Prevalence of *Clostridium difficile* and *Salmonella* spp in juvenile dogs with parvoviral enteritis and clinically healthy controls.

By

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% Percentage

= Equal to

°C Degrees Celsius

CD *Clostridium difficile*

CDI *Clostridium difficile* infection

CPV Canine parvovirus

EIA Enzyme immunoassay

ELISA Enzyme-linked immunoassay

EM Electron Microscopy

GDH Glutamate dehydrogenase

kg Kilogram(s)

n Number of animals

$NaNO_3$ Sodium nitrate

OVAH Onderstepoort Veterinary Academic Hospital

P Estimation of statistical significance

PCR Polymerase chain reaction

RV Rappaport-Vassiliadis broth

TcdA *Clostridium difficile*-associated toxin A

TcdB *Clostridium difficile*-associated toxin B

TBG Tetrathionate broth with brilliant green

XLT4 Xylose-lysine-tergitol

Summary

The Prevalence of *Clostridium difficile* and *Salmonella* spp. in Juvenile Dogs Affected with Parvoviral Enteritis and Healthy Control Dogs

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Background: *Clostridium difficile* (CD) is the most common cause of hospital-associated diarrhoea in humans and Salmonellosis is a disease of major zoonotic importance. Canine parvovirus (CPV) is a potentially fatal cause of canine enteritis with a world-wide distribution. Persistent isolation of *Salmonella* spp. during routine hospital environmental surveys of the OVAH isolation ward, reserved for the treatment of CPV positive dogs, prompted investigation into a possible source.

Hypothesis: Juvenile dogs affected by CPV will have a higher prevalence of faecal *Clostridium difficile* and *Salmonella* spp. compared to an apparently healthy juvenile cohort.

Animals: Seventy-four client-owned dogs infected with canine parvovirus and 42 apparently healthy client-owned dogs.

Methods: Prospective, longitudinal, observational study conducted at the Onderstepoort Veterinary Academic Hospital, University of Pretoria, South Africa over an 18-month period.

Fresh fecal samples were collected from dogs aged 6 weeks to 9 months that were diagnosed with CPV and admitted for treatment, and from apparently healthy dogs presenting for vaccination or routine hospital procedures. CPV shedding was confirmed using negative staining electron microscopy. *Clostridium difficile* was diagnosed using a commercially-available faecal antigen enzyme immunoassay(EIA) for the detection of *Clostridium difficile*-specific glutamate dehydrogenase and for enterotoxin TcdA and cytotoxin TcdB. Faeces were submitted for the isolation, antimicrobial susceptibility testing, and serotyping of *Salmonella* spp.

Results: The prevalence of faecal *Clostridium difficile* was 3% and 5% and for *Salmonella* spp. 22% and 31% for the CPV-cohort and apparently healthy dogs, respectively. No statistically significant associations between *Salmonella* status and possible risk factors or continuous variables such as age, body weight and duration of hospitalization were identified. Statistical analysis was not performed on *Clostridium difficile* positive dogs, due to only 2 dogs in each group testing positive. All the *Salmonella* spp. isolates (n = 32) were resistant to penicillin G, lincomycin and tylosin. Nine of the isolates were resistant to lincospectin and 21 showed intermediate (n = 20) or complete resistance (n = 1) to doxycycline/oxytetracycline. *Salmonella* spp. from nine different serotypes were identified.

Conclusions and Clinical Importance: The prevalence of *Clostridium difficile* in the CPV and healthy juvenile dogs was similarly very low and insufficient for statistical analysis. The prevalence of *Salmonella* spp. in dogs infected with CPV was not statistically different from that in an apparently healthy cohort. However, the prevalence in both groups was considerably higher than that commonly reported in adult dogs and parallels previous reports in young dogs, shelter dogs, or dogs fed a raw meat diet. Of the nine *Salmonella* serotypes identified from 32 isolates, there were several with variable resistance to a range of antibiotics including penicillin G, lincomycin, tylosin, lincospectin, and doxycycline/oxytetracycline.

Keywords: *Clostridium difficile*; Salmonellosis; Parvovirus; diarrhoea; bacterial enteropathogens; prevalence; antibiotic resistance

Chapter 1

Literature Review

Clostridium difficile

Introduction. *Clostridium difficile* (CD) is a common cause of antimicrobial-associated pseudomembranous colitis in humans and has been associated with diarrhoea in dogs and enterocolitis in foals and adult horses[1-3]. Moreover, *Clostridium difficile* and *C. perfringens* are the two most commonly incriminated bacteria in dogs and cats suffering from clostridial enteritis[4]. However, the role of CD infection (CDI) as a primary cause of diarrhoea in dogs, and the subsequent possible zoonotic potential to co-habiting humans, remains unclear.

C. difficile is a large, anaerobic, spore-forming bacillus that, in its vegetative form, is considered an important enteropathogen in many species[5-8]. The highly resistant, sporulated form of *C. difficile* is responsible for most infection transmissions[6]. The isolation of CD from healthy, non-diarrhoeic dogs is the main confounding factor as to when the presence of CD in a diarrhoeic animal can be considered pathogenic in nature. However, an outbreak of CD-associated diarrhoea has been reported in dogs at a veterinary teaching hospital in Canada[9].

Likely of greater importance, is the possible role of dogs as a source for human transmission due to the severity and prevalence of CD-associated disease in humans. *Clostridium difficile* strains of epidemiological concern in human medicine have been isolated in dogs, especially those utilised as hospital visitation dogs, raising concerns for possible zoonotic transmission of these strains to humans[10,11]. Additionally, the incidence of *C. difficile* infections in humans has markedly increased in certain parts of the world, such as Canada and the United Kingdom, over the last decade[12,13]. C. difficile expression have also been reported from humans in Sub-Saharan Africa[14]. A review of CDI in Sub-Saharan Africa concluded that an association with previous antimicrobial use is also evident in this population but relatively little literature is available to elucidate the impact of CDI on human health care[14]. Closer to the geographical area of interest, a study originating from a tertiary referral human hospital in Cape Town, reported an incidence lower (0.87 cases per 1000 hospitalisations) than that reported from countries such as Canada in the western hemisphere (7.4 cases per 1000 hospitalisations)[15]. The appearance of so-called hypervirulent strains, such as ribotype 027, also known as North

American pulsotype 1, has been implicated in the increased incidence of CDAD in North America seen in *C. difficile* infection (CDI)[16]. Ribotype 027 has also disturbingly been identified in a hospital visitation dog[10] and from environmental sites in companion animal veterinary teaching hospitals in Canada[17]. In South Africa, Asia and China, Ribotype 017 has been identified as a common cause of CDAD[18]. Although this Ribotype appears to be less pathogenic than Ribotype 027, metronidazole resistance is commonly reported and may influence appropriate empirical antimicrobial choices in cases awaiting culture and antimicrobial susceptibility results[18]. The predominant Ribotypes of CD in dogs from South Africa have not been reported to date. However, zoonotic transmission of CD has never been unequivocally demonstrated, demanding further investigation into CD epidemiology[6]. The isolation of CD from meat products for pet products have been reported from samples collected in the US, Canada and Europe[19]. This raises concerns for an additional source of exposure which has not been investigated in South Africa.

Epidemiology. The role of CDI in dogs, especially as a possible nosocomial infection and primary cause of diarrhoea is unclear and has frequently been a topic of discussion in the literature over the last decade. Several confounding factors are at play in determining the true pathogenic potential of CD when isolated from companion animals. The primary confusing factors is that *C. difficile* has been documented to be isolated from both healthy and diarrhoeic dogs[5,20-25]. In fact, carriage rates of 0-10% in healthy dogs out of the community have been reported from South Wales, Germany and Canada[5,20,21,23]. Moreover, healthy dogs used as visitation dogs in human hospitals and those that were admitted to Canadian veterinary facilities for inpatient treatment, have shown to have even higher rates at 58% and 11-48% respectively[21,24-26].

A second confounding factor is the shedding of *C. difficile* appears to be fleeting and does not follow a consistently identifiable pattern[27]. This may indicate repeated exogenous exposure with transient colonisation or the passive intestinal transit of infective material[27]. Hospital admission expression of CD, thus presumably community-acquired CD, has been reported at a rate of 11% in dogs, which is similar to that has been reported in humans in Canada[28,29]. Significant, and increasing hospital-acquired expression of *C. difficile* during hospitalisation in an intensive care unit of a Canadian veterinary teaching hospital has also been reported and may serve as a risk factor for CD expression[26]. Risk factors for the hospital-acquired

expression of CD that have been reported include antimicrobial therapy prior to hospitalisation and the use of immunosuppressive therapy during hospitalisation [26].

Thirdly, it is impossible to differentiate hospital-acquired colonisations from hospital-expressed colonisations[9]. There are widely discrepant rates between asymptomatic colonisation versus ultimate development CDI i.e. primarily *Clostridium difficile* associated diarrhoea and colonisation therefor does not necessarily indicate that CDI will develop[6]

In humans it has been suggested that primary colonisation of *C. difficile* may reduce the risk of developing *C. difficile* infection at a later stage[30]. Similar results were also found in a study from a Canadian a veterinary teaching hospital with CD expression rates on admission and a lower rate of the development of diarrhoea in CD positive animals during hospitalisation[26]. However, concerns have been expressed that individuals with pre-admissive colonisation may act as possible reservoirs for infection in the hospital environment[29]. *C. difficile* spores are highly resistant to commonly used disinfectants[31] and have been detected in veterinary hospitals [32,33]. This raises the question of possible inter-species transmission in veterinary care facilities in the United States of America treating a variety of species as CD-associated disease outbreaks have also been reported in equines[2]. However, since the environmental contamination of CD spores may be a consequence rather than a cause of infection, interpreting the significance of these results is difficult[17].

Clinical manifestation of disease. Toxin A (TcdA), toxin B (TcdB) and a binary toxin, adenosine diphosphate (ADP)-ribosyltransferase (CDT), are three toxins produced by *C. difficile*[6]. In dogs TcdA and TcdB are usually produced simultaneously, but TcdB positive-only strains have also been documented[25]. Strains of *C. difficile* producing both toxins are generally considered clinically relevant[34]. The role of CDT is unclear and nontoxigenic strains of *C. difficile* are considered irrelevant for clinical purposes[6]. The TcdA and TcdB produced with clinical CDI causes cell death by inactivation of Rho proteins by glycosylation, causing disruption of the cellular cytoskeleton due to depolymerisation of the actin filaments[35]. Conventionally, TcdA is known to cause mucosal haemorrhage and necrosis with ensuing haemorrhagic secretions whereas TcdB is not associated with histological tissue damage or fluid secretion[36]. In contrast, studies have suggested a possible synergistic activity, where the damage caused by TcdA enables the cytotoxicity of TcdB[36]. The clinical signs associated with CDI can range from subclinical carriage to a potentially fatal acute haemorrhagic diarrhoeal syndrome[37].

Clostridium difficile infection is commonly associated with concurrent signs of small and large intestine diarrhoea, suggesting involvement of the small and large intestinal tract with poor anatomical localisation based on clinical findings alone[37].

Diagnosis. A significant association between the isolation of *C. difficile*, the presence of *C. difficile* toxins in faeces and patient diarrhoea has been shown in several studies[4,23,37]. However, CDI has not been successfully experimentally reproduced [38]. Despite this, the diagnosis of CDI has been reported in 10.2-21% of diarrhoeic dogs presented to various veterinary facilities in continental North America[4,23,39]. *Clostridium difficile* is notoriously difficult to culture, and the isolation of CD alone doesn't necessarily relate to pathogenic disease without the concurrent identification of its associated toxins. For this reason, the majority of clinically utilised tests today aim to identify both CD and its associated toxins using various molecular techniques.

A cell culture cytotoxicity assay, that detects TcdB activity is considered the present gold standard for faecal toxin identification but is expensive and time-consuming to perform[6,40]. A commercially available ELISA for toxin detection, designed for use in humans, has been reported to have moderate-to-poor sensitivity and specificity in dogs[39]. At present combination testing with commercial enzyme-linked immunosorbent assay (ELISA) for faecal toxin detection and organism isolation by culture, real time PCR or antigen ELISA are recommended for the diagnosis of CDI[6]. Should a case test positive for faecal toxins in the absence of organism isolation the result must be interpreted with caution and only considered as a possible CDI, especially due to the comparatively poor specificity and sensitivity of faecal detection assays to that for organism isolation[6]. Should both faecal toxin assay and culture be positive and no other plausible explanation for the clinical signs seen be found the diagnosis of CDI is made[6]. In contrast, seeing as the isolation rates between diarrhoeic and non-diarrhoeic animals are so similar, a negative faecal culture can be clinically useful to exclude CDI as a possible diagnosis in suspected cases[37].

Treatment. The treatment of CDI in dogs is based on extrapolation from other species and anecdotal reports[6]. Metronidazole is the empirical antimicrobial agent of choice for the treatment of suspected CDI in dogs and cats combined with supportive therapy as indicated by the needs of the specific case[6]. Vancomycin is used in humans and horses as an alternative option in cases not responding to metronidazole and can be considered in dogs as well[6].

Zoonotic implications of *Clostridium difficile.* The zoonotic transmission of CD has not been unequivocally proven. However, due to the similarity in isolated strains from humans and dogs, the possibility has been raised and is of undeniable concern [6,27,41,42].

***Clostridium difficile* in veterinary facilities.** Outbreaks of suspected CDI of dogs and horses have been documented in Veterinary Teaching Hospitals in North America[2,9].

***C. difficile* in parvoviral enteritis.** Antimicrobial administration to dogs prior to admission to a veterinary facility [26], antimicrobial therapy of the dogs owners[43], living with an immunocompromised owner[27] or contact with children[43] as well as visiting human hospitals[43] have all been identified as risk factors for the *C. difficile* colonisation of the gastrointestinal tract. However, the concurrent use of antibiotics and increased *C. difficile* colonisation does not appear to be a consistent finding[21,24]. In one study the use of penicillin and streptomycin showed a positive association with antimicrobial usage and increased *C. difficile* colonisation[24]. The prophylactic use of antimicrobial drugs to guard against septicaemia following gastrointestinal bacterial translocation is one of the mainstays of therapy in parvoviral enteritis[44]. Due to the considerable loss of intestinal architecture and consequent loss of entero-protective mechanisms, dogs affected with canine parvoviral enteritis would conceptually be at an increased risk for colonisation of enteropathogenic microbes such as CD, especially when considering the substantial antibiotic use in this patient population.

Salmonella spp.

