



UNIVERSIDADE TÉCNICA DE LISBOA
Faculdade de Medicina Veterinária

RASTREIO VIROLÓGICO DE CARNÍVOROS ERRANTES E CARACTERIZAÇÃO GENÉTICA VIRAL

RICARDO CONSTANTE ROSADO

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I realize upon writing this, the first and last page of my dissertation, that I'm looking back at six years of my life at the Faculty of Veterinary Medicine in Lisbon. The first thing that comes to my mind is that I should have saved more time to write these acknowledgements, and yet the simple truth is that no matter how much time I could have saved for this page, the people to whom I am thankful would always be innumerable.

I won't dedicate this dissertation to anyone, but I would like to acknowledge that I wouldn't be here if it weren't for my dog, Juca, who passed away during the final month of my curricular training. He was the first to teach me the joys of early rising, lazy Sundays, running on the beach, and unconditional love. He chose this profession for me, always tried to show me how to communicate with animals, mostly in vain, and taught me that family doesn't have only two legs. I wouldn't dedicate this dissertation to him because he'd probably just eat it. I would like to thank the rest of my two-legged family, my parents, my brother and sister and our recent "acquisitions" for their support and patience. I am in your debt.

None of this would have been possible if Professor Luís Tavares hadn't given me the opportunity to "help out" after classes at the Virology Laboratory three years ago, much to the dismay of my colleagues in the laboratory and especially my current supervisor, Professor Ana Duarte, who has had to put up with me for the better part of those three years, and has taught me so much more than I could have hoped. Together they have shown me that there is a world within these walls, just as exciting and entertaining as working in the jungle, be it the concrete or the real one. The experience has been life-changing and they are now a part of me.

Throughout these past six years I've built relationships which are stronger than I could have dreamed and they have helped shape who I am today. I am proud to have been a part of the class of 2003-2008 and proud to be a member of the VETuna. I will always cherish the lessons I learned as part of the Students Association and the Pedagogical Council, hard as they may have been, and I would never have been able to accomplish as much as I did without the help of so many friends, teachers and colleagues. I'm especially thankful for the support of my closest friends, with whom I've traveled across oceans and shared both joys and sorrows. I will always carry you with me and be within your reach, wherever I go. Special thanks, to four dogs, Pilhas, Balú, Joca and Lady, and two cats, Bagerah and Pekenita. Together with their "person" they brought magic into my life and taught me lessons I will never forget.

Lastly, we are grateful to our colleagues and all the employees of the Lisbon Municipal Kennel for their collaboration and assistance. This work was sponsored by CIISA-FMV as part of the Integrated Masters in Veterinary Medicine.

ABSTRACT

Virological survey in stray carnivores and viral genetic characterisation

Free-roaming stray or feral dogs and cats living in urban areas can be responsible for the spread and maintenance of several infectious diseases. To investigate the presence of viral agents and the genomic diversity of canine and feline coronavirus (CCoV, FCoV) in Lisbon's Municipal kennel, a virological survey was conducted which included canine distemper virus (CDV), canine and feline parvovirus (CPV,FPV), CCoV and FCoV, feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV).

Blood samples and faecal swabs were collected from 50 dogs and 50 cats at the time of euthanasia, and 24 environmental swabs were collected at a later date. Samples were either tested using a commercial ELISA kit, or amplified by PCR and RT-PCR. All coronavirus positive samples were further characterized by RT-PCR to assess the presence of different FCoV and CCoV genotypes. All PCR products were observed on 1,5%. agarose gel.

Antibodies against FIV were found in 18% of the samples, while FeLV antigen was found in 10%. Viral nucleic acid was detected in 8.2% samples for CDV, 32.7% for CCoV, 59.6% for FPV/CPV, and 70% for FCoV. Seven (43.8%) samples were positive for CCoV type I, 9 (56.2%) for CCoV type II, and as for FCoV, 9 (25,7%), 6 (17%) and 12 (34.3%) samples were positive for FCoV type I, type II and both types, respectively. No differentiation was possible in 8 (23%) of the FCoV samples. There were positive environmental samples for CDV (50%), FPV/CPV (62.5%) and FCoV (87.5%).

The results found on this study, particularly on parvovirus and FCoV brought to our attention the need for a continued and more precise evaluation of the health status of free-roaming stray or feral animals in the municipal kennel, to correctly evaluate their role as viral reservoirs within and without the kennel premises. The high prevalence of coronavirus infection found in both dogs and cats in the Lisbon Municipal Kennel allowed the viral genetic characterization, showing a high rate of co-infection with both genotypes of FCoV and absence of co-infected animals with CCoV I and II. However, further investigation is needed in order to maintain a molecular epidemiological surveillance and help identify further CoV strains, as well as understand the pathogenic potential of these viruses.

Keywords: virological survey, molecular epidemiology, small animal viruses, coronavirus, FCoV, CCoV

RESUMO

Rastreo virológico de carnívoros errantes e caracterização genética viral

Os animais errantes ou assilvestrados que habitam áreas urbanas podem ser responsáveis pela distribuição e manutenção de diversas doenças infecciosas. Com o objectivo de investigar a presença de agentes virais e avaliar a diversidade genética de coronavírus canino e felino (CCoV, FCoV) no Canil Municipal de Lisboa, realizámos um rastreo virológico em que foram avaliados o vírus da esgana canina (CDV), parvovirus canino e felino (CPV, FPV), CCoV e FCoV, o vírus da imunodeficiência felina (FIV) e o vírus da leucemia felina (FeLV).

Foram colhidas amostras de sangue e zaragatoas rectais de 50 cães e 50 gatos na altura da eutanásia, e 24 zaragatoas ambientais numa data posterior. Avaliámos as amostras utilizando um teste comercial de ELISA ou amplificando as amostras por PCR e RT-PCR. Todas as amostras positivas para coronavírus foram caracterizadas por RT-PCR para avaliar a presença de genótipos diferentes de FCoV e CCoV. Todos os produtos de PCR foram visualizados num gel de agarose a 1,5%.

Foram encontrados anticorpos contra FIV em 18% das amostras e antigénio de FeLV em 10%. Detectámos ácido nucléico viral em 8.2% das amostras para CDV, 32.7% para CCoV, 59.6% para FPV/CPV a 70% para FCoV. Sete (43.8%) amostras foram positivas para CCoV tipo I, 9 (56.2%) para CCoV tipo II, e em relação ao FCoV, 9 (25,7%), 6 (17%) e 12 (34.3%) amostras foram positivas para FCoV tipo I, tipo II, e para ambos os tipos, respectivamente. Não foi possível obter diferenciação em 8 (23%) das amostras para FCoV. Houve amostras ambientais positivas a CDV (50%), FPV/CPV (62.5%) e FCoV (87.5%).

Os resultados deste estudo demonstraram a importância de uma avaliação contínua e mais precisa do estatuto sanitário dos animais errantes ou assilvestrados no canil municipal, principalmente em relação ao parvovirus e ao FCoV, de modo a avaliar correctamente o seu papel de reservatórios de doenças, tanto dentro como fora do canil. A alta prevalência de infecções por coronavírus em cães e gatos no Canil Municipal de Lisboa permitiu a caracterização genética viral, demonstrando uma percentagem elevada de co-infecções com os dois genótipos de FCoV, mas uma ausência de animais co-infectados com CCoV I e II. No entanto, é necessário mais investigação para manter uma vigilância epidemiológica ao nível molecular, de forma a ajudar na identificação de possíveis novas estirpes de coronavírus, assim como compreender o potencial patogénico destes vírus.

Palavras-chave: rastreo virológico, epidemiologia molecular, vírus de animais de companhia, coronavírus, FCoV, CCoV

LIST OF SCIENTIFIC COMMUNICATIONS

The work related to this dissertation resulted in the presentation of three scientific posters at two different international meetings:

- Fernandes, T., Bernardino, R., Duarte, M., Fevereiro, M., Correia, J., Duarte, A., Rosado, R., Tavares, L.. “Fatal Panleukopenia virus infection in a *Linx linx*”. International Conference on Diseases of Zoo and Wild Animals 2009, 20th to the 24th of May. Hilvarenbeek, The Netherlands.
- Ricardo C. Rosado, Ana Duarte, Augusto Baptista, Filomena Oliveira, Ana Machado, Leonel Fernandes, Luís Tavares. “Virological survey in shelter dogs and cats in Lisbon”. 8th International Congress of Veterinary Virology, 23rd to the 26th of August 2009, Budapest, Hungary.
- Ricardo C. Rosado, Ana Duarte, Augusto Baptista, Filomena Oliveira, Ana Machado, Leonel Fernandes, Luís Tavares. “Genetic characterization of coronaviruses in shelter dogs and cats in Lisbon”. 8th International Congress of Veterinary Virology, 23rd to the 26th of August 2009, Budapest, Hungary.

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1. Introduction

The present study was part of the curricular training for the Integrated Masters in Veterinary Medicine from the Faculty of Veterinary Medicine (FMV), Universidade Técnica de Lisboa. It consisted of a period of six months between 15th October 2008 and 15th April 2009 in the Virology and Molecular Biology Laboratory of the FMV under the supervision of Prof. Ana Isabel Pereira Duarte. During this time we conducted a virological survey of free-roaming carnivores at the municipal kennel in Lisbon, while participating in the diagnostic procedures at the virology laboratory.

1.1 Laboratory work

The Virology and Molecular Biology Laboratory provides diagnostic services for several clinics, including the Teaching Hospital of the FMV and the Pathology Laboratory. Samples submitted by veterinary clinicians are tested for different viral diseases using molecular biology or serological techniques. Among the different agents diagnosed in the laboratory, the most common are feline coronavirus (FCoV), feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), feline/canine parvovirus (FPV/CPV) and canine distemper virus (CDV). Other agents are tested less frequently, like feline herpesvirus (FHV), and canine coronavirus (CCoV).

Sample materials received by the laboratory include whole blood (plasma/serum), stool, intraperitoneal/intratoracic fluid and organ samples. Different materials are required and are processed accordingly to the agent to test for and the technique used (Table 1). FeLV and FIV are tested using serological techniques, namely an enzyme-linked immunosorbent assay (ELISA) for detection of Ag and Ab, respectively. Blood samples are previously centrifuged because the assay has better results with plasma/serum than whole blood.

Table 1 - Summary of agents, materials and test types performed at the Virology and Molecular Biology Laboratory at the FMV

Agents	Materials	Test type
FeLV	blood/plasma/serum	Serological (ELISA)
FIV	blood/plasma/serum	Serological (ELISA)
FCoV	blood/faeces/intraperitoneal or intratoracic fluid	RT-PCR
CPV/FPV	Blood/stool	PCR
CDV	blood/stool	RT-PCR

Samples tested for other viruses use molecular biology techniques to assess the presence of viral nucleic acid, by polymerase chain reaction (PCR) for DNA viruses and reverse transcriptase-PCR for RNA viruses. Liquid samples (blood, body fluids) are centrifuged and

the supernatant is processed for nucleic acid extraction using an appropriate commercial kit. Solid samples (stool, organs) are homogenized with Phosphate buffered Saline (PBS), centrifuged and processed as referred for liquid samples. A summary of the samples analysed at the laboratory during the course of this study is presented in tables 2 and 3.

Table 2 - Number of samples analysed at the Virology and Molecular Biology Laboratory during the period of 15th October 2008 and 15th April 2009

Agents	Samples	Positives
FeLV	14	7
FIV	10	1
FCoV	31	18
CCoV	5	2
CPV/FPV	4	2
CDV	5	0
Chlamydia	3	0
Herpesvirus	4	1
Total	76	

Table 3 - Number of samples of different biological materials received at the Virology and Molecular Biology Laboratory during the period of 15th October 2008 and 15th April 2009

Materials	Samples
blood	35
stool	30
ocular swab	3
oral swab	4
intraperitoneal/ intratoracic fluid	5

1.2 Research project

Free-roaming stray or feral dogs and cats living in urban areas can be responsible for the spread and maintenance of several infectious diseases. These animals aren't confined or owned by anyone and both their number as well as their health status is mostly unknown to veterinarians and public health services.

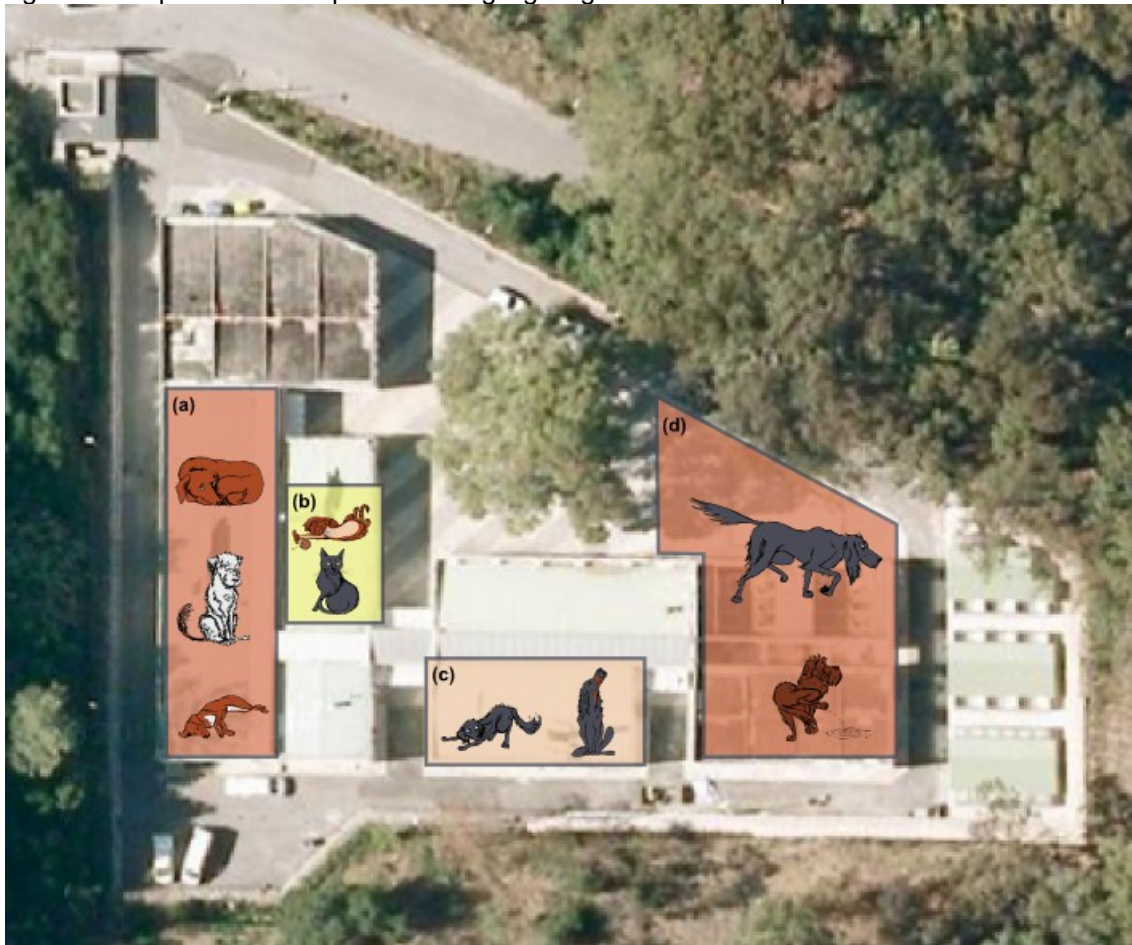
There is a lot of information throughout the scientific literature about virological surveys. These surveys can vary greatly in terms of methodology and procedures, can be very specific or very general, have different sample sizes, focus on several or only one aetiological agent and some are conducted in collaboration with TNR programs (trap, neuter, release), usually oriented to control cat population. All of them help us gain greater insight into an existing disease or its agent and the way it interacts with its host. These surveys help us paint the picture of prevalence of disease in a population, but it is a picture which is never finished, because for every question we answer, new questions arise..

