UNIVERSIDADE DE ÉVORA

ESCOLA DE CIÊNCIAS E TECNOLOGIA

DEPARTAMENTO DE MEDICINA VETERINÁRIA

Molecular Surveillance of Parvoviruses circulating in Cats in the United Kingdom

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Orientação Interna: Doutora Elsa Leclerc Duarte

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Acknowledgments

This long journey would not be possible without everyone that crossed my path and that will be part of my life forever. To choose to go for a different country and do a research project followed by an English-written dissertation was not an easy decision. However, I believe that it is outside of our comfort zone that we learn the most, and it is there where we can be challenged every single day, at every moment (in fact, even to cross the road in the UK!). Since I can remember, I always wanted to end this chapter of my life with research. We, as Veterinary Medicine students, and as future professionals, have the responsibility from day one to contribute to the improvement of the animal wellbeing and health, and through research, we can create impact in the Veterinary Science and Medicine that we know nowadays, potentially making difference to the health of thousands of animals in the future. Each small step in research is important to raise awareness, to develop more scientific knowledge regarding the animal health. Thanks to all that have contributed for making this possible during my journey.

To Dr. Elsa Leclerc Duarte, my supervisor, for the faithful guidance during the last years of this chapter. For all the advices, suggestions, patience, and for always wanting the best for her supervised students.

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Abstract

This dissertation was developed under the Curricular Traineeship scope, which took place at the University of Nottingham, between July 2016 and April 2017. Feline panleukopenia virus (FPV) and canine parvovirus (CPV), two closely related viruses, are known to cause acute enteritis in companion animals. Cats may be infected by strains of both viruses. Population-based surveillance studies have been lacking. This study investigated the prevalence of parvoviruses in a cross-sectional survey of clinically-healthy cats housed within 13 shelters across the United Kingdom, comprising 818 faecal samples. FPV/CPV DNA was detected by PCR. Overall, the prevalence of parvovirus was 3.8%. Five FPV/CPV-2a like strains and one CPV-2b were identified by sequence analysis. These results showed that parvoviruses are circulating in the UK feline population, providing an insight about parvovirus occurrence in a non-clinical population, which reinforces the possibility that asymptomatic cats may be important reservoirs for infection maintenance in companion animals.

Keywords: canine parvovirus; feline panleukopenia virus; viral genetic variants; United Kingdom

Resumo

Vigilância molecular dos Parvovírus circulantes em gatos no Reino Unido

Esta dissertação foi elaborada no âmbito do Estágio Curricular, realizado na Universidade de Nottingham, entre julho de 2016 e abril de 2017. O vírus da panleucopénia felina (FPV) e o parvovirus canino (CPV), dois vírus estreitamente relacionados, são conhecidos por causar enterite aguda em animais de companhia. Os gatos podem ser infetados por estirpes de ambos os vírus. Investigou-se a prevalência de parvovirus através de um estudo transversal em gatos clinicamente saudáveis acolhidos em 13 abrigos de distintas localizações no Reino Unido, somando 818 amostras fecais. O DNA do FPV/CPV foi detetado por PCR. Globalmente, a prevalência de infeção por parvovirus foi de 3,8%. Cinco estirpes virais compatíveis com FPV/CPV-2a e uma estirpe CPV-2b foram identificadas através de sequenciação. Os resultados comprovam que diferentes variantes de parvovirus circulam na população felina do país, reforçando a possibilidade de gatos assintomáticos constituírem reservatórios importantes para manutenção da infeção em animais de companhia.

Palavras-chave: parvovírus canino; vírus da panleucopénia felina; variantes genéticas virais; Reino Unido

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List of Abbreviations

ALT – Alanine Aminotransferase

AST – Aspartate Aminotransferase

BFPV – Blue Fox Parvovirus

bp – base pairs

BSAVA – British Small Animal Veterinary Association

CBC – Complete Blood Count

CDV – Canine Distemper Virus

CI – Confidence Interval

CNS – Central Nervous System

CPV – Canine Parvovirus

CPV-1 – Canine Parvovirus type 1

CPV-2 – Canine Parvovirus Virus type-2

DEPC – diethyl pyrocarbonate

DIC – Disseminated Intravascular Coagulation

DTT – dithiothreitol

ELISA – Enzyme Linked Immunosorbent Assay

EM – Electron Microscopy

FCV – Feline Calicivirus

FeLV – Feline Leukaemia Virus

FPV – Feline Panleukopenia Virus

GI – Gastrointestinal

HI – Haemagglutination Inhibition

ICTV – International Committee on the Taxonomy of Viruses

ID – Identification

KRV – Kilham Rat Virus

MDA – Maternally Derived Antibodies

MEV – Mink Enteritis Virus

MGB – Minor Groove Binder

MLV – Modified-live Virus

mRNA – messenger RNA

MVC – Minute Virus of Canines

Nt – Nucleotide

ORFs – Open Reading Frames

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

rfIFN-ω – Recombinant Feline Interferon-omega

SAVSNET – Small Animal Veterinary Surveillance Network

SNPs – Single Nucleotide Polymorphisms

ssDNA – single-stranded DNA

TBE – Tris-Borate-EDTA

TfR – Transferrin Receptor

UK – United Kingdom

USA – United States of America

VWD – von Willebrand's disease

WSAVA – World Small Animal Veterinary Association

Foreword

The following dissertation is based on the research work developed under the frame of the Curricular Traineeship Unit, part of the Integrated Master in Veterinary Medicine, from the University of Évora.

The traineeship took place at the School of Veterinary Medicine and Science, Faculty of Medicine & Health Sciences, University of Nottingham – Sutton Bonington Campus, United Kingdom, from 25th of July 2016 to 7th of April 2017 (8 months and 2 weeks).

The traineeship included the research planning, development of laboratory experiments and critical evaluation of results, on an independent-work basis, supervised by members of Infection and Immunity Research Theme – Virology Group, which included Dr Janet Daly, Associate Professor in Emergent Viruses, as main research supervisor, and Dr Steve Dunham, Associate Professor of Veterinary Virology, as additional research supervisor.

Laboratory training was delivered in the following laboratory techniques and procedures:

- Mammalian cell culture, cell infection and cryopreservation techniques;
- DNA Extraction and DNA purification;
- Conventional PCR and Gel Electrophoresis;
- DNA sequencing and phylogenetic analysis;
- Following COSHH and SOP protocols, at a Biosafety Level 2 Laboratory.

During the traineeship period, a contribution was made to a preliminary study about opinions of Rottweilers' owners and breeders about vaccination, through analysis and report of results, supported by the two research supervisors mentioned above and by Dr Mark Dunning, Clinical Associate Professor in Small Animal Internal Medicine.

Furthermore, training in statistics and general data management was delivered by the Stats Club of the Sutton Bonington Graduate Centre within four sessions, which took place between November 2016 and January 2017, specifically: planning research and types of data; data entry and data cleaning; basic Excel and descriptive statistics. A workshop in Academic Scientific Writing was delivered in November by Dr. Janet Daly, divided in three sessions: introduction to scientific writing; reviewing literature; managing and citing references.

Extracurricular teaching training, in facilitating group sessions and demonstrating practical sessions, was delivered by the Teaching, Learning and Assessment Office of the School of Veterinary Medicine and Science. Five sessions of the Professional and Personal Skills Module, from the Year 1 of the Veterinary Medicine course curriculum were facilitated to a group of students, which sessions contents were related with learning techniques, study methods, group

working and peer feedback, with a total duration of 10 hours, between October and November 2016. Ten practical sessions of the Veterinary Medicine curriculum were demonstrated between October and December 2016, with a total of 34 hours.

During the traineeship period, two scientific meetings were attended, namely, the International Society for Companion Animal Infectious Diseases Symposium (ISCAID) in October 2016, in Bristol, and the Glasgow Virology Workshop, at the University of Glasgow, in February 2017.

Several research seminars, associated with the Animal Infection and Immunity Research Theme, which took place at the University of Nottingham, were attended during the traineeship period, specifically:

- "*Molecular interactions underpinning packaging in viruses with segmented genomes: a novel antiviral target*", August 2016;
- "*Structural and epidemiological studies of bluetongue virus*", November 2016;
- "*Insights into the ecology and epidemiology of MERS-Coronavirus*", February 2017;
- "*Arbovirus Encephalitis: From Tissue Culture to Mosquitoes and Mice*", February 2017;
- "*Improving the use of economics in animal health – challenges in research, policy and education*", March 2017.

I. Literature Review

1. Introduction

1.1. Initial Characterization and Classification

The published history of the parvoviruses begins in 1959, after Kilham and Olivier reported the isolation of a small, stable virus, initially named rat virus (RV), later known as Kilham Rat Virus (KRV), from lysates of an experimental rat tumour.[1]

In 1966, Brailovsky first introduced to the literature the name “parvovirus”, derived from the Latin “*parvus”* meaning small, and named RV as “*Parvovirus ratti*”, in an early effort to start a Latinised binomial nomenclature system for viruses.[2] During the 1970s, a working-group from the International Committee on the Taxonomy of Viruses (ICTV) established and approved the taxonomic family *Parvoviridae*, which then advanced the taxonomic rules for the further subdivision of this rapidly expanding family.[3]

The family *Parvoviridae* nowadays includes two subfamilies: the subfamily *Densovirinae*, which contains viruses of invertebrates, and the subfamily *Parvovirinae*, with viruses that infect vertebrates.[4,5] There are five genera in the subfamily *Parvovirinae*, taxonomically grouped according to their molecular properties. The genus *Protoparvovirus* includes the Feline Panleukopenia Virus (FPV) and the closely related Canine Parvovirus (CPV).[5,6]

1.2. Viral structure

1.2.1. Genetic structure

The parvoviruses are both small and genetically simple. The genome consists of a single molecule of linear single-stranded DNA (ssDNA) of about 5,200 nucleotides (approximately 5 kilobases) in length, which has two open reading frames (ORFs). The first ORF encodes two non-structural proteins, NS1 and NS2, which are required for DNA transcription and replication.[4,7] The second ORF encodes two structural proteins of the capsid, VP1 and VP2 (Fig.1).[6] CPV and FPV only package the negative-sense DNA strand into the capsid. The genome has terminal palindromic sequences, enabling each end to form the hairpin structure required for replication of the viral DNA.[4]

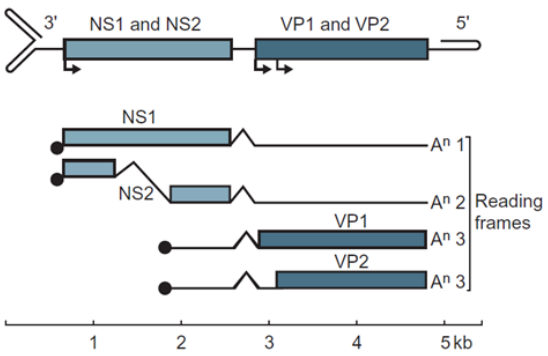

Figure 1. The genetic structure of CPV and FPV. (*Reproduced from Fenner’s Veterinary Virology, 2016*[4])

1.2.2. Capsid structure

Nearly all the biological differences between CPV and FPV are determined by the viral capsid. In fact, its nucleotide sequences are around 98% identical.[8–10]

The structures of the FPV and CPV capsids have been determined by a combination of protein analysis, electron microscopy, and lately by X-ray crystallography.[11,12] The parvovirus capsid is a icosahedral structure exhibiting T=1 symmetry that is assembled from a total of 60 copies of viral proteins, VP1 and VP2, of which approximately 90% is VP2 and 10% being the VP1 protein.[4,6]

The two proteins most likely act in a structurally equivalent way, being formed by alternative splicing of the same messenger RNA (mRNA). The entire sequence of VP2 is encoded within the VP1 gene.[12]

The surface of the capsid is structurally complex and includes features such as a raised area (spike) surrounding the three-fold axis of symmetry, a depression (dimple) spanning the two-fold axis of symmetry, and a further depressed area (canyon) surrounding the five-fold axis of symmetry of the capsid (Fig. 2).[11,12]

The receptor binding site of feline and canine parvoviruses, which determines their host and tissue tropism, is the three-fold spike, which is also the site of binding of most antibodies directed against the capsid.[4,12] The VP2 monomers make up most of the outer surface of the virus particle and are responsible for receptor binding, antigenic properties, and environmental stability.[6]

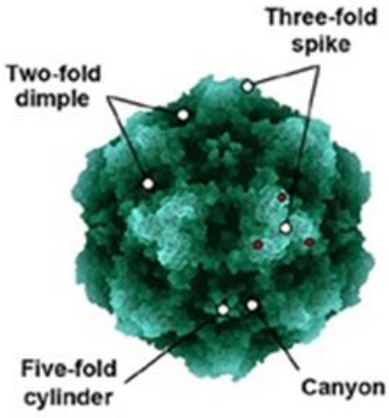

Figure 2. Structure of the Parvovirus capsid. (*Reproduced from Allison et al., 2016*[13])

Resistance to inactivation by organic solvents indicates that parvoviral capsids lack essential lipids. Indeed, parvoviruses are extremely stable to environmental conditions, including extremes of heat and pH.[4]

1.3. Viral Replication

Parvoviruses infect cells by binding to the Transferrin Receptor (TfR) type-1 on the cell membrane.[6,12] TfR has been described as the crucial and, apparently, only receptor required by parvoviruses for binding and infection of carnivore host cells.[5,13] After binding, virions are then taken up into the cell by rapid clathrin-mediated endocytosis. After entering the cell, virions traffic through the endosomal pathways within the cytoplasm.[12]

Exactly how the particles exit from the endosomal system is still unclear, however, this event causes the exposure of the capsid to lower pH as the endosome acidifies, probably causing the release of some of the capsid-bound calcium ions and movement of capsid loops, which facilitates the release of the VP1 unique region.[5,12] Additionally, the viral VP1 protein contains a phospholipase-enzyme activity, which may be involved in modifying the endosomal membrane and facilitating capsid release.[4]

The viral particles are trafficked to the nuclear pore, which allows entry into the nucleus, where the viral DNA replication and capsid assembly occurs, requiring a host-cell function of the S phase of the cell division cycle.[10] This requirement for mitotic cycling cells is explained by the need for DNA replication machinery, because the virus does not encode or package an enzymatic system for replication. Therefore, the cellular DNA-polymerases replicate the viral DNA in a double-stranded form, which is used as a template for transcription of viral mRNAs.[4]

Subsequently, alternative splicing generates several mRNA species, which are translated into four major proteins. The most abundant mRNA, which is encoded in the 5′ half of the genome, directs the synthesis of the structural proteins. The non-structural proteins are encoded in the 3′ portion of the genome.[4,5]

It is known that the 3′-terminal hairpin on the negative-sense DNA genome acts as a self-primer for the initiation of synthesis of a double-stranded DNA replicative intermediate. It is produced as two complete positive strands and two complete negative strands by a series of reopening of closed circular forms, re-initiation of replication, and single-strand endonuclease cleavages.[4,5] This complex mechanism of replication of the genome is known as a rolling-hairpin replication, and some details are still not fully understood.[4]

2. Feline Panleukopenia Virus Infection

2.1. Epidemiology

Feline panleukopenia is caused by the feline panleukopenia virus (also designated as feline parvovirus)[14,15] and was first described in 1928.[16,17] It is genetically and antigenically closely related to mink enteritis virus (MEV), blue fox parvovirus (BFPV) and canine parvovirus (CPV). FPV infects domestic cats and other *Felidae.*[14,18] Species from the families *Mustelidae*, *Procyonidae*, and *Viverridae*, which include raccoons, ring-tailed cats, minks and foxes, are also susceptible.[19,20] Minor differences in the genome have been reported, but these appear to be clinically insignificant mutations.[18,21]

The disease is characterised by severe reduction in circulating white blood cell count and enteritis with degeneration of the intestinal *villi.*[17,22] The infection causes high mortality and morbidity, and it is considered highly contagious, with concentrations of up to 10^9 viral particles shed per gram of faeces from infected animals.[17]

The virus is characterized by being ubiquitous and very stable, persisting in the environment over one year in organic material or fomites.[23] Thus, indirect transmission plays a key role in the infection of susceptible animals, it being possible that owners carry viral particles potentially infecting cats housed entirely indoors. Contaminated litter trays, food dishes, bedding and infected cages can have a particular importance in shelter environments.[17,24] Flies and other insect vectors can also be involved in the transmission of the disease.[14]

Although environmental persistence is prolonged, the shedding period in the acute phase of the disease lasts up to two days, however, the faecal shedding period can easily reach six weeks after recovery, and consequently detection of faecal viral DNA does not necessarily signify an active infection.[23]

The majority of infections are subclinical, and clinically healthy cats can demonstrate antibody titres up to one year of age.[17] Susceptible cats that are exposed and infected during the first year of life represent the most common population for clinical disease.[22,23] It is described that unvaccinated kittens that acquire maternally derived antibodies (MDAs) through colostrum are typically protected up to three months of age.[18]

2.2. Pathogenesis

As previously explained for viral replication, parvovirus needs cellular DNA polymerase, requiring rapidly multiplying cells in the S-phase of division.[4,23] Thus, lesions in feline hosts occur in tissues with a high rate of mitotic activity.[14] The most commonly affected tissues in adult animals are the bone marrow, lymphoid tissue and intestinal mucosal crypts.[17]

Transmission occurs by the faecal-oral route, with replication occurring in the oropharynx initially. A viraemic phase, occurring between two and seven days, distributes the virus throughout the different tissues.[14,25] FPV leads to immunosuppression through immune cells depletion, due to the infection of lymphoid tissues. In cats, up to nine weeks of age, thymic involution and degeneration is described.[14]

Viral replication in the bone marrow occurs in early progenitor cells, affecting all the myeloid cell populations. This pathogenesis feature actually reflects the panleukopenia designation in cats infected with FPV.[23]

FPV causes damage to the rapidly replicating cells of the intestinal mucosa crypts in clinically-affected cats, causing destruction of the intestinal villi, which may lead to diarrhoea related with malabsorption and increased permeability (Fig.3).[22,25] The most affected intestinal segments are the jejunum and ileum.[17]

