

**Retrospective evaluation of faecal PCR
results in Western Australian dogs**

Mark Kim

BVSc MVetClinStud

School of Veterinary Medicine

College of Science, Health, Engineering and Education

Murdoch University

Western Australia

This thesis is presented for the degree of Research Masters with Training (2020)

Declaration

I declare that: a) The thesis is my own account of my research b) All co-authors, where stated and certified by my principal Supervisor, have agreed that the works presented in this thesis represent substantial contributions from myself and c) The thesis contains as its main content, work that has not been previously submitted for a degree at any other university.

Chapter three has been published in a scientific journal, and chapter four has been submitted to the same journal and is currently under review. Chapter three is a duplicate of the published article, and chapter four is a duplicate of the most recent copy awaiting review at the time of thesis submission. As such, abbreviations may differ to the remaining thesis. These chapters were written with the principal supervisor and three co-authors. The majority of study designs, experimental research, and writing for publication were undertaken by myself as primary author (90%) and with guidance from the principal supervisor and other co-authors (10%). Ethical approval was not required for either retrospective study (Chapters 3 and 4).

By signing this, I hereby confirm that the above percent contributions of each author are true:

Mark Kim BVSc MVetClinStud DACVECC

Principal Author

Claire R. Sharp BSc BVMS MS DACVECC

Principal Supervisor

Abstract

Canine faecal PCR assays have recently become commercially available to investigate infectious causes of diarrhoea. Despite an increase in use, there remains uncertainty regarding the clinical relevance of positive test results. To explore this, we performed two retrospective studies: firstly, we described the faecal PCR results and clinical data of 168 dogs seen at The Animal Hospital at Murdoch University, and secondly, we established faecal PCR-based enteric organism profiles in 2025 Western Australian dogs. In the first study, 68% of dogs presented for acute diarrhoea. For the PCR results, *Clostridium perfringens* alpha-toxin gene was most frequently detected (92.9%), followed by *Campylobacter* spp. (32.7%), canine parvovirus (CPV) (17.3%), *Salmonella* spp. (8.3%), and *Giardia* spp. (5.4%). Canine enteric coronavirus and canine distemper virus were rarely detected (<2%), while no dogs tested positive for *Cryptosporidium* spp.. Antimicrobials were started or switched in 31 dogs. Forty-five dogs were CPV antigen-test negative, of which 13 were PCR-positive. In the second study, a commercial laboratory provided 2025 canine faecal PCR results from dogs in Western Australia over a three-year period. *Clostridium perfringens* alpha-toxin gene was most frequently detected (87.2%), followed by *Campylobacter* spp. (37.8%), CPV (10.5%), *Giardia* spp. (9.7%), *Salmonella* spp. (7.0%), canine enteric coronavirus (2.3%), canine distemper virus (0.3%) and no cases of *Cryptosporidium* spp.. Multiple organisms were detected in 46% of dogs. *Clostridium perfringens* alpha-toxin gene is highly prevalent in the Western Australian dog population that underwent faecal PCR testing. While *Clostridium perfringens* has been implicated in acute haemorrhagic diarrhoea syndrome, its clinical relevance is still unclear. Thus we propose a prospective clinical trial evaluating antimicrobial use in dogs with this syndrome. Also, a healthy dog PCR study will help veterinarians interpret positive faecal PCR results in dogs with acute diarrhoea.

Table of contents

Declaration	2
Abstract	3
Acknowledgements	7
List of tables and figures	8
Chapter 1: Introduction, objectives	10
Introduction	10
Objectives	11
Bibliography for literature review	11
Chapter 2: Literature review	12
Acute diarrhoea: background	12
Infectious aetiologies of acute diarrhoea	16
Clostridium perfringens	17
Campylobacter spp.	19
Canine parvovirus	21
Giardia spp.	23
Salmonella spp.	26
Cryptosporidium spp.	28
Canine enteric coronavirus	30
Gastrointestinal microbiome	31
Microbiome background	32
Microbiome and acute diarrhoea	33
Antimicrobial stewardship	34
Diagnostics	35
Background	35
Traditional diagnostic methods	35
Faecal microscopy	36
Culture-based methods	37
Immunoassays	38
Other methods	39
Molecular diagnostics	39
Faecal PCR assays - procedure	40
Faecal PCR assays - limitations	42
Canine faecal PCR literature	45
Faecal PCR assay results and causation	48
Faecal PCR assays - conclusion	50
Knowledge gap	51
Bibliography for literature review	53

Chapter 3: Faecal PCR panel results and clinical findings in Western Australian dogs with diarrhoea	70
Abstract	70
Introduction	72
Material and methods	73
Data collection	73
Laboratory results	74
Statistics	75
Results	75
Signalment	75
Vaccination and deworming history	76
Clinical signs	76
Diagnostics	77
Discussion	81
Conclusion	87
Abbreviations	88
Bibliography for Chapter 3	88
Appendix	91
Chapter 4: Prevalence of enteric organisms detected by real-time PCR assay in faeces of dogs in Western Australia	94
Abstract	94
Introduction	96
Materials and methods	97
Data collection	97
Data analysis	98
Results	98
Summary	98
Perth faecal PCR results	99
Discussion	100
Conclusion	105
Bibliography for Chapter 4	109
Chapter 5: MERINGUE: Metronidazole use in dogs with Acute Haemorrhagic Diarrhoea Syndrome. A randomised placebo-controlled clinical trial	113
Animal ethics approval: R2946/17	113
Summary	113
Abbreviations	115
Background	115
Objectives and hypothesis	117
Material and methods	117
Design and setting	117
Participants	117

Inclusion and exclusion criteria	118
Inclusion criteria	118
Exclusion criteria	118
Study procedures	118
Enrolment and study treatment administration	119
Sample and data collection	119
Statistical plan	120
Sample size calculation	120
Randomisation procedure	121
Statistical analysis plan	121
Co-enrolment in other studies	122
Feasibility	122
Timeline	122
Current progress	123
Dissemination of results	123
Bibliography for Chapter 5	123
Appendices	125
Appendix 1: Study enrolment and protocol flow chart	125
Appendix 2: Canine AHDS activity index	126
Chapter 6: Conclusion	127
Key findings	127
Contribution to the literature	128
Study limitations	129
Future directions	130
Conclusion	131

Acknowledgements

I would like to first and foremost thank my Principal Supervisor, Dr Claire Sharp, for her guidance through these four formative years of my training in emergency and critical care (ECC). Her clinical acumen, breadth of knowledge, and most importantly her commitment to teaching have given me a learning experience that I will always carry with me and strive to share with others. I consider her an exceptional role model for anyone pursuing a career in academia, and can only hope to be as generous, supportive, and inspirational as she was and is to me.

I am also very fortunate to have been part of and under the supervision of the incredible ECC academic team: Drs Corrin Boyd, Melissa Claus, and Lisa Smart, in addition to Dr Claire Sharp. I say without any hesitation that this must be one of the strongest clinical and research teams in the small world of veterinary ECC, who also happen to be wonderful people that made every day of my time at Murdoch University enjoyable, enriching, challenging, and unforgettable.

This team is also responsible for building the greater ECC team, an amazing group of veterinary professionals to whom I am also very grateful for my entire Murdoch experience.

Lastly, I want to thank the 3 most important people in my life - Leah, Noa, and Tabitha - who sacrificed so much. I promise to make up for the lost time.

List of tables and figures

Table 3-1. Clinical signs described in dogs seen at a veterinary teaching hospital for diarrhoea that had a multiplex faecal PCR panel performed as part of their diagnostic evaluation.....	73
Table 3-2. Faecal PCR results from dogs seen at a veterinary teaching hospital for diarrhoea. Prevalence is reported as the positive cases (detected + detected low level) out of a population of 166 dogs with diarrhoea.....	75
Table 3-3. Clinical data regarding adult dogs with diarrhoea that tested positive for canine parvovirus on a faecal PCR panel.....	77
Figure 4-1. Prevalence of enteric organisms detected by faecal PCR in dog faecal samples submitted to a commercial laboratory from postcodes in Western Australia (n=2025) between July 2016 and June 2019. Note that WA results comprise Perth results (n=1776) plus regional WA results (n=249). CPV, canine parvovirus; CCV, canine enteric coronavirus; CDV, canine distemper virus.....	103
Figure 4-2. Percentage of positive results by season for each organism detected by faecal PCR in dog faecal samples submitted from Perth postcodes between July 2016 to June 2019. CPV, canine parvovirus.....	104
Figure 4-3. Perth metropolitan area total number of faecal PCR tests performed on dog faecal samples submitted to a commercial laboratory between July 2016 and June 2019, by local government area. Each circle represents a local government area. Size of circle is proportional to number of tests performed.....	105

1. Chapter 1: Introduction, objectives

1.1. Introduction

Dogs are commonly presented to emergency veterinary hospitals for acute diarrhoea. Dogs may present with varying clinical signs ranging from mild diarrhoea requiring only outpatient supportive care, to more severe forms requiring hospitalised therapy for shock, pain, and other metabolic derangements.

Diagnostic testing depends on the case, and for infectious causes, faecal diagnostics are the most common tool, and can provide information to guide antimicrobial use, guide infectious disease control both in the hospital and home settings, and identify potential zoonoses.

Multiplex faecal polymerase chain reaction (PCR) assays recently became widely available to veterinarians and have augmented the traditional diagnostic tools available for investigating acute diarrhoea in dogs. These assays purport to provide high sensitivity and specificity in detecting enteric microorganisms in faecal samples.^{1,2} Consequently, we have experienced marked growth in faecal PCR use at our hospital, despite lack of high quality evidence showing that detected microorganisms are pathogenic and the primary cause of gastrointestinal disease.

Currently, a multiplex faecal PCR panel is available to our institution. The panels tests for 8 organisms: the bacteria *Clostridium perfringens* alpha toxin gene, *Campylobacter* spp., and *Salmonella* spp.; the protozoa *Giardia* spp., and *Cryptosporidium* spp.; and the viruses canine parvovirus, canine enteric coronavirus, and canine distemper virus. To better incorporate faecal PCR results into evidence-based treatment plans for dogs with acute diarrhoea, we believe further investigation into faecal PCR assays and the enteric microorganisms they detect is required.

1.2. Objectives

We performed two observational studies to examine faecal PCR assay results and their use in dogs. The objective of our first study (Chapter 3) was to describe the faecal PCR results and accompanying clinical data of dogs seen at The Animal Hospital at Murdoch University. For the second study (Chapter 4), our primary objective was to describe the enteric organisms detected in a large population of dogs in Western Australia that had a faecal PCR performed by a single commercial laboratory; the secondary objectives were to evaluate seasonal and regional prevalence patterns; and to observe rates of multiple organism detection. Finally, we started enrolment in a randomised clinical trial evaluating the effect of antimicrobial use in dogs with Acute Haemorrhagic Diarrhoea Syndrome (Chapter 5).

1.3. Bibliography for literature review

1. Decaro N, Martella V, Ricci D et al. Genotype-specific fluorogenic RT-PCR assays for the detection and quantitation of canine coronavirus type I and type II RNA in faecal samples of dogs. *J Virol Methods* 2005;130:72–78. <http://dx.doi.org/10.1016/j.jviromet.2005.06.005>.
2. Marks SL, Rankin SC, Byrne BA et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med* 2011;25:1195–1208. <http://dx.doi.org/10.1111/j.1939-1676.2011.00821.x>.

1. Chapter 2: Literature review

1.1. Acute diarrhoea: background

Diarrhoea is one of the most common gastrointestinal disorders in dogs. Diarrhoea can be classified either by its duration – acute versus chronic; anatomical origin - small versus large bowel; the pathophysiology - osmotic, secretory, inflammatory, or malabsorptive diarrhoea; or a combination of the aforementioned. The World Health Organisation defines acute diarrhoea in humans as the passing of loose or liquid stools at higher than normal frequency for less than fourteen days.¹ The definitions in veterinary medicine are similar but have durations ranging from three to fourteen days.²⁻⁴ In the acute care setting, acute rather than chronic diarrhoea is the predominant presenting complaint, and so will be the focus of this thesis.

Canine acute diarrhoea is a common condition that can cause a wide range of illness severity. Three observational studies reporting on diarrhoea prevalence found 7-44.2% of dogs were described by their owners to have recently had diarrhoea.⁵⁻⁷ Only 10-37% of these dogs were presented to veterinarians.⁵⁻⁷ Therefore, many dogs likely had a self-limiting illness not requiring veterinary care. The considerable design differences between these studies limit direct comparison. Anecdotally acute diarrhoea is one of the most common presenting complaints for dogs presenting on an emergent basis at our hospital. The same is likely true in other veterinary practices also; a suburban practice observational study in Korea reported diarrhoea as the presenting complaint in approximately 5% of 11085 dogs seen over a 12-month period.⁸

The level of treatment indicated for acute diarrhoea cases reflects the underlying aetiology and pathophysiology. Specifically, in more severe cases, diarrhoea can result in marked fluid losses from the extracellular fluid compartment,⁹ which can progress to

hypovolaemic shock. Compounding these losses, gastrointestinal illness often causes nausea, vomiting, and inappetence. As such, many dogs presenting for acute diarrhoea will require parenteral fluid therapy to correct fluid deficits and replace ongoing losses.⁹ Despite the high morbidity sometimes associated with acute diarrhoea, the prognosis is generally fair to excellent,^{2,10} although it depends not just on disease severity, but the underlying cause.

Acute diarrhoea in dogs has many known causes. Several classification systems are described, with both a pathophysiology and a causal approach most widely reported.^{11,12} There are four main pathophysiologic mechanisms of acute diarrhoea osmotic, secretory, inflammatory, and malabsorptive.¹³ Osmotic diarrhoea results from passage of osmotically active substances through the gastrointestinal tract, in particular the large bowel where definitive water absorption occurs. The most common causes of osmotic diarrhoea are dietary indiscretion of osmotically active substances or a malabsorptive process due to an underlying disease. In contrast, secretory diarrhoea develops due to disturbances in small intestinal mucosal ion channels, particularly chloride channels.¹⁴ The intestinal mucosa can remain grossly intact and unharmed. This typically results in small intestinal chloride loss accompanied by passive water and sodium losses. Secretory diarrhoea is most commonly associated with enteropathogenic *Escherichia coli* infection.¹⁵ This enterotoxin is just one of numerous bacterial enterotoxins whose effects can range from altering ion channel function as described above, to ulcerative injury to intestinal mucosa.¹³ Unlike osmotic and secretory diarrhoea, increased gastrointestinal mucosal permeability diarrhoea occurs due to disruption of the normal mucosal barrier. The main histologic findings are mucosal cellular injury, blunting of villi, as well as damage to tight junctions, which in turn allows paracellular fluid leakage. In dogs, this type of diarrhoea is primarily associated with inflammatory bowel disease

(IBD).¹² Lastly, diarrhoea can develop secondary to disorders of gastrointestinal motility. The key mechanisms described are malabsorption secondary to severely reduced intestinal transit time, as well as passage into the colon of poorly-digested particles, i.e. osmotically active substances. This clearly demonstrates the overlapping nature of all 4 mechanisms, and often more than one of these four mechanisms is present.

Alternatively, aetiologies of acute diarrhoea can be divided between primary gastrointestinal and extra-gastrointestinal origins.¹⁶ Primary gastrointestinal disorders encompass numerous aetiologies. This includes diet-related causes: dietary indiscretion or dietary intolerance. Anecdotally, these are considered one of the most common causes of acute gastrointestinal disorders in dogs. Other primary gastrointestinal aetiologies include mechanical injury due to partial obstruction or slow-moving foreign material, toxins or chemical injury such as non-steroidal anti-inflammatory (NSAID) use, gastrointestinal neoplasia (carcinoma, lymphoma, leiomyosarcoma, gastrointestinal stromal tumour, mast cell tumour, and fibrosarcoma),¹⁷ immunologic reactions (including IBD), and infectious disease. Infectious causes are often categorised by microorganism type: viral, bacterial, fungal, and parasitic (including protozoa). Also of particular interest is acute haemorrhagic diarrhoea syndrome (AHDS; also known as haemorrhagic gastroenteritis, HGE), a widely-encompassing term that describes a syndrome in dogs whereby the primary presenting complaint is severe haemorrhagic diarrhoea. The aetiology of AHDS is unknown, but proposed mechanisms include infectious microorganisms such as *Clostridium perfringens*, diet changes, and type 1 hypersensitivity reactions.^{2,3} Extra-gastrointestinal disorders traditionally include pancreatitis, hepatic or renal disease, and hypoadrenocorticism.

Many of these causes can have an uncomplicated disease trajectory. Known dietary indiscretion prior to onset of mild gastrointestinal signs can reasonably be managed with

minimal diagnostics and appropriate supportive care to ensure hydration and relief of clinical signs. Foreign object gastrointestinal obstruction can often be diagnosed with a thorough history, physical exam, and abdominal imaging. Pancreatitis, hepatic disease, or renal disease can similarly be diagnosed with a combination of a thorough history, physical exam, laboratory tests, and abdominal imaging. Some infectious aetiologies can likewise be rapidly diagnosed. Parasitism in juvenile dogs is a frequent cause of diarrhoea¹⁸ and pathologic parasites are readily detected with faecal microscopy.¹⁹ Even if such diagnostics are not pursued, endoparasitism is commonly addressed with routine antiparasitic medication.

For other infectious aetiologies however, diagnosis and determining causality is more complicated and the consequences of missed or inaccurate diagnoses include inadequate treatment with antimicrobials, inappropriate or overuse of antimicrobials, zoonotic concerns, and inappropriate infectious disease control. As such, it is vital to obtain a diagnosis for certain infectious aetiologies of acute diarrhoea. Due to limitations of traditional diagnostic tests, discussed below, there are several hurdles to achieving a definitive diagnosis.

Recently, a faecal PCR assay for eight canine enteric microorganisms performed by a commercial laboratory (Vetpath Laboratory Services, Belmont, Western Australia, Australia) became available to our institution. Anecdotally, it has gained a lot of traction as a diagnostic test for acute diarrhoea despite uncertainty about the clinical relevance of the results.¹⁰ As such, we believe the use of faecal PCR assays in clinical practice need further study. This may result in better antimicrobial stewardship and better patient care, improved infectious disease control measures in veterinary hospitals, and it may provide the impetus for the commercial veterinary laboratories to refine PCR assays available to veterinarians to ensure that they are fit for purpose.

As our research revolves around this faecal PCR assay, this review will focus on the eight microorganisms included in the multiplex PCR assay available in Western Australia. These microorganisms are *Clostridium perfringens*, *Campylobacter* spp., *Salmonella* spp., *Giardia* spp., *Cryptosporidium* spp., canine parvovirus (CPV), and canine enteric coronavirus (CCV). Although canine distemper virus is also included in this PCR assay, it will not be included in this review as it is rarely diagnosed in dogs in Australia.²⁰ Reviews of tests available for other infectious and non-infectious causes of acute diarrhoea can be found elsewhere.^{13,21,22}

The remainder of this review will explore the following literature: 2.2 Infectious aetiologies of acute diarrhoea, 2.3 Gastrointestinal microbiome, and 2.4 Diagnostic tests used to investigate acute diarrhoea, with a focus on faecal PCR assays and their limitations.

1.2. Infectious aetiologies of acute diarrhoea

Infectious aetiologies form an important area of acute diarrhoea research in dogs. Investigation of various potential pathogens has led to a better understanding of pathophysiologic mechanisms of diarrhoea such as the difference between increased permeability diarrhoea (eg. CPV enteritis) versus secretory diarrhoea (enterotoxigenic *E. coli*). This research has also identified the challenges of differentiating pathogenic from non-pathogenic microorganisms. A common thread in many published studies is the detection of microorganisms that were previously labelled pathogens in healthy control dog populations.¹⁰ Thus, it should be noted that despite the putative association between these enteric microorganisms and intestinal disease, the term pathogen is controversial given the incomplete understanding of their role in acute diarrhoea.¹⁰ As such, the term

microorganism will be used preferentially throughout this review.

1.2.1. *Clostridium perfringens*

Clostridium perfringens is one of two clostridial species commonly associated with large-bowel diarrhoea in dogs. It is a gram-positive anaerobe found ubiquitously in the environment and considered a commensal microorganism of the gastrointestinal tract in dogs, and is a well known cause of human and production animal infectious disease. The role of *C. perfringens* in canine gastrointestinal disease remains incompletely understood, but these bacteria have been implicated in acute enterocolitis.^{23,24} There are five serotypes of *C. perfringens*. Serotypes, sometimes referred to as biotypes, are typically classified by specific characteristics that allow for further subspeciation. *Clostridium perfringens* are serotyped A through E, classified on the range of enterotoxins produced. *Clostridium perfringens* enterotoxin is the most frequently implicated cause of infectious diarrhoea in both people and companion animal species.²¹ Other reported enterotoxins include alpha, beta, epsilon, iota, and more recently, the necrotic-enteritis pore-forming family of toxins.²⁵ Under certain conditions, these enterotoxins are produced following sporulation of enteric microorganisms, and possibly lead to secretory diarrhoea via disruption of chloride channels in intestinal crypt cells, and direct intestinal mucosal injury.^{13,26} In contrast to known pathogenic enterotoxins, detection of alpha toxin or its gene is of questionable clinical relevance. The role of each toxin is yet to be fully understood, but there is considerable research ongoing in this area.

Most human literature focuses on food-borne clostridiosis, an acute, potentially severe and life-threatening gastrointestinal disease.²⁷ Many companion animal studies report detection of *C. perfringens* in faecal samples, with some studies identifying *C. perfringens* enterotoxin, and infer a causative link.²⁸⁻³² Two earlier culture-based studies

from Germany and the United States (US) found higher prevalence of *C. perfringens*, higher bacterial counts, a larger variety of serotypes, and higher frequency of enterotoxin in diarrhoeic dogs as compared to non-diarrhoeic dogs.^{33,34} Furthermore, these studies found *C. perfringens* enterotoxin present only in diarrhoeic samples and not control samples, using reversed passive latex agglutination assays. Another observational study in the US used faecal PCR and ELISA for detection of bacterium and toxin producing gene, and enterotoxin respectively, for a similar comparison in 104 dogs with diarrhoea and 95 non-diarrhoeic control dogs, and detected a significantly higher prevalence of *C. perfringens* enterotoxin in diarrhoeic dogs compared with controls (only 1 control dog had the enterotoxin detected by ELISA), but dysbiosis in the diarrhoeic dogs was independent of *C. perfringens* detection.²⁹ Despite this difference, it is noteworthy that *C. perfringens* enterotoxin gene was still detected by PCR in 34% of the control dogs. Equally of note is that all tested dogs, healthy and diarrhoeic, had *C. perfringens* detected by PCR. For *C. perfringens*, these differences emphasise the importance of appropriate testing methods and critical evaluation of results to avoid over-interpretation and misguided treatment plans.

Recently, there has been greater focus on the novel pore-forming enterotoxins. Necrotic enteritis toxin E-like (NetE), F-like (NetF), and G-like (NetG) were first described in cases of canine haemorrhagic gastroenteritis and foal necrotising enterocolitis.³⁵ A follow-up prospective study characterised these toxins in 66 healthy dogs versus 54 dogs diagnosed with AHDS.³⁶ These authors found dogs with AHDS had more frequent detection of the genes that encode for NetE and NetF than healthy non-diarrhoeic dogs, but within the AHDS cohort, there was no difference in severity nor time to resolution of disease between dogs with and without NetE/NetF.³⁶ Therefore, the pathogenicity of these toxins is still incompletely understood.

A repeated finding in these studies is histologic evidence of *C. perfringens*-induced gastrointestinal mucosal injury, and this is presented as key supporting evidence for *C. perfringens*' role in disease.^{26,30,37} These studies included both postmortem investigation³⁷ following natural death, and a prospective study involving histology of endoscopic gastroduodenal mucosal biopsies.²⁶ What remains unclear however, is whether the microorganism plays a primary aetiologic role or whether secondary dysbiosis favours production of pathogenic enterotoxins. Some studies conclude a causative role, emphasising the failure to detect other known causative microorganisms in diarrhoeic dogs.^{28,32} It is likely *C. perfringens* plays a range of roles in acute diarrhoea, from sole aetiologic agent to secondary exacerbation of gastrointestinal disease. There is still no clear consensus on the best way to diagnose clostridial gastrointestinal disease. However, the detection of clostridial enterotoxins is likely the most informative method of identifying clostridiosis in dogs, understanding that the recommended ELISA has limitations, particularly a minimum limit of detection that may not correlate with disease.

1.2.2. Campylobacter spp.

Campylobacter species are Gram-negative, curved, aerophilic rods known primarily for causing mild to moderate gastrointestinal disease in dogs, however they have also been implicated in infectious cholangiohepatitis,³⁸ and acute polyradiculoneuritis.³⁹ Much veterinary research into *Campylobacter* stems from its zoonotic capabilities, being the most commonly reported zoonosis in many regions.^{40,41} In a Scandinavian case-controlled epidemiologic study, daily contact with a dog was second only to barbeque meat consumption as a source of infection.⁴² In particular, children in contact with puppies are considered most at risk for campylobacteriosis.⁴³ Given this connection, another concern is development of antimicrobial-resistant species of *Campylobacter* in

dog populations with subsequent transmission of these resistant species to at-risk human populations.⁴⁴

At least 17 *Campylobacter* species have been detected in dogs, with *C. jejuni*, *C. upsaliensis*, *C. coli*, and *C. helveticus* most frequently detected; and *C. jejuni* most frequently linked to canine gastrointestinal disease.⁴⁵ However, there are conflicting reports of *Campylobacter* prevalence in different dog populations. For example the bacteria was only detected in the faeces of 15% of hearing-assistance dogs in the United Kingdom, compared to 93% of dogs in a US shelter study.^{46,47} The variability may come from the different methodologies used in these studies - many recruiting dogs from kennels and shelters in order to explore housing density as a risk factor,⁴⁸⁻⁵⁰ and others recruiting only dogs seen at veterinary clinics.^{51,52} The majority of these studies also provide species level data. *Campylobacter upsaliensis* and *C. jejuni* are the most frequently detected species, however there is much variation reported.

Only a few studies explore the differences in *Campylobacter* prevalence between diarrhoeic and non-diarrhoeic dogs. In a smaller prospective Swedish observational study comparing 54 diarrhoeic to 54 non-diarrhoeic dogs, *Campylobacter* spp. was detected in 29.6% of diarrhoeic and 24.1% of non-diarrhoeic dogs.⁵³ Interestingly, *C. upsaliensis* was most frequently found in diarrhoeic dogs and *C. jejuni* in healthy dogs. However a larger Swiss study in 476 dogs (405 dogs with and 71 without diarrhoea) only found a difference in the prevalence of *Campylobacter* spp. in a subgroup of dogs less than one year old (44% versus 22%).⁵⁴ In a more recent Canadian study using multiplex PCR assays, 65 diarrhoeic dogs compared to 70 healthy dogs had both a much higher prevalence (97% versus 58%) and a higher variety of detected *Campylobacter* species (0-12 versus 0-7 species).⁵⁵ Many studies however, fail to identify any such differences.^{46,56} Raw meat based diets are also linked to campylobacteriosis,³⁸ but again

a causative association is confounded by similar rates of *Campylobacter* spp. detection in dogs fed raw meat versus cooked meat diets.⁴⁵ Other authors have attempted to identify seasonal variation in *Campylobacter* spp. prevalence. Again, published studies have conflicting findings with peak prevalence reported in spring,⁵⁷ autumn,⁵⁸ and summer.⁵⁹ Direct comparison between these studies is limited by the different techniques used, but it is reasonable to conclude that there are considerable geographic differences in *Campylobacter* spp. profiles in both healthy and diarrhoeic dogs.

Questions remain regarding the role of *Campylobacter* spp. in canine diarrhoea and the associated pathophysiology.¹⁰ Mucosal invasion has been demonstrated, and enterotoxins may play a role in disease,^{60,61} but other studies failed to find histopathologic changes in experimentally infected dogs.⁶² In dogs believed to have campylobacteriosis, a mixed small and large bowel diarrhoea is described, often with blood and/or mucus. More severe cases can develop vomiting and fever.⁶³ A few experimental studies in laboratory animals, including gnotobiotic puppies, demonstrated association between *Campylobacter* spp. detection and gastrointestinal signs.^{62,64} From the sparse clinical evidence, it would be reasonable to conclude that puppies are primarily at risk, that prevalence in healthy dogs is high, and further research is required to clarify if *Campylobacter* detection in diarrhoeic dogs represents campylobacteriosis, secondary dysbiosis or detection of a commensal microorganism.

