies under the age of 1 year.

Routine vaccination against CCoV is uncommon in the Netherlands and could thus not influence CCoV vulnerability. This also accounted for hEC infection. Interestingly the four puppies that were hEC infected and severely diseased were under 9,5 weeks old, suggesting that there could be an age effect but this effect was not observed between the age categories used in our study. Previously seasonality for CPV was described with the predominant occurrence of CPV in summer months while others reported spring and early summer as the seasons with the highest occurrence of CPV (Houston et al., 1996; Bagshaw et al., 2014). The prevalence of CPV and CCoV positive puppies in our study varied by season, with CPV risk increasing in winter and spring compared to summer, and CCoV increasing in winter. Possible explanations for seasonality are variable, taking into consideration the moment of first exposure. Puppies from high volume breeders will most likely get exposed at the breeder and clinical symptoms will occur shortly after purchase. In these puppies the high environmental viral load due to overcrowding combined with a lower hygiene level in the colder winter months may prone these pups to more frequent infections.

In the treatment of acute (hemorrhagic) diarrhoea in puppies the use of antibiotics is common in the Netherlands. The administration of antibiotics at the time of or prior to sampling could have interfered with the results of bacterial culture. Data about antibiotic administration at the time of sampling were available for some of the dogs and there was no significant difference between the treated and untreated animals regarding total bacterial pathogens isolated (results not shown). The use of antibiotics at the time of sampling however significantly increased the number of samples in which *C. difficile* was cultured ($p = 0.006$) and significantly decreased those in which *Campylobacter* spp. was cultured ($p = 0.037$). When the antibiotic users were discarded from the analysis *C. difficile* and *Campylobacter* spp. infections were still not significantly associated with acute diarrhoea.

5. Conclusions

Multiple enteropathogens were detected in the faeces of both diarrhoeic and healthy puppies, indicating that the clinical

relevance of enteropathogen detection needs to be interpreted with caution. In puppies suffering from (severe) acute diarrhoea routine screening should focus on CPV, CCoV and hEC. CPV and CCoV occur frequently together. Risk factors for these infections are: Originating from high-volume dog breeders, exposure in winter and/or spring and puppies being <3 months of age. Besides CPV and CCoV, puppies originating from high-volume breeders are at increased risk of *Cystoisospora* spp., and may be examined accordingly. Routine screening for *Toxocara* spp., *Giardia* spp., *Cyniclomyces guttulatus, Campylobacter* spp. *C. perfringens* and *C. difficile* may lead to inconclusive results, especially in puppies that are regularly dewormed, and may be therefore be recommendable as second priority testing.

Conflict of interest

None.

Acknowledgements

The authors acknowledge Marian Bos, Mignon 't Hooft, Laura Jesko, Melissa Weij, Miriam Koene and Marjolein Hensgens for their assistance with the laboratory work and data management.

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