Cats infected with FPV are susceptible to secondary bacterial infections with enteric bacteria, causing Gram-negative endotoxaemia and possibly bacteraemia.[17,23] It is described that co-infections increase the severity of FPV infection in cats.[26] FPV can act as an immunosuppressive agent due to leukopenia and intestinal lesions, allowing bacterial proliferation. Both *Clostridium piliforme,* which is the causal agent of Tyzzer's disease, and *salmonellae* infections are reported to increase the mortality rate in FPV clinical infections.[23]

Dual infections of viral agents, such as Feline Calicivirus (FCV) or Feline Astrovirus, can be associated with high severity of the disease.[17,27] When endotoxemia occurs, it is described that cats can develop disseminated intravascular coagulation (DIC) as a secondary complication.[17,23]

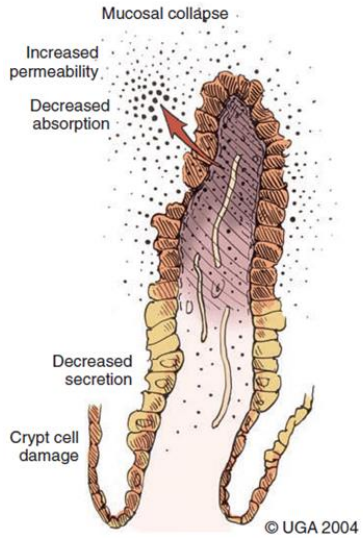

Figure 3. Parvovirus-infected villus showing collapse and necrosis of intestinal villus.*(Reproduced from Infectious Diseases of the Dog and Cat, Greene, 2012.*[52] *"Art by Dan Beisel and Kip Carter © 2004 University of Georgia Research Foundation Inc")*

In utero infection in early pregnancy can produce a spectrum of reproductive disorders, including fetal death, resorption, abortion, and mummified fetuses.[25] It is described that closer to the end of gestation, FPV infections can result in birth of live kittens with different degrees of damage to the neuronal tissue.[14] Within the same litter, some kittens are apparently unaffected (clinically healthy) due to innate resistance or acquisition of MDA, however these kittens may harbour the virus sub-clinically up to two months after birth.[18]

The central nervous system (CNS), including the cerebrum, retina and optic nerve, can be affected in late prenatal and early neonatal infection. Cerebellar damage very often results in cerebellar hypoplasia, which can be explained by the fact that the cerebellum develops during late gestation and early kittenhood.[25] It is described that the CNS may be affected by infections occurring up to nine days of age, interfering with the cortical development and resulting in reduced and distorted cell layers in the cerebellum.[14]

Parvoviral particles have been detected in the CNS of affected cats by immunochemical staining, and FPV was also detected in the cerebellum of both adult and young affected cats by polymerase chain reaction (PCR).[17] It is described that Purkinje's cells of the cerebellum are especially susceptible to FPV infection, as these cells express transferrin receptors that are used by parvoviruses for cell entry[28]; even though as post-mitotic cells they should not allow viral replication, viral antigens were detected after experimental infections in new-borns.[22,23]

Interestingly, although neurons are terminally-differentiated cells, parvoviruses seem to be able to replicate in these cells.[14,23]Parvovirus has been detected by histochemical methods

and PCR in the brains of adult cats that died of various diseases, including panleukopenia, yet the clinical significance is unclear.[29]

A link between myocarditis and parvovirus infection has been reported in neonatal infections in dogs, however, in cats, the role of FPV in myocarditis is not completely explained.[23] Data have been reported suggesting that FPV is important in the pathogenesis of cardiac disease, with one study that identified FPV DNA in a significant number of adult cats that had died from cardiomyopathy.[30] However, a recent study suggested that parvovirus was not significantly associated with myocarditis in kittens, although it is unclear so far whether the myocardium is a replication site in infected kittens.[31]

2.3. Clinical Findings

The frequency of cats that exhibit clinical disease caused by FPV infection has been reported much lower than the number of cats infected with the virus, which can be explained by the high prevalence of FPV antibodies in the cat population.[23] Thus, subclinical cases are more common in older susceptible cats.[14]

The severity of the clinical disease depends on age, immune status and concurrent infections. The clinical disease is common in young unvaccinated kittens, particularly between three and five months of age, but sudden neonatal or young death has been described in kittens of four weeks to 12 months of age in vaccinated cats. In the United Kingdom (UK), it has been reported that FPV was the cause of 25% of kitten mortality, representing a significant clinical illness in the domestic cat population.[32]

The disease is characterized by an acute self-limiting development. In the peracute form of the disease, cats can die with few or no premonitory signs, within 12 hours. In these cases, affected kittens may be found in septic shock, profoundly dehydrated, hypothermic and comatose.[23]

Typically, the disease assumes the acute form, presenting initially non-specific signs, such as fever, lethargy, anorexia and dehydration. Vomiting is a common finding in the course of the illness, which is often bile-tinged and occurs unrelated to eating.[14] Later, watery to haemorrhagic diarrhoea can be present, however it is less common.[17]

On physical examination, pain may be noted during palpation of the abdomen, and a hunched posture is frequently observed. In severely affected cats, bloody diarrhoea, oral ulceration and mucosal pallor may be present.[17] In terminal stages, affected cats may be hypothermic, bradycardic, and comatose. Cats often die of complications associated with dehydration, secondary bacterial infection and DIC.[23] When infected cats survive for longer than

five days without developing fatal complications, they usually recover, although full recovery can take several weeks.[22]

Neonatal kittens usually have neurological signs, exhibiting tremors, incoordination, ataxia, decreased postural reactions and hypermetric movements, but normal mental status, which is typical of cerebellar disease[14,17]. Not all kittens of the same litter are affected or have the same degree of neurologic deficits. Examination of the ocular fundus of kittens with neurological signs may reveal retinal lesions.[23]

2.4. Diagnosis

2.4.1. Clinical Laboratory Findings

Diagnosis of systemic feline panleukopenia is generally based on clinical signs and the presence of leukopenia, which is due to neutropenia and lymphopenia. Total leukocyte counts during the course of infection are usually between 50 and 3000 cells/µL.[17] However, leukopenia is not pathognomonic for FPV infection, as it may not occur in all clinical cases. Neutropenia often develops first as neutrophils exude into the infected intestinal tract. It is followed by leukopenia, as a result of bone marrow suppression.[23] Thrombocytopenia, which may result from damage to the bone marrow, can be found with other coagulation abnormalities in cats that develop DIC. Marked anaemia is uncommon in feline panleukopenia, except in cases with severe intestinal blood loss. Recovery may be associated with neutrophilia with a left shift, due to the resurgence of leukopoiesis.[22,23]

Serum biochemical findings are typically nonspecific. Hypoalbuminemia and hypocholesterolaemia may be found. Electrolyte abnormalities, such as hyponatremia, hypochloraemia and hyperkalaemia can also be present. In more severe cases, azotaemia is frequently found, related with pre-renal and non-renal causes such as dehydration. Increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), or hyperbilirubinemia may also be present, reflecting hepatic involvement.[17]

In kittens with neurological signs, magnetic resonance imaging or computed tomographic scanning can help to visualize cerebral or cerebellar cortical findings.[23]

2.4.2. Diagnostic Assays

2.4.2.1. Serologic Testing

Widespread exposure and immunization constrain the use of serological testing as a diagnostic tool for feline panleukopenia. Thus, serologic assays that detect FPV-antibodies are generally used to evaluate the need for vaccination. Hemagglutination inhibition (HI), which measures the ability of serum to prevent agglutination of erythrocytes by the virus, and serum neutralization assays may be used.[23] These tests can be used in outbreak situations, or in shelter environments, to assess which cats are at risk of developing disease and virus shedding, distinguishing from cats which are protected, and consequently, are at lower risk of infection.[14]

2.4.2.2. Faecal Parvovirus Antigen Testing

FPV antigen can be detected in faeces using immunological methods, with Enzyme Linked Immunosorbent Assay (ELISA) based tests, and with immunochromatographic tests, both commercially available for detection of CPV-2 antigen in faeces or intestinal contents.[17,33] Detection of the FPV antigen by CPV-2 designed tests is due to the close structural and antigenic relationship between FPV and CPV-2 strains.[34] These assays represent a practical indicator of FPV infection in kittens. However, the sensitivity and specificity of in-house test systems may vary between the commercially available assays and with the stage of infection, as virus shedding may be transient.[35]

Usually, false-negative results are more common using these assays than false-positives, therefore a positive test result in a cat exhibiting clinical signs suggests a diagnosis of feline panleukopenia. In addition, it was reported that sensitivity of commercially available tests ranged from 50% to 80%, and specificity between 94% and 100%, when compared with faecal electron microscopy using feline samples.[33] Nevertheless, these assays are usually only able to detect FPV in faeces up to 48 hours after infection thus, by the time of the onset of clinical signs, the virus may no longer be detectable. In addition, false-positive results, although infrequent, can result from the administration of attenuated live viral vaccines up to two weeks after immunization.[35,36]

2.4.2.3. Virus Isolation

FPV can be isolated in feline cells, however, isolation can be difficult and the virus usually shows minimal cytopathic effects, being more easily demonstrated in young, rapidly multiplying cells.[17] Thus, viral isolation is not often used for diagnosis, remaining an important research tool.

2.4.2.4. Faecal Electron Microscopy

Faecal Electron Microscopy (EM) can help in the diagnosis of viral enteritis, enabling the diagnosis of other infections, caused by agents such as rotavirus and coronavirus.[23] However, the application of this tool in clinical diagnosis is infrequent, as the turnaround time may be slow,

technical expertise and expensive equipment are required, resulting in large costs associated with the diagnosis.

2.4.2.5. Molecular Diagnosis

PCR can be used to identify FPV in whole blood, faecal, intestinal and tissue samples from cats.[14] Testing of whole blood is recommended in cats without diarrhoea or when no faecal samples are available. Genetic detection is considered the gold standard, being particularly valuable when viral quantities are low, allowing confirmation of the diagnosis when negative test results are obtained with commercially available in-clinic kits[23,35,36]

The sensitivity of these assays is high, so the virus can be identified for longer periods using molecular methods.[14] Also, genetic detection allows subclinical detection and positive results can be found after modified-live virus (MLV) vaccines, which are an important feature to research, but clinically, positive results should be interpreted with respect to recent MLV vaccination, relating with compatible clinical signs and haematological alterations.[23,37]

More recently, real-time PCR assays have been developed for detection of FPV and differentiation of FPV from CPV-2 strains, and to distinguish field and vaccine strains.[38]

2.5. Pathological Findings

Gross pathological changes may include focal ulceration on the surface of the tongue. The intestinal tract can be visibly dilated, with thickening, distention and hyperaemic intestinal walls, often presenting serosal haemorrhage. Intestinal contents may be bloody with a fetid odour.[22] Mesenteric lymph nodes can be oedematous. In some cats, mild pleural or peritoneal effusion may be present.[17] Prenatally infected cats commonly present a small cerebellum, and more rarely hydrocephaly. Thymic atrophy is also a common finding.[25]

Histopathological findings in the intestine include dilated crypts, necrosis of crypt epithelial cells, accumulation of cellular debris with crypt dilation and neutrophil infiltration. Shortening of *villi* occurs secondary to the necrosis of crypt cells and submucosal oedema is often present.[14] The jejunum and ileum present the most severe histopathological lesions. Acutely-affected cats often show lymphocyte depletion in lymph nodes follicles, Peyer's patches, and spleen. Usually, lymphoid atrophy is present with concomitant mononuclear phagocyte hyperplasia. In some cats, intranuclear inclusions can be found. Bone marrow examination may reveal hypoplasia.[25]

In prenatally or neonatally infected kittens, histological findings of the cerebellum include marked cerebellar degeneration and reduced population of the granular and Purkinje's cell layers. Myelin degeneration in the lateral funiculi of the spinal cord is often found.[28]

2.6. Therapy and Prognosis

Treatment of cats with feline panleukopenia is mainly supportive, particularly with intravenous crystalloids and parenteral antimicrobial therapy, as further described for CPV-2 infections in dogs. Parvovirus cases should be hospitalised in isolation facilities to avoid transmission of infection.[17,23]

With the objective of replacing lost electrolytes, correct dehydration, and meeting daily maintenance needs, parenteral fluid therapy is recommended. Ideally, lactated Ringer's solution can be used, and potassium supplementation can be valuable. Blood glucose concentration should be monitored, and dextrose supplementation may be needed accordingly.[39]

Until vomiting has ceased, oral intake of food and water should be withheld, which indirectly slows the bowel mitotic activity necessary for viral replication. To control persistent vomiting, metoclopramide or ondansetron are effective. Additionally, gastrointestinal protectants are described to coat the bowel, but should not be given to vomiting animals.[23]

Glucocorticoid therapy is not generally recommended, due to the immunosuppressive effects. In cats with severe anaemia, hypotension, or hypoproteinaemia, plasma or blood transfusion therapy is advised. In addition, a low-dose subcutaneous heparin therapy is described, when thrombocytopenia and severe coagulopathy are present.[17] Antiserum or high-titre parvoviral antiserum from vaccinated or recovered cats are useless after the onset of clinical signs.[39]

Antimicrobial agents, effective against gram-negative and anaerobic bacteria, may be administered to control secondary bacterial infection. Broad-spectrum antimicrobial agents, such as ampicillin or cephalosporin can be sufficient for non-complicated cases.[23] In severe cases with septic complications, a combination of a penicillin (penicillin or amoxicillin-clavulanate), metronidazole or clindamycin, which are chosen according to the anaerobic spectrum required, with aminoglycosides, for gram-negative spectrum, may be needed.[17] However, due to nephrotoxic potential of the aminoglycosides, their use must be avoided. In addition, quinolones can be also considered, nevertheless due to cartilaginous toxicity in growing animals and retinal toxicity, particularly in cats, their use must be carefully considered and, avoided whenever possible. Lower toxicity single agents that can be effective against both anaerobes and gram-negative bacteria, such as third-generation cephalosporins can be considered, however these agents are usually more expensive.[14,22]

In cats with feline panleukopenia, parenteral therapy with a B complex-vitamins combination is described, due to high requirements for B-vitamins, decreased food intake and loss in diuresis, preventing development of thiamine deficiency.[23]

In contrast to canine parvoviral enteritis, treatment with recombinant feline interferon (rfIFN-ω) has not been proven beneficial for treatment of feline panleukopenia.[40] However, it may be useful when administered to queens before vaccination, increasing MDA levels for passive transfer, and to kittens before they are introduced into contaminated environments, such as shelters.[41]

Response to therapy should be monitored, using leukocyte counts, as leukopoiesis usually resurges after up to 48 hours.[23] Oral intake of food should be started by frequent feeding of small quantities of bland food.[14]

Cats that survive the first 5 days of treatment generally recover, albeit it is described that recovery is frequently more prolonged than parvoviral enteritis in dogs. One study in Europe reported a survival rate of cats with feline panleukopenia of 51%.[42]

Cats with white cell counts below 1000/µL were almost twice as likely to die, compared with those with white cell counts above 2500/µL.[17] Only total leukopenia (and not lymphopenia) was correlated with mortality. In addition, both hypoalbuminemia and hypokalaemia were associated with an increased risk of mortality. Moreover, mortality in cats does not seem to be correlated with age, contrasting to what happens in dogs with parvoviral enteritis.[42]

2.7. Immunity and Prevention

Recovery from feline panleukopenia is described to confer lifelong immunity. However, the crucial factor to reduce the incidence of the disease is parenteral vaccination.[18]

Vaccination is recommended for all cats, regardless of lifestyle (indoor/outdoor), due to the gravity of the illness, its ubiquitous distribution and the high resistance of the virus in the environment. Inactivated and attenuated live viral vaccines are available to successfully prevent feline panleukopenia.[18,23] Both types of vaccines elicit protection with high antibody titres, however the attenuated live vaccine may be more rapid and efficient at inducing protection than the inactivated vaccine.[43]

With modified-live virus (MLV) vaccines, when MDA interference does not occur, immunity occurs up to 1 week after the first vaccination and lasts for at least 3 years, however it has been described that immunization can provide lifelong protection .[44,45] In addition, two doses after 3 to 4 weeks are recommended for the initial vaccination course, as maximal immunity only occurs 1 week after the second vaccination boost.[17]

Inactivated vaccines have the advantage of being safe in pregnant queens and kittens younger than 4 weeks and should be reserved for these situations. In addition, MLV vaccines can represent a risk to immunocompromised cats with acquired deficiencies, such as feline immunodeficiency virus infection, due to the possibility of vaccine-induced illness. Therefore,

inactivated FPV vaccines, which have minimal risk, should be used in immunocompromised cats.[23,46]

Oral vaccination with a MLV vaccine has been shown to be ineffective.[23,47]An intranasal MLV trivalent FPV vaccine has been available and is able to produce active immune response. However, it is not as effective as parenteral MLV vaccines, and an outbreak associated with their administration has been reported[48]. The use of intranasal vaccination against FPV is therefore controversial.[17,18]

Usually, vaccination schemes start at eight to nine weeks of age, and are followed by at least one more MLV vaccine administration or two more inactivated vaccines, between two to four weeks thereafter, with the last vaccine given between 14 to 16 weeks of age, avoiding vaccine failure by prolonged interference by MDA.[49] Although vaccination against panleukopenia was recommended annually in the past, different studies showed that vaccination protected against challenge with virulent FPV for longer than a year[44,45] The Vaccination Guidelines Group of the World Small Animal Veterinary Association (WSAVA) currently recommends, after the first vaccination course, a booster after one year and subsequent triennial vaccination thereafter. Multivalent vaccines that contain FPV antigen, combined with those of feline respiratory viruses, rabies viruses and Feline Leukaemia Virus (FeLV) are commercially available.[49,50] In shelters, the use of MLV vaccines is recommended, due to the rapid onset of immunity.[17,18]

Environments with high levels of contamination, such as breeding catteries and shelters, are high risk environments for infection.[24] New kittens should not be introduced into households that previously contained cats infected with FPV unless they are fully vaccinated.[17] In an outbreak situation, passive immunotherapy, administering high antibody serum can protect exposed and unvaccinated kittens for two to four weeks, however this strategy is only effective before the onset of possible clinical signs, and subsequent vaccination should be done after 3 weeks.[39]