1.2.3. Canine parvovirus

Canine parvovirus (CPV) is a common cause of moderate to severe gastrointestinal disease in dogs worldwide, particularly in ineffectively vaccinated puppies. Canine parvovirus is a non-enveloped DNA virus of the genus *Protoparvovirus* that is believed to have originated in wild carnivores sometime in the middle of the twentieth century, and became endemic following a global spread in the late 1970s.⁶⁵ Originally designated

CPV-2, effective vaccine and control measures helped limit the spread of the virus. This led to evolutionary pressures and two novel variants, subsequently labelled CPV-2a and CPV-2b. These mutants have mostly replaced CPV-2 from the global dog population, presumably due to selection pressures. A third variant, CPV-2c, was first reported in Italy in 2000.⁶⁶ Over the following decade, CPV-2c followed its predecessors, and is now reportedly found globally.⁶⁷

Canine parvovirus initially targets highly replicating cells, namely gastrointestinal lymphoid tissues and epithelial crypt cells, bone marrow, and less commonly myocardial cells.⁶⁸ Specifically, gastrointestinal mucosal infection results in severe crypt necrosis,⁶⁷ leading to vomiting, haemorrhagic diarrhoea, loss of mucosa defence mechanisms and subsequent bacterial translocation. Bone marrow infection similarly causes destruction of replicating progenitor stem cells, leading to leukopenia. Neutropenic dogs are likely to experience greater morbidity due to their weakened immune system and increased risk for secondary bacterial sepsis. Amongst the microorganisms covered in this review, CPV has the strongest evidence supporting its role as a primary enteric pathogen.

Despite the widespread availability of effective modified-live vaccines, the CPV variants persist in most communities, and we continue to have epizootics.⁶⁹⁻⁷¹ There are several predisposing factors including virus stability in the environment, housing density, vaccination practices, and socioeconomics. Lower vaccination rates and higher density housing are thought to account for increased CPV incidence in lower socioeconomic areas.⁷² For the role of vaccines, it is widely accepted that maternal antibodies are the most likely cause of primary vaccine failure, leading to parvovirus in puppies.⁶⁷ Also, there are two vaccine strains commercially available - one based on the original CPV2 strain, and the second based on the CPV2b strain. An experimental study from Italy demonstrated differing cross-protective titres in two groups of 18 puppies given one of

each vaccine type, with higher serum antibody titres against CPV2 and CPV2b in dogs vaccinated with the CPV2b strain vaccine.⁷³ The authors question whether older-strain vaccines provide adequate and consistent immunity against newer or heterologous strains. Raising doubts about this claim is an experimental study from the US in 30 laboratory beagle puppies demonstrating that older-strain vaccines provide sufficient protective immunity against heterologous virus strains, including CPV2c.⁷⁴ There are also numerous reports identifying possible vaccine failure in parvovirus in fully vaccinated adult and juvenile dogs.^{71,75,76} The World Small Animal Veterinary Association has subsequently published evidence-based guidelines to address these and many other concerns.⁷⁷ Certainly with our understanding of CPV thus far, it is possible selection pressures will result in further mutations, which in turn will likely increase pressure to develop vaccines based on these newer strains to maintain efficacy.⁶⁷

1.2.4. *Giardia* spp.

Giardia species are flagellated protozoa of the eukaryote domain that are also endemic, and considered the most common cause of waterborne gastrointestinal disease in humans in the US.⁷⁸ There are several *Giardia* species based on their genetic sequencing, although nomenclature has varied over the years.⁷⁸ The current known species are *G. duodenalis* (aka *G. lamblia*, *G. intestinalis*), and *G. agilis*, *muris*, *microti*, *adeae*, *psittaci*. All appear to be host-species specific (Table 1), but many can be found in more than one host. These species are sometimes instead addressed by the scientific name of their host species (Table 1). *Giardia duodenalis* can be further classified by their genetic sequence into assemblages A through H. Traditionally, assemblages A and B are known for causing disease in people, and C and D are found in dogs.

Species	Assemblage	Host(s)
---------	------------	---------

<i>G. duodenalis</i>	A	Humans, other primates, wide range of other mammals
<i>G. enterica</i>	B	Humans, other primates, dogs, cats, some other mammals
<i>G. canis</i>	C/D	Dogs and related species
<i>G. bovis</i>	E	Cattle and other hoofed animals
<i>G. cati</i>	F	Cats
<i>G. simondi</i>	G	Rats
<i>G. muris</i>	n/a	Rodents
<i>G. microti</i>	n/a	Voles and muskrats

Table 1. Species of *Giardia* in mammals. From Thompson 2012⁷⁹

Giardia has 2 life stages, the infective cyst stage, and the pathogenic trophozoites. Cysts are hardy and can survive for long times in the environment, destroyed only by desiccation. They are the means by which oro-faecal transmission is maintained. In the acidic environment of the stomach, excystation releases trophozoites, which then use flagellated motility to attach and feed on small intestinal mucosa. This then leads to malabsorption, hypersecretion, and clinical diarrhoea. Other pathophysiologic mechanisms such as secondary dysbiosis, blunting of villi, T-lymphocyte mediated epithelial cell injury, tight junction disruption, and induction of epithelial cell apoptosis are suspected but not fully understood.^{78,80} Triggered by unknown mechanisms, trophozoites undergo encystation to become cysts, which are then shed in faeces for further transmission. On the other hand, free trophozoites typically fail to survive transit through the colon, and those that do only survive for a few hours in the environment.

Dogs with giardiasis can exhibit a range of clinical signs - from acute severe diarrhoea, anorexia, and pain, to mild self-limiting diarrhoea, to chronic waxing-waning forms leading to ill-thrift. However, most dogs have only subclinical *Giardia* infections, possibly acting as asymptomatic carriers to help maintain the microorganism in the canine population.⁷⁸

Many studies compare *Giardia* prevalence between different dog populations. A meta-analysis of 127 publications found increased prevalence of *Giardia* detection in puppies less than 6 months of age, strays and kennelled dogs, and diarrhoeic dogs.⁸¹ Interestingly there was no statistically significant difference in prevalence between geographic regions. Pooled prevalence in this meta-analysis was 15.2% (95% CI 13.8 - 16.7%). A single large US study using zinc sulphate microscopy to evaluate 1.2 million faecal samples reported 4% overall *Giardia* prevalence, increasing to 13% in puppies less than 6 months old, decreasing to less than 1% in dogs over 3 years.⁸² In a second large US study using a commercial point-of-care ELISA, 15.6% of 16000 diarrhoeic dogs tested positive.⁸³ In other studies, *Giardia* prevalence ranged from as low as 4.4% in sled, shelter, and pet dogs using microscopy and IFA,⁸⁴ to 49.3% in diarrhoeic dogs tested by ELISA.⁸⁵ For the faecal microscopy analysis in this latter Japanese study, 9 non-diarrhoeic dogs had much higher prevalence compared to 68 diarrhoeic dogs (44.4% v 20.6%).⁸⁵ These conflicting results make it difficult to understand whether *Giardia* significantly contributes to acute diarrhoea in dogs. Besides prevalence, one study also attempted to compare seasonal parallels between dog and human *Giardia* infection patterns over a six-year period.⁸⁶ *Giardia* prevalence from two and a half million canine faecal samples over this time showed no seasonal variation, unlike human results, and furthermore the overall *Giardia* prevalence decreased over the six years, divergent from the unchanging human prevalence results.⁸⁶

Zoonoses are a major focus in many canine *Giardia* studies. *Giardia* has for a long time been considered a zoonotic disease, with human infections originating from other species. However, transmission from dog to human has not been definitely shown.⁷⁸ An important distinction is that only *G. duodenalis* assemblages A and B (or *G. duodenalis* and *G. enterica* respectively) are known to cause disease in humans, and only assemblages C and D (or *G. canis*) cause disease in dogs.⁷⁸ Although they do not appear to cause disease, assemblages A and B are routinely found in dogs.^{87,88} Thus, many studies focus on genotyping *Giardia* spp. in dogs, and certainly there is evidence pets and owners share the same *G. duodenalis* assemblages.⁸⁹ Conversely, only recently was *G. canis* assemblage C found in a human.⁹⁰ Most of these genotyping studies find combinations of the assemblages in individual faecal samples. Again, that direct comparison between studies is limited by methodological differences, to be discussed further below.⁸¹

1.2.5. Salmonella spp.

Salmonella spp. are gram negative, ubiquitous, facultative anaerobes of the family *Enterobacteriaceae* and are considered one of the most important infectious causes of human diarrhoea.⁹¹ *Salmonella enterica* is the only species considered relevant in veterinary medicine, although there is a single report of *S. bongori* in a diarrhoeic dog.⁹² *Salmonella enterica* is further classified into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. These are further classified into thousands of serotypes (or serovars) historically based on the Kauffman-White classification system, but newer systems have emerged, offering greater clinical relevance - typhoidal v non-typhoidal disease, or a three pronged system based on host-specificity.⁹³ These serovars are based on serologic identification of somatic (O) and flagellar (H) surface antigens. The role of *Salmonella* spp. in healthy dogs and dogs with gastrointestinal

disease is not fully understood, and it is widely accepted that *Salmonella* is often as prevalent in healthy dogs as in dogs with gastrointestinal disease. Pathogenicity likely is multifactorial involving both pathogen and host-related factors. Proposed theories include dysbiosis, and virulence factors - endotoxin production, mucosal injury, bacteraemia. Salmonellosis in dogs is typically an acute illness, with mild forms causing vomiting, diarrhoea (watery to mucoid), and more severe forms causing anorexia, fever, haemorrhagic diarrhoea, and signs of sepsis.¹⁰

There is a wide range of prevalence reported in a large number of studies, many of which are driven by zoonosis research. Although salmonellosis is primarily considered a food-borne illness in humans, there is growing interest in companion animal sources of infection.⁹⁴ Such concerns are compounded by the increasing popularity of raw meat-based diets, which has been linked to a higher prevalence of *Salmonella* spp. detection in faecal samples, as described below.

Salmonella prevalence in dogs ranges from 2.5% to 8.3%.^{95,96} One study tested 2422 faecal samples from 11 different US labs and found nearly half (45%) the *Salmonella* spp. positive samples were described as normal faeces,⁹⁵ although diarrhoeic samples had nearly twice the prevalence compared to non-diarrhoeic samples (3.8% versus 1.8%). Risk factors were raw food diet, recent antimicrobial use, probiotic treatment, and hot weather.⁹⁵ A sled dog reported over 70% prevalence of *Salmonella* spp. in dogs before and after racing.⁹⁷ A Taiwanese study reported different prevalences between stray dogs (6.3%) and pet dogs (2.1%).⁹⁸ In a Dutch prospective study establishing enteric microorganism profiles in 169 dogs, faecal culture was used and only 1% of diarrhoeic puppies, and no healthy puppies, had *Salmonella* detected.⁹⁹ All these studies used culture-based methods.

Many studies also explored associations between diet and prevalence, specifically

exposure to raw-meat diets. In 28 laboratory dogs that were fed either a contaminated raw food or uncontaminated raw food, only dogs fed contaminated raw food shed *Salmonella* spp.¹⁰⁰ Association with raw meat consumption has been duplicated in many studies.^{101–104} A large UK study of 442 *Salmonella* positive faecal samples reported serotype Typhimurium as the most common (44.3%), followed by Dublin (9%), Enteridis (6.3%), Montevideo (4.2%), Virchow (2.3%), Heidelberg (1.8%), and Derby (1.8%), and another 55 serotypes of lower frequency.¹⁰⁵ Many serotypes were strains found in other mammals. It is likely dogs carry many other serotypes that are yet to be reported.

1.2.6. Cryptosporidium spp.

Cryptosporidium is a single-cell, spore-forming coccidial protozoan parasite of the Apicomplexa phylum. It is an obligate intracellular parasite that can only survive extended periods outside a host cell as an oocyst containing sporozoites.¹⁰⁶ Until the mid-1990s, species-level classification was based on morphology and host specificity. *Cryptosporidium parvum* was the most common isolate detected in both people and other mammals, with evidence of transmission between host species.¹⁰⁷ With the advent of molecular diagnostics in the 1990s, genotype classifications for *C. parvum* are now mostly based on host-specificity, eg *C. parvum* type 1 in humans (recently renamed to *C. hominis*),¹⁰⁸ and type 2 in cattle. The dog-specific species was named *C. canis*. Only *C. parvum* has been implicated in human waterborne outbreaks, but *C. canis* has been implicated in other human infections.¹⁰⁹ Geographic variations in humans studies show *C. hominis* is most common in North America and Australia, and *C. parvum* is most common in Europe.¹¹⁰ Although there are considerable zoonotic concerns with species like *C. parvum* known to infect humans and multiple other species, detection of *C. canis* in humans is very uncommon and is mainly reported in immunocompromised humans.^{111,112}

Transmission occurs via cysts and the oro-faecal route. After ingestion, excystation occurs in the small intestine following stimulation by the acidic environment of the stomach, change to body temperature, and exposure to bile salts. Excystation releases 4 motile sporozoites per oocyst, and it is these motile sporozoites that invade gastrointestinal epithelial cells.¹⁰⁶ Sporozoites are capable of invading intestinal epithelial cells where they reside barely within the cell membrane in the apical region, but exterior to the cytoplasm. Sporozoites undergo an asexual then sexual phase, during which they are fertilised to become either thin- or thick-walled oocysts.¹⁰⁶ Thick-walled oocysts survive full transit through the gastrointestinal tract and thus become the infective stage shed into faeces, whilst thin-walled oocysts rupture within the gastrointestinal tract causing autoinfection.¹⁰⁶

Cryptosporidia are opportunistic. Most infections in dogs are considered asymptomatic or paucisymptomatic and most clinical infections occur in immunocompromised hosts.¹¹³ Mechanism of disease is not fully understood, but is often described as blunting of villi due to invasion of epithelial cells. This leads to malabsorption of electrolytes and hypersecretory diarrhoea.¹⁰⁶ Other key factors include host factors (primarily the immune response), and co-infections; reports suggest *Giardia* co-infection increases morbidity.¹¹³ Clinical signs in dogs are often reported as anorexia, weight loss, and watery small-intestinal diarrhoea without blood or mucus.¹⁰⁶

Again, many studies are driven by zoonotic concerns. One of the largest prevalence studies from Germany used direct faecal smear techniques and detected *Cryptosporidium* spp. in 3.4% of 2731 faecal samples,¹¹⁴ similar to a 677-dog UK study that used PCR assays and detected 4.6% prevalence.¹¹⁵ Over 75% of dogs in this latter UK study had formed faeces. Genotyping found 28/30 dogs had *C. canis*, and 2/30 were *C. parvum*.¹¹⁵ Another study tested 444 dog faecal samples collected from a dog park in

Vienna and found no cases using IFA and faecal microscopy.¹¹⁶ In contrast, some studies report much higher prevalence from 21 to 34%, using combinations of PCR and traditional coproscopic methods.^{117,118} These studies also found a significantly higher risk in dogs less than one year of age. *Cryptosporidium canis* is most frequently detected in dogs.^{84,118,119} Other detected species include *C. parvum*, *C. muris*, and *C. scrofarum*.^{120,121} Studies that included faecal consistency found variable rates of *Cryptosporidium* spp. detection, with some reporting no difference between diarrhoeic and non-diarrhoeic dogs.^{118,122} Recent Australian studies using PCR assays report a prevalence of *Cryptosporidium* spp. of 0-4% in dog faecal samples.^{123,124}

1.2.7. Canine enteric coronavirus

Alphacoronaviruses are enveloped RNA viruses that are traditionally classified into three antigenic groups. Canine enteric coronavirus falls under group 1, which also includes feline coronaviruses, porcine respiratory coronavirus, and human coronavirus 229E.¹²⁵ Originally identified as a canine enteric pathogen in 1974,¹²⁶ it is now found globally. There are two CCV genotypes, types I and II, distinguished by differences in spike proteins.¹²⁷ Both appear to have pathogenic capability, but their role in enteric disease is not fully understood.¹²⁸ They are often both found together. Furthermore, additional variants of type II, such as IIa and IIb, and a newer strain called CB/05, have been identified and associated with more severe forms of illness.¹²⁹⁻¹³¹ Genotyping of these more virulent strains show considerable genetic overlap, suggesting recombinant evolutionary events for which coronaviruses are renowned.¹³¹ It is this trait that gives coronaviruses their reputation for persistence and virulence.

Canine enteric coronavirus predominantly causes gastrointestinal signs including

vomiting, diarrhoea, and anorexia following oro-faecal transmission.¹²⁵ Much more prevalent in puppies, it appears to cause more severe clinical illness upon co-infection with another enteric pathogens, in particular CPV,¹³² but also canine adenovirus,¹³³ and canine distemper virus. Once infected, the virus targets enterocytes at villous tips, causing blunting of villi and secondary increase in crypt cell mitotic activity, which in turn results in malabsorption then diarrhoea.¹³⁴ A latent period of 18-72 hours has been reported.¹³⁴ Type IIa CCV causes the 'classic' coronaviriosis manifesting as enteritis and small-bowel diarrhoea. Type IIb CCV is reputed for causing disease in neonates. Shedding has been reported to occur for as long as 6 months after clinical disease, an important feature that aids persistence in the population.¹³⁴ For the more virulent newer strains, systemic disease and multi-organ failure secondary to necrosis of extra-gastrointestinal lymphoid tissue (including spleen, thymus, regional lymph nodes) is reported,^{125,131} and hence these strains have been labelled 'pantropic' CCV type IIa.¹²⁷

Prevalence reports for CCV vary depending on region, population studied, and importantly, diagnostic methodologies used. One study used PCR to detect CCV in dogs housed in 4 different UK dog kennels; prevalence in the two shelter kennels was 13.8% and 33.3%, whereas in the two boarding kennels 5.3% and 13.5%. The majority were genotyped to CCV type I.¹³⁵ Other studies report prevalence from 0.1% in 130 dogs seen at a vet clinic,¹³⁶ to 12% in 250 dogs with diarrhoea,¹³⁷ to as high as 26.7% in 101 dogs sampled from a single community in Japan.¹³⁸ Two studies comparing healthy dogs to diarrhoeic dogs found higher prevalence in the healthy dogs.^{139,140}

1.3. Gastrointestinal microbiome

Thus far we have discussed a limited list of enteric microorganisms linked to

gastrointestinal disease. It is important to note though, that the gastrointestinal microorganism profile, better known as the gastrointestinal microbiome, is composed of thousands of phylotypes, with a population far greater than the total number of cells in an adult dog.¹⁴¹ Hence, the microbiome likely plays a major role in acute infectious gastrointestinal disease in dogs, and focusing only on the above microorganisms in isolation will limit our understanding of this topic. To better understand the complexity surrounding this question, we need to first look at the field of microbiomics. However, given this is beyond the scope of our research, we will only cover the key facts.

1.3.1. Microbiome background

The canine gastrointestinal microbiome describes a diverse population of microorganisms within the canine gastrointestinal tract that is essential for nutrient metabolism, gut health, immune function, and development from birth to maturity. It consists of bacteria, archaea, protozoa, fungi, and viruses. Microbiome research has experienced huge growth this millennium coinciding with advances in molecular diagnostics, in particular gene sequencing technologies.¹⁴² There have been attempts to establish a complete microbiome, which revealed Firmicutes, Bacteroidetes, and Fusobacteria as the main bacterial phyla in the gastrointestinal tract of the adult dog.¹⁴³ Other key findings include the discovery that the microbiome changes with certain disease,¹⁴⁴ and similar to people, antimicrobials significantly alter the microbiome.¹⁴⁵ An important term that has also emerged is dysbiosis, which loosely describes any alteration in the intestinal microbiome that has putative adverse effects on host health.¹⁴² Dysbiosis can be due to changes in the microorganism population, proportion of individual microorganisms within the population, changes in population diversity, or a combination of the above.

1.3.2. Microbiome and acute diarrhoea

The microbiome cannot be evaluated with an eight-microorganism multiplex PCR assay. Although beyond the scope of our research objectives, findings from microbiome research are important considerations. First, there is much high quality evidence demonstrating large scale microbiome changes associated with gastrointestinal disease.^{146,147} One study used both gene sequencing techniques and PCR assays to compare the gastrointestinal microbiome between healthy non-diarrhoeic dogs, dogs with non-haemorrhagic and haemorrhagic acute diarrhoea, and dogs with active and controlled IBD.¹⁴⁸ The dogs with acute diarrhoea, especially haemorrhagic acute diarrhoea, demonstrated the biggest disturbances to the microbiome. Second, these 'big picture' perspectives contrast with studies investigating a single or a few microorganisms in cases of acute diarrhoea. Standard multiplex PCR assays test for five to eight microorganisms. Therefore, it is likely that important clinical information is lost by focusing on just a fraction of the complex microbiome. Furthermore, results of tests performed in one location may not directly translate to subjects from a different geographic location. This was elegantly demonstrated in a human study evaluating the faecal microbiome of 7009 people from 14 neighbouring districts.¹⁴⁹ Multiple factors affecting the faecal microbiome were evaluated, and location was the biggest source of variation, even more so than health versus disease. This clearly shows the difficulties in creating a 'reference' microbiome. The authors recommend establishing localised reference microbiomes instead.¹⁴⁹

Currently however, advanced molecular diagnostics remains the realm of research due to costs and logistics, whilst multiplex PCR assays have become widely available for clinical diagnostics. Until techniques such 454-pyrosequencing and shotgun sequencing become cheaper and technically easier to perform, PCR assays will remain the only

readily available molecular diagnostic tool. It is thus important to be mindful of the very limited scope of the gastrointestinal microbiome these PCR assays reveal, and that detection of one microorganism on a PCR assay may represent only a minute component of the dysbiosis contributing to the clinical disease.

1.3.3. Antimicrobial stewardship

Antimicrobials have a well-defined role in both the development of and treatment of severe infectious enteritis in humans.^{150,151} In veterinary medicine, there is good justification for their use in some subsets of dogs with primary gastrointestinal disease. This includes animals deteriorating to a septic state, or animals in a shelter or kennel environment involved in an outbreak of gastrointestinal disease who have certain infectious microorganisms detected in their diarrhoea, such as CPV. However, their use in otherwise routine cases of acute diarrhoea should be questioned. Many of these cases will either have self-limiting signs, or respond well to appropriate supportive care.¹⁰ This has been demonstrated in several prospective trials. In a clinical trial comparing amoxicillin-clavulanic acid to placebo in dogs with AHDS, no difference was found between treatment groups in time to resolution of clinical signs, nor in the clinical course of disease as measured by indicators such as vomiting, appetite, and hydration.² Another study used a similar study design to compare high-potency probiotics to placebo, and again found no difference in the course of disease.³

These findings underscore the importance of careful consideration of the indications for antimicrobial use in dogs and cats presenting for gastrointestinal disease. This is particularly prescient in light of the One Health Initiative, aiming for a collaborative approach to human and veterinary infectious disease, with a key goal of improving antimicrobial stewardship and stemming the rate of drug-resistance in pathogens.^{44,152}

1.4. Diagnostics

1.4.1. Background

Many dogs with acute diarrhoea have a self-limiting disease, or require only inpatient or outpatient supportive care. This is because many of these dogs will remain cardiovascularly stable and maintain euvolaemia by addressing supraphysiologic fluid losses through oral food and water intake. Even in dogs with a more severe form of illness, such as that seen in AHDS, prospective clinical trials have demonstrated good outcomes by simply addressing intravascular and interstitial fluid deficits with appropriate supportive care.² For acute diarrhoea cases where diagnostics are performed, veterinarians should be able to provide justification. For some differentials, such justification is clear. Foreign object ingestion requires diagnostic imaging to plan for possible invasive interventions (exploratory laparotomy or endoscopy), while neoplasia or IBD require a diagnosis via histology with or without concurrent imaging to plan for long term medical management.¹⁵³ Infectious causes may also need investigation to guide antimicrobial therapy, but as described above, it is unclear if a detected enteric microorganism requires antimicrobial treatment or whether detection represents pre-existing colonisation with that microorganism or changes in normal gut flora. It is important then that we have a good understanding of all diagnostics used in these cases to ensure appropriate treatment. A key component of an appropriate therapeutic plan is excellent antimicrobial stewardship, as discussed earlier. This section will discuss the current knowledge surrounding available diagnostics with a focus on faecal PCR assays.

1.4.2. Traditional diagnostic methods

Until molecular diagnostics became widely available, the diagnostic approach for

infectious causes of diarrhoea had changed minimally over the past few decades. Direct faecal microscopy, faecal flotation, antigen testing for CPV and CCV, and cultures have for a long time been widely available, and more advanced diagnostics such as virus isolation, haemagglutination assays, and electron microscopy are still restricted to university hospitals or research facilities. Available tests vary in sensitivity and specificity, which in turn impact the positive and negative predictive values,¹⁰ and disease prevalence (or pre-test probability) is equally as important in evaluating test performance. A comprehensive understanding of these details is essential when using test results to formulate evidence-based treatment plans.

1.4.2.1. Faecal microscopy

Faecal microscopy primarily entails both direct cytologic examination with or without staining, in addition to microscopic examination of samples that have undergone separation techniques utilising the different densities of faecal microorganisms centrifuged with hypertonic solutions. These are well established methodologies for detecting faecal parasites and protozoa.¹⁵

Faecal direct smear microscopy is used to identify spiral-shaped microorganisms. The classic curved or 'gull-wing' shaped microorganisms can indicate presence of *Campylobacter* spp., however it cannot accurately differentiate between similarly shaped bacteria also present in the gastrointestinal tract such as *Helicobacter* spp. and *Yersinia* spp..^{22,154} Other laboratories may also incorporate dichlorofluorescein staining to further enhance *Campylobacter* spp. detection.

For detection of *Giardia* trophozoites and cysts, some authors recommend microscopy alone or in combination with other diagnostics.⁷⁸ In one study, serial testing of faecal samples over 3-5 days was found to overcome intermittent shedding of *Giardia*

trophozoites and cysts, resulting in sensitivity over 90%, similar to point-of-care immunoassay sensitivities.¹⁵⁵ But in an experimental study, zinc sulphate floatation had a high false negative rate, even when samples were tested serially over 5 days.¹⁵⁶

1.4.2.2. Culture-based methods

Faecal cultures were the mainstay for diagnosis of bacterial gastrointestinal pathogens until the addition of molecular diagnostics. Although useful as a readily available, lower-cost tool, culture-based methods hinge on achieving exacting conditions required for target microorganisms to grow. As we have discovered with microbiomic genetic sequencing, less than 1% of the gastrointestinal microbiome can actually be cultured by current means.¹⁵⁷ For the bacterial microorganisms *C. perfringens*, *Campylobacter* spp., and *Salmonella* spp., culture methods have variable utility.

Campylobacter species' fastidious nature requires experience and rigorous technique to grow. In addition, the typical antimicrobials used in culture methods are reported to favour only *Campylobacter jejuni* and *coli*, which is unlikely to give a complete diagnostic picture as although *C. jejuni* has strong evidence for pathogenicity in mammals, other species have also been implicated in disease.^{54,55,158} For any study using only culture-based methods, false-negative rates may be high.

For *Salmonella* spp., cultures are considered a reasonable methodology as the microorganism is readily grown from a variety of standard media. In fact, current guidelines recommend enrichment of samples in broth prior to performing PCR assays.¹⁰ These observations are reflected in numerous epidemiologic studies that first culture *Salmonella* spp. from faecal samples before genotyping.^{99,159} However, widespread use does not equate to acceptable performance, and data from these studies should be critically assessed. Studies comparing culture-based methods to other diagnostics, such

as PCR assays, in dogs report higher sensitivity with PCR assays.¹⁰

1.4.2.3. Immunoassays

Immunoassays originated in the 1950s and are now considered an essential tool for investigation of several enteric microorganisms. Of the numerous immunoassays available, enzyme-linked immunoassays (ELISA) are the most widely used in veterinary medicine. An ELISA uses the specificity of antigen-antibody complexes, that together with fluorescent marker enzymes allow detection of antigens or antibodies (or other molecules of interest) in samples such as bodily fluids. These tests are available for clostridial enterotoxins, *Giardia* spp., *Cryptosporidium* spp., CPV and CCV.^{10,127,156,160,161}

Immunoassays are the most widely used test for CPV.¹⁶² Several immunoassays are available, varying from ELISAs that use antibodies to bind CPV antigen present in a suspended faecal sample, to lateral flow chromatographic assays. The point-of-care versions provide veterinarians with a rapid, bedside screening tool, which is essential in managing this highly contagious and severe disease. However, point-of-care immunoassays for CPV have been shown to under-perform, with reported sensitivities ranging from 15-65% as compared to PCR, in dogs with parvovirus.^{162,163} Also the current commercially available point-of-care immunoassays are unable to differentiate between vaccine and field strain CPV antigens, unlike fully quantitative or more advanced molecular diagnostics.¹⁶⁴ Until bedside PCR assays become available, CPV immunoassays will continue to hold an essential role managing this highly contagious disease.

Immunoassays also play a key role in the detection of *C. perfringens* enterotoxins. As *C. perfringens* is a commensal microorganism in dogs, immunoassays are used to detect clostridial enterotoxins, such as *C. perfringens* enterotoxin, the putative cause of

gastrointestinal clostridiosis.¹⁰ Current guidelines recommend testing specifically for *C. perfringens* enterotoxin using ELISA in combination with PCR assays.¹⁰ There is only one ELISA commercially available for *C. perfringens* enterotoxin testing (Techlab Inc, Blacksburg, VA); it is important to note that it has only been validated for humans, and healthy dogs also routinely test positive.^{24,165}

For *Giardia* spp., both fluorescent antibody assays and antigen-based ELISAs are routinely used, and some authors promote fluorescent antibody assays as a reference standard.¹⁵⁶ Fluorescent antibody assays use a similar principle to ELISAs and both can be direct or indirect assays. The indirect label refers to an extra step in the antibody-based assay, whereby a second antibody against the first antibody carries a fluorescent probe. This fluorescent probe allows gross detection of any antigen in the sample. Whilst these are relatively uncomplicated tests, they are limited to facilities that have the equipment and expertise to run them with minimal error.

