COMPARATIVE ANALYSIS OF THE FAECAL VIROME OF DOGS WITH VARIOUS INFLAMMATORY INTESTINAL DISEASES

Paloma Susana Moreno Méndez

https://orcid.org/0000-0002-3500-6846

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Faculty of Veterinary and Agricultural Sciences, The University of Melbourne

ABSTRACT

Little is known about the population of viruses present in the intestine of dogs, the virome, despite advances in molecular biology techniques. The viral flora in the intestine of dogs has been mostly studied in disease settings, targeted to known pathogenic viruses. There is currently a gap in our knowledge of the faecal canine virome present in healthy conditions. This study aimed to identify and characterise the virome present in faeces of healthy dogs compared to the virome present in dogs with acute diarrhoea and dogs with chronic enteropathy. Faecal samples were collected from 8 healthy dogs, 8 dogs with acute diarrhoea and 8 dogs with chronic enteropathy. A viral enrichment protocol, using a mixture of endonucleases and bacterial filtration was used to enrich viral DNA and RNA. The total DNA and RNA isolated from the stool samples were amplified using a sequence-independent single-primer amplification (SISPA) protocol and subsequently sequenced by next generation sequencing using the Illumina MiSeq platform at the Australian Genome Research Facility. Two bioinformatic pipelines were used to analyse the viral population present in each sample. After selecting high quality reads (HQRs) and removing dog and bacterial sequences, sequence information was compared against CAMERA viral reference database in one pipeline and against the NCBI non-redundant nucleotide database in the other. Faecal samples from all groups showed a large occurrence of bacteriophages, from different families mainly under the order *Caudovirales*. Eukaryotic viruses were also identified. Consistent results, obtained from both bioinformatic analysis pipelines, were found for only 8 viral eukaryotic families. Interestingly, sequences with high similarity to viruses within the *Astroviridae* and *Caliciviridae* families were identified only in samples from the dogs with acute diarrhoea. Sequences similar to those of *Picornaviridae* were identified in one dog with acute diarrhoea and in one dog with chronic enteropathy. Only one group of sequences similar to a known virus family with known pathogenic members, *Reoviridae*, was identified in all groups (healthy and diarrhoeic samples). In conclusion, the largest proportion of viruses identified belonged to bacteriophages. The eukaryotic virome detected in this study

contained viral sequences from a range of different virus families, including some with known enteric pathogens. These results were complemented with the complete genome characterisation of a canine kobuvirus, a canine astrovirus and a canine rotavirus. Furthermore, a prevalence analysis of these viruses, in a wider population, was performed. This project gives the first description of the canine faecal virome of healthy dogs and dogs with chronic enteropathy, thus contributing new knowledge that complements what was already known about the faecal virome of dogs with acute diarrhoea. Results from this study indicate that metagenomic analyses are useful for the investigation of viral populations in the faeces of dogs with various inflammatory intestinal diseases. Studies to further elucidate the epidemiological and biological relevance of these findings are warranted.

DECLARATION

I, Paloma Moreno declare that:

(i) this thesis comprises only my original work toward the PhD except where indicated in the Preface,
(ii) due acknowledgement has been made in the text to all other material used,
(iii) the thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies, and appendices

Paloma Susana Moreno

PREFACE

Two chapters of this thesis consist of original published works by Paloma Susana Moreno as the primary researcher and author, as follows:

Contributions for Chapter 2: Unpublished material not submitted for publication

Optimisation and validation of sample preparation and viral nucleic acids enrichment protocol in canine faeces for viral metagenomics

The work described in chapter 2 was performed at the Murdoch Children's Research Institute (MCRI), The Royal Children's Hospital, Melbourne. Paloma Susana Moreno contributed to 80% of the work. Experiments were performed in collaboration and under the supervision of Josef Wagner and Carl Kirkwood. Caroline Mansfield, Carl Kirkwood and Josef Wagner contributed to the editing and reviewing of the draft.

List of co-authors for Chapter 3: Chapter with publication

Characterisation of the canine faecal virome in healthy dogs and dogs with acute diarrhoea using shotgun metagenomics.

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The work described in chapter 3 was performed at the Murdoch Children's Research Institute (MCRI), The Royal Children's Hospital in Melbourne. Paloma Susana Moreno contributed to 80% of the work. Carl Kirkwood and Caroline Mansfield contributed with the conceptualization, supervision, visualization and review and editing of the draft. Josef Wagner contributed with the conceptualization, formal analysis, investigation, methodology, supervision, visualization, review and editing of the draft. Matthew Stevens contributed with the formal analysis and

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List of co-authors for Chapter 4: Chapter with publication

Characterization of the fecal virome in dogs with chronic enteropathy.

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The work described in chapter 4 was performed at the Murdoch Children's Research Institute (MCRI), The Royal Children's Hospital. Paloma Susana Moreno contributed to 75% of the work. Caroline Mansfield and Carl Kirkwood contributed with the project administration, conceptualization, supervision, visualization and review and editing of the draft. Josef Wagner contributed with the conceptualization, formal analysis, investigation, methodology, supervision, visualization, review and editing of the draft. James Gilkerson contributed with supervision and review and editing of the draft.

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Canine Rotavirus characterisation and prevalence

The work described in chapter 5 was performed at the Murdoch Children's Research Institute (MCRI), The Royal Children's Hospital. Paloma Susana Moreno contributed to 80% of the work. Laboratory work was performed under the supervision of Carl Kirkwood. Celeste Donato and Carl Kirkwood contributed with the formal analysis, methodology and editing and reviewing of the draft. Caroline Mansfield and Josef Wagner contributed to the editing and reviewing of the draft.

Presentations arising from work undertaken as part of this thesis

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1.1 Introduction

Research on enteropathies in dogs has been an ongoing task in veterinary medicine, mainly because of their high incidence and the requirement for accurate diagnosis and subsequent treatment. This literature review presents a brief analysis of our current understanding of enteropathies in dogs and a summary of what is known about the canine gut microbiota. Notably, most of the research on canine enteric viruses has been targeted to known enteric pathogens. To date, many enteropathies remain idiopathic in aetiology, possibly due to the lack of diagnostic tools to accurately determine their cause. In recent years, the advancements in viral identification technologies have facilitated the identification and discovery of new viruses, allowing a more complete understanding of the viruses that inhabit the canine gut, whilst also contributing to the development of diagnostic tools to determine the causes of enteric diseases in dogs.

1.2 Diarrhoea in dogs: the clinical presentations

Diarrhoea is defined as an increase in frequency, fluidity or volume of faeces (1). In an animal with diarrhoea due to small intestinal disease, concurrent clinical signs such as vomiting, abdominal pain, melena, and weight loss may be present(2, 3). Clinically, the causes of diarrhoea differ whether the onset is acute or chronic in nature. Diarrhoea is classified as acute when its duration is less than 14 days, and chronic when has been present for more than 3 weeks (4). In some situations, diarrhoea may be intermittent in nature. This is because the large intestine, if healthy, retains capacity to reabsorb excess water.

1.2.1.1 Pathophysiology

There are four pathophysiologic mechanisms that can produce small intestinal diarrhoea: osmotic, secretory, altered mucosal permeability and deranged motility (3, 5).

Osmotic diarrhoea is caused by an excess of osmotically active solutes, which can attract fluids into the intestinal lumen, thus increasing faecal fluidity or volume (3). Secretory diarrhoea is caused by abnormal ion transport in intestinal epithelial cells (3) that leads to an increase in intestinal fluid secretion or a decrease in intestinal absorption (5). Altered mucosal permeability is normally caused by macro and/or microscopic damage to either the epithelial cells or the epithelial junctions, producing loss of liquids, electrolytes, proteins and red blood cells into the intestinal lumen (5). Alteration of mucosal permeability is generally associated with chronic diarrhoea, but many cases of chronic diarrhoea can present initially as acute diarrhoea (3). Deranged motility is also related to chronic diarrhoea because the two major motor abnormalities in intestinal inflammation are suppression of phasic contractions and stimulation of giant migrating contractions (GMCs) (3).

1.2.2 Acute diarrhoea

Several causes for acute diarrhoea have been proposed and can be listed as follows: **dietary indiscretion**, following an abrupt change in diet or overeating; **inflammatory** causes, for example, haemorrhagic gastroenteritis; **infectious** causes, such as parasitic, bacterial, viral, fungal and rickettsial infections; **extraintestinal disorders**, for example, acute pancreatitis; **drugs and toxins**, such as NSAIDs, chemotherapeutic agents, poisons and toxaemia in pyometra or peritonitis, and partial intestinal obstruction.

As previously defined, diarrhoea is considered acute when it lasts less than 14 days (4). Sometimes acute diarrhoea does not systemically affect an animal and can be self-limiting. , These causes of diarrhoea are common in small animal practice and generally do not require

extensive diagnostic tests or therapy (3). However, sometimes acute diarrhoea causes systemic compromise and animals therefore require medical treatment, sometime with urgency. Signalment and history of the animal are vital to identify predisposing factors or aetiologies. (3, 4). Treatment of acute small intestinal diarrhoea is based on restoration and maintenance of fluids and electrolyte balance, along with nutritional support and specific interventions when indicated (3). Antibiotics are recommended only when there is evidence of intestinal compromise or specific pathogenic bacteria are isolated (3).

1.2.3 Chronic enteropathy

Chronic enteropathy (CE) in dogs has previously been referred to as inflammatory bowel disease (IBD), due to a similar presentation and chronicity to that observed in people with Crohn's disease or ulcerative colitis (4). Inflammatory bowel disease in dogs has been considered as a group of idiopathic and chronic gastrointestinal disorders involving mucosal inflammation, and characterised by persistent, more than 3 weeks, and recurrent gastrointestinal (GI) signs (2, 4, 6). This disease has been diagnosed after exclusion of any other intestinal disease and ruling out extra intestinal, infectious or parasitic diseases (2, 4). Inflammatory bowel disease can involve different parts of the GI tract and has been documented as one of the most common histopathologic diagnosis in cases of chronic vomiting and diarrhoea in dogs and cats (2, 7). Despite advances over the past three decades in our understanding of IBD, the aetiology remains poorly understood in many cases (2, 8). It has been proposed that dysbiosis, or changes in bacterial populations, altered immunological responses, environmental factors and genetic predispositions may all have an influence on the pathogenesis of IBD in people (6, 9, 10) . Several studies have shown significant differences between the microbiota of human or canine IBD patients and that of healthy control individuals; consequently, dysbiosis has been recognised as an important clinical feature of human and canine IBD (11-16). It has also been proposed that altered immunological responses in the gut could be either based on the failure to recognise

pathogenic bacteria, or alternatively, the erroneous recognition of commensal flora as pathogenic (17-19).

Inflammatory bowel disease in dogs historically was classified based on the most predominant inflammatory cell type infiltrated in the intestinal mucosa. This disease can affect either the colon, small intestine or diffusely. The histologic classifications of IBD comprised: lymphocytic - plasmocytic enteritis (LPE), eosinophilic enteritis (EE), granulomatous, regional and neutrophilic enteritis or a combination of these (20, 21). Endoscopic examination and histologic evaluation of the intestine was essential to rule out other causes of pathology such as neoplasia or lymphangiectasia. Although ideally histological examination of the intestine should definitely rule in a diagnosis of IBD, unfortunately biopsy interpretation is very subjective and varies depending on the observer and the techniques utilised in the assessment (2, 6, 7). This last observation has raised questions about the value of histopathology in the assessment of dogs with IBD (22) .

A clinical scoring system, the canine IBD activity index (CIBDAI, (23)) has been used to evaluate severity; it is based on six variables semi-objectively scored in affected dogs, namely attitude/activity, appetite, vomiting, stool consistency, stool frequency and weight loss. More recently, the canine chronic enteropathy activity index (CCECAI), has been proposed as an improved scoring system, where low serum albumin concentrations, assessment of ascites, peripheral oedema and pruritus were also evaluated (22). This last index showed strong associations between hypoalbuminemia (<20 g/L) or low concentrations of cobalamin in serum (<200ng/L) and negative clinical outcomes, defined as euthanasia due to refractoriness to treatment (22).

Clinically, the term IBD has been replaced by the term chronic enteropathy (CE) to distinguish the exclusive features of the canine disorder from that of the condition that affects humans. This new classification is based on the response to treatments and has been divided into three

different categories: food responsive enteropathy (FRE), antibiotic responsive enteropathy (ARE) and immunosuppressive responsive enteropathy (IRE) (22). Therapeutic trials are indicated, beginning with changes in the diet, followed by modification of the microbiota by the administration of antibiotics, if dietary changes do not result in clinical improvement, and lastly, immunosuppressants if the last treatment did not result in significant clinical improvement. This classification system supports the use of the term CE, rather than IBD, because not all dogs with chronic enteric clinical signs require immunosuppressant treatment (24).

In a study evaluating cases of FRE and SRE, there were no differences in the endoscopy scores between dogs affected by either type of CE. Also, no correlation between values of the old scoring index (CIBDAI) and endoscopy scores were observed (22). This shows that there is no correlation between endoscopy and clinical signs; hence, this further supports the use of the term CE, rather than IBD, to refer to this group of pathologies. Histopathological evaluation has been useful to eliminate other less common causes of enteritis, such as neoplasia or histiocytic ulcerative colitis (22).

1.2.3.1 Food responsive enteropathy (FRE)

Food responsive enteropathy is the most common CE diagnose in dogs (22, 25, 26). Dogs affected by this form of CE typically present with less severe clinical signs compared to other forms of CE (25), but this is not always the case (22). Dogs with CE, who respond to a gradual change of diet within 10 days to 2 weeks of starting the diet challenge, by decreasing or eradicating all GI signs, are classified as dogs with food responsive enteropathy (18, 22, 25). Dietary trials are indicated as the first diagnostic/therapeutic step in dogs with CE; this involves the gradual incorporation of diets with either one of two protein options, a novel protein or hydrolysed proteins (containing small peptides aimed at reducing antigenic reaction) (18, 27, 28). An ideal elimination diet includes a single or limited source of proteins that are novel for the patient, restricted fat, high digestibility and a reasonable content of fermentable fibre (29).

A dietary provocation test may be performed after some weeks, to reintroduce the old diet to evaluate if the GI signs reappear. It is suggested that the dietary regimen should be maintained for at least 12 weeks (22, 25).

The aim of including a hydrolysed protein in the diet is to avoid mast cell degranulation, thus reducing the allergenic reaction that occurs in response to intact protein (29). Several studies have not found any significant differences between the use of a novel antigen (new protein) diet and a hydrolysed protein diet, therefore, the benefit of using one over the other remains unclear (2). In general, the outcome of FRE in dogs is very good after a year, with response within 2 weeks of a strict diet (elimination diet or hydrolysed) (25).

A study in dogs comparing a highly digestible diet or elimination diet with a hydrolysed protein diet (28), found that the hydrolysed protein diet resulted in decreased clinical signs over a longer period of time. The hydrolysed protein diet had no comparative advantage over the other types of diet in the first clinical re-evaluation after treatment (median 90 days), but did have better outcome in the second (median 232 days), and third re-evaluation (median 1284 days), having a significant difference in relapse, and maintaining remission for longer time (28).

In a study evaluating risk factors associated with negative clinical outcomes, 70 dogs showing signs of CE were included and were classified as having upper gastrointestinal disease or lower gastrointestinal disease (22). All dogs were given a CIBDAI score and were treated initially with an elimination diet for 10 days. All dogs who responded to the elimination diet after these 10 days were classified as having FRE. The remaining dogs were treated with steroids. In the FRE group, 79% of dogs did not have recurrence of clinical signs after switching back to the original diet for up to 3 years of follow up (22). In these 70 dogs, the histologic score was not associated with the outcome, reaffirming the subjectivity of histopathological analyses and being consistent with results from other study (26, 30)

1.2.3.2 Antibiotic responsive enteropathy (ARE)

If after a change in the diet, gastrointestinal signs do not improve, modification of the GI microbiome is advised, usually with treatment by antibiotics. Diagnosis of ARE, are based in a positive response to antibiotic treatment trial through resolution of clinical signs, relapse of signs with withdrawal of treatment, reduction of signs with reintroduction of treatment and eradication of other etiologic agents (31). In one study a good response was obtained with antibiotic tylosin (32). Because of this, that particular entity has also been named tylosin responsive diarrhoea, but is likely to be a form of ARE (20). The most common antibiotics used for the treatment of ARE include oxytetracycline, tylosin and metronidazole (20, 31). Unfortunately, has been shown that antibiotic therapy has limited success and some dogs need ongoing treatment to avoid relapse (25, 33). Similarly, studies analysing pre and probiotics therapy have not shown systematic positive results (34, 35). Due to these difficulties and the possibility of resistance to antibiotics as a result of long-term therapy, it is required other methods to change the microbiota. Faecal transplantation is an option that has been tested in humans to treat *Clostridium difficile* (36). Regrettably, there are no published studies evaluating dogs with CE. Consequently, investigations evaluating faecal transplantation, especially in dogs with ARE, are warranted.

The role of the antibiotic is not clear, but possible effects that could explain their mechanisms of action are by producing changes in the intestinal microbiota, and consequently selection pressure and immunomodulatory effects (20, 31). The efficacy of antibiotics is unclear, except in Boxer and French bulldogs with granulomatous ulcerative colitis, where *E. coli* is clearly the cause of inflammation (37).

1.2.3.3 Immunosuppressive responsive enteropathy (IRE)

Dogs that do not respond to dietary changes or antibiotic therapy are then usually treated with steroid or immunosuppressant therapy, thus classified as having an immunosuppressive

responsive enteropathy (IRE). Hypoalbuminemia, with concentrations less than 20g/L, has been associated to negative outcome and refractoriness (22, 26), this group of dogs has been classified as having protein losing enteropathy (PLE).

Different corticosteroids have been used to treat CE in dogs, but usually prednisolone is the first line treatment(22, 25, 30, 38-40). Some studies have compared the efficacy of treatments using different steroids, but no significant differences were found (38, 40). One of such studies compared the efficacy of oral prednisone and a combination of oral prednisone and metronidazole, both treatments had a good remission rate (80%), with no significant differences between treatments observed (38). Dye *et al* (40) compared budesonide PO to prednisone PO, and both treatments decreased the CIBDAI score in dogs, without a statistical difference between them. This study had many limitations such as small sample size (34 dogs), a brief clinical evaluation period (6 weeks) and variation in dietary treatment, thus hindering the interpretation of these results. A retrospective study analysing clinical data (26) evaluated different treatments in 80 dogs with IBD. This study found that the most frequent treatments were prednisolone, sulphasalazine and azathioprine, administered at immunosuppressive and non-immunosuppressive doses. In dogs with upper GI disease, dogs that received non-immunosuppressive doses of steroids had better clinical outcomes than those receiving immunosuppressive doses, while no significant associations between treatment and outcome were observed in dogs with lower GI disease. Also, histopathological findings did not improve after treatment. Given the nature of this retrospective study, the variability of drug combinations and different diets also hinders the interpretation of results.

The immunosuppressive drug cyclosporine was evaluated in two studies by Allenspach *et al* (22, 30), in dogs that did not respond to an initial treatment with prednisolone. These dogs were predominantly affected by PLE and treatment was effective in 78% of the cases, measured as a decrease in CIBDAI scores and increased body weight; however, no significant changes were

observed in histopathological findings. Adverse effects, such as vomiting and nausea were apparent at the beginning of the treatment.

1.3 Overview of gastrointestinal microbiota in dogs

Gastrointestinal microbiota is the term used to name all microorganisms living with the host and inhabit the GI tract, including bacteria, viruses, fungi and archaea (41, 42). Coexist pathogenic microbiota and commensal, or normal, GI microbiota that lives in symbiosis with the host (43). Hence, changes in the composition of the GI microbiota produce an imbalance that would be associated with disease (41, 44, 45)

1.3.1 Bacteria

The intestinal microbiota plays a vital role in the conservation of host health. Commensal microbes act as an essential component of the intestinal barrier, protecting the host from pathogens (46). These microbes help in the digestion and gather energy from the diet, offer nutritional support for enterocytes and promote the development of the immune system (47). Gastrointestinal microbes have enzymes that digest complex carbohydrates from the diet that are not digested by the host and ferment endogenous products such as shed epithelial cells and mucus (46). The most important end-products of bacterial fermentation are the short-chain fatty acids (SCFA), particularly butyrate and acetate, which supply energy for the host. In addition, these SCFA have immune-modulatory properties and regulate colonic and intracellular pH. These properties modify the composition of gut microbes by reducing the growth and activity of potential pathogens (46). Each anatomical segment within the intestine has a characteristic ecosystem where microorganisms have specialised functions, using the host nutrients and returning metabolites for the host (48). Although the upper GI contains a large number of microbes, the colon has the most abundant microbial population, being mostly anaerobes. And it is here where the fermentation, the anaerobic digestion process, occurs (46).

1.3.1.1 GI bacterial microbiota of healthy dogs

In dogs, each intestinal compartment has a unique population of bacteria. However, this population differs between individuals, with each dog having an individualised microbial profile (48, 49). The differences occur on a species and strain level, with only slight overlap (5%-20%) of bacterial species, between individual animals (47).

Before the development of advanced methods for characterization of bacteria, the most common method used was culture (50-52). However, it is now well established that information retrieved from bacterial cultivation is not sufficient, with respect to diversity. The optimal growth requirements for numerous bacteria is not completely understood, which in turn, decreases the likelihood of identifying all bacteria present in some environments. Bacteria present in the GI tract are mostly anaerobic, which are sensitive and difficult to manage in the laboratory. Also, mutualistic interactions of bacteria with host and other microorganisms are common in the GI tract, making very difficult their growth in culture media (42).

Currently, due to bacteria are not easily cultured and their diversity is highly underestimated, bacterial DNA/RNA sequencing has been a very useful tool (48). Bacterial DNA or RNA is extracted and a specific gene is amplified with universal primers, targeting a conserved region. The most common gene used for the identification of bacteria is 16S ribosomal RNA. Likewise, some fingerprint techniques can be used to differentiate bacterial communities, separating PCR amplicons to generate a fingerprint (48). Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (T-RFLP) are some of these techniques (42, 48). To identify bacterial phylotypes, PCR amplicons require to be sequenced. Construction of 16S rRNA gene clone library is a common method used for this purpose (14, 53, 54). Currently, high-throughput sequencing platforms have been employed increasingly often, which has allowed millions of sequences to be analysed in a short time (15, 55, 56). Afterwards, to quantify bacterial groups, methods such

as quantitative real-time PCR (34), fluorescent in-situ hybridization (FISH) (57, 58) and flow cytometry of fluorescent-labelled probes, are commonly used.

Metagenomic approaches have improved the identification of GI microbiota by sequencing all DNA of a sample, without prior amplification of specific genes, allowing identification additional bacteria communities not identified with previous methods (59).

Almost 99% of all intestinal microbiota in dogs is composed by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes* and *Fusobacteria* phyla. The remaining 1% is characterised by *Tenericutes*, *Verrucomicrobia*, *TM7*, *Cyanobacteria*, *Chloroflexi* and a few unclassified bacterial lineages (48). In the stomach, *Helicobacter spp*. prevails, followed by lactic acid bacteria (*Lactobacillus* and *Streptococcus*) and *Clostridium spp* (48). The upper small intestine is more varied than stomach harbouring approximately 10 different bacterial phyla, predominantly *Clostridia*, *Lactobacillales* and *Protobacteria* (60). *Protobacteria* and *Spirochaetes* are more abundant in the proximal GI tract and normally represent <1% of sequences in the large intestine of healthy animals (48).

Assessment of duodenal biopsies of healthy dogs found that the bacterial population is composed by *Firmicutes* (46.4%), *Proteobacteria* (26.6%), *Bacteroidetes* (11.2%), *Spirochaetes* (10.3%), *Fusobacteria* (3.6%) and *Actinobacteria* (1.0%) (14). Another study by Garcia-Mazcorro et al (61) also found the prevalence of *Firmicutes* to be 46.9%, *Proteobacteria* (21.2%) and *Bacteroidetes* (6.9%) (46). Within the *Firmicutes* phylum the orders *Clostridiales* (19.6%), *Lactobacillales* (14.1%) and *Bacillales* (12%) were the most abundant bacteria in duodenal mucosa.(47, 48). In the jejunum, Suchodolski *et al.* (60) found that the most important phyla in jejunal mucosa were *Proteobacteria* (46.7%), *Firmicutes* (15.0%), *Actinobacteria* (11.2%), *Spirochaetes* (14.2%), *Bacteroidetes* (6.2%), and *Fusobacteria* (5.4%). This study indicated that there are approximately 900 bacterial phylotypes in the healthy canine jejunum (60). Other studies analysing the intestinal content from jejunum showed that *Firmicutes* was the most

abundant phylum, with *Clostridiales* (40%), *Lactobacillales* (10%) and *Enterobacteriales* (30%) being the most numerous bacterial orders (53).

The predominating bacterial orders in the ileal digesta contents from healthy dogs according Suchodolski *et al.* 2008, were *Fusobacteriales* (~30%), *Clostridiales* (25%), *Bacteroidales* (22%) and *Enterobacteriales* (~20%) (53).

Suchodolski *et al.* (53) also found that in the colonic content of healthy dogs the most abundant bacterial orders were *Fusobacteriales* (30%) and *Bacteroidales* (~30%). The following bacterial order in abundance was *Clostridiales* (~18%), being *Clostridium cluster XIV* the most predominant member (~50%) (53).

Unfortunately, all the previously mentioned studies are not comparable because they did not use the same methods and designs. Nevertheless, despite this shortcoming, different studies, with different techniques (55, 57, 62, 63), that analysed bacterial population in faeces of dogs, concluded that *Firmicutes* was the most abundant phylum in faecal samples. *Actinobacteria* and *Fusobacteria* were the second most abundant and finally *Bacteroidetes* and Proteobacteria were the least abundant bacteria in the faeces of dogs (55, 57, 62, 63). However, has been found that the order of those phyla in terms of abundance can vary (48, 59, 64).

1.3.1.2 Factors altering GI bacterial microbiota

Diverse factors can alter GI bacterial microbiota, such as acute diarrhoea, inflammatory diseases of the intestine, motility disorders, intestinal stasis, decreased gastric acid output (use of omeprazole), exocrine pancreatic insufficiency and environmental factors (use of antibiotics and change in diet) (15, 16, 60, 61, 65-67) .

Some studies analysing changes in GI bacterial microbiota in dogs, have found that GI diseases, as acute and chronic diarrhoeas, are associated with an important shift in some bacterial communities (15, 67). This change in composition and richness (the number of unique bacterial

species), called dysbiosis, has been associated with CE/IBD, and others disorders, but it is not clear if these changes are the cause or result from the disease.

1.3.1.3 GI bacterial microbiota in dogs with acute diarrhoea

Changes in the canine GI microbiota has been found in dogs with acute diarrhoea. Bell *et al* (68) using RFLP, described that *Clostridium perfringens*, *Enterococcus faecalis* and *Enterococcus faecium* populations, were more abundant in faeces of dogs with acute diarrhoea than in healthy dogs.

Using DNA sequencing some studies have revealed more accurate results of changes in GI microbiota. One study using pyrosequencing and targeting the cpn60 gene, a gene used for detection, identification and quantification of microorganism, found lower abundance of *Bacteroidetes* in faeces of dogs with diarrhoea of unspecified origin (69). One of the most common method of pyrosequencing is targeting the 16S rRNA gene of bacteria. A study using this method found that faeces of dogs with acute haemorrhagic diarrhoea had the most significant alterations in their microbiota (15). These dogs had substantial decreases in Blautia and Ruminococcaceae and increases in *Clostridium perfringens* and *Sutterella*. Furthermore, phylum Fusobacteria also increased, but not significantly (15).

Another study examining faeces of dogs, found sequences belonging to phylum Bacteroidetes were significantly decreased in dogs with acute diarrhoea, compared to healthy dogs (70). Also, sequences belonging to genus *Faecalibacterium* and no classified genus within *Ruminococcaceae,* were found to be decreased significantly in dogs with acute diarrhoea compared to healthy dogs (70). Conversely, sequences belonging to the genus *Clostridium* were significantly increased in dogs with acute diarrhoea compared to healthy dogs (70).

As the principal bacterial fermentation's end-products are the SCFA which provide energy, the dysbiosis, consequently, have a great influence on the metabolic profile of the host (70). Due to the significance of the dysbiosis and therefore, the changes in the metabolic profile, more studies are warranted; analysing a bigger population to confirm these findings and also studying the possibility of improvement in the treatment.

1.3.1.4 GI bacterial microbiota in dogs with chronic enteropathy

Evaluating duodenal samples, brush or biopsies, several studies have found that dogs with IBD have changes in their microbiota. Increases in *Proteobacteria*, *Streptococcus* and *E. coli* has been found in some studies (14, 54, 66, 71). Additionally, reduction of overall microbial diversity has been described in dogs with IBD, particularly decreases in *Fusobacteria*, *Bacteriodaceae*, *Prevotellaceae*, and *Clostridiales* (14, 56, 71).

Evaluating faecal samples, Minamoto *et al.* found that *Fusobacteria*, *Ruminococcaceae, Blautia,* and *Faecalibacterium* were significantly decreased in dogs with diarrhoea, whereas *Bifidobacterium* spp., *Lactobacillus* spp. and *E. coli* had abundance significantly increased compared to healthy dogs (72). Furthermore, Jia *et.al.* studied faecal samples using FISH and they found an increase in *Bacteroides* (57)

Other studies, analysing faecal samples in dogs with chronic enteropathy, using qPCR and pyrosequencing, showed that dogs with IBD had a significantly decreased abundance of *Faecalibacterium* spp. and *Turicibacter* spp. compared to healthy dogs (15, 34).

The dysbiosis observed in chronic enteropathies does not explain that this is the cause of this condition, likewise, is not clear that the dysbiosis is a consequence of the same. What has been established is that dysbiosis is a factor that intensifies the inflammation (16). Changes in the intestinal microbiota cause functional and immunologic alterations in the host. Therefore, a thorough understanding of the changes in the microbial population is needed to understand the

pathogenesis and offer a better and precise treatment. Probiotics, prebiotics and faecal transplantations are possible treatments that could be targeted if the dysbiosis could be thoroughly identified (41).

1.3.2 Fungi

Resident intestinal fungi have been identified in healthy dogs, as shown in a study where faeces and jejunal luminal contents from healthy dogs were cultured; yeast was isolated, more frequently from the jejunum than in faeces (25% versus 5%) (51).

Another study detected fungal DNA in 60.9% of small intestinal luminal contents from 64 healthy dogs and in 76.1% small intestinal luminal contents from 71 dogs with chronic enteropathy, but this difference was not statistically significant. The sequencing results showed 51 phylotypes, but all sequences belonged to two phyla, *Ascomycota* and *Basidiomycota*. *Saccharomycetes* were the most commonly identified fungal class, and no significant differences in the prevalence of specific fungal phylotypes were observed between healthy and diseased dogs (73).

In one study, analysing the canine intestinal microbiota of healthy dogs exposed to a fibre supplemented diet, pyrosequencing of faecal samples was used. All fungi sequences were found only in case individuals, not in controls and were classified as Dikarya. From these sequences, only three phylotypes were described, *Gibberella zeae* PH-1, *Neurospora crassa* and *Saccharomyces cerevisiae*. In overall fungi were only 0.01% of abundance of canine metagenome (59).

A more recent study (74), analysing 12 healthy dogs and seven dogs with acute diarrhoea, did not find significant differences in the percentage of fungal groups, between the diseased and healthy dogs. The most rich phylum in canine faeces was Ascomycota and the most abundant classes were Dothideomycetes and Saccharomycetes , with Candida being the most abundant genus (74). Other study also found that in dogs Saccharomycetes was the most abundant fungal

class (85.46%) mainly comprised of genus Nakaseomyces (76.72%) and Candida castellii the most abundant species (55). The rest of the class were not identified, but related to Pleosporales, Taphrinales and Trichosphaeriales (55).

More studies analysing the wide population of fungi in the intestine of dogs is warranted to evaluate the importance of fungal populations in the health and disease of the host.

1.3.3 Archaea

In one study that analysed the canine intestinal microbiome of dogs fed a fibre supplemented diet, through metagenomics, did not find differences with control dogs. Archaea comprised only approx. 1% of all sequences. All belong to Crenarcheaota and Euryarcheaota and methanogens were the most abundant and diverse. Compared to other species such as chicken, humans and mice, dogs lack of Sulfolobales, Halobacteriales and Nanoarchaeum (59)

1.3.4 Viruses

Viruses are the smallest organisms able to pass through filters that retain the smallest bacteria. Viruses have no metabolism on their own and they need to invade cells, transferring genetic material between one and another (75). Viruses have been classified in different categories e.g. enveloped and non-enveloped, genome packaging nucleic acid type, structure symmetry and shape, viruses infecting prokaryotes and infecting eukaryotes (75, 76).

Enveloped viruses have a lipid-bilayer membrane on their surface, this membrane is an impermeable barrier between their genome and the outside environment, which protects the viral capsid or protein layer. In contrast to non-enveloped viruses, enveloped viruses are easily inactivated with organic solvents (76).

Viruses contain only one type of nucleic acid; hence they can be divided as RNA or DNA viruses (76). Viruses with RNA genome, can have positive or negative polarity or sense and also, they

can have genomes of single or double stranded RNA. Likewise, DNA viruses can have genomes comprising single or double stranded DNA (76).

As obligate intracellular entities, viruses can infect eukaryotic or prokaryotic cells. Viruses that infect bacteria are called bacteriophages or phages for short. They are one of the most prevalent organisms in the planet and according to their shape are classified in tailed and non-tailed, being the tailed double-stranded DNA (dsDNA) phages the most easily identifiable. An approximate count of these tailed phages is around 10^{31} on the planet (75, 77)

Phages have been investigated since 1915 – 1917 when they were described as microorganisms that "eat" bacteria, hence, bacteriophages. Later, thanks to electron microscopy, they were more completely characterised and subsequently used in antibacterial therapy. More recently, metagenomics analyses have demonstrated that bacteriophages are rather ubiquitous on the planet (75, 77, 78).