Disinfection procedures are essential to prevent or control an outbreak, which should be done with diluted household bleach (dilution of 1:30) on all tolerable surfaces that should be exposed for at least 10 minutes. Similarly, utensils and bedding should be washed with added bleach.[23] Moreover, in shelters and catteries, it is important to isolate cats that develop gastrointestinal illness in separate housing from healthy kittens.[41]

3. Canine Parvovirus Infection

3.1. Epidemiology

Canine parvovirus is the most widely recognized cause of transmissible viral diarrhoea in dogs and one of the most common infectious diseases of dogs worldwide, being the most prevalent virus in dogs with infectious diarrhoea.[51,52] This highly contagious and often deadly disease is caused by the strains of Canine Parvovirus Virus type-2 (CPV-2), which include the 2, 2a, 2b, and 2c variants.[53,54] CPV-2 was first reported in 1978, and its variants are distinct from CPV-1, also called Minute Virus of Canines (MVC), which belongs to the genus *Bocavirus* and is assumed to have minimal pathogenic potential, although it has been related with neonatal death and gastrointestinal signs in dogs.[54–56]

It has been described that *Canidae* members are susceptible, as natural CPV infections have been reported in domestic dogs (*Canis familiaris*), bush dogs (*Speothos venaticus*), coyotes (*Canis latrans*), wolves (*Canis lupus)* and other family members.[52,57]

Although the original CPV-2 is only related with intestinal and systemic infection in dogs, the evolved strains 2a, 2b, 2c may infect felids under experimental and natural conditions.[37,58] Similarly to FPV, most infections occur as a result of contact with contaminated faeces in the environment, considering that parvovirus is highly resistant in the environment and survives at least up to 1 year in infected organic material.[22] Fomites also represents an important means of infection, and insects and rodents can serve as mechanical vectors for virus spread.[59]

The incidence of severe disease and death can be very high in susceptible animals however, acute enteritis is found in dogs of any breed, age, or sex.[60] Outbreaks of severe gastroenteritis and mortality caused by CPV-2c infections in adult dogs (more than 6 months old) have been described.[54] Nevertheless, pups between 6 weeks and 6 months of age, and especially those less than 12 weeks of age, are more likely to develop severe illness.[60] Disease can also occur in unvaccinated or improperly vaccinated adult dogs.[54,59] Rottweilers, Doberman pinschers, Labrador retrievers, American Staffordshire terriers, German shepherds, and Alaskan sled dogs seem to have an increased risk for severe CPV enteritis.[60,61] However, reasons for breed susceptibility are still unclear. The common ancestry of Doberman pinschers and Rottweilers has been implicated in their susceptibility to severe disease as both breeds have a relatively high prevalence of von Willebrand's disease (VWD), and inherited immunodeficiency.[62]

In the field, the incubation period of the original CPV-2 strains was seven to 14 days, and the emerged strains (2a, 2b, 2c) are associated with an incubation period of four to six days.[23,63] The infection does not inevitably result in apparent disease, some cases of naturally-infected dogs have been reported as never developing overt clinical signs, especially in the presence of residual MDA.[54,63]

3.2. Pathogenesis

The pathogenesis of parvovirus infections is influenced primarily by the requirement for rapidly multiplying cells, in S-phase of division, for successful infection to occur. [22,23]

Similarly to what happens in FPV infections in cats, from 18h to 24h after intranasal or oral infection, the virus initially replicates in the oropharynx, tonsils and other lymphoid tissues. Infection of lymphoid tissues leads to their necrosis. Between one and five days after infection, virus spreads systemically through viraemia.[25] Subsequently to the viraemia, a widespread infection occurs, with damage in rapidly-dividing cells of the gastrointestinal tract, thymus, lymph nodes, and bone marrow. Circulation of infected lymphocytes can carry the virus to different tissues, which allows the detection of CPV in the lungs, spleen, liver, kidney, and myocardium.[54,59]

Affected gastrointestinal tissues include the epithelium of the tongue, oral cavity, oesophagus, and intestinal tract.[52] The primary site for viral replication is the germinal epithelial cells of the intestinal crypts, causing destruction and collapse of the epithelium.[62] Cell turnover is reduced and the villi become shortened, which leads to malabsorption and increased permeability, resulting in profound enteritis and diarrhoea.[25,52] Secondary bacterial infections from gram-negative and anaerobic microflora play a key role in the pathogenesis of the disease, which may lead to bacteraemia, endotoxemia, and DIC.[52,62]

CPV infection causes destruction of the active precursors of circulating leukocytes and lymphoid cells, leading to neutropenia and lymphopenia, which is often more pronounced than neutropenia.[23,59] Neutropenia results not only from infection of the marrow but also sequestration of neutrophils in damaged gastrointestinal tissue.[25,59] Death can occur as quickly as 24 hours after the onset of clinical signs, especially in younger animals.[22]

Besides the factors related to the age of the animal, immunity and breed, other pathogenesis factors associated with the route of exposure, viral dose, and strain virulence, leading to different grades in the severity of clinical signs in affected dogs.[22]

3.3. Clinical Findings

CPV infection has been associated with three main tissues — GI tract, bone marrow, and myocardium — however, the skin and nervous tissue can also be secondary affected.[52] In addition, other clinical complications as secondary bacterial infection or thrombosis can occur.[59]

A marked variation is found in the clinical response of dogs to intestinal infection with CPV, ranging from unapparent infection to acute fatal disease. Unapparent, or subclinical, infection occurs in most dogs, mainly in puppies with intermediate MDA titres that can protect from disease but not from infection. CPV enteritis can progress rapidly, especially with the CPV-2 strains.[52,62]

Physical examination of puppies with parvoviral enteritis frequently reveals fever, lethargy, weakness, dehydration, and abdominal pain. Vomiting is frequently severe, and is followed by diarrhoea, anorexia, and rapid onset of dehydration.[52,62] Diarrhoea is often liquid, foul-smelling, and may contain streaks of blood or frank blood.[22]

Abdominal palpation may reveal a tubular mass, the consequence of intestinal intussusception. Ulcerative glossitis may occur in some puppies. Mucosal paleness, prolonged capillary refill time, or rarely hypothermia can be observed in some dogs. Septic shock may be associated with tachycardia or bradycardia, and poor pulse quality.[59]

The presence of co-infections, as seen in pups with intestinal helminths, protozoa, and enteric bacteria such as *Clostridium perfringens, Campylobacter* spp., and *Salmonella* spp. can influence the severity of the disease.[52,64]

Primary neurological disease in puppies with parvoviral enteritis may result from hypoxia secondary to myocarditis, hypoglycaemia, or intracranial thrombosis or haemorrhage, during the disease process, sepsis, or acid-base-electrolyte disturbances.[59] Co-infection with viruses such as canine distemper virus (CDV) should also be considered.[60,65] Cerebellar hypoplasia, which is common in kittens prenatally or neonatally infected with FPV, has been rarely associated with CPV *in utero* infection in dogs.[14,22] However, generalized infection has been described in neonatal pups, with haemorrhage and necrosis within the brain, liver, lungs, kidneys, lymphoid tissues, and gastrointestinal tract.[59,62] The DNA of CPV-2 variants has also been detected in the CNS of dogs without neurological signs, which could be associated with active replication of the virus in the nervous tissues, however further investigations are needed. [66] In addition, there have been sporadic reports of leukoencephalomalacia related with parvovirus.[52]

Viral myocarditis can develop from infection *in utero* or in pups up to two weeks of age, which results in signs of sudden death or congestive heart failure, presenting clinical signs that may be delayed until two months of age.[52] Clinical signs include tachypnoea and increased lung sounds, as consequence of congestive heart failure, although the spectrum of myocardial disease

in individuals is varied.[59] Cardiac signs can occur preceded by parvoviral enteritis or may occur suddenly, without apparent illness. The widespread vaccination and exposure of adult animals, which develop strong humoral immune responses, has led to a significant decline in the incidence of myocarditis and neonatal complications of parvovirus infection, because of MDA protection.[54,60] Myocarditis is sporadically found in pups that do not nurse sufficiently, or are born from unexposed or unvaccinated bitches.[62]

Erythema multiforme has been described in dogs with canine parvoviral enteritis. Skin lesions include generalized cutaneous and mucosal ulceration of the footpads, pressure points, mouth and genital mucosa. Erythematous patches on the abdomen and perigenital skin have been reported.[59,67]

The predisposition to asymptomatic urinary tract infections, which is detected in approximately 25% of pups after CPV enteritis, was attributed to faecal contamination of the genitalia associated with neutropenia. Subclinical and not treated urinary tract infection can cause chronic urinary infection, as a secondary consequence.[52]

3.4. Diagnosis

The onset of foul-smelling, bloody diarrhoea, with or without vomiting, in a young dog (under two years of age) is frequently considered indicative of CPV infection.[22,59] However, several other viral pathogens may cause diarrhoea in dogs, such as coronaviruses, adenoviruses, reoviruses and rotaviruses.[52] Enteropathogenic or parasitic infections, alone or in combination, should also be considered.[51,60] In kennels and shelters, a rapid diagnosis of CPV infection is especially important, allowing the isolation of infected dogs and preventing secondary infections of susceptible animals.[54]

3.4.1. Clinical Laboratory Findings

The most common findings in the complete blood count (CBC) are leukopenia, neutropenia, and lymphopenia. Toxic neutrophils and monocytopenia can also be present.[59] Even though leukopenia is not found in all cases, is proportional to the severity of illness at the time the blood is taken.[68]

Thrombocytosis or, less frequently, thrombocytopenia can also occur. Anaemia can be present related with the gastrointestinal blood loss, which may be non-regenerative or become regenerative, however is not a consistent feature of infection.[59]

The most frequent findings in the serum biochemistry panel in dogs with parvoviral enteritis are hypoproteinaemia, hypoalbuminemia, and hypoglycaemia. Electrolyte abnormalities such as hyponatremia, hypochloraemia, and hypokalaemia have been reported.[59] Occasionally

severe dehydration results in prerenal azotaemia. Bacterial sepsis often causes hyperbilirubinemia and increased liver enzyme activities.[68]

Coagulation abnormalities may include prolonged activated partial thromboplastin time, decreased antithrombin activity, and increased fibrinogen concentrations, leading to hypercoagulability.[69]

3.4.2. Diagnostic Assays

Suspected clinical cases should be confirmed by laboratory tests. Different methods are available for laboratory diagnosis of CPV infection. Faeces, or intestinal samples from necropsies are commonly used for the detection. Blood samples are more useful for the CPV viraemia in late stages of infection, as parvovirus viraemia can be long lasting.[54,70]

3.4.2.1. Faecal Parvovirus Antigen Testing

The faecal antigen ELISA is a commonly-used assay for diagnosis of canine parvoviral enteritis and it is available for in-hospital testing for CPV infection, performed on a rectal swab specimen. As previously described, these assays developed for CPV diagnosis can also detect FPV[35], given the close genetic and antigenic properties. Different assays are available, which detect all CPV-2 variants including CPV-2c, but their sensitivity and specificities vary.[54]

Faecal antigen ELISAs are specific but their sensitivity is particularly problematic.[59] Different studies were conducted to compare the sensitivity of three different commercially available faecal antigen assays. When compared to molecular assays, the relative sensitivity of the tests did not exceed 50%, while the specificity was 100%.[71] In another study, comparing with molecular methods and immunoelectron microscopy, the low sensitivity and high specificity of the faecal antigen ELISA kits was confirmed.[72] Although when comparing with molecular methods and immunoelectron microscopy, the specificity of all tests had been proven low; it had been demonstrated that these tests have a sensitivity between 77% and 80% on samples containing high CPV DNA loads (> 10^5 copies/mg of faeces, determined with real-time PCR).[54]

The poor sensitivity can be explained by the period of faecal virus shedding, which corresponds to the first few days of clinical illness. The incubation period from four to six days interferes with early detection, and in late stages of infection (more than 10 days) low amounts of viral particles are shed, which are not often detected by ELISA[73], especially considering that virus shedding can be intermittent and early appearance of high antibody titres in the intestinal lumen may sequestrate CPV particles.[74]

Comparing with molecular assays and immunoelectron microscopy, the specificity of the three commercially available antigen tests varied between 92% and 98%, rendering false positives unusual.[72] Generally, positive test results confirm the infection, but weak false positive

can be obtained four to eight days after administration of attenuated live CPV vaccines in dogs.[75] Negative results may be confirmed by PCR methods.[34,52]

3.4.2.2. Haemagglutination Testing

Parvoviruses agglutinate erythrocytes, making it possible to detect the presence of virus in faecal samples.[52] A haemagglutination protocol, which uses porcine erythrocytes, designated as slide agglutination test is described to indicate the presence of parvovirus strains in faecal and intestinal samples.[76] Although they can be considered a rapid method for diagnosis, haemagglutination does not seem to overcome the limitation of traditional methods[54], as it is considered to have similar sensitivity to ELISA and to be poorly specific, which can be related with the presence of isoagglutinins in faecal material, and as well other haemagglutinating viruses.[34]

Furthermore, fresh erythrocytes are constantly required for this type of assay, causing problems related to the availability and management of porcine erythrocyte donors.[54] Erythrocytes of other species (cat, rhesus monkey) can be used, however it may be difficult to obtain the required quantities and can increase the cost of the test.[34]

3.4.2.3. Faecal Electron Microscopy

As described for FPV detection, faecal electron microscopy is mostly used as a research tool, however is offered by some laboratories for the diagnosis of viral enteritis, when it is not possible to use antigen tests or PCR assays. Diagnosis of other viral agents, such as rotavirus and coronavirus may be facilitated by EM.[34,54]

Nevertheless, the use of this tool is time-consuming for diagnosis proposes and requires technical expertise. Identification of parvovirus-like morphology can be poorly sensitive, and large amounts of virus must be present for positive results.[52]

3.4.2.4. Virus Isolation

It is possible to isolate every canine parvovirus variants using canine and feline cells. This requires the availability of cell cultures, and consequently virus isolation can be carried out only in specialized laboratories.[70] Moreover, virus isolation can present low sensitivity, which is related with the intestinal lumen antibodies of infected animals that bind virions, preventing viral attachment to cell receptors. Furthermore, parvovirus strains may show minimal cytopathic effect.[34] Turnaround time can be slow, as a long incubation period (five to ten days) is required and additional testing by haemagglutination or immunofluorescence may be needed to detect viral antigens.[17] Thus, as for FPV, virus isolation is not commonly used for diagnosis, remaining an important research tool.[59]

3.4.2.5. Molecular Diagnosis

For diagnosis proposes, PCR assays are valuable when faecal antigen ELISA tests are negative, but parvovirus infection is suspected.[59] Although molecular methods can be time consuming, labour-intensive and require trained staff, because of the risk of carryover contaminations, especially with a high throughput of samples, PCR has been a reliable tool for diagnosis of CPV infection.[52,54]

Since the 1990s, a nucleic acid hybridization assay is available.[77] Since then, different PCR assays were developed, increasing sensitivity and specificity to detect CPV in faeces of infected dogs. Thus, a rapid and not expensive method is generally used for DNA extraction, based on boiling faecal homogenates, however, due to the risk of faeces inhibiting the enzymes used in the assay, it is important to optimise extraction techniques and include controls, minimising possible false negative results.[34,54]

The modified-live virus of the commercially available vaccines can be shed in the faeces, although at low titres and for a shorter time, when compared with field strains.[75,78] In a clinical case of enteritis after vaccination, this situation could cause a false-positive result in conventional PCR, causing a misdiagnosis of the disease, which is probably caused by other enteric pathogens.[52,59] Additionally, vaccine-induced disease due to reversion to virulence has been suggested but was never clearly demonstrated.[54,75]

Real-time PCR allows a higher throughput, enabling simultaneous processing of several samples, as different steps are automatied.[54,79] Another important advantage is the high sensitivity and reproducibility of the real-time PCR assay, providing an estimation of the viral DNA load, allowing the detection of virus-particles shed at low titres in faeces.[79] Moreover, this has a particular impact in kennels and shelters, making it possible to adopt measures of prophylaxis to adequately prevent CPV infection, which is often responsible for epizootic scenarios. Furthermore, using real-time PCR, it is easily possible to specifically identify CPV variants circulating in the field and to distinguish field strains from vaccine strains.[80]

3.4.2.6. Serological Testing

CPV antibodies measurement can be done in the laboratory using HI. Additionally, ELISA assays are also available for semi-quantitative measurement of antibodies to CPV in-hospital.[52,70] However, serology is not the best method to confirm the infection, as most dogs are vaccinated against CPV or had previously contact with the virus. Therefore, these assays are often used to evaluate the MDA titres in pups, assessing the need for vaccination.[54,59] ELISA test kits for semi-quantitative IgG and IgM measurements and for determining adequate IgG titres for vaccination are commercially available.[34,59]

3.5. Pathological Findings

A varied range of gross and histopathological findings are caused by parvoviruses, which can vary from minimal to severe. At necropsy, the most common finding is segmental enteritis (Fig. 4).[22,59]

Early lesions are more common in the distal duodenum, progressing through the jejunum. The large intestine is rarely affected.[52] The small intestinal wall is generally thickened and segmentally discoloured, with roughening or fibrin adhering on the serosa surfaces. Mucosa is often smooth and glassy. Lumen can contain watery to mucoid and bloody material. Gross lesions are usually classified as nonspecific enteritis.[22,59]

The histological examination is generally definitive. As the disease progresses, loss of crypt architecture and necrosis of the crypt epithelium are often observed. The villi are shortened or obliterated, resulting in collapse of the lamina propria.[52] A common finding, especially in acute cases, is intranuclear viral inclusion bodies in epithelial cells and throughout the squamous epithelia of the upper gastrointestinal tract.[22] In chronic cases, intranuclear inclusion bodies are not often present, which correlates with decreased CPV antigen detection.[22] Necrosis and depletion of the lymphoid tissue (Peyer's patches, mesenteric lymph nodes, thymus, and spleen) are frequently observed.[59] Secondary septicaemia is a common finding, and pulmonary oedema or alveolitis can be observed in these cases.[52]