1.4.2.4. Other methods

Other methods, including passive latex agglutination, virus isolation, electron microscopy, and other serologic methods are described for the detection of individual enteric microorganisms.^{11,127,162} These are rarely used in a clinical setting, whether due to scarce availability or poor performance, and will not be discussed in this review.

1.4.3. Molecular diagnostics

Molecular diagnostics were first used in veterinary medicine in the early 1990s.¹⁶⁶ This century, technological advances have broadened their availability beyond academic and research facilities, and at our institution, a faecal PCR assay became available in 2014.¹⁶⁷ One of the first publications used conventional gel-based PCR to detect CPV DNA fragments with nucleotide sequences of commercially available capsid proteins

VP1 and VP2.¹⁶⁸ Since then, PCR assays have been used extensively in identifying other infectious microorganisms associated with diarrhoea.^{55,169} The remainder of this section on PCR assays will discuss DNA testing only, but note that this process, with some variations, can equally be applied to selection and replication of RNA.

1.4.3.1. Faecal PCR assays - procedure

Polymerase chain reaction assays detect infectious microorganisms by replicating and amplifying, then detecting segments of DNA specific to the microorganism(s) in question. The entire process varies depending on the type of PCR performed, the target microorganism, and logistical limitations. Broadly, the process can be described as a process of denaturing then replicating target DNA sequences.¹⁷⁰ In more detail, the first step is preparation of a faecal sample; this is a process that varies by microorganism. For microorganisms that are readily cultured, such as *Salmonella* spp, prior enrichment of the sample in culture media such as broth is still recommended, although rarely performed clinically.¹⁰ For other microorganisms, the faecal sample can be immediately processed via addition of proteinases and chaotropic salts for lysis of cells and proteins, performed in vessels containing special membranes that allow adhesion of newly extracted DNA. This is followed by repeated washings then elution of DNA material in a solution for further processing. Laboratories now mostly use proprietary kits dedicated to faecal DNA extraction.²⁹ It is essential this extraction is performed with utmost care, firstly to prevent contamination, a recurring theme throughout the entire PCR assay process, and second to minimise damage to DNA material as this would hinder subsequent detection potentially leading to false negative results.¹⁷⁰ This solution can also be heated to denature any DNA polymerase inhibitors. Once this DNA-rich solution has been prepared, the actual PCR assay is performed.

First the appropriate primers must be chosen. This is a specific length of single-stranded

DNA precisely complementary to opposing ends of each strand of the amplicon being targeted. Primers for most common microorganisms can be commercially created using widely available software and canine gene sequencing data (GenBank). Selection of suitable primers is essential to ensure hybridisation occurs only to the targeted DNA sequence and not other similar sequences within the same microorganism or in other microorganisms. The DNA solution and primers are then combined with DNA polymerases and nucleotides in a thermocycler, which cycles the mixture through pre-programmed temperatures that denatures DNA into single strands at high temperatures, anneals primers to their complementary DNA sequence at low temperatures, followed by amplicon extension facilitated by DNA polymerases. Each cycle theoretically doubles the quantity of single-stranded target DNA, and each subsequent cycle replicates all target strands, not just the original, resulting in an exponential increase in DNA copies. One strand can result in billions of copies after 30+ cycles.¹⁷¹

Accurate reading of results is as important as the thermocycling process. There are two main methods. Conventional PCR most commonly uses gel electrophoresis - an electrical current pulls negatively charged DNA strands along a gel producing lines denoting their resting position in the gel; the distance travelled during a certain time period is determined by the length of amplified DNA strands (i.e. number of base pairs). This is compared to a 'DNA ladder' produced by known substances to identify the DNA sequences present.¹⁷⁰ However, technological advances have led to real-time PCR, which instead include short nucleotide sequences that anneal to either end of replicated target DNA strands. These sequences, or probes, are attached to a fluorescing molecule. Real-time PCR assays are faster, fully automated, and newer multiplex PCR assays allow for detection of multiple different DNA and/or RNA sequences at the same

time.¹⁷⁰ Other modalities, such as melt-curve analysis, incorporate alternative fluorescence-monitoring methods to speed up results, potentially at the cost of being able to identify multiple different DNA sequences.¹⁷² Some available assays also provide quantification of target DNA strands. This is typically achieved by combining the fluorescent probes that flag target DNA strands, with automated detection systems. These systems then use proprietary algorithms to quantify the detected target DNA in any given sample. Also, machines that track the amplification process through the exponential, linear, and plateau periods of growth are of greater utility compared to machines that can only provide counts at the end plateau period, as this diminishes their accuracy.¹⁷¹

Additionally, there are other methodologic variations that provide additional uses: restriction length fragment polymorphism was an earlier development that made gene mapping more widely available; reverse-transcription PCR allowed detection of RNA,¹⁷³ and recently isothermal PCR has been touted for potential point-of-care diagnostic use.^{174,175} Multiplex PCR use in veterinary medicine has rapidly grown this decade, with accompanying validation studies demonstrating its use for detection of bacteria, viruses, protozoa, and parasites.^{124,176}

1.4.3.2. Faecal PCR assays - limitations

With the capability to detect fragments of DNA, PCR assays are considered powerful tools in diagnostic medicine. They have overcome limitations associated with culture-based methods for microorganisms, and with vast research and development, rapid technological advances have broadened their availability and increased their capabilities. As an example, the development of gene sequencing has identified the *cpn60* universal *Campylobacter* spp. gene that has facilitated differentiation of the many *Campylobacter* spp.. Subsequently, a group of authors used these data to first create

primers for 14 different species, then they validated a real-time tandem PCR assay that identified all *Campylobacter* spp. in canine faecal samples.¹⁷⁷ However, such high sensitivity comes with limitations, to be discussed broadly in this section.

First, quality control is absolutely essential to avoid contamination and false-positive results. Contamination of samples, the thermocycler, or reagents with even a single strand of DNA can potentially result in exponential replication. Commercial laboratories in Australia are regulated by the National Association of Testing Authorities (nata.com.au), who require a minimum standard of quality control. For PCR assays, this typically involves running control samples (eg. sterile water) regularly to screen for false-positives secondary to contamination. Another consideration is careful handling of the many solutions used to run a PCR assay - nucleotides, probes, primers, polymerases, buffers, and wash solutions. Handling errors could result in failure of any of the stages of a PCR assay - denaturation, annealing, extension, identification/quantification - causing false-negative results, or contamination causing false-positive results.¹⁷⁰ Given the implications of contamination, it is recommended to separate areas of the lab where samples are handled pre-PCR to areas designated for post-PCR sample handling. Also, as mentioned earlier, primers must be carefully selected and validated to maximise specificity and precision to only the target DNA sequence. Erroneous annealing to non-target DNA sequences can result in mis-identification of a microorganism, or excessive depletion of primers during the assay, falsely lowering any quantification results.¹⁷⁰ Ideally, all PCR assays should be internally or externally validated to address these concerns. This can be a simple exercise of performing an assay on a sample known to contain the target microorganism, or also determining detection limits.¹⁷⁸ The primary purpose of any PCR assay validation would be to ensure the primer anneals only to the targeted DNA

sequence, and that the thermocycling program and polymerase combination effectively allows denaturation, annealing, and extension. Unfortunately, evaluating the validation processes in studies evaluating PCR assays is not always repeatable or even possible. This may be due to commercial confidentialities, or poor documentation.

Another important distinction are the additional considerations for using PCR to detect RNA rather than DNA. RNA is generally considered to be more fragile than DNA, and it also more readily mutates, potentially leading to poorer performance of PCR assays for RNA.

With regards to the two studies that follow (chapters 2 and 3), this vital information, including validation processes, detailing the PCR methodologies used was not available for proprietary reasons. However, the laboratory used in both studies is certified under the NATA, which as mentioned above, has validation requirements to ensure a high standard of quality control.

There are a few additional considerations when performing faecal PCR assays. First, sample handling potentially impacts results. Human studies have shown storage affects PCR results,^{179,180} and they recommend avoiding temperature fluctuations during storage and transport. Repeated temperature fluctuations has been shown to kill microorganisms and degrade any DNA present.^{179,180} Cold-storage (-80°C to 4°C) is very beneficial as it minimises changes in bacterial populations.¹⁸¹ Additionally, newer publications have also proposed sample storage with faecal stabilizers, such as commercial DNA stabilising preparations, and even 95% ethanol,¹⁷⁹ to maximise assay performance. In contrast, protozoa such as *Giardia* spp. and *Cryptosporidium* spp. require measures to disrupt cyst/oocyst walls and release DNA material. Several methods are described, including freeze-thaw cycles, and proprietary mechanical disruptors.¹⁸²

Furthermore, when reviewing PCR-based publications, data comparison is limited by methodologic differences. Differences in sample handling and preparation can affect the microorganism community in the sample, and subsequently the PCR results. The other limitations described above can equally lead to bias towards some microorganisms over others. An *in vitro* study found six different commercial extraction methods produced six significantly different DNA pools when fed a mock-microbiome sample.¹⁸³ Specifically, harsher extraction methods favoured gram-positive bacteria.

On the patient side, it is vital the veterinarian understands numerous factors will influence the faecal microbiome at the instant of sampling, including time of day, and time in relation to ingesta movement through the gut. Although microbiome alterations due to chronic diet composition are well described, short term changes also can occur in people for reasons such as regional differences, and diet changes.^{149,184}

1.4.3.3. Canine faecal PCR literature

Faecal PCR assays have been validated for *C. perfringens* toxin genes,¹⁸⁵ and their role in AHDS has been explored extensively using PCR assays.^{186,187} PCR combined with ELISA was used to establish prevalence of *C. perfringens* and associated enterotoxin in healthy versus diarrhoeic dogs.²⁹ Authors replicated PCR methodology in a previously published study, but no validation procedures are described. Newer clostridial toxins, NetE and NetF, have only been identified thus far via PCR.³⁵

Similar to studies using culture-based methods, *Campylobacter* spp. prevalence studies using PCR assays also describe a wide range, from 43% in dogs sampled at a community park to 97% in diarrhoeic dogs.^{55,188} The majority of studies however used PCR only to speciate after detection of *Campylobacter* spp. via faecal culture. In a study comparing *Campylobacter* spp. prevalence, 56% versus 97% of healthy dogs versus

diarrhoeic dogs had *Campylobacter* detected via a validated quantitative PCR assay.⁵⁵ One study used both culture-based methods and PCR assays, and although PCR had higher overall sensitivity, nine culture-positive dogs tested negative via PCR.⁵¹ Reasons for this include methodologic degradation of DNA material, or polymerase inhibitors in faecal samples. The fastidious nature of *Campylobacter* spp. means many species are difficult to grow *in vitro*, limiting the sensitivity of faecal culture to identify this microorganism. This has been overcome with new multiplex real-time PCR methods that can detect a more diverse *Campylobacter* population in dogs, and the hypothesis that diarrhoeic dogs have greater *Campylobacter* dysbiosis.⁵⁵

The use of PCR for diagnosis of *Giardia* spp. infection may be less valuable as compared to its use for other microorganisms. Since PCR assays have been reported to have a high (20%) failure rate for *Giardia* diagnosis,⁷⁸ some authors recommend using PCR assays only for genotyping to identify which assemblage is involved. Additionally, primer selection for individual assemblages must be done with caution, as some DNA sequences previously reported for *Giardia* detection are not highly specific and thus multilocus genotyping is recommended.¹¹ Furthermore, as with non-PCR techniques, intermittent shedding of cysts must be considered when encountering negative PCR results in dogs with a high index of suspicion for giardiasis. Few studies compare PCR performance to other methodologies. In one prospective healthy-dog study, PCR assay performed poorly.¹⁸⁹

As culture-based methods effectively identify *Salmonella* spp., PCR assays have played a secondary role from a diagnostic perspective. *Salmonella* is readily grown in multiple media, therefore enrichment prior to running a PCR assay is recommended.¹⁰ Few studies include validation details.¹²⁴

Canine parvovirus publications involving PCR assays have stronger data and more

rigorous methods, in part due to the longer history of PCR use for CPV investigation.¹⁶⁸ Many studies are produced by a few research groups, who routinely publish detailed PCR methodology, in addition to validation procedures.^{163,164,190} PCR has also been used to monitor for CPV mutations.⁷⁵

Historically, *Cryptosporidium* spp. were diagnosed with immunoassays or enhanced faecal microscopy. Many studies utilising PCR assays primarily did so for genotyping, after a positive test result from other, more traditional methods.^{121,191} Other studies compared PCR to other methods. One study tested 50 household dogs and reported 34% *Cryptosporidium* spp. prevalence using microscopy, and only 24% using an unvalidated nested PCR assay.¹¹⁷ Nested PCR assays use 2 sets of primers in succession, with the first set shrinking the DNA available for replication, and the second set identifying the target region within the newly-truncated sequence. This reduces replication (and subsequent amplification) of similar non-target sequences. Studies that only used PCR reported prevalence from 4.6% in 677 household dogs to 21% in 314 breeding kennel dogs.^{115,118}

Polymerase chain reaction assays are widely used for CCV investigation, and reverse-transcription PCR is reported to have the highest sensitivity compared to a reference standard post-mortem immunoassay and immunohistochemistry.¹²⁷ PCR has been validated for faecal assays,¹⁹² and more recently was used to identify pantropic severe coronaviruses.¹⁹³ Both conventional and nested PCR were shown to have much higher sensitivity compared to virus isolation and electron microscopy for the diagnosis of CCV.¹⁹⁴

Polymerase chain reaction assays clearly have a key role when investigating these enteric microorganisms as potential causes of acute diarrhoea in dogs. They have numerous advantages over traditional tests, and became commercially available to

much hype over their purported high sensitivity and accuracy. However, not only are there general limitations with PCR assays as detailed above, but limitations associated specifically with enteric microorganism investigation in dogs.

The foremost limitation of faecal PCR assays is the clinical relevance of a positive test result. This is particularly evident with *C. perfringens* and PCR assays that only test for the alpha toxin gene, as discussed earlier.¹⁰ Another issue is that for *Salmonella* spp, *Campylobacter* spp, *Giardia* spp, *Cryptosporidium* spp., and CCV, the prevalence rates vary widely between studies, and most importantly, there is not a clear difference in prevalence between healthy and diarrhoeic dogs. For *Salmonella* spp, the only consistent finding across many studies is a higher prevalence in dogs fed raw meat.^{95,100,101} For *Giardia* spp, non-specific PCR assays detect assemblages with questionable pathogenicity to dogs (A and B).⁷⁸ Similarly, only *C. jejuni* and *C. coli* are confidently described to cause acute gastrointestinal disease in dogs.⁴⁵ Unfortunately most PCR assays, including that available to veterinarians at our institution, do not have the capacity to speciate the detected *Giardia* or *Campylobacter* isolates.¹⁶⁷

These studies highlight one of the hurdles faced when investigating acute diarrhoea with faecal PCR assays - does detection of a known enteric microorganism identify a pathogenic role, is it representative of dysbiosis, or is it an incidental finding? Several factors contribute to this uncertainty - microorganism characteristics, biologic factors that impact PCR assay performance, and the complexity of the microbiome.

1.4.4. Faecal PCR assay results and causation

Several well designed prospective studies have unsuccessfully attempted to demonstrate an association of certain enteric microorganism(s) with acute diarrhoea, and imply causation. In a prospective study comparing a group of dogs with AHDS and

C. perfringens enterotoxin and a group of AHDS dogs without *C. perfringens* enterotoxin, there was no difference in the course of clinical disease, as well as time to resolution of signs.¹⁸⁶ These dogs had both ELISA to detect the actual toxin in their faeces, as well as quantitative PCR to detect *C. perfringens* enterotoxin genes. Another prospective study found similar results comparing dogs with AHDS with and without *netE* and *netF* toxins, although they did report a significantly higher prevalence of *netE* and *netF* genes in dogs with CPV compared with healthy, non-diarrhoeic dogs.³⁶ However, they detected *C. perfringens* in a larger percentage of the AHDS dogs compared to a healthy dog control group, and suggest *C. perfringens* represented either incidental dysbiosis secondary to the underlying gastrointestinal disease, or dysbiosis and enterotoxin production contributing to the clinical illness.^{36,186} A non-peer reviewed abstract proposes instead that fully quantitative PCR for *C. perfringens* alpha toxin gene may be more meaningful, having discovered that very high quantities of this otherwise innocuous toxin associated with diarrhoea in their study population.¹⁹⁵

Similar studies show frequent detection of many of these microorganisms in healthy, non-diarrhoeic dogs,^{46,118,196} whilst a general enteric microorganism study in shelter dogs using PCR found a higher percentage of CCV in healthy dogs compared to diarrhoeic dogs.¹⁴⁰ Of the microorganisms discussed in this review, only CPV has extensive evidence supporting its primary pathogenic role in gastrointestinal illness. But by no means does a positive PCR test for CPV definitively indicate parvovirus given there are numerous studies that portray a subclinical 'carrier' population of CPV-positive dogs.^{163,197}

Further complicating PCR assay performance is its inherent sensitivity. Having the ability to exponentially replicate a strand of DNA not only means laboratory contamination is an important confounder, as discussed earlier, but it may also detect incidental DNA

material in faecal samples. For example, this includes ingested DNA material of dead microorganisms that have no pathologic abilities. A microbiome study of canine duodenal flora found significant variations between samples collected a few centimetres apart, and between samples collected one week apart.¹⁹⁸ They also found a difference between samples collected from the duodenal contents versus a brush sample from the duodenal mucosa.¹⁹⁸ These findings can be extended to suggest faecal samples likely represent only the distal gastrointestinal tract, or suggest faecal samples may poorly represent the small intestines (the site of interest in many acute diarrhoea cases), or alternatively, the concurrent importance and challenges in identifying luminal versus mucosa-adherent microorganisms.

Finally, the PCR assay available to veterinarians in Perth, WA, only tests for eight microorganisms.¹⁹⁹ As such, other enteric microorganisms associated with gastrointestinal disease are excluded when using this test. These include *Escherichia coli*, *C. difficile*, *Cystoisospora* spp., and rotavirus.

1.4.5. Faecal PCR assays - conclusion

As demonstrated above, it is clear that a positive faecal PCR result does not imply that the microorganism identified is causing diarrhoea, and instead there are many factors to consider when evaluating the results of a typical faecal PCR panel. Ideally, the PCR assay should be validated and performed under strict conditions to maintain quality control and minimise lab error such as contamination, or reagent mishandling. Furthermore, stool samples should ideally be refrigerated and tested within 48 hours of collection to minimise degradation of microorganisms and their DNA.¹⁸⁰ Once a PCR has been performed, results should then be interpreted in light of a full clinical picture - including but not limited to a detailed history consisting of diet, duration and type of clinical signs, and deworming and vaccination status, including type of vaccination

protocols used.

But much more investigation is required to fully understand the role these microorganisms play in acute diarrhoea. More prospective reference studies to establish the prevalence of these microorganisms in healthy and diseased dogs is essential. Many have already been performed, but a variety of methodology limits comparisons. For those that have used comparable methods such as real-time faecal PCR assays, it is clear that there is a wide range of findings, as described above. Aside from methodologic differences that include differences in patient selection, differences in patient baseline characteristics, and disease states, other potential reasons include regional and temporal variation.

1.5. Knowledge gap

This leads to the need for descriptive PCR studies providing the accompanying clinical data, as well as the enteric microorganism profiling of specific regions, and ideally also investigating whether there are seasonal variations. Having this data on both healthy dogs and those with varying but defined gastrointestinal diseases will help veterinarians interpret positive results of a faecal PCR panel. The importance of regional variation was elegantly shown in a large study describing the gut microbiota of 7009 humans from 14 different provinces in China that found location was statistically associated with the greatest variation in the microbiota of subjects.¹⁴⁹

Although there are numerous advanced diagnostic tests for enteric microorganisms, such tests remain in the research domain only and are not yet commercially available. This includes gene sequencing, and newer variants of traditional PCR assays.²⁰⁰ A traditional real-time faecal PCR may not be capable of providing the granular data of

these more advanced diagnostics, but they are widely commercially available and thus was the methodology we focused on in our research. With the increasing use of real-time faecal PCR assays to investigate acute diarrhoea, knowledge of the regional enteric microorganism profile will contribute to a better understanding of faecal PCR results. This will have obvious benefits for patient care, and additionally, may contribute to better antimicrobial stewardship.

1.6. Bibliography for literature review

1. Diarrhoeal disease.
<http://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease>. Retrieved February 6 2020.
2. Unterer S, Strohmeyer K, Kruse BD et al. Treatment of Aseptic Dogs with Hemorrhagic Gastroenteritis with Amoxicillin/Clavulanic Acid: A Prospective Blinded Study. *J Vet Intern Med* 2011;25:973–979.
<http://dx.doi.org/10.1111/j.1939-1676.2011.00765.x>.
3. Ziese A-L, Suchodolski JS, Hartmann K et al. Effect of probiotic treatment on the clinical course, intestinal microbiome, and toxigenic *Clostridium perfringens* in dogs with acute hemorrhagic diarrhea. *PLoS One* 2018;13:e0204691.
<http://dx.doi.org/10.1371/journal.pone.0204691>.
4. Group TWIGS, The WSAVA International Gastrointestinal Standardization Group, Washabau RJ et al. Endoscopic, Biopsy, and Histopathologic Guidelines for the Evaluation of Gastrointestinal Inflammation in Companion Animals. *Journal of Veterinary Internal Medicine* 2010:10–26.
<http://dx.doi.org/10.1111/j.1939-1676.2009.0443.x>.
5. Hubbard K, Skelly BJ, McKelvie J et al. Risk of vomiting and diarrhoea in dogs. *Vet Rec* 2007;161:755–757. <https://www.ncbi.nlm.nih.gov/pubmed/18056013>.
6. Stavisky J, Pinchbeck GL, German AJ et al. Prevalence of canine enteric coronavirus in a cross-sectional survey of dogs presenting at veterinary practices. *Vet Microbiol* 2010;140:18–24. <http://dx.doi.org/10.1016/j.vetmic.2009.07.012>.
7. Pugh CA, Bronsvort BM de C, Handel IG et al. Incidence rates and risk factor analyses for owner reported vomiting and diarrhoea in Labrador retrievers--findings from the Dogslife cohort. *Prev Vet Med Elsevier*, 2017;140:19–29.
<https://www.sciencedirect.com/science/article/pii/S0167587716304354>.
8. Kim E, Choe C, Yoo JG et al. Major medical causes by breed and life stage for dogs presented at veterinary clinics in the Republic of Korea: a survey of electronic medical records. *PeerJ* 2018;6:e5161.
<http://dx.doi.org/10.7717/peerj.5161>.
9. Hume DZ. Diarrhea. *Small Animal Critical Care Medicine* 2015:639–642.
<http://dx.doi.org/10.1016/b978-1-4557-0306-7.00121-5>.
10. Marks SL, Rankin SC, Byrne BA et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med* 2011;25:1195–1208. <http://dx.doi.org/10.1111/j.1939-1676.2011.00821.x>.
11. Sykes JE, Greene CE. *Infectious Diseases of the Dog and Cat - E-Book*. Elsevier Health Sciences, 2013.
<https://market.android.com/details?id=book-eeJOAQAAQBAJ>.
12. Ettinger SJ, Feldman EC, Cote E. *Textbook of Veterinary Internal Medicine*

- eBook. Elsevier Health Sciences, 2017.

<https://market.android.com/details?id=book-57XBDQAAQBAJ>.

13. Washabau RJ, Day MJ. *Canine and Feline Gastroenterology*. Saunders, 2012.

https://books.google.com/books/about/Canine_and_Feline_Gastroenterology.html?hl=&id=-vH3ZwEACAAJ.

14. Popoff MR. Interactions between bacterial toxins and intestinal cells. *Toxicon* 1998;36:665–685. [http://dx.doi.org/10.1016/s0041-0101\(97\)00100-1](http://dx.doi.org/10.1016/s0041-0101(97)00100-1).

15. Marks SL. Diarrhea. *Canine and Feline Gastroenterology* Elsevier, 2013;99. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7151799/>.

16. Trotman TK. Gastroenteritis. *Small Animal Critical Care Medicine* 2009;558–562. <http://dx.doi.org/10.1016/b978-1-4160-2591-7.10128-6>.

17. Withrow SJ, Page R, Vail DM. *Withrow and MacEwen's Small Animal Clinical Oncology - E-Book*. Elsevier Health Sciences, 2013.

<https://play.google.com/store/books/details?id=nYYlauGf0D4C>.

18. Gates MC, Nolan TJ. Risk factors for endoparasitism in dogs: retrospective case-control study of 6578 veterinary teaching hospital cases. *J Small Anim Pract* 2009;50:636–640. <http://dx.doi.org/10.1111/j.1748-5827.2009.00814.x>.

19. Cringoli G, Rinaldi L, Maurelli MP et al. Ancylostoma caninum: calibration and comparison of diagnostic accuracy of flotation in tube, McMaster and FLOTAC in faecal samples of dogs. *Exp Parasitol* 2011;128:32–37.

<http://dx.doi.org/10.1016/j.exppara.2011.01.014>.

20. Wyllie SE, Kelman M, Ward MP. Epidemiology and clinical presentation of canine distemper disease in dogs and ferrets in Australia, 2006–2014. *Aust Vet J* Wiley, 2016;94:215–222. <http://doi.wiley.com/10.1111/avj.12457>.

21. Weese JS, Staempfli HR, Prescott JF et al. The roles of Clostridium difficile and enterotoxigenic Clostridium perfringens in diarrhea in dogs. *J Vet Intern Med* 2001;15:374–378. <https://www.ncbi.nlm.nih.gov/pubmed/11467596>.

22. Marks SL, Kather EJ. Bacterial-associated diarrhea in the dog: a critical appraisal. *Vet Clin North Am Small Anim Pract* 2003;33:1029–1060.

<https://www.ncbi.nlm.nih.gov/pubmed/14552160>.

23. Busch K, Sindern N, Hartmann K et al. The role of pore-forming toxins in dogs with acute hemorrhagic diarrhea syndrome. *J Vet Intern Med* 2017;31:1284.

24. Marks SL, Kather EJ, Kass PH et al. Genotypic and phenotypic characterization of Clostridium perfringens and Clostridium difficile in diarrheic and healthy dogs. *J Vet Intern Med* 2002;16:533–540.

[http://dx.doi.org/10.1892/0891-6640\(2002\)016<0533:gapcop>2.3.co;2](http://dx.doi.org/10.1892/0891-6640(2002)016<0533:gapcop>2.3.co;2).

25. Keyburn AL, Bannam TL, Moore RJ et al. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. *Toxins* 2010;2:1913–1927.

<http://dx.doi.org/10.3390/toxins2071913>.

26. Unterer S, Busch K, Leipig M et al. Endoscopically visualized lesions, histologic findings, and bacterial invasion in the gastrointestinal mucosa of dogs with acute hemorrhagic diarrhea syndrome. *J Vet Intern Med* 2014;28:52–58. <http://dx.doi.org/10.1111/jvim.12236>.
27. Grass JE, Gould LH, Mahon BE. Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998–2010. *Foodborne Pathog Dis* 2013;10:131–136. <http://dx.doi.org/10.1089/fpd.2012.1316>.
28. Sasaki J, Goryo M, Asahina M et al. Hemorrhagic enteritis associated with *Clostridium perfringens* type A in a dog. *J Vet Med Sci* 1999;61:175–177. <https://www.ncbi.nlm.nih.gov/pubmed/10081759>.
29. Minamoto Y, Dhanani N, Markel ME et al. Prevalence of *Clostridium perfringens*, *Clostridium perfringens* enterotoxin and dysbiosis in fecal samples of dogs with diarrhea. *Vet Microbiol* 2014;174:463–473. <http://dx.doi.org/10.1016/j.vetmic.2014.10.005>.
30. Turk J, Fales W, Miller M et al. Enteric *Clostridium perfringens* infection associated with parvoviral enteritis in dogs: 74 cases (1987–1990). *J Am Vet Med Assoc* 1992;200:991–994. <https://www.ncbi.nlm.nih.gov/pubmed/1315727>.
31. Sabshin SJ, Levy JK, Tupler T et al. Enteropathogens identified in cats entering a Florida animal shelter with normal feces or diarrhea. *J Am Vet Med Assoc* 2012;241:331–337. <http://dx.doi.org/10.2460/javma.241.3.331>.
32. Weese JS, Greenwood SJ, Staempfli HR. Recurrent diarrhea associated with enterotoxigenic *Clostridium perfringens* in 2 dogs. *Can Vet J* 2001;42:292–294. <https://www.ncbi.nlm.nih.gov/pubmed/11326633>.
33. Werdeling F, Amtsberg G, Tewes S. The occurrence of enterotoxigenic *Clostridium perfringens* strains in the feces of dogs and cats. *Berl Munch Tierarztl Wochenschr* 1991;104:228–233. <https://europepmc.org/abstract/med/1898321>.
34. Kruth SA, Prescott JF, Welch MK et al. Nosocomial diarrhea associated with enterotoxigenic *Clostridium perfringens* infection in dogs. *J Am Vet Med Assoc* 1989;195:331–334. <https://europepmc.org/abstract/med/2548985>.
35. Mehdizadeh Gohari I, Parreira VR, Nowell VJ et al. A novel pore-forming toxin in type A *Clostridium perfringens* is associated with both fatal canine hemorrhagic gastroenteritis and fatal foal necrotizing enterocolitis. *PLoS One* 2015;10:e0122684. <http://dx.doi.org/10.1371/journal.pone.0122684>.
36. Sindern N, Suchodolski JS, Leutenegger CM et al. Prevalence of *Clostridium perfringens* netE and netF toxin genes in the feces of dogs with acute hemorrhagic diarrhea syndrome. *J Vet Intern Med* 2018; <http://dx.doi.org/10.1111/jvim.15361>.
37. Schlegel BJ, Van Dreumel T, Slavić D et al. *Clostridium perfringens* type A fatal acute hemorrhagic gastroenteritis in a dog. *Can Vet J* 2012;53:555–557. <https://www.ncbi.nlm.nih.gov/pubmed/23115371>.
38. Sykes JE, Marks SL. Campylobacteriosis. *Canine and Feline Infectious*

Diseases. Elsevier Inc., 2013:452–457.