Bacteriophages have been classified in two categories: virulent and temperate and they can change between both states. Virulent phages display a lytic cycle, where the phage enters the host cell and produces bacterial lysis, releasing the free phage (77). Temperate phages in contrast, create a symbiosis with their host, called lysogenic state. In condition of lysogeny, phages are called prophages, which enter the bacterial genome and express some viral genes (79). The conditions that trigger the change from lysogenic to lytic states is poorly understood, but it appears that DNA damage, temperature, and oxidative stress can trigger the lytic cycle (80, 81).

Some prophages can disrupt some bacterial genes, which can later be restored when those phages are excised. Therefore, poly-lysogenic strains of bacteria exhibit a whole range of phenotypes, depending on which prophages are excised or induced. This shows that some bacterial populations could vary considerably depending on the temperate or lytic phages infecting them (80).

1.3.4.1 Methods of viral identification

1.3.4.1.1 Classical methods of viral identification

Viruses need cells to replicate; therefore, one of the most commonly used methods to identify and isolate viruses has been via the infection of cultured cells. Despite being a slow method, this technique allows viruses to grow and replicate in them. Viral replication is identified through the observation of changes in the cultured cells such as morphologic changes in infected cells also called cytopathic effects; adherence of erythrocytes to the virally infected cells (hemadsorption) and interference which is the capacity of some viral infections to confer resistance to infection with other viruses (75, 82).

Other method of viral identification is the direct visualisation of virions using electron microscopy. This technique has been historically used for identifying viruses in stool samples based on their morphologic characteristics, where some unknown viruses can be assigned to certain families with low error rates (75, 76). The low sensitivity, need of a skilled operator and high cost of the equipment are some of the disadvantages of this technique (75, 76).

Detection of viral antigens using virus-specific antibodies , such as immunofluorescence, immunochromatography, immunohistochemistry, immunoelectron microscopy and enzyme immunoassay methods have been widely used to identify viruses (76, 83). On the other hand, methods to detect antibodies in animals and humans have been extremely useful in the diagnosis of viral infections (76, 83).

The development of techniques that detect viral nucleic acids have facilitated the identification of viruses for diagnostic and research purposes. One of the most important advances in this field was the implementation of the polymerase chain reaction (PCR) (75, 76, 83, 84). Currently, real-time PCR techniques that provide estimations of viral load on biological specimens have been increasingly used (76).

1.3.4.1.2 Viral metagenomics, next generation sequencing and bioinformatics

Classical methods of identification of viruses have several limitations that hinder the identification of novel viral species. Numerous viruses cannot be cultured in the laboratory and not all viruses produce cytopathic effect on cultured cells; there is no single gene that is common to all viral genomes, like the ribosomal DNA of cellular organisms; moreover, there is high genetic diversity between viruses within the same family (85) (86). In light of these limitations, in recent years there has been a growing use of molecular methods like viral metagenomics. Such culture-independent methods allow the study of a set of viral populations in a sample by analysing the nucleotide sequences of a whole sample. Metagenomics techniques have been useful for detecting viral nucleotide sequences in samples without previously known information about the target (87). To perform an accurate metagenomic analysis, some steps are essential: sample preparation, amplification of viral nucleic acids, a high-throughput sequencing method and bioinformatic analysis (88, 89).

Regarding to sample preparation, viral isolation, nucleic acids extraction and removal of non-viral nucleic acid molecules is necessary (90, 91). Different strategies can be used to tackle each step, including homogenisation (91), filtration (91, 92), ultracentrifugation (91) and enzymatic digestion of non-capsid protected nucleic acids (93). A combination of strategies in each step are often required to concentrate the viral particles present in the sample (85, 90, 91).

Non-specific amplification of the viral nucleic acids can subsequently be performed in a sequence-independent fashion, yielding multiple copies of viral genomes present in the sample, regardless of their known or unknown status (90, 94). This kind of amplification technique was introduced in the 90's and one of the most used techniques to identify viruses has been sequence-independent single primer amplification (SISPA) (95). This method has been used to amplify both single and double-stranded RNA and DNA viruses, after the removal of any non-viral nucleic acid (90, 94).

After the amplification of viral nucleic acids, a sequencing method is necessary, ideally a high throughput system. Diverse technologies have been developed to determine nucleic acid sequences. Since a few decades, a sequencing method based on DNA sequencing with chain-terminating inhibitors or Sanger sequencing, has been used to analyse genomes (96). Advances to this technology, such as automated Sanger are considered first generation technology and have been very useful for many years but still have numerous limitations (97-100). These limitations involve a limited scalability, not been able to work in high-throughput systems and the high cost associated in the determination of large genomes (101).

A second generation or NGS was developed to overcome those limitations. Diverse NGS platforms have been created to produce millions of bases of sequence at a time, having the capacity to analyse large-scale projects (102, 103). The first platform was 454™ (Life Sciences/Roche, Branford, CT), based on pyrosequencing (104). This technology was subsequently followed by Genome Analyzer (GA, Solexa, Chesterford, UK), now known as Illumina® (Illumina, Inc., San Diego, CA) (105) and the SOLiD sequencing system, released by Applied Biosystems (Foster City, CA) (106). Other platforms used are Helicos sequencer (107) and Ion Torrent™ (Life Technologies, South San Francisco, CA) (108), PacBio® (Pacific Biosciences, Menlo Park, CA) (109) and more recently Oxford Nanopore Technologies (Oxford, UK) (110). All these technologies have evolved to decrease costs and generate better levels of sequencing throughput.

After a sequence has been attained, it is subjected to a search against a database of nucleotide or protein sequences to find the origin of the sequence. This task is performed with bioinformatic software tools that analyse NGS data. This analysis includes quality control, base-calling, alignment of sequence reads to a reference, *de novo* assembly (from paired and or unpaired reads) and genome browsing and annotation (111).

All these technologies and progress in sequencing, have made easier the process of identification of viromes from different environments and to the discovery of new viruses (90, 112, 113)

1.3.4.2 Virome and virus discovery

In recent years the identification and discovery of new viruses has increased considerably as a consequence of the use of NGS technologies. However, studies to identify the viral flora in animals have been in a smaller number than in humans. Studies using NGS technologies applied on animal samples may help expand our knowledge about viral evolution, cross species transmission and may offer more possibilities for animal vaccine development (114)

Since animal viruses are viewed as an important cause of novel human infections, the detection and classification of new organisms is essential to preserve both animal and human health (113). Wild animals and intensive production systems of domestic animals are frequently affected by viral epidemics. This has an important effect on human populations due to the potential of propagation of diseases. Other common sources of viral zoonosis are bats and rodents; however, the vast number of species and ubiquitous distribution of these animals makes it very difficult to have a thorough understanding of their viral genomes (114)

So far, the study of the viral microbiome (virome) has been carried out in a variety of biological and environmental samples including humans (115-118), animals (119-123), guano (124), marine sediment (125), fresh water (126), soil (127), and plants (128). Thus, viral metagenomic studies of animal and human samples will help in the genetic characterisation of viruses, facilitating the analyses of pathogenicity markers and the potential identification of control methods (90).

1.3.4.2.1 Gastrointestinal virome

The gut virome has been studied since Breitbart *et al.* 2003 analysed a faecal sample from a single healthy human adult (116). Since then, there has been a growing interest in the gastrointestinal (GI) virome in both humans and animals.

Various studies analysing the human gut virome have described the presence of viruses that infect eukaryotic cells as well as viruses that infect prokaryotic organisms, the latter being the most abundant (115, 116, 129). These reports suggest that asymptomatic individuals can carry numerous viruses and the relationship between virus and host is not always harmful for host cells (130, 131).

The GI virome has been found to be unique and individual, despite genetic relatedness, as has been shown in twins (129). It has also been shown that diet can influence the composition of GI viromes (132).

The same categories of viruses have been found in the GI virome of animals (122-124, 133-140) (119, 120, 141), where bacteriophages have been described as the most abundant component of the GI virome in certain animals (134, 140, 141).

Bacteriophages seem to be a very important component of the GI microbiota, but the true influence or role of bacteriophages in GI diseases remains poorly understood. Bacteriophages could potentially influence the shift from health to disease through dysbiosis (changes in bacterial populations), and dysbiosis has been found in GI pathologies (142). This means that some bacterial populations can vary significantly depending in whether the GI environment is predominantly populated by temperate or lytic phages. According to Reyes *et al.* 2010 (129) the viral fraction in faeces of healthy humans is predominantly composed of temperate phages, contrasting with aquatic environments where lytic phages predominate. Similar results were found by Minot 2011 (132). Conversely, more virulent phages were found in diarrhoeic individuals compared to healthy ones, as has been suggested in two reports on coliphages in human patients with diarrhoea (143, 144).

Since there is not enough data to conclude that phages have a direct influence on dysbiosis, some models that propose how phages may impact bacterial populations have been developed. These models support a potential modification of bacterial diversity and abundance as a result of phages infecting those populations. The “kill the winner” model has not been found in the gut and has only been observed in the ocean. Phages that use this strategy need a high density of prey (bacteria) to find their host, therefore, they replicate (and kill) bacteria that are present in a very high density (80). Due to the requirement of a big population of prey, this model is known as “kill the winner”. However, within the gut, it has been suggested that bacteria are refugees, possibly due to the physical characteristic (semi-solid) of the gut and are able to escape phage infection (81, 145). An alternative model has been proposed for gut bacterial populations, called bacteriophage adherence to mucus (BAM) model. This model proposes an antimicrobial defence system on mucosal surfaces provided by phages, where phages result in a beneficial effect for the host GIT they inhabit (146). The "kill the relative" model proposes that bacteria use the prophages that infect them to compete against other bacteria that occupy the same niche. Bacteria generate lytic phages from their prophages to kill the niche competitor bacteria, thus benefiting their bacterial host (147, 148). The “community shuffling” model proposes that sometimes prophages are not beneficial for their host due to a process known as prophage induction, where a prophage becomes a lytic phage. This induction can be influenced by different factors provoking stress (80). In the gut, different inducing agents can trigger this change in prophages such as, some antibiotic in sub-inhibitory concentrations, cigarette smoke and inflammation through oxidative stress (80, 149). The latter models rely on phages being lytic, but if lysogeny is favoured, phages can function as horizontal genetic information transfer vectors, thus contributing to bacterial evolution through the emergence of new strains. This model has been called “emergency of new bacterial strains”. Phages, contribute for example, to improve pathogenicity, such as increased virulence due to toxin production or help to improve host colonization due to resistance to oxidative stress, bile salts or biofilm formation (150). Also,

through this genetic material transfer, phages can contribute to conferring antimicrobial resistance (150, 151).

1.3.4.2.2 GI virome in health and disease

Toll-like receptors (TLRs) and the pathways that their engagement activates are one of the mechanisms responsible for the detection of bacteria and viruses in the gut, and the induction of inflammatory responses. These receptors recognise pathogen associated molecular patterns (PAMPs). TLR1, TLR2, TLR4, TLR5 and TLR6 are localised on the cell surface and recognise microbial membrane components, whereas TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles and recognise nucleic acids (80, 152). Viral nucleic acids act as a PAMPs and can be recognised by the latter. Given the non-specific nature of these innate immune receptors there are no TLRs that specifically recognise phages (80)

Given the high abundance of bacteriophages in the GI tract, interactions between these and the immune system have been suggested. For example, changes in the patterns of recognition of bacteria mediated by prophages, where phages could be involved in IBD pathogenesis by changing recognition patterns of bacteria have been proposed (153). Also, a direct recognition of phages by the host by antiviral innate immune sensors has been proposed, in cases where host cells directly phagocytised phage particles, or when phages were delivered to the intracellular environment by phage-producing bacteria (154). Finally, the interaction between phages and mucus acting in concert as antimicrobial defence system provided has been proposed (146).

In people, these interactions have been associated to IBD and some studies analysing phage populations found differences between healthy and individuals with Crohn's disease (CD) and ulcerative colitis (UC), where an expansion of phage populations was associated with individuals suffering IBD (142, 155, 156).

Within a person, the phageome has been identified as stable (132, 155); however, the phageome at a global scale had not been evaluated until recently. A study presented the healthy gut phageome in humans (157), where two bacteriophages dataset from two faecal samples from two persons was analysed together with available data from 62 healthy individuals from around the world. These authors proposed that the phageome could be divided in three classes: core, common and rare, where the core phageome was shared by more than half of the individuals, the common category was shared by many individuals and the rare category was in only a few individuals. In this study, the core phageome was composed by at least 23 phage species and this number was reduced in patients suffering CD and UC, suggesting that phages could contribute to human gut health (157).

Further understanding of the impact of phages on immunity could help to develop techniques such as faecal transplant (complete microbiota passed from a healthy individual to a sick one) (158) and probiotic therapy (149).

1.3.4.3 The canine faecal virome

The majority of studies analysing enteric viruses in dogs have been targeted studies, evaluating the prevalence of specific viruses. Thus, information about enteric viruses in healthy dogs has not been analysed. Only one study has investigated the faecal virome of dogs with diarrhoea. In this study two new viruses in dogs were described, canine sapovirus and canine kobuvirus, (122)as well as other known viruses of dogs such as rotavirus, densovirus, canine parvovirus (CPV2), canine coronavirus, brome mosaic virus and kashmir bee virus in this faecal virome of dogs with diarrhoea (122).

1.3.4.3.1 Enteric viruses affecting dogs

1.3.4.3.1.1 Bacteriophages

A study analysing viruses in faeces of dogs through EM, found phages with tail, possibly Caudovirales (159). This is the only report of phages in faeces of dogs.

1.3.4.3.1.2 Family Astroviridae

1.3.4.3.1.2.1 Genus Mamastrovirus, Canine astrovirus

Astroviruses are non-enveloped viruses with a star-like shape, first identified in 1975 by electron microscopy in children suffering diarrhoea (160). The family *Astroviridae* includes single stranded RNA positive sense viruses, with genomes varying in length between 6.17-7.72 kb. They comprise a 5' untranslated region (UTR), three open readings frames (ORFs), a 3' UTR and a poly (A) tail. ORF1a and ORF1b encode non-structural proteins (involved in transcription and replication of the viral genome) and ORF2 encodes the capsid polyprotein (161). They are classified in two genera, *Mamastrovirus* and *Avastrovirus* infecting mammals and avian species, respectively. Viruses belonging to *Mamastrovirus* genus include isolates from a number of mammals including humans (HAstV), pigs (162), cats (163), sheep (164), calves (165), dogs (CaAstV) (166), bats (167), rats (168), and marine mammals such as sea lions and bottlenose dolphins (169), among others. Viruses from *Avastrovirus* genus include isolates from turkeys (170), ducks (171), chicken and others and has been related with extraintestinal diseases (161). The *Mamastrovirus* genus includes two genogroups, GI and GII with 10 and 9 genotype species, respectively, where Canine astrovirus (CaAstV) belongs to genogroup I (161).

Historically, astroviruses were classified according to the host species. However, recent metagenomic analyses have revealed the existence of novel astroviruses in different animal species, sharing very closely related genomes, whilst relatively genetically diverse viruses have been identified from the same animal species. This has prompted a new classification based on the complete amino acid sequence of ORF2, which encodes the capsid polyprotein and represents the most variable region of the genome (161, 172).

The first detections of astrovirus-like particles in dogs with and without diarrhoea were reported in the 1980s, using electron microscopy (159, 166, 173). However, the first genetic analysis of the viral RNA of an astrovirus-like particle, identified by electron microscopy, was performed only in 2009 (174). In 2011 Martella *et al.* (175) isolated and characterised a canine astrovirus in cell culture. Finally, the complete genome of a canine astrovirus was elucidated for the first time by Caddy *et al.* in 2015 in the UK (176).

To date, canine astrovirus and astrovirus-like particles from canine faecal samples have been reported in USA, Australia, China, Italy, UK, France, Brazil, Korea and Japan (159, 166, 173-182). Canine astrovirus has also been found in domestic sewage samples from one Uruguayan city (183).

The clinical implications of canine astrovirus in enteric diseases of dogs, needs further analysis. Canine astrovirus has always been identified, since the first descriptions, from both healthy and diarrhoeic dogs (159, 166, 173, 175, 179). Also, in the majority of studies astrovirus has been found together with other recognised enteric pathogens.

In a study investigating kennelled puppies in France, canine astrovirus was found in 26.8% of dogs with diarrhoea, however, it also was identified in 18.7% of healthy individuals, being this difference not significant between both groups (179). In contrast, an Italian study determined that canine astrovirus was present in 24.5% (27/110) of puppies with diarrhoea, significantly higher than the proportion of positives (7/75, 9.3%) detected in healthy puppies (175). A more recent study in the UK found a 6% (4/67) of positives in dogs with diarrhoea, which was significantly higher than the 0% (0/181) detected in healthy dogs. In the same study the first complete genomes of two canine astroviruses were characterised (176). Other study analysing the prevalence of canine astrovirus in China showed a 12.02% (22/183) of positives in puppies with diarrhoea and 0% in 138 healthy dogs (177), while other two studies evaluating only dogs with diarrhoea found 37.5% (6/16) (180) and 2.2% (2/91) (181) positive samples to canine

astrovirus, respectively. In a different study where canine astrovirus was confirmed by genetic characterisation after EM, the prevalence in a population of 11 diarrhoeic puppies was 36.3% (174). As astrovirus was found in dogs which were also positive to other enteric viruses, such as parvovirus and rotavirus, the pathogenicity of astroviruses could not be confirmed in these dogs (174). Taken together all these studies are inconclusive in regard to the pathogenic potential of astroviruses in dogs.

Most of the studies investigating canine astrovirus prevalence were performed in puppies or dogs younger than 24 months old (174, 175, 177-181). One of these studies found a higher risk of astrovirus infection in younger puppies (under 7 weeks of age) (179). However, such approaches could bias the results towards a higher prevalence in puppies due to a potential preference of the virus for younger individuals. Only two studies have investigated the presence of canine astrovirus in puppies and older dogs, finding a moderate overall prevalence of 6% and 9.7% positives, respectively (176, 182).

1.3.4.3.1.3 Family Parvoviridae

1.3.4.3.1.3.1 Subfamily Parvovirinae

1.3.4.3.1.3.1.1 Genus Bocaparvovirus, Canine bocaparvovirus

Genus *Bocaparvovirus* belong to *Parvoviridae* family, and contain several species. **Bo**vine parvovirus and **Ca**nine minute virus were the first species classified in this genus, which gave its former name; bocavirus. This genus contains a third ORF, that make them different to the rest of the parvoviruses (184). Recently was added a human bocaparvovirus, that was found in children with lower respiratory tract infections (185). In animals, either cows or dogs, bocaparvoviruses affect mainly the GI and respiratory tract of young individuals and have also been associated with reproductive complications (184). The first description of canine minute virus from an apparent healthy dog, was through EM in 1970 (186). Since then, this infection has been associated to neonatal diseases and fertility disorder of dogs (187-190). Canine bocaparvovirus has been described and associated to respiratory (191) and enteric disease

(192). Currently, canine minute virus is called carnivore bocaparvovirus 1 and canine bocavirus 1 now is named carnivore bocaparvovirus 2 (193). A new bocaparvovirus, Canine bocavirus 3 (CnBoV3), has been described in the liver of a dog with severe haemorrhagic gastroenteritis, but still is an unclassified bocaparvovirus (193, 194).

1.3.4.3.1.3.1.2 Genus Protoparvovirus, Canine parvovirus

Canine parvovirus (CPV), belonging to the *Parvoviridae* family, genus *Protoparvovirus*, is a non-enveloped, ssDNA virus that causes disease in several mammalian species. CPV was first identified in the mid 70's, but a ubiquitous strain associated to enteritis was described around 1978 (195) (196-198) and was called CPV2 (199). This virus rapidly changed and two variants were later described; CPV2a and CPV2b, which replaced CPV2 (200-202). Later, in 2001, a new variant was discovered in Italy (203), this time called CPV2c. Since then, canine parvovirus has been identified as one of the most important enteric pathogens of dogs worldwide, producing high morbidity and mortality, mainly in unvaccinated dogs (204-223).

1.3.4.3.1.3.1.3 Genus Protoparvovirus, Canine Bufavirus

A new canine parvovirus has been described in an Italian outbreak of dogs with respiratory signs (224). The genetic analysis found this new virus very closely related to human bufavirus and was classified as canine bufavirus (CBuV). In a subsequent screening PCR analysis, 31% of oropharyngeal or nasal samples from dogs with respiratory signs, were positives to canine bufavirus. This study also found canine bufavirus from faecal samples of dogs with enteric signs and healthy ones, from Italy and Hungary. Nevertheless, the difference between these groups was not statistically significant (224). Canine bufavirus has been also described in China from serum and stool samples with a very low prevalence (225). Another Chinese study investigating the prevalence of CBuV found 42.15% positive faecal samples from dogs with diarrhoea and none from healthy dogs (226). Additionally, CBuV has been found in wild canids such as wolves

and foxes, from rectal swabs of dead animals deceased from causes other than enteric disease (227).

1.3.4.3.1.3.1.4 Genus Chapparvovirus

A viral metagenomic study analysing faecal samples from an outbreak of diarrhoea in Colorado, found that one pool of 3 samples were closely related to different chapparvovirus from other species (228). They characterised the near complete genome and was named cachavirus strain 1A (CachaV-1A), which could qualify as a member of a new species: Carnivore chapparvovirus species 1. Evaluating the samples individually, it was found in one more sample of the same outbreak (228). A subsequent prevalence study found 1.47% positive samples in healthy dogs and 4.35% of positives in diarrhoeic faecal samples with *p*-value of 0.05 (228).

1.3.4.3.1.4 Family Caliciviridae

1.3.4.3.1.4.1 Genus Norovirus, Canine norovirus

The genus *Norovirus* belongs to family *Caliciviridae*; these are viruses with positive sense RNA genomes, together with four other genera; *Sapovirus, Vesivirus, Lagovirus* and *Nebovirus* (75). Genus *Norovirus* is classified in six genogroups (GI – GVI) based on the capsid sequences (75). First detections of calicivirus in dogs were made in 1985 (229) (230), associated to dogs with enteritis, although one of them was linked to co-infections with other known canine enteric viruses (230). The first description of canine norovirus was in 2008, from a puppy with enteritis in Italy (231). Since then, canine norovirus has been documented in several occasions, in Portugal (232-234), China (235), Greece (236), Finland (237), USA (238), UK (239) and Japan (240). A study that evaluated seroprevalence of canine norovirus in Europe, found 36% of positive samples for IgG antibodies against canine norovirus GVI in 14 European countries (241).

1.3.4.3.1.4.2 Genus Sapovirus, Canine sapovirus

The genus *Sapovirus* are positive sense ssRNA viruses, belonging to the *Caliciviridae* family of viruses, which are divided in five genogroups (GI -GV), which affect humans, pigs and minks (242) (243). The first canine sapovirus was reported in a metagenomic study of the faecal virome of dogs with diarrhoea (122). Further analysis of this canine sapovirus in 200 dogs with diarrhoea and 200 healthy dogs, showed a very low prevalence of this virus, with only one positive dog in each group (122). So far, only two other studies have investigated the incidence of canine sapovirus. One study in Japan found a very low prevalence of canine sapovirus in dogs with diarrhoea, but there was no information about a control group of healthy dogs (240). The second study, performed in Italy, found a 2.2% of canine sapovirus positive samples in dogs with diarrhoea and 0% in healthy dogs (244).

1.3.4.3.1.4.3 Genus Vesivirus, Canine calicivirus/vesivirus

The genus *Vesivirus,* together with four other genera; *Sapovirus, Norovirus, Lagovirus* and *Nebovirus*, belongs to the *Caliciviridae* family, which are viruses with positive sense ssRNA genomes (75). The genus *Vesivirus* forms a distinct clade within the family (242). The first detections of calicivirus in dogs were made in 1985, associated with cases of enteritis (229) (230). In 1999 a strain of canine calicivirus, strain 48, was classified as a different calicivirus, with a distinct position within the animal caliciviruses (245). Later studies found other canine caliciviruses which were closely related to strain 48; this led to the suggestion that this group of viruses are closely related to the V*esivirus* or S*apovirus* genera (246). In 2015 the first canine vesivirus was described, from a study analysing dogs with diarrhoea, healthy dogs from shelters and household healthy dogs. Positive individuals were found in all three groups of dogs, but canine vesivirus had a significantly higher prevalence in sheltered healthy dogs (247). A different study investigating an acute haemorrhagic diarrhoea focal outbreak in dogs, following a stay in a pet housing facility, found that individuals that died were positive to vesivirus (248). It must

be noted that the classification of canine vesiviruses, within the *Caliciviridae* family, has been proposed to ICTV, but not yet been approved as a species (242).

1.3.4.3.1.5 Family Circoviridae

1.3.4.3.1.5.1 Genus Circovirus, Canine circovirus

Viruses in the family *Circoviridae* have a circular ssDNA genome and include two genera, *Circovirus* and *Gyrovirus*. *Circovirus* genus comprises 29 species infecting mammals and birds, with Porcine circovirus 1being the type species for this genus (249). The first description of a canine circovirus infection was in 2012, from serum samples from six dogs (250). The first characterisation of the complete genome of a canine circovirus was done from the liver of a dog with severe haemorrhagic gastroenteritis, vasculitis and granulomatous lymphadenitis (251). The same study also investigated the prevalence of this canine circovirus in faecal samples, however, there was no significant difference in the proportion of positives between dogs with diarrhoea (11.3%) and healthy dogs (6.3%). Furthermore, 68% of dogs with diarrhoea were co-infected with other pathogens (251). A different study found tissue samples from gut and liver from a dog with haemorrhagic gastroenteritis, positive to canine circovirus (252). The genome of this circovirus was characterised, and it was closely related to the American strain found in dogs with vasculitis (251), but was not related to viruses causing of gastroenteritis. Likewise, other study did not find correlation between the presence of canine circovirus and gastroenteritis, because of the difference in prevalence between healthy dogs and dogs with diarrhoea was no significant (253). Other study from Germany also did not find a strong correlation between the presence of canine circovirus and acute haemorrhagic diarrhoea syndrome (AHDS), but authors suggested a possible role as a negative co-factor in disease outcome of dogs with canine parvovirus infection (254). This hypothesis is supported with results from a second study that found dual infections of canine circovirus and CPV-2 caused

recurrent outbreaks in a breeding colony (255). Conversely, a study in Taiwan found a high prevalence of canine circovirus in dogs with diarrhoea (256). These results suggest that canine circovirus does not have a clear pathogenic role, but may make an important contribution in co-infections with other enteric viruses.

1.3.4.3.1.6 Family Coronaviridae

1.3.4.3.1.6.1 Subfamily Orthocoronavirinae

1.3.4.3.1.6.1.1 Genus Alphacoronavirus, Alphacoronavirus species 1/Canine coronavirus

Coronaviruses, are enveloped viruses that contain a positive sense, ssRNA genome, associated to enteric and respiratory diseases of mammals and birds. Canine coronavirus belongs to Alphacoronavirus 1 species (75). Canine coronavirus is classified into types I and II, related evolutionarily to feline coronavirus types I and II (257). Coronavirus in dogs with diarrhoea has been found as type I or type II or both simultaneously (258). First reports of coronavirus in dogs were made in 1970s (259, 260). Since then, several reports have been made around the world, reporting the presence of canine coronavirus as an important enteric pathogen in dogs, either alone or as co-infection (204, 209, 258, 261) (262, 263), mainly in populations of kennelled or sheltered dogs (122, 175, 264-267). Since an experimental infection performed in 1991 (268), the infection with canine coronavirus has been considered to cause mild diarrhoea. Nevertheless, as co-infection with other enteric viruses such as CPV-2 are common, canine coronavirus seems to contribute to more severe diarrhoeas (207). In addition, divergent strains of coronavirus, possibly due to point mutations, producing severe diarrhoea in dogs have been reported. These divergent strains have been described in Australia (269), UK (270), USA (271) and Sweden (272). Other study analysing a severe outbreak of fatal systemic disease in dogs from a pet shop in Italy, found canine coronavirus types I and II, and no other enteric viruses present in necropsies of affected dogs (273). The coronavirus type II found in this Italian study is the first description of a variant of canine coronavirus, being pantropic and highly pathogenic (273). Other findings of pantropic infection by coronavirus have been made in France and

Belgium (274), as well as in Greece (275)} and Italy (276). A surveillance study of pantropic canine coronavirus was performed in Europe, and was identified in France, Hungary, Italy, Greece, the Netherlands and Belgium (277). Taken together, these data indicate that canine coronavirus, despite being classified as a cause of mild enteric disease, can produce a more severe diarrhoea when combined with other enteric pathogens or as a pantropic pathogen.

1.3.4.3.1.7 Family Paramyxoviridae

1.3.4.3.1.7.1 Subfamily Orthoparamyxivirinae

1.3.4.3.1.7.1.1 Genus Morbillivirus, Canine Morbillivirus/Canine distemper virus

Canine distemper virus (CDV) are enveloped negative-stranded RNA viruses, belonging to family *Paramyxoviridae*, genus *Morbillivirus* (75). Canine Distemper Virus appears to have originated in South America and was brought into Europe around the XVIIIth century (278). This virus has an extensive host mammalian family range, including the *Canidae*, *Mustelidae*, *Procyonidae*, *Ursidae* and *Viverridae*. Furthermore, CDV has also been detected in some species of felids, primates, peccaries and seals (279). The virus enters the host via the nasal or oral routes and replicates in lymphoid tissues, resulting in severe immunosuppression (280). The initial clinical signs are lethargy, anorexia, dehydration and fever, followed by inflammation of the respiratory tract, conjunctivitis, gastroenteritis and depression (76). Some dogs develop central nervous system signs, such as seizures, cerebellar and vestibular signs, paresis and sensory ataxia with myoclonus. These neurologic clinical signs are generally progressive, with a poor prognosis (76). Canine Distemper Virus has been one of the most commonly investigated enteric viruses in dogs, being prevalent worldwide (204, 209, 222, 265, 281-285). Recently, variants of CDV have been detected causing disease in vaccinated dogs (286-288). These studies demonstrate that global surveillance of CDV strains is warranted.

1.3.4.3.1.8 Family Picornaviridae

1.3.4.3.1.8.1 Genus Kobuvirus, Aichivirus A /Canine kobuvirus

The *Kobuvirus* genus belongs to the *Picornaviridae* family; this genus comprises three species: Aichivirus A (previously Aichi virus), Aichivirus B (previously Bovine kobuvirus) and Aichivirus C (Porcine kobuvirus)(289). Three other kobuviruses have been described just recently and possibly belong to Aichivirus A, namely Canine kobuvirus (CaKV) 1, Feline kobuvirus 1 and Murine kobuvirus 1 (122, 290-292). Also, CaKV-like viruses have been found in foxes (293), these viruses may also belong to the Aichivirus A species. Other types proposed to belong to Aichivirus B are ferret kobuvirus 1 and ovine kobuvirus 1 (139, 294). *Kobuvirus* has been found in other animals as bats (124), European roller (295) and goats (296) which are unclassified yet. Porcine kobuvirus has been found in wild boars (297).

Canine kobuvirus was first described in the USA in 2011. Two groups described simultaneously the presence of CaKV in dogs with diarrhoea (122, 298). Both studies found CaKV was very closely related to human Aichi virus (Aichivirus A). In a metagenomic study (122) found that three out of 18 dogs with diarrhoea, were positive to canine kobuvirus, and one of those three dogs was co-infected with CPV2 and rotavirus.

At present, canine kobuvirus has been described in UK (299), USA (122, 298), Korea (181, 300), China (301), Italy (302), Tanzania (303) and Japan (304). In Australia the first detection of a possible kobuvirus was the identification of a Picornavirus-like particle in 1995, from three dogs with diarrhoea and one healthy dog (305).

The majority of studies evaluating canine kobuvirus have suggested that this enteric virus is more prevalent in individuals younger than 2 years (122, 181, 299-302, 304), but the universe of samples was sourced mainly from puppies, which can bias the interpretation of these data. Some of these studies have identified canine kobuvirus from both healthy dogs and dogs with diarrhoea (122, 300, 303, 304). Thus, canine kobuvirus has not been considered a causal agent in gut disease, mostly because it is regularly found in co-infections with other known enteric viruses (122, 181, 299-302, 304).

1.3.4.3.1.9 Family Reoviridae

1.3.4.3.1.9.1 Subfamily Sedoreovirinae

1.3.4.3.1.9.1.1 Genus Rotavirus, Rotavirus A/Canine rotavirus

Genus *Rotavirus* belongs to the *Reoviridae* family, with viral genomes comprising 11 segments of double stranded RNA (dsRNA). The rotaviruses are classified serologically into 7 groups (RVA -RVG) (306). In addition, a classification system has been devised to assign a genotype to each of the 11 genome segments (307). This family of viruses was first identified in Australia in 1973 (308) and since then it has been classified as the most important cause of diarrhoea in children (309). Similarly, rotaviruses are one of the most important cause of diarrhoea in farm animals (310). However, rotaviruses in dogs have been associated with mild diarrhoea, but can contribute to a more severe enteritis when they are in co-infection with other enteric viruses (311, 312). The majority of canine rotaviruses belong to group A (RVA), although canine rotavirus belonging to group C have also been described in the literature (313, 314). Lately, two canine rotavirus strains have been proposed to belong to group I (315). Rotaviruses from the same group are capable of genetic reassortment (306) producing a potential high risk of zoonosis of these new rotavirus strains (316-319).

1.4 Aims of the project

1.4.1 Overall aim

The main aim of this research project is to characterise the faecal virome of dogs with various inflammatory intestinal diseases using next generation sequencing techniques and bioinformatics pipelines.

1.4.2 Specific research aims

This project more specifically aims to:

- Develop and optimise a sample preparation protocol that produces excellent quality nucleic acids suitable for next generation sequencing techniques
- Characterise and compare the faecal virome between healthy dogs and dogs with acute diarrhoea
- Characterise and compare the faecal virome between healthy dogs and dogs with chronic enteropathy
- Characterise the full genome sequence and prevalence of selected viruses that may be associated with inflammatory intestinal disease

Chapter 2: Optimisation and validation of sample preparation and viral nucleic acids enrichment protocol in canine faeces for viral metagenomics

2.1 Introduction

Conventional methods of viral identification, including cell culture, electronic microscopy and PCR among others, have been traditionally used in virology. These have limitations, and so new technologies such as shotgun metagenomics, where all nucleic acids from a sample are sequenced without the need for culture have been developed. Shotgun metagenomic sequencing can provide information about unknown microbes in a specific sample (86, 90).