Introduction. *Salmonella* spp. are facultative anaerobic, motile, gram negative, ubiquitous, non-sporulating bacilli[6]. Two species, namely *Salmonella enterica* and *Salmonella bongori*, make up the genus with a very complex nomenclature of numerous further subdivisions of subspecies and serotypes[6,45]. *Salmonella bongori* is more prevalent in ectothermic animals and rarely an infection of warm-blooded animals although it has been isolated from a dog with diarrhoea[46]. *Salmonella enterica* is further subdivided into 6 subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*. Nearly 2500 serotypes as defined by characteristic agglutination reactions of somatic (O) and flagellar (H) antigens have been identified[47]. Specific serotypes are described using an universal antigenic formula, unless they have designated names such as with the subspecies I serotype called *S*. ser.

Typhimurium, which is implicated in almost all salmonellosis in humans and warm-blooded animals[47]. Clinical salmonellosis is rare in dogs and cats[47]. However, *Salmonella* spp. are amongst the most common causes of foodborne disease in humans and salmonellosis is a known zoonosis[47]. Inter-host transmission characteristics appear to be serotype dependent and can be host restricted or transmissible over a wide range of species[47].

Epidemiology. Similar to *Clostridium difficile* the reported prevalence of *Salmonella* in apparently healthy dogs (0-4.4%) and those affected by diarrhoea (0-3.6%) are very similar[4,48-54]. The reported prevalence rates have not shown gross temporal changes or spatial diversity from studies originating from all over the world since the early 1950's [4,48-55]. Higher prevalence has been documented in puppies (31%), shelter or kennel dogs (30%) and dogs fed a raw diet (51.4%)[54,56,57]. Alaskan sled dogs which mostly get fed raw diets and are group-housed showed a very high prevalence of 63% in one study although a prevalence exceeding 75% in dogs housed under similar circumstances have been reported[57-59]. In comparison, reports of dogs fed processed commercial pet foods have yielded faecal isolation rates of less than 1%[60-63]. A Canadian study revealed a prevalence amongst household pets as high as 23%[64]. Hospitalised dogs from continental North America, the Caribbean and Trinidad have also been reported to have a comparatively higher (2.2-19.7%) prevalence[48,65,66]. Therefore, the prevalence of *Salmonella* appears to be dependent on possible environmental sources of infection, infective agent characteristics, host-microbe interaction and predisposing factors[67].

Considerable geographical and temporal variation in the prevalence of *Salmonella* serotypes in both humans and animals have been noted[48,68]. Serotype yield within canine populations can be very diverse and more than one serotype have been simultaneously isolated from a single animal or when followed over a period of time[67]. The three most commonly isolated serotypes from dogs have recently been reported as *Salmonella* ser. Typhimurium, *Salmonella* ser. Dublin and *Salmonella* ser. Enteritidis[69]. Vulnerability to shedding and disease is associated with immunosuppression, including animals that are very young, gravid, housed in overcrowded conditions, undergoing immunosuppressive drug therapy or suffering from immunosuppressive conditions such as neoplasia, diabetes mellitus, retroviral infection and immune-mediated disease[47,70]. Additional possible risk factors have been suggested including: hospitalisation, transportation of dogs, feeding of raw meats, antimicrobial therapy and a history of environmental exposure[6,54,71].

Clinical manifestation. Even though clinical canine salmonellosis is believed to be relatively uncommon, salmonella infection is of great public health importance due to the close contact between humans and their pets[49]. Salmonellosis has a highly variable clinical presentation in dogs[6]. Case presentation can range from subclinical infections to both acute and chronic gastrointestinal disease, sometimes progressing to severe systemic illness associated with septicaemia[73,74]. Rarely, *Salmonella* spp. are incriminated as the cause of focal suppurative lesions [47]. These lesions localise to particular organs such as the lungs, conjunctiva or urinary tract[47].

Salmonella spp. cause substantial production of cytokines and chemokines by actively invading and disrupting the tight junctions of enterocytes, M cells and dendritic cells within the intestines[75]. This leads to a massive incursion of lymphocytes, macrophages and neutrophils which may be followed by severe epithelial damage and mucosal sloughing[75]. Infected individuals can shed the salmonellae for weeks because they evade the host immune response and localise within the mesenteric lymph nodes, gut-associated lymphoid tissue and intestinal epithelium[75]. Should the host become latently infected, shedding can recommence as a result of stress or immunosuppression[76,77]. In cases where the host fails to localise the infection to the gastro-intestinal tract and the organism involved possesses the properties that allows it to invade, the infection may spread systemically[77]. The virulence of an infection is considered a multi-factorial feature that is related to both the host and organism[6]. Virulent strains with an augmented ability to multiply in nonphagocytic cells may cause pyrexia, hypoglycaemia, leukopaenia and the infection process can escalate to endotoxic shock or disseminated intravascular coagulation[47].

The onset of clinical signs is thought to be 3-5 days post exposure or post onset of immunosuppression, although shorter durations of onset have also been noted[6]. Common clinical signs include pyrexia, malaise, anorexia that is followed by vomiting, abdominal pain and a watery, mucoid or even haemorrhagic diarrhoea[6]. The majority of dogs that shed *Salmonella* appear clinically normal although individual dogs may present with a history of weight loss or clinical signs evident of sepsis[73]. Abortion and stillbirth have been reported after trans-placental transmission to foetuses and fading puppies may present due to neonatal infection from the dam[74,78]. Clinicopathologic abnormalities in canine salmonellosis

are unremarkable and non-specific but may be suggestive of specific organ system involvement or disease severity[47].

Diagnosis. The diagnosis of salmonellosis in dogs is made by isolation of the organism, preferably using special culture techniques and/or by advanced molecular techniques, a compatible clinical manifestation and assessment of the presenting history for potential risk factors[6]. Experimentally-induced infections in dogs and cats via oral administration of infective material showed irregular shedding of the agent for 3 to 4 weeks and, although uncommon, for up to 100 days[79]. The intermittent and unpredictable nature of *Salmonella* spp. shedding makes the identification of true carriers difficult as they cannot be differentiated from those individuals shedding due to a transient colonisation [79]. One study comparing *Salmonella* serum antibodies to *Salmonella* faecal isolation showed that dogs negative for *Salmonella* antibodies may still be infected with *Salmonella,* limiting the usefulness of this modality in identifying *Salmonella* spp. positive animals[49].

Salmonella spp. grow readily at 37˚C on routine bacteriologic media but the use of specialised culture techniques can greatly increase the sensitivity of isolation[6,80]. The sensitivity of conventional culture techniques can also be increased by culturing serial samples [81,82]. It has been suggested that in dogs and cats six consecutive negative cultures should yield a confidence of 99% for a *Salmonella* spp. negative state[6]. It has been suggested that the gold standard for microbiologic testing should be a polymerase chain reaction (PCR) assay after overnight enrichment of a sample in a nonselective growth medium with subculture of PCR-positive samples using selective enrichment for isolation and identification of the organism involved but these tests are not widely available nor always validated for use in dogs[6,83].

Various methods of serotype identification are available today[84]. Since its development nearly 90 years ago, the Kauffman-White scheme is still widely in use[84]. This scheme utilises agglutination testing with antisera for the O and H antigens reported as an antigenic formula unique to every serotype[84]. The World Health Organisation hosts a database of the reported serotypes which is regularly updated[84]. Recent advances in molecular techniques have also led to the development of alternative techniques in serotyping employing the use of antibody microarrays or phagetyping[84]. The use of molecular typing techniques such as pulsed-field gel electrophoresis, multilocus sequence typing, multiple-locus variable-number tandem repeat analysis and whole genome or genomic marker sequencing are also on the rise but not as widely applied in practice[84].

- *S. berta*
- *S. enteritidis*
- *S. gloucester*
- *S. haardt*
- *S. lagos*
- *S. saintpaul*
- *S. tennyson*
- *S. tsevie*
- *S. typhimirium*

Figure 1 Salmonella serovars previously isolated from dogs in South Africa.

Source: Adapted from Salmonella in Domestic Animals (Wray & Wray, 2000)

Treatment. The treatment of uncomplicated canine salmonellosis is merely supportive and antimicrobial therapy is not indicated unless patients are known to be immunocompromised or systemically affected[6]. The majority of cases are self-limiting, and shedding might counterintuitively be prolonged with imprudent antimicrobial administration. If at all possible hospitalised animals should be isolated and treated with barrier control should they test positive for *Salmonella* spp.[47]. Simultaneous use of ampicillin and a fluoroquinolone is advocated as the empirical antimicrobial choice whilst awaiting the culture and susceptibility

results[47]. Supportive therapy including intravenous fluid therapy and colloidal support with intensive monitoring and resultant treatment for endotoxaemia is also recommended[47].

Zoonotic implications of *Salmonella* spp. All *Salmonella* organisms, other than that implicated in human typhoid fever, infect both animals and humans, causing them to be of major concern in zoonosis[6,79]. Dogs have been suggested to play a major role as carriers in the dissemination of salmonellae and transmit them to other healthy animals and humans[48,85]. The zoonotic transmission of *Salmonella enterica* has been associated with exposure to ill and healthy cattle, ill shelter cats and veterinary clinic inpatient cats[86-89]. On a larger scale there have been various studies linking contaminated dry pet food with salmonellosis in humans[90,91].

***Salmonella* in veterinary facilities.** *Salmonella* outbreaks have been reported in both large- and small-animal veterinary facilities[86,89,92-96]. In large-animal veterinary hospitals salmonellosis is a well-recognised nosocomial problem[92,93]. Isolation of salmonellae in veterinary facilities is of public health importance as the infected hosts may serve as a potential source of transmission to other patients or humans[48].

***Salmonella* spp in parvoviral enteritis.** The clinical presentation of salmonellosis in dogs can mimic that of dogs affected by parvoviral enteritis[47]. Resistance of *Salmonella* spp. to various antimicrobials including ampicillin, amoxicillin, gentamicin[48] and clavulanic acid has been reported[89]. Fluoroquinolone antimicrobial therapy has also been reported to fail at eliminating faecal shedding of susceptible salmonellae strains[89]. All of the abovementioned antibiotics are commonly employed in the treatment of dogs affected by parvoviral enteritis[44]. Canine parvoviral enteritis is commonly complicated by comorbid enteropathogen infections and the role of *Salmonella* spp. as a possible comorbidity has not been determined[44].

Canine parvoviral enteritis

Introduction. Viruses from the *Parvoviridae* family are commonly known as parvoviruses and can infect a variety of animal species[97]. Canine parvovirus (CPV) was initially thought to be a mutant form of feline parvovirus (FPV) but was reclassified in 2014 by the International Committee on Taxonomy of Viruses into a single genus named *Carnivore protoparvovirus 1*. However, this utilisation of this new genus name is rarely applied in clinical practice and most

reference texts still refer to CPV and FPV as separate entities. Canine parvovirus (CPV) is a globally significant enteropathogen of dogs[98,99]. Ever since its discovery in 1978, it has remained a leading cause of enteritis in dogs despite the wide availability of effective vaccines [100]. At present there are three known antigenic strains, CPV-2a, b and c[98]. In Southern Africa the prevalence of CPV-2a and CPV-2b amongst 125 dogs infected with CPV in a 1998 publication was 31% and 69% respectively[101]. CPV-2c had not been reported to be prevalent in Southern Africa as of a publication in 2013 [102] Vaccination with available modified live CPV vaccines appear to confer adequate immunity against all known subtypes[98,103,104]. Despite intensive therapy regimes, CPV infection still delivers a rather unswerving mortality rate of 16 – 25%[44,105-108]. Parvovirus is a small, ubiquitous, non-enveloped, single-stranded DNA virus that is fairly species specific[44,98]. CPV can persist in the environment for up to 5-7 months [44,109].

Epidemiology. In susceptible canine populations, parvovirus infection most commonly manifests as a severe systemic and even fatal illness[110]. Puppies from 6 weeks to 6 months of age are most commonly affected due to a suspected window of susceptibility[44,98,111]. Clinical signs relate to the virus replicating in tissues with a rapid cell turnover such as intestinal crypt epithelium, bone marrow and myocardium[98,109,110]. Haemorrhagic diarrhoea, vomiting, hyporexia, listlessness, severe dehydration, collapse and death are all characteristic symptoms of parvoviral enteritis[98,109,110]. The clinical signs usually develop within 4-5 days post oral exposure to CPV[109,110]. When considering the trend in mortality rates there is a distinct need for therapeutic agents that can assist in limiting the disease severity, shorten the duration of hospitalisation, improve survival rates and limit the costs associated with treatment[112]. Risk factors associated with parvovirus infection in dogs include inadequate protective immunity, unsanitary and overcrowded environments and enteric parasites[44,110]. Breeds including Rottweilers, Doberman Pinschers, American Pit Bull Terriers, Staffordshire Bull Terriers, Alaskan Sledge Dogs, Labrador Retrievers and German Shepherd Dogs have been reported to be at an increased risk for CPV infection and disease severity[44,98,110,113,114]. Further studies have also suggested that dogs of a purebred lineage are at increased risk for CPV infection and mortality when compared to mixed breeds[115,116]. Infection is acquired directly, transplacentally in-utero, by the faecal-oral route of transmission or indirectly via oro-nasal exposure to fomites or faecal material[99,110]. A poor humoral response to vaccination and the

persistence of maternal antibodies past the age of when the primary vaccination course would usually be completed, is thought to contribute to the predisposition of these breeds[117].

Clinical manifestation. Two clinical syndromes are described in dogs affected by CPV namely enteritis and myocardial failure[109,110]. Due to vaccination protocols allowing for protective maternal antibodies the myocardial failure syndrome, a disease of neonatal pups, is rarely reported today[99,110]. Puppies with CPV-associated myocarditis show clinical signs such as dyspnoea, crying and retching although they are often just found dead or succumb within 24 hours after appearance of clinical signs[114]. The oropharynx and local lymphoid tissue act as replication centres for the virus during the first 2 days of infection[44]. By the 3rd to 5th day viremia is marked, with CPV preferentially targeting tissues with rapid cell turnover[109,110]. Diet changes, alteration in commensal microbiota due to weaning, concurrent endoparasitism or concurrent alternative canine diarrhoea viral infections boost intestinal cell turnover which favours viral replication with increased severity in the resultant lesions and subsequent clinical disease[109,110]. Extensive destruction of progenitor cells in lymphatic tissue and bone marrow lead to a lymphopaenia and in severe cases a panleukopaenia[109,118]. In the intestinal tract, CPV replication causes necrosis of the germinal epithelium of the intestinal crypts, villous atrophy, collapse of the intestinal epithelium, with subsequent loss of its absorptive capacity, leading to vomiting and haemorrhagic diarrhoea[44,109]. Clinical signs of CPV infection are typically limited to severe gastrointestinal upset and immunosuppression, but a clinically less apparent, systemic inflammatory response can occur in many cases[44]. This is due to bacterial translocation from the damaged intestinal tract with a resultant bacteraemia and endotoxaemia[114]. Dogs with CPV have been reported to be hypercoagulable without disseminated intravascular coagulopathy and have a high prevalence of clinical thrombosis or phlebitis[119]. The inflammatory response initiated by the viral disease and associated endotoxaemia causes an increase in fibrinogen concentrations[119]. This increase, in association with vascular stasis, activation of coagulation, and vascular injury may be a risk factor for thrombosis and contribute to the hypercoagulable state in these dogs[119].