<https://ucdavis.pure.elsevier.com/en/publications/campylobacteriosis>.

39. Martinez-Anton L, Marena M, Firestone SM et al. Investigation of the role of Campylobacter infection in suspected acute polyradiculoneuritis in dogs. *J Vet Intern Med Wiley Online Library*, 2018;32:352–360.

<https://onlinelibrary.wiley.com/doi/abs/10.1111/jvim.15030>.

40. Tam CC, O'Brien SJ, Adak GK et al. Campylobacter coli—an important foodborne pathogen. *J Infect* 2003;47:28–32.

<http://www.sciencedirect.com/science/article/pii/S0163445303000422>.

41. Blaser M, Cravens J, Powers BW et al. Campylobacter enteritis associated with canine infection. *Lancet* 1978;2:979–981.

[http://dx.doi.org/10.1016/s0140-6736\(78\)92541-2](http://dx.doi.org/10.1016/s0140-6736(78)92541-2).

42. Kapperud G, Skjerve E, Bean NH et al. Risk factors for sporadic Campylobacter infections: results of a case-control study in southeastern Norway. *J Clin Microbiol* 1992;30:3117–3121. <https://www.ncbi.nlm.nih.gov/pubmed/1452694>.

43. Wolfs TF, Duim B, Geelen SP et al. Neonatal sepsis by Campylobacter jejuni: genetically proven transmission from a household puppy. *Clin Infect Dis* 2001;32:E97–9. <http://dx.doi.org/10.1086/319224>.

44. Iannino F, Salucci S, Di Donato G et al. Campylobacter and antimicrobial resistance in dogs and humans: “One health” in practice. *Vet Ital* 2019;55:203–220.

<http://dx.doi.org/10.12834/VetIt.1161.6413.3>.

45. Acke E. Campylobacteriosis in dogs and cats: a review. *N Z Vet J* 2018;66:221–228. <http://dx.doi.org/10.1080/00480169.2018.1475268>.

46. Leahy AM, Cummings KJ, Rodriguez-Rivera LD et al. Faecal Campylobacter shedding among dogs in animal shelters across Texas. *Zoonoses Public Health* 2017;64:623–627. <http://dx.doi.org/10.1111/zph.12356>.

47. Guest CM, Stephen JM, Price CJ. Prevalence of Campylobacter and four endoparasites in dog populations associated with Hearing Dogs. *J Small Anim Pract* 2007;48:632–637. <http://dx.doi.org/10.1111/j.1748-5827.2007.00367.x>.

48. Parsons BN, Williams NJ, Pinchbeck GL et al. Prevalence and shedding patterns of Campylobacter spp. in longitudinal studies of kennelled dogs. *The Veterinary Journal* 2011;249–254. <http://dx.doi.org/10.1016/j.tvjl.2010.10.006>.

49. Buogo C, Burnens AP, Perrin J et al. [Presence of Campylobacter spp., Clostridium difficile, C. perfringens and salmonellae in litters of puppies and in adult dogs in a shelter]. *Schweiz Arch Tierheilkd* 1995;137:165–171.

<https://www.ncbi.nlm.nih.gov/pubmed/7569838>.

50. Baker J, Barton MD, Lanser J. Campylobacter species in cats and dogs in South Australia. *Aust Vet J* 1999;77:662–666.

<https://www.ncbi.nlm.nih.gov/pubmed/10590795>.

51. Parsons BN, Porter CJ, Ryvar R et al. Prevalence of Campylobacter spp.

in a cross-sectional study of dogs attending veterinary practices in the UK and risk indicators associated with shedding. *The Veterinary Journal* 2010;184:66–70. <http://www.sciencedirect.com/science/article/pii/S1090023309000124>.

52. Leonard EK, Pearl DL, Janecko N et al. Factors related to *Campylobacter* spp. carriage in client-owned dogs visiting veterinary clinics in a region of Ontario, Canada. *Epidemiol Infect* 2011;139:1531–1541. <http://dx.doi.org/10.1017/S0950268810002906>.

53. Olson P, Sandstedt K. *Campylobacter* in the dog: a clinical and experimental study. *Vet Rec* 1987;121:99–101. <http://dx.doi.org/10.1136/vr.121.5.99>.

54. Burnens AP, Angéloz-Wick B, Nicolet J. Comparison of *Campylobacter* carriage rates in diarrheic and healthy pet animals. *Zentralbl Veterinarmed B* 1992;39:175–180. <http://dx.doi.org/10.1111/j.1439-0450.1992.tb01155.x>.

55. Chaban B, Ngeleka M, Hill JE. Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals. *BMC Microbiol* 2010;10:73. <http://dx.doi.org/10.1186/1471-2180-10-73>.

56. Sandberg M, Bergsjø B, Hofshagen M et al. Risk factors for *Campylobacter* infection in Norwegian cats and dogs. *Prev Vet Med* 2002;55:241–253. [http://dx.doi.org/10.1016/s0167-5877\(02\)00095-8](http://dx.doi.org/10.1016/s0167-5877(02)00095-8).

57. Carbonero A, Torralbo A, Borge C et al. *Campylobacter* spp., *C. jejuni* and *C. upsaliensis* infection-associated factors in healthy and ill dogs from clinics in Cordoba, Spain. Screening tests for antimicrobial susceptibility. *Comp Immunol Microbiol Infect Diseases* 2012:505–512. <http://dx.doi.org/10.1016/j.cimid.2012.05.002>.

58. Torre E, Tello M. Factors influencing fecal shedding of *Campylobacter jejuni* in dogs without diarrhea. *Am J Vet Res* 1993;54:260–262. <https://www.ncbi.nlm.nih.gov/pubmed/8430936>.

59. López CM, Giacoboni G, Agostini A et al. Thermotolerant *Campylobacters* in domestic animals in a defined population in Buenos Aires, Argentina. *Prev Vet Med* 2002;55:193–200. [http://dx.doi.org/10.1016/s0167-5877\(02\)00093-4](http://dx.doi.org/10.1016/s0167-5877(02)00093-4).

60. Somroop S, Hatanaka N, Awasthi SP et al. *Campylobacter upsaliensis* isolated from dog produces high titer of cytolethal distending toxin. *J Vet Med Sci Japanese Society of Veterinary Science*, 2017;16–0654. https://www.jstage.jst.go.jp/article/jvms/advpub/0/advpub_16-0654/_article/-char/ja/.

61. McDonough PL, Simpson KW. Diagnosing emerging bacterial infections: salmonellosis, campylobacteriosis, clostridial toxicosis, and helicobacteriosis. *Semin Vet Med Surg* 1996;11:187–197. [http://dx.doi.org/10.1016/s1096-2867\(96\)80032-6](http://dx.doi.org/10.1016/s1096-2867(96)80032-6).

62. Macartney L, Al-Mashat RR, Taylor DJ et al. Experimental infection of dogs with *Campylobacter jejuni*. *Vet Rec* 1988;122:245–249. <https://www.ncbi.nlm.nih.gov/pubmed/3369054>.

63. Brown C, Martin V, Chitwood S. An outbreak of enterocolitis due to *Campylobacter* spp. in a beagle colony. *J Vet Diagn Invest* 1999;11:374–376. <http://dx.doi.org/10.1177/104063879901100416>.
64. Prescott JF, Barker IK, Manninen KI et al. *Campylobacter jejuni* colitis in gnotobiotic dogs. *Can J Comp Med* 1981;45:377–383. <https://www.ncbi.nlm.nih.gov/pubmed/7337869>.
65. Carmichael LE. An annotated historical account of canine parvovirus. *J Vet Med B Infect Dis Vet Public Health* 2005;52:303–311. <http://dx.doi.org/10.1111/j.1439-0450.2005.00868.x>.
66. Buonavoglia C, Martella V, Pratelli A et al. Evidence for evolution of canine parvovirus type 2 in Italy. *J Gen Virol* 2001;82:3021–3025. <http://dx.doi.org/10.1099/0022-1317-82-12-3021>.
67. Decaro N, Buonavoglia C. Canine parvovirus—A review of epidemiological and diagnostic aspects, with emphasis on type 2c. *Vet Microbiol* 2012;155:1–12. <http://www.sciencedirect.com/science/article/pii/S0378113511005013>.
68. Decaro N, Greene CE. Canine viral enteritis. *Infectious Diseases of the Dog and Cat 4th ed Saunders, Elsevier, St Louis, MO* 2012;67–75.
69. Parker J, Murphy M, Hueffer K et al. Investigation of a Canine Parvovirus Outbreak using Next Generation Sequencing. *Sci Rep* 2017;7:9633. <http://dx.doi.org/10.1038/s41598-017-10254-9>.
70. Sutton D, Vinberg C, Gustafsson A et al. Canine parvovirus type 2c identified from an outbreak of severe gastroenteritis in a litter in Sweden. *Acta Vet Scand* 2013;55:64. <http://dx.doi.org/10.1186/1751-0147-55-64>.
71. Decaro N, Desario C, Elia G et al. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. *New Microbiol* 2008;31:125–130. <https://www.ncbi.nlm.nih.gov/pubmed/18437851>.
72. Brady S, Norris JM, Kelman M et al. Canine parvovirus in Australia: the role of socio-economic factors in disease clusters. *Vet J* 2012;193:522–528. <http://dx.doi.org/10.1016/j.tvjl.2012.01.025>.
73. Pratelli A, Cavalli A, Martella V et al. Canine parvovirus (CPV) vaccination: comparison of neutralizing antibody responses in pups after inoculation with CPV2 or CPV2b modified live virus vaccine. *Clin Diagn Lab Immunol* 2001;8:612–615. <http://dx.doi.org/10.1128/CDLI.8.3.612-615.2001>.
74. Larson LJ, Schultz RD. Do two current canine parvovirus type 2 and 2b vaccines provide protection against the new type 2c variant? *Vet Ther* 2008;9:94–101. <https://www.ncbi.nlm.nih.gov/pubmed/18597247>.
75. Decaro N, Cirone F, Desario C et al. Severe parvovirus in a 12-year-old dog that had been repeatedly vaccinated. *Vet Rec* 2009;164:593–595. <http://dx.doi.org/10.1136/vr.164.19.593>.
76. Truyen U. Evolution of canine parvovirus—A need for new vaccines? *Vet*

Microbiol 2006;117:9–13.

<http://www.sciencedirect.com/science/article/pii/S0378113506001374>.

77. Day MJ, Horzinek MC, Schultz RD et al. WSAVA Guidelines for the vaccination of dogs and cats. *J Small Anim Pract* 2016;57:E1–E45.

http://dx.doi.org/10.1111/jsap.2_12431.

78. Adam RD. Biology of *Giardia lamblia*. *Clin Microbiol Rev* 2001;14:447–475.

<http://dx.doi.org/10.1128/CMR.14.3.447-475.2001>.

79. Thompson RCA, Monis P. *Giardia*--from genome to proteome. *Adv Parasitol* 2012;78:57–95.

<http://dx.doi.org/10.1016/B978-0-12-394303-3.00003-7>.

80. Payne PA, Artzer M. The biology and control of *Giardia* spp and *Trichostrongylus axei*. *Vet Clin North Am Small Anim Pract* 2009;39:993–1007, v.

<http://dx.doi.org/10.1016/j.cvsm.2009.06.007>.

81. Bouzid M, Halai K, Jeffreys D et al. The prevalence of *Giardia* infection in dogs and cats, a systematic review and meta-analysis of prevalence studies from stool samples. *Vet Parasitol* 2015;207:181–202.

<http://dx.doi.org/10.1016/j.vetpar.2014.12.011>.

82. Little SE, Johnson EM, Lewis D et al. Prevalence of intestinal parasites in pet dogs in the United States. *Vet Parasitol* 2009;166:144–152.

<http://dx.doi.org/10.1016/j.vetpar.2009.07.044>.

83. Carlin EP, Bowman DD, Scarlett JM et al. Prevalence of *Giardia* in symptomatic dogs and cats throughout the United States as determined by the IDEXX SNAP *Giardia* test. *Vet Ther* 2006;7:199–206.

<https://www.ncbi.nlm.nih.gov/pubmed/17039442>.

84. Julien DA, Sargeant JM, Guy RA et al. Prevalence and genetic characterization of *Giardia* spp. and *Cryptosporidium* spp. in dogs in Iqaluit, Nunavut, Canada. *Zoonoses Public Health* 2019;66:813–825.

<http://dx.doi.org/10.1111/zph.12628>.

85. Mochizuki M, Hashimoto M, Ishida T. Recent epidemiological status of canine viral enteric infections and *Giardia* infection in Japan. *J Vet Med Sci* 2001;63:573–575.

<http://dx.doi.org/10.1292/jvms.63.573>.

86. Mohamed AS, Levine M, Camp JW Jr et al. Temporal patterns of human and canine *Giardia* infection in the United States: 2003-2009. *Prev Vet Med* 2014;113:249–256.

<http://dx.doi.org/10.1016/j.prevetmed.2013.11.006>.

87. Rehbein S, Klotz C, Ignatius R et al. *Giardia duodenalis* in small animals and their owners in Germany: A pilot study. *Zoonoses Public Health* 2019;66:117–124.

<http://dx.doi.org/10.1111/zph.12541>.

88. Adell-Aledón M, Köster PC, de Lucio A et al. Occurrence and molecular epidemiology of *Giardia duodenalis* infection in dog populations in eastern Spain. *BMC Vet Res* 2018;14:26.

<https://doi.org/10.1186/s12917-018-1353-z>.

89. Traub RJ, Monis PT, Robertson I et al. Epidemiological and molecular

evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. *Parasitology* 2004;128:253–262. <http://dx.doi.org/10.1017/s0031182003004505>.

90. Štrkolcová G, Mađar M, Hinney B et al. Dog's genotype of *Giardia duodenalis* in human: first evidence in Europe. *Acta Parasitol* 2015;60:796–799. <http://dx.doi.org/10.1515/ap-2015-0113>.

91. Salmonella (non-typhoidal). [https://www.who.int/en/news-room/fact-sheets/detail/salmonella-\(non-typhoidal\)](https://www.who.int/en/news-room/fact-sheets/detail/salmonella-(non-typhoidal)). Retrieved April 26 2020.

92. Giammanco GM, Pignato S, Mammina C et al. Persistent Endemicity of *Salmonella bongori* 48:z35:- in Southern Italy: Molecular Characterization of Human, Animal, and Environmental Isolates. *J Clin Microbiol* American Society for Microbiology Journals, 2002;40:3502–3505. <https://jcm.asm.org/content/40/9/3502.short>.

93. Sanderson KE, Nair S, Others. Taxonomy and species concepts in the genus *Salmonella*. *Salmonella in domestic animals* CAB International, 2013;2:1–19. https://books.google.com/books?hl=en&lr=&id=UI9cBAAAQBAJ&oi=fnd&pg=PA1&dq=sanderson+nair+taxonomy+concepts+salmonella&ots=CK52qff79r&sig=xmx_XoUO6S8scXCGZZEYz50GOk8.

94. Cherry B, Burns A, Johnson GS et al. *Salmonella* Typhimurium outbreak associated with veterinary clinic. *Emerg Infect Dis* 2004;10:2249–2251. <http://dx.doi.org/10.3201/eid1012.040714>.

95. 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. *J Clin Microbiol* 2017;55:1350–1368. <http://dx.doi.org/10.1128/JCM.02137-16>.

96. Leahy AM, Cummings KJ, Rodriguez-Rivera LD et al. Evaluation of Faecal *Salmonella* Shedding Among Dogs at Seven Animal Shelters across Texas. *Zoonoses Public Health* 2016;63:515–521. <http://dx.doi.org/10.1111/zph.12257>.

97. McKenzie E, Riehl J, Banse H et al. Prevalence of diarrhea and enteropathogens in racing sled dogs. *J Vet Intern Med* 2010;24:97–103. <http://dx.doi.org/10.1111/j.1939-1676.2009.0418.x>.

98. Tsai H-J, -J. Tsai H, -C. Huang H et al. *Salmonellae* and *Campylobacters* in Household and Stray Dogs in Northern Taiwan. *Veterinary Research Communications* 2007:931–939. <http://dx.doi.org/10.1007/s11259-007-0009-4>.

99. Duijvestijn M, Mughini-Gras L, Schuurman N et al. Enteropathogen infections in canine puppies: (Co-)occurrence, clinical relevance and risk factors. *Vet Microbiol* 2016;195:115–122. <http://dx.doi.org/10.1016/j.vetmic.2016.09.006>.

100. Finley R, Ribble C, Aramini J et al. The risk of *salmonellae* shedding by dogs fed *Salmonella*-contaminated commercial raw food diets. *Can Vet J* 2007;48:69–75. <https://www.ncbi.nlm.nih.gov/pubmed/17310625>.

101. Leonard EK, Pearl DL, Finley RL 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 Public Health* 2011;58:140–149. <http://dx.doi.org/10.1111/j.1863-2378.2009.01320.x>.
102. 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 Public Health* 2008;55:470–480. <http://dx.doi.org/10.1111/j.1863-2378.2008.01145.x>.
103. Weese JS, Rousseau J, Arroyo L. Bacteriological evaluation of commercial canine and feline raw diets. *Can Vet J* 2005;46:513–516. <https://www.ncbi.nlm.nih.gov/pubmed/16048011>.
104. Morley PS, Strohmeier RA, Tankson JD et al. Evaluation of the association between feeding raw meat and *Salmonella enterica* infections at a Greyhound breeding facility. *J Am Vet Med Assoc* 2006;228:1524–1532. <http://dx.doi.org/10.2460/javma.228.10.1524>.
105. Philbey AW, Mather HA, Gibbons JF et al. Serovars, bacteriophage types and antimicrobial sensitivities associated with salmonellosis in dogs in the UK (1954-2012). *Vet Rec* 2014;174:94. <http://dx.doi.org/10.1136/vr.101864>.
106. Barr F. Cryptosporidiosis. *J Small Anim Pract* 1997;38:319–320. <http://dx.doi.org/10.1111/j.1748-5827.1997.tb03475.x>.
107. [No title]. https://www.who.int/water_sanitation_health/. Retrieved April 30 2020.
108. Morgan-Ryan UM, Fall A, Ward LA et al. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *J Eukaryot Microbiol* 2002;49:433–440. <http://dx.doi.org/10.1111/j.1550-7408.2002.tb00224.x>.
109. [No title]. https://www.who.int/water_sanitation_health/gdwqrevision/. Retrieved May 24 2020.
110. Organization WH, Others. WHO Guidelines for Drinking Water Quality: *Cryptosporidium*. WHO Press, World Health Organization, Geneva, Switzerland. https://www.who.int/water_sanitation_health/gdwqrevision/cryptodraft2.pdf, 2006.
111. Lucio-Forster A, Griffiths JK, Cama VA et al. Minimal zoonotic risk of cryptosporidiosis from pet dogs and cats. *Trends Parasitol* 2010;26:174–179. <http://dx.doi.org/10.1016/j.pt.2010.01.004>.
112. Xiao L, Feng Y. Zoonotic cryptosporidiosis. *FEMS Immunol Med Microbiol* 2008;52:309–323. <http://dx.doi.org/10.1111/j.1574-695X.2008.00377.x>.
113. Scorza V, Tangtrongsup S. Update on the diagnosis and management of *Cryptosporidium* spp infections in dogs and cats. *Top Companion Anim Med* 2010;25:163–169. <http://dx.doi.org/10.1053/j.tcam.2010.07.007>.
114. Raue K, Heuer L, Böhm C et al. 10-year parasitological examination results (2003 to 2012) of faecal samples from horses, ruminants, pigs, dogs, cats,

rabbits and hedgehogs. *Parasitol Res* 2017;116:3315–3330.
<http://dx.doi.org/10.1007/s00436-017-5646-0>.

115. Rosanowski SM, Banica M, Ellis E et al. The molecular characterisation of *Cryptosporidium* species in relinquished dogs in Great Britain: a novel zoonotic risk? *Parasitol Res* 2018;117:1663–1667.
<http://dx.doi.org/10.1007/s00436-018-5857-z>.

116. Hinney B, Gottwald M, Moser J et al. Examination of anonymous canine faecal samples provides data on endoparasite prevalence rates in dogs for comparative studies. *Vet Parasitol* 2017;245:106–115.
<http://dx.doi.org/10.1016/j.vetpar.2017.08.016>.

117. Gharieb RMA, Merwad AMA, Saleh AA et al. Molecular screening and genotyping of *Cryptosporidium* species in household dogs and in-contact children in Egypt: risk factor analysis and zoonotic importance. *Vector-Borne and Zoonotic Diseases* Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA, 2018;18:424–432.
https://www.liebertpub.com/doi/abs/10.1089/vbz.2017.2254?casa_token=ietQlorpIFUAAAAA:eP2GsEYW8jYeIDqfNriR60SM_8fW6z5gmN6CR_Z6WB9omkYWDyX36px3vn6QtIKLlyQzGFdrYgbF.

118. Itoh N, Tanaka H, Iijima Y et al. Molecular Prevalence of *Cryptosporidium* spp. in Breeding Kennel Dogs. *Korean J Parasitol* 2019;57:197–200.
<http://dx.doi.org/10.3347/kjp.2019.57.2.197>.

119. Yu Z, Ruan Y, Zhou M et al. Prevalence of intestinal parasites in companion dogs with diarrhea in Beijing, China, and genetic characteristics of *Giardia* and *Cryptosporidium* species. *Parasitol Res* 2018;117:35–43.
<http://dx.doi.org/10.1007/s00436-017-5631-7>.

120. Ayinmode AB, Obebe OO, Falohun OO. Molecular detection of *Cryptosporidium* species in street-sampled dog faeces in Ibadan, Nigeria. *Vet Parasitol Reg Stud Reports* 2018;14:54–58.
<http://dx.doi.org/10.1016/j.vprsr.2018.08.005>.

121. Kostopoulou D, Claerebout E, Arvanitis D et al. Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece. *Parasit Vectors* 2017;10:43.
<http://dx.doi.org/10.1186/s13071-017-1989-8>.

122. Gillespie S, Bradbury RS. A Survey of Intestinal Parasites of Domestic Dogs in Central Queensland. *Trop Med Infect Dis* 2017;2.
<http://dx.doi.org/10.3390/tropicalmed2040060>.

123. Paul AEH, Stayt J. The intestinal microbiome in dogs and cats with diarrhoea as detected by a faecal polymerase chain reaction-based panel in Perth, Western Australia. *Aust Vet J Wiley Online Library*, 2019;
<https://onlinelibrary.wiley.com/doi/abs/10.1111/avj.12867>.

124. Gizzi AB da R, Oliveira ST, Leutenegger CM et al. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. *BMC Vet Res* 2014;10:23.

<http://dx.doi.org/10.1186/1746-6148-10-23>.

125. Decaro N, Buonavoglia C. An update on canine coronaviruses: viral evolution and pathobiology. *Vet Microbiol* 2008;132:221–234. <http://dx.doi.org/10.1016/j.vetmic.2008.06.007>.

126. Binn LN, Lazar EC, Keenan KP et al. Recovery and characterization of a coronavirus from military dogs with diarrhea. *Proc Annu Meet U S Anim Health Assoc* 1974;359–366. <https://www.ncbi.nlm.nih.gov/pubmed/4377955>.

127. Licitra BN, Duhamel GE, Whittaker GR. Canine enteric coronaviruses: emerging viral pathogens with distinct recombinant spike proteins. *Viruses* 2014;6:3363–3376. <http://dx.doi.org/10.3390/v6083363>.

128. Decaro N, Martella V, Ricci D et al. Genotype-specific fluorogenic RT-PCR assays for the detection and quantitation of canine coronavirus type I and type II RNA in faecal samples of dogs. *J Virol Methods* 2005;130:72–78. <http://dx.doi.org/10.1016/j.jviromet.2005.06.005>.

129. Naylor MJ, Harrison GA, Monckton RP et al. Identification of canine coronavirus strains from feces by S gene nested PCR and molecular characterization of a new Australian isolate. *J Clin Microbiol* 2001;39:1036–1041. <http://dx.doi.org/10.1128/JCM.39.3.1036-1041.2001>.

130. Evermann JF, Abbott JR, Han S. Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection. *J Vet Diagn Invest* 2005;17:610–614. <http://dx.doi.org/10.1177/104063870501700618>.

131. Buonavoglia C, Decaro N, Martella V et al. Canine coronavirus highly pathogenic for dogs. *Emerg Infect Dis* 2006;12:492–494. <http://dx.doi.org/10.3201/eid1203.050839>.

132. Decaro N, Martella V, Desario C et al. First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. *J Vet Med B Infect Dis Vet Public Health* 2006;53:468–472. <http://dx.doi.org/10.1111/j.1439-0450.2006.00974.x>.

133. Pratelli A, Martella V, Elia G et al. Severe enteric disease in an animal shelter associated with dual infections by canine adenovirus type 1 and canine coronavirus. *J Vet Med B Infect Dis Vet Public Health* 2001;48:385–392. <http://dx.doi.org/10.1046/j.1439-0450.2001.00466.x>.

134. Pratelli A. Genetic evolution of canine coronavirus and recent advances in prophylaxis. *Vet Res* 2006;37:191–200. <http://dx.doi.org/10.1051/vetres:2005053>.

135. Stavisky J, Pinchbeck G, Gaskell RM et al. Cross sectional and longitudinal surveys of canine enteric coronavirus infection in kennelled dogs: a molecular marker for biosecurity. *Infect Genet Evol* 2012;12:1419–1426. <http://dx.doi.org/10.1016/j.meegid.2012.04.010>.

136. Hackett T, Lappin MR. Prevalence of enteric pathogens in dogs of north-central Colorado. *J Am Anim Hosp Assoc* 2003;39:52–56. <http://dx.doi.org/10.5326/0390052>.

137. Costa EM, de Castro TX, Bottino F de O et al. Molecular characterization of canine coronavirus strains circulating in Brazil. *Vet Microbiol* 2014;168:8–15. <http://dx.doi.org/10.1016/j.vetmic.2013.10.002>.
138. Takano T, Yamashita S, Murata-Ohkubo M et al. Prevalence of canine coronavirus (CCoV) in dog in Japan: detection of CCoV RNA and retrospective serological analysis. *J Vet Med Sci* 2016;78:341–345. <http://dx.doi.org/10.1292/jvms.15-0347>.
139. Schulz BS, Strauch C, Mueller RS et al. Comparison of the prevalence of enteric viruses in healthy dogs and those with acute haemorrhagic diarrhoea by electron microscopy. *J Small Anim Pract* 2008;49:84–88. <http://dx.doi.org/10.1111/j.1748-5827.2007.00470.x>.
140. Tupler T, Levy JK, Sabshin SJ et al. Enteropathogens identified in dogs entering a Florida animal shelter with normal feces or diarrhea. *J Am Vet Med Assoc* 2012;241:338–343. <http://dx.doi.org/10.2460/javma.241.3.338>.
141. Suchodolski JS. Intestinal microbiota of dogs and cats: a bigger world than we thought. *Vet Clin North Am Small Anim Pract* 2011;41:261–272. <http://dx.doi.org/10.1016/j.cvsm.2010.12.006>.
142. Barko PC, McMichael MA, Swanson KS et al. The Gastrointestinal Microbiome: A Review. *J Vet Intern Med* 2018;32:9–25. <http://dx.doi.org/10.1111/jvim.14875>.
143. Suchodolski JS, Camacho J, Steiner JM. Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. *FEMS Microbiol Ecol* 2008;66:567–578. <http://dx.doi.org/10.1111/j.1574-6941.2008.00521.x>.
144. Vázquez-Baeza Y, Hyde ER, Suchodolski JS et al. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nat Microbiol* 2016;1:16177. <http://dx.doi.org/10.1038/nmicrobiol.2016.177>.
145. Igarashi H, Maeda S, Ohno K et al. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. *PLoS One* 2014;9:e107909. <http://dx.doi.org/10.1371/journal.pone.0107909>.
146. Moon CD, Young W, Maclean PH et al. Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *MicrobiologyOpen* 2018:e00677. <http://dx.doi.org/10.1002/mbo3.677>.
147. AlShawaqfeh M, Wajid B, Guard M et al. A DYSBIOSIS INDEX TO ASSESS MICROBIAL CHANGES IN FECAL SAMPLES OF DOGS WITH CHRONIC ENTEROPATHY. *J Vet Intern Med* 2016;30.
148. Handl S, Dowd SE, Garcia-Mazcorro JF et al. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol* 2011;76:301–310. <http://dx.doi.org/10.1111/j.1574-6941.2011.01058.x>.
149. He Y, Wu W, Zheng H-M et al. Regional variation limits applications of

healthy gut microbiome reference ranges and disease models. *Nat Med* 2018;24:1532–1535. <http://dx.doi.org/10.1038/s41591-018-0164-x>.