In this particular case, the original idea of a survey which would help us characterise the presence of specific agents in the environment and the risk to both wild and domestic animals, stumbled upon the difficult scenery of municipal kennels. Regulations conflict with compassion and the darkest of human nature as we try to understand the reasons why owners and citizens allow animals who share the same environment as them be relinquished to a kennel and ultimately euthanized after all other options are spent. In the process, we came across problems with which our colleagues at the municipal kennel have had to deal for a long time.

For the purpose of this study, “free-roaming” describes homeless animals, including socialized strays and unsocialized feral. In the case of the municipal kennel, most of the dogs were socialized, while practically all of the cats caught by the kennel employees were feral or barely socialized.

The kennel is placed in the middle of the Monsanto Natural Park, yet the facilities soon proved to be too small to contain the growing pet overpopulation in the Lisbon urban area, in spite of the abundant space surrounding it. Dogs are housed in three kennels: the main one (Figure 1-a) where most of the dogs are kept in open cells, alone, but with some contact with each others; another smaller room where aggressive animals are kept in containment cages (Figure 1-c); and an exterior kennel where more social dogs are housed in pairs, or in small groups (Figure 1-d). All cats are housed together with other animals captured in the same day, in a single room with a number of different cages (Figure 1-b). The same room also has cages for smaller dogs and young puppies. Cats considered fit for adoption, are also housed in groups in an exterior kennel.

Figure 1 - Map of the municipal kennel highlighting the rooms sampled.



Main dog kennel (a); cat kennel (b); aggressive dogs kennel (c) and exterior kennel (d).

Animals are delivered at any time of the day or night (when delivered by police forces), or captured by kennel employees and taken directly to one of the kennels after the entry record has been made. Owners who relinquish their animals sign a statement and provide identification. Veterinarians fill in details of the clinical examination and tend to any animals which require medical treatments. Although surgeries are performed, most of the treatments are supportive. When possible, animals are photographed to be advertised on the kennel website for adoption. Euthanasia is usually on a weekly basis for the simple reason of keeping the kennel population at an acceptable number. During this study an average of 26 animals per week were euthanized.

Among the problems identified at the beginning of the study by the veterinarians and employees of the kennel were the lack of proper ventilation in the interior kennels, the high number of animals present at all times, which prevented any sanitary break from taking place and the difficulty in providing better health care to the animals who needed it, due to lack of financing support.

Both veterinarians and employees mentioned the presence of infectious diseases among the kennel population, specially canine distemper and parvovirus among the dogs and panleukopenia among the cats.

1.3 Objectives

In order to conduct a virological survey of both dogs and cats 100 samples were collected from animals euthanized at the Lisbon municipal kennel. For the purpose of this study, canine distemper virus (CDV), canine parvovirus (CPV), canine coronavirus (CCoV), feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV), feline coronavirus (FCoV), and feline parvovirus (FPV) were selected. All coronavirus positive samples were further characterized to assess the presence of different FCoV and CCoV genotypes within the animal population.

Most of these animals were captured in urban areas in and around Lisbon and a few were delivered by their owners to be euthanized for health or other issues. Since background information on the health status of these animals was lacking, antibody detection was only performed for FIV, because of its specific pathogenesis which produces persistent infection, but low level virus replication, and the fact that it has no commercial vaccine in Portugal. All other viruses were tested for the presence of antigen by enzyme-linked immunosorbent assay (ELISA) or nucleic acid by polymerase chain reaction (PCR).

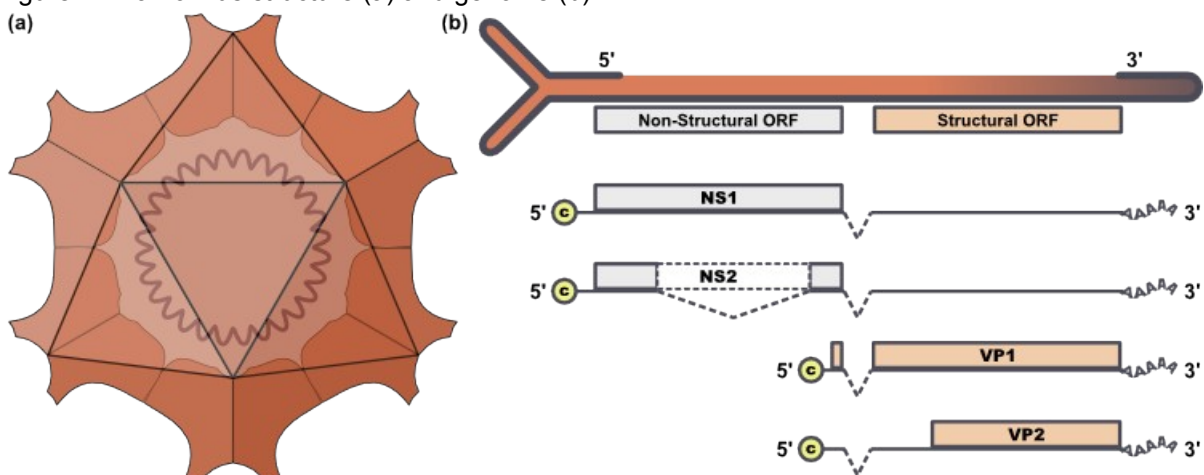
This information was used to estimate the prevalence of these diseases in dogs and cats at the Lisbon Municipal Kennel and evaluate the sanitary conditions in the kennel. As a secondary objective, we tried to estimate the role of stray and feral animals in the spread and maintenance of infectious disease in both domestic populations and wild animals.

1.4 DNA Virus

1.4.1 Parvovirus

Viruses from the family *Parvoviridae* infect a wide variety of hosts, ranging from insects to primates (Hueffer and Parrish, 2003). The current classification of parvoviruses is based primarily on their host range and their dependence on help from other viruses for replication, traditionally separating them into autonomous viruses of vertebrates, helper-dependent viruses of vertebrates, and autonomous viruses of insects. Viruses from insects and other arthropods are included in the subfamily *Densovirinae*, while viruses from vertebrates are contained in the subfamily *Parvovirinae* (Lukashov & Goudsmit, 2001). Among these, members of the genus *Parvovirus* are among the smallest DNA virus to include important pathogens of both dogs and cats (Patel & Heldens., 2009). The viruses are all classified as members of the feline parvovirus subgroup of the family *Parvoviridae* and are named for the host from which they are isolated—hence CPV, FPV, raccoon parvovirus, mink enteritis virus (MEV), as well as bluefox parvovirus (BFPV) from Arctic foxes (Parrish, 1995). Feline panleukopenia virus (FPV) is considered the prototype parvovirus of carnivores (Truyen et al., 2009) and Canine parvovirus (CPV) is a host range variant which acquired the ability to infect dogs. While FPV infects all felids, as well as raccoons, minks and foxes, CPV infects members of the *Canidae* family and recently regained the ability to infect cats (Truyen, Evermann, Vieler & Parrish, 1996). Current taxonomy defines canine parvovirus and feline panleukopenia virus as one single taxonomic entity (Tattersall, 2006 cited by Truyen et al., 2009), but in the present study we refer to CPV as parvovirus in dogs and FPV parvovirus in cats.

Figure 2 - Parvovirus structure (a) and genome (b).



Adapted from <http://www.expasy.ch/viralzone/>.

Parvoviruses are small and spherical, roughly 25 nm in diameter, without an envelope (Parrish, 1995) (Figure 2-a). FPV and CPV isolates may differ by as little as 0.5% in their DNA sequence (Hueffer et al., 2003). Their genome is a single-stranded DNA, roughly 5 kb long with hairpin structures at both ends and encodes two genes (Figure 2-b), which result in the expression of two non-structural proteins, NS1 and NS2, coded by the 3' end open reading frame. The capsid proteins are coded by an open reading frame towards the 5' end of the (negative sense) genomic DNA. The structural proteins VP1 and VP2 are formed by alternate splicing of the messenger RNA from the viral DNA, so that the complete sequence of VP2 is present in VP1 (Agbandje, Parrish and Rossman, 1995; Parrish, 1999). The number of capsid protein species per virion varies among parvoviruses. There are three types of polypeptides in CPV, VP1, VP2 and VP3. VP3 is formed by cleavage of 15 to 20 amino acids from the amino terminus of VP2 after virion assembly, and is not present in empty particles (Agbandje et al., 1995). The full capsid contains 60 copies of a combination of VP1, VP2 and VP3, with VP2 being the dominant protein (54-55 copies) (Parrish, 1999). The structure of the capsid defines three separate regions surrounding the threefold axes of symmetry, termed threefold spike, which are determinant in the host range differences between CPV and FPV by allowing it to bind to different transferrin receptors in the host cell (Hueffer and Parrish, 2003). The carnivore group of parvoviruses shows high conservation of DNA sequences, with less than 1.3% sequence variation between diverse isolates collected 30 years apart (Truyen et al., 1995). However, several sites in VP2 of CPV-2 display a nucleotide substitution rate similar to that of RNA viruses, which caused them to undergo a complex series of host range changes in the past three decades, and determined the appearance of diverse antigenic epitopes in naturally occurring variants of the virus (CPV-2a, 2b, 2c), (Truyen et al., 1995; Truyen et al., 1996; Shackelton, Parrish, Truyen & Holmes 2005).

All parvoviruses are extremely stable and resistant to adverse environmental influences, being able to persist on inanimate objects for 5 months or longer (McCaw & Hoskins, 2006). FPV resists heating at 56° C for 30 minutes and most common detergents and disinfectants, such as 70% alcohol, organic iodines, phenolics and quaternary ammonium compounds. One noteworthy exception is inactivation of both CPV and FPV by 6% sodium hypochlorite for 10 minutes at room temperature, which still has to be thorough, (Greene & Addie, 2006). Feline panleukopenia virus (FPV) has been known to cause disease in cats, raccoons and some related carnivores for more than a hundred years (Lamm & Rezabek, 2008), while canine parvovirus (CPV) was first isolated in 1978 (Carmichael & Binn, 1981¹). The new virus spread globally in a pandemic of disease during that same year and has since remained endemic in dogs throughout the world. There is no serological or other evidence for infection

1 cited by Reed, Jones & Miller, 1988

of dogs by a related virus prior to the mid-1970s (Parrish, 1990²) and its exact evolution and origin remains elusive to date. One of the hypothesis about its sudden emergence suggested that CPV arose as a host range mutant directly from FPV in the dog or cat populations, similar to the scenario proposed for the emergence of MEV in the 1940s. Another hypothesis was that CPV emerged from a FPV vaccine virus after propagation in tissue culture and was initially spread in vaccine, which would explain the almost simultaneous appearance of CPV in the dog populations worldwide. Various isolates from wild carnivores support the hypothesis that CPV arose in a different host from the cat or dog, and that another carnivore may have harboured its immediate ancestor. DNA and amino acid analysis from one Arctic fox from Finland and red foxes from Germany revealed intermediate sequences between the FPV and CPV viruses, providing evidence of interspecies transmission between domestic and wild carnivores for the first time (Truyen, Müller, Heidrich, Tackmann & Carmichael, 1998; Truyen 1999).

Since the emergence of CPV it has continued to grow at an exponential rate, contrary to FPV, which maintains a population growth rate compatible with its endemic nature. CPV growth is characteristic of an epidemic, as it adapted to its canine host in a matter of years, spread across the world in a couple of months and subsequently generated a number of antigenic and host-range variants (Shackelton et al., 2005). CPV-2 was replaced by CPV-2a in 1981 and again by CPV-2b between 1984 and 1990 (Patel & Heldens, 2009). Interestingly, these variants show only a few amino acid substitutions in its genomic sequence. According to Truyen (2006) the adaptation to a new host was most likely due to the virus gaining the ability to bind the canine transferrin receptor, which is used to enter and infect canine cells. A new strain, emerged in the end of the 1990s, was characterised by a mutation in a major antigenic site of the viral capsid (position 426 Asp to Glu) and was named CPV-2c (Buonavoglia et al., 2001; Decaro et al., 2007a). This strain has already spread worldwide, yet it is still more prevalent in Europe (Nakamura et al., 2004; Decaro et al., 2007a; Hong et al., 2007; Kapil et al., 2007; Pérez et al., 2007). While the virulence and pathogenicity of CPV-2c were first deemed to be low, more recent reports are conflicting (Decaro et al., 2005; Decaro et al., 2008), just like reports on the efficacy of current vaccines (Decaro et al., 2008; Spibey, Greenwood, Sutton, Chalmers & Tarpey, 2008; Calderon et al., 2009).