Diagnosis. History and clinical findings can be used to make a tentative diagnosis of CPV infection[98]. Leukopaenia characterised by a lymphocytopaenia and neutropaenia is commonly seen associated with CPV infection[98]. Lymphocyte number usually increases again rapidly after the initial lymphocytolysis during initial viral replication[98]. Neutropaenia

secondary to peripheral neutrophil consumption at the onset of gastrointestinal signs along with the destruction of progenitor cells is very suggestive of canine parvoviral enteritis[98]. However salmonellosis, or any other overwhelming infection, has been reported to cause similar haematological findings[98]. Definitive diagnostic tests include detection of CPV in the faeces of affected dogs by electron microscopy, virus isolation, haemagglutination, and latex agglutination, as well as by serology and at necropsy by histopathology[44,110]. Faecal enzyme-linked immunosorbent assay (ELISA) tests for antigen are available for cage-side testing for acute CPV[98,114] False positive results using faecal antigen ELISA assays may occur 5-15 days after vaccination with a modified live CPV vaccination[98] False negative results may also occur due to binding of test antigen with serum neutralizing antibodies in bloody diarrhoea[110].

Treatment. To date, no definitive treatment has been established[44], therefore, treatment remains symptomatic and supportive[98]. Without treatment, CPV infection is often fatal with mortality rates of up to 91%[44] being reported. The survival of acute CPV cases is largely dependent on the intensive treatment given when an infected puppy is hospitalized[118]. Fluid therapy, electrolyte supplementation, antimicrobials, antiemetics, analgesia and nutritional therapy are the mainstay of supportive therapy[98]. Fluid therapy is aimed at improving perfusion, correcting dehydration and to pre-empt ongoing losses due to vomiting and diarrhoea[98]. Commonly seen sequelae of canine parvoviral enteritis such as hypokalaemia, hypoglycaemia, hypoproteinaemia and anaemia are monitored for and treated as indicated[98]. Severe peripheral neutropaenia and bacterial translocation necessitates the use of antimicrobials of which first generation cephalosporins or penicillins such as amoxicillin or ampicillin are common empirical choices[98]. Potentiated penicillins, metronidazole and fluoroquinolones such as enrofloxacin or pradofloxacin can be added additionally as indicated[98]. Maropitant, metoclopramide and ondansetron are used as anti-emetics to limit further fluid and electrolyte loss due to vomiting[98]. Pain associated with acute gastroenteritis, or less frequently intestinal intussusception, is treated with partial opioid agonists such as buprenorphine[98,99]. Early enteral nutritional therapy is essential for intestinal mucosal growth and repair with improved local immunity and subsequent reduced bacterial translocation[112]. Naso-oesophageal and nasogastric tubes can aid in feeding adequate nutritional requirements[98]. Assessing gastric residual volume and therefore gastrointestinal motility is an additional advantage of nasogastric intubation[98]. Recombinant feline interferon is a

cytokine with inhibitory effects on viral and cell proliferation which has been shown to reduce mortality and palliate clinical signs in CPV infection[120]. Cross-protection between antibodies against mutant *Salmonella typhimirium* bacterin-toxoid, in the form of hyper-immune equine serum, has been proposed to provide protection against gram-negative toxins present in CPV infections[98]. Cost, potential hypersensitivity and questionable efficacy have been expressed as limiting factors for its use[98]. Several biomarkers for prognostication and diagnostic use during the CPV infection disease process have been identified[99].

CPV infection and bacteria. Coliform septicaemia has been demonstrated in CPV infected dogs[121]. *Escherichia coli* has been recovered from lung and liver tissue of the majority of puppies that died following severe CPV infection[121,122]. Secondary *Salmonella* spp., *Clostridium perfringens* and *Campylobacter* spp. have also been reported in CPV infection[123,124]. A study determining the prevalence of bacterial colonisation of intra-venous catheters and the bacteria involved was published in 2002 at the [Onderstepoort Veteirnary Academic Hospital (OVAH)] intended for the current study[125]. This study yielded a prevalence of 22% catheter colonisation with organisms involved mainly of environmental or gastrointestinal origin such as *Serratia* spp., *Staphylococcus intermedius*, *Streptococcus* spp., *Acinetobacter anitratus*, *Klebsiella* spp., *Citrobacter freundii*, *Escherichia coli* and *Enterobacter* spp.[125]. The isolated bacteria were also resistant to antimicrobials such as penicillin, cloxacillin, lincomycin, erythromycin and cephalexin but susceptible to amikacin, chloramphenicol, enrofloxacin, potentiated sulphonamides and potentiated penicillins amongst others[125].

Outcome of literature review

Canine parvovirus (CPV) is a universally prevalent and potentially fatal cause of canine enteritis, which is often exacerbated by concurrent infections with other enteropathogens[44]. Salmonellosis is a well-established major zoonotic disease which is commonly associated with foodborne disease in humans[90,91]. Zoonotic transmission of *Salmonella* spp. within a veterinary practice and outbreaks of salmonellosis in both large and small animal facilities have been reported[86,94,95]. In large-animal veterinary hospitals, *Salmonella* spp. are also well-recognized nosocomial contaminants[96]. Animals have been infected with *Salmonella* spp. via oral exposure under experimental conditions but the transmission pathway under natural conditions remains unclear, and it is thus important to evaluate the risk factors that increase

the likelihood of infection[75]. *Clostridium difficile* (CD) is a common cause of antimicrobial-associated diarrhoea and pseudomembranous colitis in humans and has been associated with diarrhoea in dogs and enterocolitis in foals and adult horses[1-3]. The role of CD infection as a cause of diarrhoea in dogs, however, remains unclear. An outbreak of CD-associated diarrhoea has been reported in dogs at a Canadian veterinary teaching hospital[9]. Moreover, CD strains of epidemiological concern in human medicine, have been isolated in dogs from Canada, raising concerns for possible transmission of these strains to humans from dogs[10,11]. However, zoonotic transmission of CD has never been unequivocally demonstrated. The prevalence of CD and *Salmonella* spp. from healthy dogs and in dogs with parvoviral enteritis in South Africa is unknown and is the aim of the current study.

Project justification

Salmonella spp., methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and methicillin-resistant *Staphylococcus aureus* (MRSA) have been cultured during several environmental surveys of the Onderstepoort Veterinary Academic Hospital (unpublished data – Dr. CH Annandale). After the implementation of more stringent infection control measurements across the hospital, *Salmonella* spp. have not been cultured from previously affected areas, save for the small animal isolation ward in which dogs with parvoviral enteritis, and rarely cats with upper respiratory tract disease are treated. *Salmonella* spp. have been repeatedly cultured from this ward over a 2-year period during routine environmental surveillance. Infection control protocols for the isolation ward have always been strict, especially when compared to those in other areas in the hospital. These measures, for example, include a foot bath on entrance and exit, wearing of protecting gowns and gloves when working in the ward, washing and cleansing of hands using a chlorhexidine-based scrub and a high percentage alcohol disinfectant in between patients and upon exiting the ward. Anecdotal reports of mild staff diarrhoea, intermittent bouts of diarrhoea in hospitalised patients in wards and units besides the isolation ward, which were undiagnosed, along with the repeated isolation of *Salmonella* spp. from the environment have prompted further investigation.

Clostridium difficile is considered the most common cause of hospital-acquired (HA) and anti-microbial associated diarrhoea. CD has been recognised as a serious, recently emerging and

evolving disease in the USA, Canada and Europe, but the few studies in southern Africa, to date, have shown a much lower incidence in humans with no known reports in companion animals[14]. Both Salmonellosis and *Clostridium difficile* infection (CDI) have varying clinical presentations in humans, ranging from an asymptomatic carrier status to severe clinical signs. Although Salmonellosis is a known zoonosis, the inter-species transmission of Clostridium *difficile* between owners and their pets has not been proven to date. Several studies have however suggested its potential as a zoonotic agent[6,22]. A number of risk factors and possible risk factors have been identified for the colonisation or development of clinical disease related to both these bacteria[22]. Dogs infected with canine parvovirus are exposed to ample opportunity for colonisation, overgrowth or shedding of bacteria such as *Clostridium difficile* and *Salmonella* spp. when the pathogenesis and overlapping risk factors for disease are considered. Many of the anti-microbial agents used in the supportive treatment of parvoviral enteritis cases are also indicated for clinical cases of salmonellosis or CDI. Antimicrobial resistance to nearly all of these antimicrobial agents has been reported at one stage or another in different parts of the world for both bacteria. To the knowledge of the author, no studies have been done in South Africa on the prevalence or antimicrobial susceptibility of either one of these bacteria in apparently healthy dogs or in dogs affected by parvoviral enteritis.

Chapter 2

Aims and Objectives

The primary aim of the project was to determine the prevalence of *Clostridium difficile,* and the prevalence and predominant molecular types of *Salmonella* spp. in juvenile dogs affected by parvoviral enteritis admitted to the small animal isolation ward for treatment. Additionally, these prevalences and predominant molecular types were then to be compared to those of a cohort of apparently healthy dogs presenting for routine hospital visits. This information might then provide baseline Salmonella and CD prevalence values and serve to demonstrate if there is a significant difference in prevalence of these two organisms between juvenile dogs affected with CPV and an apparently healthy age-matched cohort.

A secondary aim of the project was to re-evaluate the prevalence of these microbes again on discharge for comparison to the admission samples. By this means, valuable information might be gained of the epidemiology and expression or colonisation rates of these microbes within the parvoviral isolation ward.

Hypotheses

Primary hypothesis

H_0: There is no difference in the prevalence of *Clostridium difficile* and *Salmonella* spp. in juvenile dogs affected with parvoviral enteritis when compared to a control group of apparently healthy juvenile dogs.

H_1: *Clostridium difficile* and *Salmonella* spp. has a higher prevalence in juvenile dogs affected with parvoviral enteritis when compared to a control group of apparently healthy juvenile dogs.

Secondary hypothesis

H_0 There is no difference in the prevalence of *Clostridium difficile* and *Salmonella* spp. in juvenile dogs affected with parvoviral enteritis on discharge when compared to admission.

H_1 There is a higher prevalence of *Clostridium difficile* and *Salmonella* spp. in juvenile dogs affected with parvoviral enteritis on discharge when compared to admission.

Benefits arising from the study

Clostridium difficile is a global and progressive cause of concern of nosocomial and antimicrobial-associated diarrhoea in humans while *Salmonella* spp. are omnipresent agents of zoonotic significance. Baseline knowledge of the prevalence of *Clostridium difficile* and *Salmonella* spp. in apparently healthy dogs in the Onderstepoort and surrounding regions of Gauteng Province of South Africa was obtained from this research. The admission prevalence, or increased risk of hospital-acquired expression of *Clostridium difficile* and *Salmonella* spp. in dogs affected by parvoviral enteritis may have been fundamental in the alteration and further application of biosecurity measurements whilst caring for these patients. In addition, local epidemiological data regarding the antimicrobial susceptibility patterns and serotype prevalence were previously undetermined for *Salmonella* spp. in dogs in South Africa. This information may guide empirical antibiotic choices and promote prudent antimicrobial choices in future whilst awaiting antimicrobial susceptibility results in affected cases.

Chapter 3

Material and Methods

Model system

This project was a prospective, longitudinal, observational study on clinical cases of juvenile dogs naturally infected with CPV enteritis that presented to the Onderstepoort Veterinary Academic Hospital (OVAH). The apparently healthy cohort for relative prevalence comparison was collected prospectively at a single time-point on presentation only. The study was approved by the animal ethics committee of the University of Pretoria (V 091-15; See Appendix A)

Experimental design

A minimum of 70 client-owned juvenile dogs that presented to the Outpatients clinic, OVAH, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, diagnosed with parvoviral enteritis, that would be admitted to the isolation unit were to be included in the study (affected cohort). The apparently healthy cohort comprised at least 40 clinically healthy puppies presented to the Outpatients clinic, OVAH, for routine vaccination, ovariohysterectomy, orchidectomy and blood donor screening and were collected to allow for comparable prevalence analysis of *Clostridium difficile* and *Salmonella* spp. in healthy juvenile dogs of a matching age interval to the affected cohort (6 weeks to 9 months of age).

Juvenile dogs from the affected cohort, admitted for treatment, were to have a second faecal sample collected at discharge to investigate possible change in CD/*Salmonella* spp. status from admission to discharge. All isolates were then to be submitted for antimicrobial susceptibility testing and serotyping.

Inclusion criteria:

Affected (CPV positive) cohort

- The juvenile dogs could be of any breed, sex or weight but aged older than 6 weeks and younger than 9 months.
- The juvenile dogs had to exhibit clinical signs of parvovirus infection such as lethargy, vomiting, haemorrhagic diarrhoea, anorexia and dehydration.

- Affected dogs had to be admitted to the Onderstepoort Veterinary Academic Hospital isolation ward due to the severity of their clinical signs as decided by the clinician on duty at Outpatients and were not to have received any treatment prior to admission.
- The diagnosis of canine parvoviral enteritis had to be confirmed using a commercial antigen ELISA or faecal electron microscopy (EM) (Philips CM 10 transmission electron microscope, Philips Electron Optical Division, Eindhoven, The Netherlands) within 24 hours of being admitted.

Apparently healthy cohort

- The juvenile dogs could be of any breed, sex or weight but aged older than 6 weeks and younger than 9 months.
- Dogs presenting for vaccinations, routine surgical procedures and blood donor screening could be included.
- Dogs had to be deemed clinically healthy based on their clinical parameters (temperature, pulse, respiration rate, abdominal palpation, capillary refill time and mucous membrane colour), with no obvious signs of an inflammatory process or disease present.
- Collected dogs were not collected a second time when they presented for follow-up vaccinations or visits within the study period.

Owners of the dogs that were included in the study (both affected and apparently healthy) were informed of the nature of the study and were requested to sign a consent form prior to sample collection (See Appendix B).

Experimental procedure

Affected (CPV positive) cohort

Upon admission, prior to any treatment, each dog underwent a clinical examination and the data was captured on a form (See Appendix B). This data was collected by the primary investigator. A drop of blood was taken from the ear of the dog to make a peripheral blood smear. This was done by the student responsible for the case and examined by the on-duty clinician.

A fresh faecal sample was collected at admission. A sterile lubricated 1ml syringe was inserted into the rectum and faeces aspirated.