150. Tsai M-H, Huang Y-C, Lin T-Y et al. Reappraisal of parenteral antimicrobial therapy for nontyphoidal *Salmonella* enteric infection in children. *Clinical Microbiology and Infection* 2011;300–305. <http://dx.doi.org/10.1111/j.1469-0691.2010.03230.x>.

151. Squire MM, Riley TV. *Clostridium difficile* Infection in Humans and Piglets: A “One Health” Opportunity. *Current Topics in Microbiology and Immunology* 2012;299–314. http://dx.doi.org/10.1007/978-3-662-45792-4_237.

152. Robinson TP, Bu DP, Carrique-Mas J et al. Antibiotic resistance is the quintessential One Health issue. *Transactions of The Royal Society of Tropical Medicine and Hygiene* 2016;377–380. <http://dx.doi.org/10.1093/trstmh/trw048>.

153. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. *Vet Clin North Am Small Anim Pract* 2011;41:381–398. <http://dx.doi.org/10.1016/j.cvsm.2011.02.003>.

154. Rossi M, Hänninen ML, Revez J et al. Occurrence and species level diagnostics of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in healthy and diarrheic dogs and cats. *Vet Microbiol* 2008;129:304–314. <http://dx.doi.org/10.1016/j.vetmic.2007.11.014>.

155. Saleh MN, Heptinstall JR, Johnson EM et al. Comparison of diagnostic techniques for detection of *Giardia duodenalis* in dogs and cats. *J Vet Intern Med* 2019;33:1272–1277. <http://dx.doi.org/10.1111/jvim.15491>.

156. Rishniw M, Liotta J, Bellosa M et al. Comparison of 4 *Giardia* diagnostic tests in diagnosis of naturally acquired canine chronic subclinical giardiasis. *J Vet Intern Med* 2010;24:293–297. <http://dx.doi.org/10.1111/j.1939-1676.2010.0475.x>.

157. Deng P, Swanson KS. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *British Journal of Nutrition* 2015;S6–S17. <http://dx.doi.org/10.1017/s0007114514002943>.

158. Goossens H, Vlaes L, De Boeck M et al. Is “*Campylobacter upsaliensis*” an unrecognised cause of human diarrhoea? *Lancet* 1990;335:584–586. [http://dx.doi.org/10.1016/0140-6736\(90\)90359-d](http://dx.doi.org/10.1016/0140-6736(90)90359-d).

159. Botha WJ, Schoeman JP, Marks SL et al. Prevalence of *Salmonella* in juvenile dogs affected with parvoviral enteritis. *J S Afr Vet Assoc* 2018;89:e1–e6. <http://dx.doi.org/10.4102/jsava.v89i0.1731>.

160. Rimhanen-Finne R, Enemark HL, Kolehmainen J et al. Evaluation of immunofluorescence microscopy and enzyme-linked immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. *Vet Parasitol* 2007;145:345–348. <http://dx.doi.org/10.1016/j.vetpar.2007.01.008>.

161. Esfandiari J, Klingeborn B. A comparative study of a new rapid and one-step test for the detection of parvovirus in faeces from dogs, cats and mink. *J Vet Med B Infect Dis Vet Public Health* 2000;47:145–153.

<http://dx.doi.org/10.1046/j.1439-0450.2000.00328.x>.

162. Desario C, Decaro N, Campolo M et al. Canine parvovirus infection: which diagnostic test for virus? *J Virol Methods* 2005;126:179–185.
<http://dx.doi.org/10.1016/j.jviromet.2005.02.006>.

163. Schmitz S, Coenen C, König M et al. Comparison of three rapid commercial Canine parvovirus antigen detection tests with electron microscopy and polymerase chain reaction. *J Vet Diagn Invest* 2009;21:344–345.
<http://dx.doi.org/10.1177/104063870902100306>.

164. Decaro N, Elia G, Desario C et al. A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus. *J Virol Methods* 2006;136:65–70.
<http://dx.doi.org/10.1016/j.jviromet.2006.03.030>.

165. Sykes JE, Marks SL. Enteric Clostridial Infections. *Canine and Feline Infectious Diseases* 2014:458–464.
<http://dx.doi.org/10.1016/b978-1-4377-0795-3.00048-x>.

166. Hirasawa T, Kaneshige T, Mikazuki K. Sensitive detection of canine parvovirus DNA by the nested polymerase chain reaction. *Vet Microbiol* 1994;41:135–145. <https://www.ncbi.nlm.nih.gov/pubmed/7801517>.

167. Vetpath. Vetpath > Home. <http://vetpath.com.au>. Retrieved May 16 2020.

168. Mochizuki M, San Gabriel MC, Nakatani H et al. Comparison of polymerase chain reaction with virus isolation and haemagglutination assays for the detection of canine parvoviruses in faecal specimens. *Res Vet Sci* 1993;55:60–63.
[http://dx.doi.org/10.1016/0034-5288\(93\)90035-e](http://dx.doi.org/10.1016/0034-5288(93)90035-e).

169. 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* 2007:3692–3700. <http://dx.doi.org/10.1128/jcm.00896-07>.

170. Stirling D, Bartlett JMS. PCR Protocols (Methods in Molecular Biology). Humana Press, New Jersey, 2003.

171. Garibyan L, Avashia N. Polymerase chain reaction. *J Invest Dermatol* 2013;133:1–4. <http://dx.doi.org/10.1038/jid.2013.1>.

172. Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. *Hum Mutat* Wiley, 2009;30:857–859.
<http://doi.wiley.com/10.1002/humu.20951>.

173. Elia G, Decaro N, Martella V et al. Detection of canine distemper virus in dogs by real-time RT-PCR. *J Virol Methods* 2006;136:171–176.
<http://dx.doi.org/10.1016/j.jviromet.2006.05.004>.

174. Wilkes RP, Lee P-YA, Tsai Y-L et al. An insulated isothermal PCR method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need. *J Virol Methods* 2015;220:35–38.

<http://dx.doi.org/10.1016/j.jviromet.2015.04.007>.

175. 搖Tsai, Lo CF, Tang-Nelson K et al. Validation of a commercial insulated isothermal PCR-based POKKIT test for rapid and easy detection of white spot syndrome virus infection in *Litopenaeus vannamei*. *PLoS One* 2014;9:e90545.
176. Hu W, Wu S, Yu X et al. A Multiplex PCR for Simultaneous Detection of Three Zoonotic Parasites *Ancylostoma ceylanicum*, *A. caninum*, and *Giardia lamblia* Assemblage A. *Biomed Res Int* 2015;2015:406168. <http://dx.doi.org/10.1155/2015/406168>.
177. Chaban B, Musil KM, Himsworth CG et al. Development of cpn60-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. *Appl Environ Microbiol* 2009;75:3055–3061. <http://dx.doi.org/10.1128/AEM.00101-09>.
178. Kildare BJ, Leutenegger CM, McSwain BS et al. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: a Bayesian approach. *Water Res* 2007;41:3701–3715. <http://dx.doi.org/10.1016/j.watres.2007.06.037>.
179. Song SJ, Amir A, Metcalf JL et al. Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. *mSystems* 2016;1. <http://dx.doi.org/10.1128/mSystems.00021-16>.
180. Wu W-K, Chen C-C, Panyod S et al. Optimization of fecal sample processing for microbiome study — The journey from bathroom to bench. *Journal of the Formosan Medical Association* 2019:545–555. <http://dx.doi.org/10.1016/j.jfma.2018.02.005>.
181. Choo JM, Leong LEX, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. *Sci Rep* 2015;5:16350. <http://dx.doi.org/10.1038/srep16350>.
182. Hawash Y. DNA extraction from protozoan oocysts/cysts in feces for diagnostic PCR. *Korean J Parasitol* Korean Society for Parasitology, 2014;52:263–271. <http://parasitol.kr/journal/view.php?doi=10.3347/kjp.2014.52.3.263>.
183. Yuan S, Cohen DB, Ravel J et al. Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLoS One* 2012;7:e33865. <http://dx.doi.org/10.1371/journal.pone.0033865>.
184. David LA, Maurice CF, Carmody RN et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014;505:559–563. <http://dx.doi.org/10.1038/nature12820>.
185. Albini S, Brodard I, Jaussi A et al. Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. *Vet Microbiol* 2008;127:179–185. <http://dx.doi.org/10.1016/j.vetmic.2007.07.024>.
186. Busch K, Suchodolski JS, Kühner KA et al. *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A/B do not play a role in acute

haemorrhagic diarrhoea syndrome in dogs. *Vet Rec* 2015;176:253.
<http://dx.doi.org/10.1136/vr.102738>.

187. Suchodolski JS, Markel ME, Garcia-Mazcorro JF et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* 2012;7:e51907. <http://dx.doi.org/10.1371/journal.pone.0051907>.

188. 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 Public Health* 2014;61:208–218.
<http://dx.doi.org/10.1111/zph.12062>.

189. Uehlinger FD, Naqvi SA, Greenwood SJ et al. Comparison of five diagnostic tests for *Giardia duodenalis* in fecal samples from young dogs. *Vet Parasitol* 2017;244:91–96. <http://dx.doi.org/10.1016/j.vetpar.2017.07.030>.

190. Decaro N, Elia G, Martella V et al. A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. *Vet Microbiol* 2005;105:19–28. <http://dx.doi.org/10.1016/j.vetmic.2004.09.018>.

191. Alves MEM, Martins FDC, Bräunig P et al. Molecular detection of *Cryptosporidium* spp. and the occurrence of intestinal parasites in fecal samples of naturally infected dogs and cats. *Parasitol Res* 2018;117:3033–3038.
<http://dx.doi.org/10.1007/s00436-018-5986-4>.

192. Decaro N, Pratelli A, Campolo M et al. Quantitation of canine coronavirus RNA in the faeces of dogs by TaqMan RT-PCR. *Journal of Virological Methods* 2004;145–150. <http://dx.doi.org/10.1016/j.jviromet.2004.03.012>.

193. Zappulli V, Caliari D, Cavicchioli L et al. Systemic fatal type II coronavirus infection in a dog: pathological findings and immunohistochemistry. *Res Vet Sci* 2008;84:278–282. <http://dx.doi.org/10.1016/j.rvsc.2007.05.004>.

194. Pratelli A, Buonavoglia D, Martella V et al. Diagnosis of canine coronavirus infection using nested-PCR. *J Virol Methods* 2000;84:91–94.
[http://dx.doi.org/10.1016/s0166-0934\(99\)00134-2](http://dx.doi.org/10.1016/s0166-0934(99)00134-2).

195. Animal--Cardiology S, Echocardiography D, Echocardiography T et al. 2012 ACVIM Forum Research Abstracts Program. *J Vet Intern Med* 2012;26:690–822.
https://static-curis.ku.dk/portal/files/44753472/side_713_konferenceAbstracts_ACVI_M2012.pdf.

196. Uiterwijk M, Nijse R, Kooyman FNJ et al. Host factors associated with *Giardia duodenalis* infection in dogs across multiple diagnostic tests. *Parasit Vectors* 2019;12:556. <http://dx.doi.org/10.1186/s13071-019-3810-3>.

197. Freisl M, Speck S, Truyen U et al. Faecal shedding of canine parvovirus after modified-live vaccination in healthy adult dogs. *Vet J* 2017;219:15–21.
<http://dx.doi.org/10.1016/j.tvjl.2016.11.011>.

198. Suchodolski JS, Ruaux CG, Steiner JM et al. Application of molecular

fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. *J Clin Microbiol* 2004;42:4702–4708.
<http://dx.doi.org/10.1128/JCM.42.10.4702-4708.2004>.

199. Vetpath. Vetpath > Products & Services > Products & Services > Test Information.

<http://www.vetpath.com.au/ProductsServices/ProductsServices/TestInformation/tabid/432/filter/F/currentpage/1/Default.aspx>. Retrieved March 29 2020.

200. Decaro N, Desario C, Amorisco F et al. Detection of a canine parvovirus type 2c with a non-coding mutation and its implications for molecular characterisation. *Vet J* 2013;196:555–557.

<http://dx.doi.org/10.1016/j.tvjl.2012.12.017>.

2. Chapter 3: Faecal PCR panel results and clinical findings in Western Australian dogs with diarrhoea

This chapter provides faecal PCR results from a single institution, together with relevant clinical data collected from electronic medical records. Together, they provide insight on how the test is being used by veterinarians, and how faecal PCR results may impact clinical management of dogs with diarrhoea. This allows us to identify aspects of faecal PCR use that need further exploration, with the ultimate goal of establishing an evidence base that allows veterinarians to use faecal PCR results with greater confidence.

This chapter was published in the peer-reviewed Australian Veterinary Journal (<https://doi.org/10.1111/avj.13008>), and was presented in abstract form at the International Veterinary Emergency and Critical Care Society annual congress in New Orleans, Louisiana, US, September 2018.

2.1. Abstract

Aim

To describe faecal PCR (fPCR) results and clinical findings of dogs seen at a university teaching hospital for diarrhoea.

Design

Retrospective case series (April 2015 to July 2018)

Procedure

Data were collected from the hospital electronic medical records. Data extracted included signalment, history, clinical signs, treatment, fPCR panel results, other faecal diagnostic test results, and antimicrobial use.

Results

One hundred and sixty-eight dogs with diarrhoea had a fPCR panel submitted. Most dogs (115, 68.5%) had diarrhoea of three days or less duration. *Clostridium perfringens* alpha toxin gene was most frequently detected (156, 92.9%) by fPCR, followed by *Campylobacter* spp. (55, 32.7%), canine parvovirus (CPV) (29, 17.3%), *Salmonella* spp. (14, 8.3%), and *Giardia* spp. (9, 5.4%). For the 45 dogs that had a negative point-of-care CPV test, 13 were CPV fPCR positive; some of which were adult dogs with current vaccination status. A total of 94/168 (56%) dogs received antimicrobials at some time during the treatment of diarrhoea.

Conclusion

Faecal PCR panels can identify dogs with enteric organisms in their faeces that traditional faecal diagnostics may miss, thus contributing additional information to the diagnostic process. Nonetheless, fPCR results should be interpreted in light of the clinical findings, and particular consideration given to avoiding inappropriate use of antimicrobials. This study highlights that testing for *C. perfringens* alpha toxin gene is not likely to be diagnostically helpful, and that adult dogs with diarrhoea might be identified as CPV positive with PCR testing, despite a negative point-of-care CPV test result and a current vaccination status.

2.2. Introduction

Faecal real-time PCR assays (fPCR) have become widely commercially available as a diagnostic tool in the investigation of dogs with diarrhoea.¹⁻⁵ These assays can detect bacterial, viral, and protozoal organisms with high sensitivity. However, the clinical significance of detected organisms in dogs with diarrhoea remains poorly understood,⁶ and the integration of fPCR results into clinical case management has not been described. Furthermore, enteric organism strains and prevalence vary in different geographic locations.^{4,7,8} Establishing regional enteric organism prevalence provides baseline data for future studies of pathogenicity and allows comparisons to investigations performed elsewhere.

In Western Australia (WA), the main commercially available fPCR panel^a tests for the bacteria *Salmonella* spp., *Campylobacter* spp., and the *Clostridium perfringens* alpha toxin gene, the protozoa *Giardia* spp. and *Cryptosporidium* spp., and the viruses canine parvovirus (CPV), canine enteric coronavirus (CCV), and canine distemper virus (CDV). A recent study reported the results of this fPCR panel in 405 canine and 289 feline faecal samples in WA over a 12 month period from 2014 to 2015.⁵ That study described only case signalment and fPCR results; no clinical information about the patients was included, thus providing limited clinical context. Additionally, while the authors reported that these cases had diarrhoea, how this was determined was unclear since their data presumably originated from the commercial laboratory, and not the submitting veterinary practices. Another limitation of this recent study is that given the laboratory receives samples from all over the state, and also interstate, it is unlikely the cases only represent Perth, WA. Thus, further studies with more background information are needed to understand the fPCR results in a clinical context.

In order to explore the role of fPCR in the diagnostic approach to diarrhoea in dogs, the objective of this study was to describe the fPCR results and clinical findings of dogs with diarrhoea seen at a university teaching hospital that had fPCR performed. Based on clinical experience and a recent publication⁵, we hypothesised that fPCR testing would commonly identify the *C. perfringens* alpha toxin gene, *Campylobacter* spp. and CPV.

2.3. Material and methods

2.3.1. Data collection

Electronic medical records for all dogs with a fPCR panel submitted from The Animal Hospital at Murdoch University (TAHMU) from April 2015 to July 2018 were identified by a fee code search. The specific fee codes used in the search were “Faecal Multiplex PCR” and “Faecal Multiplex PCR Plus”. April 2015 was chosen as the start date of the study as this was the first time TAHMU submitted a fPCR panel. Cases were included if they had a multiplex tandem fPCR performed by a single, nationally accredited (nata.com.au) commercial laboratory,^a with or without faecal wet microscopy and zinc sulphate floatation. Information on assay validation and primers used are considered proprietary information by the laboratory and were not available for publication. Cases whose fPCR was outsourced to another external laboratory were excluded. Hospital medical records were then reviewed. Cases were excluded if they did not have diarrhoea reported in the history, initial physical exam, or the inpatient medical record for hospitalised cases. Acceptable alternative terminology for diarrhoea included reference to the stools as ‘unformed’, ‘liquid’, or ‘watery’.

The following data were extracted from the medical records if available: signalment (date of birth, sex, breed), date of first presentation for illness event in question, diet history,

vaccination history, deworming history, clinical signs, comorbidities, date of fPCR sample submission and receipt by the commercial laboratory, the date the fPCR results were reported, fPCR results, results of in-house point-of-care faecal antigen testing (Anigen Rapid CPV/CCV Antigen Test, Bionote, South Korea) for CPV or CCV, and antimicrobial use (including type and duration) before and after fPCR results were reported. Additionally, the TAHMU service providing patient care was recorded. Vaccination status was defined according to published vaccination guidelines,⁹ and refers only to the core vaccines - CPV, CDV, and canine adenovirus. Current deworming status was defined as regular administration of registered dewormers given to manufacturer recommendations.

2.3.2. Laboratory results

The fPCR used by this laboratory includes primers for *Salmonella* spp., *Campylobacter* spp., *Clostridium perfringens* alpha toxin gene, *Cryptosporidium* spp., *Giardia* spp., CPV, CCV, and CDV, with the results listed as being either not detected, detected, or detected low level. Low level detection is a semi-quantitative reading based on the number of PCR cycles at which fluorescence reached a pre-determined threshold level. Faecal microscopy results, when performed, included the presence or absence of erythrocytes, leucocytes, spiral-shaped organisms, yeast, protozoa, ova, and cysts. Data were entered into a web-based database application (REDCap, redcap.org).¹⁰ Functions within REDCap were used to calculate a variety of time parameters. Age in months was calculated as the difference between the date of first presentation for the illness event in question, and the date of birth. Time to fPCR results, in days, was calculated as the difference between the date of sample submission and reporting by the commercial laboratory.

2.3.3. Statistics

Summary statistics are presented as median (range) or number (percentage) where appropriate. For the purposes of overall enteric organism prevalence data, “Detected” was combined with “Detected low level”, and the sample considered positive for the organism in question.

2.4. Results

One hundred and seventy dogs seen at TAHMU had a fPCR panel performed by Vetpath Laboratory Services between April 2015 and July 2018. Two dogs were excluded as they had fPCR performed at a different laboratory or did not have diarrhoea as a presenting complaint, leaving 168 dogs for analysis. Eighteen dogs had the fPCR alone performed, while the remaining 150 dogs also had faecal wet microscopy and floatation performed.

2.4.1. Signalment

The median age was 39.5 months (3 years, 3.5 months), with a range from 1 month to 16 years. The population included 57 neutered males (33.9%), 57 spayed females (33.9%), 28 intact males (16.7%), and 26 intact females (15.5%). The majority of dogs were purebreds (114, 67.9%), with 45 individual breeds presented. The most commonly represented breeds were the Labrador retriever (n=11/114, 9.6%), Dachshund (7, 6.1%), German shepherd dog (5, 4.4%), Cavalier King Charles spaniel (5, 4.4%), great Dane (5, 4.4%), French bulldog (5, 4.4%), border collie (4, 3.5%), Weimaraner (4, 3.5%), kelpie (4, 3.5%), and miniature schnauzer (4, 3.5%). The remaining 36 breeds were represented by three or fewer dogs. The remaining 54 dogs (32.1%) were mixed breeds. Median bodyweight was 11.6 kg (range 0.8 to 69.0kg). For the majority of dogs (88/168,

52.4%), medical records noted no obvious dietary cause of diarrhoea such as history of recent dietary indiscretion, scavenging behaviour, recent diet changes, nor history of consuming raw meat.

2.4.2. Vaccination and deworming history

The vaccination status was reported as current in 128/168 dogs (76.2%); this included 58 dogs for which the last vaccination date was recorded, 68 for which the date was not recorded, and two that received booster vaccines during the management of their diarrhoea. In the 58 dogs where the most recent vaccination date was recorded, the median time from last vaccination to diarrhoea presentation was four months (range 0 – 86 months). Fourteen dogs (8.3%) were not current on vaccinations, and vaccination status was unknown or not recorded for 26/168 dogs (15.5%). Routine deworming was recorded as being current for 94/166 dogs (56.6%), although specific details were rarely recorded.

2.4.3. Clinical signs

Based on our inclusion criteria, diarrhoea was present in all cases (Table 1). The duration of diarrhoea prior to fPCR submission was less than three days in the majority of dogs (115/168, 68.5%), three to seven days in 29/168 (17.3%), between 7 and 30 days in 15/168 (8.9%) and greater than 30 days duration in 9/168 dogs (5.4%). A majority of dogs had concurrent loss of appetite (103/168, 61.3%), and vomiting (108/168, 64.3%). The nature of the diarrhoea, vomiting, and loss of appetite is displayed in more detail in Table 1, as are other clinical signs in the population described.

Table 1. Clinical signs described in dogs seen at a veterinary teaching hospital for diarrhoea that had a multiplex faecal PCR panel performed as part of their diagnostic evaluation

Clinical signs	Number of dogs /168 total
Diarrhoea	168
Haematochezia	91
Melaena	8
Both haematochezia and melena	6
Vomiting	108
Haematemesis	9
Digested blood (“coffee grounds” appearance)	1
Haematemesis and digested blood	1
Loss of appetite	103
Inappetence	80
Complete anorexia	23
Other clinical signs	
Tenesmus	21
Abdominal pain / discomfort	11
Ptyalism	8
Acute collapse	6

2.4.4. Diagnostics

The median time from sample collection to reporting of fPCR results was three days (range one to eight days). The majority of dogs were managed by the emergency and critical care service (134/168, 79.8%) at TAHMU, with lesser numbers by the primary care (28/168, 16.7%), internal medicine (5/168, 3.0%), and surgery (1/168, 0.6%)

services. One hundred and fifteen dogs (109/168, 64.9%) were hospitalised for a median duration of two days (range 1-16 days). The other 59 dogs were managed as outpatients.

Clostridium perfringens alpha toxin gene was most frequently detected with 156/168 dogs (92.9%) positive (Table 2). *Campylobacter* spp. (55/168, 32.7%) and CPV (29/168, 17.3%) were the next most frequently detected enteric organisms, followed by *Salmonella* spp. (14/168, 8.3%) and *Giardia* spp. (9/168, 5.4%). Canine enteric coronavirus and CDV were rarely detected (<2%), and no dogs tested positive for *Cryptosporidium* spp. (Table 2).

Of the 29 dogs that had CPV detected on faecal PCR, 19 dogs were less than or equal to 4 months of age. One dog was six months, and another nine months old. The remaining eight dogs were 13-150 months old. Summary data for these eight adult dogs that were CPV positive on fPCR are summarised in Table 3; one dog aged 13 months had not received its “one year booster”, two dogs had received their first annual vaccination following puppy vaccinations and were current, three had no vaccine history provided, one had protective titres documented in the 12 months prior, and the remaining dog had been vaccinated as a puppy and at 14 months with a three-year vaccine, and tested CPV positive at 41 months of age. There were 7/29 dogs (24.1%) with low-level detection of CPV. Two of this subset were less than four-month old puppies without a vaccination date recorded, one was a nine-month puppy who had last been vaccinated at 12 weeks of age, two had current vaccination records according to guideline definitions, one had CPV titres of 1:80 measured three months prior to fPCR testing, and the last was a 7-year old dog recorded as being current on vaccinations but without an actual date in the medical record.

Table 2. Faecal PCR results from dogs seen at a veterinary teaching hospital for diarrhoea. Prevalence is reported as the positive cases (detected + detected low level) out of a population of 168 dogs with diarrhoea.

Enteric organisms on faecal PCR panel	Not detected (N)	Detected low level (DL)	Detected (D)	Prevalence
Bacteria				
<i>Salmonella</i> spp.	154	1	13	14/168 (8%)
<i>Campylobacter</i> spp.	113	2	53	55/168 (33%)
<i>Clostridium perfringens</i> alpha toxin	12	4	152	156/168 (93%)
Protozoa				
<i>Giardia</i> spp.	159	0	9	9/168 (5%)
<i>Cryptosporidium</i> spp.	168	0	0	0/168 (0%)
Viruses				
Canine parvovirus (CPV)	139	7	22	29/168 (17%)
Canine enteric coronavirus (CCV)	167	0	1	1/168 (0.6%)
Canine distemper virus (CDV)	166	1	1	2/168 (1%)

In house CPV point-of-care antigen testing was performed on 53/168 dogs (31.5%). For these dogs, seven (13.2%) tested positive, 45 (84.9%) tested negative, and the test result was not recorded for one dog. The seven dogs that tested positive also had CPV detected on fPCR. For the 45 dogs with a negative antigen test, 13 (28.9%) had CPV detected on fPCR. Most recent vaccination date was recorded for six of these 13 dogs, of which five had been vaccinated within fourteen days of fPCR submission. Further testing has been performed in one of these dogs to determine if it was a vaccine or field strain. This case was a 16-week old golden retriever vaccinated (Nobivac DHP, MSD

Animal Health, Bendigo VIC) 14 days prior to presentation for 24 hours of severe vomiting and haemorrhagic diarrhoea. Testing performed at the University of Adelaide, according to previously published protocols,¹¹ confirmed infection with a field strain of CPV-2c.

For the 49 *Campylobacter* spp. fPCR positive dogs that had faecal cytology, only 15/49 (30.6%) had spiral-shaped organisms detected; for the 100 *Campylobacter* spp. fPCR negative dogs with faecal cytology results, 11 (11.0%) had spiral-shaped organisms detected. Of eight *Giardia* spp. fPCR positive dogs that had faecal cytology, *Giardia* cysts, trophozoites, or flagellates were detected in five of these dogs. Conversely, for the 141 dogs that had a negative fPCR for *Giardia* spp., only one dog had *Giardia* trophozoites detected on microscopy.

Antimicrobials were administered both before and after receipt of fPCR results to 26/168 dogs (15.5%). An additional 51 dogs (30.4%) were administered antimicrobials only during the treatment period prior to fPCR results, and 17 dogs (10.1%) were administered antimicrobials only after fPCR results were received. For the 26 dogs that received antimicrobials both before and after fPCR results, drug selection was altered for 14 dogs upon receipt of the fPCR results. Antimicrobials used before fPCR results were metronidazole (49 dogs), fenbendazole (36), amoxicillin-clavulanic acid (10), piperacillin-tazobactam (4), toltrazuril (4), erythromycin (3), azithromycin (1), tylosin (1), clindamycin (1), cephazolin (1), and ceftazidime (1). Antimicrobials used after fPCR results were received were erythromycin (15), metronidazole (12), amoxicillin-clavulanic acid (7), azithromycin (3), amoxicillin (3), fenbendazole (2), ampicillin (1), toltrazuril (1), pradofloxacin (1), piperacillin-tazobactam (1), and tylosin (1). Thirty-seven dogs were treated with more than one antimicrobial.

Table 3. Clinical data regarding adult dogs with diarrhoea that tested positive for canine parvovirus (CPV) on a faecal PCR panel.