Both CPV and FPV are highly contagious and most infections are a result of contact with contaminated faeces, but also fomites, insects and rodents. Animals may carry the virus in their hair coat for extended periods (McCaw & Hoskins, 2006). Infection in domestic dogs doesn't always result in apparent disease, as many dogs which are naturally infected never develop overt clinical signs (McCaw & Hoskins, 2006). Clinical illness is usually most severe in young puppies, especially when they harbour other pathogens such as parasites or enteric

² cited by Hueffer et al. 2003

bacteria. Incubation periods can vary between 7-14 days for CPV-2 and as brief as 4-6 days for newer variants (McCaw & Hoskins, 2006) and infected carnivores shed virus at high titres in their faeces (Truyen et al., 2009).

The pathogenesis of parvovirus infections is influenced primarily by the requirement of DNA replication for mitotic cells. Following oronasal exposure, the virus first replicates in the mucosa and lymphoid tissue of the buccal cavity and spreads next via cell-free viraemia to other internal organs. In vivo tissue tropism of FPV and CPV varies, but after progeny virus spread it can be found in virtually all organ tissues in both dogs and cats (Decaro et al., 2007c; Truyen et al., 2009). In puppies secondary sites of infection are typically the liver and heart, and animals often succumb to heart failure associated with pulmonary oedema, hepatomegaly and ascites. Older dogs commonly present with vomiting, diarrhoea and leucopenia with a high morbidity, but a much lower mortality than in puppies. The disease induced by the Glu-426 mutant CPV-2c has been described as mild in pups, with mucoid diarrhoea and relative leucopenia and lymphopenia (Decaro et al., 2005), but a more severe clinical course with higher mortality rates was also described in a breeding kennel in Italy (Decaro et al., 2008).

Pathogenesis of FPV infection in cats is similar and the infection is systemic. Replication causes shortening of the intestinal villi due to a sometimes complete loss of the rapidly dividing epithelial cells in the gut. Thus, the most common clinical sign is diarrhoea followed by leucopenia and anaemia caused by replication of the virus in early progenitor cells of the bone marrow. Kittens infected during pregnancy may present cerebellar ataxia and intention tremor due to impaired development of the cerebellum caused by lytic virus replication in the Purkinje cells (Truyen et al., 2009).

The clinical diagnosis of CPV-2 infection is indecisive, since several other pathogens may cause diarrhoea in dogs. Several methods are available for the laboratory diagnosis of CPV infections. Virus isolation from blood or faeces in cell cultures and the demonstration of haemagglutination of porcine erythrocytes have been extensively used for diagnosis, but these methods are too labour-intensive and time-consuming for routine diagnostic testing (Desario et al., 2005). In practice, antigen detection in faeces is usually carried out using commercially available latex agglutination or immunochromatographic tests (Truyen et al., 2009), and recent advances in molecular technique have led to the development of highly sensitive and specific PCR and real-time PCR assays for the detection and quantisation of CPV-2 DNA in the faeces of diarrhoeic dogs (Desario et al., 2005). However, the period of faecal viral shedding is brief and virus is seldom detected by 10 to 12 days after natural infection. Furthermore, vaccine virus can yield false-positive results up to 12 days after vaccination (McCaw & Hoskins, 2006). On the other hand, the use of an antibody test such

as ELISA or indirect immunofluorescence is of limited value, because serological tests do not differentiate between infection and vaccination induced antibodies (Truyen et al., 2009).

Both CPV and FPV vaccines are considered core vaccines by the Vaccinations Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA). Modified live vaccines have been available for some time and are recommended because of the more rapid and effective immunity they produce in comparison to inactivated virus vaccines. These are only recommended in the case of pregnant queens and kittens younger than 4 weeks, because no danger exists of post-vaccinal virus spread or clinical illness as a result of reversion to virulence (Greene & Addie, 2006; Day, Horzinek & Schultz, 2007). The recent emergence of CPV variants which infect even animals which have been repeatedly vaccinated has raised concerns that the antigenic differences between the original type 2 and its variants may decrease the effectiveness of the CPV-2 based vaccines and it would be useful to prepare vaccines using the CPV variants circulating in the field (Decaro et al., 2008).

1.5 RNA Virus

1.5.1 Canine Distemper Virus

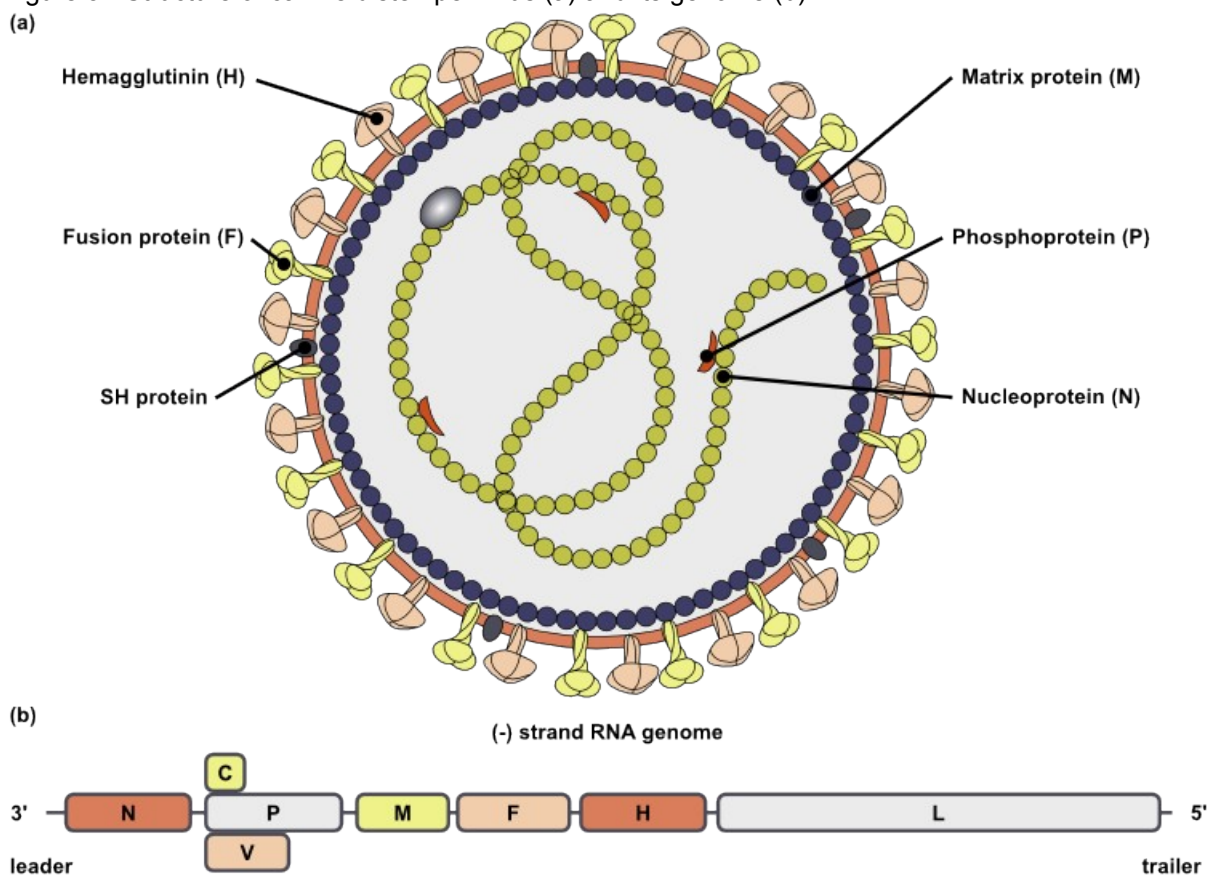
Canine distemper (CD) is considered by many the most important worldwide infectious disease of domestic dogs, and its fatality rate is second only to that of rabies. This morbillivirus infection of dogs and other carnivores, has been recognized for at least 250 years and the first report of CD was from South America by Ulloa in 1746. Heusinger was convinced that the disease was introduced in 1760 from Peru to Spain, from where it spread to other parts of Europe and Russia within a few years. The epidemic spread of CD through Europe started around the 1760s, but the disease may have occurred earlier and was possibly confused with rabies. In 1815 Jenner observed that CD among dogs is as contagious as smallpox, measles and scarlet fever among humans and Karle succeeded in experimentally transmitting CD in 1844, by brushing the lips of young dogs with discharge from diseased dogs. The aetiology of the disease remained controversial until 1905, when Henri Carré demonstrated it was caused by a filterable virus (Carré, 1905³; Blancou, 2004). Clinical distemper has been largely controlled by routine vaccination in domestic dogs since the 1950s, but evidence of vaccinal failure caused by the emergence of viral variants in the recent years has raised concerns and renewed investigation efforts. Vaccine-induced infections have occurred in a variety of species, as have large-scale epidemics in felids and CDV may have the most far reaching implications of any infectious agent for susceptible free-living and captive carnivores. The discoveries of related viruses, such as phocine and

³ cited by Rikula, 2008

delphine morbilliviruses, and CDV's similarity to the measles virus suggest viral mutability and a zoonotic potential for CDV (Deem, Spelman, Yates & Montali, 2000).

The genus morbillivirus within the family Paramyxoviridae includes important, highly contagious pathogens of animals and man. Until recently the genus comprised four distinct members: measles virus (MV) of man, canine distemper virus (CDV) of canines and their relatives, rinderpest virus (RPV) of cloven-hoofed animals, and peste-des-petits-ruminants virus (PPRV) mainly of small ruminants. All of these may cause severe disease in their respective host and MV is considered the prototype virus for the genus. Knowledge of the natural host range is a major factor in differentiating between these closely related morbillivirus species (Blixenkrone-Möller et al., 1992), and the natural host range for CDV has recently been proven to comprise all families of the order Carnivora (Deem et al., 2000).

Figure 3 - Structure of canine distemper virus (a) and its genome (b).



N – nucleocapsid protein; P – phosphoprotein; C, V – transcriptional units; M – matrix protein; F – fusion protein; H – hemagglutinin; L – large protein. Adapted from <http://www.expasy.ch/viralzone/>.

CDV has a variable diameter between 150-250 nm, with negative-sense, single-stranded RNA, containing six non-overlapping genes encoding six structural proteins and enclosed in a nucleocapsid of helical symmetry (Greene & Appel, 2006) (Figure 3). It is surrounded by a

lipoprotein envelope derived from the host cell membrane, where the virus incorporates the matrix protein (M) and two glycoprotein spikes, the hemagglutinin (H) and fusion protein (F). The nucleocapsid protein (N) encapsulates and protects the RNA and is associated with the phosphoprotein (P) and the large protein (L) which assists in transcription and replication (Blixenkron-Möller et al., 1992; Barrett, 1999; Martella, Elia & Buonavoglia, 2008). Two additional proteins, C and V, are found as transcriptional units within the P gene (Beineke, Puff, Seehusen & Baumgärtner, 2009). The viral protein M is responsible for connecting the nucleocapsid and the surface glycoproteins H and F during viral maturation (Beineke et al., 2009), while the H protein is responsible for attachment to cell receptors and the F protein promotes fusion of the cell membrane with the viral envelope, and also between host cells (Martella et al., 2008). While CDV is considered to have one antigenic type, CDV strains can be divided into distinct lineages, based on phylogenetic analysis of subgenomic F, P, and complete H gene sequences, which are mainly associated with the geographical area from which the strain is isolated (Lednicky et al., 2004, Lan et al., 2005, Martella et al., 2006).

CDV is extremely susceptible to heat and drying and is destroyed by temperatures greater than 50° C for 30 minutes. It may survive in excised tissues and secretions for up to 3 hours, but does not usually persist in kennels after infected dogs have been removed, especially in warm climates. The virus remains viable between pH 4.5 and 9.0 and is inactivated by ultraviolet light. As an enveloped virus, it is susceptible to ether and chloroform, dilute formalin solution, phenol and quaternary ammonium disinfectant. Routine disinfection procedures are usually effective in destroying the virus in kennel, clinic or hospital environments (Greene & Appel, 2006).

Transmission occurs mainly by direct animal-to-animal contact and the virus can be detected from most body tissues, including urine (Elia et al., 2006; Greene & Appel, 2006) However, it is most abundant in respiratory exudates and commonly spread by exposure to infectious aerosol or droplets (Green and Appel, 2006). Viral shedding occurs by 7 days postinfection and can last 60 to 90 days, although shorter periods are more typical. Transplacental infection can occur and the virus is maintained in a population by contact among infected animals and a constant supply of susceptible puppies for infection. Although immunity after exposure to the wild-type virus is prolonged or life-long, it is not as absolute after vaccination and there are estimates that 25-75% of susceptible animals become subclinically infected, but clear the virus. However, some dogs aren't able to clear the virus completely and may harbour it in their central nervous system. Spontaneous distemper is correlated in most cases with the loss of maternal antibodies, affecting dogs between 3 and 6 months the most, but disease can be severe and widespread, affecting all ages, in susceptible isolated populations and different strains may have different pathogenicity and clinical evolution (Green and Appel, 2006).

Great variations in duration, severity and clinical presentation of distemper have been found in experimentally infected dogs as well as in animals suffering from natural disease. The incubation period may vary from 1 to 4 weeks and depends on viral strain, age of the animal at the time of infection and immune status of the host. Disease manifestation ranges from virtually no clinical signs to severe disease with approximately 50% mortality. Tissue macrophages and monocytes located in or along the respiratory epithelium and tonsils represent the first cell type to pick up and propagate the virus after natural exposure by aerosol droplets. Following this local burst of virus replication the pathogen is then disseminated by lymphatics and blood to distant hematopoietic tissues during the first viraemic phase (Beineke et al., 2009). The virus multiplies from 4-6 days PI in the lymphoid follicles of the spleen, in the lamina propria of the stomach and small intestine, and in the Kupffer's cells in the liver, which is accompanied by an initial fever 3-6 days PI. Further spread of CDV to epithelial and central nervous system (CNS) tissues 8-9 days PI depends on the immune status of the dog, and most likely takes place both as a cell-associated and plasma-phase viraemia (Green and Appel, 2006). The clinical picture in all susceptible species manifests most frequently in respiratory, gastro-intestinal, integumentary, and CNS systemic signs, which may emerge several months later, and without any preceding systemic signs. Biphasic fever and general malaise are often associated with viraemia (Deem et al., 2000).