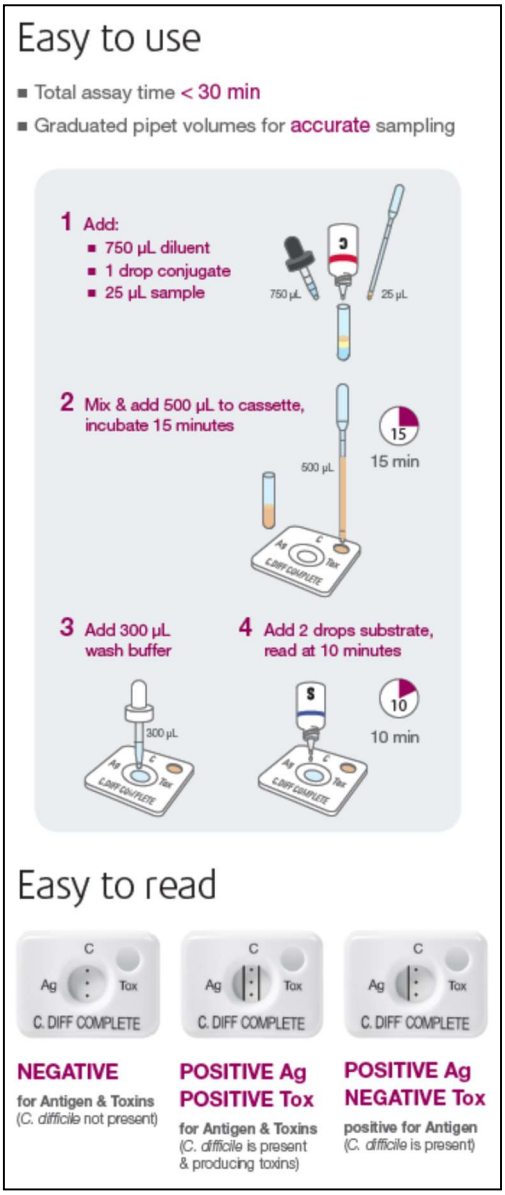
The collected faecal sample was used to do:

A faecal flotation using the Kyron disposable faecal flotation kit and Kyron egg flotation fluid ($NaNO_3$) (Kyron Laboratories, Johannesburg, South Africa), examined under a light microscope by the on-duty clinician;2. A faecal wet preparation using a drop of faeces mixed with 2 – 3 drops of saline and covered with a cover slip, examined under a light microscope by the on-duty clinician;

3. A commercial CPV ELISA SNAP test (SNAP® Parvo, IDEXX Laboratories, Westbrook, ME, USA) according to manufacturer's recommendations;

4. EM analysis (Philips CM 10 transmission electron microscope, Philips Electron Optical Division, Eindhoven, The Netherlands; Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria) with 0.1ml of faeces submitted in a capped sterile 1ml syringe to confirm the presence of CPV and screening for other viral pathogens (Canine Distemper Virus, Corona Virus and Rota Virus);

5. A rapid membrane enzyme immunoassay for simultaneous detection of Clostridium difficile glutamate dehydrogenase antigen and toxins A and B (*C. DIFF QUIK CHEK COMPLETE* ®, TechLab Inc, Blacksburg, VA, USA) as per manufacturer recommendations (See Figure 2);



6. *Salmonella* spp. culture submitted in a capped sterile 1ml syringe (Bacteriology Laboratory, Faculty of Veterinary Science, University of Pretoria);

7. The remaining faeces was stored for use in future studies at - 70°C in Eppendorf tubes.

Affected dogs all received standard treatment for parvovirus infection as set out by the isolation unit, OVAH. This includes intravenous fluid therapy, electrolyte replacement,

antibiotic, anti-emetic and gastro-protectant treatment, anthelminthic treatment, enteral feeding and blood or plasma transfusions if needed.

The on-duty clinician decided when a dog could be discharged from the hospital in consultation with the primary investigator. If a patient was to be euthanised for any reason it was also to be done in consultation with the primary investigator. Data regarding the outcome of the hospitalisation was recorded in an excel spread sheet.

A second faecal sample was collected at discharge or death when possible. The same collection protocol as for admission samples were followed.

The second faecal samples were used to do:

1. A rapid membrane enzyme immunoassay for simultaneous detection of Clostridium difficile glutamate dehydrogenase antigen and toxins A and B (*C. DIFF QUIK CHEK COMPLETE* ®, TechLab Inc, Blacksburg, VA, USA) as per manufacturer recommendations;
2. *Salmonella* spp. culture submitted in a capped sterile 1ml syringe (Bacteriology Laboratory, Faculty of Veterinary Science, University of Pretoria);
3. The remaining faecal sample was stored for use in future studies at - 70°C in Eppendorf tubes.

Apparently healthy cohort

Each dog underwent a clinical examination and the data was captured on a form (See Appendix B). This data was collected by the primary investigator. A fresh faecal sample was collected using a gloved finger and sterile lubrication.

The collected faecal samples were used to do:

1. A faecal flotation using the Kyron disposable faecal flotation kit and Kyron egg flotation fluid ($NaNO_3$), examined under a light microscope by the on-duty clinician;
2. A faecal wet preparation using a drop of faeces mixed with 2 – 3 drops of saline and covered with a cover slip, examined under a light microscope by the on-duty clinician;
3. EM analysis (Philips CM 10 transmission electron microscope, Philips Electron Optical Division, Eindhoven, The Netherlands; Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria) with 0.1ml of faeces submitted in a capped sterile 1ml syringe to exclude the presence of

CPV and screening for other viral pathogens (Canine Distemper Virus, Corona Virus and Rota Virus);

4. A rapid membrane enzyme immunoassay for simultaneous detection of Clostridium difficile glutamate dehydrogenase antigen and toxins A and B (*C. DIFF QUIK CHEK COMPLETE* ®, TechLab Inc, Blacksburg, VA, USA) as per manufacturer recommendations;
5. *Salmonella* spp. culture submitted in a capped sterile 1ml syringe (Bacteriology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria).

Detection of *Clostridium difficile*

CD was detected via a commercial fecal antigen enzyme immunoassay (*C. DIFF QUIK CHEK COMPLETE* ®, TechLab Inc, Blacksburg, VA, USA) performed according to the manufacturer's recommendation (See Figure 1). The antigen enzyme immunoassay (EIA) tested for the communal CD antigen, glutamate dehydrogenase (GDH), and CD toxins, toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin). All CD EIA's were performed by the primary investigator.

Culture of Salmonella spp.

Faeces was submitted for the selective isolation of *Salmonella* spp. using a previously reported technique[80]. The recovered isolates were stored at - 70°C in brain-heart infusion broth, pending serotyping at a reference laboratory.

The submitted fecal specimens were incubated in an enrichment broth of buffered peptone water at 37°C for 24 hours. The specimens were then vortexed, and 1-mL incubated in a selective tetrathionate broth with brilliant green (TBG) for a further 24 hours at 43 °C. 0.1 mL of vortexed TBG was then transferred to 10 mL of Rappaport-Vassiliadis broth (RV) and incubated for 24 hours at 43 °C after which a vortexed sample was plated onto xylose-lysine-tergitol (XLT4) agar. After overnight incubation at 43 °C, suspect colonies (pink colonies with or without black centers) were plated onto Columbia blood agar plates and incubated for 24 hours at 37 °C. *Salmonella enterica* isolation was confirmed by biochemical testing using a commercial kit.

Antimicrobial Susceptibility testing

Antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method[126]. Antimicrobial agents used in susceptibility testing included a standard panel of amikacin, amoxycillin/ampicillin, doxycycline/oxytetracycline, enrofloxacin, gentamicin, penicillin G, trimethoprim/sulphamethoxazole, chloramphenicol, cephalexin/cephalothin, kanamycin, lincomycin, lincospectin, orbifloxacin, amoxicillin clavulanic acid, tylosin and polymixin B.

Additional diagnostics

Negative staining transmission electron microscopy (Philips CM 10 transmission electron microscope, Philips Electron Optical Division, Eindhoven, The Netherlands) was performed by the Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria using the negative staining technique. Processing and interpretations were performed by the technicians employed by the laboratory. Samples were noted for the presence of CPV, Canine Distemper Virus, Corona Virus and Rota Virus.

Serotyping of the *Salmonella* isolates was performed by the General Bacteriology Laboratory, Agricultural Research Council-Onderstepoort Veterinary Institute, Onderstepoort, South Africa and reported using the Kauffman-White-Le Minor scheme.

Data analyses

The data obtained was captured onto specially formulated data sheets and the results transferred into a Microsoft Excel® spreadsheet. The data was then transferred into a statistical software program (IBM SPSS Statistics Version 24, Chicago, IL, USA) for statistical analysis.

The data was assessed for normality using Shapiro-Wilk testing and descriptive statistics were calculated. The Chi-Square or Fisher's exact tests were performed to test for significance between proportions as required by the specific data sets for *Salmonella* status, sex, age, body weight, diet fed, antibiotic use in the home environment, previous hospital-visits, vaccination status, treatment outcome and *Salmonella* status upon discharge. A 5% level of significance was considered statistically significant for all comparisons. Statistical analysis was not performed on the data collected for CD positive animals due to the low prevalence of CD detection in this study. Only two animals in either cohort tested positive for CD.

Project Management

Experimental animals

All the animals involved in the study were client-owned dogs. The clients were informed of the nature of the study and were requested and then required to sign a consent form (Appendix A) before the animal was entered into the study. The apparently healthy cohort were dogs that had an appointment for routine prophylactic care, that matched the age interval of the study. The affected group were clinical cases that presented to the Outpatients clinic of the Onderstepoort Veterinary Academic Hospital and were diagnosed with parvoviral enteritis from the history, clinical examination and supporting canine parvovirus antigen ELISA or EM results. These dogs had to be admitted to the isolation ward.

Staff, laboratories, facilities, equipment and supplies

Staff

Project leader and Promoter: Dr CH Annandale (Department of Production Animal Studies, University of Pretoria, Pretoria, RSA)

Involvement:

- Project conception and design
- Data analysis
- Manuscript drafting

Primary Investigator: Dr WJ Botha (Department of Companion Animal Clinical Studies, University of Pretoria, Pretoria, RSA)

- Project design
- Protocol drafting
- Data collection
- Perform and interpret EIA for CD detection
- Data analysis
- Manuscript drafting
- Mini-dissertation drafting

Co-Investigator and Co-Promoter: Prof JP Schoeman (Department of Companion Animal Clinical Studies, University of Pretoria, Pretoria, RSA)

- Project design
- Data analysis
- Manuscript drafting

Co-Investigator: Dr Z Whitehead (Department of Companion Animal Clinical Studies, University of Pretoria, Pretoria, RSA)

- Project conception and design
- Data collection

Co-worker: Dr SL Marks (Department of Medicine and Epidemiology, University of California, Davis, School of Veterinary Medicine, Davis, USA)

- Project conception and design
- Data interpretation
- Manuscript drafting

Clinicians, nurses and students of the Isolation ward:

- Responsible for standard admission and treatment protocols regarding cases admitted to the isolation unit
- Monitoring and treatment of the cases in the isolation unit
- Contact primary investigator regarding management of cases enrolled into the project

Laboratories

Faecal EM processing and analysis were performed by the technicians of the Electron Microscopy unit at the Dept. of Anatomy & physiology, Faculty of Veterinary Science, University of Pretoria.

Faecal culture, organism isolation, identification and antimicrobial susceptibly testing using culture were performed by technicians of the Bacteriology Laboratory, Faculty of Veterinary Science, University of Pretoria.

Salmonella spp. serotyping were performed by the technicians of the General Bacteriology Laboratory, Agricultural Research Council - Onderstepoort Veterinary Institute, Agricultural Research Council.

Facilities

All the admitted dogs were housed in cages in the Small Animal Isolation Ward (See Figure 3), OVAH. Dogs included in the apparently healthy cohort were seen by the Outpatients, Small Animal Surgery and Blood Bank Clinics of the Onderstepoort Veterinary Academic Hospital.

Equipment and supplies

All equipment used belonged to the Faculty of Veterinary Science, University of Pretoria.

Figure 3 The isolation ward of the Onderstepoort Veterinary Academic Hospital where the study dogs affected by canine parvoviral enteritis were housed and treated.

Chapter 4

Results

Study prevalence of *Salmonella* spp. and *Clostridium difficile*

The study comprised of 74 CPV infected dogs and 42 apparently healthy dogs. The most significant finding of this study was the relatively high prevalence of *Salmonella* spp. in both cohorts (See Table 1). The prevalence of *Salmonella* spp. was 22% (n = 16/74) and 31% (n = 13/42) in infected and apparently healthy dogs, respectively, and the difference was not significant. The study did not identify any significant associations ($P < 0.05$) between the assessed historical and clinical variables and the isolation of *Salmonella* spp. All *Salmonella* spp. isolates were resistant to at least 3 antibiotics. The prevalence of CD was 3% (n = 2/74) and 5% (n = 2/42) in CPV-infected and apparently healthy dogs, respectively, and the small number of animals from which CD was detected (two from each cohort) precluded statistical analysis of these animals.

Organism	**Prevalence (Affected)**	**Prevalence (Healthy)**
Clostridium difficile	3%	5%
Salmonella spp.	22%	31%

Table 1 Prevalence of *Salmonella* spp. and *Clostridium difficile* recovered from 74 juvenile dogs infected with canine parvovirus and 42 apparently healthy age-matched controls.

Study population descriptors

The infected cohort comprised 45% (n = 33/74) female and 55% (n = 41/74) male dogs and the apparently healthy cohort 62% (n = 26/42) female and 38% (n = 16/42) male dogs. There was no significant difference in sex ratio between the groups (P=0.07), nor any association between sex and the isolation of *Salmonella* spp. (P = 0.623). The median age of both the infected and apparently healthy cohorts was 3 months (range: 6 weeks to 8 months). The median body weight for the infected and apparently healthy cohorts was 5.9kg (range = 0.8-30.8 kg) and 6.3kg (range = 1.9-22kg) respectively. Neither age (P = 0.241) nor body weight (P = 0.223) were significantly associated with the isolation of *Salmonella* spp.. Both cohorts comprised various breeds with the most common being mixed breed 19% (n = 14/74), American Pitbull terriers 15% (11/74) and Boerboels 14% (n = 10/74). The rest of the breeds

included 6 Jack Russell terriers, 5 Dachshunds, 4 Belgian Mallinois Shepherds, 4 Staffordshire Bullterriers, 3 Rottweilers and 3 Yorkshire terriers, 2 Labrador Retrievers, and one each of the following breeds: Maltese, Miniature Pinscher, Border Collie, German Shepherd Dog, Golden Retriever, Pekingese, Pomeranian, Pug, Rhodesian Ridgeback, Scottish terrier and Siberian Husky.