Study ID	Age (months)	Days since last vaccination	Vaccine type/brand	Concurrent enteric organisms detected ^b	POC CPV test result
074	9	187	Protech C3	<i>C.perfringens</i>	Negative
097	13	272	Protech C3	<i>C.perfringens</i> , <i>Campylobacter</i> spp.	Not performed
150	15	No vaccine history recorded		<i>C.perfringens</i> , <i>Campylobacter</i> spp.	Not performed
060	17	248	Companion C5	<i>C.perfringens</i>	Not performed
039	19	110	Protech C3 + Pi2/BB Oral	<i>C.perfringens</i>	Negative
040	21	543 since vaccination 103 since last protective titres	Protech C3	<i>C.perfringens</i>	Not performed
091	41	812	Nobivac C5	<i>C.perfringens</i>	Not performed
035	85	No vaccine history recorded		<i>C.perfringens</i>	Not performed
112	150	No vaccine history recorded		<i>C.perfringens</i>	Negative

2.5. Discussion

This study further expands our understanding of the use of a fPCR panel as part of the diagnostic approach to diarrhoea in dogs seen at a university teaching hospital in Perth, WA. The panel was performed predominantly in hospitalised dogs with acute diarrhoea treated by the emergency and critical care service, but was also used as a diagnostic

tool in outpatients and those with chronic diarrhoea. Consistent with our hypothesis, the most commonly detected enteric organisms were the *C. perfringens* alpha toxin gene, *Campylobacter* spp., and CPV. The key findings to be discussed include the almost universal detection of the *C. perfringens* alpha toxin gene, high prevalence but lack of speciation of *Campylobacter* spp., and the high proportion of dogs with CPV, including adult dogs, those current on vaccinations, and those that tested negative with a point-of-care CPV antigen test.

The near universal detection of *Clostridium perfringens* alpha toxin gene in dogs in our study raises questions regarding its clinical relevance. Many fPCR assays, including that used in this study, test for *C. perfringens* spp. by detecting DNA of the alpha toxin gene. This has been shown to have limited clinical significance as it is frequently found in healthy dogs,¹² however fully quantitative fPCR assays may provide some predictive capability.¹³ Furthermore, the alpha toxin gene is found in all 5 biotypes of *C. perfringens* spp., but only biotype A has been implicated as a canine enteropathogen.^{14,15} Ideally, investigation of clostridial enteropathogens in dogs with diarrhoea should focus on the *C. perfringens* enterotoxin.^{12,15,16} The ACVIM guidelines on canine enteropathogens, including *C. perfringens*, provide evidence-based guidance on the interpretation of faecal diagnostics, including fPCR.⁶ The guidelines recommend that investigation of the role of *C. perfringens* in canine diarrhoea should involve both a PCR assay to detect the *C. perfringens* enterotoxin gene, together with an ELISA to detect the actual enterotoxin.⁶ The latter is not commercially available in Australia. The guidelines also emphasise the need to interpret positive test results in light of test methodology and clinical signs. At this time, we recommend that no treatment decisions are made based on the detection of *C. perfringens* alpha toxin gene via fPCR. It is likely that some patients in our study received inappropriate antimicrobials based on a positive *C. perfringens* fPCR result.

The prevalence of enteric organisms in our study varies considerably from previously published studies, reiterating that there is often geographic variation in prevalence of enteric organisms. One large study compared the prevalence of seven enteric organisms by fPCR across six countries.⁴ In their Australian data, *C. perfringens* alpha toxin was found in 46% of dogs,⁴ a much lower proportion than the 93% of dogs in our study. Even when comparing to the previously published study from WA, the prevalence of *C. perfringens* alpha toxin was higher in our study (93% vs. 81%).⁵ Further investigation is warranted to better understand these differences.⁴

One third of dogs in this study were fPCR positive for *Campylobacter* spp. This is comparable to prevalence data from other studies,¹⁷ although this ranges from 93% in North American shelter dogs,¹⁸ to 10% in dog faeces collected in a South American community park.¹⁹ One of the limitations with such prevalence data both in ours and many other studies, is the lack of *Campylobacter* speciation. Over 17 *Campylobacter* species have been reported in dogs, of which *C. jejuni*, *C. upsaliensis*, *C. coli*, and *C. helveticus* are most commonly found. Many of these *Campylobacter* species are likely commensal organisms, and in fact, only *C. jejuni* has reasonable evidence linking it to gastrointestinal illness in dogs.²⁰ However, one study proposed that diarrhoea samples with greater *Campylobacter* species diversity, perhaps representing dysbiosis, could be used to diagnose canine campylobacteriosis.²¹ Other *Campylobacter* species such as *C. upsaliensis* and *C. coli* have also been detected in diarrhoeic dogs, but typically with a prevalence not statistically different to non-diarrhoeic dogs.^{22,23}

Investigation for *Campylobacter* spp. via faecal microscopy is fraught with even more limitations, as shown in our results. Only 58% of dogs with spiral-shaped bacteria on faecal microscopy had *Campylobacter* spp. detected via fPCR, highlighting the low sensitivity and specificity of faecal microscopy consistent with previous studies.¹² Other

spiral-shaped organisms that could be evident on faecal microscopy include enteric *Helicobacter* spp., *Brachyspira* spp., and *Vibrio* spp..²⁴ Given the uncertainty about the role of *Campylobacter* spp. in diarrhoea in dogs,^{21,24} detection of *Campylobacter* spp. and the other enteric organisms on a fPCR panel should always be interpreted in light of the clinical findings.

The prevalence of fPCR CPV positivity in this study was considerably higher than previously reported rates in studies using fPCR,^{4,5} even after excluding low-level detection cases. Positive CPV results could be true positive findings due to primary or secondary vaccine failure²⁵ or false positive results, due to vaccine interference, laboratory error, or subclinical infection. Primary vaccine failure is defined as the inability of the vaccinee to mount an appropriate and protective antibody response after vaccine administration.^{26,27} Primary vaccine failure is most commonly due to maternally derived antibody interference during the first 12-16 weeks of age,²⁸⁻³⁰ although could be due to other factors such as breach of the vaccine cold chain. Consequently, the World Small Animal Veterinary Association (WSAVA) Vaccination Guidelines Group recommends giving the last puppy booster vaccination at or after 16 weeks of age.⁹ For the CPV-positive dogs in our study, 19/29 were less than 16 weeks of age, making primary vaccine failure due to maternally derived antibody interference likely. False positive fPCR CPV results due to detection of vaccine-strain rather than field-strain CPV is also possible in these puppies and would require genotyping.^{11,31} This was only performed in one case in our study but such assays are not readily available commercially and mostly remain the realm of research laboratories.³² Since vaccine strain shedding has been associated with low copy numbers of viral DNA in faecal samples, fully quantitative fPCR could also play a role in ruling in or out vaccine interference.¹

Secondary vaccine failure, or waning of antibody-mediated protection over time, could also lead to CPV infection, and may account for some of the CPV positive adult dogs in our study. Given the presence of diarrhoea at the time that these adult dogs tested CPV positive we do believe that these cases had true parvovirus, however other possible explanations include that CPV infection was present but they had another cause for their diarrhoea (i.e. the CPV itself was subclinical), or a false positive result due to laboratory error. Regardless, given the highly contagious nature of CPV, the authors recommend that CPV positive dogs with gastrointestinal signs requiring hospitalisation should still be managed in isolation to minimise risk of transmission to other dogs. None of these adult CPV-positive dogs had been recently vaccinated against CPV (Table 3), ruling out vaccine interference. The identification of one CPV positive result in an adult dog with acute haemorrhagic diarrhoea that had 'protective' anti-CPV serum antibody titres documented 103 days prior to the fPCR (Study ID 040, Table 3), may support the hypothesis proposed by other authors that serum antibody titres do not equate to immunity. Rather, gastrointestinal mucosal antibodies are responsible for protection against CPV-induced diarrhoea.^{29,33} False positive CPV results due to laboratory error, including sample mis-handling, and contamination of equipment with errant DNA, must also be considered. However, we used an accredited laboratory that operates under strict guidelines for sample handling and quality control making this less likely. Additional supporting data about assay performance is considered proprietary information by the commercial laboratory and was not available for publication.

The poor performance of the CPV point-of-care tests in our study is consistent with previous studies comparing point-of-care tests to fPCR assays that report sensitivities ranging from 15-65%.¹⁻³ As point-of-care tests require large quantities of faecal antigen,^{1,34} false negatives can occur with samples containing low quantities of viral antigen - during early or late stages of disease, or with gastrointestinal mucosal antibody

binding of faecal CPV antigen. We still consider these tests useful given their immediate turnaround time, but recommend caution interpreting a negative result, particularly in patients with high index of clinical suspicion for parvovirus. This caution should be extended to adult vaccinated dogs with haemorrhagic diarrhoea awaiting fPCR results given our study findings.

One of the indications for performing fPCR is to detect the presence of enteropathogens that might warrant treatment with a specific antimicrobial. As such, we explored antimicrobial use in this population of dogs with diarrhoea that had faecal PCR performed. While the impact of fPCR results on treatment decisions cannot be completely understood from a retrospective study such as this, we did note that antimicrobial use changed following receipt of fPCR results in some cases, including changes to antimicrobial selection or addition of antimicrobials to treatment plans. Given the uncertainty of a detected organism's role in diarrhoea as described above, it is vital that fPCR results are interpreted in context of the clinical findings. Consistent with the ACVIM guidelines,⁶ clinicians at our institution are advised to start antimicrobial treatment for an enteric organism only if the patient is septic or if the diarrhoea has failed to respond to supportive care by the time the fPCR results are available. Further investigation is warranted in the form of prospective clinical studies evaluating the efficacy of antimicrobials, in addition to supportive care, in the treatment of diarrhoea in dogs. The decision to restrict antibiotic use as above is facilitated by the median three-day turnaround time for fPCR assays. In our experience we found value in this delay by allowing the clinician to consider a dog's response to supportive care (IV fluids, anti-emetics, antacids, and analgesia) prior to deciding on antimicrobial use.

It was interesting to see fPCR performed in the investigation of chronic diarrhoea (greater than 7 days duration) for 24 dogs. Of the eight organisms on the fPCR panel,

only giardiasis is reliably associated with chronic waxing and waning gastrointestinal signs.³⁵ Even PCR use for giardiasis investigation could be questioned, as immunofluorescence assays are considered the reference standard for *Giardia* spp. detection by some publications, with fPCR recommended to be used only for additional genotyping.³⁶

While this study enhances our understanding of the use of fPCR panels in the management of dogs with diarrhoea, the retrospective case-series study design is a major limitation and allows only for reporting of observational data. For example, the majority of dogs were recorded as having no history of recent dietary indiscretion, scavenging behaviour, recent diet changes, nor history of consuming raw meat. However, this data recording may be inconsistent between clinicians, limiting interpretation. Additionally, as above, the components of the fPCR panel may not be the most ideal; for example testing for *C. perfringens* enterotoxin is likely more informative than the alpha toxin gene. Lastly, additional molecular diagnostics of CPV positive dogs would have aided in differentiating vaccine interference from wild-type infection.

2.6. Conclusion

Faecal PCR panels can identify dogs with enteric organisms in their faeces that traditional faecal diagnostics may miss, thus contributing additional information to the diagnostic process. Nonetheless, fPCR results should be interpreted in light of the clinical findings, and particular consideration given to avoiding inappropriate use of antimicrobials. This study provides a reminder that testing for *C. perfringens* alpha toxin gene is not likely to be diagnostically helpful, and that adult dogs with diarrhoea may be

CPV positive despite a negative point-of-care CPV test and being considered to have a current vaccination status.

2.7. Abbreviations

ACVIM American College of Veterinary Medicine; CCV Canine Enteric Coronavirus;

CDV Canine Distemper Virus; CPV Canine Parvovirus; fPCR Multiplex Tandem

Real-time Faecal PCR; GI gastrointestinal; TAHMU The Animal Hospital at Murdoch

University; WA Western Australia; WSAVA World Small Animal Veterinary Association

^aVetpath Laboratory Services, Belmont, Western Australia, Australia

2.8. Bibliography for Chapter 3

1. Decaro N, Desario C, Amorisco F et al. Detection of a canine parvovirus type 2c with a non-coding mutation and its implications for molecular characterisation. *Vet J* 2013;196:555–557.
<http://dx.doi.org/10.1016/j.tvjl.2012.12.017>.
2. Desario C, Decaro N, Campolo M et al. Canine parvovirus infection: which diagnostic test for virus? *J Virol Methods* 2005;126:179–185.
<http://dx.doi.org/10.1016/j.jviromet.2005.02.006>.
3. Schmitz S, Coenen C, König M et al. Comparison of three rapid commercial Canine parvovirus antigen detection tests with electron microscopy and polymerase chain reaction. *J Vet Diagn Invest* 2009;21:344–345.
<http://dx.doi.org/10.1177/104063870902100306>.
4. Gizzi AB da R, Oliveira ST, Leutenegger CM et al. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. *BMC Vet Res* 2014;10:23.
<http://dx.doi.org/10.1186/1746-6148-10-23>.
5. Paul AEH, Stayt J. The intestinal microbiome in dogs and cats with diarrhoea as detected by a faecal polymerase chain reaction-based panel in Perth, Western Australia. *Aust Vet J Wiley Online Library*, 2019;
<https://onlinelibrary.wiley.com/doi/abs/10.1111/avj.12867>.
6. Marks SL, Rankin SC, Byrne BA et al. Enteropathogenic bacteria in dogs

and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med* 2011;25:1195–1208. <http://dx.doi.org/10.1111/j.1939-1676.2011.00821.x>.

7. He Y, Wu W, Zheng H-M et al. Regional variation limits applications of healthy gut microbiome reference ranges and disease models. *Nat Med* 2018;24:1532–1535. <http://dx.doi.org/10.1038/s41591-018-0164-x>.

8. Holtz LR, Cao S, Zhao G et al. Geographic variation in the eukaryotic virome of human diarrhea. *Virology* 2014;468-470:556–564. <http://dx.doi.org/10.1016/j.virol.2014.09.012>.

9. Day MJ, Horzinek MC, Schultz RD et al. WSAVA Guidelines for the vaccination of dogs and cats. *J Small Anim Pract* 2016;57:E1–E45. http://dx.doi.org/10.1111/jsap.2_12431.

10. Harris PA, Taylor R, Thielke R et al. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42:377–381. <http://dx.doi.org/10.1016/j.jbi.2008.08.010>.

11. Woolford L, Crocker P, Bobrowski H et al. Detection of the Canine Parvovirus 2c Subtype in Australian Dogs. *Viral Immunol* 2017;30:371–376. <http://dx.doi.org/10.1089/vim.2017.0019>.

12. Marks SL, Kather EJ. Bacterial-associated diarrhea in the dog: a critical appraisal. *Vet Clin North Am Small Anim Pract* 2003;33:1029–1060. <https://www.ncbi.nlm.nih.gov/pubmed/14552160>.

13. Leutenegger CM, Marks SL, Robertson J. Toxin quantification of clostridium perfringens is a predictor for diarrhea in dogs and cats. *JOURNAL OF VETERINARY INTERNAL MEDICINE*. WILEY-BLACKWELL COMMERCE PLACE, 350 MAIN ST, MALDEN 02148, MA USA, 2012:794–794.

14. Mehdizadeh Gohari I, Parreira VR, Nowell VJ et al. A novel pore-forming toxin in type A Clostridium perfringens is associated with both fatal canine hemorrhagic gastroenteritis and fatal foal necrotizing enterocolitis. *PLoS One* 2015;10:e0122684. <http://dx.doi.org/10.1371/journal.pone.0122684>.

15. Schlegel BJ, Van Dreumel T, Slavić D et al. Clostridium perfringens type A fatal acute hemorrhagic gastroenteritis in a dog. *Can Vet J* 2012;53:555–557. <https://www.ncbi.nlm.nih.gov/pubmed/23115371>.

16. Silva ROS, Lobato FCF. Clostridium perfringens: A review of enteric diseases in dogs, cats and wild animals. *Anaerobe* 2015;33:14–17. <http://dx.doi.org/10.1016/j.anaerobe.2015.01.006>.

17. Acke E. Campylobacteriosis in dogs and cats: a review. *N Z Vet J* 2018;66:221–228. <http://dx.doi.org/10.1080/00480169.2018.1475268>.

18. Leahy AM, Cummings KJ, Rodriguez-Rivera LD et al. Faecal Campylobacter shedding among dogs in animal shelters across Texas. *Zoonoses Public Health* 2017;64:623–627. <http://dx.doi.org/10.1111/zph.12356>.

19. Toledo Z, Simaluiza RJ, Ochoa S et al. Occurrence and antimicrobial susceptibility of *Campylobacter jejuni* and *C. coli* in dog feces from public parks in Southern Ecuador. *Acta Sci Vet* 2015;43:1284.
https://www.academia.edu/download/49323502/2015-_Occurrence_and_Antimicrobial_Susceptibility_of_C_jejuni___C_coli_in_dog_feces_from_public_parks_Ecuador.pdf.
20. Macartney L, Al-Mashat RR, Taylor DJ et al. Experimental infection of dogs with *Campylobacter jejuni*. *Vet Rec* 1988;122:245–249.
<https://www.ncbi.nlm.nih.gov/pubmed/3369054>.
21. Chaban B, Ngeleka M, Hill JE. Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals. *BMC Microbiol* 2010;10:73.
<http://dx.doi.org/10.1186/1471-2180-10-73>.
22. Sandberg M, Bergsjø B, Hofshagen M et al. Risk factors for *Campylobacter* infection in Norwegian cats and dogs. *Prev Vet Med* 2002;55:241–253. [http://dx.doi.org/10.1016/s0167-5877\(02\)00095-8](http://dx.doi.org/10.1016/s0167-5877(02)00095-8).
23. Olson P, Sandstedt K. *Campylobacter* in the dog: a clinical and experimental study. *Vet Rec* 1987;121:99–101.
<http://dx.doi.org/10.1136/vr.121.5.99>.
24. Rossi M, Hänninen ML, Revez J et al. Occurrence and species level diagnostics of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in healthy and diarrheic dogs and cats. *Vet Microbiol* 2008;129:304–314. <http://dx.doi.org/10.1016/j.vetmic.2007.11.014>.
25. Sheppeard V, Forssman B, Ferson MJ et al. Vaccine failures and vaccine effectiveness in children during measles outbreaks in New South Wales, March-May 2006. *Commun Dis Intell Q Rep* 2009;33:21–26.
<https://www.ncbi.nlm.nih.gov/pubmed/19618764>.
26. Heininger U, Bachtiar NS, Bahri P et al. The concept of vaccination failure. *Vaccine* 2012;30:1265–1268. <http://dx.doi.org/10.1016/j.vaccine.2011.12.048>.
27. Wiedermann U, Garner-Spitzer E, Wagner A. Primary vaccine failure to routine vaccines: Why and what to do? *Hum Vaccin Immunother* 2016;12:239–243.
<http://dx.doi.org/10.1080/21645515.2015.1093263>.
28. Truyen U. Evolution of canine parvovirus—A need for new vaccines? *Vet Microbiol* 2006;117:9–13.
<http://www.sciencedirect.com/science/article/pii/S0378113506001374>.
29. Decaro N, Buonavoglia C. Canine parvovirus—A review of epidemiological and diagnostic aspects, with emphasis on type 2c. *Vet Microbiol* 2012;155:1–12.
<http://www.sciencedirect.com/science/article/pii/S0378113511005013>.
30. Altman KD, Kelman M, Ward MP. Are vaccine strain, type or administration protocol risk factors for canine parvovirus vaccine failure? *Vet Microbiol* 2017;210:8–16. <http://dx.doi.org/10.1016/j.vetmic.2017.08.019>.

31. Decaro N, Cirone F, Desario C et al. Severe parvovirus in a 12-year-old dog that had been repeatedly vaccinated. *Vet Rec* 2009;164:593–595. <http://dx.doi.org/10.1136/vr.164.19.593>.
32. Decaro N, Elia G, Desario C et al. A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus. *J Virol Methods* 2006;136:65–70. <http://dx.doi.org/10.1016/j.jviromet.2006.03.030>.
33. Freisl M, Speck S, Truyen U et al. Faecal shedding of canine parvovirus after modified-live vaccination in healthy adult dogs. *Vet J* 2017;219:15–21. <http://dx.doi.org/10.1016/j.tvjl.2016.11.011>.
34. Decaro N, Desario C, Beall MJ et al. Detection of canine parvovirus type 2c by a commercially available in-house rapid test. *Vet J* 2010;184:373–375. <http://dx.doi.org/10.1016/j.tvjl.2009.04.006>.
35. Adam RD. Biology of *Giardia lamblia*. *Clin Microbiol Rev* 2001;14:447–475. <http://dx.doi.org/10.1128/CMR.14.3.447-475.2001>.
36. Rishniw M, Liotta J, Bellosa M et al. Comparison of 4 *Giardia* diagnostic tests in diagnosis of naturally acquired canine chronic subclinical giardiasis. *J Vet Intern Med* 2010;24:293–297. <http://dx.doi.org/10.1111/j.1939-1676.2010.0475.x>.

2.9. Appendix

Abstract presented at the International Veterinary Emergency and Critical Care Society annual symposium in New Orleans, Louisiana, US September 2018.

EVALUATION OF FAECAL PCR PANEL RESULTS IN DOGS IN WESTERN AUSTRALIA

M.W. Kim¹; C.R. Sharp¹; C.J. Boyd¹; L. Twomey²

¹Murdoch University, Murdoch, WA, Australia; ²Vetpath Laboratory Services, Ascot, WA, Australia

Introduction

Real-time PCR assays for common canine enteropathogens have become widely available as a diagnostic tool to investigate acute diarrhea in dogs. The prevalence of

enteropathogens in dogs in Western Australia is currently unknown. The primary objective of this study was to describe and compare the fecal real-time PCR (qPCR) results of dogs presenting to veterinary facilities in Western Australia.

Methods

Electronic medical records at The Animal Hospital at Murdoch University (TAHMU) were searched for dogs who had fecal qPCR submitted. Results were compared with those from a major regional reference laboratory (Vetpath Laboratory Services). Prevalence of each pathogen was compared between groups using Fisher's exact test; a p value <0.05 was considered significant.

Results

In the TAHMU cohort (n=92), *Clostridium perfringens* α toxin gene (CP) was the most frequently detected enteropathogen (in 90% cases), followed by *Campylobacter* spp. (27%), canine parvovirus (CPV; 26%), *Salmonella* spp. (11%), and *Giardia* (3%). In the VetPath cohort (n=951), 86% were CP positive, 32% *Campylobacter* spp. positive, 13% CPV positive, 8% *Giardia* positive and 8% *Salmonella* spp. positive. The TAHMU cohort included significantly more cases of CPV (p<0.001) and CP (p=0.044). In the TAHMU cohort, diarrhea was a presenting complaint in 90/92 dogs. The majority (68/92) of dogs were recorded as current on vaccinations. 60/92 dogs were treated as inpatients, and 59/60 survived to discharge. 57/92 dogs received antimicrobials, 47 of which started antimicrobials prior to receipt of qPCR results. Following qPCR results, 32/92 continued antimicrobial therapy, with drug selection unchanged in 11 of these dogs. Of the 24 CPV-positive dogs, 21 were current on vaccinations, with a 2 week (range 3 days to 22 months) median duration from time of last vaccination to presentation. 29/92 dogs had CPV fecal antigen testing performed, with 11 testing negative in contrast to positive CPV

detection on qPCR; 9/11 of these dogs were current on vaccinations.

Conclusion

The prevalence of CPV was significantly higher in the TAHMU cohort, and CP was frequently detected in both populations of dogs. The potential role of the identified pathogens in disease requires further elucidation.

3. Chapter 4: Prevalence of enteric organisms detected by real-time PCR assay in faeces of dogs in Western Australia

This chapter provides a large set of faecal PCR results from a single commercial laboratory running fPCR assays for veterinary clinics within the state of Western Australia (WA). We hoped to support the validity of, as well as expand on results reported in chapter 3. We also aimed to seek seasonal or geographic patterns.

This chapter was submitted to the peer-reviewed Australian Veterinary Journal (<https://doi.org/10.1111/avj.13008>), and is awaiting the second round of responses from reviewers.

3.1. Abstract

Aim

The primary objective was to describe the prevalence of enteric organisms in dogs in Western Australia that had a faecal PCR (fPCR) submitted to a commercial veterinary laboratory. Secondary objectives were to identify any seasonal or regional prevalence patterns, and document co-detection rates with more than one organism.

Design

Retrospective observational study (July 2016-June 2019)

Procedure

Faecal PCR results were downloaded into a spreadsheet. Results included date of sample submission and sample origin postcode. Faecal samples from interstate or with a post-office box origin were excluded. Faecal PCR results were recorded as not detected, detected, or low-level detection for each organism.

Results

From 2025 fPCR results, *Clostridium perfringens* alpha toxin gene was most frequently detected (87.2%), followed by *Campylobacter* spp. (37.8%), canine parvovirus (10.5%), *Giardia* spp. (9.7%), *Salmonella* spp. (7.0%), canine enteric coronavirus (2.3%), canine distemper virus (0.3%) and no cases of *Cryptosporidium* spp.. Most samples (87.2%) originated from metropolitan Perth; of these, 45.6% had multiple organisms, and 8.7% had no organisms detected. There was no statistically significant seasonal variation. It was difficult to establish regional prevalence patterns, despite a large sample size. *C.perfringens* alpha toxin gene and *Campylobacter* spp. were the most common organisms co-detected.

Conclusion

Clostridium perfringens alpha toxin gene was frequently detected, and the majority of faecal samples had two or more organisms detected. Given our incomplete understanding of the pathogenic role of the detected organisms further studies are required to elucidate the role these organisms play in gastrointestinal disease in dogs.

3.2. Introduction

Faecal real-time PCR assays (fPCR) are available for the investigation of diarrhoea in dogs.¹⁻³ The commercially available veterinary fPCR assays typically test for viruses, bacteria, and protozoa. Theoretically these assays could be used to identify causative infectious agents of diarrhoea, and target antimicrobial treatment, however, merely the detection of an organism on a fPCR assay does not imply causation of diarrhoea.^{4,5}

Numerous factors should be taken into consideration when interpreting fPCR assay results in a given patient, including the test resolution (including whether the assay provides genus or species level results), seasonal variation,^{6,7} regional enteric organism profiles, and the presence of co-infections. Given the reported significance of geographic variation,^{8,9} establishing a regional enteric organism profile may help veterinarians interpret fPCR results, and facilitate more informed comparison of studies from different geographic regions.

Currently there are few studies describing enteric organism prevalence data in Australian dogs, with only three utilising fPCR as the primary diagnostic tool.¹⁰⁻¹² Firstly, an observational study included retrospective fPCR results from Australian dogs, listing the prevalence of eight enteric organisms.¹⁰ However, no time or location data were included, precluding evaluation of regional or seasonal variation. Another study evaluated fPCR results of dogs in Western Australia (WA), but although the testing laboratory receives samples from interstate, the authors did not clarify if interstate faecal samples were excluded.¹¹ Furthermore, with only twelve months of data, they were unable to identify any seasonal variation. The last of these studies reported clinical data alongside fPCR results from a smaller cohort of dogs seen at a single institution.¹²

The primary objective of this study was to describe the prevalence of enteric organisms in a large population of dogs in WA that had a fPCR submitted to a commercial veterinary laboratory. The secondary objectives were to identify any seasonal or regional prevalence patterns, and document co-detection rates with more than one organism.

3.3. Materials and methods

3.3.1. Data collection

All fPCR panel results performed on dogs from July 2016 to June 2019 performed at a single, nationally accredited (nata.com.au) commercial laboratory^a were downloaded into a single spreadsheet (Microsoft Excel). The spreadsheet contained the date the sample was received, the patient's name, the postcode the sample originated from, and the fPCR results. Cases were excluded if the sample was outsourced to a different external laboratory for fPCR, or if the sample originated from a post office mailbox or a postcode outside WA.

The fPCR used by this laboratory^a includes primers for the bacteria *Salmonella* spp., *Campylobacter* spp., and *Clostridium perfringens* alpha toxin gene, the protozoa *Cryptosporidium* spp., and *Giardia* spp., and the viruses canine parvovirus (CPV), canine enteric coronavirus (CCV), and canine distemper virus (CDV). For CPV, this assay is reported to detect strains 2a, 2b, and 2c. The results report the organism being either not detected, detected, or low level detection. Low level detection is a semi-quantitative reading based on the number of PCR cycles at which fluorescence of the target DNA sequence reaches the threshold level for detection. References to general fPCR results will be described as enteric organism detection, including *C. perfringens*, for which this fPCR actually detects the gene encoding the alpha toxin.

Information on PCR equipment, reagents, primers, cycle thresholds, and assay validation procedures are considered proprietary by the laboratory and were unavailable for publication.

3.3.2. Data analysis

Summary statistics are presented as number (percentage) or median (range) where appropriate. Data sorting and statistical analysis were performed using open-source statistical software (R version 3.3.3). Prevalence for each enteric organism was defined as the proportion of dogs that had an enteric organism or toxin gene detected by faecal PCR performed at a commercial laboratory^a during the study period. For the purposes of prevalence data, low level detection results were combined with full detection results. To evaluate seasonal variation of the prevalence of each organism, we combined prevalence data for each season then used chi-square tests for comparison. Significance was set at $P < 0.05$. Local government area boundaries were used to identify within-state prevalence patterns, and regional prevalence was mapped using an online mapping tool (plot.ly).

3.4. Results

3.4.1. Summary

A total of 2423 fPCR results for the three-year period of July 2016 to June 2019 were available. Three hundred and ninety-eight were excluded, leaving 2025 fPCR results for analysis in the study. The majority of exclusions were for interstate submission ($n=358$), 32 had been outsourced to another commercial laboratory, and eight entries had a blank fPCR results template. For the included results, *C.perfringens* alpha toxin gene was most frequently detected (1765/2025, 87.2%), followed by *Campylobacter* spp.