Practical diagnosis of canine distemper is primarily based on clinical suspicion, but there are a number of specific confirmatory laboratory tests available which detect either CDV or a specific immune response in samples from the affected animal. Virus isolation is still considered the gold standard, but it's not straight forward since it requires that virulent CDV is adapted before it grows in routinely-used epithelial or fibroblast cell lines. The best results are achieved by direct cultivation of buffy coat cells or other target tissues from the infected host together with mitogen-stimulated dog lymphocytes (Greene & Appel 2006). RT-PCR can demonstrate CDV from buffy coat cells of dogs with acute infection and from serum, whole blood, cerebrospinal fluid (CSF) or urine of dogs with systemic or neurological CD (Shin et al., 1995; Frisk, Konig, Moritz & Baumgartner, 1999, Saito et al., 2006). RT-PCR can be applied for the detection of CDV from smears of epithelial cells and from other tissue samples. A positive RT-PCR result is indicative of CD infection, whereas a negative one can result from various reasons. Other confirmatory test available for antemortem diagnostic are immunofluorescence assays, ELISA and immunohistochemistry performed on blood, CSF and tissue samples (Greene & Appel 2006).

The first vaccine against CD was made in 1923 by Puntoni from the formalin-inactivated brain tissue of a dog suffering from CD encephalitis (Appel, 1999⁴). The protection obtained

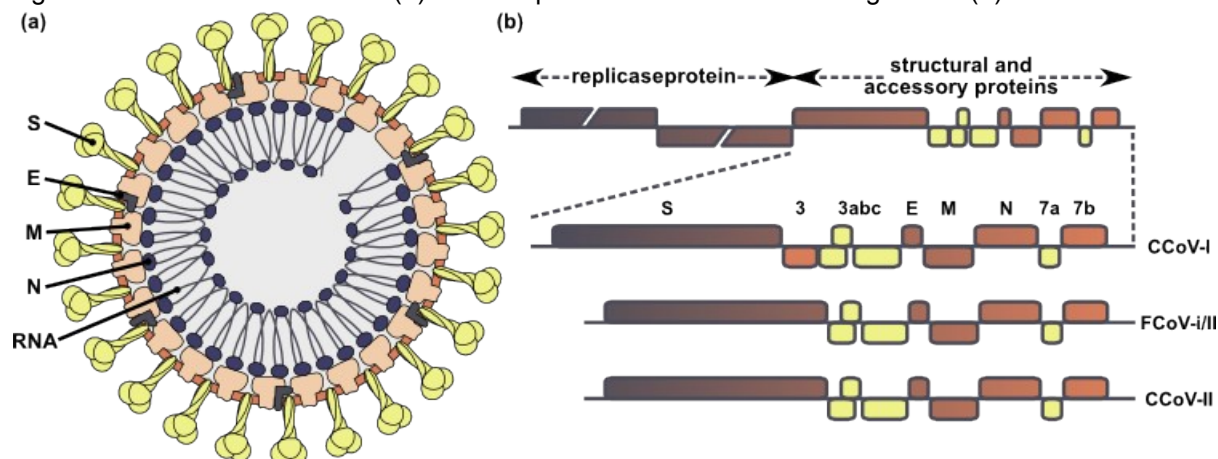
4 cited by Rikula, 2008

with inactivated vaccines was limited, and they are no longer used. However, active immunization was not successful before MLVs became available in the 1950s. All commercial CD vaccines available for dogs are multivalent vaccines and are considered core vaccines for both domestic dogs and animals in shelter environments (Day et al., 2007). Vaccinal virus is shed by animals for a little over a week after vaccination (Kim, Cho, Youn, Yoo & Han, 2001). Not all vaccine produce the same level of protection and increased potency of protection is often associated with induced illness, especially in wild or immunocompromised domestic carnivores (Greene & Appel 2006).

1.5.2 Coronavirus

This virus genus belongs to the family *Coronaviridae*, within the order *Nidovirales* and can be grouped into three clusters, as defined by their antigenic and genomic properties (Addie et al., 2009). Of interest to this study, feline coronavirus (FCoV) and canine coronavirus (CCoV), belong to subgroup 1a coronaviruses (Addie et al., 2009; Decaro and Buonavoglia, 2008; Pratelli et al., 2002; Lorusso et al., 2008), together with transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCoV) and porcine epidemic diarrhoea virus (PEDV). Group 2 is subdivided into groups 2a and 2b, depending whether they are bovine-like coronaviruses or severe acute respiratory syndrome (SARS)-like. The recent canine respiratory coronavirus (CRCoV) is included in the latter. The third coronavirus (CoV) group comprises CoVs of avian origin, such as the avian bronchitis virus.

Figure 4 - Coronavirus structure (a) and comparison of CCoV and FCoV genome (b).



S – spike protein; E – envelope protein; M – membrane protein; N – nucleocapsid protein; RNA – genomic RNA. Virion structure adapted from <http://www.expasy.ch/viralzone/>. Genome structure adapted from Decaro and Buonavoglia, 2008.

Coronaviruses are the largest RNA viruses known thus far (Gorbalenya, Enjuanes, Ziebuhr & Snijder, 2006), with genome sizes ranging between 27 and 31 kb. They are roughly spherical enveloped viruses 100-200 nm in diameter, with a fringe of circa 20 nm long petal-shaped

spikes with single-stranded positive sense RNA (Figure 4) and different CoVs have a similar genome organization, with a set of five genes in conserved order (de Vries, Horzinek, Rottier & de Groot, 1997). More than 70% of the genome in the 5' end is taken up by two overlapping open reading frames (ORFs) encoding two replicase polyproteins from which up to 16 mature products are derived. These proteins are required for RNA synthesis and other aspects of viral replication (de Vries et al., 1997; Lorusso et al., 2008). Four structural proteins are encoded by genes in a 3'-coterminal nested set of subgenomic mRNAs, namely the spike, envelope, membrane and nucleocapsid proteins (de Vries et al., 1997). The spike (S) protein forms the characteristic viral peplomers which mediate viral attachment to specific cell receptors and fusion between the envelope and the plasma membrane (Enjuanes et al., 2000⁵). The envelope protein (E) is an integral membrane protein and thought to be important for viral envelope assembling (Vennema et al., 1996). The membrane protein (M), a type III glycoprotein, is the most abundant structural protein. The nucleocapsid protein (N) is a highly basic phosphoprotein which modulates viral RNA synthesis, binding to it and forming a helical nucleocapsid. In addition to these structural proteins, CoVs may have a varying number of ORFs encoding non-structural proteins also expressed from subgenomic mRNAs (Lorusso et al., 2008). Most of these proteins aren't essential for replication and their exact function is unknown, but may be connected to virulence and host range (Yamanaka, Crisp, Brown & Dale, 1998; Haijema, Volders & Rottier, 2004).

CoVs lose infectivity after approximately 40 hours at room temperature or 60 hours when refrigerated (Tennant, Gaskell & Gaskell, 1994), but virus has been shown to survive outside cats for up to 7 weeks in dry conditions (Addie and Jarret, 2006). They are readily inactivated by most commercial detergents and disinfectants (McCaw & Hoskins, 2006).

Coronaviruses are important pathogens of mammals and birds. Clinical signs are usually enteric or respiratory, but can also be systemic. Feline infectious peritonitis (FIP) was first described as an important disorder of cats in 1963, and though the disease was thought to be infectious, no specific aetiologic agent was identified until 1970, when the close similarities of FIP virus (FIPV) in tissues to members of the family *Coronaviridae* was recognized. The close genetic relationship of FIPV to coronaviruses of dogs and swine was first reported by Pedersen et al. in 1978 and the existence of two serotypes, feline coronavirus (FCoV)-like and canine coronavirus (CCoV)-like, of feline coronaviruses was first reported in 1984. The incidence of the disease increased in the 1960s onward, and it is currently one of the leading infectious causes of death among young cats from shelters and catteries. The reason for its sudden emergence is not known, but some suggest the virus may have speciated into cats within the last half century. TGEV was first described in North America a decade earlier and at least one strain of CCoV can induce mild enteritis in cats

5 cited by Decaro & Buonavoglia, 2008

and enhance a subsequent infection with FIPV (Pedersen, Allen & Lyons, 2008). Recombinants between these three viruses are known to occur, favoured by the ease with which transcription units (RNAs) can be gained or lost during the divergent evolution of coronaviruses. (de Groot, Ter Haar, Horzinek & Van Der Zeijst, 1987). Another hypothesis suggests that the FIP mutation may be selective to a variant FCoV that appeared in the 1950s, which could have arisen because of the intra- and inter-species mutability of coronaviruses (Pedersen et al., 2008).

The first report on CCoV infection is dated 1971, when the virus was first isolated a coronavirus from faeces of dogs with suspected infectious enteritis in a canine military unit in Germany (McCaw & Hoskins, 2006). Since then, several CCoV outbreaks have been reported worldwide, although its true importance as a cause of infectious enteritis in the dog population is still unknown. Serological and virological investigations have demonstrated that CCoV is widespread in dog population, mainly in kennels and animal shelters (Decaro and Buonavoglia, 2008) and serological evidence suggest CCoV has been present indefinitely in the dog population and is an infrequent cause of infectious enteritis (McCaw & Hoskins, 2006).

Feline coronavirus may be subdivided serologically and by nucleotide sequencing into two types. Type I virus is the most prevalent (Hohdatsu, Okada, Ishizuka, Yamada & Koyama, 1992; Addie, Schaap, Nicolson, & Jarrett, 2003; Vennema, 1999; Kummrow et al., 2005; Shiba, Maeda, Kato, Mochizuki & Iwata, 2007). Type II virus is less common, resulting from recombination between type I feline coronavirus and canine coronavirus involving the spike gene (Herrewegh, Smeenk, Horzinek, Rottier & de Groot, 1998), and can be readily propagated in cell cultures, unlike type I virus (Pedersen et al., 1984). Both types of virus can induce FIP and feline coronavirus strains have also been subdivided into two distinct "biotypes": Feline Enteric Coronavirus (FECV) and Feline Infectious Peritonitis Virus (FIPV). Technically, FCoV includes all strains (numerous), serotypes (types I and II) and biotypes (enteric or infectious peritonitis viruses) of the genus (Pedersen et al., 2008), but since all FCoV may induce systemic infection, such descriptions are perhaps best avoided (Addie et al., 2009).

Genetic analysis of several CCoVs detected in pups with diarrhoea in Italy revealed a number of point mutations affecting a fragment of the M gene, which led to the designation of these atypical CCoVs as FCoV-like CCoVs (Pratelli et al., 2002). Phylogenetic analysis showed that this new strain segregates with FCoVs type I rather than reference CCoVs and FCoVs type II and it has since been designated as the prototype of the newly recognised CCoV type I, whereas reference CCoVs have been referred to as CCoV type II. Unlike group 1 CoVs, CCoV type I shares with members of groups 2 and 3 a potential cleavage site in the

S protein, although the potential implications in viral pathobiology have not been determined (Pratelli et al., 2003a).

Coronaviruses are highly contagious and spread rapidly through groups of susceptible animals. Faeces are the major source of both FCoV and CCoV and the major mode of transmission is believed to be the faecal-oral route, though contamination via saliva and transplacental transmission have been described. Susceptible cats are most likely to be infected with FCoV from asymptomatic cats and litter boxes represent the main source of infection in groups of cats (Addie et al., 2009). Following natural infection with FCoV cats begin to shed virus in the faeces within one week (Pedersen, Sato, Foley & Poland, 2004) and shedding continues for weeks to months, while a small proportion of carrier cats may shed virus for life (Addie et al., 2009). Young animals are at greater risk of developing clinical disease and FIP (Hartmann 2005; McCaw & Hoskins, 2006). Dogs shed the virus starting at 1 to 4 days post-infection and it can be isolated in faeces of infected dogs for weeks to months (Tennant et al., 1994; McCaw & Hoskins, 2006; Decaro and Buonavoglia, 2008).

Most FCoV infections proceed asymptotically or only with minor signs of enteritis, but about 12% of infected cats (Addie et al., 2003), 70% of them less than a year old (Hartmann, 2005), develop a lethal systemic pyogranulomatous disease called FIP (Addie et al., 2009). Its precise cause is still unclear, but could either be a mutation favouring viral replication in monocytes and macrophages (Haijema et al., 2004; Rottier, Nakamura, Schellen, Volders & Haijema, 2005; Cornelissen, Dewerchin, Van Hamme & Nauwynck, 2007), or the interaction between viral load and the cat's immune response (Meli et al., 2004; Dewerchin, Cornelissen & Nauwynck, 2005; Rottier et al., 2005; Kipar et al., 2006; Dye & Siddell, 2007) and it is likely that both viral genetics and host immunity play a role in the development of FIP. The two forms of the disease described are called "wet", or effusive, and "dry", or non-effusive, and whether a cat develops the wet or dry form is thought to depend on strength of the T-cell-mediated immune response, which is probably the only efficient immune response against disease progression (Cornelissen et al., 2007). While virus replicates to high titres in monocytes and can be found in many organs in cats with FIP, it is mainly confined to the intestine in asymptomatic cats, even though a low-level monocyte-associated viraemia can still be detected by RT-PCR (Herrewegh et al., 1995; Meli et al., 2004; Addie et al., 2009). Activation of monocytes and perivascular macrophages may lead to the development of typical widespread pyogranulomatous and vasculitis/perivasculitis lesions in various tissues and organs, including lung, liver, spleen, omentum, and brain of cats with FIP (Addie et al., 2009).

CCoV infections are usually also restricted to the alimentary tract, leading to the onset of clinical signs typical of the gastroenteric involvement including loss of appetite, vomiting, fluid diarrhoea, dehydration and, only occasionally, death. Usually, systemic disease is not

observed during CCoV infection, although the virus has been isolated from several tissues (tonsils, lungs and liver) of pups infected experimentally (Tennant, Gaskell, Kelly, Carter & Gaskell, 1991) and a highly virulent strain has been reported in Italy, where it caused a systemic disease followed by a fatal outcome in pups (Buonavoglia et al., 2006; Decaro et al., 2007b). Clinical signs consisted of fever, lethargy, loss of appetite, vomiting, haemorrhagic diarrhoea, severe leucopenia and neurological signs (ataxia, seizures) followed by death within 2 days after the onset of the symptoms. Fatal disease commonly occurs as a consequence of mixed infections with CCoV together with CPV-2, canine adenovirus type 1 or CDV (Decaro and Buonavoglia, 2008).