Possible risk factors and *Salmonella* spp. status

Of the CPV-infected cohort, 3% (n = 2/74) of dogs were fed home cooked diets, 68% (n = 50/74) store-bought commercial diets, 1% (n = 1/74) premium veterinary-specific diets, and 28% (n = 21/74) mixed diets. None of the dogs in the apparently healthy cohort were fed a home cooked diet and 45% (n = 19/42) were fed commercial diets, 33% (n = 14/42) premium diets and 22% (n = 9/42) mixed diets. Eleven percent (n = 8/74) of owners of the CPV-infected cohort indicated that antibiotics were being used at home at the time of presentation with none reporting antibiotic use in the apparently healthy cohort. Fifty-nine percent (n = 44/74) of the CPV-infected cohort and 22% of the apparently healthy cohort reported prior visits to a veterinary practice. The nature of these visits was not recorded for every individual. Of the 74 CPV-infected dogs, 3% (2/74) had had three vaccinations, 16% (n = 12/74) had had two vaccinations, 35% (n = 26/74) had had a single vaccination, and 46% (n = 34/74) had never been vaccinated. There was no significant association between the type of diet fed (P = 0.335), antibiotic use in the home environment (P = 0.483), previous hospital visits (P = 0.678) or previous vaccinations (P = 0.177) and the isolation of *Salmonella* spp..

Thirty-eight percent (n = 28/74) of the CPV-infected cohort had a follow-up faecal specimen collected at discharge and *Salmonella* spp. were isolated from 7% (n = 2/28). One dog was positive for the isolation of *Salmonella* spp. on both admission and discharge samples, and the second dog was positive on the discharge sample only. Additionally, three dogs that were positive for *Salmonella* spp. at admission were negative at discharge. None of these fecal samples were positive for *Clostridium difficile*.

The mortality rate in the CPV-infected cohort was 18% (n = 13/74), which was similar to the mortality rates for CPV infection in general and for CPV infected dogs from the same institution, in particular [99,105,107]. Five of these dogs were euthanized due to poor prognosis and 8 dogs died naturally. The median hospitalization duration was 5 days (range = 2-11).

No significant association between isolation of *Salmonella* spp and length of hospitalization (*P* = 0.72) or survival (P = 0.328) was identified in the CPV-infected cohort.

Antimicrobial susceptibility of *Salmonella* spp. isolates

All *Salmonella* spp. isolates (n = 32) were resistant to penicillin G, lincomycin and tylosin. Nine of the isolates were resistant to lincospectin and 21 showed intermediate (n = 20) or complete resistance (n = 1) to doxycycline/oxytetracycline. All the isolates were sensitive to amikacin, amoxicillin/ampicillin, enrofloxacin, gentamicin, trimethoprim sulphamethoxazole, chloramphenicol, cephalexin/cephalothin, orbifloxacin, amoxicillin clavulanic acid and polymixin B.

Salmonella spp. serotypes

Four serotypes were identified amongst the 32 isolates of *Salmonella* spp.. The serotype of 16 isolates could not be determined by the reference laboratory and seven could only be partially serotyped. A minimum of nine different serotypes is therefore considered to have been present. The serotyping results are listed in Table 2.

Serotype	**Number of isolates**
S. Heidelberg 4,5:r:1,2	4
Salm II 16:z:e,n,x	1
Salm II 30:b:z6	1
Salm Poly OMD	16
Salm II 4,5:z:1,5	1
S. Braenderup 6,7,14:e,h:e,n,z1	1
S. Chile 6,7:z:1,5	2
Salm II 18:z10:z6	4
S. Cotia 18:-:1,6	2

Table 2 Serotypes of *Salmonella* spp. recovered from 32 faecal isolates of juvenile dogs co-infected with canine parvovirus (total n= 16 /74) and an apparently healthy cohort of age-matched controls (total n= 13/42).

Salmonella spp. serotypes as identified by a reference laboratory using commercial antisera. Salm Poly OMD isolates could not be completely serotyped and Salm II isolates were only partially identified.

Chapter 5

Discussion

This study identified a prevalence of *Salmonella* spp. of 22% in CPV-infected dogs and 31% in apparently healthy dogs. This is in-line with that previously described for juvenile dogs[56] CD was detected in 3% of CPV-infected and 5% of apparently healthy dogs which is similar to that previously described for dogs from Canada[21,23,24]. The comparative prevalence of CD and *Salmonella* spp. was not statistically different between a cohort of dogs diagnosed and admitted for the treatment of canine parvoviral enteritis and a clinically healthy cohort. This study did not identify any risk factors of those included for the isolation of *Salmonella* spp..

CD has been isolated from healthy dogs at a prevalence of 0-10%[5,20,21,23]. The diagnosis of CD infection relies on demonstrating the presence of both CD and its associated toxins with supporting clinical findings in the absence of other possible causes[6]. PCR is employed to detect a common antigen (CD specific glutamate dehydrogenase (GDH)) produced by all CD strains and cell culture cytotoxicity assay (CTA) to detect TcdB[6]. However, these tests are not always universally and readily available to practitioners. The single membrane enzyme immunoassay employed in this study has been shown to be an adequate assay in humans when screening for CD, with a sensitivity of 78.3% and specificity of 100% when compared to PCR and cytotoxigenic culture on human fecal samples[127]. The same enzyme immunoassay has previously been employed for the detection of CD in several studies using canine faeces[128-130]. However, this test has not been validated in dogs and a previous study evaluating the use of enzyme immunoassays designed for use in humans was shown to have moderate-to-poor diagnostic sensitivity for the detection of toxin activity when used in dogs[39]. Nonetheless, the use of an enzyme immunoassay for the detection of the common GDH antigen is still considered the standard of care to demonstrate the presence of CD and should be combined with additional diagnostics (ELISA/CTA) for TcdA and TcdB to confirm toxigenicity[6].

Previous isolation of CD isolates commonly implicated in human CD infections from dog faeces have raised concerns for possible zoonotic transmission to humans[10,25]. However, no clear proof of interspecies transmission of CD has been documented to date and little is known about the significance of the detection of CD in dogs[6]. The reported prevalence of CD

in dogs ranges between 0-40% in healthy and diarrhoeic dogs[21,23,24] and the prevalence of CD identified in our study falls within this range. However, the lack of a standardized approach for the detection of CD and the variety of methods employed in the literature hampers the direct comparison of different reported prevalences. Previously identified risk factors for the detection of CD in dogs include increasing age, concurrent antibiotic usage and previous visits to human hospitals[21,25]. CD was detected in only four dogs in this study, which precluded investigation into possible risk factors.

The reported prevalence of *Salmonella* spp. in dogs ranges between 0-76%[48,131,132]. However, most non-diarrhoeic dogs have a reported prevalence below 4.4%[48,54,133,134]. Higher prevalence rates have been reported from dogs housed in a shelter, stray populations of strays, working dogs used at an abattoir or on farms, and hunting dogs[54,131,135,136]. The reported risk factors for the isolation of *Salmonella* spp. include contact with livestock, a multiple dog household, the use of a prebiotic within the last 30 days, administration of antibiotics, hospitalization, and the feeding of raw diets or treats including raw meat and eggs[137-139]. A German study reported a prevalence of 25% in dogs younger than 6 months of age compared to a prevalence of 5.2% in older dogs[56]. The prevalence of *Salmonella* spp. identified in this study supports the prevalence reported in juvenile dogs possibly have a higher prevalence of Salmonella spp., but failed to identify or sanction any of the previously reported risk factors. Murine studies have shown a 100,000-fold decrease in the 50% implantation dose for *Salmonella* colonization following the disruption of the intestinal microbiota by streptomycin treatment[140]. This would suggest that all juvenile animals may have a greater susceptibility to *Salmonella* spp. colonization associated with the lack of a well-established intestinal microbiota[67]. However, CPV-infected dogs would be expected to suffer from a greater degree of dysbiosis compared to healthy individuals, as a major reason for the higher prevalence of *Salmonella* in juvenile animals when compared to adult animals.

Although not statistically significant, the apparently healthy group did have a higher prevalence of *Salmonella* spp.. *Salmonella* spp. are facultative intracellular organisms with an affinity for the specialized epithelial cells (M cells) overlying intestinal lymphoid tissue[141]. After passing through the M cells, *Salmonella* spp. infect the underlying mononuclear phagocytes of the gut associated lymph tissue and then replicate and spread via the

reticuloendothelial system[67]. On the other hand, CPV enteritis is associated with severe gastrointestinal epithelium loss, widespread loss of lymphocytes and lymphoid tissue involution[142]. This would suggest that CPV infection destroys the cells necessary for *Salmonella* spp. colonization and possibly explains the lower prevalence of *Salmonella* spp. documented in the CPV-infected cohort in our study.

The transmission of *Salmonella* spp. is thought to occur most likely via the fecal-oral route[143]. Dogs, and particularly young dogs, have indiscriminate eating habits and are more likely to consume contaminated feed, water or faeces and this may likely explain the higher prevalence found in young dogs. Feed sources such as offal, raw meat and sometimes commercial dog feed have been found to be contaminated with *Salmonella* spp. and may serve as possible sources of exposure[90,144,145]. Interestingly, in our study most dogs were fed solely commercial or premium pelleted diets and only 28% and 22% of dogs in the CPV-infected and apparently healthy cohorts, respectively, were fed chicken, pet mince or table scraps in addition to their staple diet. One dog was fed a diet of raw meat only and *Salmonella* spp. was not isolated from this dog. Most dogs from which *Salmonella* spp. was isolated were fed a commercial diet (41%; 13/32), followed by a mixed diet (28%; 9/32), with few being fed premium diets (16%; 5/32). Consequently, further studies may be indicated to evaluate commercial diets as a possible source of exposure. Natural treats and chews have been implicated as a possible source of exposure to both pets and owners[146]. Unfortunately, the use of these products in our population was not assessed, but may serve as an additional source due to their frequency of use in puppies. Juvenile dogs may further have increased exposure via coprophagia, contact with wildlife species and ingestion of carrion, considering their inquisitive nature.

Salmonellosis is considered an important nosocomial disease in large-animal veterinary hospitals[80]. The use of targeted environmental surveillance is central to the monitoring and management of *Salmonella* spp. in large-animal veterinary hospitals to prevent and mitigate nosocomial infections and outbreaks[80]. The persistent isolation of *Salmonella* spp. during targeted environmental surveillance of the OVAH small animal isolation ward, suggested that the population of dogs housed in this environment may be a persistent possible source of environmental contamination. Contamination of this area was thought to then act as a nidus of infection and consequent spread to other parts of the hospital. In this study, *S.*

Heidelberg was the only serotype also recovered from the environment in the large-animal section within the same facility[80]. This finding suggests that there is no significant cross contamination between the two sections of the hospital. However, the relatively high prevalence of *Salmonella* spp. in juvenile dogs may raise concern for possible contamination by this population of patients within the small animal hospital. Further studies are needed to determine the significance of this notion, especially considering that targeted environmental surveillance for *Salmonella* spp. may not be as stringent as that in large animal hospitals.

S. braenderup was isolated from one dog from the CPV-infected cohort and has previously been implicated in a 1959 report, in an outbreak of salmonellosis in humans in South Africa[147]. The isolation of this serotype raised concerns for possible transmission to the students and personnel treating these patients and emphasizes the need of strict hygiene practice. None of the serotypes isolated in our study were also identified in a previous study reporting *Salmonella* serotypes in dogs [i]from South Africa (See Table 1). However, the inferences made regarding the serotypes recovered in this study were hampered by the relatively large number of unidentified serotypes recovered. Further studies using alternative typing methods such as multilocus sequence typing will be needed to better clarify the possible clinical implications of the serotypes recovered.

All *Salmonella* spp. isolates in this study were resistant to at least three antibiotics. This prevalence of resistance amongst the isolates in this study is higher than that previously reported for isolates from dogs[48]. All the isolates were resistant to tylosin, lincospectin and penicillin G. Resistance to tylosin is unsurprising considering their limited efficacy against gram-negative bacteria[148]. Lincospectin is not commonly used in small animal practice, hence, resistance to these antibiotics are of little clinical significance. Despite the fact that all isolates were resistant to penicillin G, no resistance was reported to other beta-lactam antibiotics commonly used in practice. In conclusion, none of these antibiotics are routinely used in the empirical treatment of suspected salmonellosis and, therefore, these resistance patterns are unlikely to have therapeutic implications. However, a few isolates did show intermediate resistance to doxycycline, which may need to be closely monitored in the future.

Salmonella spp. were identified from two CPV-recovered dogs at discharge. No *Salmonella* sp. was isolated in the one dog at admission and the second dog had *Salmonella* spp. isolated at both admission and discharge. Possible explanations for the negative isolation of *Salmonella* spp. at admission and positive isolation at discharge include sampling or isolation error or colonization during the hospitalization. The use of antimicrobials in the treatment of CPV may aid in the colonization of *Salmonella* spp. by transiently disrupting the normal microbiota and weakening the colonization resistance offered by these microbes. However, in both cases with positive *Salmonella* spp. isolated at discharge, antibiotic therapy was in effect unsuccessful in preventing colonization or clearing the dog from *Salmonella* spp. despite sensitive susceptibility patterns being reported to the antibiotics routinely used in the treatment of CPV cases. However, this is again negated by the three dogs that were positive for isolation of *Salmonella* spp. at admission and were negative for isolation of *Salmonella* spp. at discharge.

Study Limitations

There were several limitations to this study. Firstly, dogs diagnosed with CPV infection and treated on an outpatient-basis were not included in this study. Therefore, the prevalence of CD and *Salmonella* spp. identified in this study may not reflect that for the whole population of juvenile dogs infected with CPV. In addition, the study of CPV-infected dogs that were hospitalized and treated introduced a potential population bias reflecting dogs that were owned by people who were able to afford the costs of hospitalization and treatment. Secondly, the inherent limitations of diagnostic testing of CD in dogs utilizing human-based immunoassays was an unavoidable limitation and reflects the current status of testing of CD in veterinary reference laboratories world-wide. Thirdly, the relatively small number of dogs in which *Salmonella* spp. was isolated limited the investigators' ability to assess risk factors for this bacterial enteropathogen.

Conclusion

The prevalence of *Clostridium difficile* in the CPV and healthy juvenile dogs was similarly very low and insufficient for statistical analysis. The prevalence of *Salmonella* spp. in juvenile dogs infected with CPV (22%) was not statistically different from that in an apparently healthy cohort (31%) from a matching age range. However, the prevalence in both groups was considerably higher than that commonly reported in adult dogs and parallels previous reports in young dogs, shelter dogs, or dogs fed a raw meat diet. Of the nine *Salmonella* serotypes identified from 32 isolates, there were several with variable resistance to a range of antibiotics including penicillin G, lincomycin, tylosin, lincospectin, and doxycycline/oxytetracycline. To the authors' knowledge, this is the first report of the prevalence of CD and *Salmonella* spp. in dogs diagnosed with canine parvoviral enteritis and only the second to focus on a population of juvenile dogs.