However, protocols developed for bacterial analysis are inadequate for viral analysis, and so optimised protocols are required to increase the yield of viral nucleic acids from a complex biological sample. The rate-limiting step for all protocols to enhance viral isolation is the removal of background prokaryotic (bacteria) and eukaryotic (host) nucleic acids in the sample preparation (85, 86, 90, 320).

This chapter describes the validation of an optimisation protocol for preparation of canine faecal samples and enrichment for viral nucleic acids, as a part of the research investigating the faecal virome of healthy dogs and dogs with acute diarrhoea (321). A limitation of this protocol is that it is a summary of a step by step process rather than a structured experimental study, aiming to produce an adequate protocol which yields the biggest amount of viral DNA/RNA.

Filtration, enzymatic removal of non-protected nucleic acids and density gradient ultracentrifugation to pellet viruses were used to increase the levels of viral nucleic acids while reducing background bacteria and host nucleic acid presence. To evaluate qualitatively the presence of bacteria in samples, a bacterial 16S rRNA gene PCR was performed after each procedure (appendix 8.5).

2.2 Optimisation of sample preparation and viral enrichment protocol in canine faeces

A faecal sample from a healthy young adult male dog was collected and frozen at -80℃ until used. Dilution of the faecal sample was performed using a 20% method of faecal extraction preparation; briefly, 800 µL of 0.01 M tris HCl (pH 7.5 + NaCl (0.1 M) + $CaCl^2$ (0.01 M)) was added to 200mg of faecal sample, vortexed until homogenised, and centrifuged at 16,600 x *g* for 3 minutes. The supernatant was collected and filtered through a 0.44µm syringe driven filter unit Millex® HV and then through a 0.22 µm Millipore® ultrafree-MC centrifugal filter unit and centrifuged at 12,000 x *g* for 4 minutes. The filtration step removed host cells, cellular debris and bacteria or bacterial products.

Three samples and one extraction control were generated. These three samples were created by spiking the original samples described in the paragraph above, with different amounts of viral nucleic acids from vaccines to evaluate the ability of virus identification of this protocol. Vaccines used were: CANVAC® 3 (Pfizer, Australia), a combination of living attenuated vaccine against canine distemper (paramyxovirus), infectious canine hepatitis (adenovirus) and canine parvovirus; and RotaTeq® (MSD, Australia), a live, oral vaccine containing 5 live human rotavirus strains.

Two of the samples and the extraction control were subjected to digestion of non-encapsulated DNA and RNA for viral enrichment with DNase and RNase treatment using DNase I recombinant RNase-free (10 U/µL), Roche® and RNase A (100 mg/mL), QIAGEN. All samples were incubated

at 37°C for 2 hours in a water bath, shaking samples every 30 minutes. DNase was inactivated by adding 0.5 M EDTA (pH 8.0) AMRESCO® (E522-100mL) to the samples to a final concentration of 8 mM and incubated at 75°C for 10 min in a water bath. The inactivated samples then had viral nucleic acid extracted using the QIAamp® Viral RNA mini kit, according to the manufacturer instructions (QIAamp® Viral RNA mini kit Handbook April 2010, third edition, QIAGEN).

To evaluate the presence of the spiked canine parvovirus and human rotavirus and to evaluate the presence of bacteria, three PCRs were performed and visualised in a 1% agarose gel with TBE buffer. For broad range bacterial 16S ribosomal gene, the following primers were used: 27f AGAGTTTGATCMTGGCTCAG, *E. coli* position 8 – 27 and 149r TACGGYTACCTTGTTACGACTT *E. coli* position 1492 – 1513; PCR conditions were 94ºC for 5 minutes, 30 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1 minute 30 seconds, then an extension phase of 72ºC for 7 minutes (322). We detected bacterial 16S DNA in all samples and positive control, indicating that bacterial DNA did not decrease with this protocol. For canine parvovirus PCR the following primers were used: JS1F AGCTACAGGATCTGGGAACG, position 2843 – 2862 and JS2R CCACCCACACCATAACAACA, position 4799 – 4818. Nucleotide position are referred to the sequences of CPV-2 strain CPV-b (208). PCR conditions were 94ºC for 5 minutes, 40 cycles of 94ºC for 1 minute, 52ºC for 1 minute and 72ºC for 2 minutes and a final elongation step of 72ºC for 7 minutes. All samples presented the same band as the positive control, showing that canine parvovirus could be easily detected with this protocol. For rotavirus VP6 gene PCR, the following primers were used: WC3F GAYGGNGCDACNACATGGT, position 747 – 765, and WC3R GTCCARTTCATNCCTGGYGG, position 1126 – 1107. PCR condition were 45ºC for 30 minutes, 95ºC for 15 minutes, 40 cycles of 94ºC for 30 seconds. 45ºC for 45 seconds and 70ºC for 75 seconds, with a final step of 70ºC for 7 minutes. All samples had the same band as the positive control, showing that VP6 gene of rotavirus could be easily detected with this protocol.

A new experiment with a new faecal sample from a young adult female dog was performed. A different extract dilution protocol (10% dilution) was used and a third filtration step, using a 0.22µm syringe driven filter unit Millex®HV, was added between the two filtration steps. Additionally, different types of DNases (ds DNase, HL ds DNase, Heat and run enzyme, all from ArcticZymes®) were tested, trying to decrease presence of bacteria and isolate the presence of viruses. These samples were not spiked with vaccines and the protocol was aimed only to identify bacterial and viral nucleic acids in dog faeces. The purification of viral DNA was carried out using the same kit and protocol as the previous experiments (QIAamp® Viral RNA mini kit, QIAGEN). A bacterial 16S gene PCR was performed, and no big differences were found between different DNases treatments but a slight difference, compared to control, was observed in all treated samples. As there was insufficient reduction of bacteria compared to the control, a new protocol was evaluated.

A further attempt to decrease presence of bacteria in faecal samples was tested using ultracentrifugation of larger volumes of faecal matter. One faecal sample from the same dog used in the last two experiments in addition to a faecal sample from a third dog (adult, female, healthy). The 20% faecal extract preparation method was used. Briefly, 8 grams of faeces were mixed with 32 mL of 0.01M tris HCl (pH 7.5 + NaCl (0.1M) + CaCl2 (0.01M)). Both samples were spiked with 250µL of CANVAC® and RotaTeq® vaccines each. Glass beads were added to the mixture and vortexed for 5 minutes, followed by a centrifugation step of 20 minutes at 13,000 x *g* at 10 C°. The supernatant of each sample was divided in two aliquots and centrifuged in 14 x 95 mm (14mL) Beckman tubes at 13,000 x *g* for 15 min at 4°C in an Optima L-90K® ultracentrifuge. The supernatants were then filtered through a 0.45µm syringe driven filter unit. Four tubes were loaded with 7 mL of the filtered supernatant and then 2mL of 25% sucrose was added to the bottom of the tube. Finally, those tubes were filled with the rest of the sample and all samples were centrifuged for 3 hours at 200,000 x *g* at 4 C° in the same ultracentrifuge. The supernatant was discarded and the pellet was resuspended in 450µL of NFW until dissolved; 400

µL from each sample was collected and pooled, then separated in four aliquots. Two of these samples were filtered, this time through a 0.22µm column filter to evaluate differences with filtration diameter. Subsequently, all four samples were subjected to nucleic acid digestion and incubated at 37°C for 2 hours in a water bath, shaking samples every half an hour. DNase was inactivated by adding 0.5M EDTA (pH 8.0) AMRESCO® (E522-100mL) to the samples to a final concentration of 8 mM and incubated at 75°C for 10 min in a water bath. After this step, the purification of viral RNA was carried out using the QIAamp® Viral RNA mini kit, according to the manufacturer instructions. Bacterial 16S DNA was again detected in all samples. Furthermore, this protocol was not able to detect parvovirus from any sample except the positive control. Therefore, this ultracentrifugation protocol was also discarded.

A new protocol following a published protocol for viral enrichment with a mixture of DNases was then evaluated (122, 136, 323). This protocol included zirconia/silica beads, only one filtration step, 0.45µm filter, and the addition of three different DNases and one RNase. The published protocol was modified by adding a different buffer to dissolve faeces, one more DNase (DNase I, Roche®) and a larger amount of each DNase.

Four new faecal samples, from four different adult healthy dogs, were tested by adding the latter mixture of DNases. The same four samples were also used without the DNases mixture, which served as controls. None of these samples were spiked with vaccines, as the aim of this step was to compare if there were differences in viral and bacterial isolation between samples with the added mixture of DNases. After nucleic acid digestion and purification of viral nucleic acids all samples were subjected to broad range bacterial 16S ribosomal gene PCR.

There was a markedly improved result compared with previous protocols. All samples that were subjected to DNases treatment showed a much weaker or no visible bacterial 16S band in the gel, compared to the non-treated sample, indicating a reduced presence of bacteria in them. After this improved result, this protocol was used to identify the virome of faecal samples in

healthy dogs, dogs with acute diarrhoea (321) and dogs with chronic enteropathy (324) (Fig. 1 and appendix 8.2).

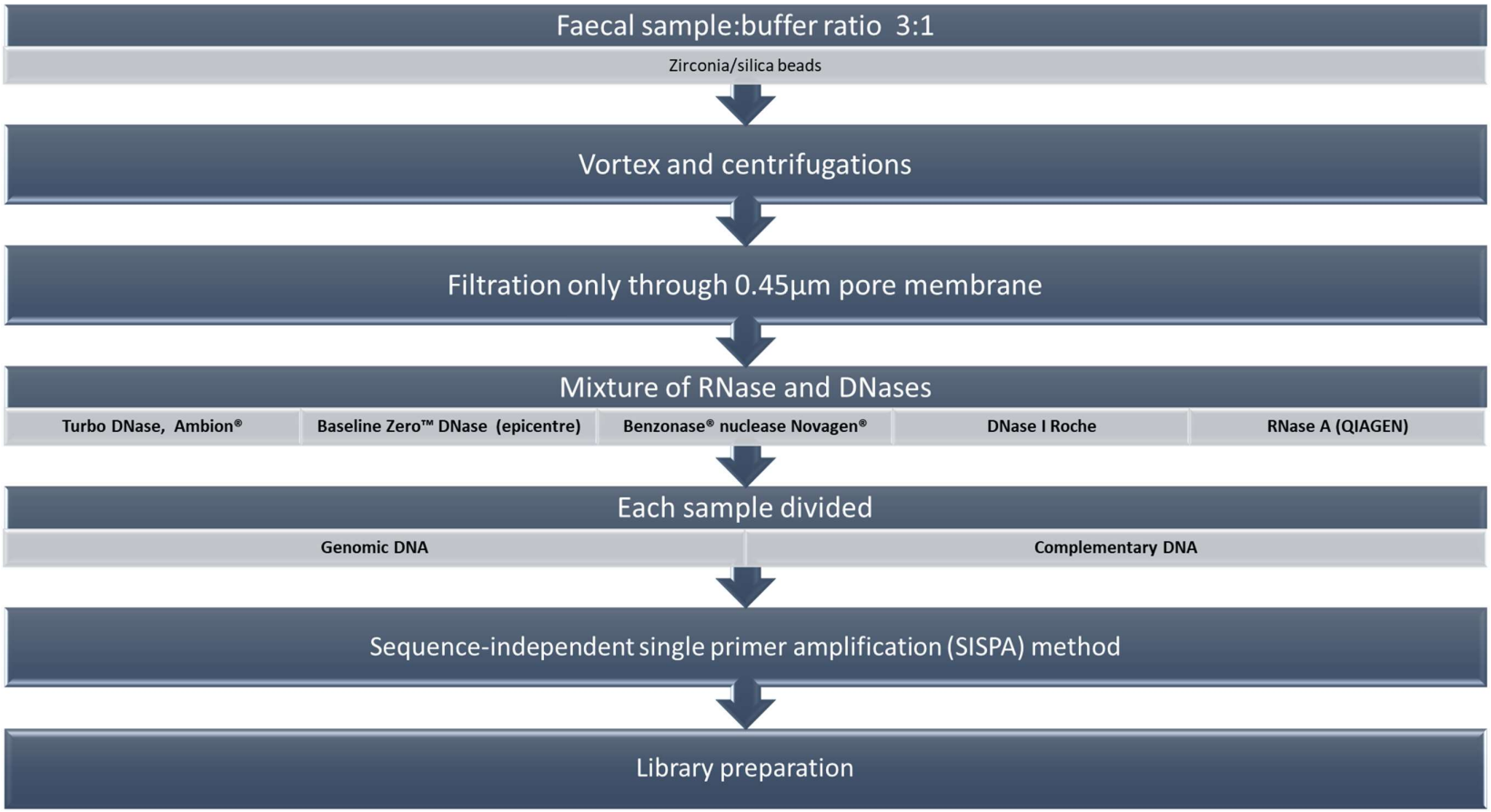

Figure 1: *Final protocol used in viral metagenomic to evaluate canine faecal samples*

2.3 Discussion

Enrichment for viral nucleic acids is essential during sample preparation to improve the subsequent identification of viral sequences and decrease contamination from other sources (host, bacteria, diet). Different protocols were performed in this study, to decrease the amount of bacterial DNA in faecal samples. After several attempts, the best result was obtained after the addition of a mixture of nucleases, which decreased the presence of bacteria according to gel electrophoresis imaging. To complete the protocol and improve the identification of viral sequences after sequencing, separation of each sample in genomic DNA and complementary DNA before random amplification, using the sequence-independent single primer amplification (SISPA) method, were performed (Fig 1). One of the advantages of this protocol is to allow identification of big viruses, through eliminating the 0.22µm filtration step, but this could also result in loss of smaller viruses. Despite this, parvoviruses were detected, being one of the smallest viruses.

Chapter 3: Characterisation of the canine faecal virome in healthy dogs and dogs with acute diarrhoea using shotgun metagenomics

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RESEARCH ARTICLE

Characterisation of the canine faecal virome in healthy dogs and dogs with acute diarrhoea using shotgun metagenomics

Paloma S. Moreno[1,2]*, Josef Wagner[1,3¤a], Caroline S. Mansfield[2], Matthew Stevens[4], James R. Gilkerson[5], Carl D. Kirkwood[1,3¤b]

1 Enteric Viruses Group, Murdoch Childrens Research Institute, Melbourne, Victoria, Australia, **2** Translational Research and Animal Clinical Trial Study (TRACTS) group, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Melbourne, Victoria, Australia, **3** Department of Paediatrics, The University of Melbourne, Melbourne, Victoria, Australia, **4** Australian Genome Research Facility, Melbourne, Victoria, Australia, **5** Centre for Equine Infectious Diseases, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Melbourne, Victoria, Australia

¤a Current address: Pathogen Genomics, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom
¤b Current address: Enteric and Diarrheal Diseases, Global Health, Bill and Melinda Gates Foundation, Seattle, Washington, United States of America
* pmmoreno@student.unimelb.edu.au

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Abstract

The virome has been increasingly investigated in numerous animal species and in different sites of the body, facilitating the identification and discovery of a variety of viruses. In spite of this, the faecal virome of healthy dogs has not been investigated. In this study we describe the faecal virome of healthy dogs and dogs with acute diarrhoea in Australia, using a shotgun metagenomic approach. Viral sequences from a range of different virus families, including both RNA and DNA families, and known pathogens implicated in enteric disease were documented. Twelve viral families were identified, of which four were bacteriophages. Eight eukaryotic viral families were detected: *Astroviridae*, *Coronaviridae*, *Reoviridae*, *Picornaviridae*, *Caliciviridae*, *Parvoviridae*, *Adenoviridae* and *Papillomaviridae*. Families *Astroviridae*, *Picornaviridae* and *Caliciviridae* were found only in dogs with acute diarrhoea, with *Astroviridae* being the most common family identified in this group. Due to its prevalence, characterisation the complete genome of a canine astrovirus was performed. These studies indicate that metagenomic analyses are useful for the investigation of viral populations in the faeces of dogs. Further studies to elucidate the epidemiological and biological relevance of these findings are warranted.

Introduction

Interest in the virome, or the entire population of viruses present in a biological sample, has increased recently due to improved availability of high throughput sequencing or next generation sequencing (NGS) technologies, and improved metagenomic analytical methods [1, 2].

data collection and analysis, decision to publish, or preparation of the manuscript.

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The virome comprises all types of viruses, including those that infect prokaryotic and eukaryotic organisms, DNA or RNA viruses, and viruses that cause acute or chronic infections. Many of these viruses are difficult or impossible to propagate in cell culture, and molecular detection is difficult as no common gene such as the ribosomal 16S gene that is present in bacterial species exists in viruses. These limitations have hindered the identification and characterisation of uncultured viruses [3, 4]. Recently, due to the advent of molecular enrichment protocols, high throughput sequencing and new metagenomic analytical methods we are now able to explore, identify and characterise viruses from different biological and environmental samples with a greater capacity [2, 5–11]

In studies of human faeces, the virome has been shown to include viruses that infect eukaryotic organisms and viruses that infect prokaryotes (bacteriophages) [2, 5, 12–18]. Bacteriophages have been reported in many studies to be the most frequently detected viral constituent in the gut of humans [1, 2, 5, 8, 16, 19, 20]. The faecal virome has been characterised for several animal species including pigs, bats, cats, pigeons, horses and ferrets [2, 6, 7, 9–11, 21–31]. In dogs, the presence of enteric viral pathogens such as canine parvovirus, coronavirus, rotavirus and distemper virus (*Paramyxoviridae*) have been identified only through targeted studies [32–35]. To date, only one published study has used high throughput sequencing to investigate the faecal viral population in diarrhoeic dogs [6]. These investigators analysed faeces from dogs with acute diarrhoea and detected two new virus species, canine sapovirus and canine kobuvirus; known canine enteric viruses such as canine coronavirus, canine parvovirus, canine rotavirus as well as plant and insect viruses were also reported [6].

The aim of this study was to describe the faecal virome of samples collected from healthy dogs, and compare these findings to the faecal virome of dogs with acute diarrhoea in Australia, using an Illumina MiSeq shotgun metagenomic sequencing approach.

Results

Overview of the canine faecal virome

A total of 16 faecal samples (8 from healthy and 8 from diarrhoeic dogs) were subjected to viral nucleic acid extraction, followed by nucleic acid enrichment, reverse transcription, random amplification and the creation of two libraries for each sample (DNA and cDNA), before being sequenced by Illumina MiSeq platform (Table 1). After sequencing, a total of 93,744,624 raw sequences were generated. All raw sequences are available in NCBI, (Bioproject ID: PRJNA380672). After trimming by quality 80,414,313 high quality reads (HQRs) were available. All sequences corresponding to dog and cellular organisms (383,785 and 27,825,631 respectively) were removed and the resultant reads were *de novo* assembled (Fig 1) generating in total 1,672,615 contigs and singletons (reads). From these contigs/singletons 1,285,171 (76.8%) had no hits in the database (S1 Table). Further analysis of contigs/singletons with no hits confirmed most sequences had no hits, while a limited number matched bacterial, human or animal sequences with a very low coverage. In addition to the contigs/singletons with no hit in the database, some contigs/singletons matching to cellular organisms and some with low complexity were identified, however, were not analysed any further (S1 Fig).

Sequences similar to twelve viral families were identified in faecal samples from healthy and diarrhoeic dogs after analyses with two different bioinformatic pipelines and comparison against viral and NCBI databases (Fig 1). Eight of these viral families infect eukaryotic organisms, and the remaining four infect prokaryotes.

Despite the known bias of SISPA in the resultant sequences after *de novo* assembly [36], we report the number of contigs/singletons matching viral families and the subsequent analysis of alignments with the lowest common ancestor according to MEGAN V5.2.1 [37].

Table 1. Summary of clinical information and contigs/singletons of eukaryotic viral families detected by metagenomic sequencing in faeces of dogs.

SAMPLE	SEX	BREED	AGE	TOTAL N° PROKARYOTICCONTIGS/ SINGLETONS	EUKARYOTIC VIRAL FAMILIES (N° OF CONTIGS/SINGLETONS DETECTED)	LOWEST COMMON ANCESTOR ACCORDING TO MEGAN (N° OF CONTIGS/SINGLETONS DETECTED)
ND4	Male Neutered	Maltese cross	7 years	1012	*Reoviridae* (39)	Rotavirus A (22)
ND5	Male Neutered	Jack Russell	2 years	209	None	None
ND6	Female	Maltese/Shih Tzu	5 years	953	None	None
ND7	Female Neutered	Staffordshire	4 years, 7months	731	None	None
ND8	Male Neutered	Labrador cross	7 years	82	None	None
ND9	Male Neutered	Mastiff cross	8 months, 1week	5	*Parvoviridae* (3)	Canine parvovirus (3)
ND10	Male	Maltese cross	8 months	2	*Coronaviridae* (912)	Alphacoronavirus 1, FIPV (76)
ND11	Male Neutered	Maltese/Shih Tzu	2 years, 6months	18	*Adenoviridae* (1)	Human adenovirus C (1)
					Papillomaviridae (1)	Human papillomavirus type 118 (1)
DD1	Male	Mastiff cross	2 years	1777	*Astroviridae* (18)	Canine astrovirus (5)
					Picornaviridae (3)	Canine kobuvirus (3)
DD2	Male	Mastiff cross	4 months	2179	*Parvoviridae* (41)	Canine parvovirus (41)
					Astroviridae (3)	Canine astrovirus (3)
DD3	Female Neutered	Wirehair Dachshund	5 years	1559	*Reoviridae* (2)	Rotavirus A (2)
					Astroviridae (3)	Canine astrovirus (3)
					Coronaviridae (4)	Alphacoronavirus 1, FIPV (3)
DD5	Male	Husky	10 weeks	1387	*Astroviridae* (3)	Canine astrovirus (3)
					Caliciviridae (8)	Canine norovirus (6)
DD7	Male	Husky	10 months	2593	*Reoviridae* (3)	Rotavirus A (3)
DD8	Female Neutered	German shorthair pointer	7 months	3230	*Parvoviridae* (3)	Canine parvovirus (3)
					Coronaviridae (211)	Alphacoronavirus 1, FIPV (36)
DD9	Female	Bull dog	4 years	3319	*Reoviridae* (5)	Rotavirus A (1)
DD10	Male	Rhodesian ridgeback	2 years	886	*Reoviridae* (4)	Rotavirus A (4)
					Astroviridae (1)	Canine astrovirus (1)

ND: normal dogs, **DD**: dogs with acute diarrhoea.

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Virome of healthy dogs

Faecal samples were collected from eight healthy dogs (Table 1). Genetic analyses identified 659,696 contigs/singletons with no hits and 3968 contigs/singletons were classified as viral, matching to five viral families that infect eukaryotes and four that infect prokaryotes. 75.9% (3012 contigs/singletons) of the total number of viral contigs/singletons were classified as bacteriophages in the healthy canine faecal virome. Bacteriophages were detected in the faeces of all dogs in this group and belonged to *Caudovirales* order and *Microviridae* family.

Viral contigs/singletons from five eukaryotic virus families were identified in faecal samples from 4 of the 8 healthy dogs (Table 1). Three out of five viral families detected were DNA viruses. *Adenoviridae* and *Papillomaviridae* were detected in a single sample containing only

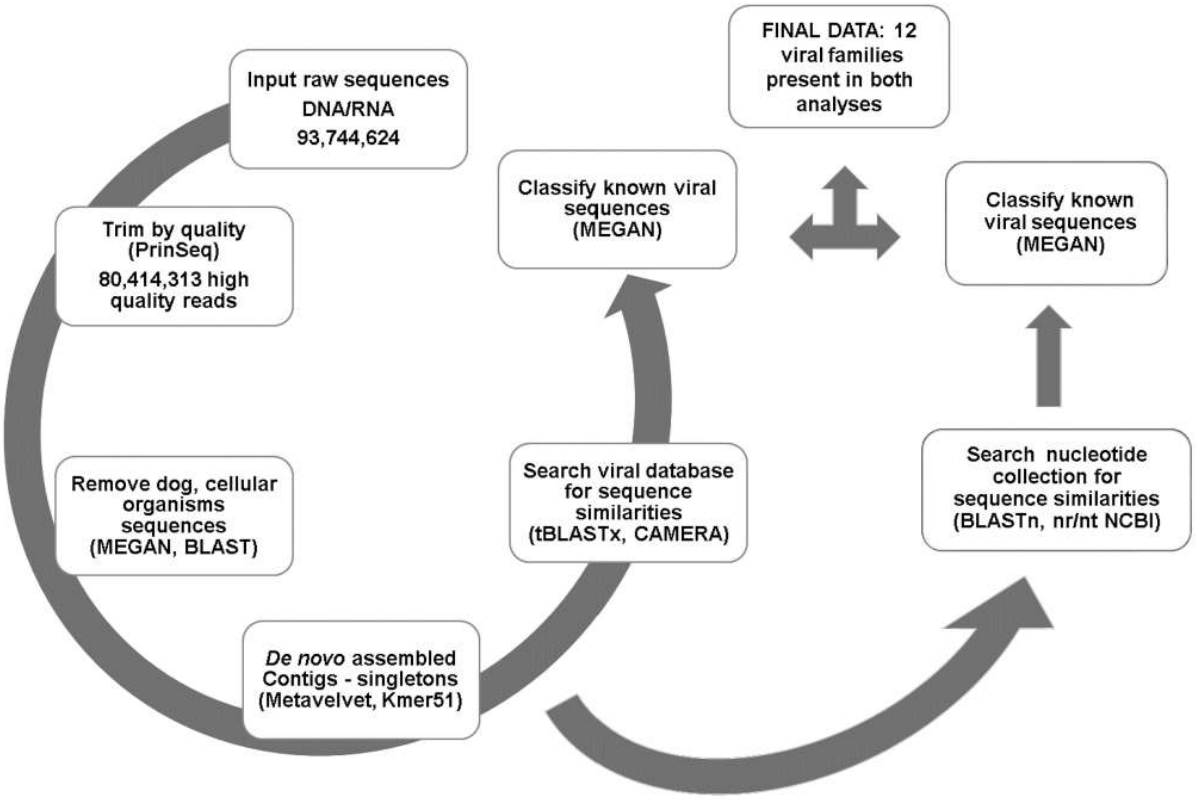

Fig 1. Summary of the bioinformatic pipelines after sequencing, showing results obtained in some steps.

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one contig/singleton each. The contig corresponding to *Papillomaviridae* family aligned with human papillomavirus type 118 (GQ246951.1), and covered 1.7% of complete genome (minimum match: 70% and minimum overlap: 30). A similar situation was identified for the Adenovirus contig. Genetic analysis revealed it matched human adenovirus C (NC_001405.1) and only covered 0.34% of the genome. This healthy individual dog sample (ND11) was the only one that had co-infection with different eukaryotic virus families in this group.

The highest number of contigs/singleton detected (n = 912) were from the family *Coronaviridae* (Table 1), however, these were all detected in one sample. After analysis, only 76 contigs/singletons matched the reference sequence of Alphacoronavirus 1 (Feline infectious peritonitis virus, NC_002306.3) and covered only 0.5% of the complete genome (minimum match: 75% and minimum overlap: 50), which represented 3.2% of FIPV_gp02 (receptor binding molecule) region.

Contigs/singletons belonging to the *Reoviridae* family were found in only one sample. Genetic analysis revealed they covered between 11%–35.5% of VP1, VP2, VP3 and VP4 genes of reference sequences of Rotavirus A (NC_011506–NC_011510).

Another eukaryotic viral family found in one healthy dog sample was *Parvoviridae*, genetic analysis of the 3 contigs/singletons showed a coverage of approximately 3.5% of the complete genome of canine parvovirus reference sequence (NC_001539), or 9.3% of the polyprotetin Ns1-Ns2.

Virome of dogs with acute diarrhoea

In eight faecal samples from dogs with acute diarrhoea, a total of 625,475 contigs/singletons had no hits and 17,242 were identified as viral contigs/singletons comprising 6 eukaryotic and 4 prokaryotic viral families (Table 1). Bacteriophages comprised 98.19% of the total of viral contigs/singletons and they were present in all individuals and were identified as belonging to order *Caudovirales* and *Microviridae* family. Eukaryotic families found in this group were *Coronaviridae*, *Parvoviridae*, *Reoviridae*, *Caliciviridae*, *Astroviridae*, and *Picornaviridae* (Table 1). The most common eukaryotic viruses identified were RNA viruses (5/6 viral families). Interestingly, all 8 samples in this group contained at least one eukaryotic family each. Co-infection was identified in 6 individual dog samples from this group. From the 8 samples from dogs with acute diarrhoea, 2 different eukaryotic virus families were detected in five samples and 3 eukaryotic families were detected in one sample (Table 1). The most prevalent family identified was *Astroviridae*, present in 5 dogs followed by *Reoviridae* present in 4 of 8 dogs with acute diarrhoea (Table 1).

Astroviridae contigs/singletons from 5 dogs were compared with reference sequence of canine astrovirus (NC_026814.1), the lowest common ancestor according to MEGAN, and they covered between 2.4% and 5% of compete genome and between 6.3% and 12.9% of the ORF2. The sample with the most contigs/singletons was later characterised.

Reoviridae contigs/singletons found in 4 dogs were compared with reference sequence of Rotavirus A (NC_011503.2) and 3 of them covered between 10.5% and 23.4% of the VP4 gene and the other sample covered 23.6% of the VP7 gene.

Furthermore, contigs/singletons matching to canine parvovirus, were found in 2 dog samples with acute diarrhoea. One of the samples covered approximately 5.8% of the complete genome of canine parvovirus reference sequence (NC_001539), corresponding to 17.6% of VP2. The other sample contained 41 contigs/singletons that matched to this same reference sequence and in total they cover 100% of VP1, 66.2% of polyprotein NS1 and NS2 (CPVgp1) and 87.9% of VP2 genes.

In this group were also found contigs/singletons matching to the *Coronaviridae* family, covering between 0.6% and 1.7% of the complete genome of reference sequence Alphacoronavirus 1 (FIPV, NC_002306.3).

One dog with acute diarrhoea contained contigs/singletons similar to a canine norovirus (JF930689.1), covering approximately 7.9% of the complete genome. Other dog sample had contigs/singletons similar to a canine kobuvirus (JN387133.1), covering 2.2% of complete genome.

Canine astrovirus characterisation

To further explore the high abundance of contigs/singletons from *Astroviridae* family in dogs with acute diarrhoea and their absence in healthy dogs, a near complete full genome of a representative canine astrovirus was generated through Sanger sequencing (DD1, Table 1). The genome encoded the complete three open reading frames (ORFs): ORF1a, ORF1b and ORF2. The total length was 6513 nucleotides, excluding the 3' poly (A) tail and the nucleotide composition was 28% A, 22% G, 26% T, 23% C. The G/C composition was 45%. The GenBank accession number for the canine astrovirus sequence is KX756441.

A phylogenetic tree was constructed using the protein alignment from the conserved region of the capsid (ORF2) of the astrovirus characterised in this study (DD1) and other canine astrovirus ORF2, together with *Mamastrovirus* sequences from different mammalian species, including a chicken astrovirus as an outgroup. The phylogenetic analysis grouped our canine astrovirus within the canine astrovirus clade. The closest canine astroviruses to our Australian sample were from UK and China with an identity between 98.82%–99.41% (Fig 2).

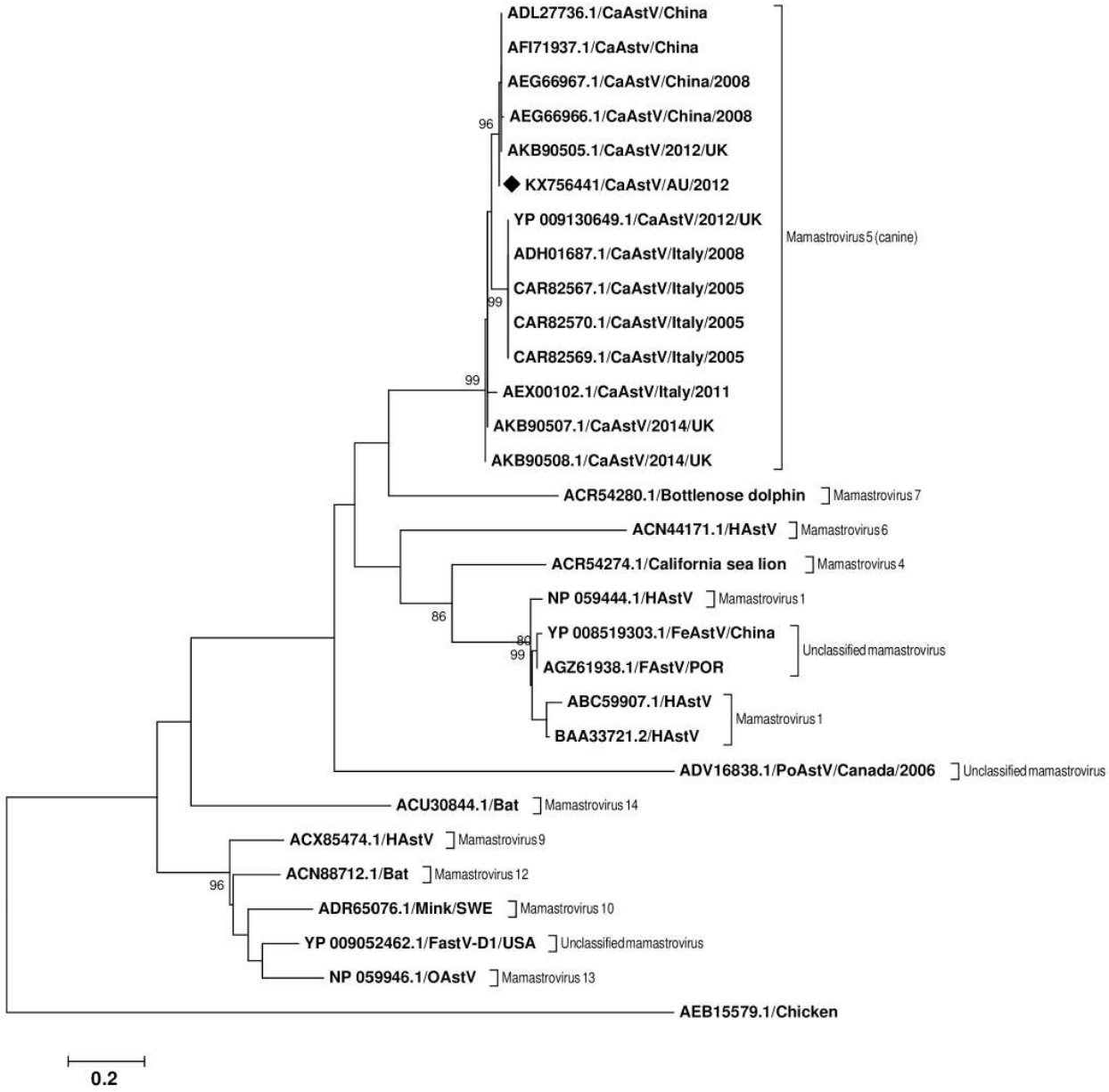

Fig 2. Phylogenetic analysis based on the conserved region of amino acid sequence of the capsid region of astroviruses from various mammalian species. GenBank accession numbers are shown for all sequences analysed and the sequence determined in this study is noted with a black diamond. The tree was constructed using the Maximum Likelihood method, based on the JTT matrix-based model with 1000 bootstrap replications. Bootstrap values $\geq$ 70% are indicated at each branch. Evolutionary analyses were conducted in MEGA6.