Diagnosis of these diseases was difficult before the development of sensitive molecular techniques and diagnosis of FIP *intra vitam* is still extremely challenging in the absence of effusion (McCaw & Hoskins, 2006; Addie et al., 2009). Coronavirus particles can be observed in electron microscopy, but false-negatives are possible. The original virus strains do not grow well in tissue or cell culture systems, which renders virus isolation difficult. Positive serum titres detected by ELISA or serum neutralization only confirm exposure to the viruses, though the presence of IgM antibodies against CCoV is indicative of recent infection (Naylor, Monckton, Lehrbach & Deane, 2001; McCaw & Hoskins, 2006). Viral detection in blood, faeces, or effusions using highly sensitive RT-PCR assays are becoming more common, though the test is not a useful prognostic indicator for the development of FIP, and false-negatives in FIP-positive cats are possible (Hartmann et al., 2003; McCaw & Hoskins, 2006; Addie et al., 2009).

CCoV vaccination is not recommended at the moment due to the questionable efficiency of available vaccines and the frequency of adverse reactions (Pratelli et al., 2004a). Vaccine development has been especially aimed at preventing FIP, but the phenomenon of antibody-dependent enhancement has foiled many attempts so far, because cats with pre-existing antibodies, especially those directed against the spike protein, developed an enhanced form of disease in experimental infections, typified by an earlier development and a shortened disease course leading to a more rapid death (Addie et al., 2009). However, the role of ADE in natural infection is not clear since in field studies cats were most likely to develop FIP on first exposure to FCoV (Addie et al., 2003). There is one commercial vaccine available for cats which contains a temperature sensitive mutant of the type 2 FCoV and is designed to be administered intranasally, thus inducing local mucosal immune responses through the induction of IgA and cell mediated immunity. Unfortunately there is considerable controversy regarding the efficacy and safety of this vaccine (Addie et al., 2009). Recent efforts oriented towards the development of a vaccine which protects by cellular mediated immunity have been successful in creating a live attenuated vaccine by deletion of genes implicated in viral virulence which is ready to be tested in the field and prepared for the market if successful,

even if no explanation for this success has been found (Haijema, Schellen, Egberink & Rottier, 2009).

1.5.3 Retroviruses

Among the three genera to cause common infection in pets, feline foamy virus is a transmissible retrovirus considered non-pathogenic, but feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) can cause a variety of life-threatening conditions in both domestic as well as wild felines, unless properly managed (Levy et al., 2008b). Dogs, on the contrary, have no well-characterized retroviral infections, in spite of periodic reports compatible with this type of infections (Dunham & Graham, 2008). Additionally in the cat, a number of endogenous, nonpathogenic retroviruses (enFeLV and RD-114) are reported, derived from ancient retroviral infections of their ancestors, which cannot be induced to produce infectious virus particles and are inherited by transmission through germ line. Retroviruses are enveloped viruses with single-stranded RNA genome which is reverse transcribed by a specialized enzyme into dsDNA and integrated into the host cell genome. The virus depends on the enzymatic machinery of the host cell for replication and so infection doesn't normally lead to cell death (Lutz et al., 2009).

Feline leukaemia virus was first described in 1964 when virus particles were observed budding from the membrane of malignant lymphoblasts from a cat with naturally occurring lymphoma. The virus was proven to be capable of transmitting lymphocytic neoplasia when experimentally injected into healthy cats and was thought to account for most disease-related deaths in pet cats for many years (Hartmann 2006). The isolation of a T-lymphotropic virus possessing the characteristics of a lentivirus from pet cats in Davis, California was reported in 1987 and it was evident that in causing an acquired immunodeficiency syndrome in cats, FIV was of substantial veterinary importance. The virus has remained the object of intense investigation as a model of lentiviral pathogenesis and prevention (Hartmann, 1998; Sellon & Hartmann, 2006). Both are among the most common infectious diseases of cats (Levy et al., 2008b).

FeLV is a γ -retrovirus of the subfamily *Orthoretrovirinae* typically associated with the long-latency induction of leukaemia and lymphoma in cats. It occurs in nature not as a single genomic species, but as genetically complex families of closely related viruses (Levy, 2008). FIV is part of the same subfamily as FeLV, *Orthoretrovirinae*, but belongs to the genus *Lentivirus*, which shares many properties with human immunodeficiency virus (HIV). For this reason it has become a model of lentiviral pathogenesis and prevention and remains until today the object of intense investigation (Sellon & Hartmann, 2006).

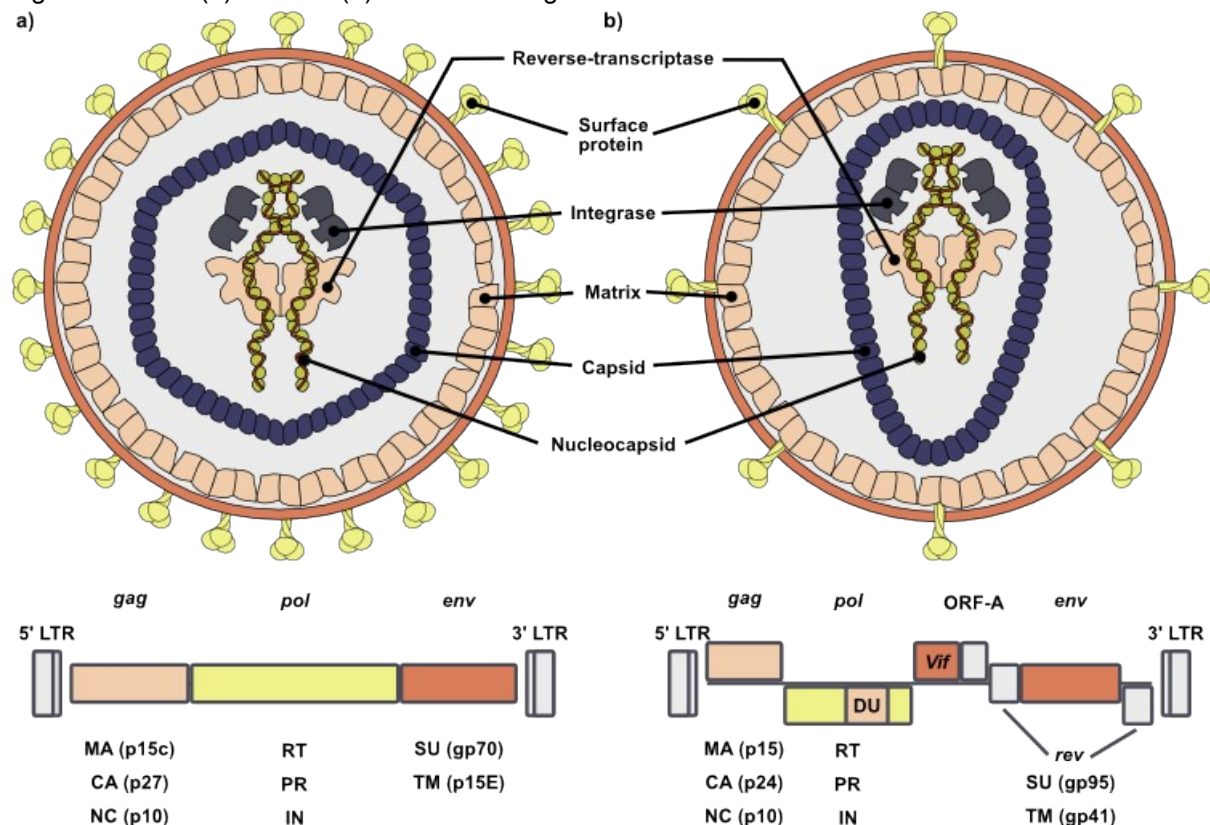
The diameter of FeLV virus is ~127 nm (DeBlois & Wesley, 1977) and the extracellular virion shows no symmetry of the nucleocapsid (Sarkar, Nowinski & Moore, 1971). Its genome is

similar to other retroviruses in the family (Figure 5-a). The single-stranded RNA transcribed into a ~8.5kb dsDNA (Soe, Devi, Mullins & Roy-Burman, 1983) by the viral reverse transcriptase (RT) is called provirus, and is integrated into the host cell genome. The gene sequence includes the group specific antigen group gene (*gag*), the polymerase gene (*pol*) and the envelope gene (*env*). These genes are flanked on both sides by long terminal repeat sequences (LTRs), with a regulatory function of the viral expression and replication (Hartmann, 2006). The complete FIV virion is spherical to ellipsoid in shape, 105-125 nm in diameter with short, poorly defined envelope projections (Pedersen et al., 1987⁶). Each virion contains two copies of the positive-sense, polyadenylated RNA genome and a number of molecules of viral proteins (Bendinelli et al., 1995). Its genome organization is similar to other retro viruses and lentiviruses, with ~9400 base pairs (Hartmann, 1998) and additional ORFs encoding accessory proteins allowing the virus to regulate its life cycle more tightly or productively (Dunham & Graham, 2008) (Figure 5-b). The *gag* gene codes internal virion proteins, such as the matrix, capsid and nucleocapsid protein. The capsid protein p27 of FeLV and p24 of FIV are both important for diagnosis purposes (Hosie et al., 2009) and p27 is produced in infected cells abundantly, which means it can be detected in the cytoplasm of infected cells, as well as in the plasma of infected cats (Hartmann, 2006). The *pol* gene codes the viral enzymatic proteins namely, the protease determinant for the virion maturation, the reverse transcriptase responsible for copying the viral genomic RNA into a complementary dsDNA molecule and the integrase that modulate the viral integration into the cell genome as provirus. The FIV *pol* gene encodes an additional protein, dUTPase (DU), which is a characteristic enzyme of non-primate lentiviruses (Elder et al., 1992) and minimizes the incorporation of uracil into viral DNA, proving essential for an efficient viral replication with stable and correct DNA synthesis (Inoshima, Miyazawa & Mikami, 1998). The *env* gene codes the surface and transmembrane glycoprotein, which are responsible for defining the virus subgroup in FeLV, and subtypes in FIV (Hartmann, 2006; Hosie et al., 2009). The genetic variation for FIV is greater than that seen for FeLV (Dunham & Graham, 2008), and the clinical relevance of this variability is that different FIV *env* sequences may confer different cell tropism and have different pathogenic consequences (Verschoor et al., 1995; Pancino, Castelot & Sonigo, 1995). Based on these sequence variability within the *env* gene five subtypes, or clades, have been recognized – A, B, C, D and E (Sodora et al., 1994; Pecoraro et al., 1996; Nishimura et al., 1998). A sixth subtype – F, has been proposed, but not yet fully characterised (Duarte & Tavares, 2006) Among the auxiliary genes of FIV, three have been identified and partially characterized. The *vif* gene is essential for productive FIV infection *in vitro* (Lockridge, Himathongkham, Sawai, Chienand & Sparger, 1999) and required for efficient viral replication *in vivo* during the early stage of infection in cats

6 cited by Hartmann, 1998

(Inoshima et al., 1996). ORF-A is involved in multiple steps of the FIV cycle including both virion formation and infectivity (Gemeniano, Sawai, Leutenegger & Sparger, 2003) and the *rev* gene encodes a protein which controls the cytoplasmic expression of other FIV viral proteins essential for productive infection (Phillips et al., 1992; Tomonaga et al., 1993).

Figure 5 - FeLV (a) and FIV (b) structure and genome.



Major genes: *gag* – group specific antigen; *pol* – polymerase; *env* – envelope. Major viral proteins: MA – matrix; CA – capsid; NC – nucleocapsid; RT – reverse-transcriptase; PR – protease; IN – integrase; SU – surface protein; TM – transmembrane protein. Virion structure adapted from <http://www.expasy.ch/viralzone/>. Genome structure adapted from Dunham and Graham, 2008.

FeLV occurs in nature in four subgroups, designated A, B, C and T, classified first on the basis of superinfection interference testing, and later distinguished genetically. Superinfection interference is the ability retroviruses have to downmodulate the presence of a particular membrane receptor on the infected host cell, thus preventing successive rounds of infection of the same cell. (Mendoza, Anderson & Overbaugh, 2006). This interference reflects differences in the genetic sequence of the *env* gene, more specifically, in the surface protein, which binds to different host cell receptors for entry. Only FeLV-A is contagious and represents the predominant agent horizontally transmitted from cat to cat (Hartmann, 2006; Levy, 2008). Amino acid sequence in the surface proteins of known isolates of FeLV-A has been reported to be ~97% identical, in spite of isolation over more than a decade from widely different geographic locations (Donahue et al., 1988; Levy, 2008). Subtype B originates from

recombination of FeLV-A with the endogenous retrovirus enFeLV and subtype C is the result of mutations within the *env* gene (Lutz et al., 2009). Subtype T is a highly cytopathic form which evolves from FeLV-A as a result of multiple mutations on the *env* gene and is characterised by its tropism for T lymphocytes (Hartmann, 2006).

None of these retroviruses survive more than a few minutes outside the host and both are readily destroyed by disinfectants, soap, heating and drying. Transmission via fomites is unlikely, but there is potential for iatrogenic transmission to occur via contaminated needles, surgical instruments or blood transfusions (Hosie et al., 2009; Lutz et al., 2009).

FeLV and FIV infections are life-threatening infections which occur globally. Risk factors for infection include male gender, adulthood, and outdoor access, whereas indoor lifestyle and sterilization are associated with reduced infection rates (Levy, Scott, Lachtara & Crawford, 2006). FeLV prevalence may be influenced by the density of cats and there may be noticeable geographical and local variation. In some European countries, the USA and Canada, the prevalence of FeLV infection in individually kept cats seems to be very low, usually less than 1 %, but prevalence may be greater than 20 % in large multi-cat households without specific preventive measures for introduction of FeLV. Over the last 25 years, the prevalence and importance of FeLV infection in Europe has greatly diminished due to the availability of reliable tests, the test and removal programmes initiated, improved understanding of the pathogenesis and the introduction of highly efficacious FeLV vaccines (Lutz et al., 2009). In contrast, the prevalence of FIV has not changed since the virus was discovered, in 1986, and is highly variable between regions, with estimates of 1 to 14% in cats with no clinical signs and up to 44% in sick cats (Hartmann 1998).

Cats with FeLV viraemia act as a source of infection. Virus is shed from an infected cat in saliva, nasal secretions, faeces or milk and infection is transmitted mainly by mutual grooming, but also through bites. In viraemic queens, pregnancy usually results in embryonic death, stillbirth or in viraemic kittens which fade away rapidly. In latently infected queens, transmission does not usually take place during pregnancy. Young kittens are especially susceptible to FeLV infection while with age, cats become increasingly resistant to infection, although they can still be infected provided the challenge is sufficiently severe (Lutz et al., 2009).