References

1. Bouza E, Munoz P, Alonso R. Clinical manifestations, treatment and control of infections caused by Clostridium difficile. Clinical Microbiology and Infection 2005;11:57-64.

2. Madewell BR, Tang YJ, Jang S, et al. Apparent outbreaks of Clostridium difficile-associated diarrhea in horses in a veterinary medical teaching hospital. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 1995;7:343-346.

3. Perk J, Cosmetatos I, Gallusser A, et al. Clostridium difficile associated with typhlocolitis in an adult horse. Journal of veterinary diagnostic investigation 1993;5:99-101.

4. Cave NJ, Marks SL, Kass PH, et al. Evaluation of a routine diagnostic fecal panel for dogs with diarrhea. Journal of the American Veterinary Medical Association 2002;221:52-59.

5. al Saif N, Brazier JS. The distribution of Clostridium difficile in the environment of South Wales. Journal of medical microbiology 1996;45:133-137.

6. Marks SL, Rankin SC, Byrne BA, et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2011;25:1195-1208.

7. Weese JS, Weese HE, Bourdeau TL, et al. Suspected Clostridium difficile-associated diarrhea in two cats. Journal of the American Veterinary Medical Association 2001;218:1436-1439, 1421.

8. Frazier KS, Herron AJ, Hines ME, 2nd, et al. Diagnosis of enteritis and enterotoxemia due to Clostridium difficile in captive ostriches (Struthio camelus). Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 1993;5:623-625.

9. Weese JS, Armstrong J. Outbreak of Clostridium difficile-associated disease in a small animal veterinary teaching hospital. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2003;17:813-816.

10. Lefebvre SL, Arroyo LG, Weese JS. Epidemic Clostridium difficile strain in hospital visitation dog. Emerging infectious diseases 2006;12:1036-1037.

11. Lefebvre SL, Weese JS. Contamination of pet therapy dogs with MRSA and Clostridium difficile. The Journal of hospital infection 2009;72:268-269.

12. Pepin J, Valiquette L, Alary ME, et al. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne 2004;171:466-472.

13. Valiquette L, Low DE, Pepin J, et al. Clostridium difficile infection in hospitals: a brewing storm. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne 2004;171:27-29.

14. Keeley AJ, Beeching NJ, Stott KE, et al. Clostridium difficile: A healthcare-associated infection of unknown significance in adults in sub-Saharan Africa. Malawi Medical Journal 2016;28:66-69.

15. Rajabally N, Pentecost M, Whitelaw A, et al. The Clostridium difficile problem: A South African tertiary institution's prospective perspective. South African Medical Journal 2013;103:168-172.

16. Hubert B, Loo VG, Bourgault AM, et al. A portrait of the geographic dissemination of the Clostridium difficile North American pulsed-field type 1 strain and the epidemiology of C. difficile-associated disease in Quebec. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2007;44:238-244.

17. Murphy CP, Reid-Smith RJ, Boerlin P, et al. Escherichia coli and selected veterinary and zoonotic pathogens isolated from environmental sites in companion animal veterinary hospitals in southern Ontario. The Canadian veterinary journal La revue veterinaire canadienne 2010;51:963-972.

18. Kullin B, Brock T, Rajabally N, et al. Characterisation of Clostridium difficile strains isolated from Groote Schuur Hospital, Cape Town, South Africa. European Journal of Clinical Microbiology & Infectious Diseases 2016;35:1709-1718.

19. Gould LH, Limbago B. Clostridium difficile in food and domestic animals: a new foodborne pathogen? Clinical Infectious Diseases 2010;51:577-582.

20. Perrin J, Buogo C, Gallusser A, et al. Intestinal carriage of Clostridium difficile in neonate dogs. Zentralblatt fur Veterinarmedizin Reihe B Journal of veterinary medicine Series B 1993;40:222-226.

21. Struble AL, Tang YJ, Kass PH, et al. Fecal shedding of Clostridium difficile in dogs: a period prevalence survey in a veterinary medical teaching hospital. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 1994;6:342-347.

22. Weese JS. Bacterial enteritis in dogs and cats: diagnosis, therapy, and zoonotic potential. The Veterinary clinics of North America Small animal practice 2011;41:287-309.

23. Weese JS, Staempfli HR, Prescott JF, et al. The roles of Clostridium difficile and enterotoxigenic Clostridium perfringens in diarrhea in dogs. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2001;15:374-378.

24. Riley TV, Adams JE, O'Neill GL, et al. Gastrointestinal carriage of Clostridium difficile in cats and dogs attending veterinary clinics. Epidemiology and infection 1991;107:659-665.

25. Lefebvre SL, Waltner-Toews D, Peregrine AS, et al. Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. Journal of Hospital Infection 2006;62:458-466.

26. Clooten J, Kruth S, Arroyo L, et al. Prevalence and risk factors for Clostridium difficile colonization in dogs and cats hospitalized in an intensive care unit. Veterinary microbiology 2008;129:209-214.

27. Weese JS, Finley R, Reid-Smith RR, et al. Evaluation of Clostridium difficile in dogs and the household environment. Epidemiology and infection 2010;138:1100-1104.

28. Hutin Y, Casin I, Lesprit P, et al. Prevalence of and risk factors for Clostridium difficile colonization at admission to an infectious diseases ward. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 1997;24:920-924.

29. Kyne L, Warny M, Qamar A, et al. Asymptomatic carriage of Clostridium difficile and serum levels of IgG antibody against toxin A. The New England journal of medicine 2000;342:390-397.

30. Shim JK, Johnson S, Samore MH, et al. Primary symptomless colonisation by Clostridium difficile and decreased risk of subsequent diarrhoea. Lancet 1998;351:633-636.

31. Weese JS, Staempfli HR, Prescott JF. Isolation of environmental Clostridium difficile from a veterinary teaching hospital. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2000;12:449-452.

32. Berry AP, Levett PN. Chronic diarrhoea in dogs associated with Clostridium difficile infection. The Veterinary record 1986;118:102-103.

33. Gröschel DH, Toye B. Clostridium difficile infection. Critical reviews in clinical laboratory sciences 1996;33:203-245.

34. Alfa MJ, Kabani A, Lyerly D, et al. Characterization of a toxin A-negative, toxin B-positive strain of Clostridium difficile responsible for a nosocomial outbreak of Clostridium difficile-associated diarrhea. Journal of clinical microbiology 2000;38:2706-2714.

35. Just I, Wilm M, Selzer J, et al. The enterotoxin from Clostridium difficile (ToxA) monoglucosylates the Rho proteins. Journal of Biological Chemistry 1995;270:13932-13936.

36. Mitchell T, Ketley J, Haslam S, et al. Effect of toxin A and B of Clostridium difficile on rabbit ileum and colon. Gut 1986;27:78-85.

37. Marks SL, Kather EJ, Kass PH, et al. Genotypic and phenotypic characterization of Clostridium perfringens and Clostridium difficile in diarrheic and healthy dogs. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2002;16:533-540.

38. Clooten JK, Kruth SA, Weese JS. Genotypic and phenotypic characterization of Clostridium perfringens and Clostridium difficile in diarrheic and healthy dogs. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2003;17:123; author reply 123.

39. Chouicha N, Marks SL. Evaluation of five enzyme immunoassays compared with the cytotoxicity assay for diagnosis of Clostridium difficile-associated diarrhea in dogs. Journal of

veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2006;18:182-188.

40. Landry ML, Topal J, Ferguson D, et al. Evaluation of biosite triage Clostridium difficile panel for rapid detection of Clostridium difficile in stool samples. Journal of clinical microbiology 2001;39:1855-1858.

41. Arroyo LG, Kruth SA, Willey BM, et al. PCR ribotyping of Clostridium difficile isolates originating from human and animal sources. Journal of medical microbiology 2005;54:163-166.

42. Rupnik M. Is Clostridium difficile-associated infection a potentially zoonotic and foodborne disease? Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2007;13:457-459.

43. Lefebvre SL, Reid-Smith RJ, Waltner-Toews D, et al. Incidence of acquisition of methicillin-resistant Staphylococcus aureus, Clostridium difficile, and other health-care-associated pathogens by dogs that participate in animal-assisted interventions. Journal of the American Veterinary Medical Association 2009;234:1404-1417.

44. Prittie J. Canine parvoviral enteritis: a review of diagnosis, management, and prevention. Journal of Veterinary Emergency and Critical Care 2004;14:167-176.

45. Brenner FW, Villar RG, Angulo FJ, et al. Salmonella nomenclature. Journal of clinical microbiology 2000;38:2465-2467.

46. Lefebvre SL, Reid-Smith R, Boerlin P, et al. Evaluation of the risks of shedding Salmonellae and other potential pathogens by therapy dogs fed raw diets in Ontario and Alberta. Zoonoses and public health 2008;55:470-480.

47. Sykes JE, Marks SL. Chapter 45 - Salmonellosis. In: Sykes JE, ed. Canine and Feline Infectious Diseases. Saint Louis: W.B. Saunders; 2014:437-444.

48. Seepersadsingh N, Adesiyun AA, Seebaransingh R. Prevalence and antimicrobial resistance of Salmonella spp. in non-diarrhoeic dogs in Trinidad. Journal of veterinary medicine B, Infectious diseases and veterinary public health 2004;51:337-342.

49. Fukata T, Naito F, Yoshida N, et al. Incidence of Salmonella infection in healthy dogs in Gifu Prefecture, Japan. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 2002;64:1079-1080.

50. Kozak M, Horosova K, Lasanda V, et al. Do dogs and cats present a risk of transmission of salmonellosis to humans? Bratislavske lekarske listy 2003;104:323-328.

51. Weber A, Wachowitz R, Weigl U, et al. [Occurrence of Salmonella in fecal samples of dogs and cats in northern Bavaria from 1975 to 1994]. Berliner und Munchener tierarztliche Wochenschrift 1995;108:401-404.

52. Hackett T, Lappin MR. Prevalence of enteric pathogens in dogs of north-central Colorado. Journal of the American Animal Hospital Association 2003;39:52-56.

53. Procter TD, Pearl DL, Finley RL, et al. A cross-sectional study examining Campylobacter and other zoonotic enteric pathogens in dogs that frequent dog parks in three cities in south-western Ontario and risk factors for shedding of Campylobacter spp. Zoonoses and public health 2014;61:208-218.

54. Shimi A, Keyhani M, Bolurchi M. Salmonellosis in apparently healthy dogs. The Veterinary record 1976;98:110-111.

55. Marks SL, Kather EJ. Bacterial-associated diarrhea in the dog: a critical appraisal. The Veterinary clinics of North America Small animal practice 2003;33:1029-1060.

56. Förster D, Holland U, Tesfamariam H. Occurrence of canine Salmonella infections. Zentralblatt fur Veterinarmedizin Reihe B Journal of veterinary medicine Series B 1974;21:124.

57. Cantor GH, Nelson S, Jr., Vanek JA, et al. Salmonella shedding in racing sled dogs. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 1997;9:447-448.

58. McKenzie E, Riehl J, Banse H, et al. Prevalence of Diarrhea and Enteropathogens in Racing Sled Dogs. Journal of Veterinary Internal Medicine 2010;24:97-103.

59. Morley PS, Strohmeyer RA, Tankson JD, et al. Evaluation of the association between feeding raw meat and Salmonella enterica infections at a Greyhound breeding facility. Journal of the American Veterinary Medical Association 2006;228:1524-1532.

60. Spain CV, Scarlett JM, Wade SE, et al. Prevalence of enteric zoonotic agents in cats less than 1 year old in central New York State. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2001;15:33-38.

61. Van Immerseel F, Pasmans F, De Buck J, et al. Cats as a risk for transmission of antimicrobial drug-resistant Salmonella. Emerging infectious diseases 2004;10:2169-2174.

62. Stavisky J, Radford AD, Gaskell R, et al. A case–control study of pathogen and lifestyle risk factors for diarrhoea in dogs. Preventive Veterinary Medicine 2011;99:185-192.

63. Murphy C, Reid-Smith RJ, Prescott JF, et al. Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. The Canadian veterinary journal La revue veterinaire canadienne 2009;50:1047-1053.

64. Leonard EK, Pearl DL, Finley RL, et al. Comparison of antimicrobial resistance patterns of Salmonella spp. and escherichia coli recovered from pet dogs from volunteer households in Ontario (2005-06). Journal of Antimicrobial Chemotherapy 2012;67:174-181.

65. Mackel DC, Galton MM, Gray H, et al. Salmonellosis in dogs. IV. Prevalence in normal dogs and their contacts. The Journal of infectious diseases 1952;91:15-18.

66. Ball MR. Salmonella in dogs and cats of the Los Angeles, Honolulu, and Bermuda areas. Journal of the American Veterinary Medical Association 1951;118:164-166.

67. Wray C, Wray A. Salmonella in domestic animalsCabi; 2000.

68. Leonard F. Salmonella infection and carriage: the importance of dogs and their owners. The Veterinary record 2014;174:92-93.

69. Philbey AW, Mather HA, Gibbons JF, et al. Serovars, bacteriophage types and antimicrobial sensitivities associated with salmonellosis in dogs in the UK (1954-2012). The Veterinary record 2014;174:94.

70. Moazed T, Deeb B, DiGiacomo R. Subcutaneous abscess due to Salmonella adelaide in a grey collie with cyclic hematopoiesis. Laboratory animal science 1990;40:639.

71. McVey DS, Chengappa MM, Mosier DE, et al. Immunogenicity of chi4127 phoP-Salmonella enterica serovar Typhimurium in dogs. Vaccine 2002;20:1618-1623.

72. Venter B. Epidemiology of salmonellosis in dogs--a conceptual model. Acta veterinaria Scandinavica Supplementum 1988;84:333.

73. Allison CJ. The dog as a symptomless carrier of Salmonella typhimurium. The Veterinary record 1969;85:564.

74. Morse EV, Duncan MA. Canine salmonellosis: prevalence, epizootiology, signs, and public health significance. Journal of the American Veterinary Medical Association 1975;167:817-820.

75. Grassl GA, Finlay BB. Pathogenesis of enteric Salmonella infections. Current opinion in gastroenterology 2008;24:22-26.

76. Delaloye J, Merlani G, Petignat C, et al. Nosocomial nontyphoidal salmonellosis after antineoplastic chemotherapy: reactivation of asymptomatic colonization? Eur J Clin Microbiol Infect Dis 2004;23:751-758.

77. Lomborg SR, Agerholm JS, Jensen AL, et al. Effects of experimental immunosuppression in cattle with persistently high antibody levels to Salmonella Dublin lipopolysaccharide O-antigens. BMC veterinary research 2007;3:17.

78. Redwood D, Bell D. Salmonella panama: isolation from aborted and newborn canine fetuses. Veterinary Record 1983;112:362-362.

79. Bagcigil AF, Ikiz S, Dokuzeylu B, et al. Fecal shedding of Salmonella spp. in dogs. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 2007;69:775-777.