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We also included the phylogenetic tree made with the full length of the ORF2 as a supplementary figure (S3 Fig).

Discussion

Using next generation sequencing and metagenomics analysis, the virome in faecal samples from 8 healthy dogs and 8 dogs with acute diarrhoea is described. Only a single previous shotgun metagenomic study investigating the faecal virome of dogs with diarrhoea has been reported. In that study, mammalian viruses were found in 15 samples and two new virus species were described [6]. Our study analysed 16 faecal samples from dogs (8 healthy and 8 diarrhoeic), and identified eukaryotic viruses in 12 samples, including all diarrhoeic samples and 50% of the healthy samples. Thus, 70% of canine faeces contained eukaryotic viruses, suggesting that mammalian viruses are a common component of the enteric microbial population in dogs.

Our results must be interpreted with caution, due to bias created by SISPA. Areas of exaggerated depth appear when the SISPA method is used, creating artefacts during *de novo* assembly. This results in regions of repetitive sequences [36]. In order to overcome this bias, all contigs/singletons were analysed at family level and only for viral eukaryotic families were the results further analysed to evaluate what percentage they covered to some specific viral species.

The most common viral contigs/singletons identified in both groups were bacteriophages, similar to previous findings from human and other animal faecal virome studies [2, 5, 16, 19, 22, 27, 30]. Bacteriophages belonging to families *Myoviridae*, *Siphoviridae* and *Podoviridae* (dsDNA viruses from order *Caudovirales*) and ssDNA family *Microviridae* were identified, which is similar to other studies on faeces from humans [2, 5, 16], cats [31], horses [29], sea lions [24], pine martens and European badgers [26], ferrets [21] and small carnivores [11].

Bacteriophages modify diversity of bacterial populations due to their lytic life cycle and also promote different characteristics in the bacterial population due to their lysogenic life cycle transferring genes such as encoding toxins or resistance to antibiotic [38]. This life-cycle may lead to bacteriophages conferring advantage to some bacterial species in the environmental niche [39]. Therefore, it is possible that the greater amount of contigs/singletons corresponding to bacteriophages identified in the group of dogs with acute diarrhoea, when compared to healthy dogs, means a higher amount of bacteriophages. If so, bacteriophages could have generated a change in the normal balance of bacterial population resulting in dysbiosis, and ultimately causing diarrhoea. Conversely, it could be that an initial change in the bacterial population in these dogs resulted from the acute diarrhoea [40, 41] is the cause of the variation in the bacteriophage population. In our sample population, the latter explanation is most likely, because in a shelter environment a higher number of circulating pathogens, changes in diet and a stressful environment could contribute to the dysbiosis associated with acute diarrhoea [42]. The bacterial microbiome and the analysis of contigs/singletons matching specific bacteriophages were not assessed in this study, therefore further microbiome/virome cross analysis is necessary to elucidate the association between bacteria and bacteriophages in dogs. However, even this analysis would be unlikely to determine the cause or effect relationship between bacteriophages and dysbiosis at a single point in time.

The analysis of the lowest common ancestor of eukaryotic viral families, according to MEGAN, identified eight eukaryotic virus species (Table 1). However, each of these results require validation by targeted PCR, or whole genome characterisation of each species, as the NGS results after SISPA amplification may be biased and not accurate depiction at a species level [36].

Sequences matching those of human viruses (adenovirus and papillomavirus) were found in one sample from a healthy dog. Only one contig from each virus covering a very small

percentage of the genome in both cases. This finding could suggest contamination during collection or processing.

Known enteric pathogenic families *Parvoviridae* and *Coronaviridae* were identified in samples from both healthy dogs and dogs with acute diarrhoea. Interestingly, almost all positive samples were from puppies (between 4–8 months) that had been vaccinated less than one month prior to sampling. The lowest common ancestry analysis in MEGAN of the contigs/singletons matching *Parvoviridae* family, suggested they were canine parvovirus (CPV), but as CPV positive dogs (as tested by faecal antigen tests) were not included in this study it is highly likely these results represent vaccine derived sequences not detected by the CPV antigen detection kit, or represent a virus load below the level of detection. Previous studies have demonstrated that modified live vaccine virus can be detected in faecal samples for extended periods of time after vaccination [43]. Further genome characterisation of these canine parvovirus is warranted to confirm this hypothesis.

Three individual samples contained *Coronaviridae* contigs/singletons, two of which were from puppies [ND10 and DD8] (Table 1) and one from an adult dog [DD1] (Table 1). Our results are consistent with Li et al 2011, who also reported the highest number of *Coronaviridae* reads in one sample collected from a puppy [6]. Canine coronavirus can be shed in faeces in high numbers for up to 156 days [44, 45]. These findings validate the affinity of the *Coronaviridae* viral family to infect young individuals [46], and present as a common enteric pathogen in a shelter environment [42, 45, 47].

The uncommon viruses, canine kobuvirus and canine norovirus, were identified only in samples from dogs with acute diarrhoea. Previous studies have suggested these viruses may have some association with enteric disease in dogs, however, both viral species have been detected in both healthy dogs and dogs with diarrhoea [6, 48]

Our shotgun metagenomic sequence data indicated that the most frequent RNA viral family in dog samples with acute diarrhoea was *Astroviridae*, being identified in more than half of the diarrheal samples. [49]. In dogs, astrovirus has been previously detected mainly in puppies with diarrhoea, but has also been occasionally reported in healthy dogs [50–54]. The only previous report of a possible canine astrovirus in Australia was described in canine faeces in the 1984, where astrovirus–like particles were detected using electronic microscopy in healthy dogs [55].

To date, canine astrovirus has been reported in USA [56], China [51], Italy [50, 57, 58], UK [52], France [53], Brazil [59], Korea [60] and Japan [54]. The first description of the complete genome of two canine astroviruses was reported by a group of researchers from the UK in 2015 [52]. The current study contributes the first description of the complete genome of a canine astrovirus identified in Australia. In our study, using Sanger sequencing a near complete genome of a canine astrovirus was assembled from one dog with acute diarrhoea. A phylogenetic tree, analysing the capsid region (ORF2) of this Australian canine astrovirus and other astrovirus sequences present in GenBank, determined that it belonged to the canine astrovirus clade, very closely related to the canine astrovirus strains from the UK and China (Fig 2).

It is interesting to note that all canine astrovirus positive samples, were collected from the same shelter and obtained within a short period of time (Sept–Nov 2012). We could infer that this virus was endemic at that time in that shelter, and or maybe could represent an outbreak of diarrhoea in the shelter within that period of time. A more sensitive test (i.e.: quantitative PCR) in a larger number of samples from cases and controls may be useful to better understand the potential role of astroviruses as an aetiological agent in acute diarrhoea of dogs.

Conclusion

In this study we analysed the faecal virome in healthy dogs and compared these findings with the faecal virome of dogs with acute diarrhoea. Known DNA and RNA viruses were found, together with different proportions of bacteriophages in each group. In addition, we described and characterised the first complete genome of a canine astrovirus in Australia. Future longitudinal studies analysing viruses, bacteria and other potential pathogens should be performed to assess the aetiology of diarrhoea in dogs and further elucidate the pathological importance of viruses found in dog intestines.

Material and methods

Animals and sample collection

Faecal samples from a total of 16 dogs were obtained between September 2012 and March 2013. All dogs were aged between 2.5 months and 7 years; and comprised 5 females and 11 males of various breeds (Table 1).

All faecal samples were collected from a single shelter in Melbourne (Lost Dogs Home), Australia. All samples were maintained at 4˚C for up to 24 hrs, then were transported on dry ice before storing up to five aliquots of 500 mg of faeces each at -80˚C until further analyses. Information about age, sex, breed, diet, vaccination and deworming status was recorded for each dog (University of Melbourne Animal Ethics Committee approval IDs 1413272.2 and 1112035.1).

Animals were determined to be healthy based on physical examination by a veterinarian and absence of any clinical signs of disease. Faecal consistency was considered normal as per published criteria (Faecal scoring chart, PURINA), and all dogs had been treated with deworming drugs for prophylaxis (Ilium Pyraquantal, TROY or Milbemax, Novartis). All samples were lifted from the floor, first thing in the morning before cleaning, during November 2012.

Faecal samples from 8 dogs with an acute onset of diarrhoea (less than 3 days of duration), were collected by a veterinarian from within the animal's enclosure. All dogs with acute diarrhoea were tested for the presence of canine parvovirus antigen in faeces using the Anigen rapid CPV/CCV Ag Test kit, (Bionote). Positive samples were excluded from the study.

None of the dogs had been treated with antimicrobial drugs within the previous 8 weeks of sample collection. The majority of healthy dogs were receiving commercial dry food and some of the dogs with diarrhoea were being fed a high-fibre prescription veterinary diet (Hill's i/d diet).

Sample preparation and faecal extract preparation

Faecal samples were processed as described previously [6, 12]. Briefly, aliquots of 500 mg of faecal sample were thawed and re-suspended in saline buffer (0.01M Tris solution (pH7.5), 0.15M NaCl, 0.01M $CaCl_2$) at 3:1 ratio of solid mass. One mm zirconia/silica beads were added to the stool solution, filling around 150μL of an Eppendorf tube, and vortexed vigorously for 3 minutes. The samples were then centrifuged at 17900 x *g* for 5 min, collecting the supernatant and repeating this step three more times. To reduce solid faecal matter and bacterial contamination, 500 μl of this solution was filtered through a 0.45 μm tube filter (Corning Costar Spin X) by centrifugation at 3800 x *g* for 5 minutes, then the filtrate was transferred to 2 mL tubes.

Pre-extraction nucleic acid digestion

To enrich for viral DNA and RNA, a DNase/RNase step was incorporated using a modified protocol described previously [6, 12]. Each sample was treated with a cocktail of DNases

(Turbo DNase, from Ambion, Baseline-ZERO from Epicentre, Benzonase from Novagen and DNase I from Roche) and RNase A (QIAGEN). This mixture was incubated in a water bath at 37˚C for 3 hours. To stop the enzymatic activity, EDTA (AMRESCO) was added in a final concentration of 15 mM to each sample and incubated at 75˚C for 10 min.

Nucleic acid extraction and reverse transcription

Viral DNA/RNA protected from digestion within viral capsids were extracted using QIAamp Viral RNA mini kit (QIAGEN), according to manufacturer's recommendations. A second DNase/RNase step was performed on the extracted viral RNA for elimination of genomic DNA, using DNase I recombinant, RNase free (10U/μl) (Roche) and Protector RNase inhibitor (40 U/μl) (Roche). After digestion of the DNA, the viral RNA was transcribed with Sensiscript Reverse Transcriptase kit (QIAGEN; Sensiscript RT kit) to generate cDNA, according to manufacturer's instructions with minor modifications. Briefly, for a more sensitive detection in the subsequent PCR, a mixture of oligo-dt primers (Oligo (dT)15 primer, Promega) and random primers (Random hexamers, TaqMan Reverse Transcription Reagents, Roche, Applied Biosystems) were used and a RNA denaturation step (95˚ for 3 minutes) was added.

Random amplification, Sequence-Independent Single Primer Amplification (SISPA) method

Viral cDNA and genomic DNA were randomly amplified using a modified SISPA protocol [61, 62]. Briefly, a second strand synthesis was performed with Large (Klenow) Fragment (New England Biolabs) and random hexamers (Roche, Biosystems, 50μM) followed by digestion of the second strand product with the restriction enzyme *CviQI* (Csp6.1), (New England Biolabs). Then a *CSp11*/*NBam24* adaptor was ligated to the digested DNA using T4 DNA ligase (Invitrogen) followed by PCR amplification of the adaptor-ligated product with NBam24 PCR primers. An aliquot of the PCR product was validated on a 1% agarose TBE gel, where a positive smear with multiple bands confirmed the random SISPA amplification of nucleic acid products.

Viral library preparation and sequencing

The amplified PCR products were cleaned up using WIZARD SV Gel and PCR clean-up system (Promega) following manufacturer's recommendations and two libraries with dual indexing for each sample were generated (DNA and cDNA) with Illumina Nextera XT DNA Sample Preparation kit, according to manufacturer indications. After visualise it with Agilent 2200 Tape Station System (Agilent Technologies), the libraries were submitted to the Australian Genome Research Facility (AGRF) for a 250 bases paired-end sequencing on the MiSeq Illumina platform.

Bioinformatic analyses

All raw sequences were deposited under Bioproject ID: PRJNA380672 at NCBI database. Raw sequences were trimmed by quality score with PrinSeq software (v0.20.3) [63], filtering for low quality reads from both ends using the DUST score [64] with a threshold of 7. Poly A/T tails in both ends (ten nucleotides of each end) and SISPA primers sequences were also removed using this software. The Mothur software v.1.31.2 [65] was applied and the sequences were trimmed again, eliminating homopolymers, ambiguous bases and sequences less than 100bp. After these trimming steps, high quality reads (HQR) were obtained and all bad quality reads were removed from the group file (Fig 1).

The HQR were then compared against a dog chromosome database (CanFam3.1) using the BLASTn (Blast 2.2.29+ standalone) algorithm with an 80% identity cut off. The BLASTn files were analysed by MEGAN V5.2.1 [37] and all dog sequences were removed using Mothur v.1.31.2.

Subsequently, these dog free sequences were compared against a bacterial database (CAMERA prokaryotic nucleotide database 10572.V7, Nov 2012; http://camera.calit2.net/) [66] to eliminate bacterial sequences, using the BLASTn (Blast 2.2.29+ standalone) algorithm with an 80% identity cut off. To extract cellular organism sequences from the group file, MEGAN V5.2.1 and Mothur software were used as described above (Fig 1).

The host and bacteria free sequence reads, were *de novo* assembled with MetaVelvet (velvet 1.2.08, KMER51) [67] using Kmer size 51 and contigs and singletons were created. These singletons were clustered with a 98% similarity using CD-HIT-est (version.4.5.4 2011) [68] (Fig 1).

All contigs and singletons clusters were analysed through two pipelines. (1) Contigs and singletons clusters were compared against the CAMERA Viral Nucleotide Sequence database 10570.V9, using tBLASTx search with an E-value cut off 10^{-5}; (2) Contigs and singletons clusters were compared against the NCBI nucleotide database (2012) using BLASTn search with an E value cut off 1. All blast searches were performed in Blast 2.2.29+ standalone. These files were then analysed by MEGAN V5.2.1 [37] and the lowest common ancestor of known viral sequences were identified (Fig 1).

Finally, all viral contigs and singletons of eukaryotic organisms present in both analyses were aligned and compared with the NCBI reference sequence of the lowest common ancestor given by MEGAN V5.2.1. All alignments were made using Sequencher version 5.0.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA) with minimum match percentage of 70%–80% and minimum overlap 50 as assembly parameters, evaluating the percentage of coverage of the genome.

All contigs and singletons with no hits were re-evaluated using online BLASTn with an E value cut off 10 and visualised with MEGAN V5.2.1 to evaluate the alignment with its lowest common ancestor.

Sequencing of canine astrovirus genome

In order to acquire the complete genome of canine astrovirus, multiple sets of primers were selected from the literature or designed based on sequences obtained from Illumina reads (S2 Table). Nucleic acids from a single faecal sample from a dog with acute diarrhoea (DD1), which had 18 contigs/singletons of canine astrovirus (after tBLASTx analysis) was used to determine the complete genome sequence. RNA was extracted directly from the centrifuged sample after faecal extraction, previous to enrichment of viral nucleic acids as outlined before.

RT-PCR was performed with SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen™). PCR conditions used were: 45˚C for 60 min and 95˚C for 5 min, 35 cycle of 94˚C for 40 sec, 55˚C for 1min and 72˚C for 5 min, and a final elongation step of 72˚C for 5 min, followed by final hold at 4˚C. PCR products were run on a 1.2% agarose TBE gel stained with RedSafe™ nucleic acid staining solution (iNtRON Biotechnology). All PCR products were excised and cleaned up with WIZARD SV Gel and PCR clean-up system (Promega) following manufacturer's protocol and sequenced using Sanger sequencing at the AGRF.

The near complete genome of the canine astrovirus was assembled using Sequencher version 5.0.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA) with minimum match percentage 80 and minimum overlap 50 as assembly parameters.

Phylogenetic analysis

Phylogenetic analysis of this canine astrovirus was performed aligning protein sequences of the 172 conserved amino acids of the capsid region (ORF2) from different species (S2 Fig), using CLUSTAL W, from MEGA version 6.0 [69] with default settings. A phylogenetic tree with 1000 bootstrap was generated using the Maximum likelihood method based on the JTT matrix-based model [70], using MEGA version 6.0. The percentage of identity was calculated with CLUSTALO 1.2.4 [71]

Supporting information

S1 Fig. MEGAN taxonomic tree showing distribution and number of contigs/singletons in each sample.
(PDF)

S2 Fig. Multiple alignment of ORF2, conserved region. Disagreements to consensus sequence are highlighted.
(PDF)

S3 Fig. Phylogenetic analysis based on the full length amino acid sequence of the capsid region of astroviruses from various mammalian species.
(PDF)

S1 Table. Number of reads at each step during the bioinformatic pipeline; number and classification of contigs/singletons; and minimum (Min), mean and maximum (Max) size of contigs/singletons for each eukaryotic viral family.
(PDF)

S2 Table. Oligonucleotides used in characterisation of canine astrovirus.
(PDF)

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Author Contributions

Conceptualization: JW CSM CDK.

Formal analysis: PSM.

Funding acquisition: CSM.

Investigation: PSM MS.

Methodology: JW.

Project administration: CDK CSM.

Resources: CSM.

Supervision: CSM CDK.

Visualization: PSM.

Writing – original draft: PSM.

Writing – review & editing: JW CSM MS JRG CDK.

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Table S1: number of reads at each step during the bioinformatic pipeline; number and classification of contigs/singletons; and minimum (Min), mean and maximum (Max) size of contigs for each eukaryotic viral family

Sample name	Paired-end file names	Nº of raw sequences	High Quality Reads (HQR) Trimmed by quality	Dog sequences extracted	HQR cellular and viral sequences (Dog free sequences)	Cellular organisms sequences extracted	HQR viral sequences Dog and cell. org. free
ND4	ND4_DNA_R1	1311100	1134390	1605	1132785	923407	209378
	ND4_RNA_R1	1866166	1573342	3903	1569439	1353026	216413
	ND4_DNA_R2	1311100	1158643	1583	1157060	938453	218607
	ND4_RNA_R2	1866166	1547022	4489	1542533	1314009	228524
ND5	ND5_DNA_R1	1736359	1536868	3729	1533139	1249437	283702
	ND5_RNA_R1	1990867	1795532	4557	1790975	1611306	179669
	ND5_DNA_R2	1736359	1500716	3332	1497384	1206251	291133
	ND5_RNA_R2	1990867	1667959	4393	1663566	1480099	183467
ND6	ND6_DNA_R1	815204	743183	1434	741749	461093	280656
	ND6_RNA_R1	1645140	1493204	8841	1484363	1353584	130779
	ND6_DNA_R2	815204	750404	1701	748703	459550	289153
	ND6_RNA_R2	1645140	1347374	8192	1339182	1207044	132138
ND7	ND7_DNA_R1	1198122	1054259	117	1054142	934316	119826
	ND7_RNA_R1	1025856	952260	1207	951053	871329	79724
	ND7_DNA_R2	1198122	1082375	129	1082246	956680	125566
	ND7_RNA_R2	1025856	908242	1253	906989	826788	80201
ND8	ND8_DNA_R1	1465187	1314951	803	1314148	177130	1137018
	ND8_RNA_R1	1786048	1512166	147300	1364866	277672	1087194
	ND8_DNA_R2	1465187	1343643	808	1342835	148393	1194442
	ND8_RNA_R2	1786048	1568078	148497	1419581	245946	1173635
ND9	ND9_DNA_R1	1208162	1062399	1908	1060491	55277	1005214
	ND9_RNA_R1	1727061	1470574	85947	1384627	502996	881631
	ND9_DNA_R2	1208162	1065512	2031	1063481	25847	1037634
	ND9_RNA_R2	1727061	1428391	80566	1347825	425831	921994
ND10	ND10_DNA_R1	1164583	1004863	174	1004689	58792	945897
	ND10_RNA_R1	2065597	1820777	89560	1731217	788411	942806
	ND10_DNA_R2	1164583	1070826	169	1070657	33792	1036865
	ND10_RNA_R2	2065597	1706077	84174	1621903	643891	978012
ND11	ND11_DNA_R1	1657053	1392154	8164	1383990	514156	869834
	ND11_RNA_R1	1594165	1365243	18018	1347225	701964	645261
	ND11_DNA_R2	1657053	1383952	8381	1375571	441348	934223
	ND11_RNA_R2	1594165	1265384	18037	1247347	595568	651779
DD1	DD1_DNA_R1	927568	811717	129	811588	457413	354175
	DD1_RNA_R1	1403863	1249563	119	1249444	1231056	18388
	DD1_DNA_R2	927568	802403	132	802271	428809	373462
	DD1_RNA_R2	1403863	1201423	99	1201324	1190116	11208
DD2	DD2_DNA_R1	1377334	1207914	61	1207853	50603	1157250
	DD2_RNA_R1	1780885	1516340	274	1516066	1489247	26819
	DD2_DNA_R2	1377334	1145968	56	1145912	29040	1116872
	DD2_RNA_R2	1780885	1480746	313	1480433	1461423	19010
DD3	DD3_DNA_R1	490858	426251	160	426091	193750	232341
	DD3_RNA_R1	955369	857384	603	856781	831042	25739
	DD3_DNA_R2	490858	422577	174	422403	184050	238353
	DD3_RNA_R2	955369	848432	696	847736	826371	21365
DD5	DD5_DNA_R1	1052108	951196	79	951117	927185	23932
	DD5_RNA_R1	1544169	1323961	369	1323592	1299445	24147
	DD5_DNA_R2	1052108	860548	69	860479	842992	17487
	DD5_RNA_R2	1544169	1328612	416	1328196	1309927	18269
DD7	DD7_DNA_R1	1565728	1308876	724	1308152	673563	634589
	DD7_RNA_R1	2101608	1803874	287	1803587	1773208	30379
	DD7_DNA_R2	1565728	1189953	724	1189229	581705	607524
	DD7_RNA_R2	2101608	1679440	292	1679148	1655439	23709
DD8	DD8_DNA_R1	1629252	1366799	1688	1365111	682270	682841
	DD8_RNA_R1	2127325	1885864	472	1885392	1846841	38551
	DD8_DNA_R2	1629252	1294015	1545	1292470	568200	724270
	DD8_RNA_R2	2127325	1769648	426	1769222	1740760	28462
DD9	DD9_DNA_R1	1385737	1251037	114	1250923	1151356	99567
	DD9_RNA_R1	1961904	1755877	662	1755215	1713698	41517
	DD9_DNA_R2	1385737	995565	97	995468	900714	94754
	DD9_RNA_R2	1961904	1576436	639	1575797	1541926	33871
DD10	DD10_DNA_R1	900679	815499	439	815060	416921	398139
	DD10_RNA_R1	1411255	1281734	338	1281396	1256344	25052
	DD10_DNA_R2	900679	767363	413	766950	362251	404699
	DD10_RNA_R2	1411255	1216535	347	1216188	1200225	15963
Total		93744624	80414313	757958	79656355	53601276	26055079

* R1: paired end 1
§ R2: paired end 2

Nº total contigs/singletons *de novo* assembly R1* + R2§ + DNA + RNA	Nº total contigs/singletons having no hits	Nº total viral contigs/ singletons	Nº prokaryotic viral contigs/ singletons	Nº eukaryotic viral contigs/ singletons	Contig sizes of eukaryotic viral families																				
					Adenoviridae			Papillomaviridae			Reoviridae			Parvoviridae			Astroviridae			Caliciviridae			Coronaviridae			Picornaviridae		
					Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max
64004	44668	1051	1012	39							101	251.00	865														
66919	43601	209	209	0																							
98776	67498	953	953	0																							
51350	36074	731	731	0																							
163899	90422	82	82	0																							
122239	96287	8	5	3										251	251	251											
106884	85702	914	2	912																			101	209	251		
227873	195444	20	18	2	129	129	129	192	192	192																	
103195	87031	1798	1777	21													112	199	263							154	184	221
162010	141255	2223	2179	44										101	240	519	193	270	365								
64487	50642	1568	1559	9							208	228	248				193	220	244				113	236	292		
20379	6426	1398	1387	11													199	219	251	196	267	493					
128635	110895	2596	2593	3							125	191	251														
144578	118010	3444	3230	214										242	248	251							101	222	380		
52034	31718	3324	3319	5							138	216	251														
95353	79498	891	886	5							202	284	433				126	126	126								
1672615	1285171	21210	19942	1268																							

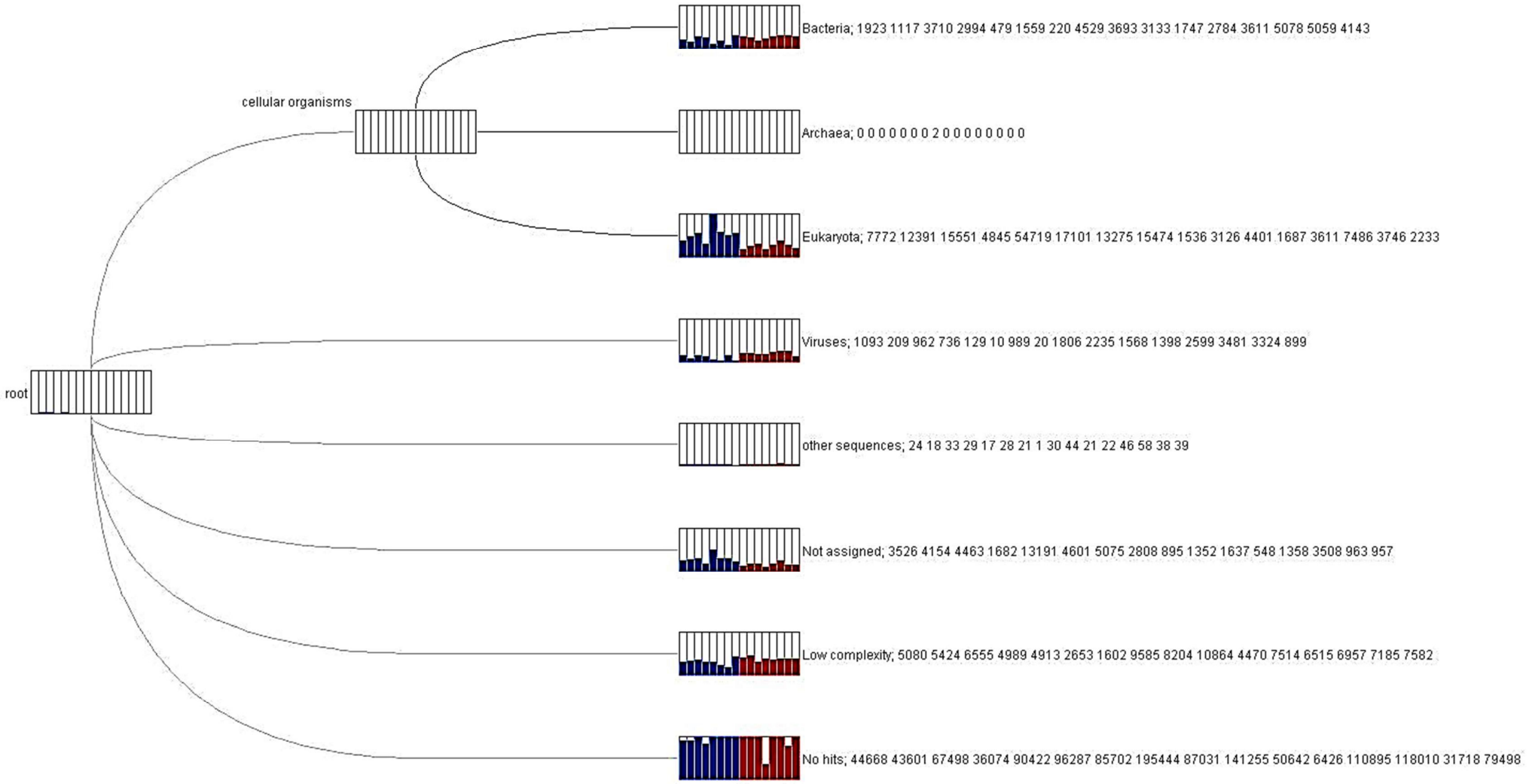

Figure S1: MEGAN taxonomic tree showing distribution and number of contigs/singletons in each sample. Numbers next to the charts indicate the numbers of contigs/singletons in each sample at every taxon. Root: contigs/singletons of all samples after *de novo* assembly. Blue columns: healthy samples, Red columns: dogs with acute diarrhoea samples.

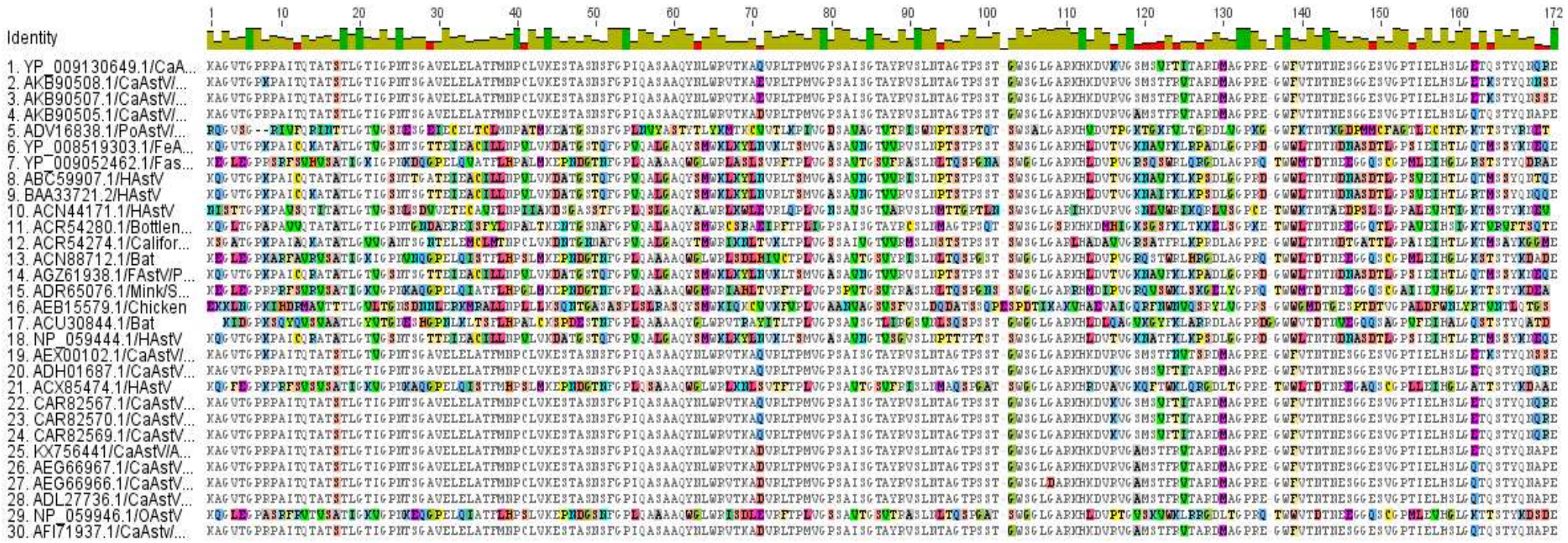

Figure S2: Multiple alignment of ORF2, conserved region. Disagreements to consensus sequence are highlighted.