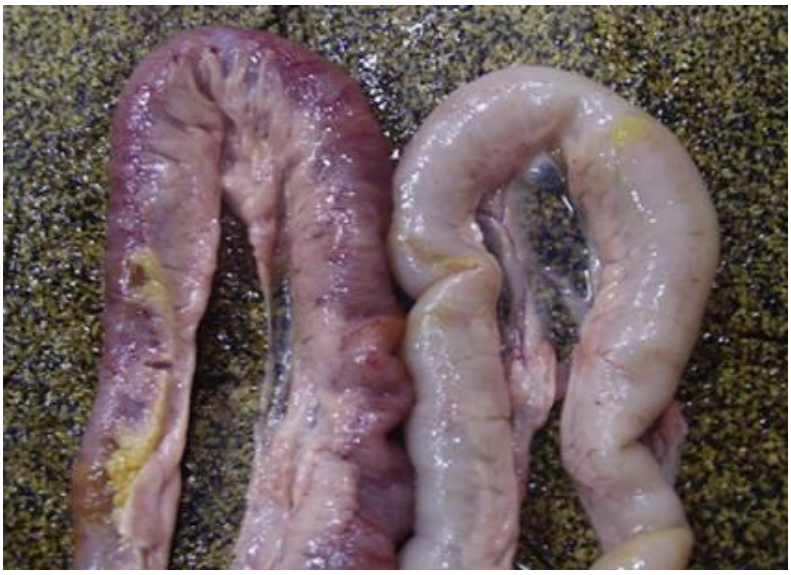

Figure 4. Intestine from a dog with acute parvovirus infection. There is segmental enteritis, with the affected segment on the left and the unaffected segment on the right. *(Reproduced from Lamm C. et al., 2008[22])*

Myocarditis is present especially in cases of younger animals, and is recognized grossly as pale streaks in the myocardium. The lesions are often classified as a non-suppurative myocarditis with multifocal infiltration of lymphocytes and plasma cells within the myocardium.[22] Basophilic intranuclear inclusion bodies are observed in cardiac muscle fibres and parvovirus-like virus particles in the inclusion bodies can be seen by EM and by *in situ* hybridization.[22,59]

Specific identification of parvovirus in tissue specimens is achieved by immunofluorescence or other immunochemical methods.[59] Because of the loss of detectable antigen, immunochemical methods can give false negative results in later stages. Moreover, quantitative PCR methods have been described as the most specific and sensitive tool for virus identification in tissue specimens, detecting more than 90% of infected animals.[52] In fact, virus detection may be more prevalent in the tongue compared to other tissues, when there is autolysis or freezing and thawing before necropsy.[52]

3.6. Therapy and Prognosis

The therapy of parvovirus infection is supportive and symptomatic. The patient should be hospitalized in isolation.[22] The primary goals of symptomatic treatment for parvoviral enteritis are restoration of fluid and electrolyte balance, and prevention of secondary bacterial infections.[52] Intravenous fluid therapy is indicated and should be continued for as long as vomiting or diarrhoea, or both, persists. A balanced electrolyte solution such as lactated Ringer's solution supplemented with potassium and 2.5% glucose is often used.[81]

Single agent antimicrobial therapy with anaerobic coverage, such as metronidazole or ampicillin may be sufficient in many cases.[82] However, in cases with haemorrhagic diarrhoea a combination of antimicrobial drugs is recommended.[22] Thus, antimicrobial agents with activity against gram-negative and anaerobic bacteria are recommended in combination due to the severe disruption of the intestinal epithelium allowing bacteria into the blood stream and peripheral neutropenia increases the risk of sepsis.[59] The most common bacteria appear to be *Escherichia coli* and *C. perfringens.*[52,64] A common combination used is a penicillin and an aminoglycoside for gram-negative aerobic and anaerobic bacteria spectrum. The patient should be fully hydrated before aminoglycoside administration, because of their potential nephrotoxicity.[59]

When nephrotoxicity of aminoglycosides is a concern, parenteral penicillin or third-generation cephalosporines may be used as alternatives to achieve the desired spectrum.[59] The use of quinolones, which have a good gram-negative aerobic antibacterial spectrum, must be avoided in young growing animals, as it has been associated with cartilage damage in prolonged use.[52,81]

Anti-emetic drugs are needed to manage severe vomiting. Frequent or persistent vomiting can be managed with metoclopramide hydrochloride and prochlorperazine or administration of maropitant.[81] The serotonin receptor antagonists are also described as efficacious anti-emetics. Both ondansetron and dolasetron have been used in dogs. However, the use of these anti-emetic agents does not always limit the vomiting, and their use should be closely monitored, because it can lead to hypotension.[52]

Usually, oral intake may be suspended until vomiting has stopped for at least 24 hours, which could take up to three to five days in severe cases. It has been recommended, instead of avoiding the oral route completely, to trickle-feed small amounts of glutamine-containing solutions with the objective of reducing bacterial translocation.[81] Also, when dogs with parvoviral enteritis were fed, starting on the first day of treatment via naso-oesophageal tube, their recovery time is shorter, and body weight was maintained, when compared to dogs treated with the conventional methods of food withholding until signs had ceased (12 hours).[83] After vomiting is controlled, it is advised to introduce initially small amounts of a bland diet.[59]

The loss of blood caused by parvovirus enteritis can lead to severe anaemia in some puppies. Transfusion of whole blood is of benefit in these cases. When hypoproteinaemia is present a whole blood transfusion will help, however, a plasma transfusion is more appropriate.[52,62] Plasma can provide immunoglobulins and colloidal albumin, however antibody titres may not be sufficient to be beneficial, and the animal usually develops is own effective antibody response within three days after onset of clinical signs. Thus, hyperimmune plasma administration is described but has been questioned because, after the onset of clinical signs, the level of antibodies may be increased.[52,59] The use of synthetic colloids such as hetastarch is described when oedema is present because of decreased proteins and is not corrected by plasma transfusion. Hetastarch has anticoagulant properties, which can be useful considering hypercoagulable states. However, their use remains controversial as it has been associated with acute kidney injury.[59,84]

Glucocorticoids may be used to treat early sepsis or endotoxemia. However, their use should only happen when dehydration is correct and repeated doses are not advised.[52] Other authors consider that the use of glucocorticoids is of unproven benefit in parvoviral enteritis secondary complications.[81]

Other adjuvant therapies have been investigated, including anti-endotoxin sera, recombinant bactericidal permeability-increasing (BPI) protein[85], recombinant granulocyte colony-stimulating factor (G-CSF)[86] and oseltamivir.[87] However, these therapies remain controversial as no significant difference in the clinical outcome was found and further investigation is required.[59] In contrast, treatment of pups with recombinant feline interferon-omega (rfIFN-ω) has been associated with significantly reduction of disease severity and mortality.[52,59,88]

The prognosis may vary with the severity of illness and owners' ability to afford appropriate treatment. It is described that pups that survive the first three to four days of parvoviral enteritis typically make a rapid recovery, up to 1 week in uncomplicated cases.[52,59] Severe cases, which are characterized by development of secondary sepsis or other complications, require further hospitalization and aggressive treatment.[59,89] Moreover, it is reported that with appropriate care, 75% of parvovirus cases should respond to medical therapy.[22]

3.7. Immunity and Prevention

The environment is the focus of concern to prevent the virus spreading. Disinfection procedures are as described for FPV. Additionally, as parvoviruses are resistant to heat, surviving at 70ºC for 30 minutes, steam cleaning can be used on surfaces that do not tolerate hypochlorite.[41,52]

In shelters, quarantine periods of two weeks are recommended to pups that might be shedding the virus.[41] Rodent and insect vector control is recommended to prevent dissemination of virus in the environment.[59] The regular removal of faecal contamination and minimization of possible stressors, such as overcrowding, poor nutrition, and parasitism can also help to decrease the prevalence and impact of enteric viral infections, such as the parvoviral enteritis.[41,52]

Vaccination is widely available and is the most effective method to prevent the CPV infection in dogs. Both live attenuated and inactivated vaccines are available.[52,59] However, commercially inactivated CPV vaccines have been extensively replaced by live attenuated vaccines, which provide superior and longer duration immunity. Without the interference of MDAs, the protection against CPV infection is as early as three days post-vaccination.[90] MLV CPV vaccines replicate in the intestinal tract and lower quantities of virus are shed after vaccination, usually up to 10 days. However, a recent study demonstrated faecal shedding up to 28 days after vaccination.[75,78] Although concerns about the possible reversion of virulence which could cause apparent disease have been raised, different studies have shown that attenuated live vaccines are safe.[52,54,91]

Maternal antibody interference and the lack of sufficient seroconversion response are the primary causes of vaccination failure.[54] The window of susceptibility is the period when MDA interferes with the ability of the vaccine to stimulate an effective immune response, but does not prevent infection with virulent wild-type virus.[59] The window of susceptibility was reported to be between the age of 40 to 69 days; however, this may vary.[92]

Serological tests to determinate MDAs titres can help to predict the age at which a pup should be vaccinated successfully. Nevertheless, this could represent an extra-cost in the vaccination approach.[54] Several strategies have been proposed to overcome MDA interference, such as high-titre vaccines and intranasal vaccination. Nowadays, the WSAVA Vaccination

Guidelines Group recommends delaying the primary CPV vaccination course to 14-16 weeks of age, with the aim of ensuring effective immunization even in pups with long-lasting MDA.[49,52,54]

Therefore, pups of unknown immune status are usually vaccinated initially at six to eight weeks of age, with three to four weeks interval, continuing through to 14 to 16 weeks of age (16 to 20 weeks in breeding kennels).[52] At 1 year of age, a booster should be given, repeating every 3 years. In Europe, multivalent vaccination is often used, which is known as DHPPi, providing immunity against canine distemper, canine parvovirus, canine adenoviruses, and canine parainfluenza.[49] To confirm the protection against CPV, serum antibody titres of at least 1:80 are well correlated with protection after vaccination.[90,93]

4. Epidemiological Evolution of Canine and Feline Parvoviruses

4.1. Host Range and Evolution Perspective

Feline panleukopenia-like disease was recognized in cats more than 100 years ago[22], and was first reported in the literature in 1928[16]. The virus has remained stable, with minor variations in its genome.[18] Moreover, it is not only able to cause significant disease in domestic cats, but also infect an extensive variety of wild felids and other wild carnivores. Until the late 1970s, domestic dogs and close relatives, such as wolves and coyotes, were resistant to FPV-like virus infection.[7]

In 1978, a new virus variant emerged infecting domestic dogs, causing a pandemic disease and spreading worldwide in the late 1970s, being also able to infect wildlife species, such as coyotes and raccoons. This virus was identified as CPV-2, distinguishing the virus from the distantly-related minute virus of canines (MVC), also known as CPV type 1 (CPV-1).[55,56] Phylogenetic analysis has shown that CPV-2 was derived from a common ancestor, shared with FPV and other parvoviruses such as raccoon parvovirus (RPV) and blue fox parvovirus (BFPV).[7,58]

As previously mentioned, although CPV and FPV are around 98% identical in DNA sequence, they have specific host ranges and antigenic properties that are controlled by the capsid protein gene.[58] The new canine host range of CPV-2 can be explained by a small number of mutations in the capsid protein gene, which altered residues in the surface of the capsid, and it is believed that wild animals, such as raccoons and foxes, are associated with the viral evolution. The capsid uses the transferrin receptor (TfR) type-1 to bind and enter the cells for infection, and the residues on the surface of the capsid controls the interaction with the TfR.[12] Thus, as the FPV is unable to bind canine TfR, the specific host range for dogs is related to a few amino-acid changes, which allow effective binding by the virus. This successful viral adaption involved six genomic changes associated with the threefold spike of the capsid, allowing CPV-2 to infect the canine host range, but losing the ability to replicate in cats.[57]

Therefore, the changes at VP2 residues 93 (Lys to Asn), 103 (Val to Ala) and 323 (Asp to Asn) between FPV and CPV-2 explain the acquisition of the canine host and range, whereas the differences of VP2 residues 80 (Lys to Arg), 564 (Asn to Ser) and 568 (Ala to Gly) can explain the loss of the feline host range. Other changes at the residues 232 (Val to Ile) and 375 (Asp to Asn) were found in some isolates of the original strain of CPV-2, however it is suggested that these variations are not critical to the success of CPV-2 in nature.[19,57]

In the early 1980s, CPV-2 was replaced by a genetic and antigenic variant, named CPV-2a, which spread worldwide within a period of three years, suggesting that CPV-2a had an epidemiological advantage over CPV-2.[94] In fact, CPV-2a had reacquired the ability to infect cats,

becoming the dominant lineage of the virus (Fig. 5). The variation in the residues 87 (Met to Leu), 300 (Ala to Gly) and 305 (Asp to Tyr) allowed the replication in cats, and other changes on the residues 101 (Ile to Thr), 297 (Ser to Ala) and 555 (Val to Ile) are described between the original CPV-2 and CPV-2a viruses.[57]

In 1984, only a few years after the original CPV-2 emerged, another antigenic variant was detected in the United States of America (USA), named CPV-2b with a change at residue 426 (Asn to Asp), and in the VP2 residue 555 (Ile to Val). However, only the difference at residue 426 is antigenically significant.[19,57]

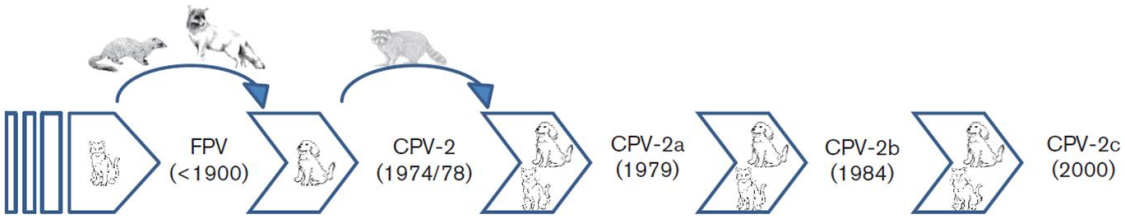

Figure 5. Feline and canine parvoviruses host range evolution. *(Reproduced from Miranda C. et al., 2016* [57]*)*

In 2000, a CPV-2c variant was reported in Italy[95], however CPV-2c was found in archived samples from 1996, in Germany, revealing that this strain was circulating in Europe before the first reported case in Italy.[96] CPV-2c strains have an antigenic change in the same residue as CPV-2b. Thus, antigenic differences between the 3 variants are presented at residue 426 – Asn in CPV-2a, Asp in CPV-2b and Glu in CPV-2c – affecting the major antigenic region, located at the threefold spike in the VP2 protein (Table 1). While some authors disagree with the strain nomenclature, due to the strains differing only at one position, and other designations were proposed recently, the reference to CPV stains 2a, 2b and 2c is prevalent in the literature.[6,57,97]

Other VP2 mutations were reported in the antigenic variants and include the amino acid change at VP2 position 297 (Ser to Ala) both in CPV-2a and 2b. These variants have been also designated as New CPV-2a and 2b.[57,98] The 300Asp variation, of CPV-2a and 2b has been reported more recently, and may be related to extended adaptation of the virus in the feline or raccoon hosts.[13,99] In fact, although CPV is a DNA virus, its genomic substitution rate is about 10^{-4} per site per year, which is comparable to RNA viruses.[6,100]

The three antigenic variants of CPV-2 are distributed worldwide, being able to infect a variety of different hosts. Thus, the emergence of the CPV-2 strains and reacquiring the capacity to replicate in the feline host, represent a selective advantage for the virus.[57]

4.2. Canine Parvovirus Infection in Cats

As previously described, CPV-2 variants can induce disease in infected cats, with clinical signs indistinguishable from feline panleukopenia. However, the natural infection is often asymptomatic.[101] Thus, the onset of clinical illness may be associated with the general condition of the affected cats at the time of exposure.[23]

Milder clinical signs were associated with CPV-2a or CPV-2b infection in cats, when compared to those with FPV infection, suggesting that CPV-2 variants were less virulent in cats.[102] However, several reports of CPV-2c isolated from cats claim that this strain is most pathogenic to felids.[37,103]

Similar to the common alterations in dogs, pronounced lymphopenia and mild leukopenia are often described in cats infected with CPV-2 strains.[17] These findings frequently contrast to the marked leukopenia and mild lymphopenia found in cats with FPV infection.[22]

Parvoviral infection can be clinically diagnosed using the tests already described for FPV infection in cats and CPV infection in dogs, however, none of the routine clinical tests can accurately distinguish FPV from CPV-2 variants. Thus, to distinguish FPV and CPV-2 isolates, HI with specific monoclonal antibodies, sequence analysis of sizable portions of the VP2 gene, or the use of Minor Groove Binder (MGB) probes in quantitative PCR, which uses different fluorophores to detect Single Nucleotide Polymorphisms (SNPs) existing between the CPV-2 strains, is recommended (Table 1).[38,54]

Therapy should be the same as described for treating feline panleukopenia. Preventive measures, including vaccination, are the same described for feline panleukopenia infections, as it has been reported that attenuated FPV vaccines are able to elicit protection in domestic cats against FPV and CPV strains.[50,104] However, the cross-reactivity of FPV vaccines against CPV-2 strains has been under discussion.