80. Lyle CH, Annandale CH, Gouws J, et al. Comparison of two culture techniques used to detect environmental contamination with Salmonella enterica in a large-animal hospital. Journal of the South African Veterinary Association 2015;86:01-05.

81. Schuurman T, de Boer RF, van Zanten E, et al. Feasibility of a molecular screening method for detection of Salmonella enterica and Campylobacter jejuni in a routine community-based clinical microbiology laboratory. Journal of clinical microbiology 2007;45:3692-3700.

82. van Duijkeren E, Flemming C, Sloet van Oldruitenborgh-Oosterbaan M, et al. Diagnosing salmonellosis in horses. Culturing of multiple versus single faecal samples. The Veterinary quarterly 1995;17:63-66.

83. Ward MP, Alinovi CA, Couetil LL, et al. Evaluation of a PCR to detect Salmonella in fecal samples of horses admitted to a veterinary teaching hospital. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2005;17:118-123.

84. Wattiau P, Boland C, Bertrand S. Methodologies for Salmonella enterica subsp. enterica subtyping: gold standards and alternatives. Applied and environmental microbiology 2011;77:7877-7885.

85. Kocabiyik AL, Cetin C, Dedicova D. Detection of Salmonella spp. in stray dogs in Bursa Province, Turkey: first isolation of Salmonella Corvallis from dogs. Journal of veterinary medicine B, Infectious diseases and veterinary public health 2006;53:194-196.

86. Cherry B, Burns A, Johnson GS, et al. Salmonella Typhimurium outbreak associated with veterinary clinic. Emerging infectious diseases 2004;10:2249-2251.

87. Rice DH, Hancock DD, Roozen PM, et al. Household contamination with Salmonella enterica. Emerging infectious diseases 2003;9:120-122.

88. Outbreaks of multidrug-resistant Salmonella typhimurium associated with veterinary facilities--Idaho, Minnesota, and Washington, 1999. MMWR Morbidity and mortality weekly report 2001;50:701-704.

89. Wright JG, Tengelsen LA, Smith KE, et al. Multidrug-resistant Salmonella Typhimurium in four animal facilities. Emerging infectious diseases 2005;11:1235-1241.

90. Behravesh CB, Ferraro A, Deasy M, 3rd, et al. Human Salmonella infections linked to contaminated dry dog and cat food, 2006-2008. Pediatrics 2010;126:477-483.

91. Imanishi M, Rotstein DS, Reimschuessel R, et al. Outbreak of Salmonella enterica serotype Infantis infection in humans linked to dry dog food in the United States and Canada, 2012. Journal of the American Veterinary Medical Association 2014;244:545-553.

92. Ewart SL, Schott HC, 2nd, Robison RL, et al. Identification of sources of Salmonella organisms in a veterinary teaching hospital and evaluation of the effects of disinfectants on detection of Salmonella organisms on surface materials. Journal of the American Veterinary Medical Association 2001;218:1145-1151.

93. Alinovi CA, Ward MP, Couetil LL, et al. Detection of Salmonella organisms and assessment of a protocol for removal of contamination in horse stalls at a veterinary teaching hospital. Journal of the American Veterinary Medical Association 2003;223:1640-1644.

94. Hartmann FA, Callan RJ, McGuirk SM, et al. Control of an outbreak of salmonellosis caused by drug-resistant Salmonella anatum in horses at a veterinary hospital and measures to prevent future infections. Journal of the American Veterinary Medical Association 1996;209:629-631.

95. Ketaren K, Brown J, Shotts EB, et al. Canine salmonellosis in a small animal hospital. Journal of the American Veterinary Medical Association 1981;179:1017-1018.

96. Timoney JF, Neibert HC, Scott FW. Feline salmonellosis. A nosocomial outbreak and experimental studies. The Cornell veterinarian 1978;68:211-219.

97. Hueffer K, Parker JSL, Weichert WS, et al. The Natural Host Range Shift and Subsequent Evolution of Canine Parvovirus Resulted from Virus-Specific Binding to the Canine Transferrin Receptor. Journal of Virology 2003;77:1718-1726.

98. Bird L, Tappin S. Canine parvovirus: where are we in the 21st Century? Companion Animal 2013;18:142-146.

99. Schoeman JP, Goddard A, Leisewitz AL. Biomarkers in canine parvovirus enteritis. New Zealand veterinary journal 2013;61:217-222.

100. Nappert G, Dunphy E, Ruben D, et al. Determination of serum organic acids in puppies with naturally acquired parvoviral enteritis. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire 2002;66:15-18.

101. Steinel A, Venter EH, Van Vuuren M, et al. Antigenic and genetic analysis of canine parvoviruses in southern Africa. 1998.

102. Dogonyaro BB, Bosman A-M, Sibeko KP, et al. Genetic analysis of the VP2-encoding gene of canine parvovirus strains from Africa. Veterinary microbiology 2013;165:460-465.

103. Wilson S, Illambas J, Siedek E, et al. Vaccination of dogs with canine parvovirus type 2b (CPV-2b) induces neutralising antibody responses to CPV-2a and CPV-2c. Vaccine 2014;32:5420-5424.

104. Wilson S, Stirling C, Borowski S, et al. Vaccination of dogs with Duramune DAPPi+LC protects against pathogenic canine parvovirus type 2c challenge. The Veterinary record 2013;172:662.

105. Goddard A, Leisewitz AL, Christopher MM, et al. Prognostic usefulness of blood leukocyte changes in canine parvoviral enteritis. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2008;22:309-316.

106. McClure V, van Schoor M, Thompson PN, et al. Evaluation of the use of serum C-reactive protein concentration to predict outcome in puppies infected with canine parvovirus. Journal of the American Veterinary Medical Association 2013;243:361-366.

107. Schoeman JP, Herrtage ME. Serum thyrotropin, thyroxine and free thyroxine concentrations as predictors of mortality in critically ill puppies with parvovirus infection: a model for human paediatric critical illness? Microbes and Infection 2008;10:203-207.

108. Mann F, Boon G, Wagner-Mann C, et al. Ionized and total magnesium concentrations in blood from dogs with naturally acquired parvoviral enteritis. Journal of the American Veterinary Medical Association 1998;212:1398-1401.

109. Pollock R, Coyne M. Canine parvovirus. The Veterinary clinics of North America Small animal practice 1993;23:555-568.

110. SmithCarr S, Macintire DK, Swango LJ. Canine parvovirus. 1. Pathogenesis and vaccination. Compendium on Continuing Education for the Practicing Veterinarian 1997;19:125-&.

111. Jeoung S-Y, Kim D, Ahn S-J, et al. Epidemiological observation on recent outbreaks of canine parvoviral enteritis in Korea. The Korean Journal of Veterinary Clinical Medicin 2006;23:223-229.

112. Mohr AJ, Leisewitz AL, Jacobson LS, et al. Effect of early enteral nutrition on intestinal permeability, intestinal protein loss, and outcome in dogs with severe parvoviral enteritis. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 2003;17:791-798.

113. Houston DM, Ribble CS, Head LL. Risk factors associated with parvovirus enteritis in dogs: 283 cases (1982-1991). Journal of the American Veterinary Medical Association 1996;208:542-546.

114. Hoskins JD. Update on canine parvoviral enteritis. Veterinary medicine (1985)(USA) 1997.

115. Kalli I, S Leontides L, E Mylonakis M, et al. Factors affecting the occurrence, duration of hospitalization and final outcome in canine parvovirus infection. Research in veterinary science 2010;89:174-178.

116. Ling M, Norris JM, Kelman M, et al. Risk factors for death from canine parvoviral-related disease in Australia. Veterinary microbiology 2012;158:280-290.

117. Rewerts J, McCaw D, Cohn L, et al. Recombinant human granulocyte colony-stimulating factor for treatment of puppies with neutropenia secondary to canine parvovirus infection. Journal of the American Veterinary Medical Association 1998;213:991-992.

118. Johnson R, Smith J. Epidemiology and pathogenesis of canine parvovirus. In: Australian Veterinary Practitioner 1983;31-31.

119. Otto CM, Rieser TM, Brooks MB, et al. Evidence of hypercoagulability in dogs with parvoviral enteritis. Journal of the American Veterinary Medical Association 2000;217:1500-1504.

120. de Mari K, Maynard L, Eun HM, et al. Treatment of canine parvoviral enteritis with interferon-omega in a placebo-controlled field trial. The Veterinary record 2003;152:105-108.

121. Turk J, Miller M, Brown T, et al. Coliform septicemia and pulmonary disease associated with canine parvoviral enteritis: 88 cases (1987-1988). Journal of the American Veterinary Medical Association 1990;196:771-773.

122. Otto CM, Drobatz KJ, Soter C. Endotoxemia and tumor necrosis factor activity in dogs with naturally occurring parvoviral enteritis. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 1997;11:65-70.

123. Turk J, Fales W, Miller M, et al. Enteric Clostridium perfringens infection associated with parvoviral enteritis in dogs: 74 cases (1987-1990). Journal of the American Veterinary Medical Association 1992;200:991-994.

124. Sandstedt K, Wierup M. Concomitant occurrence ofCampylobacter and parvoviruses in dogs with gastroenteritis. Veterinary research communications 1980;4:271-273.

125. Lobetti RG, Joubert KE, Picard J, et al. Bacterial colonization of intravenous catheters in young dogs suspected to have parvoviral enteritis. Journal of the American Veterinary Medical Association 2002;220:1321-1324.

126. Bauer A, Kirby W, Sherris JC, et al. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology 1966;45:493.

127. Quinn CD, Sefers SE, Babiker W, et al. C. Diff Quik Chek complete enzyme immunoassay provides a reliable first-line method for detection of Clostridium difficile in stool specimens. Journal of clinical microbiology 2010;48:603-605.

128. Orden C, Blanco JL, Álvarez-Pérez S, et al. Isolation of Clostridium difficile from dogs with digestive disorders, including stable metronidazole-resistant strains. Anaerobe 2016.

129. Álvarez-Pérez S, Blanco J, Peláez T, et al. Faecal shedding of antimicrobial-resistant Clostridium difficile strains by dogs. Journal of Small Animal Practice 2015;56:190-195.

130. Álvarez-Pérez S, Blanco JL, Harmanus C, et al. Prevalence and characteristics of Clostridium perfringens and Clostridium difficile in dogs and cats attended in diverse veterinary clinics from the Madrid region. Anaerobe 2017.

131. Khan A. Salmonella infections in dogs and cats in the Sudan. British Veterinary Journal 1970;126:607-612.

132. Stone GG, Chengappa M, Oberst RD, et al. Application of polymerase chain reaction for the correlation of Salmonella serovars recovered from greyhound feces with their diet. Journal of Veterinary Diagnostic Investigation 1993;5:378-385.

133. Timbs D, Carter ME, Davis G, et al. The Salmonella excretor incidence of dogs in Hawke's Bay. New Zealand veterinary journal 1975;23:54-56.

134. Adesiyun A, Campbell M, Kaminjolo J. Prevalence of bacterial enteropathogens in pet dogs in Trinidad. Zoonoses and public health 1997;44:19-27.

135. Stucker CL, Galton MM, Cowdery J, et al. Salmonellosis in Dogs: II. Prevalence and Distribution in Greyhounds in Florida. The Journal of infectious diseases 1952:6-11.

136. Frost A, Eaton N, Gilchrist D, et al. The incidence of Salmonella infection in the dog. Australian veterinary journal 1969;45:109-110.

137. Leonard E, Pearl D, Finley R, et al. Evaluation of pet-related management factors and the risk of Salmonella spp. carriage in pet dogs from volunteer households in Ontario (2005–2006). Zoonoses and public health 2011;58:140-149.

138. Uhaa I, Hird D, Hirsh D, et al. Case-control study of risk factors associated with nosocomial Salmonella krefeld infection in dogs. American journal of veterinary research 1988;49:1501-1505.

139. Reimschuessel R, Grabenstein M, Guag J, et al. Multilaboratory survey to evaluate Salmonella prevalence in diarrheic and nondiarrheic dogs and cats in the United States between 2012 and 2014. Journal of clinical microbiology 2017;55:1350-1368.

140. Que JU, Hentges D. Effect of streptomycin administration on colonization resistance to Salmonella typhimurium in mice. Infection and immunity 1985;48:169-174.

141. Kohbata S, Yokoyama H, Yabuuchi E. Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. Microbiology and immunology 1986;30:1225-1237.

142. Meunier PC, Cooper BJ, Appel MJG, et al. Pathogenesis of Canine Parvovirus Enteritis: The Importance of Viremia. Veterinary pathology 1985;22:60-71.

143. Tanaka Y, Katsube Y, Imaizumi K. Experimental carrier in dogs produced by oral administration of Salmonella typhimurium. Japanese Journal of Veterinary Science 1976.

144. Chengappa M, Staats J, Oberst R, et al. Prevalence of Salmonella in raw meat used in diets of racing greyhounds. Journal of Veterinary Diagnostic Investigation 1993;5:372-377.

145. Selmi M, Stefanelli S, Bilei S, et al. Contaminated commercial dehydrated food as source of multiple Salmonella serotypes outbreak in a municipal kennel in Tuscany. Vet Ital 2011;47:175-190.

146. Finley R, Reid-Smith R, Weese JS, et al. Human health implications of Salmonella-contaminated natural pet treats and raw pet food. Clinical Infectious Diseases 2006;42:686-691.

147. Bokkenheuser M. A review of salmonellosis in South Africa. South African Medical Journal 1959;33:702-706.

148. Kim HB, Singer RS, Borewicz K, et al. Effects of tylosin administration on C-reactive protein concentration and carriage of Salmonella enterica in pigs. American journal of veterinary research 2014;75:460-467.

Addendums

Appendix A: University of Pretoria Animal Ethics Approval Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The prevalence of Clostridium difficile and Salmonella spp in juvenile dogs infected with parvoviral enteritis**
PROJECT NUMBER	**V091-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. WJ Botha**

STUDENT NUMBER (where applicable)	**UP_04415957**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

A copy of the Informed Consent Form is required

ANIMAL SPECIES	**Canine**	
NUMBER OF ANIMALS	**250**	
Approval period to use animals for research/testing purposes		**October 2015-October 2016**
SUPERVISOR	**Prof. J Schoeman**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	.28 September 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

Appendix B: Data collection and consent forms

Consent form for control dogs – parvoviral enteritis study

PATIENT LABEL

Your dog has been selected to serve as a healthy control dog for a study to aid us in evaluating the blood clotting abnormalities as well the prevalence of *Clostridium difficile* and *Salmonella* spp. in the stool of dogs suffering from parvo viral enteritis (cat flu). We would appreciate your consent to collect a stool sample and 3 blood samples.

The volume of stool and blood we will collect will in no way harm your pet or change the procedure for which your pet is seen or was admitted for.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial you are welcome to contact us.