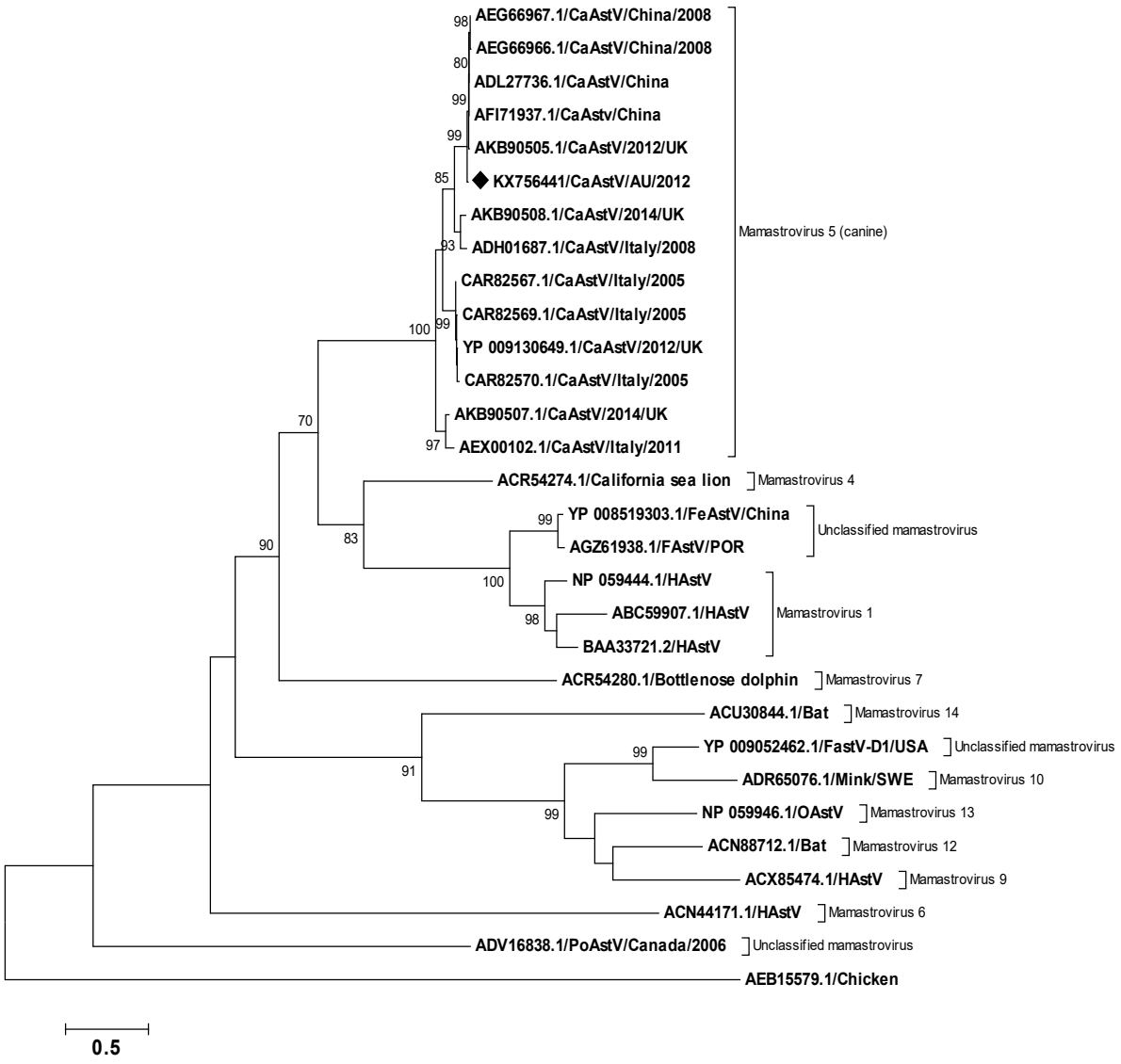

Figure S3: Phylogenetic analysis based on the full-length amino acid sequence of the capsid region of astroviruses from various mammalian species. GenBank accession numbers are shown for all sequences analysed and the sequence determined in this study is noted with a black diamond. The tree was constructed using the Maximum Likelihood method, based on the JTT matrix-based model with 1000 bootstrap replications. Bootstrap values ≥70% are indicated at each branch. Evolutionary analyses were conducted in MEGA6.

Table S2: Oligonucleotides used in characterisation of canine astrovirus

Oligonucleotide	Sequence	Reference
625F-1	GTACTATACCRTTCTGATTTAATT	(325)
626R-1	AGACCAARGTGTCATAGTTCAG	(325)
501F20	CTAACAATCGTGGTCGCAAG	(179)
1156R21	TTGATTTGTGCATCCTTGTCC	(179)
ORF1a	TGAAGGACTGCTCAGAGTG	
PM_ASTRO_DD1_1F	CCAAGAGTTGGTTTGGGTGATTAAA	This study
PM_ASTRO_DD1_349R	GCACTCTGAGCAGTCCTTCA	This study
PM_ASTRO_DD1_1201F	CTGATGTCCTCTGTCGGTAACAA	This study
PM_ASTRO_DD1_3185R	TGCTCCGGACATAATCCTTGAA	This study
PM_ASTRO_DD1_3527F	TGCCGAACAGAGGAGGAAAT	This study
PM_ASTRO_DD1_4908R	GGGAGCATTCTGGTAGGTGG	This study
PM_ASTRO_DD1_4357F	CGTGGTCGCAAGAGAGTTGA	This study
PM_ASTRO_DD1_5854R	AGTTGGAGTAGCGTATCTGGC	This study
PM_ASTRO_DD1_5525F	AATACCAGCAGAACCCACCG	This study
PM_ASTRO_DD1_6526R	AAAAGAAAAGAGTGAAAGTGAACCT	This study

Chapter 4: Characterization of the faecal virome in dogs with chronic enteropathy

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Characterization of the fecal virome in dogs with chronic enteropathy

Paloma S. Moreno[a,d,*], Josef Wagner[a,b,1], Carl D. Kirkwood[b,2], James R. Gilkerson[c], Caroline S. Mansfield[d]

[a] Enteric Viruses Group, Murdoch Children's Research Institute, VIC, Australia
[b] Department of Pediatrics, The University of Melbourne, VIC, Australia
[c] Centre for Equine Infectious Diseases, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, VIC, Australia
[d] Translational Research and Animal Clinical Trial Study (TRACTS) group, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, VIC, Australia

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ABSTRACT

The fecal virome has been investigated in humans and various animal species using next generation sequencing. However, limited information is available about the fecal virome of dogs with chronic enteropathy (CE). We aimed to characterize the canine fecal virome of dogs with CE and compare it with the virome of previously analyzed healthy dogs.A total of 16 adult dogs; 8 healthy dogs (data from a parallel study) and 8 dogs with CE had fecal samples assessed by viral shotgun sequencing. Fecal samples were subjected to enrichment of viral nucleic acids prior to sequencing and metagenomic analyses. Characterization of the complete genome of a canine kobuvirus was performed by Sanger sequencing. An additional 21 healthy dogs and 14 dogs with CE were further analyzed for the prevalence of canine kobuvirus.Three fecal samples from dogs with CE contained in total 3 eukaryotic viral families. In contrast, 4/8 fecal samples previously identified from healthy dogs, contained 5 eukaryotic viral families with 2 families exclusive to this group. Bacteriophages were identified in all fecal samples from CE and healthy dogs. Canine kobuvirus was identified in one dog with CE, by shotgun sequencing, and the complete genome was then characterized. This kobuvirus was classified within canine kobuvirus group, being similar to strains from Korea and China. The larger prevalence study did not detect additional samples positive for canine kobuvirus. The fecal virome of dogs with CE differs in number and type of viral families from healthy dogs. The first Australian canine kobuvirus sequence was identified and characterized from a dog with CE.

1. Introduction

The virome comprises all viruses, including those infecting eukaryotic and prokaryotic organisms within a biological or environmental sample. Recent improvements in molecular diagnostic techniques, have allowed better identification and discovery of known and new viruses (Delwart, 2007; Rosario and Breitbart, 2011). Viruses infecting bacteria (bacteriophages) are the predominant viral component in feces of multiple animal species (Breitbart et al., 2003; Moreno et al., 2017; Norman et al., 2015; Reyes et al., 2010). The interplay between intestinal bacteria, viruses and the host immune system has been a major focus of research in host health and disease, especially in conditions such as inflammatory bowel disease (IBD) (Babickova and Gardlik, 2015; De Paepe et al., 2014; Wagner et al., 2013).Chronic enteropathy (CE) is the clinical term for what used to be called IBD in dogs, and is characterized by gastrointestinal clinical signs that persist longer than 3 weeks without any specific pathogenic, mechanical or other extra-intestinal cause of diarrhea (Dandrieux, 2016). Canine CE has recently been classified according to the method by which clinical resolution is achieved: food-responsive (FRE) (Allenspach et al., 2007), antibiotic-responsive (ARE) (Hall, 2011) and immunosuppressant-responsive enteropathies (IRE) (Allenspach et al., 2007; Dandrieux, 2016). Dysbiosis in dogs with CE has been well established (Suchodolski, 2016), but it is unknown if there are differences in the microbiome between the types of CE, whether the changes are biologically relevant and indeed whether the dysbiosis is a cause or consequence of the inflammation (Suchodolski, 2016). Similarly, no information about the intestinal virome in dogs with CE has been published.

The purpose of this study was to characterize the virome in feces collected from dogs with CE and compare it to the fecal virome present in healthy dogs (Moreno et al., 2017). A secondary aim was to

* Corresponding author: Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, 250 Princes Hwy, Werribee, Melbourne, Victoria, 3030, Australia.
E-mail address: pmmoreno@student.unimelb.edu.au (P.S. Moreno).
[1] Dr Wagner's current address is Infection Genomics, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom.
[2] Dr. Kirkwood current address is Enteric and Diarrheal Diseases, Global Health, Bill and Melinda Gates Foundation, Seattle, Washington, United States of America.

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determine the sequence and prevalence of a unique canine kobuvirus that was identified during initial assessment by viral shot gun sequencing.

2. Material and methods

2.1. Clinical CE cases

Eight client-owned dogs were recruited into the study by U-Vet Hospital, The University of Melbourne, for investigation of chronic gastrointestinal clinical signs, vomiting, diarrhea, weight loss, for more than 3 weeks of duration. Full, written owner consent was obtained and the project was approved by the University of Melbourne Animal Ethics Committee (study ID: 1112072.2), which operates according to the National Health and Medical Research Council (NHMRC) guidelines for use of animals in research. No dog had received dietary, antibiotic or anti-inflammatory treatment in the preceding 4 weeks, and all had active clinical signs of CE at the time of assessment. Each dog had full hematology, biochemistry, including serum cobalamin and canine pancreatic lipase immunoreactivity, urinalysis, fecal analysis and abdominal ultrasound performed. After no specific abnormalities were identified, the gastrointestinal tract was assessed by upper and lower gastrointestinal endoscopy and endoscopic biopsies obtained from the stomach, duodenum, ileum and colon for histopathology. Dogs were then recruited fully into the study if no disease other than CE was observed. Treatment comprised initially of a hydrolyzed/hypoallergenic prescription veterinary diet, followed by antibiotics, and then immunosuppressives based on clinical response. Response to a treatment was defined by a 75% reduction in canine chronic enteropathy clinical activity index (CCECAI) (Allenspach et al., 2007) within 2–3 weeks of the commencement of treatment. The type of chronic enteropathy (CE) was ultimately defined by at least an 8-week reduction (> 75%) in CCECAI to either diet, antibiotic or immunosuppressive treatment.

Fecal samples from these dogs were used for metagenomic sequencing and were collected at the first visit prior to diagnostic or therapeutic interventions, between June 2010 and March 2013. All dogs were aged between 1.5 and 5 years, median 2.8 years; and comprised 3 females and 5 males of different breeds. The final CE classification was 4 FRE, 3 ARE and 1 IRE (Table 1). An additional 14 dogs with CE,7 FRE, 5 ARE and 2 IRE, (Table S1) were recruited under the same inclusion criteria as the initial 8, to specifically evaluate prevalence of canine kobuvirus (Table S1). These 14 dogs were recruited between June 2010 and Sept 2015 and fecal samples were also obtained at the first visit, from 7 males and 7 females of different breeds. The age range varied between 7 months and 11 years old, median 4.8 years.

2.2. Control dogs

Eight fecal samples from healthy dogs kept in a shelter were collected for a parallel project analyzing the fecal virome of healthy dogs and dogs with acute diarrhea (Moreno et al., 2017). These dogs were 6 males, 2 females, different breeds, age range: 6 months to 7 years, median 3.5 years (Table 1).

An additional 21 healthy dogs had fecal samples collected to evaluate the prevalence of canine kobuvirus. These samples were collected from client or staff-owned dogs from the U-Vet Hospital, between September 2014 and February 2015, from 8 males and 13 females of different breeds (Table S1). The age range was between 3 months and 15 years old (median 5 years). All healthy dogs were considered to be healthy based on absence of signs of clinical disease with no antibiotic administration for more than 6 months, and full worming and vaccination prophylaxis completed.

All fecal samples were initially stored at 4 °C before being stored at − 80 °C within 6 h of collection. Information about age, sex, breed, diet, vaccination and deworming status was recorded for each dog.

2.3. Sample preparation and fecal extract preparation

Fecal samples were processed as described previously (Moreno et al., 2017). Briefly, fecal samples were dissolved in saline buffer, vortexed and filtered through a 0.45 µm filter by centrifugation at 3800 × *g* for 5 min. Before nucleic acid extraction all samples were subjected to a viral enrichment protocol using a mixture of DNases and RNase A, as described previously (Moreno et al., 2017).The nucleic acid extraction was performed using QIAamp® Viral RNA mini kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations. Each sample was then divided into two aliquots. One aliquot was used for genomic DNA analysis and the second aliquot was used for a second DNase step to remove genomic DNA. Following the second DNase step, the leftover viral RNA was transcribed with Sensiscript Reverse Transcriptase kit (Sensiscript RT kit; QIAGEN, Hilden, Germany) to generate complementary DNA (cDNA), according to manufacturer's instructions with the same modifications as specified previously (Moreno et al., 2017).

Table 1
Summary of signalment and the contigs/singletons of prokaryotic and eukaryotic viral families detected by metagenomic sequencing in feces of dogs.

SAMPLES	AGE	SEX	BREED	DIAGNOSTIC	PROKARYOTIC VIRAL CONTIGS/ SINGLETONS	EUKARYOTIC VIRAL FAMILIES (Nº OF CONTIGS/SINGLETONS DETECTED)
HD4	7 y	MN	Maltese X	Healthy	1012	*Reoviridae* (39)
HD5	2 y	MN	Jack Russell	Healthy	209	None
HD6	5 y	F	Maltese/Shih Tzu	Healthy	953	None
HD7	4 y 7 m	FS	Staffy	Healthy	731	None
HD8	7 y	MN	Labrador X	Healthy	82	None
HD9	8 m 1 w	MN	Mastiff/Staffy	Healthy	5	*Parvoviridae* (3)
HD10	8 m	M	Maltese X	Healthy	2	*Coronaviridae* (912)
HD11	2 y 6 m	MN	Maltese/Shih Tzu	Healthy	18	*Adenoviridae* (1) *Papillomaviridae* (1)
CE1	2y 8m	FS	Labrador	FRE	214	*Papillomaviridae* (1) *Reoviridae* (2)
CE4	5y	M	Rottweiler	IRE	449	None
CE5	2y 5m	MN	Bull Terrier	FRE	227	None
CE8	1y 6m	F	German Shepherd	ARE	499	None
CE9	1y 9 m	M	Weimaraner	FRE	8777	*Picornaviridae* (9)
CE10	2y	M	Basset hound	ARE	319	None
CE11	4y 5m	MN	Labradoodle	ARE	468	*Reoviridae* (4)
CE13	5y	FS	Terrier cross	FRE	202	None

HD: healthy dogs, CE: dogs with chronic enteropathy, MN: male neutered, M: male, FS: female spayed, F: female, FRD: food responsive enteropathy, IRE: immunosuppressive responsive enteropathy, ARE: antibiotic responsive enteropathy.

2.4. Random amplification, sequence-independent single primer amplification (SISPA) method

Both viral cDNA and genomic DNA were randomly amplified using the SISPA protocol modified from Allander et al (2001) (Allander et al., 2001) and Reyes and Kim, (1991) (Moreno et al., 2017; Reyes and Kim, 1991). Briefly, a second strand synthesis was performed, followed by digestion of the second strand product with a restriction enzyme. Then an adaptor was ligated to the digested DNA followed by PCR amplification of the adaptor-ligated product.

2.5. Viral library preparation and sequencing

The amplified PCR products were cleaned up with WIZARD® SV Gel and PCR clean-up system (Promega®) following manufacturer's indications. Two libraries with dual indexing for each sample were generated with Illumina Nextera® XT DNA Sample Preparation kit and submitted to the Australian Genome Research Facility (AGRF) for a 250 base paired-end sequencing on the MiSeq® Illumina platform.

2.6. Bioinformatic analyses

Raw sequences were trimmed by quality. The final high-quality reads (HQRs) were used for screening against the dog and bacterial genomes and all dog and bacterial sequences were removed. The host and bacteria free sequences were *de novo* assembled and the resulting contigs and singletons were used for further analysis (Moreno et al., 2017). All contigs and singletons were analyzed through two different bioinformatic pipelines. Initially, contigs and singletons were compared against the CAMERA Viral Nucleotide Sequence database 10,570.V9, using tBLASTx search with an E-value cut off 10^{-5}. In the second pipeline, contigs and singletons were compared against the NCBI nucleotide database (2012) using BLASTn search with an E-value cut off 1. The tBLASTx and BLASTn files were then analyzed by MEGAN V5.2.1 (Huson et al., 2011) which identified the lowest common ancestor of known viral sequences.

Finally, all viral contigs/singletons from eukaryotic organisms present in both analyses were assembled and compared against the NCBI reference sequence to evaluate the genome coverage. Sequence analysis software (Sequencher® version 5.0.1, Gene Codes Corporation, Ann Arbor, MI USA) with minimum match percentage 70 and minimum overlap 50 as assembly parameters were used.

2.7. Canine kobuvirus study

The nucleic acid from the fecal sample from the dog with chronic enteropathy (CE9), which had 9 confirmed contigs/singletons identified as canine kobuvirus (according to MEGAN) was used to generate the complete genome sequence (Table S2)

Reverse transcription polymerase chain reaction (RT-PCR) was performed with SuperScript® III One-Step RT-PCR System with Platinum® Taq (Invitrogen™) and PCR conditions used were: 45 °C for 60 min and 94 °C for 2 min, 40 cycles of 94 °C for 45 s, 48 °C for 1 min and 72 °C for 2 min, and a final elongation step of 72 °C for 10 min, followed by final hold at 4 °C. PCR products were separated on a 1.2% agarose and Tris/Borate/EDTA buffer (TBE) gel stained with nucleic acid staining solution RedSafe™ nucleic acid staining solution (iNtRON Biothecnology). All PCR products were gel excised and cleaned up with WIZARD® SV Gel and PCR clean up system (Promega®) following manufacturer's protocol and sequenced using Sanger sequencing at AGRF. The near complete genome of the canine kobuvirus was assembled using the sequence analysis software (Sequencher® version 5.0.1, Gene Codes Corporation, Ann Arbor, MI USA) with minimum match percentage 80 and minimum overlap 50 as assembly parameters.

Phylogenetic analysis of the polyprotein region of this canine kobuvirus was performed together with its closest viral relatives (best BLAST hits). Twenty three amino acid sequences of the polyprotein region identified in GenBank were aligned using a multiple sequence alignment program CLUSTAL W, from MEGA7 version 7.0 (Kumar et al., 2016) with default settings. A phylogenetic tree with 1000 bootstrap was generated using the Maximum Likelihood method based on the JTT matrix-based model, using MEGA7 version 7.0.

The prevalence of canine kobuvirus was evaluated using a PCR targeting a fragment of the 3D region identified from the confirmed positive sample detected by metagenomic analysis (CE9). DNA and RNA from the additional 35 fecal samples (14 case and 21 control) (Table S1) were then extracted. Briefly, 800 µL of virus dilution buffer (0.01 M Tris solution (pH 7.5), 0.15 M NaCl, 0.01 M $CaCl_2$) was added to 0.2*g* of fecal material, vortexed until homogenized, centrifuged at 20,000 × g for 3 min and the supernatant harvested. The RNA was extracted from 140 µL of the supernatant using QIamp® Viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. In order to amplify a 504 bp fragment from the canine kobuvirus 3D region, two primers were used: CaKV/F and CaKV/R (Di Martino et al., 2013), and RT-PCR was performed using QIAGEN One-Step RT-PCR kit (QIAGEN, Hilden, Germany). The following PCR conditions were used: a RNA denaturation step at 97 °C for 3 min in a heat block, prior to the addition of master mix. Subsequently, all samples were placed in the thermo cycler and subjected to 45 °C for 60 min and 94 °C for 2 min, 40 cycles of 94 °C for 45 s, 48 °C for 1 min and 72 °C for 50 s, and a final elongation step of 72 °C for 10 min, followed by final hold at 4 °C. All positive samples were visualized on a 1.2% agarose TBE gel.

3. Results

3.1. Overview of the fecal virome of dogs with CE

As a result of bioinformatic analyses, over 52 million MiSeq reads were generated from the eight fecal samples from dogs with chronic enteropathy, of which 45,190,173 sequences were HQRs. From these HQR, all sequences matching to the dog genome and cellular organisms (15,640 and 42,402,966 respectively) were removed. The remaining cleaned up reads, or dog and cellular organisms free sequences, were *de novo* assembled and 290,225 contigs and singletons were generated (Table S3). Out of the 290,225 contigs and singletons, 11,173 contigs and singletons were identified as viral sequences (Table S3), comprising three viral families that infects eukaryotes, *Papillomaviridae*, *Picornaviridae* and *Reoviridae,* present only in three CE samples. Four families that infect bacteria (bacteriophages) were also identified, *Myoviridae, Siphoviridae,* and *Podoviridae* from the dsDNA order *Caudovirales* and family *Microviridae* (ssDNA), being present in all CE samples. Overall, bacteriophages comprised 99.8% (11,157 contigs and singletons) of the total number of viral contigs and singletons and were present in all CE samples (Table S3). At the genus level different bacteriophage genera were present in samples from dogs with CE. Bacteriophages that could not be classified to the genus level were also identified (Fig. 1a and b).

Further analysis of eukaryotic viruses identified that one sample (CE1, Table S1 and S3) covered 3.4% of the complete genome of Human papillomavirus type 109 (NC_012485.1) with the largest proportion identical to E1 protein (10.8%) and E2 protein (9%). *Reoviridae* contigs and singletons were found in two samples (Table S3). Further analysis identified, that these contigs and singletons covered between 13.7% and 15.4% of the VP4 gene segment of the Rotavirus reference sequence for VP4 gene (NC_011510.2). Contigs and singletons matching the *Picornaviridae* family were used to characterize the complete genome of canine kobuvirus, as explained below.

The fecal virome of eight healthy dogs has been previously described by our group (Moreno et al., 2017). The healthy dog studies identified 3968 viral contigs/singletons. Bacteriophage were found in all samples and 75.9% of viral contigs and singletons were

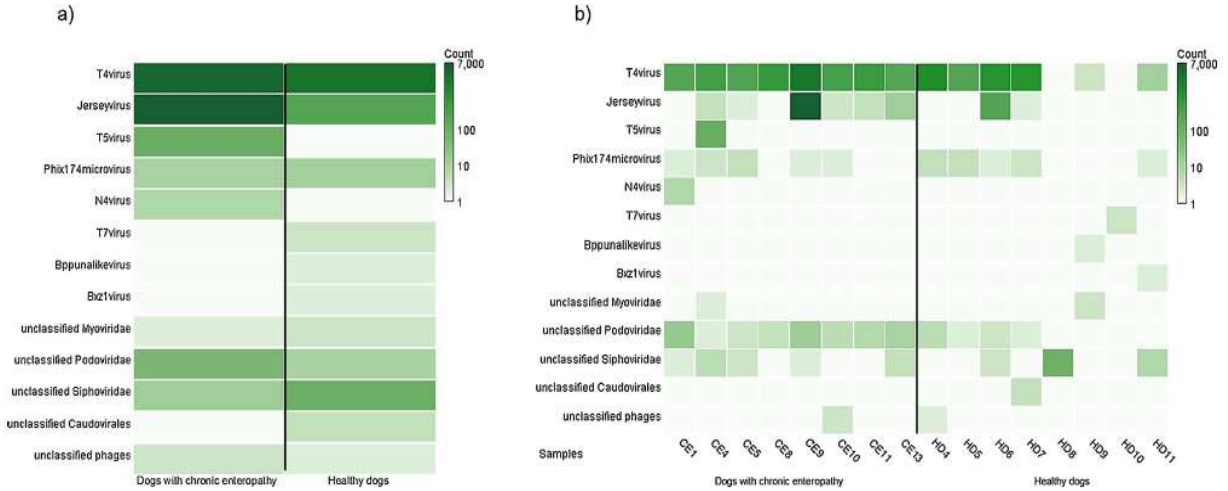

Fig. 1. Heat maps of taxonomy profiles of bacteriophage genera and unclassified bacteriophages identified in the feces of dogs with CE and healthy dogs, shown for groups (1a) and for individuals (1b). The Y axis shows bacteriophage taxa and the X axis indicates the name of the groups or individual dog identification. Colors indicate number of contigs/singletons belonging to a specific taxon in a logarithmic scale.

bacteriophages. The same bacteriophage families were detected in healthy dogs and in dogs with CE, but at the genus level there were some differences between both groups (Fig. 1a and b).Eukaryotic viral families found in the healthy group were *Adenoviridae*, *Papillomaviridae*, *Parvoviridae*, *Reoviridae* and *Coronaviridae* (Table S3). *Papillomaviridae and Reoviridae* families were also present in dogs with CE.

3.2. Canine kobuvirus characterization and prevalence

In one dog with CE we detected, by shotgun sequencing, 9 contigs/singletons which were annotated as *Picornaviridae*. This sample, a 21-month-old, male, Weimaraner dog with FRD (CE9, Table S1), was chosen for a more detailed follow up. Using Sanger sequencing the near-complete genome of a canine kobuvirus was determined. The total aligned Sanger sequenced reads generated one contig which was 8058 nucleotides, excluding the first 109 nucleotides from the noncoding region and the 3' poly (A) tail. The nucleotide composition was 19.7% A, 21% G, 22.1% T, and 37.1% C. The G/C composition was 58.1%. The genome encoded the complete large open reading frame (ORF) for a polyprotein. The total length was 7335 nucleotides, which encode the polyprotein of 2444 aa. The nucleotide composition was 19.8% A, 20.7% G, 21.9% T, and 37.7% C. The G/C composition was 58.3%.

A phylogenetic tree was constructed by multiple alignment of the full sequence of the polyprotein from the canine kobuvirus characterized in this study (Canine kobuvirus_CE9/AUS/2012, GenBank accession number: MH052678) and 23 other polyprotein kobuvirus sequences reported from different animal species (Table S4). The phylogenetic analysis revealed that the canine kobuvirus identified in our study clustered within the canine kobuvirus clade, with similarities between 96.8% and 99.14%. The most closely related sequences were those previously identified from South Korea and China (Oem et al., 2014) (Fig. 2). To evaluate prevalence of canine kobuvirus in a larger population, PCR screening was performed in all dogs used for metagenomics and extra 35 individual dogs with a similar background, 21 healthy client-owned dogs and 14 client-owned dogs with CE. As a result, only one dog was confirmed positive, the same sample already identified as positive (CE9) by shotgun sequencing.

4. Discussion

In this study we described the fecal virome from dogs with chronic enteropathy. We identified both prokaryotic and eukaryotic viruses, whereby prokaryotic viruses (bacteriophage) comprised 93.6% off all contigs/singletons. This high percentage of bacteriophages in dogs with CE is consistent with previous fecal virome studies from humans with IBD or CE (Lepage et al., 2008; Norman et al., 2015). Even though it is known that the SISPA method has some inherent bias with regions of repetitive sequences being generated (Rosseel et al., 2013), we believe that it was the best available technology at the time of the study and the overall findings are consistent and reliable.

Bacteriophages have the ability to modify bacterial populations, both in number and function (De Paepe et al., 2014) and are classified depending on their life cycle as lytic or lysogenic. In the lysogenic cycle, phages are called temperate phages or prophages, and bacteria and bacteriophages live in symbiosis (De Paepe et al., 2014). Nevertheless, bacteriophages can be induced to become lytic or pathogenic, where they then replicate and kill their host (De Paepe et al., 2014). Bacteriophages thus have the ability to transfer their genome into their host bacteria, conveying genetic information and changing the pathogenicity of gut microbial flora or ultimately, modifying bacterial populations through their virulent state (De Paepe et al., 2014).

Recent research analyzing the potential importance of bacteriophages in the immune response of the host and their influence in the dysbiosis present in humans with IBD has been published (Babickova and Gardlik, 2015; Lepage et al., 2008; Norman et al., 2015; Wagner et al., 2013).Various studies analyzing phage populations have found differences between healthy and IBD individuals (Crohn's disease, CD) (Lepage et al., 2008; Norman et al., 2015) and similarly, differences between phage populations between CD and ulcerative colitis (UC) individuals have been detected (Norman et al., 2015). Despite all these investigations it is not yet clear the exact role that phages may play. It is also possible that bacteriophages may exert a beneficial effect by conferring fitness to a subset of bacteria.

Our current study found different bacteriophage populations between dogs with CE and healthy dogs (i.e.: T7 virus, Bppunalikevirus, Bxz1virus); hence, it is possible that the difference between bacteriophage populations may be an important factor in preserving or restoring canine intestinal health.

Among eukaryotic viral sequences both, known pathogenic and nonpathogenic viral species were identified in healthy dogs and dogs with CE. Sequences identified as *Reoviridae* and *Papillomaviridae* were found in both groups and further analysis identified them as human rotavirus and human papillomavirus. Sequences representing the viral family *Picornaviridae* were identified in one dog with chronic enteropathy. After further sequence analysis, a canine kobuvirus was characterized. Previous studies using electron microscopy identified picornavirus-like particles in canine fecal samples in 1995 in Australia (Finlaison, 1995). Our study is the first description of a canine

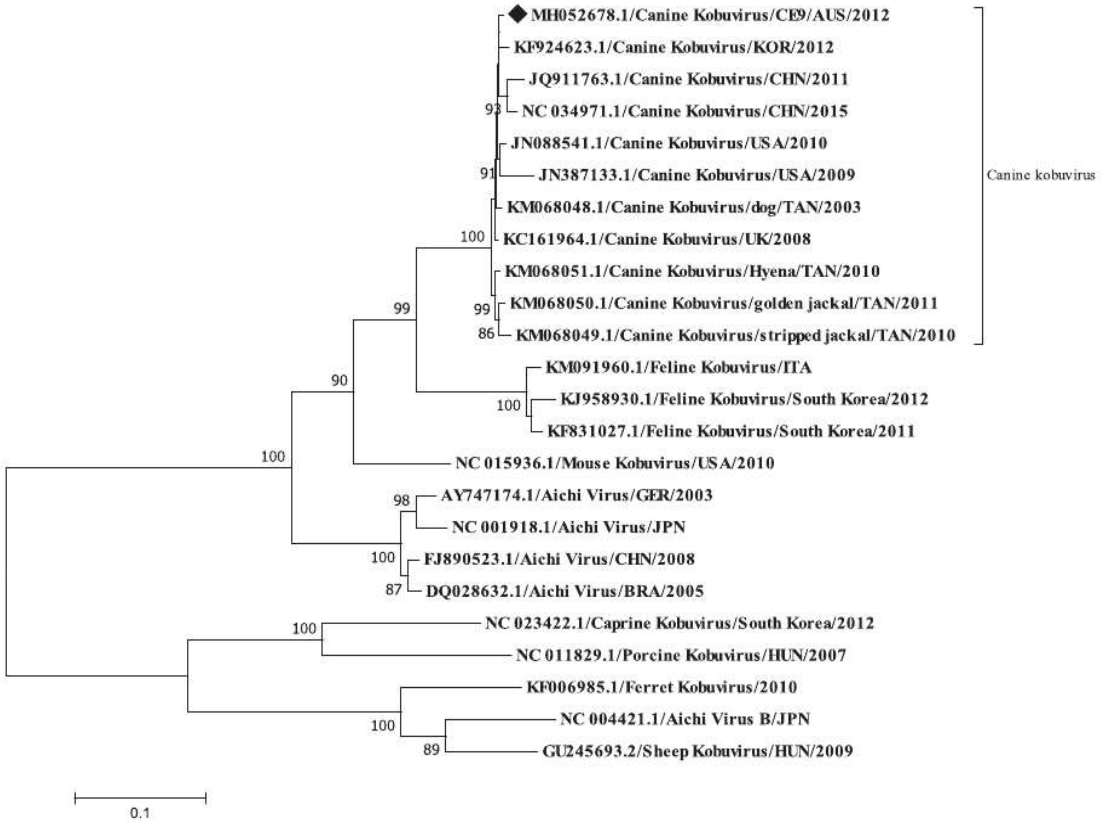

Fig. 2. Phylogenetic analysis of the polyprotein region of kobuviruses from various mammalian species. GenBank accession numbers are shown for all sequences analysed (Table S4) and the sequence determined in this study is noted with a black diamond. The tree was constructed using the Maximum Likelihood method, based on the JTT matrix-based model with 1000 bootstrap replications. Evolutionary analyses were conducted in MEGA7.

kobuvirus genome in Australia. A phylogenetic tree, analyzing the complete polyprotein region of this canine kobuvirus and other kobuvirus sequences from different species present in GenBank, showed that it belonged to the canine kobuvirus clade, close to sequences from South Korea (Oem et al., 2014) and China (Li et al., 2016).

The majority of studies evaluating canine kobuvirus have suggested that this enteric virus is more prevalent in animals younger than 2 years (Choi et al., 2014; Di Martino et al., 2013; Li et al., 2011; Oem et al., 2014). Some of these studies have identified canine kobuvirus from both healthy dogs and dogs with diarrhea (Li et al., 2011; Oem et al., 2014). Thus, canine kobuvirus has not been considered a primary causal agent of diarrhea in dogs, mostly because it is regularly found in co-infection with other known enteric viruses (Choi et al., 2014; Di Martino et al., 2013; Li et al., 2016, 2011; Oem et al., 2014). It is currently unknown whether kobuvirus may induce disease in a genetically susceptible animal or interact with bacteria to worsen inflammation. The prevalence study performed in our larger cohort of healthy dogs and dogs with CE did not found any other samples positive for kobuvirus. Further epidemiological studies in a larger cohort may help to better understand the epidemiology of this virus in the canine population and its association with disease.

Although only a small number of dogs with CE were analyzed in this study, there was a difference in the fecal virome when compared to that present in healthy dogs. As a result, it can be hypothesized that the intestinal virome is an important component of the complex interaction between the intestinal immune system, and luminal contents. Further evaluation of the type and function of bacteriophages in particular is warranted alongside evaluation of the intestinal bacteria in future studies of CE in dogs. This is of particular importance when considering the efficacy of potential therapies, including phage, probiotics and fecal microbial transplantation.

Accession number

The GenBank accession number for the Canine kobuvirus sequence is MH052678.

Declarations of interest

None.

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Appendix A. Supplementary data

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Supplementary data

Table S1: Clinical details of dogs recruited for the canine kobuvirus prevalence study.