(766/2025, 37.8%), then CPV (213/2025, 10.5%), *Giardia* spp. (197/2025, 9.7%), *Salmonella* spp. (141/2025, 7.0%), CCV (46/2025, 2.3%), and CDV (7/2025, 0.3%) (Figure 1). *Cryptosporidium* spp. was not detected in any sample.

The faecal samples for these fPCR assays were submitted from 94 different postcodes, of which 65 postcodes were from the Perth metropolitan area, and the remaining 29 from regional WA. The Perth metropolitan area of approximately 6400 square kilometres represents less than 0.3% of the state of WA,¹³ but was the source of 1776/2025 (87.7%) fPCR samples. With these numbers, we focused only on the Perth metropolitan area results for the most commonly detected organisms *Clostridium perfringens* alpha toxin gene, *Campylobacter* spp., CPV, *Giardia* spp., and *Salmonella* spp., for evaluation of seasonal prevalence, regional variation, and co-detection.

3.4.2. Perth faecal PCR results

A total of 1776 fPCR assays were performed during the study period from Perth postcodes. We detected a sharp decline in the monthly median number of tests performed over the study period, with a median of 67 (range 35-106) per month up to September 2017, and a median of 38 (range 25-49) tests per month thereafter.

Seasonal Prevalence No significant differences were evident in organism prevalence when statistically compared among seasons (Figure 2). The variation in *Campylobacter* spp. among seasons approached significance ($p=0.058$), with a range of 41.2% in the spring to 32.8% in the autumn.

Geographical Variation Faecal samples were submitted from 65 postcodes in the Perth metropolitan area. There was considerable variation in the number of fPCR assays submitted from each local government area in Perth (Figure 3), regardless of proximity to Perth CBD. These differences did not clearly follow population density in

Perth. The majority of samples (1018/1776, 57.3%) were submitted from eleven postcodes, of which four postcodes submitted over 100 samples each. Most postcodes (54/65, 83.1%) submitted less than 1.2 samples per month over the three-year period.

Detection of multiple organisms Multiple organisms were detected by fPCR in 810/1776 (45.6%) dogs, while 811 (45.7%) had just one organism detected, and 154 (8.7%) were negative for all organisms. Two organisms were detected in 582 fPCR panels, three organisms in 174 cases, four organisms in 46 cases, five organisms in 6 cases, and six organisms in 2 cases. For dogs with multiple organisms detected, *C.perfringens* alpha toxin gene and *Campylobacter* spp. formed the most common co-detection (619/810, 76.4%). For the 180 dogs with CPV detected, 161/180 (89.4%) also had *C.perfringens* alpha toxin gene detected. Furthermore, 101/180 (56.1%) dogs had CPV and *Campylobacter* spp., of which most also had *C. perfringens* alpha toxin gene (91/180, 50.6%). For the 41 dogs with both CPV and *Giardia* spp. detected, all but one dog (40/41, 97.6%) had concurrent *C.perfringens* alpha toxin gene detected. Twenty-nine dogs had CPV, *C.perfringens* alpha toxin gene, *Campylobacter* spp., and *Giardia* spp. detected concurrently.

3.5. Discussion

Our study describes the enteric organism profile in faecal samples from WA dogs as detected by a fPCR assay, with *C.perfringens* alpha toxin gene detected in 87.2%, followed by *Campylobacter* spp. in 37.8%. Canine parvovirus, *Giardia* spp. and *Salmonella* spp. were detected in 10.5%, 9.7%, and 7.0% of samples respectively.

Prior enteric organism studies in dogs show considerable variation in the prevalence of *C.perfringens* alpha toxin gene, *Campylobacter* spp., CPV, *Giardia* spp., and *Salmonella*

spp..^{4,10,11,14,15} Many factors may contribute to this variation, including test methodology, sample collection and handling methods, true geographic variation in prevalence, as well as patient factors and patient selection. With regard to test methodology numerous factors must be considered when interpreting results in studies of enteric organism prevalence. Firstly, fPCR is widely considered more sensitive and specific than traditional faecal tests, such as culture and direct microscopy.^{1,16,17} As such, it would not be surprising for organism detection rates to be higher in studies using fPCR than those that employ traditional faecal tests. Even between studies using fPCR assays, use of different primers and other reagents involved in the assay, as well as differing methods of detection such as qualitative conventional gel PCR versus fully quantitative real-time PCR assays, can all have considerable impact on the results. Guidelines exist for optimising the collection, handling, and shipping of faecal samples for fPCR assays, however for retrospective studies such as this, it is unknown whether or not submitted faecal samples were handled appropriately. Inappropriate sample handling could result in degradation of organism genetic material and reduce the rates of detection of enteric organisms.¹ Patient factors, such as day-to-day variation in enteric organisms found in faecal samples, will also influence results.

The prevalence values we report are similar to results from an earlier time period at the same laboratory,¹¹ as well as the prevalence data from diarrhoeic dogs seen at our institution.¹² By demonstrating similar results in this larger cohort we have established a more rigorous prevalence profile of these enteric organisms in dogs in WA that had a fPCR performed. Comparisons to other non-WA regions show considerable variation, but the aforementioned differences in diagnostic methodologies must be considered, in addition to geographic variation, when making direct comparisons.

The prevalence of the *C.perfringens* alpha toxin gene in this study (87.2%), and another WA prevalence study (85.4%) was markedly higher than the reported eastern Australia fPCR prevalence (45.6%).¹⁰⁻¹² In fact, when expanding comparison data globally, only a cohort of diarrhoeic dogs in Germany reported *C.perfringens* prevalence (78%) comparable to that of WA dogs.¹⁴ However, in the German study, *C.perfringens* detection was performed via faecal culture rather than fPCR assays. The very high *C.perfringens* alpha toxin gene prevalence in our cohort must be viewed in light of our understanding that the alpha toxin is of questionable relevance in dogs with gastrointestinal disease,¹ and this emphasises our limited ability to interpret the detection of *C.perfringens* alpha toxin gene. The current recommendation to assess the role of *C.perfringens* in dogs with gastrointestinal disease is to test for the presence of the main enterotoxin implicated in clostridiosis in dogs, *Clostridium perfringens* enterotoxin.¹ While fPCR can identify the enterotoxin gene, an ELISA is required to determine the presence of the enterotoxin itself, and it is recommended to use the two tests concurrently.¹

Giardia prevalence varies widely among reported studies, including in Australia.¹⁵⁻¹⁷ Two PCR-based studies describe *Giardia* prevalence of 6.2% and 10.8% in Australian dogs.^{10,11} Two additional Australian studies reported faecal-microscopy based *Giardia* prevalence from 5.5% in dogs seen at veterinary clinics to 22% in a heterogeneous population.^{15,16} In addition to methodological differences, direct comparison is limited by testing only to genus level. Classification of *Giardia* has evolved, and species nomenclature is now mostly based on assemblages A to G, with species names representing host specificity.¹⁸ Only assemblages C and D, or *Giardia canis*, are suspected to cause gastrointestinal disease in dogs, however, assemblages A and B, or *G. duodenalis* and *G. enterica* respectively, have been frequently detected in dogs.^{19,20} These two species have not been implicated in gastrointestinal disease in dogs. If there

are true differences between the different cohorts in these studies, the main consideration would be contamination of bodies of water accessed by the dog population, housing density, and socioeconomic and sanitary infrastructure differences.

A recent study reported a prevalence of CPV of 9.4% in WA dogs tested by fPCR, which similar to our findings.¹¹ The previously reported prevalence of CPV ranged from 6.8% in Australian dogs tested by fPCR,¹⁰ to 35% in a cohort of diarrhoeic dogs in Brazil.¹⁰ Interestingly, *Campylobacter* prevalence was highest (97% in diarrhoeic, and 56% in non-diarrhoeic, faecal samples) in a study that used custom-designed primers to identify 14 species of *Campylobacter*.⁴ For CCV, CDV, and *Cryptosporidium spp.*, previous Australian data report much higher prevalence.¹⁰ This may represent true regional variation, although again direct comparison is limited given the two comparator studies did not use the same PCR assay. Given the low detection rates for CCV, CDV, and *Cryptosporidium spp.* in this population, their removal from the fPCR panel could be considered by the commercial laboratory. It may be more appropriate for veterinarians to order tests for these pathogens in specific patients in which these diseases are a relevant differential diagnosis, rather than having them as a part of a panel.

Statistically significant seasonal variation in prevalence was not evident for any of the enteric organisms detected by fPCR in this study. This may be due to lack of seasonal variation of enteric organisms in dogs in Perth, or due to type II error. For some of the detected organisms seasonal variation has been suggested in other parts of the world, although findings are rarely consistent across regions. For example, *Campylobacter spp.* showed higher rates of detection in the warmer months of spring and summer in Spain and Norway,^{21,22} but not in Switzerland.²³

The drop in number of tests per month in the second half of 2017 may represent the 'hype-cycle' phenomenon in diagnostic medicine.^{24,25} This phenomenon sees clinicians

eagerly adopt a newly promoted diagnostic test at a rate that outpaces supportive evidence for its use, until a combination of anecdotal experience and published evidence reveals the limitations. This then leads to reduced use of such tests as clinicians more critically assess the benefits versus risks. For fPCR assays, a better understanding of their limitations ideally results in a more considered response to positive detection of an organism, and therefore better antimicrobial stewardship.

Similar to other studies, we found 46% of dogs had two or more organisms detected by fPCR.^{10,11} Co-detections may represent the presence of multiple organisms causing no pathogenicity in that individual host, multiple primary pathogens, a primary pathogen with secondary dysbiosis resulting in co-detection of additional organisms that may or may not have a pathogenic role, or dysbiosis secondary to a non-infectious aetiology. Only primary pathogens may require treatment in the form of antimicrobials, however a fPCR panel does not facilitate determination of which if any of the identified organisms are pathogenic. Nonetheless, the authors believe that prescribing specific antimicrobials targeted against each enteric organism identified on a fPCR panel is likely to contribute to excessive use of antimicrobials. Further research is required to determine how enteric organisms, alone or in combination, contribute to gastrointestinal disease in dogs in order to aid veterinarians in translating fPCR results into treatment decisions.

The retrospective study design and lack of clinical information on the dogs whose fPCR results were included in this study restricts our interpretation of the data. It is a study limitation that our target population for describing enteric organism prevalence was specific to dogs who had a fPCR performed, not a true prevalence of all dogs in WA. Inclusion of only cases that had fPCR performed introduces bias with regards to patient selection, reason for testing, and location insight. For example, patient selection bias likely resulted from not knowing the clinical reasoning that led to fPCR submission.

Other factors such as owner finances, may also have influenced such decisions. The latter is an important component given the relatively high cost of fPCR assays here in WA (230AUD cost to client at our institution at time of writing). Additionally, information that would have aided data interpretation includes patient signalment, diet, environment, nature and duration of clinical signs, and any prior treatment. Based on anecdotal evidence, it is likely most dogs that had a fPCR assay presented for gastrointestinal signs, but again, lack of clinical information prohibits any such assumptions.

False positive or false negative test results must also be considered a source of variation. Factors ranging from stool collection method, storage conditions, and quality control procedures in the laboratory can produce erroneous or misleading results.²⁶ It is also worth noting that the fPCR assay used in this study does not test for several known enteropathogens, namely *E.coli* and *Clostridium difficile* enterotoxin A and B.²⁷⁻²⁹ A comprehensive investigation of infectious causes of diarrhoea in dogs would ideally include these organisms. Another study limitation is that our ability to identify geographical patterns is restricted by having only postcodes, which only represent the location of the submitting veterinary clinic, not the dog's place of residence. Clearly, there is a real possibility that dogs from a wider area are seen at a single veterinary clinic. Furthermore, breaking down the large sample size into location (postcode) removed our ability to establish meaningful temporo-geographical patterns (Figure 3) and we were unable to compare regional prevalence.

3.6. Conclusion

Clostridium perfringens alpha toxin gene is highly prevalent in the faeces of WA dogs that had a fPCR performed. Nearly half the dogs in this large cohort had two or more

organisms detected, highlighting the need for further investigation into each organism's role in disease and in health. There were no statistically significant seasonal variations in detection rates. Additionally, despite a large sample size, the concentration of results by region was too small to establish regional prevalence patterns. Ideally, a large prospective cross-sectional study of diarrhoeic versus healthy dogs, with higher resolution testing to species or subspecies level, is needed to establish the prevalence of known enteric pathogens in WA dogs. This may elucidate both the relevance of these organisms in disease, as well as the optimal treatment approach. Until then, careful consideration of antimicrobial stewardship is essential to minimise their unnecessary or inappropriate use.

^a Vetpath Laboratory Services, Ascot, Western Australia, Australia

Figure 1. Prevalence of enteric organisms detected by faecal PCR in dog faecal samples submitted to a commercial laboratory from postcodes in Western Australia (n=2025) between July 2016 and June 2019. Note that WA results comprise Perth results (n=1776) plus regional WA results (n=249). CPV, canine parvovirus; CCV, canine enteric coronavirus; CDV, canine distemper virus.

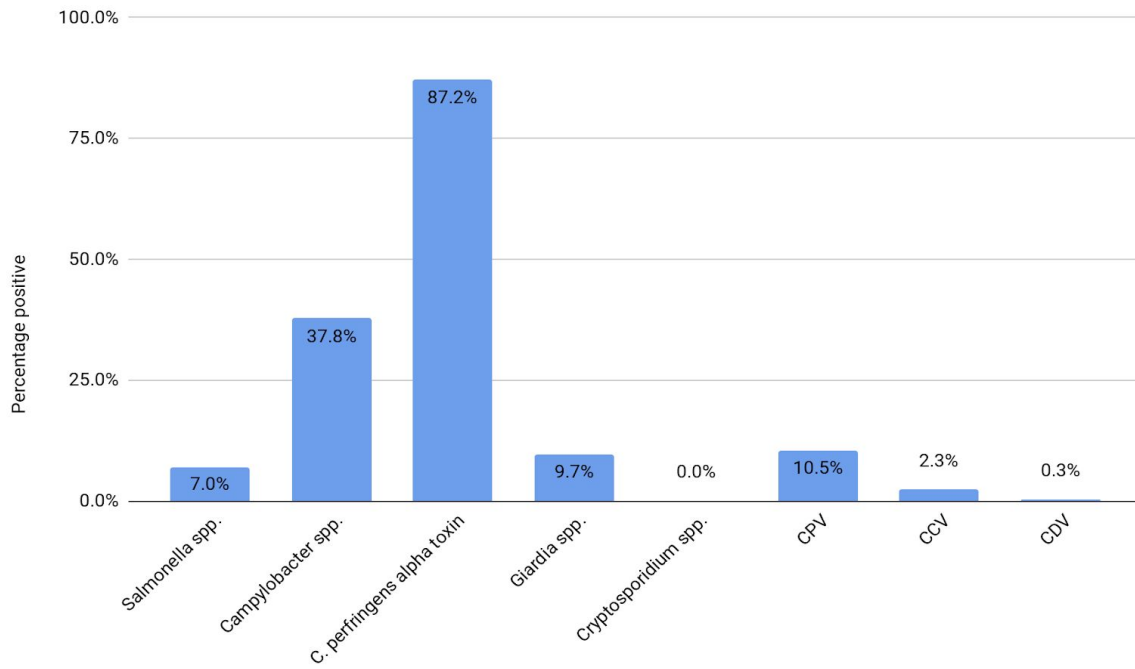


Figure 2. Percentage of positive results by season for each organism detected by faecal PCR in dog faecal samples submitted from Perth postcodes between July 2016 to June 2019. CPV, canine parvovirus.

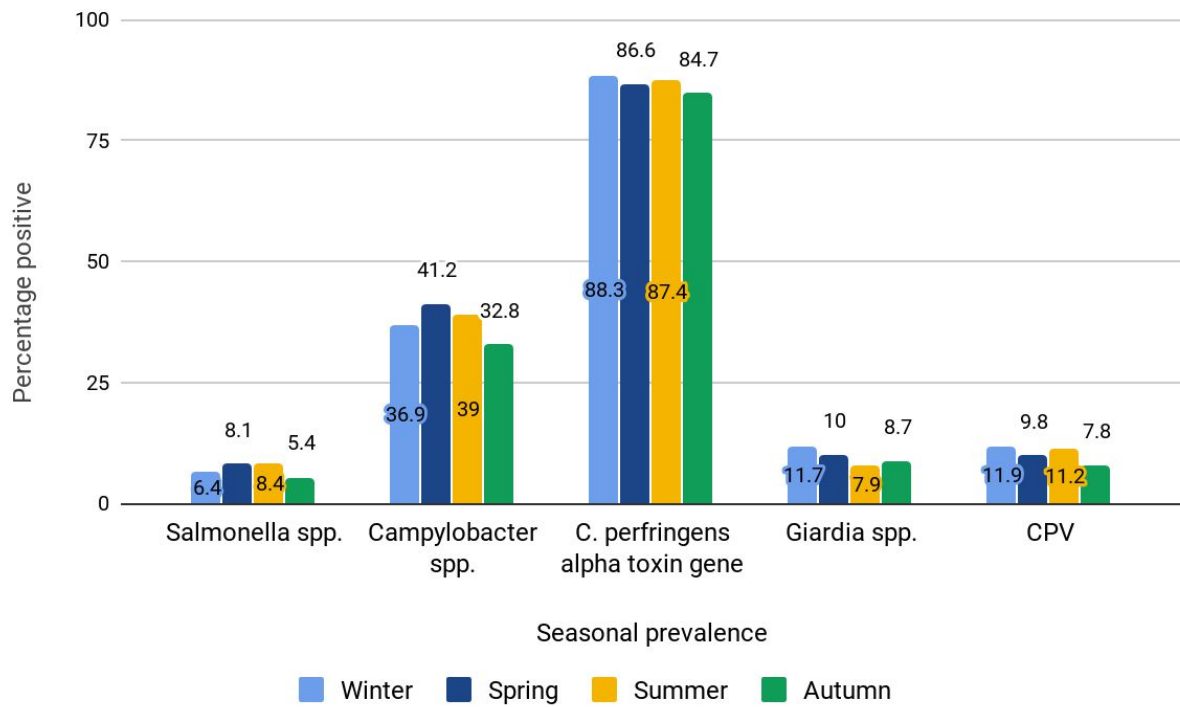
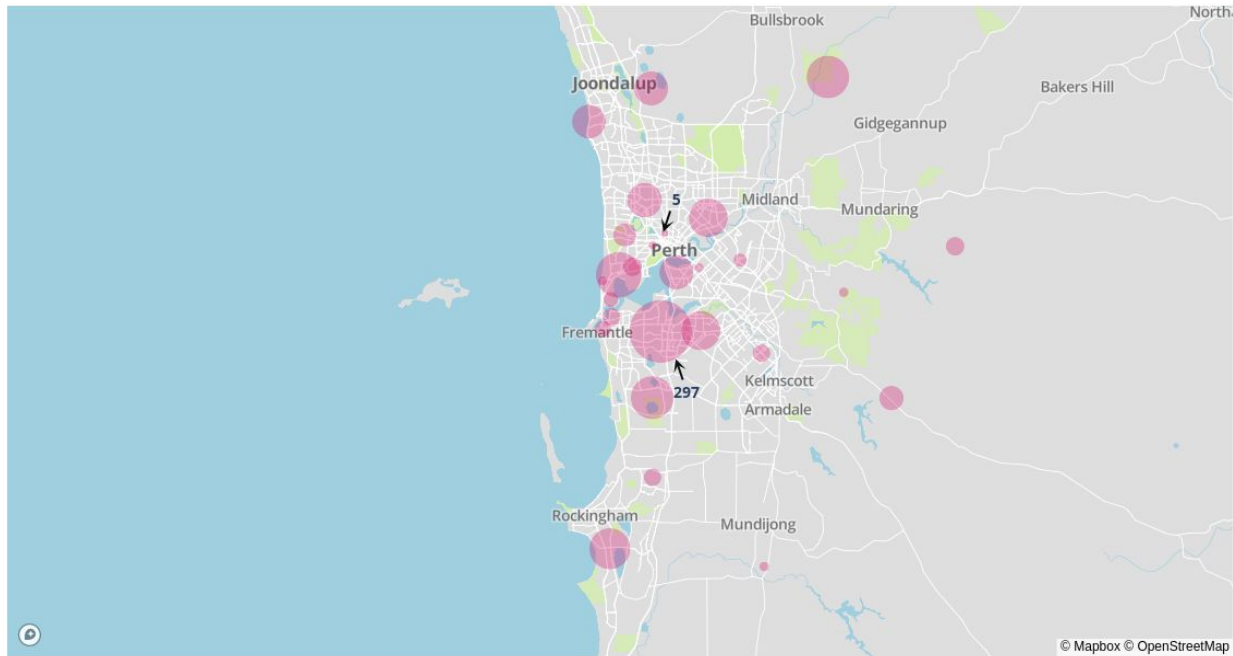


Figure 3. Perth metropolitan area total number of faecal PCR tests performed on dog faecal samples submitted to a commercial laboratory between July 2016 and June 2019, by local government area. Each circle represents a local government area. Size of circle is proportional to number of tests performed.



3.7. Bibliography for Chapter 4

1. Marks SL, Rankin SC, Byrne BA et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med* 2011;25:1195–1208. <http://dx.doi.org/10.1111/j.1939-1676.2011.00821.x>.
2. Ettinger SJ, Feldman EC, Cote E. *Textbook of Veterinary Internal Medicine - eBook*. Elsevier Health Sciences, 2017. <https://market.android.com/details?id=book-57XBDQAAQBAJ>.
3. Marks SL. Diarrhea. *Canine and Feline Gastroenterology* Elsevier, 2013;99. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7151799/>.
4. Chaban B, Ngeleka M, Hill JE. Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals. *BMC Microbiol* 2010;10:73. <http://dx.doi.org/10.1186/1471-2180-10-73>.
5. Bagcigil AF, Ikiz S, Dokuzeylu B et al. Fecal shedding of *Salmonella* spp. in dogs. *J Vet Med Sci* 2007;69:775–777.

<https://www.ncbi.nlm.nih.gov/pubmed/17675814>.

6. Tangtrongsup S, Scorza AV, Reif JS et al. Seasonal distributions and other risk factors for *Giardia duodenalis* and *Cryptosporidium* spp. infections in dogs and cats in Chiang Mai, Thailand. *Prev Vet Med* 2020;174:104820. <http://dx.doi.org/10.1016/j.prevetmed.2019.104820>.
7. 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. *J Clin Microbiol* 2017;55:1350–1368. <http://dx.doi.org/10.1128/JCM.02137-16>.
8. He Y, Wu W, Zheng H-M et al. Regional variation limits applications of healthy gut microbiome reference ranges and disease models. *Nat Med* 2018;24:1532–1535. <http://dx.doi.org/10.1038/s41591-018-0164-x>.
9. Sanchez-Thevenet P, Carmena D, Adell-Aledón M et al. High Prevalence and Diversity of Zoonotic and Other Intestinal Parasites in Dogs from Eastern Spain. *Vector Borne Zoonotic Dis* 2019;19:915–922. <http://dx.doi.org/10.1089/vbz.2019.2468>.
10. Gizzi AB da R, Oliveira ST, Leutenegger CM et al. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. *BMC Vet Res* 2014;10:23. <http://dx.doi.org/10.1186/1746-6148-10-23>.
11. Paul AEH, Stayt J. The intestinal microbiome in dogs and cats with diarrhoea as detected by a faecal polymerase chain reaction-based panel in Perth, Western Australia. *Aust Vet J Wiley Online Library*, 2019; <https://onlinelibrary.wiley.com/doi/abs/10.1111/avj.12867>.
12. Abstracts from the International Veterinary Emergency and Critical Care Symposium, the European Veterinary Emergency and Critical Care Annual Congress, and the ACVECC VetCOT Veterinary Trauma & Critical Care Conference 2018. *J Vet Emerg Crit Care* 2018;28:S1–S37. <https://onlinelibrary.wiley.com/doi/abs/10.1111/vec.12758>.
13. Kennewell C, Shaw BJ. Perth, Western Australia. *Cities Elsevier*, 2008; <https://www.sciencedirect.com/science/article/pii/S0264275108000176>.
14. Werdeling F, Amtsberg G, Tewes S. The occurrence of enterotoxigenic *Clostridium perfringens* strains in the feces of dogs and cats. *Berl Munch Tierarztl Wochenschr* 1991;104:228–233. <https://europepmc.org/abstract/med/1898321>.
15. Bugg RJ, Robertson ID, Elliot AD et al. Gastrointestinal Parasites of Urban Dogs in Perth, Western Australia. *The Veterinary Journal* 1999;157:295–301. <http://www.sciencedirect.com/science/article/pii/S1090023398903276>.
16. Palmer CS, Andrew Thompson RC, Traub RJ et al. National study of the gastrointestinal parasites of dogs and cats in Australia. *Veterinary Parasitology* 2008;181–190. <http://dx.doi.org/10.1016/j.vetpar.2007.10.015>.
17. Ortuño A, Scorza V, Castellà J et al. Prevalence of intestinal parasites in

shelter and hunting dogs in Catalonia, Northeastern Spain. *The Veterinary Journal* 2014;199:465–467.

<http://www.sciencedirect.com/science/article/pii/S109002331300614X>.

18. Thompson RCA, Ash A. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections. *Infect Genet Evol* 2016;40:315–323.

<http://dx.doi.org/10.1016/j.meegid.2015.09.028>.

19. Adell-Aledón M, Köster PC, de Lucio A et al. Occurrence and molecular epidemiology of *Giardia duodenalis* infection in dog populations in eastern Spain. *BMC Vet Res* 2018;14:26.

<https://doi.org/10.1186/s12917-018-1353-z>.

20. Julien DA, Sargeant JM, Guy RA et al. Prevalence and genetic characterization of *Giardia* spp. and *Cryptosporidium* spp. in dogs in Iqaluit, Nunavut, Canada. *Zoonoses Public Health* 2019;66:813–825.

<http://dx.doi.org/10.1111/zph.12628>.

21. Carbonero A, Torralbo A, Borge C et al. *Campylobacter* spp., *C. jejuni* and *C. upsaliensis* infection-associated factors in healthy and ill dogs from clinics in Cordoba, Spain. Screening tests for antimicrobial susceptibility. *Comparative Immunology, Microbiology and Infectious Diseases* 2012:505–512.

<http://dx.doi.org/10.1016/j.cimid.2012.05.002>.

22. Sandberg M, Bergsjø B, Hofshagen M et al. Risk factors for *Campylobacter* infection in Norwegian cats and dogs. *Prev Vet Med* 2002;55:241–253.

[http://dx.doi.org/10.1016/s0167-5877\(02\)00095-8](http://dx.doi.org/10.1016/s0167-5877(02)00095-8).

23. Wieland B, Regula G, Danuser J et al. *Campylobacter* spp. in dogs and cats in Switzerland: risk factor analysis and molecular characterization with AFLP. *J Vet Med B Infect Dis Vet Public Health* 2005;52:183–189.

<http://dx.doi.org/10.1111/j.1439-0450.2005.00843.x>.

24. Culbreath K, Petti CA. Balancing Enthusiasm for Innovative Technologies with Optimizing Value: An Approach to Adopt New Laboratory Tests for Infectious Diseases Using Bloodstream Infections as Exemplar. *Open Forum Infect Dis* 2015;2:ofv075.

<http://dx.doi.org/10.1093/ofid/ofv075>.

25. Thompson M, Plüddemann A, Price CP et al. Emerging diagnostic technologies in primary care: what's on the horizon? *Br J Gen Pract* 2013;63:177–178.

<http://dx.doi.org/10.3399/bjgp13X665107>.

26. Markoulatos P, Sifakos N, Moncany M. Multiplex polymerase chain reaction: A practical approach. *J Clin Lab Anal* 2002;16:47–51.

<http://doi.wiley.com/10.1002/jcla.2058>.

27. Schmidt VM, Pinchbeck GL, Nuttall T et al. Antimicrobial resistance risk factors and characterisation of faecal *E. coli* isolated from healthy Labrador retrievers in the United Kingdom. *Prev Vet Med* 2015;119:31–40.

<http://dx.doi.org/10.1016/j.prevetmed.2015.01.013>.

28. Busch K, Suchodolski JS, Kühner KA et al. *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A/B do not play a role in acute haemorrhagic diarrhoea syndrome in dogs. *Vet Rec* 2015;176:253.

<http://dx.doi.org/10.1136/vr.102738>.

29. Álvarez-Pérez S, Blanco JL, Peláez T et al. Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs. *J Small Anim Pract* 2015;56:190–195. <http://dx.doi.org/10.1111/jsap.12311>.

4. Chapter 5: MERINGUE: Metronidazole use in dogs with Acute Haemorrhagic Diarrhoea Syndrome. A randomised placebo-controlled clinical trial

This is the study protocol for a clinical trial currently underway (enrolment ongoing) at The Animal Hospital at Murdoch University. The two observational studies in the previous two chapters identified the challenges of interpreting a faecal PCR panel for dogs. In chapter two we also observed the controversial use of antimicrobials in the majority of dogs, despite lack of strong evidence that certain microorganisms cause diarrhoea in dogs, or that antimicrobial use improves outcome. Although clinical trials exist in this area, none evaluate metronidazole use alone compared to placebo. A non-inferiority design was deemed the most appropriate study design to demonstrate whether appropriate supportive care alone was no worse than supportive care plus metronidazole.