Sincerely
Dr. W.J. Botha & Dr. Z. Whitehead
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria
Onderstepoort
0110
Tel 012 529 8128

I, ……, hereby give permission that my dog (Dog's name)……..,
a (breed)…… (colour) ……..
(sex)…… (age) ……
may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.
The trial has been explained to be and I understand that this study will in no way harm my dog. Furthermore I understand that no additional costs will be incurred to me in respect of this trial for the collection of blood and stool samples or the blood test and stool analysis required over and above the normal vaccination and deworming, ovariohysterectomy or castration costs.

Signed at Onderstepoort on the …… day of …… 20….
Signature Owner/Agent ……..
Home Tel: ……..
Work Tel: …… Cell No: ……

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Toestemmingsvorm vir kontrole honde - parvovirus enteritis studie

U hond is gekies om te dien as 'n gesonde kontrole hond vir 'n studie wat ons instaat stel om die bloedstolling abnormaliteite en die voorkoms van *Clostridium difficile* en *Salmonella* spp. In die stoelgang te bepaal in honde met parvovirus enteritis (katgriep). Ons sal u toestemming waardeer om een stoelgang monster en drie bloed monsters te versamel.

PATIENT LABEL

Die volume stoelgang en bloed wat versamel word sal in geen opsig u troeteldier skade doen of die prosedure verander waarvoor u troeteldier hier is of opgeneem word nie.

Dankie vir u bereidwilligheid om deel te neem in hierdie kliniese studie. U is welkom om ons te kontak indien u enige verdere vrae het aangaande die studie.

Die Uwe,
Dr. W.J. Botha en Dr. Z. Whitehead
Departement Geselskapsdier Kliniese Studies

Fakulteit Veeartsenykunde

Universiteit van Pretoria

Onderstepoort

0110

Tel 012 529 8128

Ek, ……, gee hiermee toesteming dat my hond (Naam)……,

'n (ras)…….. (kleur)……

(geslag)…… (ouderdom)……...

mag deelneem aan hierdie kliniese studie uitgevoer by die Onderstepoort Veterinêre Hospitaal.

Die doel van hierdie studie is aan my verduidelik en ek verstaan dat hierdie studie in geen opsig my hond sal skade doen nie. Ek verstaan ook dat geen addisionele kostes om my onthalwe aangegaan sal word nie ten opsigte van die vereiste monster versameling en ontleding bo en behalwe die normale inenting en ontwurming, sterlisasie of kastrasie kostes.

Geteken te Onderstepoort op hierdie ……. dag van …… 20….

Handtekening Eienaar/Agent ……..

Huis Tel: ……..

Werk Tel: …… Sel No: ……

Control dog Questionnaire and Check list:

Collector: WB / ZW / PD

Date of collection: ……../……../……

PATIENT LABEL

Habitus	
Body condition	
Skin	
Eyes	
Mucous membranes	
CRT	
Lymph nodes	
Thoracic auscultation	
Pulse Rate	
Respiratory Rate	
Abdominal palpation	
Temperature	

1.	**Reason for visit to OVAH**	Vaccination/deworming	Sterilization/castration
2.	**Has the dog had any previous illnesses?**	YES	NO
3.	**Has the dog had any previous visits to the vet/ hospital?**	YES	NO
4.	**Has the dog had any previous blood transfusions**	YES	NO
5	**Has the dog vomited or had diarrhoea in the last 14 days?**	YES	NO
6.	**Are there other pets at home?**	YES	NO
7.	**Are any other dogs on the property ill (parvo symptoms)?**	YES	NO
8.	**What diet is fed at home?**		
9.	**Is anybody in the household using antibiotics?**	YES	NO

Bloodsmear	Parasites		RBC	WBC	PLT
Faecal appearance					
Faecal float					
Faecal wetprep					
Faecal smear					
***C. difficile* SNAP**	GDH	Positive / Negative		Toxin assay	Positive / Negative
***Salmonella* Culture**		Result:			
***Salmonella* Seroptype**		Result:			

Haematology:

	Collect minimum 500uL blood
	Request standard complete blood count
	Store EDTA

Consent form for Parvovirus enteritis trial – CPV infected dogs

PATIENT LABEL

I, (Full name)……

Hereby give permission for the dog under my care,

(Dogs name)……

a (breed)……(colour)……

(sex)…… (age)……

to participate in the clinical study on parvoviral enteritis at the Onderstepoort Veterinary Academic Hospital.

I have received and understand the client information sheet regarding the parvo study. The trial has been explained to me and I understand that the study will in no way harm my dog and that the costs of the additional tests and additional treatments will be borne by the trial fund. I will only be liable for costs pertaining to the treatment that would in any event be required by my dog.

Signed at: Onderstepoort on the …….day of (month)……(year)……

Signature owner/ authorised person……

Home tel:……

Cell:……

Work tel:……

Thank you for allowing your pet to be entered into this study.

Toestemmingsvorm vir parvovirus enteritis studie – geaffekteerde honde

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

UNIQUE NUMBER

PATIENT LABEL

Ek, (Volle naam en van) ……..

gee hiermee toestemming dat my hond in my sorg,

(hond se naam)……..

(ras)……(kleur)…….

(geslag)……..(ouderdom)……

mag deelneem aan die studie op parvovirus

by Onderstepoort Veterinêre Akademiese Hospitaal.

Ek verstaan die inligtingsvorm wat ek ontvang het aangaande die katgriep studie. Die doel van die projek is aan my verduidelik, en ek verstaan dat die studie nie my hond op enige manier skade sal aandoen nie, en die koste van die addisionele toetse en addisionele behandeling deur die projekfonds gedra sal word. Ek verstaan dat ek aanspreeklik is vir die koste van standaard behandeling en diagnostiese toetse wat in elk geval deur my hond benodig sou word.

Geteken te Onderstepoort op die …….dag van (maand)……(jaar)……

Handtekening van eienaar / gemagtigde persoon…….

Huis tel:……

Sel:……..

Werk tel:……..

Dankie vir u bereidwilligheid om u hond te laat deelneem aan hierdie studie.

Client information sheet: Canine parvovirus study

From the history, clinical examination and tests done to date, it seems that your dog has contracted an infectious viral disease called canine parvovirus or "cat flu". This virus causes severe damage to the intestinal tract resulting in intestinal bleeding, decreased food absorption, vomiting, diarrhoea and fever. The virus also causes suppression of the bone marrow, which leads to a decrease in white blood cells and in turn decreases the dog's ability to fight infections. Unfortunately there is no antivirus treatment that exists at this time so we have to treat the dogs symptomatically.

Your dog has been admitted to the isolation unit at the Onderstepoort Veterinary Academic Hospital where he/she will receive intensive treatment. Your dog will receive intravenous fluid (drip), antibiotics, medication to control the nausea and vomiting, deworming and other treatments such as blood or plasma transfusions that may be needed.

We are conducting several studies on dogs with parvoviral enteritis.

One study is done to determine the prevalence of certain bacteria (*Clostridium difficile* and *Salmonella* spp.) in the stool of dogs affected with parvoviral enteritis. Both of these bacteria are very important in both human and veterinary medicine and will enable us to better understand these infections, their interactions and implications on public health. A second study will evaluate the clotting ability of the blood in affected dogs at admission and after treatment with a drip to determine if dogs with parvoviral enteritis are more prone to form blood clots and if the administration of a drip affects the clotting ability. In some cases an additional treatment with dog plasma will be given at no extra costs. This treatment will be evaluated to see whether it will be able to increase the antibodies against the virus. We will also perform blood tests to determine if the administration of plasma has any effect on the clotting ability of blood. This will allow us to investigate the potential benefit of plasma use in dogs with parvoviral enteritis.

This trial will not harm your pet in any way as he/she will still receive the same treatment were he/she not involved in the trial. You will also not endure any extra costs for the trial, you will only be liable for the usual cost of treatment as discussed with you by the admitting clinician. This study has been approved by the Ethics Committee of the Faculty of Veterinary Science, University of Pretoria.

Thank you for allowing your pet to be included in our study. If you have any further questions you can contact us on (012) 529-8000.

Dr. Wilco Botha (BSc BVSc) & Dr. Zandri Whitehead (BSc BVSc)

Eienaar Inligtingsvorm: Katgriep Studie

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Vanuit die geskiedenis, kliniese ondersoek en laboratorium
toetse sover uitgevoer op u hond, blyk dit asof u hond aan ‘n virusinfeksie lei, genaamd honde parvovirus of die sogenaamde “katgriep” virus. Hierdie virus veroorsaak skade aan die dermkanaal wat lei tot dermbloeding, swak absorpsie van voedingstowwe, braking, diarree en koors. Dit veroorsaak ook ander probleme soos ‘n verlaging in die witseltelling as gevolg van beenmurgonderdrukking wat veroorsaak dat die dier nie infeksies doeltreffend kan beveg nie. Ongelukkig is daar tans geen antivirus behandeling nie en moet ons die diere simptomaties behandel.

U hond word opgeneem in die isolasie-eenheid by die Onderstepoort Veterinêre Akademiese hospital waar hy/sy intensiewe behandeling sal ontvang. U hond sal behandel word met binne-aarse vloeistowwe (drip), antibiotika, middels wat naarheid en braking onderdruk, ontwurming, en indien nodig, bloed- of plasma oortappings.
Ons onderneem verskeie studies op honde met parvovirus enteritis.
Een studie ondersoek die voorkoms van sekere bakterieë (*Clostridium difficile* en *Salmonella* spp.) in die stoelgang van honde wat met parvovirus enteritis (katgriep) siek is. Beide die bogenoemde bakterieë is van groot belang in beide mens en veterinêre geneeskunde. Met behulp van hierdie studie sal ons dié infeksies, hulle interaksies en implikasie op publieke gesondheid beter kan verstaan. ‘n Tweede studie evalueer die stollings vermoë van die bloed in geaffekteerde honde met opname en na behandeling met ‘n drip om te bepaal of honde met parvovirus enteritis meer geneig is daartoe on bloedklonte te vorm en of die toediening van ‘n drip die stollingvermoë beïnvloed. In sekere gevalle sal ‘n addisionele behandeling met plasma aan honde gegee word teen geen ekstra koste nie. Hierdie behandeling sal geevalueer word om te sien of dit die teenliggampies teen die virus kan verhoog. Die potensiële voordele van plasma gebruik sal hiermee ondersoek kan word.

Die studie sal u dier geen skade aandoen nie en hy/sy sal steeds dieselfde behandeling ontvang sou hy/sy nie in die studie ingesluit wees nie. U sal nie verantwoordelik gehou word vir die koste van die addisionele bloedtoetse nie. U sal slegs vir die koste van behandeling verantwoordelik wees soos met u bespreek is deur die dokter aan diens. Hierdie studie is goedgekeur deur die Etiese komittee van die Fakulteit Veeartsenykunde, Universiteit van Pretoria.
Dankie dat u toelaat dat u hond ingesluit kan word in die studie. Indien u enige verdere navrae het kan u gerus die klinikus aan diens vra of ons kontak by: (012) 529 8196.

Dr. Wilco Botha (BSc BVSc) & Dr. Zandri Whitehead (BSc BVSc)

History & Clinical Examination

Collector: WB / ZW / PD

Date of Admission:_____

Time of Admission:_____

Vaccination status (parvo)	0	1	2	3	
Number of days depressed	1	2	3	>3	
Number of days anorectic	1	2	3	>3	
Number of days vomiting	1	2	3	>3	
Vomiting episodes per day	1	2	3	>3	
Description of vomitus	WHITE / YELLOW / BROWN	FOAMY / CLOTS	OTHER:		
Number of days diarrhoea	1	2	3	>3	
Diarrhoea episodes per day	1	2	3	>3	
Description of diarrhoea	WATERY / MUCOID	BLOODY / BLACK / WHITE / YELLOW / GREEN	OTHER:		
Diet fed at home					
Other pets at home	YES	NO			
Other dogs on the property ill	YES	NO			
Owners using antibiotics	YES	NO			
Previous visit to vet / hospitals	YES	NO			
% Dehydrated	<5%	5%	7%	10%	12%
Mentation status	1+	2+	3+	4+	
Mucosa	PALE / PALE PINK / PINK / CONGESTED / YELLOW / BLUE	MOIST / TACKY	OTHER:		
Oral ulcerations	YES	NO			
Capillary refill time	< 1 SEC	1-2 SEC	➢ 2 SEC		
Pulse quality	ABSENT	WEAK	STRONG	WATERHAMMER	Other:
Pulse rhythm	REGULAR	IRREGULAR			
Respiratory pattern & Depth	ABDOMINAL / COSTAL / COSTOABDOMINAL	NORMAL / SHALLOW / LABOURED	OTHER		
Lung sounds	ABSENT	NORMAL	ABNORMAL:		
Abdominal palpation	NO PAIN / TENSE / PAIN	THICKENED / GAS / FLUID / INTUSSUSCEPTION	OTHER:		
Any signs of inflammation	YES	NO			
Temperature		**Pulse Rate:**		**Respiratory Rate:**	

DATA COLLECTION & RESULT CHECK LIST

Collector: WB / ZW / PD

	Client consent form signed
	Client information leaflet handed out

	Full clinical examination – clinical examination form completed					
	Bloodsmear	Parasites:	RBC:	WBC:	PLTS:	
	CPV Elisa SNAP test:		positive negative			
	EM –sample collected and sent to EM		positive negative Other viruses:			
	Faecal float					
	Faecal wetprep					
	Faecal smear					
	***Clostridium difficile* SNAP**	GDH	Positive / Negative	Toxin assay	Positive / Negative	
	Salmonella culture		Result:			
	Salmonella serotype		Result:			
	Faecal sample stored					

Pre-treatment blood Collection:

Haematology:

	Collect minimum 500uL blood
	Request standard complete blood count
	Store EDTA

Serum Biochemistry and Coagulation:

	Collect 1 serum tube (minimum 1mL)										
	Request TSP, Alb, Na, K, Cl	TSP:		Alb:		Na:		K:		Cl:	
	Collect 1 citrate tube (2.7mL)										
	Request TEG	Collection Time: ____:____ TEG Start Time: ___:___									
	Store serum and citrate										

Appendix C: Presentations and publications arising from this study

The following presentations and publications have resulted from this study:

Presentations

Event	Venue	Date	Title	Type
ECVIM Congress	St Julian's, Malta	15/09/2017	Prevalence of Clostridium difficile and Salmonella spp. In Juvenile Dogs affected with Canine Parvoviral Enteritis	Abstract presentation

Publications

Botha WJ, Schoeman JP, Marks SL, Whitehead Z, Annandale CH. The prevalence of Clostridium difficile and Salmonella spp. in juvenile dogs affected with canine parvoviral enteritis. *Presubmission for Journal of Veterinary Internal Medicine*.