SAMPLES	SEX	AGE	BREED	TYPE OF CHRONIC ENTEROPATHY
CE1	Female Neutered	2 years, 8 months	Labrador	Food responsive enteropathy
CE4	Male	5 years	Rottweiler	Immunosuppressant-responsive enteropathy
CE5	Male Neutered	2 years, 6 months	Staffordshire / Bull terrier	Food responsive enteropathy
CE8	Female	1 year, 6 months	German Shepherd	Antibiotic responsive enteropathy
CE9	Male	1 year, 9 months	Weimaraner	Food responsive enteropathy
CE10	Male	2 years	Basset hound	Antibiotic responsive enteropathy
CE11	Male Neutered	4 years, 5 months	Labradoodle	Antibiotic responsive enteropathy
CE13	Female Neutered	5 years	Terrier cross	Food responsive enteropathy
CE2	Male Neutered	2 years, 6 months	Great Dane	Food responsive enteropathy
CE6	Male Neutered	2 years, 8 months	Poodle	Antibiotic responsive enteropathy
CE14	Male	5 years	poodle	Steroid responsive enteropathy
CE15	Male Neutered	5 years	Border Collie	Antibiotic responsive enteropathy
CE16	Male Neutered	5 years	Golden Retriever	Food responsive enteropathy
CE17	Male	9 years	Flat coated retriever	Immunosuppressant-responsive enteropathy
CE18	Female	9 months	Maltese	Food responsive enteropathy
CE19	Female Neutered	10 years	Labrador	Food responsive enteropathy

CE20	Female Neutered	4years, 7months	Rottweiler	Antibiotic responsive enteropathy
CE21	Female Neutered	11 years	Terrier	Food responsive enteropathy
CE22	Female Neutered	11 years	German Shepherd	Food responsive enteropathy
CE23	Female	4 years	Whippet	Antibiotic responsive enteropathy
CE24	Male Neutered	9 years	Chihuahua cross	Food responsive enteropathy
CE25	Female Neutered	2 years	Greyhound	Antibiotic responsive enteropathy
HD4	Male Neutered	7 years	Maltese X	Healthy, from shelter
HD5	Male Neutered	2 years	Jack Russell	Healthy, from shelter
HD6	Female	5 years	Maltese/Shih Tzu	Healthy, from shelter
HD7	Female Neutered	4 years 7 months	Staffy	Healthy, from shelter
HD8	Male Neutered	7 years	Labrador X	Healthy, from shelter
HD9	Male Neutered	8 months 1 week	Mastiff/Staffy	Healthy, from shelter
HD10	Male	8 months	Maltese X	Healthy, from shelter
HD11	Male Neutered	2 years 6 months	Maltese/Shih Tzu	Healthy, from shelter
HD46	Male	4 years 8 months	Labrador	Healthy
HD47	Female	7 years	Leonberger	Healthy
HD48	Female	7 years	Leonberger	Healthy
HD49	Male	6 years	German Wirehaired pointer	Healthy
HD50	Female	1 years 2 months	German Wirehaired pointer	Healthy
HD51	Female Neutered	9 years	Kelpie	Healthy

HD52	Female	6 months	Havanese	Healthy
HD53	Female Neutered	5 years	Terrier X	Healthy
HD54	Female Neutered	3 years	Nova scotia duck tolling retriever	Healthy
HD55	Female Neutered	5 years	Cairn Terrier	Healthy
HD56	Male Neutered	11 years	Siberian husky cross	Healthy
HD57	Female	4 months	Kelpie X	Healthy
HD58	Male Neutered	6 years	Labrador	Healthy
HD59	Male Neutered	4 years	Miniature poodle bichon frise X	Healthy
HD60	Female Neutered	9 years	Cocker spaniel	Healthy
HD61	Male Neutered	8 years	Cattledog X	Healthy
HD62	Female Neutered	4 years 6 months	Kelpie	Healthy
HD63	Female Neutered	15 years	Poodle	Healthy
HD64	Female Neutered	11 months	Pomeranian/Terrier X	Healthy
HD65	Male	12 weeks	Griffon Bruxellois	Healthy
HD66	Male Neutered	5 years	Kelpie X	Healthy

HD: healthy dogs, **CE**: dogs with chronic enteropathy. Sample names in bold were also used for metagenomic analysis.

Table S2: Oligonucleotides used in the characterization of canine kobuvirus.

Oligonucleotide	Sequence	Reference
Cakobu 11F	TCCTCGGTGGTCTCATTGACTA	(300)
Cakobu 11R	TAATCCAGATCATACACCTGAGA	(300)
CaKV/F	CCCTGGAACACCCAAGGCCGCT	(302)
CaKV/R	TCTGGTTGCCATAGATGTGGTG	(302)
Dogkobu F	CGGATTCCATATTGGCCCTTG	(122)
Dogkobu R	ACAGCGTGAAATTGCGGTGG	(122)
Cakobu 1R	GCATGCAGTGGCGTCACTAGTTG	(300)
Cakobu 2F	TTATTGCCACTGCGATGAGGAA	(300)
Cakobu 5R	CCACTGTCTTCCAGTAGGACT	(300)
Cakobu 6F	AGATGCTCTCCAACTTCTTCAT	(300)
Cakobu 10R	GGTTGATGTTGACACCGGGTT	(300)
PM_KOBU_CDD9_90F	CCTCCCTTTCCTCTGGGCTT	This study
PM_KOBU_CDD9_916R	TGGAGTCCAGAAGCCAGTCT	This study
Cakobu5F	AGTCCTACTGGAAGACAGTGG	(300)
Cakobu6R	ATGAAGAAGTTGGAGAGCATCT	(300)
Cakobu 10F	AACCCGGTGTCAACATCAACC	(300)
Cakobu 12R	AAGAACAGTTAGAAAAG	(300)
Cakobu 9R	TGGAGTCCTCACGCTGTTGCCA	(300)
Cakobu 9F	TGGCAACAGCGTGAGGACTCC	(300)
PM_KOBU_CDD9_1918F	TGCAGGTTACCGTCAACGGCT	This study
Cakobu 7R	TTGGTGTCAGCGTCAGCGGTCA	(300)
PM_KOBU_CDD9_5678F	CCACCACAAGCCTGTCTCTT	This study
PM_KOBU_CDD9_6522R	GCAAACTGGGTGATTTGGGG	This study

Table S3: number of reads at each step during the bioinformatic pipeline; number and classification of contigs; and minimum (Min), mean and maximum (Max) size of contigs for each eukaryotic viral family

Sample name	Paired-end file names	N° of raw sequences	High Quality Reads (HQR) Trimmed by quality	Dog sequences extracted	HQR cellular and viral sequences (Dog free sequences)	Cellular organisms sequences extracted	HQR viral sequences Dog and cell. org. free	N° total contigs/singletons *de novo* assembly R1* + R2§ + DNA + RNA	N° total contigs/singletons having no hits	N° total viral contigs/singletons	N° prokaryotic viral contigs/singletons	N° eukaryotic viral contigs/singletons
CE1	CE01_DNA_R1	1562796	1342026	1457	1340569	1271458	69111	20513	15693	217	214	3
	CE01_RNA_R1	1400151	1178960	145	1178815	1158396	20419					
	CE01_DNA_R2	1562796	1365302	1682	1363620	1282079	81541					
	CE01_RNA_R2	1400151	1204767	167	1204600	1270875	23725					
CE4	CE04_DNA_R1	1676264	1421886	297	1421589	1225966	195723	48679	41613	451	451	0
	CE04_RNA_R1	1763049	1587207	192	1587015	1563914	23101					
	CE04_DNA_R2	1676264	1457591	321	1457270	1228492	228778					
	CE04_RNA_R2	1763049	1559977	186	1559791	1536720	23071					
CE5	CE05_DNA_R1	1528365	1315788	123	1315665	1275372	40293	5932	2848	227	227	0
	CE05_RNA_R1	805981	696672	20	696652	686891	9761					
	CE05_DNA_R2	1528365	1363434	135	1363299	1316307	46992					
	CE05_RNA_R2	805981	744591	37	744554	734376	10178					
CE8	CE08_DNA_R1	1627075	1373845	164	1373681	1253565	120116	22389	19154	493	493	0
	CE08_RNA_R1	1494732	1337802	15	1337787	1315180	22607					
	CE08_DNA_R2	1627075	1467373	180	1467193	1320593	146600					
	CE08_RNA_R2	1494732	1363066	10	1363056	1339507	23549					
CE9	CE09_DNA_R1	1703141	1417250	857	1416393	1133376	283017	105341	42190	8786	8777	9
	CE09_RNA_R1	2118470	1889744	1281	1888463	1863268	25195					
	CE09_DNA_R2	1703141	1318051	847	1317204	1017947	299257					
	CE09_RNA_R2	2118470	1812774	1146	1811628	1785783	25845					
CE10	CE10_DNA_R1	1856770	1414630	50	1414580	1071985	342595	67403	63867	319	319	0
	CE10_RNA_R1	2134448	1955127	51	1955076	1923158	31918					
	CE10_DNA_R2	1856770	1539323	56	1539267	1305643	423624					
	CE10_RNA_R2	2134448	1821050	51	1820999	1790145	30854					
CE11	CE11_DNA_R1	1663438	1402030	2187	1399843	1367291	32552	9803	6565	473	468	4
	CE11_RNA_R1	2156235	1968275	469	1967806	1944203	23603					
	CE11_DNA_R2	1663438	1283967	2114	1281853	1248304	33549					
	CE11_RNA_R2	2156235	1815716	488	1815228	1792633	22595					
CE13	CE13_DNA_R1	1478450	1247564	371	1247193	1211553	35640	10105	6835	203	203	0
	CE13_RNA_R1	1460113	1202292	69	1202223	1182685	19538					
	CE13_DNA_R2	1478450	1135461	400	1135061	1082044	35017					
	CE13_RNA_R2	1460113	1126532	72	1126460	1105257	21203					
Total CE samples		**52819356**	**45190173**	**15640**	**45174533**	**42402966**	**2771567**	**290225**	**188753**	**13173**	**13157**	**16**
HD4	HD4_DNA_R1	1311100	1134390	1605	1132785	923407	209378	64004	44668	1051	1012	39
	HD4_RNA_R1	1866166	1573342	3903	1569439	1353026	216413					
	HD4_DNA_R2	1311100	1158643	1583	1157060	938453	218607					
	HD4_RNA_R2	1866166	1547022	4489	1542533	1314009	228524					
HD5	HD5_DNA_R1	1736359	1536868	3729	1533139	1249437	283702	66919	43601	209	209	0
	HD5_RNA_R1	1990867	1795533	4557	1790975	1611306	179669					
	HD5_DNA_R2	1736359	1500716	3332	1497384	1206251	291133					
	HD5_RNA_R2	1990867	1667959	4393	1663566	1480099	183467					
HD6	HD6_DNA_R1	815204	743183	1434	741749	461093	280656	98776	67498	953	953	0
	HD6_RNA_R1	1645140	1493204	8841	1484363	1353584	130779					
	HD6_DNA_R2	815204	750404	1701	748703	459550	289153					
	HD6_RNA_R2	1645140	1347174	8192	1338982	1207044	132138					
HD7	HD7_DNA_R1	1198122	1054259	117	1054142	934316	119826	51350	36074	731	731	0
	HD7_RNA_R1	1025856	952260	1207	951053	871329	79724					
	HD7_DNA_R2	1198122	1082375	129	1082246	956680	125566					
	HD7_RNA_R2	1025856	908242	1253	906989	826788	80201					
HD8	HD8_DNA_R1	1465187	1334951	803	1334148	1177130	1137018	163899	90423	82	82	0
	HD8_RNA_R1	1786048	1512166	147300	1364866	277672	1087194					
	HD8_DNA_R2	1465187	1343643	808	1342835	148393	1194442					
	HD8_RNA_R2	1786048	1568078	148497	1419581	245946	1173635					
HD9	HD9_DNA_R1	1308162	1062399	1928	1060491	55277	1005214	132239	96287	8	5	3
	HD9_RNA_R1	1727061	1470574	85947	1384627	502996	881631					
	HD9_DNA_R2	1308162	1065512	2031	1063481	25847	1037634					
	HD9_RNA_R2	1727061	1428191	80568	1347825	425831	921994					
HD10	HD10_DNA_R1	1364583	1004863	174	1004689	58792	945897	106384	85703	914	2	913
	HD10_RNA_R1	2065597	1820777	89560	1731217	788411	942806					
	HD10_DNA_R2	1364583	1070826	169	1070657	33792	1036865					
	HD10_RNA_R2	2065597	1706077	84174	1621903	643891	978012					
HD11	HD11_DNA_R1	1657053	1392154	8164	1383990	534156	849834	227873	195444	20	18	2
	HD11_RNA_R1	1594165	1365243	18018	1347225	701964	645261					
	HD11_DNA_R2	1657053	1383952	8381	1375571	441348	934223					
	HD11_RNA_R2	1594165	1265384	18037	1247347	595568	651779					
Total HD samples		**48533240**	**42020763**	**745002**	**41275761**	**22783386**	**18492375**	**901944**	**659696**	**3968**	**3032**	**956**
Total		**101353696**	**87210936**	**760642**	**86450294**	**65186352**	**23263942**	**1192169**	**848449**	**15141**	**14169**	**972**

CE: Dogs with chronic enteropathy
HD: healthy dogs
* **R1**: paired end 1
§ **R2**: paired end 2

Contig sizes of eukaryotic viral families

Adenoviridae			Papillomaviridae			Reoviridae			Picornaviridae			Parvoviridae			Coronaviridae		
Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max	Min	mean	Max
			251	251	251	251	251	251									
									155	308	723						
						251	251	251									
						101	251	865									
												251	251	251			
															101	209	251
129	129	129	192	192	192												

Table S4: GenBank accession numbers, names and references of 23 kobuvirus sequences used to create phylogenetic tree of the polyprotein region.

KF924623.1 Canine kobuvirus 1 isolate 12D049, complete genome (300)

JQ911763.1 Canine kobuvirus CH-1, complete genome, direct submission

NC_034971.1 Canine kobuvirus isolate SMCD-59, complete genome, direct submission.

JN088541.1 Canine kobuvirus US-PC0082, complete genome (298)

JN387133.1 Kobuvirus dog/AN211D/USA/2009 polyprotein gene, complete cds (122)

KM068048.1 Canine kobuvirus 1 isolate DD2 polyprotein mRNA, complete cds (303)

KC161964.1 Canine kobuvirus strain UK003, complete genome (299)

KM068051.1 Canine kobuvirus 1 isolate B103 polyprotein mRNA, complete cds (303)

KM068050.1 Canine kobuvirus 1 isolate 75 polyprotein mRNA, complete cds (303)

KM068049.1 Canine kobuvirus 1 isolate 82 polyprotein mRNA, complete cds (303)

KM091960.1 Feline kobuvirus strain FeKoV/TE/52/IT/13, complete genome (326)

KJ958930.1 Feline kobuvirus isolate 12D240, complete genome (327)

KF831027.1 Feline kobuvirus strain FK-13, complete genome (328)

NC_015936.1 Mouse kobuvirus M-5/USA/2010, complete genome (134)

AY747174.1 Aichi virus isolate BAY/1/03/DEU from Germany polyprotein gene, complete cds (329)

NC_001918.1 Aichi virus, complete genome (330)

FJ890523.1 Aichi virus isolate Chshc7, complete genome (331)

DQ028632.1 Aichi virus isolate Goiania/GO/03/01/Brazil, complete genome (329)

NC_023422.1 Caprine kobuvirus isolate 12Q108, complete genome (296)

NC_011829.1 Porcine kobuvirus swine/S-1-HUN/2007/Hungary, complete genome (332)

KF006985.1 Ferret kobuvirus isolate MpKoV38 polyprotein gene, complete cds (139)

NC_004421.1 Aichivirus B genomic RNA, complete genome, strain:U-1 (333)

GU245693.2 Kobuvirus sheep/TB3/HUN/2009, complete genome (294)

Chapter 5: Canine Rotavirus characterisation and prevalence

5.1 Introduction

Rotavirus are viral enteric pathogens affecting humans and animals. Rotavirus infection is the main cause of diarrhoea in infants and young children worldwide (334, 335). Rotavirus have been recovered from diarrhoeal faeces of the young of several animal species, including sheep, cattle, horses, goats, dogs, cats, rabbits, rats, mice, non-human primates and birds (306, 336). In all species the severity of diarrhoea can vary from asymptomatic disease to very severe enteritis that could cause death (310).

In dogs, rotavirus has been regarded as a minor pathogen, with clinical infection predominantly detected in puppies younger than 12 weeks with mild diarrhoea (311). Fatal cases have only been observed sporadically in very young puppies (311). Clinical presentation commonly includes non-severe, watery to mucoid diarrhoea of between 8 -10 days duration. Rotavirus in dogs is generally found in mixed enteric infections, together with other enteric viruses (311)

Rotaviruses are members of genus *Rotavirus* within the family *Reoviridae*. The mature virus particle possesses a triple-layered protein capsid and is approximately 75nm in diameter. The virus genome is comprised of 11 segments of double-stranded RNA (dsRNA) encoding 6 viral structural proteins (VPs) and 6 non-structural proteins (NSPs) (306). Rotavirus are classified as species (interchangeably referred to as groups) based on sequence of the VP6 gene. Nine species have been described (A-I) with a tenth tentative species recently described (J) (337). Rotavirus are capable of reassortment of genome segments, among different strains, within the same virus species (338). Epidemiologically, rotavirus group A (RVA) is the most important group related to gastroenteritis in people and a wide range of animal species (306). A classification system, based on nucleotide identity cut-off percentages, was created to assign a genotype to each of 11

genome segments (307). Whole genome classification is also used, the nomenclature Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx represents the genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 respectively (339).Currently there are 36 G, 51 P, 26 I, 22 R, 20 C, 20 M, 31 A, 22 N, 22 T, 27 E and 22 H types according to Matthijnssens J. Rotavirus Classification Working Group: RCWG; https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification.

To date, most canine rotavirus reported belong to group A (RVA), however, some Group C canine rotavirus isolates have been described from dogs (313, 314), and more recently two strains identified in sheltered dogs have been proposed as group I (315). The canine group A strains have been reported to be mostly G3P[3](338), with fewer G8P[1] reported (338, 340).

In this study we aimed to characterise the full genome of a canine rotavirus found in an asymptomatic dog and also evaluate the prevalence of rotavirus from group A in healthy dogs, dogs with acute diarrhoea and chronic enteropathy.

5.2 Material and Methods

5.2.1 Animals and sample collection

To evaluate the prevalence of rotavirus in dogs, samples were collected from dogs housed in a single shelter in Melbourne (Lost Dogs Home) and from the U-Vet Werribee Animal Hospital, University of Melbourne, with varied clinical presentation/signs between June 2010 and Sept 2015.

Faecal samples were collected from 57 healthy dogs, 36 dogs with acute diarrhoea and 14 dogs with chronic enteropathy. In addition, samples from the 24 dogs (8 of each group) previously characterised by next generation sequencing (NGS) analysis (321, 324), were also analysed.

Information about age, sex, breed, diet, vaccination and deworming status was recorded for each dog (University of Melbourne Animal Ethics Committee Approval IDs 1413272.2, 1112072.2 and 1112035.1).

5.2.2 Faecal extract preparation

Faecal samples were processed as described previously (122, 321, 341). Briefly, faecal sample aliquots of 500 mg were thawed and resuspended in saline buffer (0.01 M Tris solution [pH7.5], 0.15 M NaCl, 0.01 M $CaCl_2$) at a 3:1 ratio of solid mass. One-millimetre zirconia/silica beads were added to the stool solution, filling approximately 150 µL of an Eppendorf tube, and vortexed vigorously for 3 min. The samples were then centrifuged at 17900 x *g* for 5 min, collecting the supernatant and repeating this step three times. To reduce solid faecal matter and bacterial contamination, 500 µL of this solution were filtered through a 0.45 µm tube filter (Corning® Costar® Spin X®, CLS8162-96EA SIGMA) by centrifuging at 3800 x *g* for 5 min, then the filtrates were transferred to 2 ml tubes and stored at minus 20ºC degrees.

5.2.3 RNA extraction and amplification of the genome of canine rotavirus

RNA was extracted directly from the purified faecal preparation, using spin protocol of QIamp® Viral RNA mini kit, according to the manufacturer instructions (QIamp® Viral RNA mini kit Handbook April 2010, third edition, QIAGEN).

The complete genome of the 11 genes of canine rotavirus, were amplified using multiple sets of primers selected from the literature or designed based on sequences obtained from Illumina reads (Table 4). RT-PCR reactions were performed using the SuperScript® III One-Step RT-PCR System with Platinum® Taq (Invitrogen™). PCR conditions used were: 45 °C for 30 min and 95 °C for 15 min, 40 cycles of 94 °C for 45 s, 45 °C for 45 s and 70 °C for 5 min, and a final elongation step of 68 °C for 5 min, followed by final hold at 4 °C.

5.2.4 Agarose Gel Electrophoresis and sequencing

The 50 µL PCR product was combined with 10 µL of dye (0.2% bromethyl phenol blue) and loaded on a 1.2% w/v agarose gel (Bioline) (dissolved in 0.5X TBE), containing RedSafe™ nucleic acid staining solution (iNtRON Biotechnology). All gels were electrophoresed in 0.5X TBE buffer for 60 min at 100 V. Each gel also contained a 100-base pair (bp) DNA ladder (1.0 µg/µL) (Invitrogen) for size estimation. PCR products were visualised on an ultra violet (UV) transilluminator (Gene Genius, Bio Imaging Systems, Syngene).

PCR products were excised and purified using the WIZARD ® SV Gel and PCR clean-up system (Promega®) following manufacturer's protocol. Briefly, membrane binding solution was added to each excised band at a 10 µL to 10 mg ratio and heated at 60 °C until the gel melted. This solution was then added to a spin column and incubated for 1 min at room temperature. The column was centrifuged at 16,000 x *g* for 1 min and filtrate discarded. Membrane wash solution (700 µL) was added to the column, centrifuged and filtrate discarded again. The column as washed a second time, membrane wash solution (500 µL) was added, and the column was centrifuged for 5 min and filtrate discarded. The column was centrifuged again for 1 min to remove traces of ethanol. Sample DNA was eluted from the column using 50 µL of NFW following a 2 min incubation period at room temperature and centrifugation for 1 min. The concentration of all purified DNA was determined using a spectrophotometer Nanodrop® 2000. Samples were sent and sequenced using Sanger sequencing at the Australian Genome Research Facility (AGRF), at the Walter and Eliza Hall Institute (WEHI) of Medical Research using an ABI PRISM BigDye® Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, CA) in a 3730xl DNA Analyser (Applied Biosystems, CA).

The near complete genome of the canine rotavirus was assembled using Sequencher® version 5.0.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA) with minimum match percentage 80 and minimum overlap 50 as assembly parameters. Chromatograms were

visually analysed for base calling errors and forward and reverse sequence reads were assembled into contiguous DNA sequences. Contiguous sequences were used to query the nucleotide database in GenBank using the Basic Local Alignment Search Tool (BLAST®) at the NCBI, USA (http://www.ncbi.nlm.nih.gov/blast).

5.2.5 Phylogenetic analysis

Maximum likelihood phylogenetic trees were constructed at the nucleotide level using selected models of nucleotide substitution GTR+G $_{G4}$+I (VP1 and NSP1), GTR+G$_{G4}$ (VP2, VP3, VP7, NSP2 and NSP3), HKY+G $_{G4}$ (NSP4) and TrN+G $_{G4}$ (VP6), TrN+G $_{G4}$+I (VP4) and TrN +I (NSP5), using MEGA6.0. Optimal evolutionary models were selected based upon the Akaike information criterion (corrected) (AICc) ranking implemented in jModelTest (342). Phylogenetic trees were statistically supported by bootstrap analysis with 1000 pseudoreplicate runs. Nucleotide and amino acid distance matrixes were calculated using the *p*-distance algorithm in MEGA6.0 (343).

5.2.6 Assignment of genotype

The genotype of each gene segment was determined using the online RotaC$^{2.0}$ rotavirus genotyping tool (http://rotac.regatools.be/) (344) as recommended by the RCWG. RotaC$^{2.0}$ utilises defined nucleotide identity cut-off percentages to assign a genotype to each rotavirus gene segment allowing the determination of complete genome constellations for rotavirus strains investigated in this study (307, 344).

5.2.7 Prevalence study

The prevalence of RVA in all canine samples was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA, ProSpecT). Briefly, new sample aliquots (0.2 g) from the 24 individuals already enrolled for NGS study and an additional 107 faecal samples were analysed. Faecal samples were thawed and prepared with the 20% (w/v) method. Briefly 800 µL of virus dilution buffer (0.01 M Tris solution [pH 7.5], 0.15 M NaCl, 0.01 M $CaCl_2$) was added to 0.2 g of

faecal material, vortexed until homogenised, centrifuged at 20,000g x *g* for 3 min and the supernatant harvested. Aliquots of 100 µL of faecal samples were analysed using the ProSpecT™ Rotavirus test, for detection of rotavirus group A, following manufacturer's instructions.

5.3 Results

5.3.1 Characterisation of canine rotavirus

The whole genome of a G3P[3] canine rotavirus was characterised from a healthy dog faecal sample. All 11 gene segments were classified as belonging to established genotypes: G3-P [3]-I2-R3-C2-M3-A9-N2-T3-E3-H6 (Table 1). The genotype constellation of this strain was almost identical to other previously characterised canine rotavirus strains including RVA/Dog-tc/ITA/RV198-95/1995/G3P[3], RVA/Dog-tc/ITA/RV52-96/1996/G3P[3], RVA/Dog-tc/USA/CU-1/1982/G3P[3, RVA/Dog-tc/AUS/K9/1981/G3P[3], RVA/Dog-tc/USA/A-79-10/1979/G3P[3, feline rotavirus RVA/Cat-tc/AUS/Cat97/1984/G3P[3] and human rotavirus strains RVA/Human-tc/ISR/Ro1845/1985/G3P[3] and RVA/Human-tc/USA/HCR3A/1984/G3P[3] (Table 2). The sole exception was the VP6 gene, which was not closely related to canine or feline strains and shared 97.5 % nucleotide similar to RVA/Human-wt/USA/6235/2003/G3P[3] and classified at I2 (Table 2).

Compared to the genome constellations of previously characterised G3P[3] strains from a variety of species, the complete genome of RVA/Dog-wt/AUS/ND4/2013/G3P[3] shares ten genes with all G3P[3] strains from canine/feline origin. Also, the strain shares nine genes with RVA/Simian-tc/USA/RRV/1975/G3P[3], RVA/Human-wt/USA/6212/2003/G3P[3] and RVA/Human-wt/USA/6235/2003/G3P[3], shares 8 genotypes with RVA/Bat-tc/MSLH14/2012/G3P[3], RVA/Horse-wt/ARG/E3198/2008/G3P[3], RVA/Dog-wt/HUN/135/2012/G3P[3], RVA/Human-wt/CHN/M2-102/2014/G3P[3] and RVA/Human-tc/ITA/PA260-97/1997/G3P[3]. Finally, shares from 4 – 7 genotypes with RVA/Bat-

wt/ZMB/LUS12-14/2012/G3P[3], RVA/Human-tc/AUS/RCH272/2012/G3P[14], RVA/Cat-tc/AUS/Cat2/1984/G3P[9] and RVA/Cat-wt/ITA/BA222/2005/G3P[9] (Table2)

Table 1: Genotype characterisation of RVA/Dog-wt/AUS/ND4/2013/G3P[3] according to RotaC$^{2.0}$ rotavirus genotyping tool.

Gene	*Genotype*	*Similarity*
VP1	**R3** (RNA dependent RNA polymerase)	**R3-RVA/Human-tc/JPN/AU-1/1982/G3P9** (87.1%)
VP2	**C2** (core shell protein)	**C2-RVA/Human-tc/USA/DS-1/1976/G2P4** (89.9%)
VP3	**M3** (Methyltransferase)	**M3-RVA/Human-wt/BEL/B4106/2000/G3P14** (86.1%)
VP4	**P[3]** (protease sensitive)	**P3-RVA/Dog-tc/AUS/K9/1981/G3P3**(95.4%)
VP6	**I2** (intermediate capsid)	**I2-RVA/Human-tc/ITA/PA169/1988/G6P14** (92.9%)
VP7	**G3** (Glycosylated)	**G3-RVA/Human-wt/BEL/B4106/2000/G3P14** (87.5%)
NSP1	**A9** (Interferon agonist)	**A9-RVA/Rabbit-tc/ITA/30-96/1996/G3P14** *(*81.8%)
NSP2	**N2** (NTPase)	**N2-RVA/Cow-tc/GBR/UK/1973/G6P5** (85.8%)
NSP3	**T3** (Translation enhancer)	**T3-RVA/Simian-tc/USA/RRV/1975/G3P3** (86.4%)
NSP4	**E3** (Enterotoxin)	**E3-RVA/Human-tc/THA/T152/1998/G12P9** (84.7%)
NSP5	**H6** (Phosphoprotein)	**H6-RVA/Simian-tc/USA/RRV/1975/G3P3** (93.0%)

Table 2: Genome constellation and nucleotide similarities of RVA/Dog-wt/AUS/ND4/2013/G3P[3], compared to strains with similar genome constellation

Strain name	Genotypes										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Dog-wt/AUS/ND4/2013/G3P[3]	**G3**	**P[3]**	**I2**	**R3**	**C2**	**M3**	**A9**	**N2**	**T3**	**E3**	**H6**
RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]	G3 (91.0%)	P[3] (93.0%)	I3 (83.1%)	R3 (94.5%)	C2 (92.2%)	M3 (83.4%)	A9 (91.5%)	N2 (84.8%)	T3 (93.6%)	E3 (93.9%)	H6 (95.8%)
RVA/Dog-tc/ITA/RV52-96/1996/G3P[3]	G3 (82.6%)	P[3] (94.1%)	I3 (82.9%)	R3 (94.6%)	C2 (92.5%)	M3 (83.3%)	A9 (91.8%)	N2 (85.4%)	T3 (93.1%)	E3 (85.5%)	H6 (95.4%)
RVA/Dog-tc/USA/CU-1/1982/G3P[3]	G3 (91.1%)	P[3] (93.2%)	I3 (81.6%)	R3 (96.3%)	C2 (92.0%)	M3 (91.7%)	A9 (91.1%)	N2 (96.1%)	T3 (93.8%)	E3 (94.3%)	H6 (97.5%)
RVA/Dog-tc/AUS/K9/1981/G3P[3]	G3 (91.7%)	P[3] (94.9%)	I3 (81.6%)	R3 (96.3%)	C2 (92.7%)	M3 (92.1%)	A9 (91.8%)	**N2 (97.4%)**	T3 (94.8%)	E3 (94.7%)	H6 (98.1%)
RVA/Dog-tc/USA/A-79-10/1979/G3P[3]	G3 (91.5%)	P[3] (93.5%)	I3 (89.0%)	**R3 (96.5%)**	C2 (92.6%)	M3 (91.2%)	A9 (91.9%)	N2 (95.8%)	T3 (94.5%)	E3 (94.5%)	H6 (97.5%)
RVA/Cat-tc/AUS/Cat97/1984/G3P[3]	G3 (90.1%)	P[3] (93.3%)	I3 (81.6%)	R3 (94.3%)	**C2 (92.9%)**	M3 (91.4%)	A9 (91.3%)	N2 (95.9%)	T3 (94.4%)	E3 (94.5%)	H6 (97.1%)
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	G3 (91.7%)	P[3] (92.5%)	I3 (81.6%)	R3 (94.9%)	C2 (92.7%)	M3 (91.1%)	A9 (91.3%)	N2 (96.3%)	T3 (94.4%)	E3 (94.1%)	H6 (97.1%)
RVA/Human-tc/USA/HCR3A/1984/G3P[3]	G3 (90.7%)	P[3] (95.2%)	I3 (80.8%)	**R3 (96.5%)**	C2 (92.4%)	M3 (92.1%)	A9 (90.4%)	N2 (96.9%)	**T3 (95.5%)**	E3 (92.8%)	H6 (97.6%)
RVA/Simian-tc/USA/RRV/1975/G3P[3]	G3 (85.0%)	P[3] (79.4%)	I2 (92.2%)	R2	C3	M3 (82.9%)	A9 (81.7%)	N2 (85.1%)	T3 (85.7%)	E3 (84.8%)	H6 (92.9%)
RVA/Human-wt/USA/6212/2003/G3P[3]	G3 (89.4%)	P[3] (95.9%)	I3 (81.2%)	R1	C2	M3 (90.2%)	A9 (90.5%)	N2 (97.0%)	T3 (93.8%)	E3 (93.7%)	**H6 (98.3%)**
RVA/Human-wt/USA/6235/2003/G3P[3]	**G3 (98.1%)**	**P[3] (97.9%)**	**I2 (97.5%)**	R1	Cx	M3 (93.6%)	**A9 (95.7%)**	N2 (91.7%)	T3 (94.6%)	**E3 (98.1%)**	H6 (96.1%)
RVA/Bat-tc/MSLH14/2012/G3P[3]	G3 (84.7%)	P[3] (80.0%)	I8 (81.8%)	R3 (84.9%)	C3	M3 (82.1%)	A9 (81.3%)	N3	T3 (85.5%)	E3 (84.0%)	H6 (92.9%)

RVA/Horse-wt/ARG/E3198/2008/G3P[3]	G3 (82.4%)	P[3] (79.3%)	I3 (80.8%)	R3 (86.3%)	C3	M3 (81.9%)	A9 (80.8%)	N3	T3 (85.5%)	E3 (86.1%)	H6 (94.6%)
RVA/Human-wt/CHN/M2-102/2014/G3P[3]	G3 (82.9%)	P[3]	I3 (82.3%)	R3 (85.8%)	C3	M3 (83.4%)	A9 (80.3%)	N3	T3 (83.9%)	E3 (84.8%)	H6 (94.4%)
RVA/Dog-wt/HUN/135/2012/G3P[3]	G3 (82.7%)	P[3] (94.0%)	I3 (93.0%)	R3 (86.8%)	C3	M3 (83.6%)	A15	N2 (85.5%)	T3 (93.4%)	E3 (85.5%)	H6 (96.1%)
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	G3 (83.2%)	P[3] (94.6%)	I3 (83.1%)	R3 (86.6%)	C3	M3 (83.4%)	A15	N2 (85.4%)	T3 (94.5%)	E3 (85.1%)	H6 (96.1%)
RVA/Bat-wt/ZMB/LUS12-14/2012/G3P[3]	G3 (83.2%)	P[3] (92.9%)	I3 (81.6%)	R2	C2 (85.4%)	M3 (83.5%)	A9	N2 (85.6%)	T3 (83.1%)	E2	H3
RVA/Human-tc/AUS/RCH272/2012/G3P[14]	G3 (82.9%)	P[14]	I2 (91.6%)	R3 (84.5%)	C3	**M3 (98.1%)**	A9 (82.8%)	N2 (83.7%)	T6	E2	H3
RVA/Cat-tc/AUS/Cat2/1984/G3P[9]	G3 (79.2%)	P[9]	I3 (81.6%)	R3 (94.5%)	C2 (92.6%)	M3 (91.7%)	A3	N1	T6	E3 (93.9%)	H3
RVA/Cat-wt/ITA/BA222/2005/G3P[9]	G3 (78.4%)	P[9]	I2 (87.6%)	R2	C2 (85.6%)	M2	A3	N1	T3 (84.9%)	E2	H3

Shaded cells indicate strains with the same genotype as **RVA/Dog-wt/AUS/ND4/2013/G3P[3]**

Bold letters and numbers represent strains within each genotype with the highest nucleotide similarity to **RVA/Dog-wt/AUS/ND4/2013/G3P[3]**

5.3.2 Phylogenetic analyses

Phylogenetic analyses were conducted on the nucleotide sequences of the entire open reading frames for all 11 gene segments to examine genetic relatedness of RVA/Dog-wt/AUS/ND4/2012/G3P[3] to global rotavirus strains. Analyses included various human and animal rotavirus strains with similar genome constellation that had been previously described and available in GenBank.