Table 1. Amino acid variations in the VP2 protein of feline and canine parvoviruses *(a). (Reproduced from Decaro N. et al., 2012* [54]*)*

Aa Residue	***80***	***87***	***93***	***101 (b)***	***103***	***232***	***297***	***300***	***305***	***323***	***375***	***426 (c)***	***555***	***564***	***568***
Nt (d) position	3024-3026	3045-3047	3063-3065	3087-3089	3093-3095	3480-3482	3675-3677	3684-3686	3699-3701	3753-3755	3909-3911	4062-4064	4449-4451	4476-4478	4488-4490
Codon observed	AAA (Lys) AGA (Arg)	ATG (Met) TTG (Leu)	AAA (Lys) AAC (Asn) AAT (Asn)	ATT (Ile) ACT (Thr)	GUA (Val) GCA (Ala)	GTA (Val) ATA (Ile)	TCT (Ser) GCT (Ala)	GCT (Ala) GGT (Gly)	GAT (Asp) TAT (Tyr)	GAC (Asp) AAC (Asn)	AAT (Asn) GAT (Asp)	AAT (Asn) GAT (Asp) GAA (Glu)	GTA (Val) ATA (Ile)	AAT (Asn) AGT (Ser)	GCT (Ala) GGT (Gly)
FPV	Lys	Met	Lys	Ile	Val	Val	Ser	Ala	Asp	Asp	Asp	Asn	Val	Asn	Ala
CPV-2	Arg	Met	Asn	Ile	Ala	Ile	Ser	Ala	Asp	Asn	Asn	Asn	Val	Ser	Gly
CPV-2a	Arg	Met	Asn	Thr	Ala	Ile	Ser	Gly	Tyr	Asn	Asp	Asn	Ile	Ser	Gly
CPV-2b	Arg	Leu	Asn	Thr	Ala	Ile	Ser	Gly	Tyr	Asn	Asp	Asp	Val	Ser	Gly
New CPV-2a	Arg	Leu	Asn	Thr	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Asn	Val	Ser	Gly
New CPV-2b	Arg	Leu	Asn	Thr	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Asp	Val	Ser	Gly
Asp-300 (2a/2b)	Arg	Leu	Asn	Thr	Ala	Ile	Ala	Asp	Tyr	Asn	Asp	Asn (2a) Asp (2b)	Val	Ser	Gly
CPV-2c	Arg	Leu	Asn	Thr	Ala	Ile	Ala	Gly	Tyr	Asn	Asp	Glu	Val	Ser	Gly

Table 1 footnotes:

(a) Positions are referred to the amino acid and nucleotide sequences of strain CPV-b (GenBank accession no. M38245)

(b) Codon affected by SNPs used to design type-specific probes distinguishing CPV-2 from CPV-2a/2b/2c

(c) Codon affected by SNPs used to design type-specific probes distinguishing CPV-2a from CPV-2b, and CPV-2b from CPV-2c

(d) Nucleotide (Nt)

4.3. Vaccination Efficacy: heterologous strain protection challenge

Concerns about the efficacy of CPV type 2-based vaccines against the antigenic variants have been raised, as many commercially modified live virus vaccines are based on the original type CPV-2, which was isolated in the early 1980's.[90,105] Although cross-protection against the different strains was shown, generally, vaccines should contain the newest antigenic types of a given virus, implying the most complete possible protection.[105] Consequently, different studies were conducted to address this topic.

On one side, CPV-2 vaccines were shown to induce immunity against the homologous (vaccine) virus, but presented significantly lower cross-reactivity with its variants, which presumably can allow infection of these strains in vaccinated dogs.[54,106,107] Thus, the divergence between the vaccine and field strains can contribute to increase the risk of an outbreak in the canine population.[108]

Moreover, in most CPV-2c outbreaks involving adult dogs, the vaccination schedules were completed, including a yearly booster vaccination.[54,109,110] In young dogs, it is also described that they have undergone the vaccination protocol within the first year of life.[54,111,112] Regardless of these reports, different studies have demonstrated that type-2 vaccines are able to protect dogs against the CPV-2c challenge, under experimental conditions.[54,91] However, there is a lack of information regarding the immunity elicited by the original CPV-2 vaccines against the 2c strain after an extended interval between vaccination and challenge, when the type-2 antibody titres could be lower than necessary to prevent infection and disease caused by strains circulating in the field.[54,113]

Considering the concerns about the antigenic differences between the original CPV-2 strain and the field strains, which may decrease the effectiveness of vaccination, a wider use of vaccines type 2b-based has been suggested.[54,106,112]

Recently, studies conducted by a vaccine manufacturer have demonstrated that a recent multivalent vaccine containing a CPV-2b strain was able to induce cross-neutralising antibody responses to CPV strains, and can protect against a virulent CPV-2c infection in dogs.[91,114]

The emergence of the CPV-2 variants in cats causes the same concerns about the effective efficacy of the traditional FPV-based vaccines against the infections of the strains 2a, 2b and 2c in the feline population.[46] It has been reported that a common FPV-based vaccine was able to elicit cross-protection against CPV-2b, however, antibody titres against these CPV strains were significantly lower, when compared to FPV-antibodies. Additionally, it has been reported that the CPV-2c strain had the lowest degree of cross-protection, which could explain the higher degree of virulence of this variant in cats.[46,54] Recently, a study conducted by a vaccine manufacturer has shown that a multivalent vaccine was able to protect cats against infection and

associated lymphocytopenia following challenge with CPV-2b and 2c, under experimental conditions, up to 20 days post-infection.[50] However, further studies are needed to address this question and multivalent vaccines containing FPV combined with a CPV strain variant should be considered, the CPV-2c strain being a logical candidate for the vaccine antigen, to offer cross-immunity in field conditions.[23,37]

Despite the distinct positions described about this topic, it is important to highlight that continuous surveillance studies should be done, allowing the record of distribution of CPV-2 strains and assessment of vaccine efficacy in both canine and feline populations.[18,113,115] In addition, a recent study reinforces the need to develop specifically-designed clinical trials, which should include adequate size sample and follow-up, to obtain reliable information about the cross-protection from currently licensed vaccines.[108]

4.4. Worldwide distribution of Canine Parvovirus variants

In late 1970s and early 1980s, the early evolutionary events of CPV-2 were characterized by global dissemination and replacement by CPV-2a. After the emergence of CPV-2b and CPV-2c, it was reported that all the CPV strains were globally distributed in the canine population. The prevalence of CPV-2 strains may vary, depending on geographical region (Fig. 6).[57]

Therefore, epidemiological reports shown that CPV-2a is predominant in Australia, most of Asian countries and Europe, and is the only variant reported in New Zealand, Nigeria and some Eastern European countries.[96,116,117] However, CPV-2b prevalence has been reported in several countries within the five continents, being the predominant strain in Ireland, UK, USA and in a few countries of Asia. Other reports have shown that CPV-2a and 2b were equally distributed in Belgium, Switzerland and Austria. In addition, the CPV-2c has been mainly found in South American and European countries.[57,96]

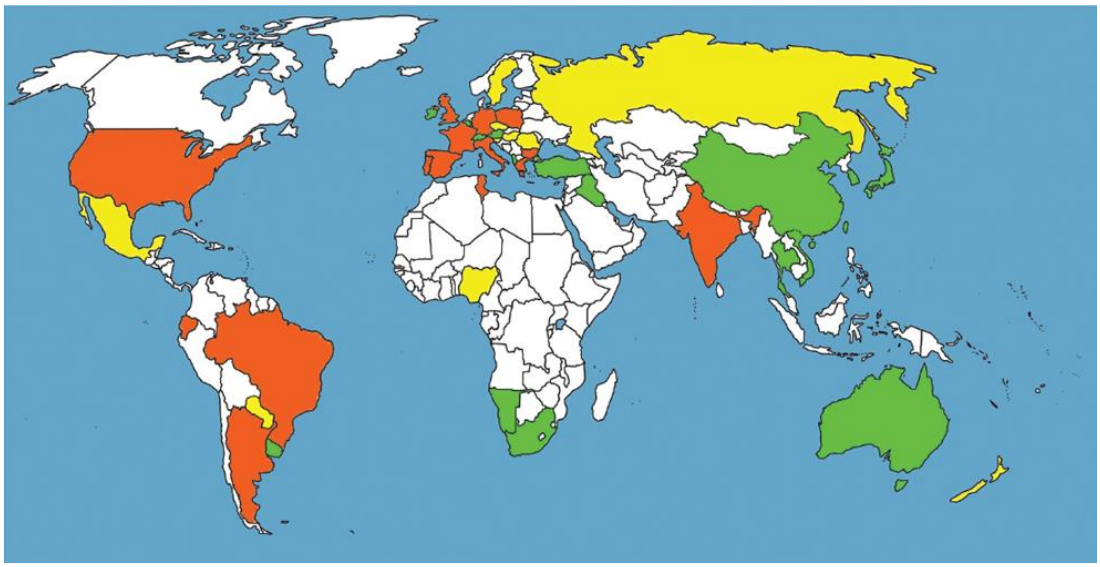

Figure 6. Geographical distribution of three CPV variants based on 426 residue detected in dogs population worldwide.

Footnotes: Orange: presence of three CPV variants; green: presence of two of three CPV variants; yellow: presence of one of three CPV variants. *(Reproduced from Miranda C. et al., 2016 [57])*

In addition, the prevalence of the different CPV-2 strains may also vary across different regions of the same country where the samples are collected, as was reported in Portugal, where in the continental south and the island regions, CPV-2c was reported to be the predominant strain, in contrast with the north and central continental region, CPV-2b was reported to be the prevalent variant.[118,119]

The prevalence of CPV infection in clinically-healthy cats or in cats with panleukopenia remains unknown, however the isolation of CPV-2 variants has been reported worldwide.[57] In one study in Vietnam and Taiwan more than 80% of the isolates from cats with panleukopenia were types of CPV.[99] In Germany, one study found that around 10% of viruses isolated from cats with panleukopenia were CPV-2a or a CPV-2a-derived variant.[19,120] Several studies reported the isolation of CPV-2a and 2b in domestic cats from Europe, Japan, to India.[37,121,122] CPV-2c was also been detected in clinically ill cats[103,123], and as well in co-infections by multiple CPV variants, and mixed infections with FPV and CPV-2 strains.[26,124]

The frequency of distribution of the CPV strains has shown differences associated with the year of collection of samples. Moreover, the reasons behind the differences found in the world distribution of CPV-2 variants are not completely understood, which is associated with the genetic diversity and its dynamic changes during the CPV evolution, suggesting that field parvoviruses are under strong selection pressure.[54,57]

Therefore, the three CPV-2 strains are circulating worldwide, being difficult to state which variant is predominant. In addition, the geographical variation in CPV distribution highlights the importance of surveillance reports, providing more data to the study of the parvovirus epidemiology.[54,113,115]

4.5. Epidemiological Surveillance in the United Kingdom

Disease surveillance has been described as the ongoing systematic collection and analysis of data to characterize the current health and disease situation within a given population. In the UK, disease surveillance has been a standard practice in farmed livestock and equine populations.[125] However, and despite the estimated UK population of 11.6 million dogs and 10.1 million cats in 2011, disease surveillance of the UK small companion animal population has been lacking.[125,126]

Although vaccination is widely available, clinical illness due to CPV infection is still a common finding by veterinary practices across the UK. In fact, accordingly to the Small Animal Veterinary Surveillance Network (SAVSNET), which has the aim of collecting data from veterinary practices across the UK, to increase the understanding of current disease burden in small companion animal population, a mean percentage of 13.2% of samples testing positive for CPV-2 strains was found by laboratory-based surveillance of submitted samples from dogs, between October 2013 and September 2014, and a mean percentage of 10.7% of positive samples for CPV-2 variants was reported between October 2014 and September 2015.[125]

In 2008, a study using samples from clinically-ill dogs was conducted with the objective of determining which strains of CPV were circulating in the UK. It was found that CPV-2b was the most prevalent strain, followed by CPV-2a; no CPV-2c was found in this study.[127] The CPV-2b strain was the most prevalent strain in a previous study using UK samples, which found only one CPV-2c isolate in the country.[96] In another study, a significant geographic clustering in some areas was found, suggesting that CPV-2a and 2b may have different distributions across different areas of the UK.[128]

Additionally, the prevalence of parvoviruses in clinically-healthy cats and dogs was investigated in two rescue shelters in the UK. A prevalence of 32.5% of CPV-2 strains, in faeces, was found in a cross-sectional study of 50 cats from a feline-only shelter. Also, 33.9% of cat faecal samples tested positive in a longitudinal study of 74 cats from a mixed canine and feline shelter. Randomly selected positive samples were identified as CPV-2a or 2b.[101]

Longitudinal sampling of the mixed shelter, showed that all cats shed the same virus strain at each date they were positive, despite the lack of clinical signs. Although many cats were shedding CPV-2 strains, canine faecal samples from this shelter were negative for CPV-2 variants. In addition, half of the sequences of the feline strains were similar to those obtained from a previous study of clinically-affected dogs in the UK.[101,128]

Therefore, it was shown that clinically healthy cats can shed CPV for prolonged periods of time, suggesting that cats can represent an important reservoir for the maintenance of infection in both cat and dog population.[101] For that reason, it is important to continue to conduct surveillance studies, providing information on what extend is parvovirus circulating in the UK and what antigenic variants are found in the field.

II. Research Project

5. Aim

A previous study from 2007 has reported CPV-2a and CPV-2b isolated from dogs in the UK, and only one CPV-2c isolate from the same population.[96] Subsequently to this study, in 2008, CPV-2a and CPV-2b strains were identified in a clinically-ill dog population.[127] In 2011, another report shared the same findings on the CPV-2 variants in the UK.[128] In all these studies, CPV-2b was the most prevalent strain reported. Therefore, to date, only one CPV-2c isolate from dogs was reported, and no CPV-2c was previously detected in cats in the UK.

No study has been documented in the UK to determine the circulating variants of parvoviruses in asymptomatic cats since 2012.[101] It would be interesting to know to what extent currently-circulating CPV variants infect cats.

The aim of this study was to investigate the prevalence of feline and canine parvoviruses infection in cats, and to genetically characterise possible positive samples, generating data on the molecular surveillance of parvoviruses circulating in a non-clinical cat population in the UK.

6. Materials and Methods

6.1. Sample Collection and Study Population

The samples used in this project were kindly made available by Dr. Allison German and Prof. Kenton Morgan, from the Institute of Infection and Global Health, University of Liverpool, and resulted from a study on molecular epidemiology of rotavirus in cats in the UK.[129]

The collection included a total of 1,727 faecal samples, from a total of 25 adoption centres in the UK run by Cats Protection, the largest feline welfare charity in the UK. The samples were collected during the winter and summer months of 2012.[129]

Shelters are widely distributed geographically in the UK, and vary in size and construction. The number of cat accommodation spaces ranges from 16 to 202 per centre, and the populations of cats are in constant flux.[129]

The 818 faecal samples of this cross-sectional study were collected in 2012 from cats held in 13 adoption centres across the UK (Figure 7), and originally part of a larger biobank of 1,727 samples.

Map Legend:

1. York

2. St Helens

3. Mansfield

4. Birmingham

5. Downham Market

6. Dereham

7. Hereford

8. Bridgend

9. Truro

10. Exeter

11. Ferndown

12. Isle of Wight

13. Haslemere

Figure 7. Geographical location of the cat rehoming centres (n=13). Map generated with ArcGIS™ (Esri®).

6.2. Sample Preparation and DNA Extraction

Each faecal sample was maintained at -80ºC in the Biobank at the University of Nottingham's School of Veterinary Medicine and Science until testing. An inventory was constructed with an alphanumeric code that was assigned to each box of samples during entry into the Biobank, the number of samples in each box, the shelter location and the individual numeric code of each sample.

Template DNA from the faecal samples was prepared by adding approximately 1g of faecal material to a universal faecal sample tube containing 5ml of a 25% weight/volume mix of kaolin (Sigma-Aldrich) and 0.01M dithiothreitol (DTT) (Sigma-Aldrich) in Phosphate Buffered Saline (PBS). The preparation was thoroughly mixed for several minutes using a vortex. The tube was placed in a water bath at 37ºC for 15 minutes.

Subsequently, 1.5ml of mixture from each tube was taken into a labelled microfuge tube by pipetting twice 0.75ml. The tubes were then centrifuged at 1300rpm for 5 minutes. Each batch of DNA extractions performed up to 48 samples.

A volume of 5 µl of each sample supernatant was added to a labelled conical-bottomed 1ml tubes containing 95 µl of diethyl pyrocarbonate (DEPC) treated water. The tubes were then boiled at 100ºC in a water bath for 10 minutes.

After the DNA extraction steps were completed, the products of the DNA extraction were kept in the 1ml tubes labelled with an individual serial number, matching with the inventory, and were stored in plastic boxes, labelled with the box code, extraction serial number and date.

6.3. Amplification by PCR

To plan each PCR assay in 96-well plates, a MS Excel® (Microsoft®) spreadsheet with the plate layout was prepared before the PCR assay. Each PCR had an individual code, and each assigned well number corresponded to the individual numeric code from the sample and extraction number, as in the example presented in the Tables 2 and 3.

To ensure PCR reliability, a negative control consisting of a master mix of the PCR template was separately prepared, made up with the volume for a 96-well plate, to run with each plate. Subsequently, the DNA template was separately and carefully added to each well. The components of each PCR reaction are described in Table 4. The DNA polymerase used was the MyFi™ DNA polymerase (Bioline®), which has higher fidelity than native *Taq* polymerase, accordingly to the manufacturer. The MyFi™ reaction buffer contains 1 mM dNTPs, 3 mM $MgCl_2$, DNA polymerase and enhancers, which means that it was not necessary to add these components separately, when preparing the PCR master mix.

Table 4. PCR master mix contents per reaction tube.

Master mix contains	Per tube (µl)
DEPC treated water	9.0
MyFi polymerase mix	12.5
Forward primer (felVP2-3820)	0.5
Reverse Primer (VP2-4247R)	0.5
DNA Sample	2.5
Total	25.0

Primers (Sigma-Aldrich®) were designed by Dr. Stephen Dunham to allow both FPV and CPV detection (Table 5), spanning the amino acid 426 region of the VP2 gene, which determines the CPV-2 variants. The product size of the reaction was 428 base pairs (bp).

The PCR cycling conditions were defined as follows: initial denaturation at 95ºC for 1 minute; 40 cycles of: denaturation of 15 seconds at 95ºC, followed by a primer annealing at 60ºC for 15 seconds; extension at 72ºC for 15 seconds; and a final extension of 2 minutes at 72ºC. A Life ECO™ thermal cycle (Bioer®) was used to run the reactions.

Table 2. Example of 96-well PCR plate layout planning (PCR individual code: P8.15.02.)

Row/Column	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**	**9th**	**10th**	**11th**	**12th**
A	89	81	73	65	57	49	41	33	25	17	9	1 (JD11[a])
B	90	82	74	66	58	50	42	34	26	18	10	2[b]
C	91	83	75	67	59	51	43	35	27	19	11	3
D	92	84	76	68	60	52	44	36	28	20	12	4
E	93	85	77	69	61	53	45	37	29	21	13	5
F	94	86	78	70	62	54	46	38	30	22	14	6
G	95	87	79 (JD12[a])	71	63	55	47	39	31	23	15	7
H	Neg Control[c]	88	80	72	64	56	48	40	32	24	16	8

Table 2 footnotes: (a) alphanumeric code of biobank box of samples; (b) number assigned per each well; (c) negative control.