4.1. Animal ethics approval: R2946/17

4.2. Summary

Title: Metronidazole use in dogs with Acute Haemorrhagic Diarrhoea Syndrome: A randomised, controlled clinical trial

Subject: To evaluate the effect of metronidazole versus placebo on time to resolution of diarrhoea in dogs with Acute Haemorrhagic Diarrhoea Syndrome (AHDS).

Significance: Despite lack of evidence of an infectious aetiology in AHDS dogs, and strong evidence demonstrating the effectiveness of appropriate supportive care, antimicrobials continue to be prescribed to this population of sick dogs.

Hypothesis/Objectives: Our objective is to compare metronidazole and supportive care to placebo and supportive care as treatment for AHDS and their effect on clinical markers including time to resolution of diarrhoea. We hypothesise that placebo and supportive care will be non-inferior to metronidazole and supportive care. A secondary objective will be to determine the faecal enteric organism profile of enrolled dogs.

Study Design: A prospective, randomised, blinded, placebo-controlled non-inferiority phase II clinical trial. Enrolled dogs will receive standard supportive care in addition to either metronidazole 10mg/kg IV/PO q12 hours *OR* 0.9% sodium chloride at an equivalent volume IV/PO q12 hours. Study drug will be given for a total of five days (ten doses). A faecal sample will be collected at admission prior to study drug and submitted for a multiplex real-time PCR assay. All clinical and laboratory data will be recorded. A daily AHDS index will be scored. All faeces passed will be photographed. Data will be recorded for 24 hours past the last dose of study drug for a total of six days of data collection.

Expected Results: We expect no difference in time to resolution of diarrhoea between the two groups.

Anticipated Outcomes: If placebo is shown to be non-inferior to treatment with metronidazole, it would provide high quality evidence to consider standard supportive treatment as the standard-of-care in dogs with AHDS, and limit antimicrobial use only when infectious causative pathogen has been demonstrated. It would also weaken support for metronidazole-susceptible organisms as the cause of AHDS. If placebo is shown to be inferior to treatment with metronidazole, it would justify pursuit of an infectious aetiology with further studies.

4.3. Abbreviations

AHDS Acute Haemorrhagic Diarrhoea Syndrome

CPV Canine parvovirus

CCV Canine enteric coronavirus

CDV Canine distemper virus

4.4. Background

Acute haemorrhagic diarrhoea syndrome (AHDS) in dogs is a frequent presenting complaint to the emergency veterinary hospital. Severe gastrointestinal fluid and protein losses often lead to marked dehydration, hypovolaemic shock, and high morbidity.¹ There are several proposed aetiologies of AHDS, including infectious disease and type I hypersensitivity reactions to food,^{1,2} but a definitive cause remains elusive due to the limitations of available diagnostic tools.^{3,4} Thus dogs with a known cause of haemorrhagic diarrhoea, such as pancreatitis, canine parvoviral enteritis, coagulopathies, hypoadrenocorticism, and other infectious diseases, are not considered to fall under the umbrella term AHDS. Recently, the development of molecular diagnostics has revealed the significant changes to the microbiome associated with AHDS as compared to dogs without diarrhoea and dogs with inflammatory bowel disease.⁵ Ongoing investigations have subsequently focused on an infectious aetiology, thus far without success.

Treatment of AHDS primarily involves aggressive supportive care to restore euvolaemia and ensure adequate organ perfusion, in addition to correcting dehydration and metabolic derangements. Other commonly used treatments address other clinical signs, such as antiemetics for nausea and vomiting, and analgesia for abdominal pain.

Although antimicrobials are also frequently used, there is minimal evidence that they provide clinical benefit to dogs with AHDS. In a randomised, placebo-controlled German clinical trial of antimicrobial use in 60 dogs with AHDS, no difference was found between the dogs that received a broad spectrum antimicrobial (amoxicillin-clavulanic acid) versus placebo.² These authors also evaluated probiotics versus placebo in a similar clinical trial, again finding no difference in clinical progress between groups. Furthermore, in the 2011 American College of Veterinary Internal Medicine Consensus Statement on enteropathogenic bacteria, antimicrobial use is only recommended for dogs with acute diarrhoea that have confirmed Clostridial enteritis alongside evidence of sepsis.³

Metronidazole is often the antimicrobial of choice to treat dogs with AHDS, despite the absence of any supporting evidence. Metronidazole's antimicrobial spectrum, particularly against clostridial organisms, antiprotozoal properties, or its immunomodulatory properties, are frequently cited to justify its use. Metronidazole's immunomodulatory properties have been demonstrated in people with gastrointestinal disorders, as well as several experimental rodent models.^{6,7} However, there is no strong evidence these immunomodulatory properties extend to the canine gastrointestinal tract, nor is there evidence that AHDS has an infectious aetiology.^{2,8-10} Metronidazole can also cause adverse effects in dogs including neurotoxicity of unknown mechanism, anorexia, nausea, vomiting, diarrhoea, hepatotoxicity, and neutropaenia.¹¹⁻¹³

Another emerging area of concern is the effect of antimicrobials on the gastrointestinal microbiome. The microbiome is closely linked to both health and disease. Research has shown antimicrobial use has adverse effects on commensal intestinal flora;¹⁴ a change that has implications ranging from acute gastrointestinal to chronic extra-gastrointestinal disease. Furthermore, inappropriate or unnecessary use of antimicrobials in veterinary

patients has been linked to antimicrobial resistance. This forms part of the basis of antimicrobial stewardship, a rapidly growing discipline in veterinary medicine. Lastly, it is unclear whether enteric organisms detected on faecal PCR panels need to be treated with antimicrobials.³

4.5. Objectives and hypothesis

Objective: To evaluate time to resolution of diarrhoea in dogs with AHDS treated with metronidazole and standard medical care as compared to placebo and standard medical care. A secondary objective will be to determine the faecal enteric organism profile of enrolled dogs, as detected by a faecal real-time PCR assay.

We hypothesise that treatment of dogs with AHDS with placebo and standard medical care will be non-inferior to treatment with metronidazole and standard medical care as determined by time to resolution of diarrhoea (days).

4.6. Material and methods

4.6.1. Design and setting

This study is a prospective, randomised, blinded, placebo-controlled, single-centre, non-inferiority phase II clinical trial in dogs treated by the Emergency and Critical Care (ECC) service of The Animal Hospital at Murdoch University. Enrolled dogs will be randomised to receive either Metronidazole 10mg/kg *or* 0.9% sodium chloride IV/PO q12 hours for five days (ten total doses). Ethics approval was obtained from Murdoch University (R2946/17) prior to study enrolment.

4.6.2. Participants

Dogs presenting to the ECC service for haemorrhagic diarrhoea of less than or equal to three days duration will be eligible for inclusion. Informed owner consent will be required

for enrolment. We expect some dogs will have an underlying condition known to cause haemorrhagic diarrhoea that will be missed during clinical investigation.

4.6.3. Inclusion and exclusion criteria

4.6.3.1. Inclusion criteria

All dogs presenting for acute haemorrhagic diarrhoea of less than or equal to three days duration that are deemed to require hospitalisation by the attending clinician are eligible for inclusion.

4.6.3.2. Exclusion criteria

1. Age less than 18 weeks
2. Bodyweight 2.9kg or less
3. Administration of any steroid or non-steroidal anti-inflammatory medications
4. Known severe kidney *OR* liver disease
 - a. includes severe kidney or liver disease diagnosed during initial investigation for current illness event
5. Positive in-house parvoviral antigen test (if performed)
6. Known IBD or gastrointestinal neoplasia
7. Known hypoadrenocorticism
8. Primary or secondary coagulopathy
9. Known or suspected GI foreign body
10. Administration of metronidazole in the last seven days

4.6.4. Study procedures

See appendix 1 for study flow chart.

4.6.4.1. Enrolment and study treatment administration

Once the attending clinician has identified the eligible dog, the attending clinician will obtain opt-in written or verbal client consent. Written consent will be obtained at a later time for verbal consent obtained over the phone. A faecal sample will be collected by the attending clinician or nurse prior to administration of study drug.

Study treatment will be randomly allocated via a computer-generated schedule, and attending nurses, clinicians, researchers, and the owners will be blinded to treatment allocation. The in-hospital study treatment will be covered by an opaque bag, and various-sized unlabelled capsules will be prescribed for continued treatment at home after discharge. Setup or prescribing of treatment will be performed by a hospital staff member not directly involved in the care of the patient.

In hospital, either metronidazole or 0.9% sodium chloride will be administered according to the randomisation schedule at 2mL/kg IV every 12 hours after the initial faecal sample has been collected. This equates to 10mg/kg metronidazole or 2mL/kg 0.9% sodium chloride IV every 12 hours. All other treatments and all diagnostics will be at the attending clinician's discretion. At the time of discharge, the study treatment will be prescribed at the same dosage to be given per os, to complete a total five day course of study treatment.

4.6.4.2. Sample and data collection

Baseline data collected will include signalment, body weight, diet history, vaccination and deworming history, and gastrointestinal clinical signs.

All stools passed in hospital will be photographed to scale with a timestamp. An AHDS activity index (Appendix 2) will be completed at admission, then once daily until and including day six.

Part of the faecal sample will be submitted to a commercial laboratory (VetPath Laboratories) for faecal microscopy and flotation, as well as a multiplex real-time faecal PCR. The remainder of the sample will be stored at -80°C for further evaluation at a later time.

Faecal scoring (Appendix 2) will also be used to monitor clinical progress, in particular resolution of diarrhoea. This index has been previously used in studies investigating AHDS in dogs² and was based on a similar index used in the investigation of canine inflammatory bowel disease¹⁵ and has been adapted in other clinical trials investigating AHDS in dogs.^{8,16} Enrolled dogs will be scored at admission, then every 24 hours. The last scoring will occur the day after the last dose of study medication, for a total of six days. Timing of the scoring once the patient is discharged may be adjusted to a suitable time following discussion with the owner, to maximise owner compliance.

Follow up communication will be done as needed to ensure compliance with scoring of daily AHDS activity index, and taking time stamped photos of all stools passed at home. These will be forwarded to the primary investigator.

All data will be entered into a secure, web-based data capture application.

4.6.5. Statistical plan

4.6.5.1. Sample size calculation

An anticipated mean of three days to resolution of diarrhoea with a standard deviation of one day is consistent with data presented in prior clinical trials,^{2,10} as well as our clinical experience. Non-inferiority margin of one day was determined to be a clinically relevant improvement if associated with a treatment intervention, in our case metronidazole use. This would be associated with reduced morbidity, shorter time in hospital, and considerable financial benefit to owners. These numbers together with an α of 0.05 and

power of 0.9 were used to calculate sample sizes using a standard non-inferiority sample size calculator (<http://www.hwasoon.kim/NISSC/#!/continuous>). This resulted in 21 dogs in each group. This was increased by 50 percent to a total of 30 dogs in each group, to allow for dogs lost to follow up and dogs that drop out of the study.

4.6.5.2. Randomisation procedure

Randomisation will be generated using an online tool available at <http://www.randomizer.org>. Randomisation will occur in blocks of ten to ensure even distribution over time.

4.6.5.3. Statistical analysis plan

All personnel involved in the study, or treatment of enrolled dogs will be blinded to treatment allocation. Baseline patient and laboratory data will be presented as median (range) or mean (95% confidence intervals) for descriptive purposes. We will report the faecal PCR results for all dogs enrolled in the study.

A subset of stool photographs will be evaluated by blinded clinicians to validate faecal consistency scores using Kappa statistics. The distribution of AHDS scores over time will be compared between treatment groups using Friedman's test for repeated measures. Time to resolution of diarrhoea will be graphically represented by Kaplan-Meier plots for visual comparison.

The distribution of time to resolution of diarrhoea (days until normal stool) will be compared between groups using non-inferiority analysis, with a one-day non-inferiority margin as described above. A standard deviation that falls within the non-inferiority margin of one day will demonstrate standard supportive care is non-inferior to the addition of metronidazole in the treatment of AHDS in dogs. Significance will be set at $P < 0.05$. All data will be analysed using RStudio and a commercial software package.

Data will be analysed on an intention-to-treat basis. Dogs will be censored if lost to follow up, if owner’s elect to discontinue involvement in the study, or if the dog dies.

4.7. Co-enrolment in other studies

Co-enrolment in other studies will be made on a study-by-study basis by the investigators.

4.8. Feasibility

The study will be conducted in The Animal Hospital at Murdoch University, a university teaching and tertiary referral hospital that saw more than 9,000 emergency and critical care cases in 2016. We estimate that two to three dogs with acute haemorrhagic diarrhoea are seen per week. A conservative estimation for enrolment of two dogs per week is five to six months. Excluded dogs will be recorded together with reason for non-recruitment.

4.9. Timeline

	J	J	F	M	A	M	J	J	A	S	C	N	D	J	F	M	A	M	J	J	A	S
	a	a	e	a	p	a	u	u	e	t	t	v	c	a	e	a	p	a	u	u	u	e
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	9	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
Enrol patients	█	█	█	█	█	█	█	█	█	█	█	█	█	█								
Analyse data														█	█							
Write draft paper and revisions														█	█	█	█	█	█	█	█	█
Submit paper																					█	█
Present at IVECCS 2021																						█

4.10. Current progress

We have currently enrolled 38/60 dogs. Follow up data, including faecal scoring and photos, are missing for 9/38 dogs.

4.11. Dissemination of results

We plan to present the results as an oral abstract presentation at the 27th International Veterinary Emergency and Critical Care Symposium in Nashville, Tennessee USA. We plan to submit the manuscript to an appropriate veterinary journal.

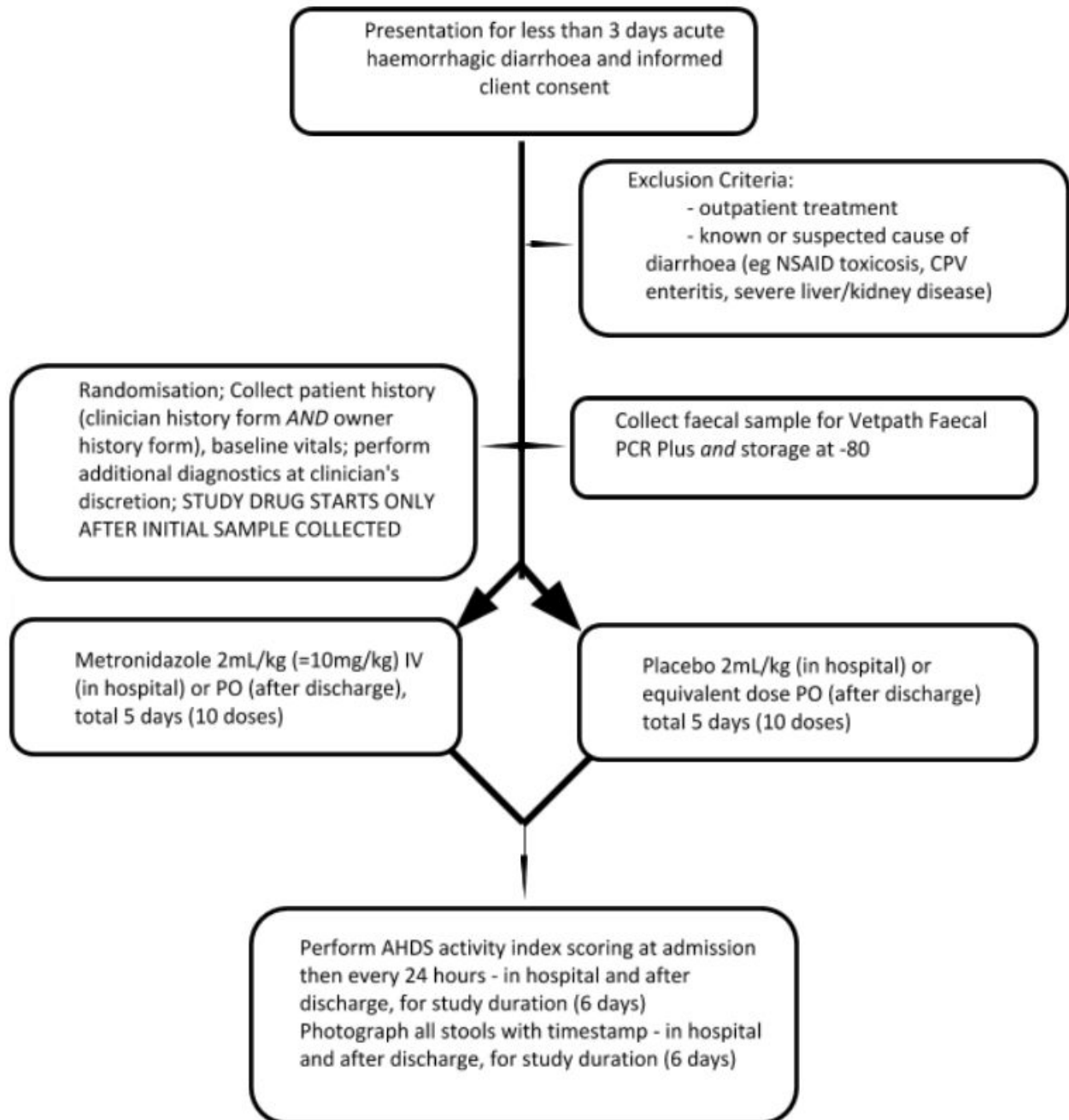
4.12. Bibliography for Chapter 5

1. Mortier F, Strohmeyer K, Hartmann K et al. Acute haemorrhagic diarrhoea syndrome in dogs: 108 cases. *Vet Rec* 2015;176:627. <http://dx.doi.org/10.1136/vr.103090>.
2. Unterer S, Strohmeyer K, Kruse BD et al. Treatment of Aseptic Dogs with Hemorrhagic Gastroenteritis with Amoxicillin/Clavulanic Acid: A Prospective Blinded Study. *J Vet Intern Med* 2011;25:973–979. <http://dx.doi.org/10.1111/j.1939-1676.2011.00765.x>.
3. Marks SL, Rankin SC, Byrne BA et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med* 2011;25:1195–1208. <http://dx.doi.org/10.1111/j.1939-1676.2011.00821.x>.
4. Unterer S, Busch K, Leipig M et al. Endoscopically visualized lesions, histologic findings, and bacterial invasion in the gastrointestinal mucosa of dogs with acute hemorrhagic diarrhea syndrome. *J Vet Intern Med* 2014;28:52–58. <http://dx.doi.org/10.1111/jvim.12236>.
5. Suchodolski JS, Markel ME, Garcia-Mazcorro JF et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* 2012;7:e51907. <http://dx.doi.org/10.1371/journal.pone.0051907>.
6. Shakir L, Javeed A, Ashraf M et al. Metronidazole and the immune system. *Pharmazie* 2011;66:393–398. <http://dx.doi.org/10.1691/ph.2011.0790>.
7. Miyachi Y. Potential antioxidant mechanism of action for metronidazole: implications for rosacea management. *Adv Ther* 2001;18:237–243. <https://www.ncbi.nlm.nih.gov/pubmed/11841193>.

8. Busch K, Suchodolski JS, Kühner KA et al. Clostridium perfringens enterotoxin and Clostridium difficile toxin A/B do not play a role in acute haemorrhagic diarrhoea syndrome in dogs. *Vet Rec* 2015;176:253. <http://dx.doi.org/10.1136/vr.102738>.
9. Sindern N, Suchodolski JS, Leutenegger CM et al. Prevalence of Clostridium perfringens netE and netF toxin genes in the feces of dogs with acute hemorrhagic diarrhea syndrome. *J Vet Intern Med* 2018; <http://dx.doi.org/10.1111/jvim.15361>.
10. Ortiz V, Klein L, Channell S et al. Evaluating the effect of metronidazole plus amoxicillin-clavulanate versus amoxicillin-clavulanate alone in canine haemorrhagic diarrhoea: a randomised controlled trial in primary care practice. *J Small Anim Pract* 2018;59:398–403. <http://dx.doi.org/10.1111/jsap.12862>.
11. Plumb DC. *Plumb's Veterinary Drug Handbook*. CreateSpace Independent Publishing Platform, 2017. <https://market.android.com/details?id=book-m2hGswEACAAJ>.
12. Tauro A, Beltran E, Cherubini GB et al. Metronidazole-induced neurotoxicity in 26 dogs. *Australian Veterinary Journal* 2018:495–501. <http://dx.doi.org/10.1111/avj.12772>.
13. Evans J, Levesque D, Knowles K et al. Diazepam as a treatment for metronidazole toxicosis in dogs: a retrospective study of 21 cases. *J Vet Intern Med* 2003;17:304–310. <https://www.ncbi.nlm.nih.gov/pubmed/12774970>.
14. Igarashi H, Maeda S, Ohno K et al. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. *PLoS One* 2014;9:e107909. <http://dx.doi.org/10.1371/journal.pone.0107909>.
15. Jergens AE, Schreiner CA, Frank DE et al. A scoring index for disease activity in canine inflammatory bowel disease. *J Vet Intern Med* 2003;17:291–297. <https://www.ncbi.nlm.nih.gov/pubmed/12774968>.
16. Ziese A-L, Suchodolski JS, Hartmann K et al. Effect of probiotic treatment on the clinical course, intestinal microbiome, and toxigenic Clostridium perfringens in dogs with acute hemorrhagic diarrhea. *PLoS One* 2018;13:e0204691. <http://dx.doi.org/10.1371/journal.pone.0204691>.

4.13. Appendices

4.13.1. Appendix 1: Study enrolment and protocol flow chart



4.13.2. Appendix 2: Canine AHDS activity index

	Score			
	Normal	Mild	Moderate	Severe
Vomiting	0: No	1: 1/day	2: 2-3/day	3: >3/day
Stool consistency	0: Normal	1: slightly soft	2: very soft	3: watery diarrhoea
Stool frequency	0: Normal	1: 2-3x/day	2: 4-5x/day	3: >5x/day
Appetite	0: Normal	1: mildly reduced	2: markedly reduced	3: not eating

5. Chapter 6: Conclusion

5.1. Key findings

In order to expand upon our knowledge of faecal PCR (fPCR) panel results in Western Australian dogs, we first described fPCR results and clinical findings in a group of 168 dogs seen at a university teaching hospital for diarrhoea. Acute diarrhoea was the most common reason for veterinarians at our hospital to submit a faecal PCR, accounting for 68.5% of cases. The majority of dogs (56%) received antimicrobials. Our second study reported the fPCR results of a larger cohort of 2025 dogs. Of these, nearly half (46%) the dogs had more than one enteric microorganism detected, with *Clostridium perfringens* alpha toxin gene and *Campylobacter* spp. the most common co-detection. No variation in seasonal prevalence was found.

In both studies, faecal PCR assays detected *Clostridium perfringens* alpha toxin gene in a large majority of dogs. Furthermore a large percentage of dogs had *Campylobacter* spp. detected. Canine parvovirus was also detected frequently in adult dogs, many of whom had a complete vaccination history. Twenty-nine percent of dogs that had a negative parvovirus antigen test had parvovirus detected via a contemporaneous PCR assay. Lastly, veterinarians frequently changed antimicrobial treatment decisions (for 83/168 (49%) dogs, antibiotics were stopped, started, or changed) upon receiving PCR results. For the second state-wide study, minor seasonal variations were noted, but there were insufficient data to evaluate regional variation patterns. Nearly half the faecal samples had multiple organisms detected (45.6%), with the majority of these (32.8%) having two organisms co-detected. *Clostridium perfringens* alpha toxin gene and *Campylobacter* spp. were the most common co-detection.

5.2. Contribution to the literature

We report the first state-wide canine enteric organism profile in Western Australia, in addition to reporting the first clinical dataset of dogs with diarrhoea that had a faecal PCR assay performed. These findings are an important first step to improve our understanding of the clinical relevance of faecal PCR results - our high rates of *C. perfringens* and *Campylobacter* spp. detection questions the clinical relevance of these microorganisms in dogs with diarrhoea.

Our first study also reported a much higher proportion of canine parvovirus detection in adult dogs, many of whom were vaccinated, than other parvovirus studies. It is unclear if these cases represented true parvovirus or incidental findings in dogs with non-parvovirus gastrointestinal disease. Due to the time lapsed between last vaccination and faecal PCR submission, vaccine interference was considered unlikely in those with appropriate vaccination data.

Our second study exposed the challenges of establishing seasonal and geographical prevalence patterns, even with a relatively large dataset. Also, similar to previous publications, co-detection of two or more microorganisms was common. However the study design did not allow for comparison of co-detection findings to clinical disease. Regardless, this evidence either suggests a pathogenic role of co-detected microorganisms or reflects dysbiosis.

Both studies question the utility of this multiplex faecal PCR assay in its current form as a diagnostic tool for acute diarrhoea in dogs. Lack of species level information, and high prevalence of many of the included organisms in non-diarrhoeic samples, and long turnaround times may be associated with inappropriate antimicrobial use.

5.3. Study limitations

The limitations of these studies mostly relate to the retrospective design. For the first study, the decision to perform a faecal PCR assay was unlikely random, and instead factors including client finances, medical history, veterinarian preference, and results of other diagnostics resulted in non-random patient selection. For example, in an incompletely vaccinated puppy with moderate to severe clinical signs, a positive point-of-care parvovirus antigen test would likely preclude the need for further testing, whereas a negative antigen test would warrant confirmation with a more sensitive test. For this scenario, ideally all such cases and comparable control dogs would be enrolled and tested prospectively, allowing for both evaluation of PCR sensitivity in addition to providing better data evaluating the possibility of subclinical carriers. This patient selection bias equally applies to the data from the second study, which is confounded further by the lack of any clinical data. Another fundamental limitation of the second study was that no clinical information was available regarding the included dogs. We thus limited our study to observational reporting of fPCR results.

The inherent selection bias of retrospective datasets also restricts assessment of any geographical or seasonal variation patterns. Even if a grossly obvious spike in a single microorganism was detected in one region (or one time period), numerous scenarios - such as a veterinarian who strongly favours faecal PCR assays compared to colleagues in the region, or if most of the positive tests were from a single breeder in a closed population of dogs - blunt or completely nullify any meaningful interpretation.

However, regardless of these above limitations, we developed concerns regarding the relevance of genus-level testing for some microorganisms. The current literature clearly identifies differences in prevalence and pathogenicity between different species of *Campylobacter*, *Giardia*, *Salmonella* serotypes, and clostridial enterotoxins. It is clear

that more targeted fPCR assays would greatly benefit both frontline veterinarians treating dogs with acute diarrhoea, but also researchers investigating the pathogenic role of these microorganisms.

5.4. Future directions

Reference studies to establish the enteric organism profile of healthy, non-diarrhoeic dogs in various regions will help elucidate the role these organisms play in acute diarrhoea or other gastrointestinal diseases. Enrolment for such a study is ongoing at our institution. This should provide some context for whether the prevalence of the microorganisms we reported needs further exploring. The antimicrobial use described in the first study highlights the need for further prospective and microbiologic studies to evaluate if these enteric microorganisms are primary pathogens. This may provide evidence to aid treatment decisions, in particular with regards to antimicrobials. Due to the challenges of performing a suitably designed study, prospective clinical trials looking at the efficacy of antimicrobials in dog populations with acute diarrhoea (or other gastrointestinal disease) could provide high-quality evidence supporting or undermining antimicrobials use. The study proposal in chapter 5 aims to do this.

Based on our research, we propose the following panel for further research as the ideal diagnostic tool to investigate diarrhoea in dogs:

- faecal microscopy for ova, cysts, parasites
- ELISA for *C. perfringens* enterotoxin
- Faecal culture add-on option if high index of suspicion for *Salmonella* spp.

- Real time fully-quantitative PCR for *C. perfringens* enterotoxin, *Campylobacter upsaliensis* and *jejuni*, and CPV. Expanded panels should be available for CCV and *Cryptosporidium canis* and *parvum*.

Lastly, we suggest more research exploring a CPV ‘carrier’ population would greatly enhance our epidemiologic understanding of this disease. This would require screening tests of both healthy dogs and dogs with gastrointestinal disease, but also more advanced molecular diagnostics to genotype any parvovirus strains detected.

5.5. Conclusion

As faecal PCR assays become part of the core diagnostics used to investigate canine acute diarrhoea, further research and data will be essential to ensure they positively contribute to patient outcomes. It is clear in many scenarios that they can perform extremely well and in general by nature of their design, they can be very sensitive and specific. However, it is equally important we recognise the limitations of faecal PCR assays, which requires a solid understanding of the processes involved (such as the importance of validation procedures). Additionally, it is essential clinicians approach PCR results with caution and incorporate the entire clinical picture before formulating treatment plans involving antimicrobials. It is clear that *C. perfringens* alpha toxin gene, as detected by the faecal PCR assay available to us, has minimal clinical relevance. For the other organisms, species level testing could be much more informative. Further studies that establish enteric microorganism profiles of various subpopulations of dogs with and without acute diarrhoea, as well as studies that elucidate species-level data on detected microorganisms will greatly enhance our ability to interpret faecal PCR results.