The majority of natural FIV infections are acquired by biting, presumably through the inoculation of virus, or virus-infected cells, from the saliva of persistently infected cats. Transmission from mother to kittens may occur but only a proportion of the offspring become persistently infected, depending on the viral load of the queen during pregnancy and birth. Cats can be infected by experimental inoculation of virus into the nose, mouth, vagina and rectum and virus can be recovered from semen following natural or experimental infection,

although none of these has been documented in nature. Queens may still be infected at mating however, if bitten by an infected tomcat (Hosie et al., 2009).

Even though FeLV infection has been considered more of a concern for cats that are 'friendly' with other cats, because of the close, intimate contact which facilitates transmission, and FIV infection has been viewed as a concern for cats that are 'unfriendly' with other cats, because of the mode of transmission through bite wounds, both viruses can be spread among cats that are not known to fight as well as those that are prone to aggressive behaviour. Characteristics such as gender, age, lifestyle, and health status can be used to assess the likely risk of FeLV and FIV infections, but most cats have some degree of infection risk and less than one quarter of all cats have ever been tested (Levy et al., 2008b). Although infected cats may experience a prolonged period of clinical latency, a variety of disease conditions are associated with retroviral infections, including anaemia, lymphoma, chronic inflammatory conditions, and susceptibility to secondary and opportunistic infections. FeLV usually infects individual lymphocytes in the oropharynx that are transported to the bone marrow, where large amounts of virions are then produced. Viraemia develops within a few weeks of infection, leading to the infection of salivary glands and intestinal linings, but may often develop only several months after constant exposure to shedding cats. A functioning immune system may frequently overcome the development of viraemia, which is then transient, and so-called "regressor" cats are generally not at risk of developing disease. In a multi-cat household without control of FeLV infection, 30-40 % of the cats develop persistent viraemia, 30-40 % exhibit transient viraemia and 20-30 % seroconvert without ever being detectably viraemic. In spite of overcoming viraemia, these cats remain latently infected and reactivation may take place in vivo in case of immune suppression or chronic severe stress, though this is generally believed to be a rare occurrence. It appears likely that no cat can completely clear FeLV infection from all cells. Although recovered cats appear to have the same life expectancy as cats that have never been exposed to FeLV, proviral DNA has been found in the tumours of FeLV-free cats. The typical clinical signs of FeLV infection usually develop in viraemic cats, sometimes not until after several years of viraemia (Lutz et al., 2009).

Acute FIV infection is associated with transient fever, lymphadenopathy, and leucopenia but frequently goes unnoticed by cat owners. Virus is detected in high concentrations in the blood by culture and PCR within 2 weeks of infection and both CD4+ (helper) and CD8+ (cytotoxic-suppressor) T-lymphocytes decline within the first few weeks of infection (Levy et al., 2008b). Latent infection arises when a cell carries an integrated copy of provirus but does not produce new virus particles and these cells represent a "reservoir" of infection that is not susceptible to neutralising antibodies, posing an obstacle for effective vaccination. The decrease in viral replication controlled by the immune response marks the beginning of the

so-called 'asymptomatic' phase that can last for many years, or may be lifelong and during which the infected cat remains relatively free of clinical signs (Hosie et al., 2009). During this time, progressive dysfunction of the immune system occurs, and it is generally recognized that cell-mediated immunity is more profoundly affected than humoral immunity. Chronic inflammatory conditions, neoplasia, and infections with intracellular organisms, therefore, are more common than infections controlled by antibodies in FIV-infected cats (Levy et al., 2008b).

Identification and segregation of infected cats is considered to be the single most effective method for preventing new infections with FeLV and FIV and the retroviral status of all cats should be known because of the serious health consequences of infection influencing patient management both in illness and wellness care (Levy et al., 2008b). In general terms, diagnosis of FeLV infection involves the detection of antigen, usually the capsid protein p27, which is produced abundantly in most infected cats, while the diagnosis of FIV is aimed at detecting antibodies to the capsid protein p24, because of its persistent, life-long infection with low viral loads (Hartmann, 2006; Levy et al., 2008b). The most commonly used tests are commercially available ELISA assays or immunochromatography tests, but the recent introduction of a commercial FIV vaccine, as well as the advances in molecular techniques mean that increasing efforts are being made to develop more sensitive PCR and RT-PCR assays, which can provide significantly more information (Hofmann-Lehmann et al., 2008; Hosie et al., 2009; Lutz et al., 2009).

The first FeLV vaccine was introduced in the USA in 1984 and protected cats from viraemia. Since then many breakthroughs have been achieved in recombinant DNA technology, which most vaccines on the market today make use of, but different studies still report varying levels of efficacy of protection for many of these (Lutz et al., 2009). Recent studies have demonstrated without exception that cats that are able to overcome p27 antigenaemia become provirus positive in the blood and also positive for viral RNA in plasma, although at very low levels. These experiments confirm that FeLV vaccination neither induces sterilising immunity nor protects from infection and no FeLV vaccine is likely to provide 100 % efficacy of protection or prevent infection (Hofmann-Lehmann et al., 2007).

There is a FIV vaccine made from whole inactivated virus preparations available in the USA, Australia and New Zealand for some time, but its efficacy has not been tested against a range of European field isolates (Hosie et al., 2009). In one study vaccination was shown not to protect cats against a virulent UK primary isolate of FIV (Dunham et al., 2006). Neither the European Advisory Board for Cat Diseases (ABCD), nor the World Small Animal Veterinary Association (WSAVA) recommend vaccinating cats against FIV given the problems associated with serological diagnosis of infections and lack of evidence of efficacy against European isolates (Day et al., 2007; Hosie et al., 2009).

2. Materials and methods

2.1 Study population

For the purpose of this study, a sample of 50 dogs and 50 cats from the Lisbon municipal kennel was chosen. The animals were euthanized during the period of the study and the sample limit of 100 animals was reached after 4 weeks, between October and November 2008. No living animals took part in this study because of lack of authorization. Information regarding sex, race, origin (captured/relinquished), clinical history and examination was collected from kennel records where available, after the sampling procedure.

2.2 Sample collection

Euthanasia was performed according to the following protocols: Dogs weighing up to 15kg were administered 10ml of sodium pentobarbital (Eutasil, Sanofi Veterinária, Portugal) intravenously, followed by 10ml of ether intravenously. Dogs weighing over 15kg were administered 15-20ml of sodium pentobarbital (Eutasil, Sanofi Veterinária, Portugal) intravenously, followed by 15-20ml of ether intravenously. Cats were anaesthetized with intramuscular ketamine followed by intracardiac administration of 10ml sodium pentobarbital (Eutasil, Sanofi Veterinária, Portugal).

Around 2ml blood was collected from the cephalic vein of dogs using butterfly needles into a 5ml syringe prior to pentobarbital administration and then placed in blood tubes with EDTA. From cats, blood was collected by cardiac puncture into a 5ml syringe prior to pentobarbital administration and then placed in blood tubes with EDTA. Faecal swabs were collected from dogs and cats after euthanasia. Samples were taken immediately back to the laboratory and kept refrigerated until processed.

In March 2009, taking into account preliminary results of the survey, environmental swabs were collected from cages and cells at the municipal kennel. These cages were randomly selected from the same rooms which sampled animals occupied prior to euthanasia. In total, 24 environmental swabs were collected from 6 occupied cells and cages. The chosen cells were sampled every 4 days until we had 4 samples from each.

2.3 Sample processing

Blood samples were centrifuged for 5 minutes at 5000 rpm to separate plasma from blood cells and were frozen at -20 °C. Faecal samples were suspended in phosphate buffered saline (PBS) solution and centrifuged for 10 minutes at 10000 rpm. Environmental samples were also suspended in PBS and centrifuged for 10 minutes at 10000 rpm.

2.4 Antibody detection

An ELISA kit was chosen for the detection of antibodies to FIV because of its high sensitivity. Plasma from all 50 cat samples was analysed using a commercially available kit (Viracheck[®]/FIV, Synbiotics), following manufacturer's directions.

Dog samples were also tested for antibodies to CDV and CPV using ELISA test kits. Without any vaccination history, the information gathered from IgG antibody detection alone would be of little use. For this purpose, an ELISA test kit which detects IgM specific to CDV (Ingezim Moquillo IgM/ Ingenasa) was used to test samples for the presence of antibody titres which would indicate recent CDV infection. A CPV test kit for IgM was unfortunately not available at the time, so samples were tested for the presence of IgG using a different kit (Ingezim Parvo Canino/ Ingenasa).

2.5 Antigen detection

The detection of FeLV was performed using a commercial ELISA kit (Viracheck®/FeLV, Synbiotics) which detects the presence of core viral antigen p27. This antigen is produced abundantly in most infected cats (Levy et al., 2008b) and is detected here in its soluble form. Because of this, it is a marker of infection, but not always of viraemia (Lutz et al., 2009). Though it is possible that about 10% of the cats which test negative to the presence of p27 antigen are positive by PCR (Hofmann-Lehmann et al., 2001), the specificity of the test is close to 100% .

2.6 Nucleic Acid Extraction

Both viral DNA and RNA were extracted from samples of faecal and environmental swabs using QIAmp MiniElute Virus Spin Kit (Qiagen), following the manufacturer's procedures. Nucleic acid was kept at -80°C until analysed.

2.7 PCR and RT-PCR

Faecal and environmental swabs were tested according to various protocols, depending on the virus to detect and were all performed in 25µl reactions. DNA samples were amplified using PCR Fidelity Taq 2x Master Mix (USB) and RNA samples were amplified with one step RT-PCR Fidelity Taq 2x Master Mix (USB). Primers used for the different assays are summarized in Table 4 and thermal cycles in Table 5.

The detection of Parvovirus was performed on both dog and cat samples using primer pair 555for/555rev (Buonavoglia et al., 2001) which amplifies a 583bp fragment of the gene encoding the capsid protein (Desario et al., 2005). Primers and magnesium (MgCl₂) concentration was 50pmol/µl and 2mM, respectively and 5µl of DNA template were used.

The primers used for detection of CDV in dog samples were described by Frisk et al. (1999). The sequence amplifies a 287bp sequence of the nucleoprotein (NP) gene and is localized in a highly conserved region of the Ond-CDV strain, sharing high homology among different morbiliviruses (Frisk et al., 1999). Primers and MgCl₂ concentration were the same as used for CPV PCR and 10µl RNA template was added.

Table 4 - Primers, position and fragment sizes used for PCR amplification of different viruses

Virus	Primer	Sequence	Position	Fragment
CPV/FPV ¹	555for 555rev	CAGGAAGATATCCAGAAGGA GGTGCTAGTTGATATGTAATAAACA	4003-4022 4561-4585	583 bp
CDV ²	p1 p2	ACA GGA TTG CTG AGG ACC TAT CAA GAT AAC CAT GTA CGG TGC	769-789 1055-1035	287 bp
CCoV I ³	CCoV1a CCoV2	GTGCTTCCTCTTGAAGGTACA TCTGTTGAGTAATCACCAGCT	6900-6920 7118-7138	239 bp
CCoV II ³	Can1F CCoV2	TAACATTGCTCTCAGGGAATTTG TCTGTTGAGTAATCACCAGCT	6937-6959 7118-7138	202 bp
FCoV ⁴	a	GGCAACCCGATGTTTTAAACTGG	205-184	223 bp
	b	CACTAGATCCAGACGTTAGCTC	1-23	
	c	CCGAGGAATTACTGGTCATCGCG	213-192	177 bp
	d	GCTCTCCATTGTTGGCTCGTC	29-51	
FCoV I ⁵	lffs	GTTTCAACCTAGAAAGCCTCAGAT	3921-3944	376 bp
	lubs	CCACACATACCAAGGCC	4280-4296	
	nffles	CCTAGAAAGCCTCAGATGAGTG	3928-3949	360bp
	nlubs	CCAAGGCCATTTTACATA	4270-4287	
FCoV II ⁵	lcfs	GCCTAGTATTATACCTGACTA	3978-3998	283 bp
	lubs	CCACACATACCAAGGCC	4280-4296	
	nlcfs	CAGACCAAAGTGGACTGTAC	4041-4060	218 bp
	nlubs	CCAAGGCCATTTTACATA	4270-4287	

1 – Desario et al., 2005

2 – Frisk et al., 1999

3 – Pratelli et al, 2004b

4 – Herrewegh et al., 1995. Primer numerical position as determined from the termination codon of ORF 7b

5 – Addie et al., 2003

Two pairs of CCoV primers were used, in separate reactions, to amplify a fragment of the gene encoding the membrane protein M (Pratelli et al., 2004b). These have the advantage of amplifying two fragments of different size depending whether CCoV type I or type II is present in the sample, respectively, one with 239bp and another with 202bp. Primer concentration was 50pmol/μl, MgCl₂ was 1.75mM and 5μl RNA template was used.

For detection of FCoV all cat samples were amplified by RT-PCR followed by nested PCR (nPCR) (Herrewegh et al., 1995). The primers for this assay amplify a fragment within ORF-7b, located at the 3' end of the genome and encode a highly conserved non-structural glycoprotein of unknown function (Lin et al., 2009). The first PCR amplifies a 223bp fragment and the nested a 177bp one. Primer concentration was the same as above mentioned and MgCl₂ concentration was 1.5mM and 2mM in the RT-PCR and the nPCR. Ten micro-liters of template were used for the first RT-PCR and 1 μl for the nPCR.

Positive samples to FCoV RNA were subjected to a second RT-PCR and nPCR assay (Addie et al., 2003) for differentiation between FCoV type I and II. A universal reverse primer, lubs, directed at a conserved region of the spike gene S, and to forward primers, lffs and lcfs, directed at specific sequences of the S gene yielded fragments of different size depending on

the type of FCoV present, I or II. The nPCR is intended to increase both the sensitivity and specificity of the reaction using nested primers nlubs, nlffles and nlcfs, for the same regions. Fragment sizes for FCoV type I and II were 376bp and 283 bp for the initial RT-PCR, and 360 bp and 218 bp for the nPCR. Primers concentration was 10pmol/μl for both forward primers and 15pmol/μl for the reverse primer and MgCl₂ concentration was raised from 1.5mM to 2.25mM in the nPCR. The amount of template used was 5 μl in the RT-PCR and 1 μl in the nPCR.

All PCR products were detected by electrophoresis in a 1,5% agarose gel and visualized using UV illumination after ethidium bromide staining. Comparison of CCoV type I and II positives were analysed on 2,5% agarose gel.