Table 3. Example of the 96-well PCR plate layout for the assay P8.15.02.

	1st	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**	**9th**	**10th**	**11th**	**12th**
A	26339013_11	26339003_3	22696076_73	22696065_65	22696057_57	22696049_49	22696041_41	22696033_33	22696025_25	22696017_17	22696009_9	22696001[a]_1[b]
B	26339014_12	26339004_4	22696077_74	22696066_66	22696058_58	22696050_50	22696042_42	22696034_34	22696026_26	22696018_18	22696010_10	22696002_2
C	26339015_13	26339005_5	22696078_75	22696067_67	22696059_59	22696051_51	22696043_43	22696035_35	22696027_27	22696019_19	22696011_11	22696003_3
D	26339018_14	26339006_6	22696079_76	22696068_68	22696060_60	22696052_52	22696044_44	22696036_36	22696028_28	22696020_20	22696012_12	22696004_4
E	26339019_15	26339008_7	22696080_77	22696069_69	22696061_61	22696053_53	22696045_45	22696037_37	22696029_29	22696021_21	22696013_13	22696005_5
F	26339020_16	26339010_8	22696081_78	22696070_70	22696062_62	22696054_54	22696046_46	22696038_38	22696030_30	22696022_22	22696014_14	22696006_6
G	26339021_17	26339011_9	26339001_1	22696071_71	22696063_63	22696055_55	22696047_47	22696039_39	22696031_31	22696023_23	22696015_15	22696007_7
H	Neg Control[c]	26339012_10	26339002_2	22696075_72	22696064_64	22696056_56	22696048_48	22696040_40	22696032_32	22696024_24	22696016_16	22696008_8

Table 3 footnotes: (a) individual numeric code of the sample; (b) DNA extraction serial number; (c) negative control.

Each PCR plate had a negative control in one of the wells. An aliquot of the master mix was frozen down at -20ºC and was used to run a positive control in a separate reaction (to avoid cross-contamination).

Table 5. Primers used accordingly.

Primer [(a)]	Name	Tm (ºC)	Sequence (5´- 3´)
Forward	felVP2-3820F	62.8	TTGARGCRTCTACACAAGGG
Reverse	VP2-4247R	66.7	TGGTGCATTTACATGAAGTCTTGG

Table 5 footnotes: (a) Primers (Sigma-Aldrich®) designed by Dr. Stephen Dunham.

6.4. Agarose gel electrophoresis

Tris-Borate-EDTA (TBE) 1x was used to prepare a 1.5% TBE agarose gel with Nancy-520 fluorescent stain (Sigma-Aldrich®). TBE 1x buffer was also used in the electrophoresis tank.

Each PCR product was prepared with 2 µl of gel loading dye (New England Biolabs®), making up a total volume of 6 µl. The agarose gel was usually prepared with 48-wells; a volume of 6 µl of 1Kb DNA ladder (HyperLadder™; Bioline®) was added to the first well and the PCR negative control to the last well. A positive control was run separately from the gel of the samples at the same time, to avoid cross-contamination.

The gel electrophoresis ran for one hour and 30 minutes at 80V. The result of each electrophoresis was visualised under UV illumination using a gel imaging system (ImageQuant™ 300; GE Healthcare®).

6.5. DNA Sequencing

Following gel electrophoresis, 10 positive PCR products (25 µl) were purified using QIAquick® PCR Purification Kit (QIAGEN), according to the manufacturer's instructions.

The DNA was then quantified using NanoDrop™ 2000 spectrometer (Thermo Scientific™), obtaining the concentration of DNA (ng/µl). The purified DNA products were sent for the Sanger Sequencing Service of the Source BioScience laboratories, along with the reverse primer VP2-4247R. Source BioScience laboratories required a concentration of 10 ng/µl for purified PCR samples and 3.2 pmol/µl for the primer sent, with a volume of 5 µl per reaction.

6.6. Sequence analysis

FinchTV™ software (Geospiza®) was used to visualize and obtain sequencing results. The reverse complement of the nucleotide sequences was obtained using a reverse complement tool available online (http://reverse-complement.com/), according to the fact that the reverse primer was used for sequencing.

Sequences were then compiled into a MS Word® document with a reference sequence of CPV-2 (GenBank accession number: M38245) at the start, followed by the nucleotide sequence of each sample.

The sequences were aligned using the multiple sequence alignment tool Clustal Omega© (EMBL-EBI) available online (http://www.ebi.ac.uk/Tools/msa/clustalo/).

6.7. Statistical analysis

Prevalence is defined as the proportion of a population affected by instances of disease or related attributes, such as infection, at a given point in time. It can be interpreted as the probability of an individual from the same population having the disease at this given point in time. Although prevalence can be defined simply as the number of affected animals, it is most meaningful when expressed in terms of the number of diseased animals in relation to the number of animals in the population at risk of developing the disease.[130,131] Accordingly:

$$\text{Prevalence}^{131} = \frac{\text{number of individuals infected}}{\text{number of individuals in the population at risk}}$$

A 95% confidence interval (CI) was calculated using the QuickCalcs® tool (GraphPad® Software).

7. Results

7.1. Gel Electrophoresis Results

From the total of 818 faecal samples extracted and PCR-screened for FPV and CPV infection, 31 were PCR-positive. All the specific electrophoresis bands were expected to have 428bp, thus specific bands matching with the 400bp ladder were considered positive (Fig. 8). The HyperLadder™ 1kb weight marker scale is shown in the Figure 9.

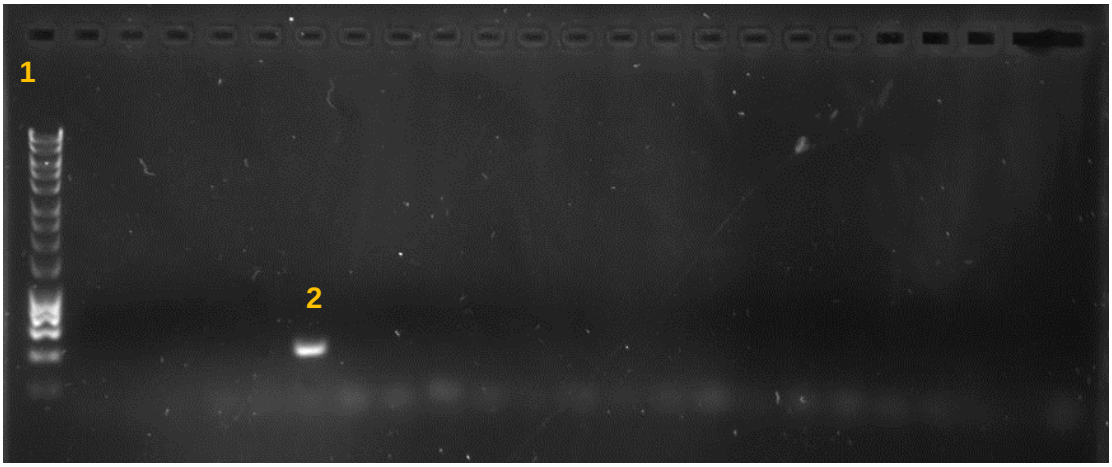

Figure 8. Example of a PCR-positive product in the electrophoresis gel, visualized under UV light. Visualization and image captured using ImageQuant™ 300 system; GE Healthcare®.

Footnotes: 1 – HyperLadder™ 1kb (Bioline®); 2 – PCR-positive product.

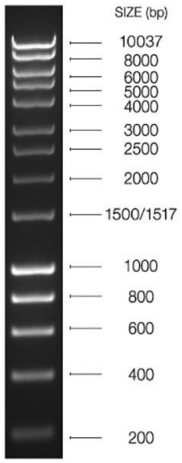

Figure 9. HyperLadder™ 1kb run on a 1% TBE-agarose gel and visualized using ethidium bromide. *(Source: Bioline®, available at https://www.bioline.com/sg/hyperladder-1kb.html#RL)*

Furthermore, some electrophoresis bands did not show high intensity under UV light, albeit specific (approximately 400bp). For that reason, fainter specific bands were considered as positive PCR-products (Fig. 10).

The positive samples were distributed by Centre location and identification (ID) as presented in Table 6.

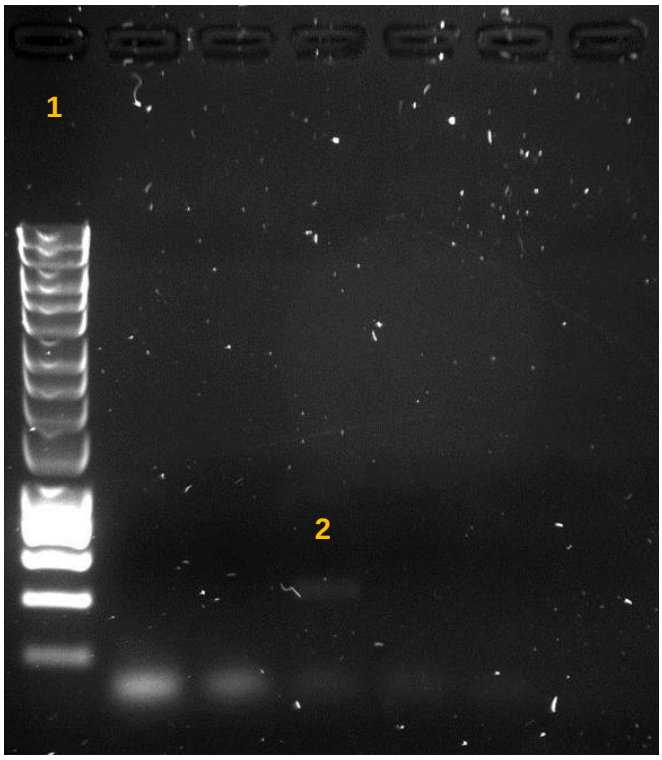

Figure 10. Example of a specific positive faint band in the electrophoresis gel, visualized under UV light. Visualization and image captured using ImageQuant™ 300 system; GE Healthcare®.

Footnotes: 1 – HyperLadder™ 1kb (Bioline®); 2 – PCR-positive band.

Table 6. Positive samples ID distributed by Centre, with an assigned positive number (n=31).

Centre and Sample ID	Sample Purified (SP) No.	Total of Positive Samples per Centre
York		1
13476035	1	
Dereham		15
12166002	2	
12166017	3	
12166025	4	
12166032	5	
12166033	6	
12166038	7	
12166043	8	
12166044	9	
12166052	10	
12166054	11	
12166058	12	
12166059	13	
12166060	14	
12166069	15	
12166073	16	
Birmingham		4
6817004	17	
6817012	18	
6817039	19	
6817061	20	
Hereford		2
35179027	21	
35176006	22	
Truro		1
14076015	23	
Haslemere		1
24736009	24	
Bridgend		2
6847095	25	
6847099	26	
Isle of Wight		1
6836050	27	
Downham Market		3
22696004	28	
22696009	29	
22696081	30	
Exeter		1
26339006	31	
Total		**31**

7.2. Genetic Sequencing Results

Sequencing results of ten positive samples were sent by Source Bioscience, and were then visualized using FinchTV™. An example of a good sequence trace is shown in Figure 11, with little baseline interference (baseline “noise”).

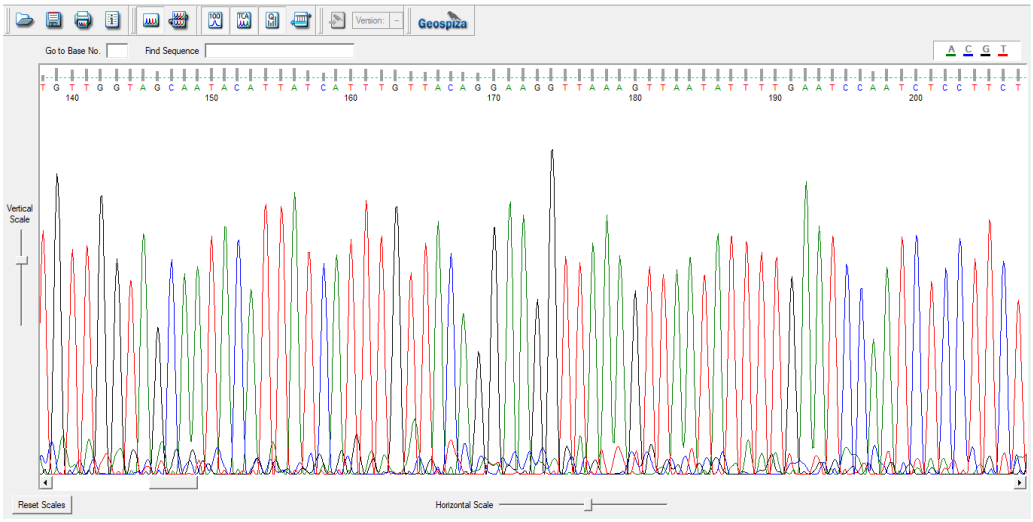

Figure 11. Section of a good sequence chromatogram, with little baseline interference. (Visualized with FinchTV™; Geospiza®)

Other sequence products did not work so well, producing traces with high baseline interference, with multiple peaks at a given nucleotide position (Fig. 12). Thus, due to the low confidence in determining the right nucleotides and the presence of unreadable bases, the sequence traces that presented this condition were excluded from the analysis.

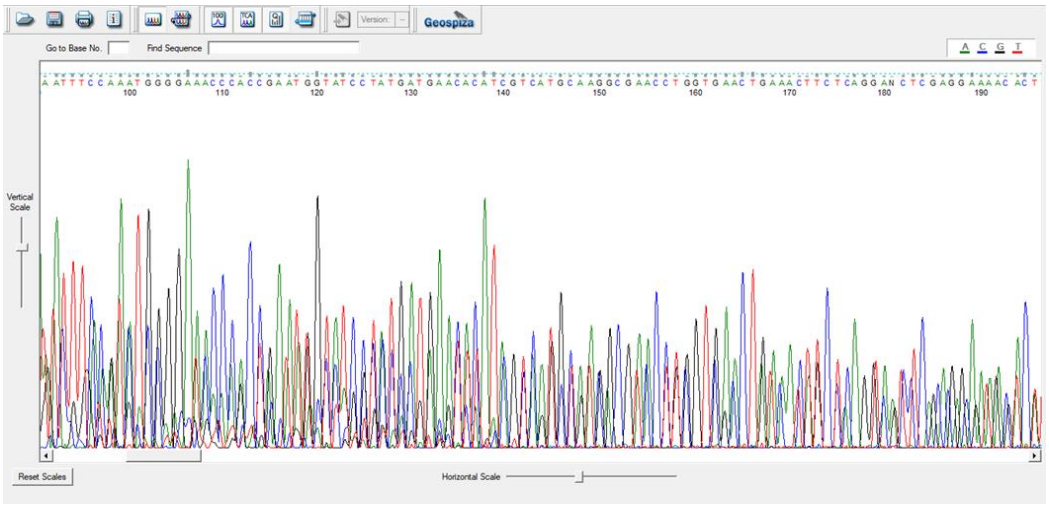

Figure 12. Section of a sequence chromatogram with high baseline interference, presenting multiple peaks at a given nucleotide position. (Visualized with FinchTV™; Geospiza®)

After the reverse complement for each valid nucleotide sequence, a multiple alignment along with a reference sequence for CPV-2 (GenBank accession number: M38245) was done and it is presented in Figure 13. The sequence analysis considered the Nt position 4062-4064, which is the codon of the amino acid residue 426 (Table 1), spanned by the PCR assay.

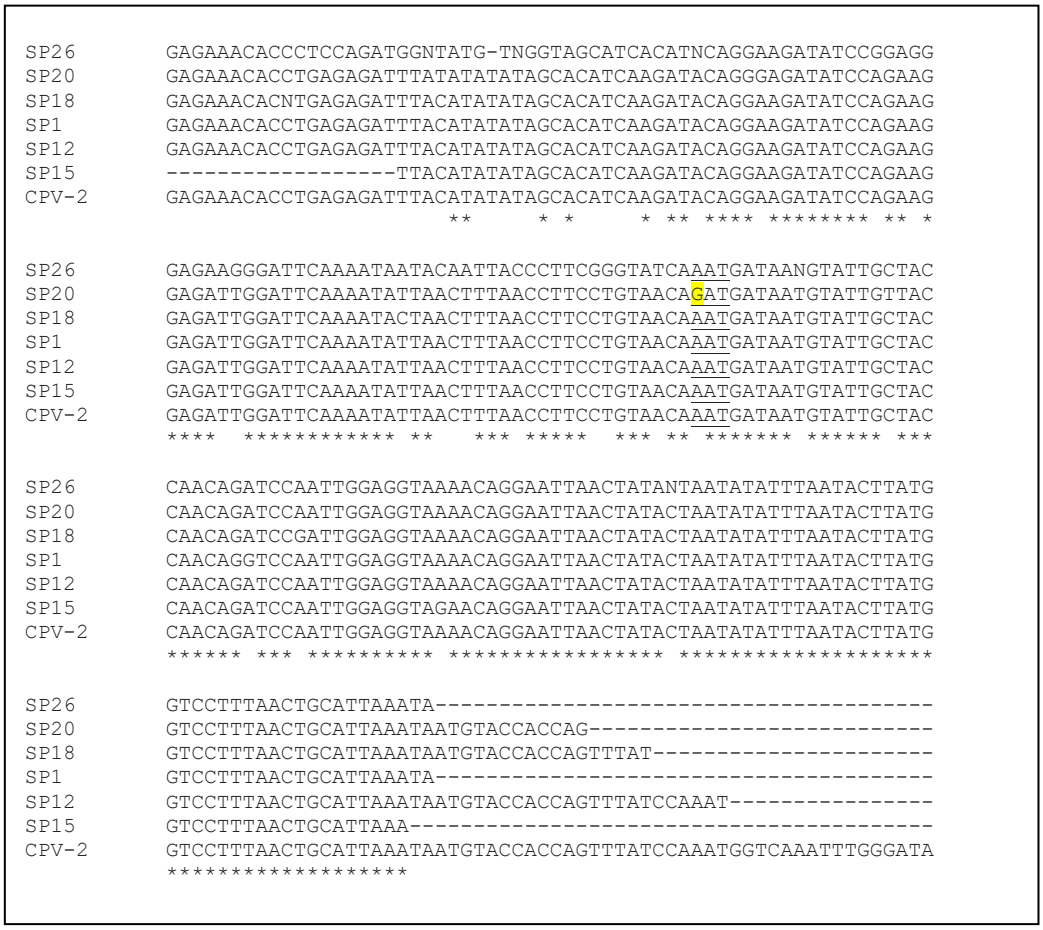

Figure 13. Section of the multiple alignment of positive samples successfully sequenced, along with the CPV-2 reference sequence (GenBank accession number: M38245). (Multiple aligned with Clustal Omega© tool; EMBL-EBI).