Analysis of the VP7 gene revealed that the strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] clustered in a lineage comprised of animal strains from various species including cats, dogs, horses, bats and alpacas; and human strains of zoonotic origin. The VP7 clustered within a sub-lineage comprised historic canine and feline strains, and shared the highest nucleotide similarity with the human strain RVA/Human-wt/USA/6235/2003/G3P[3] (98.1%) which also has the same G3P[3] genome constellation (Fig. 2). Similarly, the VP4, NSP1 and NSP4 genes also shared the highest nucleotide similarity with RVA/Human-wt/USA/6235/2003/G3P[3] (97.9%, 95.7% and 95.7% respectively), these genes clustered within a lineage predominantly comprised of genes from canine and feline strains (Fig. 3, 8 and 11).

In the VP6 gene analysis, RVA/Dog-wt/AUS/ND4/2012/G3P[3] clustered distantly from the other VP6 genes of canine/feline origin, as most of these strains belong to the V6 I3 genotype. RVA/Dog-wt/AUS/ND4/2012/G3P[3] clustered within the I2 different lineage, closer to strains from bovine and human origin. Interestingly, the gene clusters with the VP6 from a G3P[3] strain which also has genotype V6 I2, RVA/Human-wt/USA/6235/2003/G3P[3], sharing 97.5% of nucleotide similarity suggesting the possible widespread circulation canine/feline origin strains exhibiting this unusual genotype constellation (Fig. 4).

Phylogenetic analysis of the VP1 gene of RVA/Dog-wt/AUS/ND4/2012/G3P[3] with the R3 VP1 from global strains revealed our VP1 gene clustered within a sub-lineage comprised Vp1 from

strains with a canine/feline origin, sharing between 94.3% - 96.5% nucleotide similarity (Fig. 5). Similarly, in the VP2 gene analysis, our gene formed an isolated node within the same lineage containing VP2 of strains with the same origin and same constellation in the VP2 C2 genotype (Fig 6). The VP3 gene clustered with a gene from the human RVA/Human-wt/AUS/RCH272/2012/G3P[14] sharing a 98.1% nucleotide similarity, within a lineage containing other strains of feline/canine origin (Fig. 7).

The NSP2 and NSP5 genes each formed separate clusters with genes from the RVA/Human-wt/USA/6212/2003/G3P[3] strain, but within a larger gene lineage that includes strains from the same origin (Fig. 9 and 12).

The NSP3 gene is clustered together with RVA/Human-tc/USA/HCR3A/1984/G3P[3], and NSP3 genes from strains of canine/feline G3P3 origin (Fig. 10).

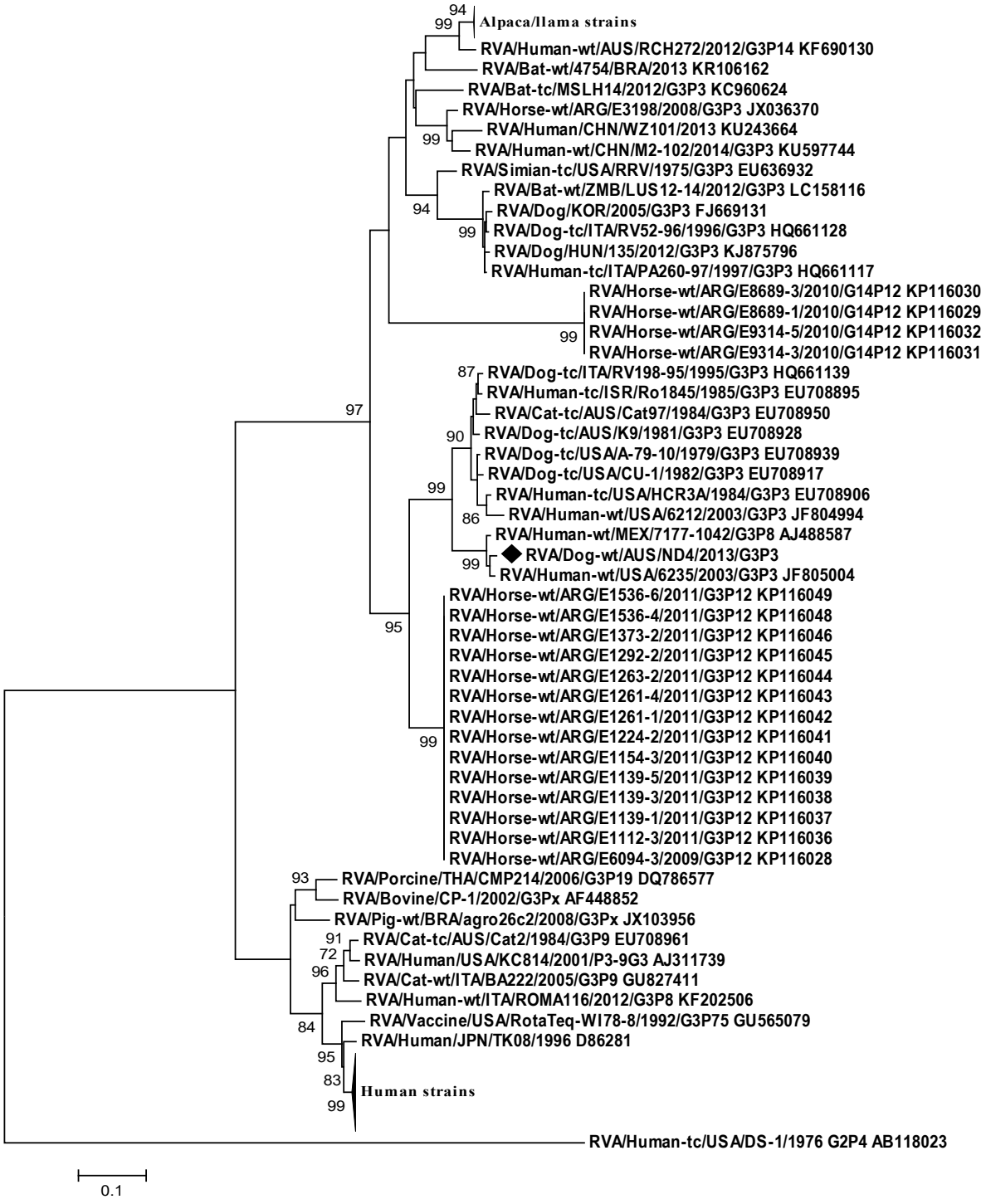

Figure 2: *Maximum likelihood phylogenetic tree of VP7 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other G3 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345)

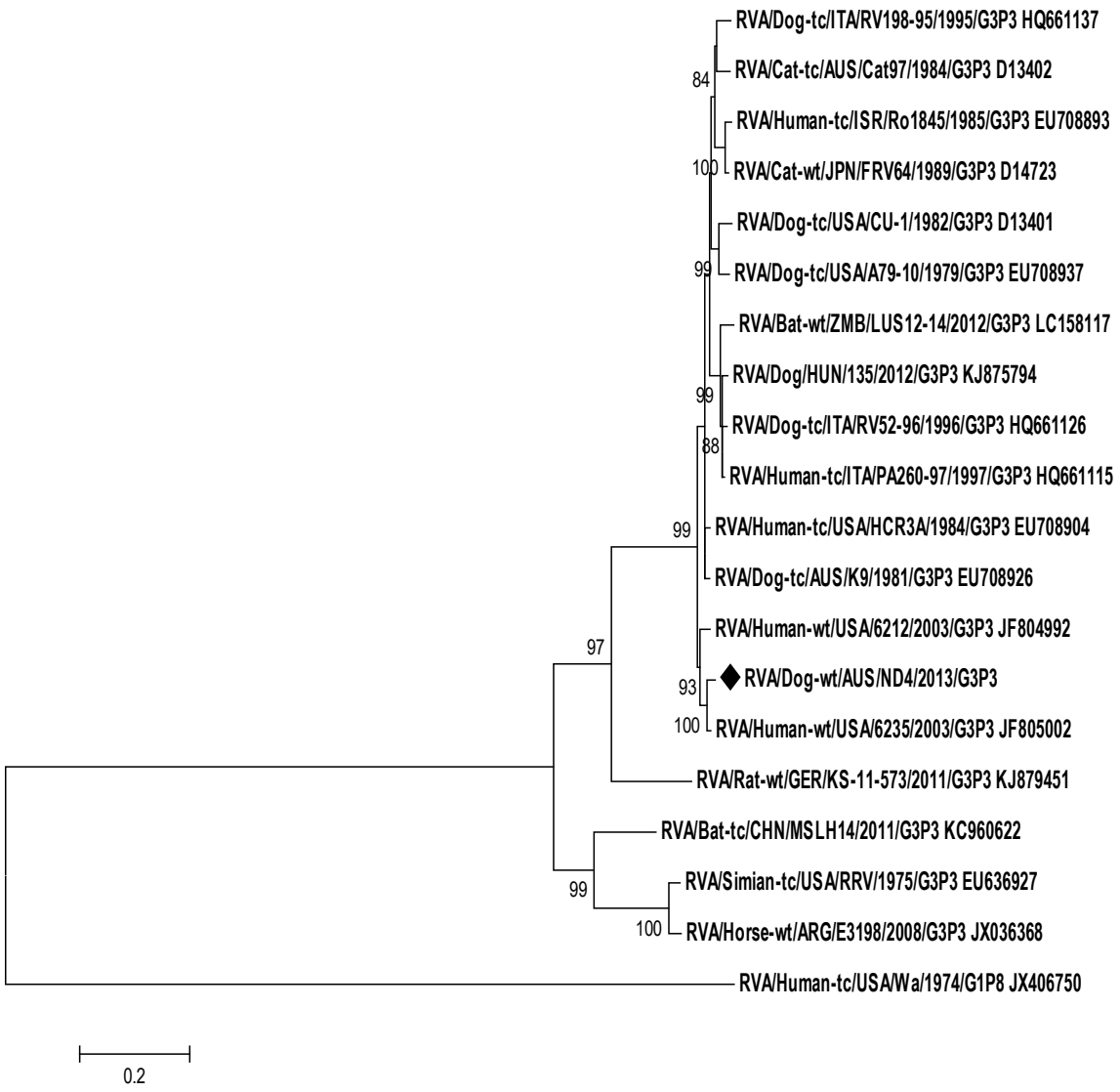

Figure 3: *Maximum likelihood phylogenetic tree of VP4 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other P[3] RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

Figure 4: *Maximum likelihood phylogenetic tree of VP6 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other I2 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

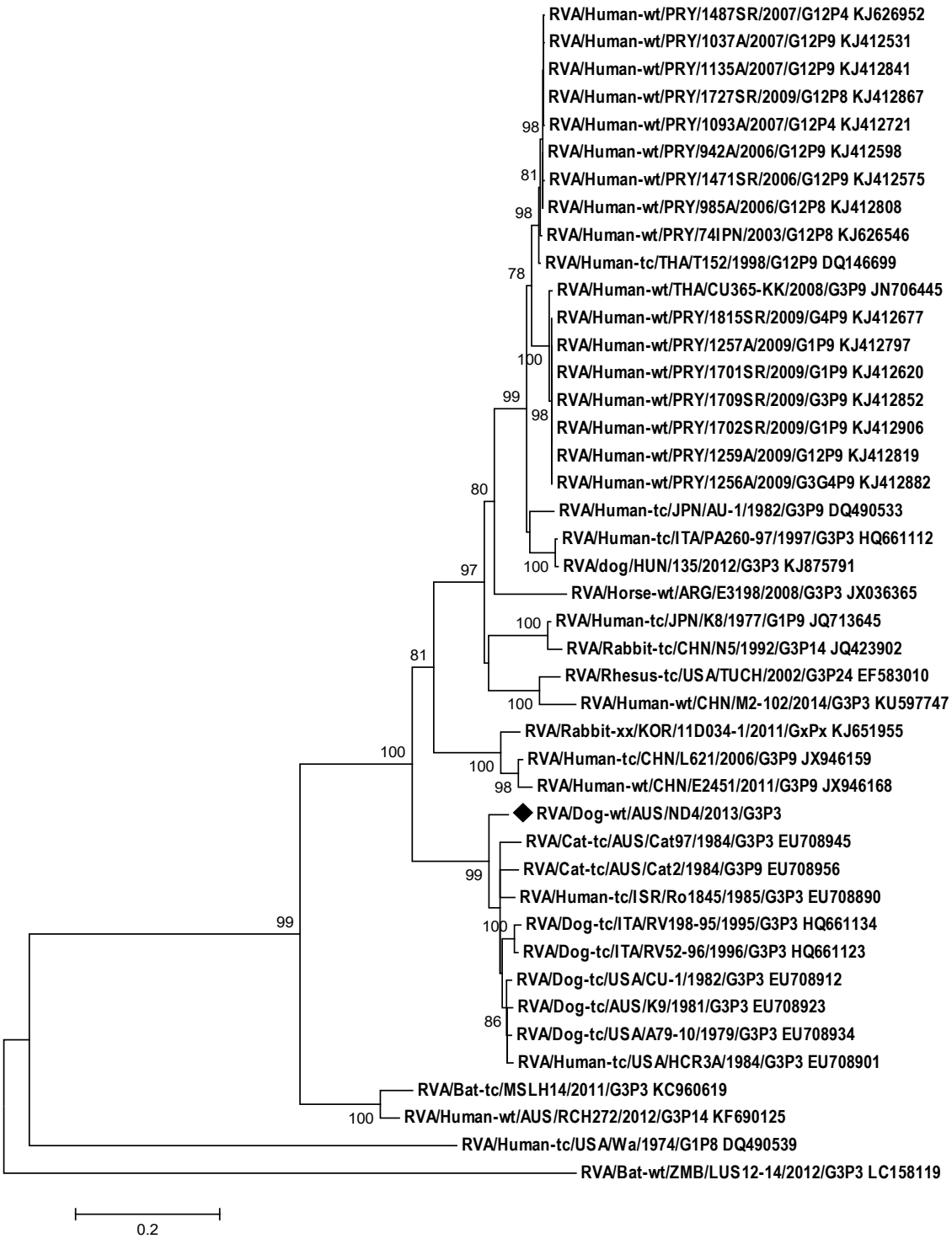

Figure 5: *Maximum likelihood phylogenetic tree of VP1 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other R3 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

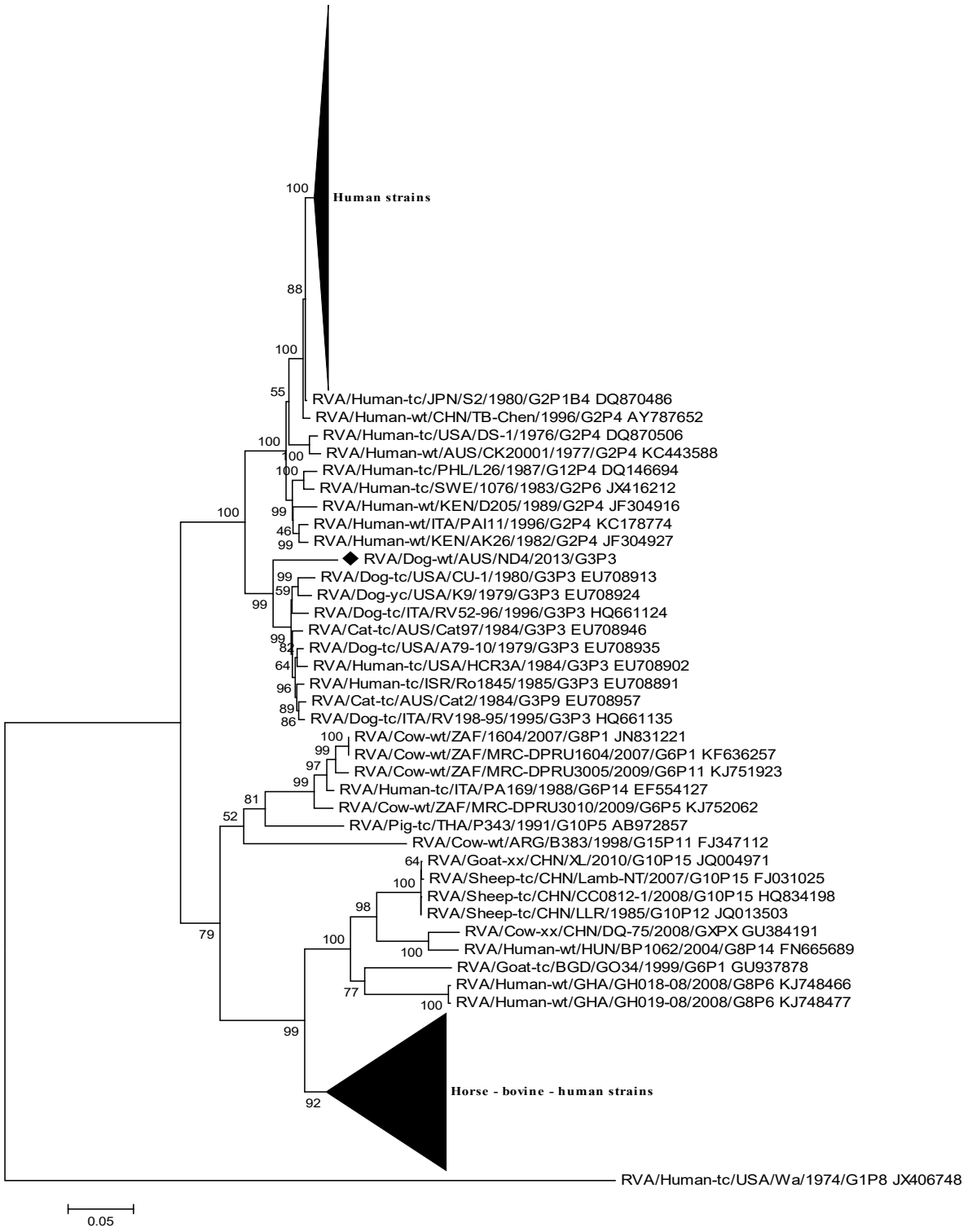

Figure 6: *Maximum likelihood phylogenetic tree of VP2 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other C2 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

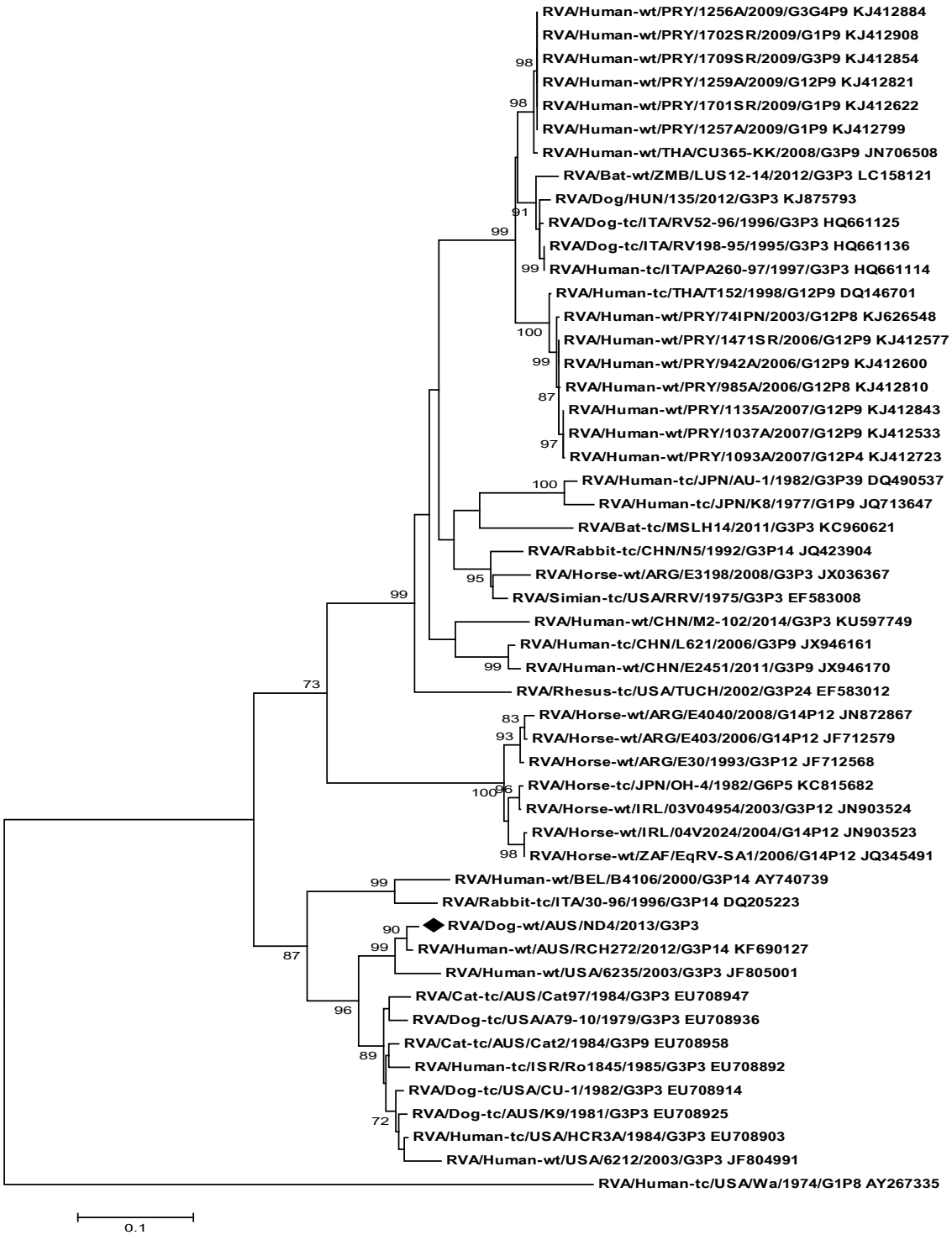

Figure 7: *Maximum likelihood phylogenetic tree of VP3 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other M3 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

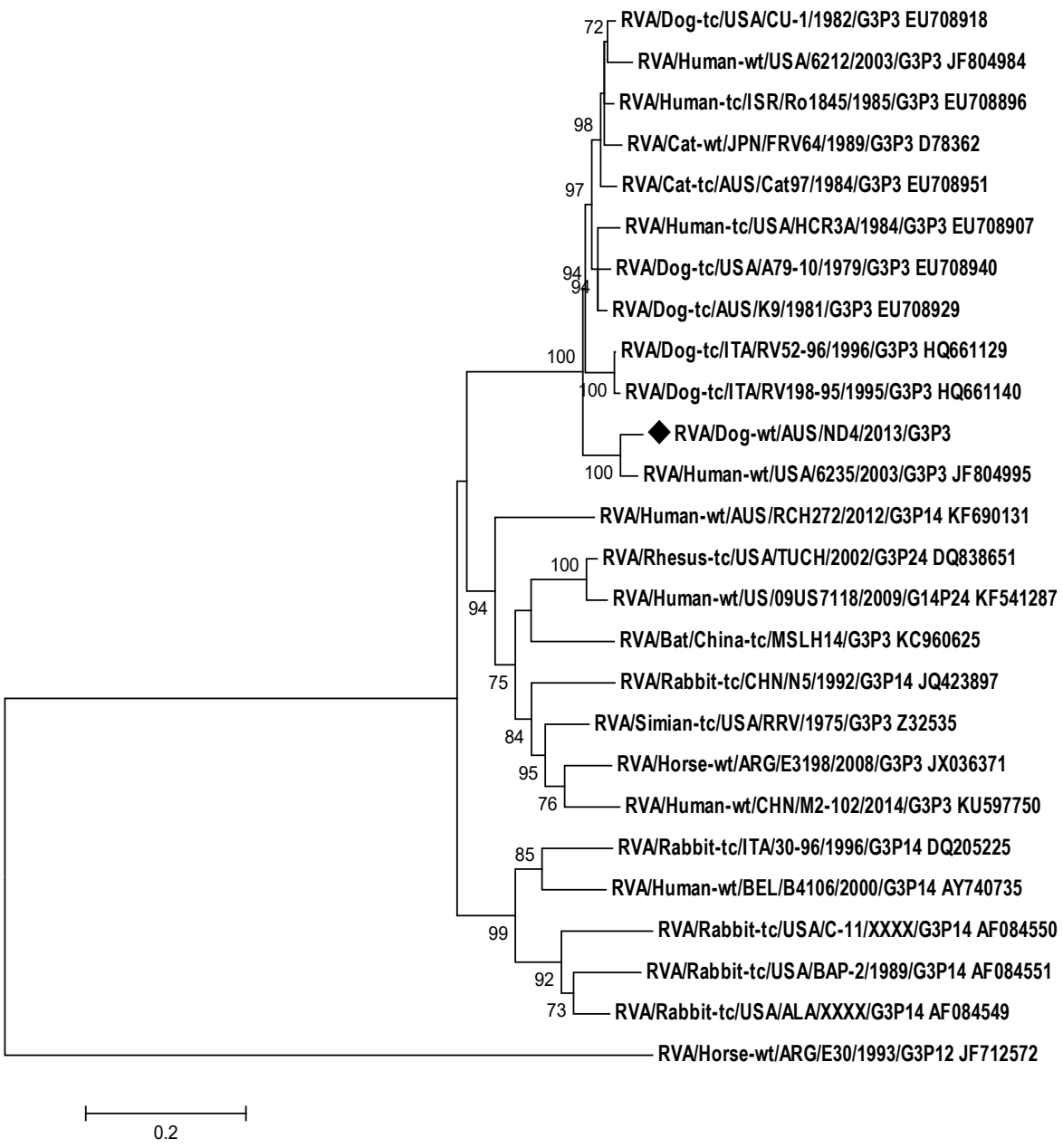

Figure 8: *Maximum likelihood phylogenetic tree of NSP1 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other A9 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

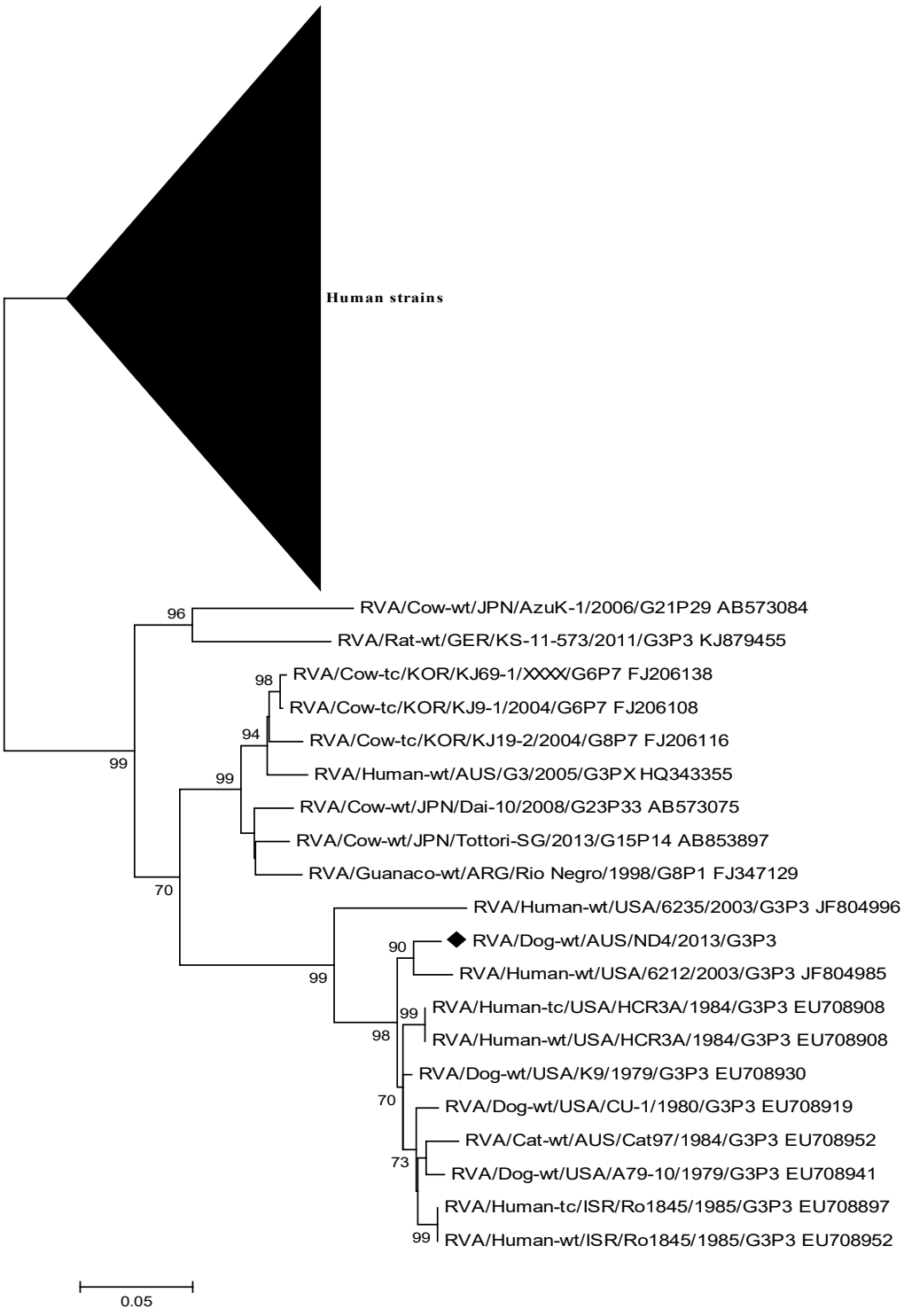

Figure 9: *Maximum likelihood phylogenetic tree of NSP2 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other N2 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

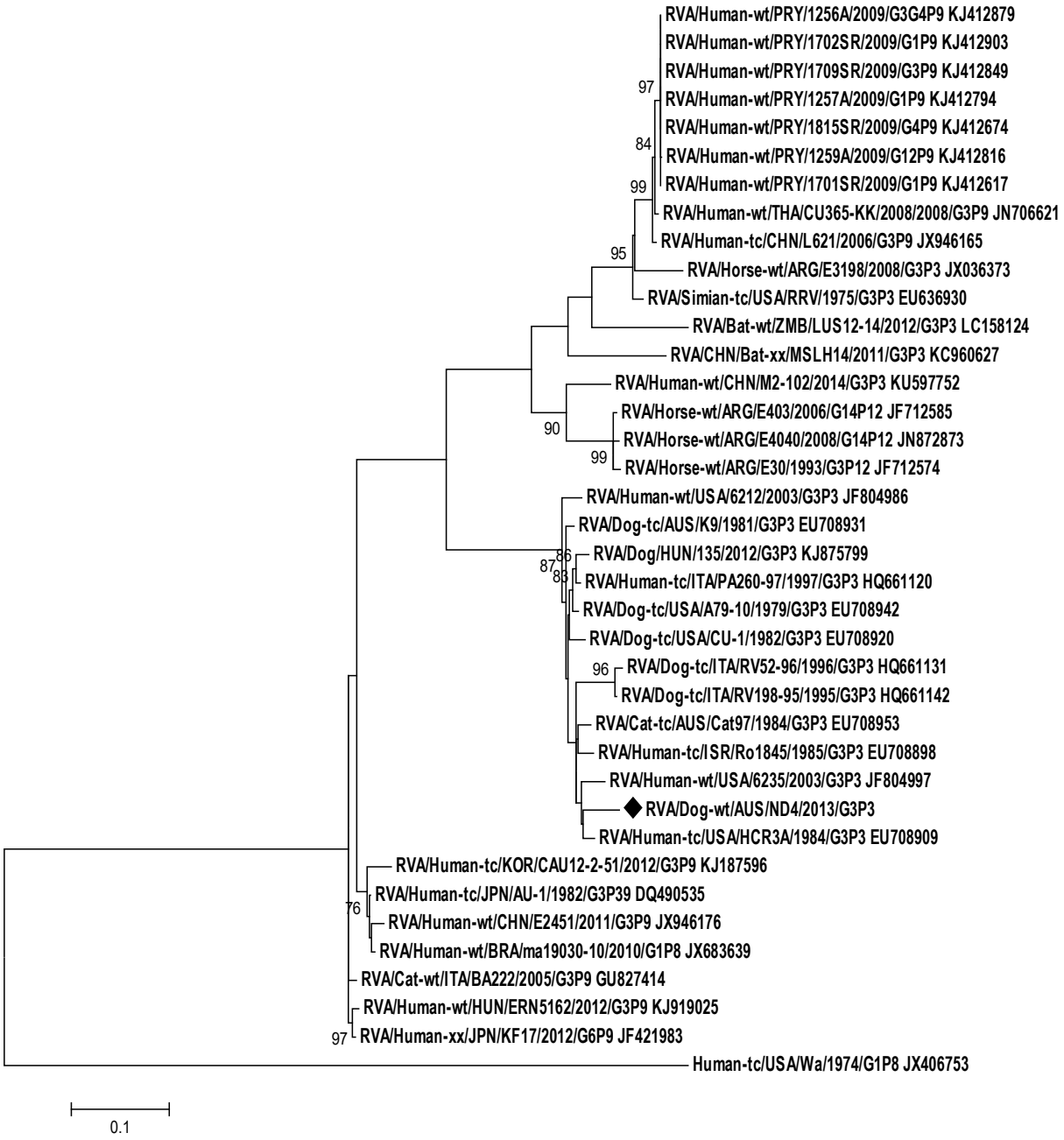

Figure 10: *Maximum likelihood phylogenetic tree of NSP3 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other T3 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