Table 5 - Summary of temperature cycles used for PCR for different viruses

Viruses	Reverse transcription	Initial denaturation	Denaturation	Annealing	Extension	Final extension
CPV/FPV		94° C / 10 min	94° C / 30 sec	50° C / 1 min	68° C / 1 min	68° C / 10 min
			40 cycles			
CDV	42° C / 45 min	94° C / 2 min	91° C / 45 sec	52° C / 45 sec	68° C / 1 min	68° C / 10 min
			40 cycles			
CCoV	42° C / 30 min	94° C / 2 min	94° C / 30 sec	50° C / 45 sec	68° C / 30 sec	68° C / 10 min
			35 cycles			
FCoV	42° C / 45 min	94° C / 5 min	94° C / 30 sec	47° C / 1 min	68° C / 1 min	68° C / 5 min
			35 cycles			
		nPCR	94° C / 1 min	47° C / 1 min	68° C / 1 min	68° C / 5 min
	5 cycles					
	94° C / 30 sec		51° C / 45 sec	68° C / 1 min	68° C / 5 min	
		30 cycles				
FCoV genotype	42° C / 45 min	94° C / 2 min	94° C / 20 sec	47° C / 45 sec	68° C / 45 sec	68° C / 10 min
			35 cycles			
	nPCR	94° C / 2 min	94° C / 20 sec	47° C / 45 sec	68° C / 45 sec	68° C / 10 min
			35 cycles			

3. Results

3.1 Kennel records

A total of 100 samples from 50 dogs and 50 cats were collected. Kennel records were available only for the dogs in the study, because cats are housed in groups and could not be identified from their records. In some cases, records were incomplete. Out of the dogs tested, 19 (38%) were female and 28 (56%) were male. Twenty-six (52%) were of indeterminate race, 13 (26%) were pitbull and 11 (22%) were of other identifiable races. Eighteen (36%) animals were relinquished by their owners and 32 (64%) were captured by municipal services or police forces. Records from clinical examination showed that 28 (56%) were “apparently normal”. The most common previous conditions described in examination records were dermatitis and diverse injuries (fractures and haemorrhages), followed by conjunctivitis and claudication. Dogs spent on average 24 days at the kennel before euthanasia. These numbers are summarized in table 6.

Table 6 - Details from kennel records and distribution of race, origin and clinical examination per gender.

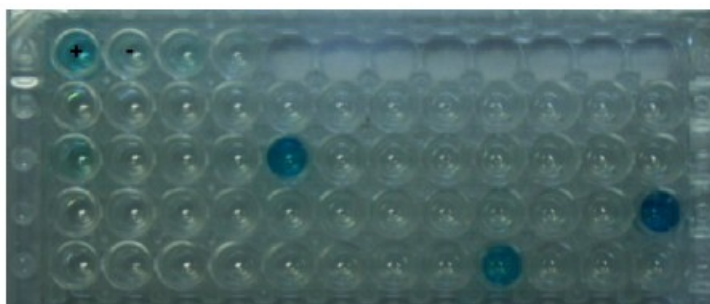
	Race			Origin		Clinical examination		Total
	Indeterminate race	Pitbull	Others	Captured	Relinquished	Apparently normal	Previous conditions	
Male	14	7	7	17	11	17	11	28
Female	9	6	4	13	6	9	10	19
Unknown	3	0	0	2	1	2	1	3
Total	26	13	11	32	18	28	22	50

3.2 Laboratory results

3.2.1 Serological results

The ELISA kits for CDV and CPV were used according to the manufacturer's instructions, yet the measured optical densities of the negative controls did not validate the tests, therefore no conclusions could be drawn about the serological status of these animals.

Figure 6 - ELISA assay for FeLV with positive (+) and negative (-) controls marked.

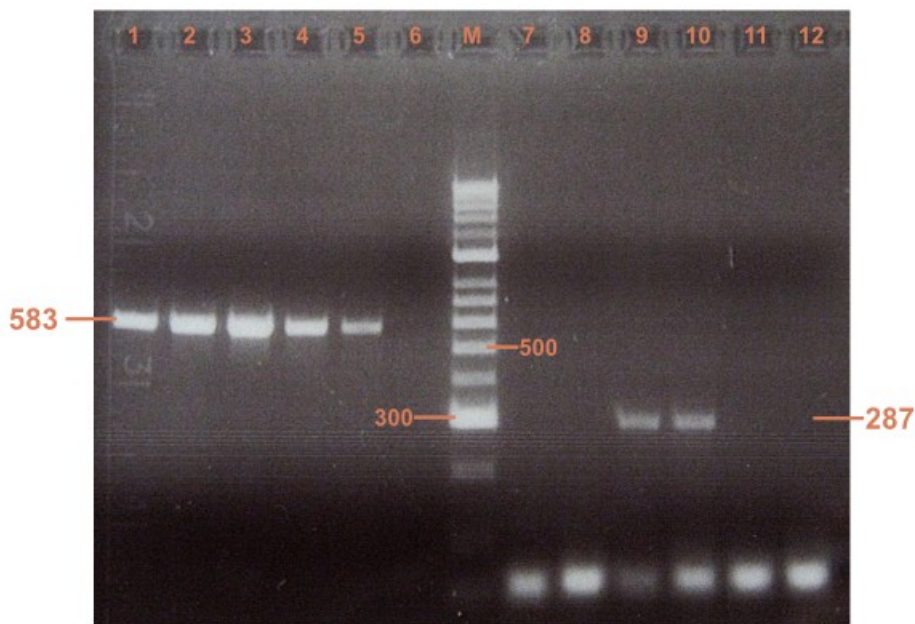


All of the blood serum samples tested by ELISA for the presence of FeLV p27 core antigen were run in a single plate. The test yielded 5 positive samples (5/50= 10%). The ELISA for FIV antibodies resulted in 9 positive samples and 12 inconclusive results were confirmed by a second run (9/50= 18%) (Figure 6).

3.2.2 Nucleic acid amplification results

To assess the sensibility of viral nucleic acid detection several dilutions of Hexadog vaccine (Merial), were used for parvovirus PCR and CDV RT-PCR. Both viruses are present in the vaccine in a concentration of 10^3 tissue culture infectious dose 50 (TCID₅₀), and 1/10 serial dilutions were performed until 10^{-4} . We detected amplification until the 10^{-4} dilution in the case of CPV and until 10^{-3} for the CDV (Figure 7). No positive control was available for any of the coronaviruses.

Figure 7 - PCR and RT-PCR results of Hexadog vaccine dilutions used to test sensibility of parvovirus and CDV detection.



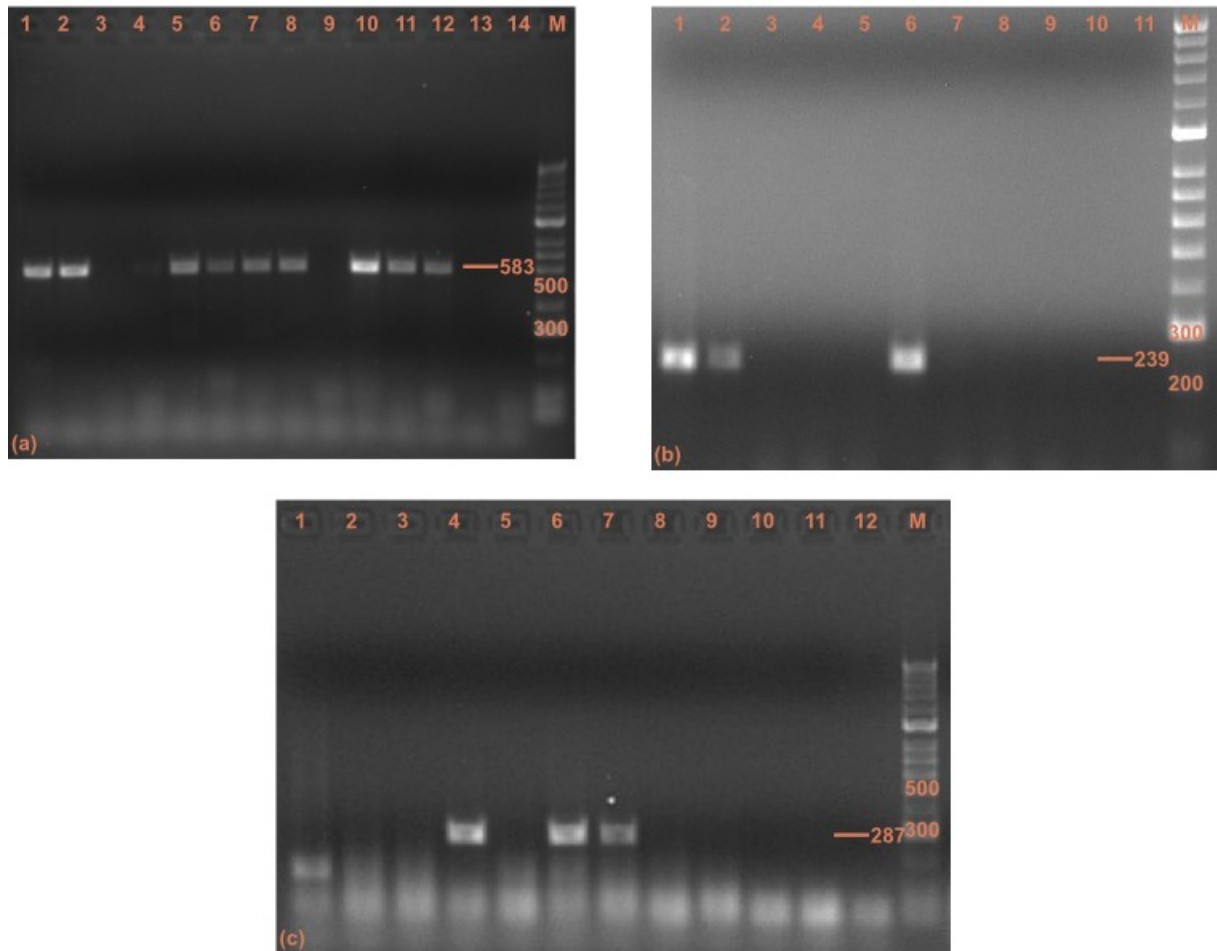
Lanes 1-5, detection of parvovirus DNA in 1/10 serial dilutions until 10^{-4} ; lanes 7-11, detection of CDV RNA in 1/10 serial dilutions until 10^{-4} with amplification only in lanes 9 (10^{-2} dilutions) and 10 (10^{-3} dilutions); lanes 6 and 12, negative controls; M, molecular size marker (100 bp ladder).

3.2.2.1 Dog samples

Faecal samples collected from dogs at the time of euthanasia were processed and tested for the presence of CPV DNA, CCoV and CDV RNA. None of the dogs evaluated had clinical signs of haemorrhagic diarrhoea, though a few samples had visible traces of blood at the time of collection. Out of the 49 dog samples tested, 25 (51%) were positive for CPV nucleic acid. Samples were evaluated for the presence of both genotypes of CCoV, and in total there

were 16 (33%) positives. The assay for the presence of CDV RNA yielded 4 (8.2%) positive samples (Figure 8).

Figure 8 - PCR results for CPV (a), CCoV type I (b) and CDV (c) in dog samples.

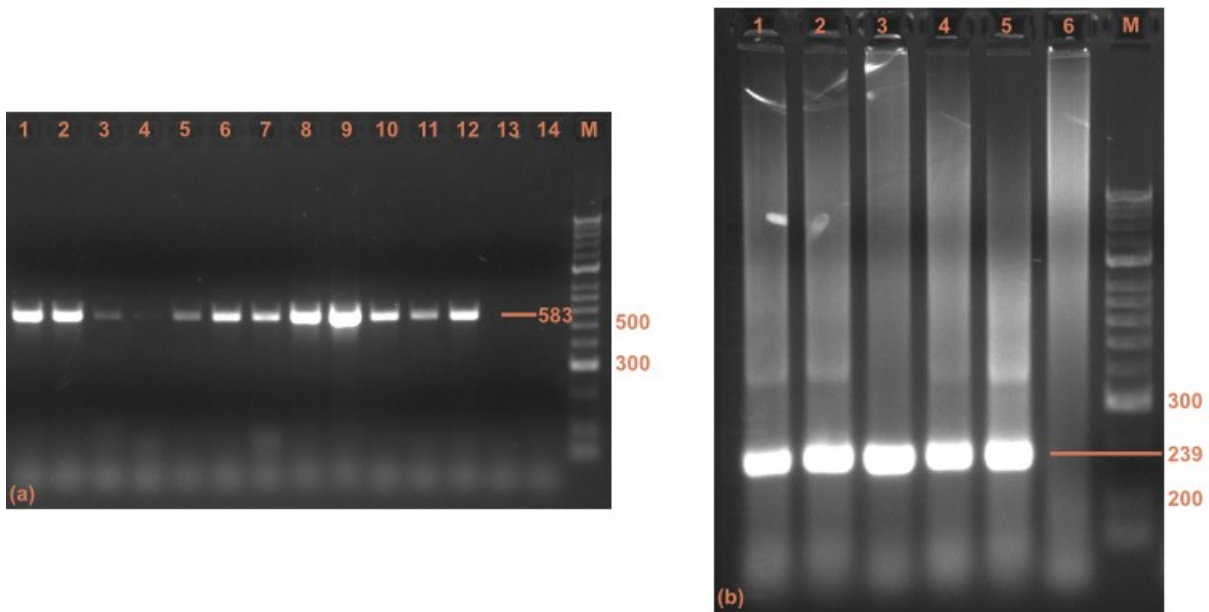


CPV (a): lanes 1-12, samples; lanes 13-14, negative controls. CCoV type I (b): lanes 1-10, samples; lane 11, negative control. CDV (c): lanes 1-11, samples; lane 12, negative control. M, molecular size marker (100 bp ladder).

3.2.2.2 Cat Samples

Faecal samples were collected from cats immediately following euthanasia and these samples were tested for the presence of FPV DNA and FCoV RNA. The PCR assay for FPV revealed the presence of viral nucleic acid in 34 samples (68%) and the RT-nPCR assay for FCoV RNA resulted in 35 (70%) positive samples. Results were observed on agarose gel visualized with UV illumination as seen in Figure 9.

Figure 9 - PCR results for FPV (a) and FCoV (b) in cat samples.