Footnotes: underlined nucleotides: codon at Nt position 4062-4064, amino acid residue 426; yellow: different nucleotide highlighted, originating the codon “GAT”; *: same nucleotide presented in the nucleotide position.

Considering the sequence results, it was possible to group the sequenced samples under two strain groups, given the amino acid variation of canine and feline parvoviruses, at the 4062-4064 nucleotide position (amino acid residue 426), which is a codon affected by SNPs (Table 7).

Table 7. Parvovirus strain classification of the sequenced samples, accordingly to the codon observed at the amino acid residue 426, Nt position 4062-4064 (n=6).

Strain [(a)]	**FPV / CPV-2a**	**CPV-2b**	**CPV-2c**
Codon observed [(b)]	*AAT*	*GAT*	*GAA*
Samples ID	SP1; SP12; SP15; SP18; SP26	SP20	-
Number of sequenced samples (n=6)	5	1	0

Table 7 footnotes: (a), (b): modified from Table 1. Amino acid variations in the VP2 protein of feline and canine parvoviruses *(Adapted from Decaro N. et al., 2012 [54]).*

7.3. Parvovirus Prevalence

7.3.1. Overall Prevalence

Considering the study population of cats held in the 13 rehoming centres, from the total of 818 faecal samples extracted and screened for FPV and CPV infection, 31 were PCR-positive.

Prevalence = 31/818 = 0.0379 (95% confidence interval [CI], 0.0259 to 0.0534).

The overall estimated prevalence of PCR-parvovirus positive faecal samples in the population was 3.8% (95% CI, 2.59 to 5.34% [31/818]).

7.3.2. Centre Prevalence

The prevalence of parvovirus PCR-positive faecal samples was calculated for each cat rehoming centre. Parvovirus was detected in at least one sample from 10 out of the 13 centres (95% CI, 46.19 to 94.96%), as represented in the Figure 14. No positive samples were detected in the cat shelters of St Helens, Mansfield, and Ferndown.

The estimated prevalence in the sampled centres, which presented at least one positive, ranged between 20.5% (95% CI, 11.98 to 31.62%) in Dereham, and 1.28% (95% CI, 0.03 to 6.94%) in Truro (Table 8).

Table 8. Prevalence of parvovirus positive faecal samples in different centres.

Centre	Prevalence (% [no. parvovirus positive/total no.])	95% CI (%)[a]
Dereham	20.5 (15/73)	11.98 – 31.62
Hereford	4.65 (2/43)	0.57 – 15.81
Downham Market	3.53 (3/85)	0.73 – 9.97
Haslemere	3.23 (1/31)	0.08 – 16.70
York	2.63 (1/38)	0.07 – 13.81
Bridgend	2.50 (2/80)	0.30 – 8.74
Birmingham	2.43 (4/164)	0.67 – 6.13
Exeter	2.38 (1/42)	0.06 – 12.57
Isle of Wight	1.37 (1/73)	0.03 – 7.4
Truro	1.28 (1/78)	0.03 – 6.94
St Helens	0 (0/54)	0 – 6.6
Mansfield	0 (0/30)	0 – 11.57
Ferndown	0 (0/27)	0 – 12.77

Table 8 footnotes: (a) 95% CI, 95% Confidence Interval.

Map Legend:

Parvovirus detected in cats of the centres of:
(green)

1. York

4. Birmingham

5. Downham Market

6. Dereham

7. Hereford

8. Bridgend

9. Truro

10. Exeter

12. Isle of Wight

13. Haslemere

Parvovirus not detected in cats of the centres of:
(red)

2. St Helens

3. Mansfield

11. Ferndown

Figure 14. Geographical location of the cats rehoming centres, according to the detection of parvovirus. Map generated with ArcGIS™ (Esri®).

Footnotes:
numbers in green: location of the cat rehoming centres where parvovirus was detected (n=10); numbers in red: location of the cat rehoming centres where parvovirus was not detected (n=3).

8. Discussion

This study represents the first population-based study of parvovirus prevalence in the UK since 2012. It is known that CPV strains have evolved and can infect and cause disease in cats. This study provides evidence of infection by parvoviruses in a non-clinical cat population across UK.

Parvoviruses were detected at a low prevalence (3.8%) in this apparently healthy cat population in the UK, considering the 13 rehoming centres sampled, which comprised 818 samples.

Parvoviral infections were reported in asymptomatic cats in the UK in a previous study[101], and a high prevalence of parvoviral shedding was reported in apparently healthy cats (33%), which contrasts sharply with overall prevalence found in this study. The prevalence found in the present study, in centres that presented at least one positive sample, ranged between 20.50% and 1.28%, with twelve of the 13 centres presenting a prevalence for parvovirus faecal shedding of less than 5%, suggesting that the results could be highly dependent on timing and location of sampling. Significantly, ten out of 13 centres housed at least one cat shedding parvovirus (parvovirus-positive cat).

In other countries, such as India and China, the prevalence found for CPV/FPV infections in cats ranged between 11% and 12.5%. Considering that these studies have included both asymptomatic and clinical affected cats, the prevalence reported is more in line with the estimated prevalence found in the present study in the UK. Additionally, these studies mentioned that preventive measures and vaccination are still uncommon in those areas, which is likely to contribute for a higher prevalence.[121,122]

Interestingly, it is possible to compare the low prevalence of parvoviruses asymptomatic infection found in this study with other viral agents in the same cat population, namely the feline rotaviruses. Indeed, this specific sample collection was previously included in a broad study of rotaviruses, which included not only the 13 rehoming centres tested in this study for FPV/CPV, but the total of the 25 rehoming centres in the UK. The estimated prevalence of feline rotaviruses in the cat population was 3.0%.[129] This is interestingly similar to the prevalence of 3.8% for parvoviral asymptomatic infection found in this study, considering that the present study reflects a substantial proportion of the same population.

The study suggests a low national prevalence of parvovirus in the cat population as the prevalence of infection found was 3.8% and the cross-sectional sampling provided a snapshot view across the UK.

Longitudinal sampling methods based on this estimated prevalence could be used to better understand transmission. Additionally, longitudinal sampling could be useful to determine whether the cats of non-clinical populations act as long-term carriers.

The higher prevalence for parvovirus faecal shedding was found in the Dereham centre (20.5%) may arise from a spread within a centre or reflect the income of cats already infected and shedding parvoviral particles due to an outbreak in the local area. It has been suggested that high shelter prevalence is mainly driven by incoming infected cats, which reflects the FPV/CPV infection in the general feline population.[101] Limited evidence for transmission within the centre is also consistent with previous studies of FCV, an important respiratory pathogen of cats, in similar shelters.[132] In addition, this result cannot be related to sample contamination, since the samples were carefully processed, with a rigorous use of negative controls, and all the positive samples were double-checked in a separate reaction using the initial DNA extraction

Viral diseases, especially gastrointestinal pathogens are not easily contained, and biosecurity procedures are essential to infection spread control within shelters environments.[41] Biosecurity measures for the cat rehoming centres are described to be high standard across the country, as all shelters apply strict hygiene guidelines.[129] Therefore, that may also reflect the low prevalence found for FPV/CPV faecal shedding in the tested shelters.

Further investigation would allow completion of the analysis of the collection of samples from the 25 rehoming cat centres across the UK, which would allow inference of a prevalence of all centres. Additionally, a more reliable and broad conclusion about the national prevalence of parvoviruses asymptomatic infection in the UK cat population could be estimated, as more distinct locations can be included in the study, such as Northern Ireland (shelter of Belfast) and Scotland (shelter of Glasgow). Additionally, epidemiological data, such as the age of the cats and faecal consistency of the samples can be associated with the population, which may be considered as valuable epidemiological variables to further characterize the parvoviral infection in this non-clinical feline population.

The use of a shelter cat population as a sentinel for the cat population in the UK may be considered a study limitation, as a shelter feline population rather than owned cats may have associated stressors and increased opportunities for disease spreading, considering the densely populated environment with cats from diverse backgrounds, and the constant flux of animals. However, it has been described that the majority of the study population is comprised of healthy cats that have been abandoned from homes due to socioeconomic factors, and consequently, true strays represent a minority of the population. Feral cats are only sporadically and temporarily housed in the shelters for trap-neuter-release programmes. Therefore, the use of these centres is considered a valid comparison and a reflection of the cat owned population in the UK.[129]

The molecular analysis of positive samples provided an insight into the molecular characterisation of the circulating field variants of parvoviruses in the feline population across the country. In the present study, five FPV/CPV-2a-like variants and one CPV-2b strain were identified, considering the spanning of the amino acid residue 426 position. Therefore, the identification of one CPV-2b variant evidences that CPV-2 variants are present in clinically healthy cats in the United Kingdom. Further studies would allow to obtain additional genetic information on these isolates, particularly the differentiation between FPV and CPV-2a variants, using a PCR assay specifically designed to obtain genetic data on different and extended key-position sites of the VP2 viral gene. The development of other PCR assays, specifically designed to obtain full-length amplification of the VP2 gene, would also be useful in an attempt to identify possible mutations and to proceed to viral phylogenetic analysis, which could be advantageous to better understand the epidemiological aspects such as field selection pressure on parvoviruses of the cat population.

The use of a previously-designed conventional PCR assay to screen and detect FPV/CPV-2 strains in a large number of tested samples was convenient due to the limited time available to this project, and it fulfilled the main goal of generating an insight about the epidemiological situation of parvoviruses in the shelter cat population. In-house experiments before the study course have attested the reliability of the established assay conditions. None the less, in further studies, the use of real-time PCR technology could be beneficial to obtain quantitative data, since low shedding titres are expected in this type of population, without overt clinical disease. Further investigation would allow measurement of viral DNA load in faeces and to determine whether DNA viral loads correlate with clinical signs, such as diarrhoea, to be determined through further inputs of the epidemiological data. Additionally, it would be possible to determine whether DNA sequencing information and certain genotypes are related to disease severity.

The presence of faint but specific electrophoresis bands, as shown in the results analysis, can be explained by the presence of low parvoviral DNA load, which is indeed expected in a population without overt clinical signs. Further investigation using real-time PCR methods would be beneficial, as it is more sensitive than conventional PCR coupled with agarose electrophoresis gels.

Vaccination status of the population was not considered in this study, however it is described that post-vaccinal faecal shedding can be detected, interfering with diagnostic assays.[43] Further investigation could allow determining to what extent low titres of MLV vaccines are shed by cats in this feline population, which could potentially cause an overestimated prevalence of parvoviral infection Additionally, further studies are needed to better understand the interaction in the field between CPV infection and FPV vaccination in cats.

It has been described that cats may be able to shed CPV/FPV for relatively prolonged periods of time, as an asymptomatic infection, and are more likely to be chronically infected rather than experiencing repeated rounds of infection-clearance-reinfection.[101] Indeed, although parvoviruses are normally associated with acute infections, longer infection courses have been reported.[133] It was shown in experimentally infected cats that FPV can be shed in urine and faeces up to 42 days post infection.[133] In mink, faecal shedding of MEV, a closely related virus of both CPV and FPV, has been demonstrated for up to one year, suggesting that adult mink may become asymptomatically infected, and may act as reservoirs.[134] The virus hiding in intestinal epithelial cells beyond the reach of antibodies is eliminated in the faeces, as these cells are continuously desquamated.[101] Infection persistence is also a characteristic of human parvovirus B19.[101,135]

Clinically normal long-term shedders have been considered important to identify in attempts to control or eradicate these diseases in both dog and cat populations.[101] Further studies are needed to clarify the potential of cats as reservoirs for parvoviruses, and to determine its mechanism and dynamics.

9. Conclusion

Diseases surveillance has been lacking in UK companion animal populations, despite the estimated population of more than 11 million dogs and 10 million cats in the country.

In summary, the present study provides an updated and valuable insight into the surveillance of parvoviruses in the cat population in the UK. The surveillance of parvoviruses in feline populations is important for investigating parvovirus genetic diversity, elucidating the role of asymptomatic carriage, and monitoring the risk of infection and outbreak situations of this evolving and emerging disease in the pet population in the UK.

Moreover, this study raises different concerns about the control of parvovirus infection in both cats and dogs, namely, whether these observations are reproductible in other cat populations, which transmission dynamics occur in the field, particularly, whether the viruses are transmissible between cats and dogs, and whether they are able to cause disease in unvaccinated populations. Intensifying surveillance of parvoviruses in both cat and dog population is required.

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III. Appendix Section

A1. A study of UK Rottweiler owners/breeders' opinions about vaccination – Preliminary Report of Results

Information Notice

During the traineeship at the School of Veterinary Medicine and Science, University of Nottingham, a contribution was made to this project. Dr. Mark Dunning developed and conducted the survey, and the Trainee (Ivo Fins) have conducted the result analysis as part of his training activities, resulting in the present preliminary report of results.

Further analysis, discussion of results, and development of the project are planned, however outside of the traineeship scope.

Additionally, an abstract has been submitted, with the participation of the Trainee (Ivo Fins) as first author, and it has been accepted for oral presentation at the British Small Animal Veterinary Association (BSAVA) Congress 2018.

Introduction

Among other dog breeds, Rottweilers have been reported to have a high susceptibility to parvovirus infection, however evidence to support this have been lacking. Whilst, in the United Kingdom, the 2017 PDSA Animal Wellbeing (PAW) Report have stated that 25% of the dogs have not had a primary vaccination course when young, compromising 1814 dog owners surveyed in the country.

Aim

To better understand vaccination practices in UK Rottweilers, according to the responses of Rottweiler owners/breeders.

Methods

The survey was conducted from March 2016 to May 2017. It registered 624 total respondents, all of which were Rottweilers' owners/breeders.

A simple quantitative method was used in the majority of the results' analysis, with the exception of the open-ended questions, where the tendency of answers by topic was identified. Whenever possible, the answers were categorized.

The results of each question and a keynote analysis are presented below.

Results

1. Please state how many dogs you currently own:

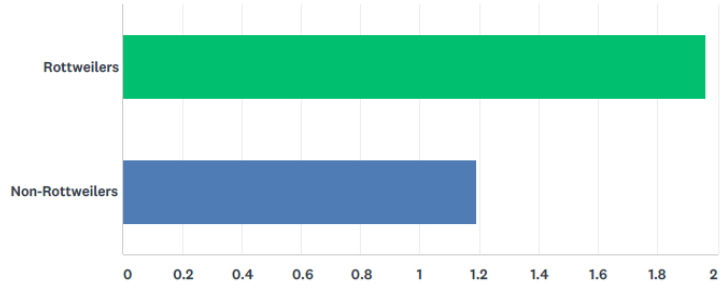

Chart 1. Average number of dogs owned by the respondents.

Table 9. Average and total number of dogs and relative frequencies of answers to the question one.

ANSWER CHOICES	AVERAGE NUMBER	TOTAL NUMBER	RESPONSES
Rottweilers	2	1,214	620
Non-Rottweilers	1	528	444
Total Respondents: 624			

The total number of Rottweilers included in this study is 1214 (Table 9), with an average number of 2 Rottweilers per owner (Chart 1). 71% of the owners have also other dogs' breeds, classified as Non-Rottweilers.

2. Have ever had dogs / litters affected by parvovirus?

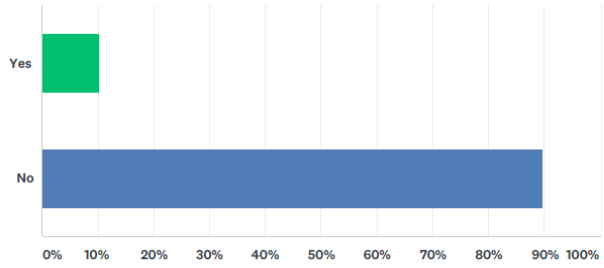

Chart 2. Percentage of dogs/litters affected by parvovirus.

Table 10. Relative frequencies of answers to the question two.

ANSWER CHOICES	RESPONSES	
Yes	10.34%	64
No	89.66%	555
TOTAL		619

The majority of respondents have never had dogs or litters affected by parvovirus (89.7%) (Chart 2 and Table 10).

3. If yes, please give details including the outcome.

From a total of 69 responses, approximately 63% describe successful recovery of parvovirus cases, with hospitalization.

In several answers is possible to know in what age the parvovirus infection occurred, being possible define the most common time frame when parvovirus cases occur, which was between the 6 weeks and 1-year old.

Nine responses from the 69 answers were excluded due to not presenting any information (N/A), or due to not give details on the clinical outcome or age of the dogs.