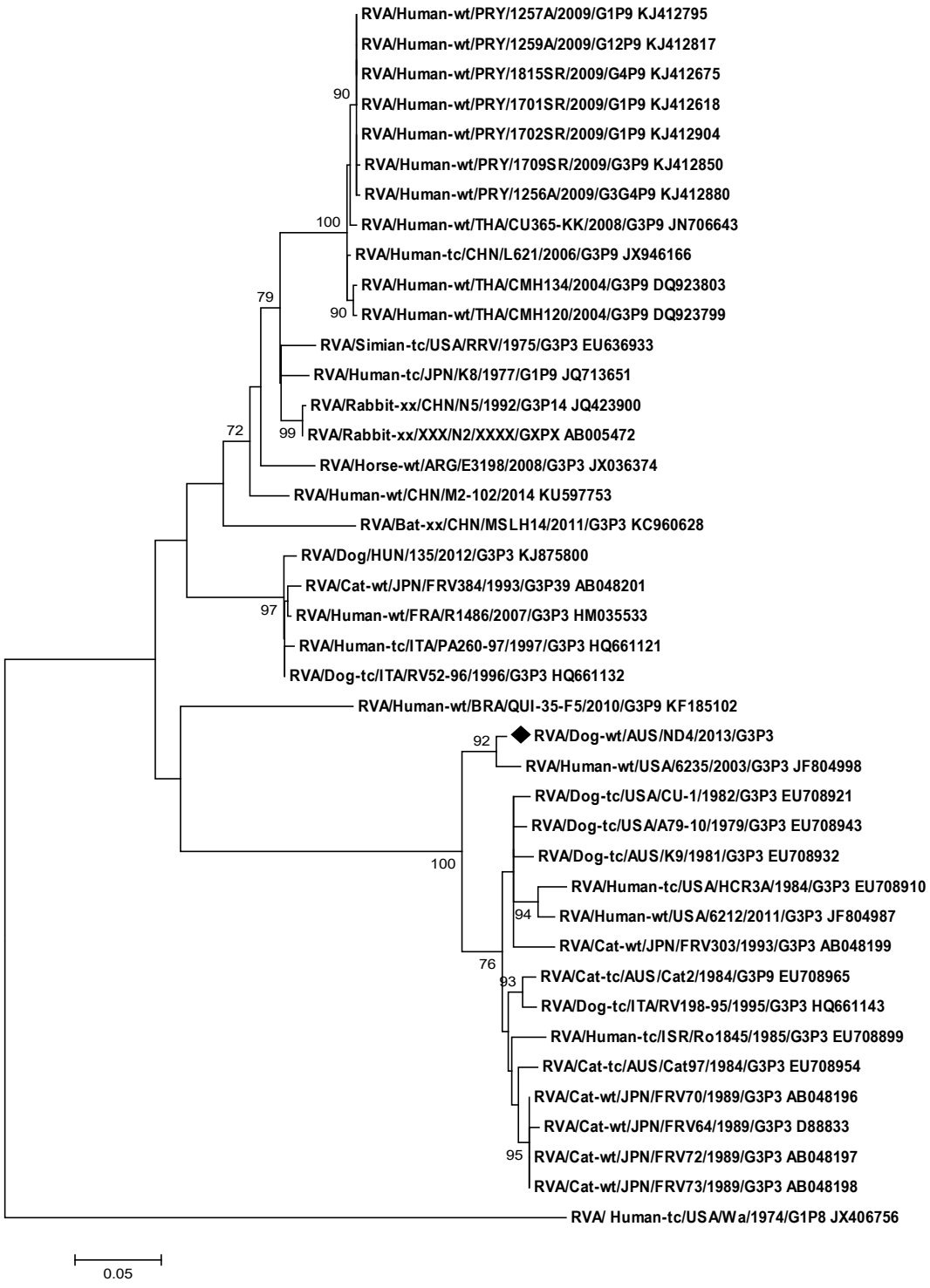

Figure 11: *Maximum likelihood phylogenetic tree of NSP4 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other E3 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

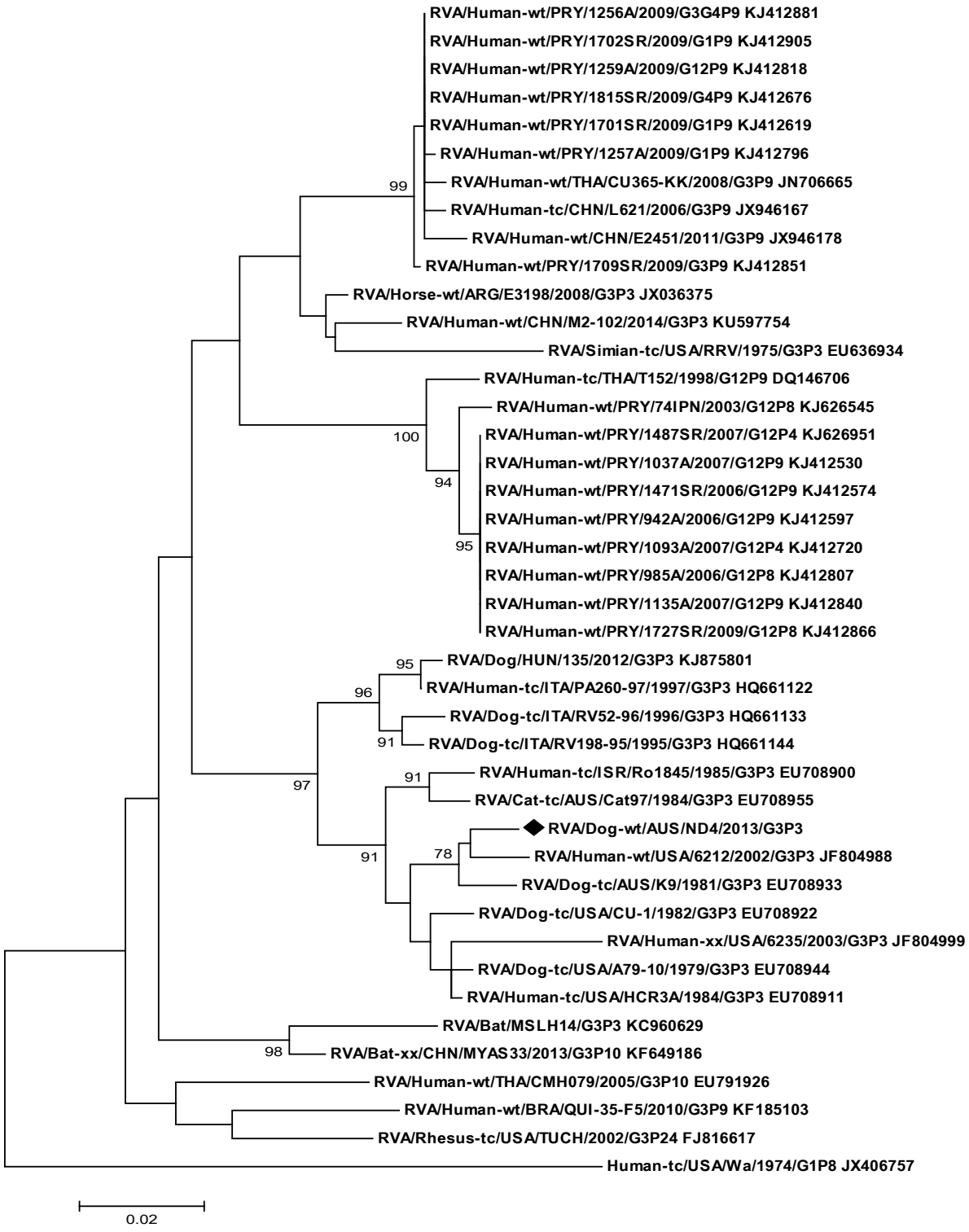

Figure 12: *Maximum likelihood phylogenetic tree of NSP5 gene of rotavirus G3P[3], strain RVA/Dog-wt/AUS/ND4/2012/G3P[3] with other H6 RVA strains.*

The position of strain ND4 is indicated by a ◆ symbol. Bootstrap values ≥ 70% are shown. Scale bar shows number of substitutions per site. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (345).

5.3.3 Prevalence of rotavirus group A

An ELISA test was then performed on all 131 faecal samples collected from the 3 different groups of dogs; healthy, acute diarrhoea and chronic enteropathy; to identify the presence of group A rotavirus. Statistical analyses showed that there was no significant difference in the prevalence of rotavirus detection between the healthy dogs and those with diarrhoea (Table 3). This result indicates that rotavirus is not associated with symptoms of diarrhoea in our group of dogs.

Table 3: **Proportion of rotavirus positive faecal samples collected from healthy dogs, dogs with acute diarrhoea and dogs with chronic enteropathy, as determined by ELISA testing**.

Group	ELISA-positive proportion (percentage)
Healthy dogs	3/65 (4.62 %)
Dogs with acute diarrhoea	4/44 (9.1 %)
Dogs with chronic enteropathy	0/22 (0 %)
Total	7/131 (5.34 %)

Pearson Chi-Square = 2.532, DF = 2, P-Value = 0.282; Likelihood Ratio Chi-Square = 3.507, DF = 2, P-Value = 0.173

Differences in prevalence were found between the different methods used to detect rotavirus. The ELISA test did not identify two samples from dogs in the chronic enteropathy group that were identified as RVA positive through NGS. Similarly, three samples from dogs in the acute diarrhoea group were negative when tested with the ELISA but positive through NGS. Furthermore, the ELISA test identified two samples from dogs with acute diarrhoea as rotavirus positive that were negative by NGS.

5.4 Discussion

Rotaviruses are enteric pathogens that affect people and a wide range of animal species. In dogs, rotavirus is not an important enteric pathogen and mainly participate in co-infections with other enteric viruses and are most often detected in immunosuppressed individuals (312, 346). The

risk associated with canine rotavirus is its zoonotic potential (318, 347, 348). Some human rotavirus strains, found in children with diarrhoea, have a close genetic relationship to G3 canine rotavirus strains, possibly generated through reassortment with animal strains (316, 347-349). This also creates a challenge for current vaccines due to the rise of new strains (350).

In our study, metagenomic analysis of 24 faecal samples identified sequences matching viruses belonging the Reoviridae family, with the full genome determine for an RVA G3P[3] virus identified in a sample from a healthy dog. Our G3P[3] strain differed from previously characterised G3P[3] feline/canine strains only in the VP6 gene, which was genotype I2 and exhibited closest genetically similar to bovine and human strains. The VP6 gene exhibited 97.5% of nucleotide similarity to a human G3 strain: RVA/Human-wt/USA/6235/2003/G3P[3]. This human strain also exhibited the highest genetic similarity across 5 other genes, (G3, P3, I2, A9 and E3) to our canine G3P[3] strain. The RVA/Human-wt/USA/6235/2003/G3P[3] strain was detected from an American Indian child with gastroenteritis, and was also suggested to have been derived via reassortant with animal strains (348). This, also suggests that our G3P[3] strain is a canine/human reassortant. G3P[3] strains have been isolated mainly in dogs and cats, but also in humans, owning some canine/feline characteristics (338).

To develop a full picture of the diversity of rotaviruses, surveillance is needed of both animal and human strains. Due to reassortment, new rotavirus strains containing novel genome constellations are appearing, and have been identified as causing severe disease in humans (318, 348). The Australian rotavirus surveillance program, has demonstrated an emergence of novel strains circulating in children and adults in the past decade [29]. These novel strains often share high genetic similarities to animal strains, specifically feline/canine-like G3P[3] (351). Although uncommon, the identification of novel strains in humans and animals emphasise the importance of understanding diversity and distribution of animal rotaviruses (349, 351-356). It is critical to understand the emergence and spread of novel rotavirus strains, both in animal and human

species as these could pose significant challenge to the rotavirus vaccine program which is currently included in the national childhood immunization programs of more than 90 countries across the world (350).

We used an ELISA test to identify the presence of rotavirus because this is the most commonly used test in veterinary and human medicine. Nevertheless, our results suggest that identification of RVA in animals, specifically dogs, could be an underestimate and PCR screening may be a more reliable detection assay. This could provide a more reliable approach to identify infections and assess a zoonotic potential.

In conclusion, this study highlights the importance of understanding rotavirus disease in different animal species, as it can provide crucial insight to reassortment between human and animal rotavirus strains, and emergence of new strains. Rotavirus has been found previously in healthy dogs and understanding the presence of canine rotavirus (340) can help to evaluate the possible transmission of zoonotic disease.

Table 4: List of primers used for full genome characterisation of G3P[3] canine rotavirus

VP1	Name	Sequence	Reference
	PM_ND4_VP1_2120 F	GAGGACAAAGTACACAATGGG	This study
	3304 Rev g1 RV3	GGTCACATCTAAGCGCTCTA	(357)
	PM_ND4_VP1_1786 F	GCATCAGGTGAAAAGCAAAC	This study
	PM_ND4_VP1_2585 R	GGAAATTTGTGCGCGACG	This study
	PM_ND4_VP1_755F	CACCAATGTCAATTCTAGTTGC	This study
	PM_ND4_VP1_1909R	CTCCATCAACTCGAATTATCTTAG	This study
	GEN VP1 F	GGCTATTAAAGCTRTACAATGG	(358)
	PM_ND4_VP1_1180R	CTTGAAGCAGATGATCATCATGC	This study
	PM_ND4_VP1_401R	GAATCAGTGTATTCTTCAGCTGTTGG	This study

VP2	Name	Sequence	Reference
	PM_ND4_VP2_1199R	CTCAATAGACATAGTACGTTGAC	This study
	GEN VP2 F	GGCTATTRAAGGYTCAATGG	(358)
	PM_ND4_VP2_1893F	GCCGCCAATAGATTGAATC	This study
	GEN VP2 R	GGTCATATCTCCACARTGG	(358)
	PM_ND4_VP2_692F	AGATGAGACAACGTGTACAGGC	This study
	PM_ND4_VP2_2100R	GCACGTTCAATTTGATTCC	This study
	PM_ND4_VP2_263R	GATGTTCTTCCTTCGTTTTTAGCAC	This study

VP3	Name	Sequence	Reference
	CD-G10P14-VP3-23F	GTGTGTTTTACCTCTGATGG	(356)
	CD_G3P14_VP3_603R	GGTCCAACGTTCACTACA	(349)
	VP3 P4	CAGTTGGTTTTGAGGCTA	(349)
	CD-G10P14-VP3-917F	ATCAGCACCATCGTACTGGA	(356)
	GEN VP3 R	GGYCACATCATGACTAGTG	(358)

VP4	Name	Sequence	Reference
	1 For g4 RV3	GGCTATAAAATGGCTTCACTC	(357)
	End G4	TAAGGTTATGTATGGTCACATC	(359)
	PM_ND4_VP4_1876F	GAAATGGCAACACAAACTGACGG	This study
	PM_ND4_VP4_423R	CCATTGCGTCTGTGAAGTATTCTC	This study

VP6	Name	Sequence	Reference
	GEN-VP6F	GGCTTTWAAACGAAGTCTTC	(358)
	GEN-VP6R	GGTCACATCCTCTCACT	(358)
	PM_ND4_VP6_969F	ATCATGCTACAGTGGGAC	This study
	PM_ND4_VP6_538R	GATCCTGCGTTTAGCCACAT	This study

VP7	Name	Sequence	Reference
	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	(360)
	End9	GGTCACATCATACAATTCTAATCTAAG	(360)

NSP1	Name	Sequence	Reference
	GEN_NSP1F	GGCTTTTTTTTATGAAAAGTCTTG	(307)
	GEN_NSP1R	GGTCACATTTTATGCTGCC	(307)
	VARG_VF5 R	GGTCACATTTTWTGCTYCCTAGG	(349)
	PM_ND4_NSP1_767F	GAAATCTCACTGATTTTACTTGGG	This study

NSP2	Name	Sequence	Reference
	GEN_NSP2F	GGCTTTTAAAGCGTCTCAG	(307)
	GEN_NSP2R	GGTCACATAAGCGCTTTC	(307)

NSP3	Name	Sequence	Reference
	GEN_NSP3F	GGCTTTTAATGCTTTTCAGTG	(307)
	GEN_NSP3R	ACATAACGCCCCTATAGC	(307)

NSP4	Name	Sequence	Reference
	10.1	GGCTTTTAAAAGTTCTGTCC	(356)
	10.2	GGTCACACTAAGACCATTCC	(356)

NSP5	Name	Sequence	Reference
	GEN_NSP5F	GGCTTTTAAAGCGCTACAG	(358)
	GEN_NSP5R	GGTCACAAAACGGGAGT	(358)

Chapter 6: General discussion and conclusions

The gut virome is increasingly being studied in humans and animals, leading to the discovery of new viruses and the establishment of models of healthy gut viromes (155, 361). In dogs, this field has not been studied in depth. The work described in this thesis includes the second study analysing the faecal virome of dogs with diarrhoea (122) and the first one to describe the canine faecal virome in healthy dogs, and dogs with chronic enteropathy (CE).

Virus discovery and detection for diagnostic purposes has progressively increased with new and improved viral identification techniques, such as next generation sequencing, metagenomics and bioinformatic analyses (89, 90, 118). The study of the virome has been crucial for the discovery of new viruses, while also providing new information about viruses present in samples or environments where they were previously unaccounted (112, 362). To achieve this, appropriate sample preparation protocols capable of producing high concentrations of high quality viral nucleic acids and low numbers of bacteria are essential (88, 90).

Chapter 2 describes the optimisation of the sample preparation and nucleic acid extraction protocol that was used in the studies here presented. There were several attempts at optimising exiting protocols routinely used in viral detection and diagnostic of human and animal faecal samples, with the objective of reducing the bacterial load as much as possible, while simultaneously enriching the viral acid nucleic concentration. Finally, a sample preparation protocol that included vortexing and filtration before the addition of a mixture of nucleases was used. Thanks to this combination of mechanical exclusion and enzymatic degradation, the bacterial concentration as evaluated through bacterial 16S gene PCR, decreased noticeably. This led to the establishment of a defined protocol, which was subsequently used to prepare all samples subjected to shotgun sequencing and bioinformatic analyses.

In the current study, 24 faecal samples from dogs were analysed using shotgun metagenomic analysis. Three groups were analysed: healthy dogs, dogs with acute diarrhoea and dogs with CE, each group containing 8 individuals. Healthy dogs and dogs with acute diarrhoea were recruited from an inner-city animal shelter and dogs with CE were recruited from a referral veterinary hospital. Comparisons between the faecal viromes of healthy dogs and dogs with acute diarrhoea or CE were done separately.

The comparisons between the faecal virome of healthy dogs and dogs with acute diarrhoea were published (321) and were presented in chapter 3. Viruses that infect eukaryotes and prokaryotes were found in healthy individuals, suggesting to be an important part of the GI microbiota, as seen in gut viromes of other species (115, 116, 119, 129, 134). Likewise, the presence of pathogenic or potentially pathogenic viruses found in this group is not unexpected. The occurrence of pathogenic viruses in healthy individuals may be accounted for different causes, such as the shedding of an earlier infection (206, 261, 363), shedding from vaccination (364), infection of animals with acquired genetic resistance, chronic infections in immunodeficient individuals (261) and the presence of age-restriction patterns that influence the course of the infection (175, 261)

In both groups of individuals, the most common component of the virome were bacteriophages, similar to other findings in human and animal viromes (115, 116, 134, 155). In fact, the families of bacteriophages found in healthy dogs and dogs with acute diarrhoea were the same. Nevertheless, there was a different diversity of eukaryotic viruses between both groups.

The comparisons between the faecal virome of healthy dogs and dogs with CE was also published (324) and is presented in chapter 4. As in healthy dogs and dogs with acute diarrhoea, bacteriophages were the most common viral component of the faecal virome in dogs with CE. This result was also reported in people with IBD (142, 155, 365). Viruses that infect eukaryotes were found in both groups, however, dogs with CE had lower diversity of families compared to

healthy dogs. The importance of the analysis of eukaryotic viruses in chronic enteropathies, laid in findings from studies that found some specific strains of an eukaryotic virus, murine norovirus (MNV), induced intestinal pathologies related to Crohn's disease in mice, in genetically susceptible individuals (366). Inversely, it has been shown that the same MNV strains in non-susceptible individuals, can replace the beneficial function of commensal bacteria (367).

The combined analysis of the results from the three groups is further discussed here. Viruses infecting eukaryotes and prokaryotes were found in all groups (Appendix 8.2). The most ubiquitous viruses present in faeces of dogs were bacteriophages, found in all samples from all three groups of dogs, and every individual dog. This finding, where bacteriophages are the most prevalent viral component, is similar to what has been observed in human and other animal faecal virome studies (116, 129, 134, 140, 141, 155). Bacteriophage families found in our study were identical in all groups of dogs. In contrast, differences in the families of eukaryotic viruses were observed between all three groups, where dogs with CE had the least diversity of eukaryotic viruses. This result is consistent with a study analysing viruses in people with Crohn's disease (368). In healthy dogs, and dogs with CE, 4/8 and 3/8 samples harboured eukaryotic viral contigs/singletons, respectively. However, samples from dogs with acute diarrhoea contained at least one eukaryotic viral family in each sample (Appendix 8.2). Likewise, dogs from this group also had more viral co-infections (contigs/singletons from more than one viral family) than the other two groups (Appendix 8.2).

A possible cause of these differences might be the different health status, as has been shown in several publications (155, 368). Nevertheless, also could be the different background of these dogs, as while healthy dogs and dogs with acute diarrhoea came from a shelter environment, dogs with CE were recruited from a veterinary hospital and lived with their owners, confirming findings of microbial load in sheltered dogs (369).

In this study, the genomes and prevalence of three eukaryotic viruses were individually analysed and characterised. We confirmed the presence of a canine kobuvirus, a canine astrovirus and a canine rotavirus in the samples analysed. Although, the presence of canine rotavirus and canine astrovirus-like have been identified in canine faeces before in Australia using EM (159), this is the first genome characterisation of both viruses and first description of canine kobuvirus in the country. A prevalence study evaluating the presence of these three viruses was performed in a bigger cohort. Both shelter dogs and client-owned dogs were recruited for this prevalence study, to give a true representation of prevalence.

A low prevalence of canine astrovirus was found in healthy dogs and dogs with acute diarrhoea, and no astrovirus was identified in dogs with CE. This difference between groups was not significant (Appendix 8.3). Contrary to expectations, a difference in the results of screening PCR compared to NGS results was detected, where not all positive samples identified with the sequencing method were detected by the directed-PCR prevalence study. A possible explanation for these results might be the inherent limitations of our screening PCR designed to amplify a large product (1381bp), which may have had a negative impact on the sensitivity of the assay (Appendix 8.4).

Each group of dogs had at least one positive result for canine kobuvirus. When healthy dogs and dogs with CE from the same background (client-owned) were compared, the only positive sample was the same one identified with NGS (Table S1, chapter 4). When dogs from shelter environment were included, a low prevalence was also detected both in healthy dogs and dogs with acute diarrhoea. In summary, all three groups had a very low prevalence of canine kobuvirus and the difference between groups was not significant (Appendix 8.3).

The analysis of the full genome of the canine rotavirus characterised in this study (chapter 5), identified from a healthy dog, determined that it was a G3P[3] canine rotavirus strain from group A. This strain, belonged to the canine/feline constellation, but differed in one gene, which was

genetically similar to that of human strains. This difference suggests that the viral strain here characterised is a canine/human reassortant. Reassortment is a common evolutionary strategy in rotaviruses and explains the emergence of new viral strains. Novel strains may be capable of producing disease or escaping the immunity induced by existing vaccines. To evaluate the prevalence of this viral strain in the sample collection used in this study, an antigen detection ELISA test was used. Results from this analysis determined a low prevalence for this virus, indicating that a more sensitive and specific detection technique, such as PCR screening, may be required to have a more reliable assessment of the prevalence of this virus and follow the possible emergence of this reassortant. Surveillance of rotavirus in humans and animals are warranted to evaluate the potential transmission of this zoonotic agent. Prevalence of canine rotavirus was very low and no significant difference between groups was found.

It is interesting to note that all healthy dogs from a client-owned background, were negative to any of these viruses (Table S1, chapter 4). Likewise, all positive healthy dogs belonged to a shelter environment. Therefore, the difference in the virus prevalence of these dogs may well be associated with environmental differences, such as diet, vaccination and deworming status, and intensive housing system such as shelter conditions, which could lead to differences in the immunocompetent status of the dogs (369). Overall, these findings suggest that the animals' environment plays an important role in the prevalence of enteric viruses, raising the possibility that differences observed in the viromes of these dogs, as determined by shotgun sequencing techniques, may occur as a consequence of the environment where they live rather than due to the disease.

As discussed in chapter 3, our results biased by the SISPA method, which did not allow us to do a more accurate quantitative analysis of the viral populations observed. Also, not all eukaryotic viruses found in the metagenomic shotgun analysis study were confirmed through PCR. The selection of the three viral genera that were further characterised was based on the novelty of

the viruses detected or their potential implication as causative agents of gastrointestinal disease.

Future studies, based on the samples and data gathered during this study may want to focus on other viral species. Future studies could also evaluate the faecal virome in combination with the faecal bacteriome in a longitudinal study, using a larger number of samples to evaluate more closely the relationships between populations of bacteria and bacteriophages, and their association with gastrointestinal disease in dogs. Studies evaluating some specific populations of bacteriophages and their role in immunity are warranted, particularly in studies performed in dogs with chronic enteritis and studies evaluating faecal transplantation. Furthermore, one of the most important research that needs to be implemented and expanded is the use of bacteriophages therapy as a potential solution to antibiotics resistance.

This thesis makes a significant contribution to our current understanding of the canine intestinal virome in sickness and in health. Future research should analyse all components of the microbiome, including eukaryotic viruses, bacteriophages, bacteria, archaea and fungi. Such studies of the GI ecosystem, would allow a better understanding of the complex interactions that take place between the different participants (host and microbiome) and the determinants that are associated with health and disease, where the physio-pathological processes may be recognised as ecological interactions within a rich and diverse environment.

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Appendices

8.1 Appendix: Canine faecal sample preparation and viral enrichment protocol used in viral metagenomic analysis

I. Faecal sample preparation

A. - Stool: solid mass

Fume hood:

1) Add buffer (0.01M Tris HCl pH7.5 +NaCl 0.1M + $CaCl_2$ 0.01M) in approximately 3:1 ratio of solid mass (500mg stool + 1.5ml buffer) in a 2ml screw cap tube for each sample.
2) Add 1 mm zirconia/silica beads to stool solution (fill between 100 and 200µl graduation of a 1.5ml tube)
3) Vortex solution vigorously for ~1min per sample. Repeat 3 times
4) Clarify by centrifugation at 13000 rpm (17900g) for 5 min

Dirty hood:

5) Remove supernatant onto fresh tube (2ml screw cap tube),
6) Spin solution at 13.000rpm (17900g) for 5 min and transfer supernatant into clean 2ml screw cap tube.
7) Spin solution additional time at 13.000rpm for 5 min
8) Remove 500µl of solution and put the supernatant through a Corning® Costar® Spin X® centrifuge tube filter 0.45 µm, sterile (CLS8162-96EA SIGMA). Use a new column for the rest of the sample.
9) Centrifuge at 6.000rpm (3800g) for 5 min, transfer all filtrate to a 2ml screw cap tube.
10) Proceed with nuclease treatment

B. - Stool: small volume solid mass or diarrhoea

Essentially follow identical protocol as **A,** with following exceptions:

(a) Step 1: use 1:1 ratio of buffer

(b) Omit step 2

II. Nuclease treatment

315µl	Sample
28µl	Turbo DNase
12µl	Baseline Zero DNase
12µl	Benzonase
5µl	DNase I Roche
6µl	RNase A (QIAGEN)
42µl	10x Turbo buffer
420µl	**Volume total**

Incubate at 37ºC for 3 hrs (mix by gentle vortex every 45min) in a water bath.

To stop nuclease activity, add 12.6 µl EDTA 0.5M (AMRESCO®), pH 8.0, to a final concentration of 15mM.

Incubate at 75℃ for 10 minutes in a heater block

Finalize with a short spin and the samples are ready to extract viral nucleic acids with QIamp® Viral RNA mini kit; spin protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

8.2 Appendix: Summary of clinical information and number of contigs/singletons of eukaryotic and prokaryotic viral families detected by metagenomic sequencing in faeces of dogs.

SAMPLES	AGE	SEX	BREED	DIAGNOSTIC	PROKARYOTIC VIRAL CONTIGS/SINGLETONS	EUKARYOTIC VIRAL FAMILIES (Nº OF CONTIGS/SINGLETONS DETECTED)
HD4	7 y	MN	Maltese X	Healthy	1012	*Reoviridae* (39)
HD5	2 y	MN	Jack Russell	Healthy	209	None
HD6	5 y	F	Maltese/Shih Tzu	Healthy	953	None
HD7	4 y 7 m	FS	Staffy	Healthy	731	None
HD8	7 y	MN	Labrador X	Healthy	82	None
HD9	8 m 1 w	MN	Mastiff/Staffy	Healthy	5	*Parvoviridae* (3)
HD10	8 m	M	Maltese X	Healthy	2	*Coronaviridae* (912)
HD11	2 y 6 m	MN	Maltese/Shih Tzu	Healthy	18	*Adenoviridae* (1) Papillomaviridae (1)
CE1	2 y 8m	FS	Labrador	FRE	214	*Papillomaviridae* (1) *Reoviridae* (2)
CE4	5 y	M	Rottweiler	IRE	449	None
CE5	2 y 5m	MN	Bull Terrier	FRE	227	None
CE8	1 y 6m	F	German Shepherd	ARE	499	None

CE9	1 y 9 m	M	Weimaraner	FRE	8777	*Picornaviridae* (9)
CE10	2 y	M	Basset hound	ARE	319	None
CE11	4 y 5m	MN	Labradoodle	ARE	468	*Reoviridae* (4)
CE13	5 y	FS	Terrier cross	FRE	202	None
DD1	2 y	M	Mastiff cross	Acute diarrhoea	1777	*Astroviridae* (18) *Picornaviridae* (3)
DD2	4 m	M	Mastiff cross	Acute diarrhoea	2179	*Parvoviridae* (41) *Astroviridae* (3)
DD3	5 y	FS	Wirehair Dachshund	Acute diarrhoea	1559	*Reoviridae* (2) *Astroviridae* (3) *Coronaviridae* (4)
DD5	10 w	M	Husky	Acute diarrhoea	1387	*Astroviridae* (3) *Caliciviridae* (8)
DD7	10 m	M	Husky	Acute diarrhoea	2593	*Reoviridae* (3)
DD8	7 m	FS	German shorthair pointer	Acute diarrhoea	3230	*Parvoviridae* (3) *Coronaviridae* (211)
DD9	4 y	F	Bull dog	Acute diarrhoea	3319	*Reoviridae* (5)
DD10	2 y	M	Rhodesian ridgeback	Acute diarrhoea	886	*Reoviridae* (4) *Astroviridae* (1)

HD: healthy dogs, CE: dogs with chronic enteropathy, DD: dogs with acute diarrhoea, MN: male neutered, M: male, FS: female spayed, F: female, FRD: food responsive enteropathy, IRE: immunosuppressive responsive enteropathy, ARE: antibiotic responsive enteropathy.

8.3 Appendix: Proportion of canine astrovirus and canine kobuvirus positive faecal samples collected from healthy dogs, dogs with acute diarrhoea and dogs with chronic enteropathy, as determined by PCR testing.

Canine astrovirus

Group	PCR-positive proportion (percentage)
Healthy dogs	4/65 (6.15 %)
Dogs with acute diarrhoea	3/44 (6.82 %)
Dogs with chronic enteropathy	0/22 (0 %)
Total	7/131 (5.34 %)

Pearson Chi-Square = 1.515, DF = 2, P-Value = 0.469; Likelihood Ratio Chi-Square = 2.672, DF = 2, P-Value = 0.263

Canine kobuvirus

Group	PCR-positive proportion (percentage)
Healthy dogs	3/65 (4.62 %)
Dogs with acute diarrhoea	2/44 (4.55 %)
Dogs with chronic enteropathy	1/22 (4.55 %)
Total	6/131 (4.58 %)

Pearson Chi-Square = 0.000, DF = 2, P-Value = 1.000; Likelihood Ratio Chi-Square = 0.000, DF = 2, P-Value = 1.000

8.4 Appendix: material and methods used for the determination of canine astrovirus prevalence in healthy dogs, dogs with acute diarrhoea and dogs with chronic enteropathy

DNA and RNA from additional 93 faecal samples were extracted. Faecal samples were thawed and prepared with the 20% (w/v) method. Briefly 800µl of virus dilution buffer (0.01M Tris solution (pH 7.5), 0.15M NaCl, 0.01M $CaCl_2$) was added to 0.2g of faecal material, vortexed until homogenised, centrifuged at 20,000g for 3 minutes and the supernatant harvested. The RNA was extracted from 140µl of the supernatant using QIamp® Viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations.

In order to amplify the last part of ORF1b and the start of ORF2, due to this region is highly conserved among most astroviruses (172), two primers were used: PM_Astro_DD1_3527F and PM_Astro_DD1_4908R (Table S2, chapter 3), resulting in the amplification of a 1381bp fragment.

RT-PCR was performed with QIAGEN One-Step RT-PCR kit (QIAGEN, Hilden, Germany). PCR conditions used were an RNA denaturation step at 97°C for 3 minutes in a heater block, prior to the addition of master mix. Afterwards, on the thermo cycler, 45°C for 60 min and 95°C for 5 min, 35 cycle of 94°C for 40 sec, 55°C for 1min and 72°C for 5 min, and a final elongation step of 72°C for 5 min, followed by final hold at 4°C.

All positives samples were visualized in a 1.2% agarose TBE gel, where 20µl of PCR product sample and 10µl of dye (0.2% bromethyl phenol blue) were subjected to electrophoresis at 100V for 70 minutes.

8.5 Appendix: Diagram step-by-step of optimisation of the final protocol