4. Do you routinely have your Rottweilers vaccinated against any of the following diseases? (please select all that apply)

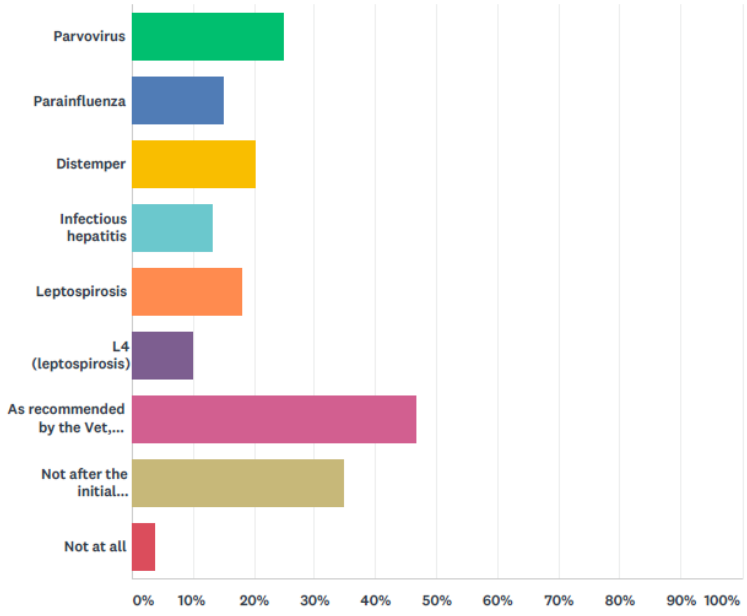

Chart 3. Percentage of answers distributed by vaccination option/disease.

Table 11. Relative frequencies of answers to the question four.

ANSWER CHOICES	RESPONSES	
Parvovirus	24.96%	142
Parainfluenza	15.11%	86
Distemper	20.39%	116
Infectious hepatitis	13.18%	75
Leptospirosis	17.93%	102
L4 (leptospirosis)	10.02%	57
As recommended by the Vet, details not known	46.75%	266
Not after the initial vaccination course	34.80%	198
Not at all	3.87%	22
Total Respondents: 569		

Approximately 47% from a total of 569 respondents answered as recommended by the Vet (Table 11).

It is important to highlight that almost 35% of the respondents said that they do not routinely vaccinate their Rottweilers after the initial vaccination course (Chart 3).

Parvovirus was the most frequent individual disease vaccinated against with around 25% of responses.

Approximately 4% of the owners/breeders respond that do not routinely vaccinate their dogs.

5. If not, please give details as to why not.

Around 38% of the respondents agree with the initial course of puppy vaccination.

33% of the 214 respondents describe worries about over vaccination and vaccination boosters, affirming that vaccination boosters are not necessary, and could be just a money-maker by veterinarians and pharmacological companies.

Around 14% of the answers mention titre testing to decide the vaccination course.

11% of the respondents mentioned cases of adverse reaction to vaccination or immunity disorders caused by vaccination.

It is important to highlight that some owners/breeds present their worries about the use of chemicals and possible links with autoimmune diseases and cancer.

Less than 5% of the answers support the use of natural medicines and homeopathic nosodes instead of vaccination.

Several respondents allude to evidence that could support the dangers of over vaccination, including vaccination studies, seminars and other sources of knowledge.

6. Do you vaccinate your dog with the Kennel cough vaccine against *Bordetella*?

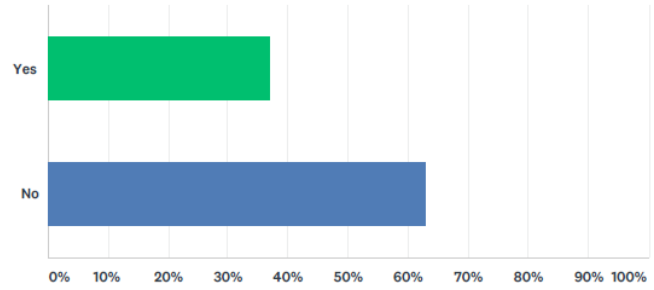

Chart 4. Percentage of answers regarding Kennel cough vaccination.

Table 12. Relative frequencies of answers to the question six.

ANSWER CHOICES	RESPONSES	
Yes	36.99%	226
No	63.01%	385
TOTAL		611

63% of the 611 responders answered that they do not vaccinate their dogs with the Kennel cough vaccine against *Bordetella* (Table 12).

Approximately 37% responders indicate that they vaccinate their dogs with the Kennel cough vaccine (Chart 4).

7. Please give details as to why/why not.

The majority of the 455 respondents affirm this vaccination is not needed, or it is only necessary if their dogs are going into kennels or in contact with several dogs regularly (e.g. dog shows).

Owners with dogs that are kennelled regularly answered that they vaccinate their dogs with the Kennel Cough vaccine, as a requirement of boarding kennels and as a precaution.

Around 13% of the respondents believe the Kennel Cough vaccine is not very effective, because just cover a minimal number of strains, which do not convince the respondents that the vaccination is in fact need it.

8. Do you have the same strategy for vaccinating any non-Rottweiler dogs you own?

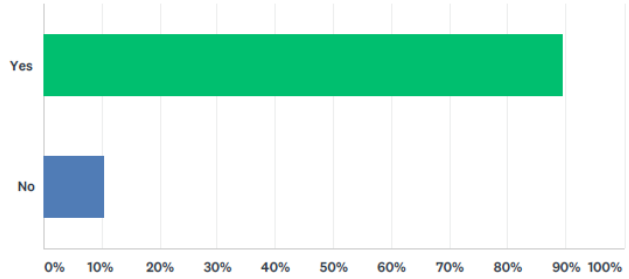

Chart 5. Percentage of answers regarding the strategy for vaccination non-Rottweiler dogs owned by the respondents.

Table 13. Relative frequencies of answers to the question eight.

ANSWER CHOICES	RESPONSES	
Yes	89.58%	404
No	10.42%	47
TOTAL		451

89.6% of the 195 respondents affirm that they have the same strategy for vaccinating any non-Rottweiler dog they own (Chart 5, Table 13).

9. If no, please give details of the difference.

For the majority of the respondents, the question is not applicable as they do not own other dogs.

In the considered answers are identified several reasons that describe non-vaccination, such as only vaccinate due to dog shows, the owners no longer vaccinate any dogs they own, non-Rottweiler dog has a compromised immune system, and the non-Rottweiler dog is not insured.

10. Do you follow the vaccination schedule recommended by your vet?

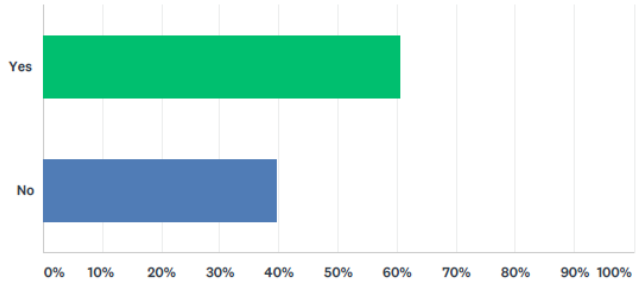

Chart 6. Percentage of answers regarding the vaccination schedule recommended by the vet.

Table 14. Relative frequencies of answers to the question ten.

ANSWER CHOICES	RESPONSES	
Yes	60.46%	370
No	39.54%	242
TOTAL		612

Approximately 60% of the respondents affirm that they follow the vaccination schedule recommended by their Vet, against almost 40% that affirm not follow the schedule recommended by the Vet (Chart 6, Table 14).

11. If no, please comment on why not and give details of any other schedule you follow:

The majority of the respondents consider that dogs are over vaccinated if they would follow a vaccination schedule. Thus, most of the respondents affirm to follow a first course vaccination to puppies, but not the recommended boosters.

Around 14% of the 219 answers mention titre testing instead of following a restricted vaccination schedule.

Homeopathic nosodes are also mentioned as an alternative to vaccination.

12. How often do you re-vaccinate after the initial puppy vaccination course?

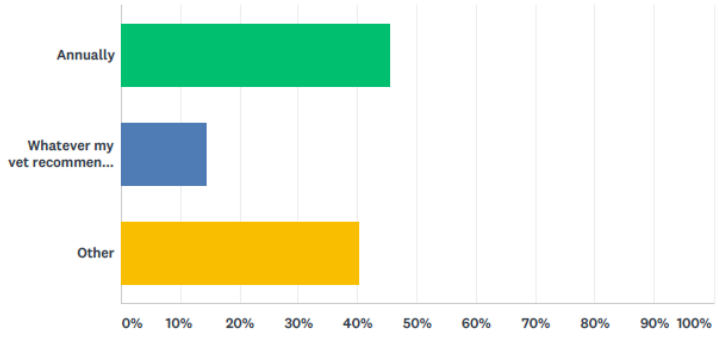

Chart 7. Percentage of answers regarding the re-vaccination after the initial vaccination course.

Table 15. Relative frequencies of answers to the question twelve.

ANSWER CHOICES	RESPONSES	
Annually	45.50%	278
Whatever my vet recommends me to do	14.40%	88
Other	40.10%	245
TOTAL		611

Approximately 46% of the 611 respondents affirm that they re-vaccinate annually after the initial vaccination, and about 14% follow the Vet recommendations (Chart 7, Table 15).

Around 40% of the respondents identify their situation as other.

13. If you have answered 'other', please comment (e.g. no boosters, every 3 years):

The main tendency of answer is no boosters after the puppy initial course, including some answers that mention the inclusion of the first-year booster, and no more vaccination after that.

In 12% of the 250 answers is possible to identify titre testing habits or intention in have immunity levels checked before deciding for vaccination.

Isolate answers (not a tendency of answer) mention no vaccination at all; cases of vaccination just when is required by kennels policy; the use of homeopathic products, or to follow advices given by holistic "veterinarians".

Five (5) responses were excluded due to mention that the answer was the same replied on other questions (report produced using aggregated data per question).

14. **Have you ever had blood samples taken before or instead of vaccinations to determine if your dog/s have an adequate level of immunity?**

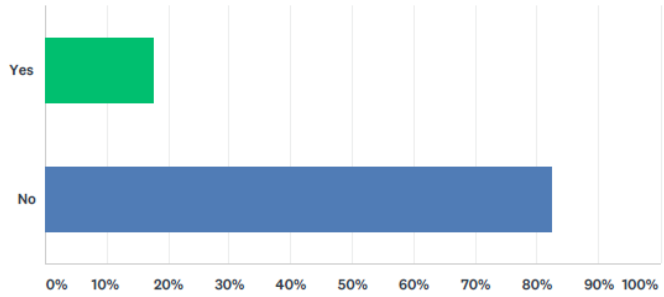

Chart 8. Percentage of answers regarding the determination of level of immunity before vaccination.

Table 16. Relative frequencies of answers to the question fourteen.

ANSWER CHOICES	RESPONSES	
Yes	17.53%	108
No	82.47%	508
TOTAL		616

Approximately 83% of the 616 respondents did never have blood samples taken before or instead of vaccinations to determine if their dog/s have an adequate level of immunity (Chart 8, Table 16).

15. **Has your veterinarian ever recommended having blood samples taken before vaccination to determine if your dog/s have an adequate level of immunity?**

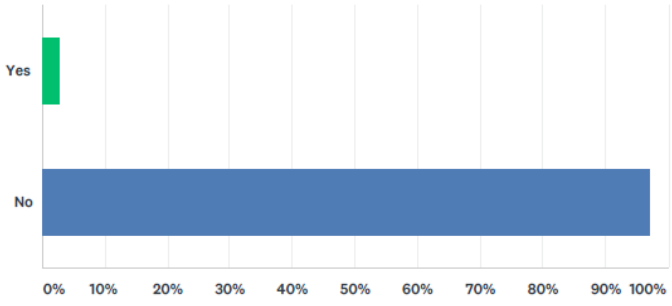

Chart 9. Percentage of answers regarding the recommendation for determine the level of immunity before vaccination.

Table 17. Relative frequencies of answers to the question fifteen.

ANSWER CHOICES	RESPONSES	
Yes	2.79%	17
No	97.21%	593
TOTAL		610

97% of the 610 respondents affirm that their veterinarian never had recommended having blood samples taken before vaccination to determine if their dog/s have an adequate level of immunity (Chart 9, Table 17).

16. **Do you use any non-veterinary based products/natural remedies to aid with preventing any of the diseases usually protected with vaccinations?**

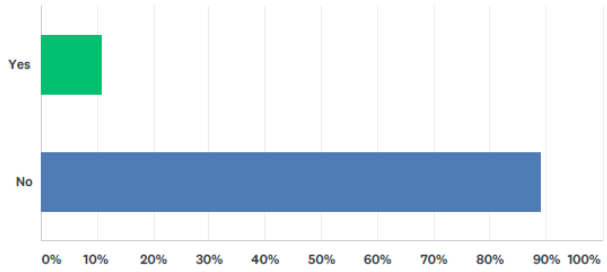

Chart 10. Percentage of answers regarding the use of non-veterinary based products/natural remedies.

Table 18. Relative frequencies of answers to the question sixteen.

ANSWER CHOICES	RESPONSES	
Yes	10.78%	66
No	89.22%	546
TOTAL		612

Approximately 89% of the 612 respondents affirm that do not use any non-veterinary based products/natural remedies to aid with preventing any diseases usually protected with vaccinations, against 11% that affirm to use non-veterinary products (Chart 10, Table 18).

17. If yes, please provide further details of what these are and why these are used?

The majority of the 64 answers mention the use of homeopathic nosodes, although is also possible identify the use of supplementation like as pro-biotics, Diatomaceous Earth, Turmeric, Ainsworth, Hilton supplement, Benelyn, Golden Paste, Omnious and MSM Riaflex.

Natural supplements given for miscellaneous conditions include coconut oil, flaxseed and pumpkin seeds, seaweed, garlic, soya bean, banana extract and honey.

18. Have you ever felt your dog has been unwell after their vaccinations?

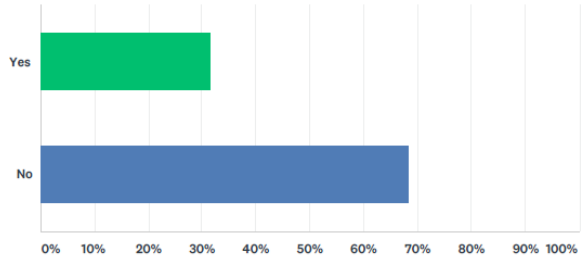

Chart 11. Percentage of answers regarding the malaise of dogs after vaccination.

Table 19. Relative frequencies of answers to the question eighteen.

ANSWER CHOICES	RESPONSES	
Yes	31.60%	195
No	68.40%	422
TOTAL		617

Around 68% of the 617 respondents had never felt their dog has been unwell after their vaccinations, against approximately 32% affirm that had happen (Chart 11, Table 19).

19. If yes, please give details of the clinical signs exhibited, when they started and for how long the signs persisted?

Considering the 197 answers, the most frequently clinical sign is lethargy. Other frequent clinical signs include anorexia, vomits, diarrhoea, and oedema at the vaccine inoculation site. Mainly, the described signs persisted for 1 to 2 days.

Secondary consequences (presumably related with vaccination) and adverse reactions include immune-mediated meningitis, paralysis, immune-mediated arthritis, collapse followed by recover, colitis, skin hotspots, ulcerated skin lesions, scratching, and interdigital cysts.

20. If you are a breeder do you recommend vaccinating any puppies that you sell on as pets?

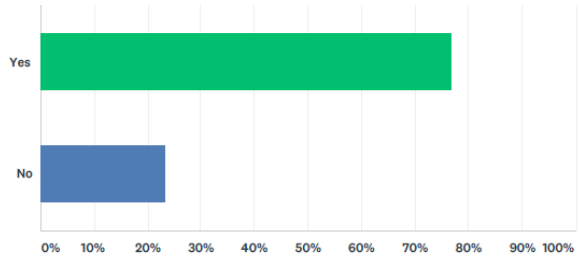

Chart 12. Percentage of answers regarding the recommendation of vaccinate sold puppies.

Table 20. Relative frequencies of answers to the question twenty.

ANSWER CHOICES	RESPONSES	
Yes	76.68%	148
No	23.32%	45
TOTAL		193

Around 77% of the 193 respondents, as breeders, say that they recommend vaccinate puppies they sell as pets. Even so, 23% do not recommend vaccinate the puppies they sell (Chart 12, Table 20).

21. If no, please give further details as to why and what you recommend instead:

Around 33% of the breeders recommend the first vaccination course (puppy vaccination) only.

Additionally, approximately 27% of the answers recommend titre testing instead of booster vaccination.

24% of the respondents says that they leave to the owners' decision, or advise them to do their own search for information about vaccination, sometimes presenting their personal point of view.

10% of the respondents describe worries about compatibility of different vaccine brands used by different vets.

6% of the respondents recommend the use of homeopathic nosodes, or do not vaccinate.

Twenty-five (25) of the 74 answers were excluded due to not applicable information (N/A) (non-breeders).

22. If you bought your Rottweiler from a breeder, did they provide any advice about vaccination?

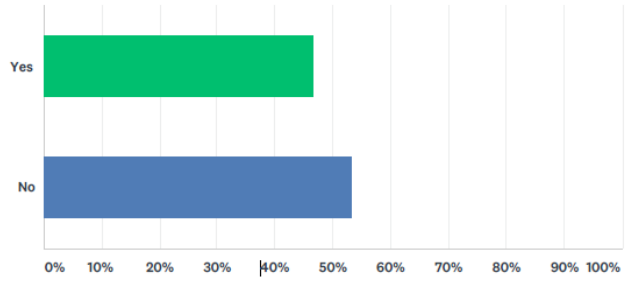

Chart 13. Percentage of answers regarding the provision of advises about vaccination.

Table 21. Relative frequencies of answers to the question twenty-two.

ANSWER CHOICES	RESPONSES	
Yes	46.72%	214
No	53.28%	244
TOTAL		458

53% of 458 respondents affirm that they did not receive any advice about vaccination from the breeder, against 47% that affirm received advices from the breeder about vaccination (Chart 13, Table 21).

23. If yes, please give further details.

39% of the respondents affirm that the breeders only provide information about the vaccination scheme on course and/or when was due the next vaccine.

Approximately 25% of the owners mention that it was recommended the initial vaccination course only.

Around 13% of the owners were advised to have yearly boosters done.

Additionally, around 12% of the owners have received advices to discuss with the vet the vaccination theme, or to follow the vet recommendations on the matter.

Approximately 6% of the owners affirm that they have received recommendations for titre testing.

Less than 5% of the answers include advices to use homeopathic products, or no vaccinate their dogs, or do not vaccinate against Leptospirosis/do not use Lepto4.

Thirty-seven (37) of the 202 answers were excluded due to no present any applicable information (N/A), or declare that do not have received any advice, or the dog does not have come from a breeder (i.e. shelters, rescues).