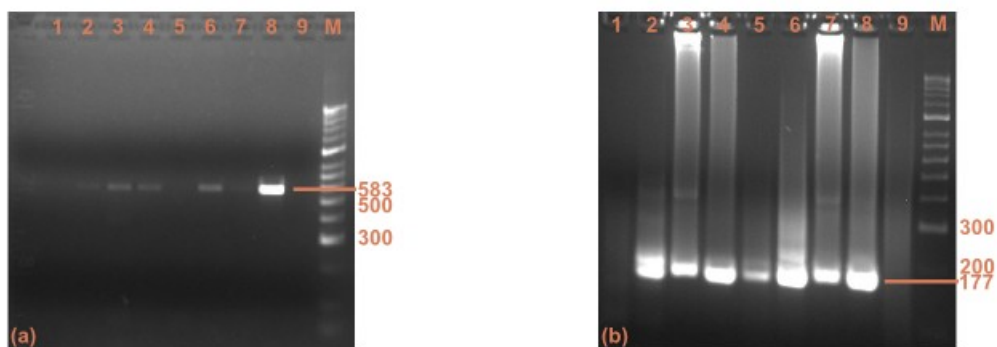


FPV (a): lanes 1-12, samples; lanes 13-14, negative controls. FCoV (b): lanes 1-5, samples; lane 6, negative control. M, molecular size marker (100 bp ladder).

3.2.2.3 Environmental samples

The high percentage of positive samples for parvovirus DNA and coronaviruses RNA led us to evaluate the environmental presence of these viruses. Sixteen samples were collected from 4 cells occupied by dogs and 8 samples from 2 cat cages. Eight samples originating from 3 different cells were positive for the presence of CDV RNA. Parvovirus DNA was present in 15 samples out of 24, including samples from the same 3 dog cells and all of the cat cages. None of the environmental samples tested positive for the presence of CCoV, but 7 samples out of 8 from both cat cages showed amplification of FCoV RNA (Figure 10). A summary of the laboratory results is presented in table 7.

Figure 10 - PCR results for CPV and FCoV in environmental samples.



CPV (a): lanes 1-8, samples; lane 9, negative control. FCoV (b): lanes 1-8, samples; lane 9, negative control. M, molecular size marker (100 bp ladder).

Table 7 - Distribution of positive results per tested samples

	dog	cat	environment
CDV	4/49 (8.2%)		8/16 (50%)
CCoV	16/49 (32.7%)		0/16 (0%)
Parvovirus	25/49 (51%)	34/50 (68%)	15/24 (62.5%)
FCoV		35/50 (70%)	7/8 (87.5%)
FIV		9/50 (18%)	
FELV		5/50 (10%)	

3.2.3 Genetic characterisation of coronaviruses

One of the objectives of the virological survey at the municipal kennel was also the genetic characterisation of the viruses found. While there are plans to continue this beyond the scope of this dissertation, the only viruses which we had the possibility to genotype during this time were the canine and feline coronaviruses. The CCoV were readily characterized using the method and primers described by Pratelli et al. (2004b). Withinin the 49 total samples 16 (32.6%) were positive for CCoV RNA, from which 7 (43.8%) samples were genotyped for type I, and 9 (56.2%) for type II. None of the samples tested positive for both genotypes. All of these positive samples were re-amplified and visualized on a 2.5% agarose gel for better resolution between CCoV type I and type II (Figure 11-a).

Regarding FCoV, 35 positive samples for FCoV RNA (70%) out of the 50 collected from cats were subjected to a second RT-PCR for discrimination between genotype I and II. This resulted in 9/35 (25,7%) samples which amplified FCoV type I, 6/35 (17%) where FCoV type II was detected and 12/35 (34.3%) samples which showed co-infection with both types. No amplification was obtained from 8 (23%) samples (Figure 11-b).

Figure 11 - PCR results of genetic characterisation of CCoV (a) and FCoV (b).



CCoV (a): lanes 1-2, negative controls; lanes 3-11, CCoV type II; lanes 12-18 CCoV type I. FCoV (b): odd lanes, RT-PCR sample prior to nested PCR, with no amplification; even lanes, nPCR results showing infection with type I (lanes 2, 4 and 8), type II (lane 10) and both types (lane 6). M, molecular size marker (100 bp ladder).

4. Discussion and conclusions

A quick search for articles containing the term “virological survey” on PubMed results in more than ten thousand hits, with almost one tenth of them written in the last year. A more detailed search shows there is a lot of diversity among the published works which attests to the variety of information which is possible to gather from these studies.

However, the number of these surveys which aim to ascertain the presence of a particular disease in a population is actually diminishing, in favour of molecular surveys, which genetically characterize and classify the viruses found. This happens because the technology for phylogenetic analysis is cheaper and easier to perform with each and every day, and because we are more and more certain that there is no way of escaping the presence of these viruses in any population. Studies regarding viral evolution and phylogeny can help determine how we can best deal with these pathogens, especially true in the case of high risk populations, such as animal shelters, kennels and wildlife conservation.

In our study we determined by RT-PCR a prevalence of 8% for CDV, which means that 4 animals out of 49 tested had not only come in contact, but were actually shedding the virus in the faeces at the time of sampling. The published literature typically reports the prevalence of antibodies to CDV, which means the animals have come in contact with the disease, but may or may not be shedding virus. Since the 1950's there have been live attenuated vaccines for CDV which have effectively reduced the incidence of the disease, especially in Europe and North America, and so most of the virological surveys have focused on stray and unvaccinated dogs, or wild animals. In Turkey Gencay et al. (2004) evaluated sera collected from 609 dogs by virus neutralization assay and reported a seroprevalence of 9.03%. Most of the animals were housed in shelters or rehabilitation centres with no clear vaccination history, but the highest antibody titres were also below the considered protective level. In Zimbabwe unvaccinated dogs in communal lands were tested by ELISA for antibodies to CDV, resulting in 82% prevalence, which means that not only are these rural dogs commonly exposed to the virus, but also that a substantial number survive (Kelly, Musuka, Eoghin, Tebje-Kelly & Carter, 2005). The prevalence of antibodies detected by indirect fluorescent assay in Iran was 17.52% in 97 samples taken from unvaccinated dogs in the rural areas surrounding the city of Ahvaz between 2004 and 2005 (Avizeh, Shapouri & Akhlaghi, 2007). A higher prevalence of CDV detected by RT-PCR (73.8%) was reported by Calderon et al. (2007), by analysing 99 samples submitted during 2003 and 2004 for diagnostic purposes. In spite of the fact that most of the samples were obtained from animals which had been vaccinated at least once, 89% of the positive animals showed typical symptoms of CDV, confirming clinical disease which might be caused by the emergence of viral variants, or by improper vaccination. Dezengrini, Weiblen and Flores (2007) reported an antibody prevalence of 27.3% in 817 sera

collected from unvaccinated animals in several neighbourhoods of Santa Maria, Brazil, which were submitted to a virus neutralization assay. On Isabela island, in the Galapagos, vaccination of dogs and cats, as well as movement between the islands and continent is forbidden due to the Galapagos unique biodiversity and conservation status, which believes that these and other introduced species are the greatest threat to the local wildlife. Levy et al. (2008a) hypothesized that due to this isolation, the population of dogs and cats would be protected from diseases common in the mainland, but nonetheless reported a prevalence of 22% for antibodies to CDV, determined by virus neutralization.

Taking into account previous studies and characteristics of the study population, which has a high density and a high turnover, the prevalence of this virus in the animals at the municipal kennel is not particularly high. One of the reasons for this finding might be the fact that animals in and around Lisbon area are commonly vaccinated. However, another explanation could be that the younger dogs which are more at risk of developing the disease weren't part of this study because of the sampling method (euthanasia). It is possible that the positive animals we found had chronic infections characterized by viral persistence and low shedding exacerbated by the stress of capture. It would have been interesting to evaluate antibodies titres to CDV by ELISA, but the lack of information regarding the vaccinal status of the animals could have lead to a misleadingly high number. In spite of the high lability of CDV virions, we found evidence of environmental contamination by viral RNA in a cell with a possibly infected animal, which was euthanized, but not tested. During the following two weeks after this dog's euthanasia, environmental swabs taken from the cell tested positive for viral RNA, indicating that sanitization procedures weren't optimal.

Recent reports of vaccination failures and detection of viral variants distinct from the vaccine strains have resulted in an effort by researchers to characterise the CDV from different populations. The high genetic diversity of RNA viruses has been implicated as responsible for this phenomenon, but only recent phylogenetic studies have proved without a doubt that there are genetically different strains of CDV in circulation worldwide. An interesting report by Lednicky et al. (2004) identified more virulent strains responsible for a higher mortality rate among free-ranging raccoons around a large Chicago zoo, which were distinct from typical American CDV lineages. The same distinct strains may also have been responsible for a serious CDV outbreak among shelter dogs in the Chicago area in late 2004, affecting also vaccinated dogs, according to Kuehn (2004). Pardo, Johnson and Kleiboeker (2005) also characterised CDV strains which caused the death of puppies in the USA and concluded that none of the strains had ever been previously detected in continental America and might have originated from non-canine species with phylogenetic relationship to European seals, minks and even Chinese pandas, thus explaining why the vaccinated puppies were not immunised. In Italy, several strains from dogs and one fox were analysed phylogenetically and clustered

in three distinct lineages (Martella et al., 2006). The sequence of the H gene was used to identify lineages, named for the geographic location of their isolates, and subsequent studies have mostly used the same nomenclature. The majority of samples displayed high genetic homogeneity with the European lineage, but unexpectedly, two unrelated samples, including one from a vaccinated pup, were more closely related to the Arctic lineage, raising questions on the epidemiology of these unusual strains and whether they represent occasional findings. The fox strain was only distantly related to the European lineage suggesting the existence of non-urban epidemiological cycles responsible for the maintenance of these atypical CDV strains in wildlife. Further studies added more details, and more branches to the CDV phylogenetic tree. Argentinian isolates formed a separate lineage, which was more related to Asian strains (Calderon et al., 2007), while Hungarian isolates were diverse, with the most prevalent lineage being the Arctic (Demeter et al., 2007). The authors suggested that this heterogeneity may be caused by the lack of geographical barriers and uncontrolled movement of domestic and wild animals throughout Europe. Kapil et al. (2008) analysed seven samples from dogs which had died of CDV in the USA and also grouped them in the European wildlife and the Arctic lineage, showing that movement of dogs infected with different strains is also intercontinental. Further studies are needed in order to better understand if the rise of novel CDV strains caused by antigenic drift and shift are truly responsible for the recent reports of vaccination failure.

Canine coronavirus RNA was present in the faeces of 33% of the 49 animals sampled from the municipal kennel in our study. As for CDV, most of the studies published investigate the presence of antibodies to CCoV. Vaccination against coronavirus is still uncommon in most countries and in fact controversial due to adverse postvaccinal reactions and low efficacy of engendered immunity (Pratelli 2006). The duration of immunity is still to be determined, but antibodies caused by natural exposure seem to decay rapidly (Zarnke et al., 2001). In a UK rescue kennel, the virus was isolated by Tennant, Gaskell, Jones and Gaskell (1993) in 45% of faecal samples collected, from dogs with and without diarrhoea, but the seroprevalence in the same kennel was 76%. In 1999, Bandai et al. performed an extensive virological and epidemiological study to characterise the situation in Japan regarding CCoV infections. The seroprevalence reported for 467 dogs in animal shelters was 44.1% and viral RNA was detected by RT-nPCR in 16% of 100 stool samples from diarrhoeic dogs. Naylor et al. (2001) developed an indirect ELISA for the detection of IgG and IgM with the objective of surveying antibodies to CCoV among different dog populations in Australia and evaluating the role of this virus as a causative agent of gastroenteritis. Out of 1396 serum samples analysed, 40.8% of the animals housed in kennels had antibodies to CCoV, as opposed to only 15.8% of the animals housed in the open. The role of IgM to help determine recent infections was

shown by the high prevalence of these antibodies in animals with clinical signs of gastroenteritis (85%), in comparison to 15% in dogs without any clinical history of gastroenteritis, which still had a 30% seroprevalence of IgG. In Turkey, Yeşilbağ, Yılmaz, Torun and Pratelli (2004) tested 179 dogs by serum neutralization assay, yielding 62.5% positive samples, and further 90 samples from diarrhoeic puppies were assayed by RT-PCR resulting in 15.5% positives. In 2005, Sokolow et al. (2005) performed an epidemiological evaluation of diarrhoea in dogs in an animal shelter in California, USA, collecting faecal specimens from 60 case dogs (with diarrhoea) and 60 control dogs. Detection of CCoV was done by RT-PCR and 73.3% of case dogs tested positive, as opposed to 59.3% of the control dogs, showing that there was no significant difference and CCoV was not associated with diarrhoea, but instead was nearly ubiquitous in the kennel population. Priestnall, Pratelli, Brownlie & Erles (2007) assessed the serological prevalence of CCoV in southern Italy and analysed its epidemiologic relationship with CRCoV. They concluded that the seroprevalence of CCoV depends heavily on the sampled dog population with 97% of positive pups from shelters with outbreaks of enteric disease, compared to 86.1% of adult dogs and 73.4% from a previous study in the same area. The authors also suggested that the high prevalence was caused by continuous reinfections from the environment or by the development of chronic infections in some animals. In Santa Maria, Brazil the seroprevalence of CCoV was 50.4% in the previously mentioned study by Dezengrini et al. (2007). Recently, Stavisky et al. (2009) carried out a cross-sectional study survey of randomly selected veterinary practices from throughout the UK targeting both diarrhoeic and asymptomatic dogs for detection of CCoV RNA by RT-PCR in faecal samples. This resulted in a prevalence of 2.8% positive dogs (7 out of 249), of which 3 dogs lived in the same household and none had presented at the veterinary practices with clinical signs of diarrhoea. Confronted with a prevalence which was lower than expected, the authors suggested that the variability of prevalence estimates depend on factors such as the detection methods used, the characteristics and disease status of the dog population under study and the possibility that CCoV prevalence in a population might fluctuate over time.

The prevalence of CCoV infections at the municipal kennel in Lisbon is in agreement to other studies which state that the virus is practically endemic in kennel populations. While the lack of widespread vaccination means that positive serological results will almost surely indicate exposure to the pathogen, the fact that antibodies to CCoV seem to decay rapidly, questions the usefulness of these methods. The high turnover rates of animals in shelters seems to assure the establishment of the virus in susceptible populations, either by chronic infections with continuous viral shedding, or by repeated reinfection from the environment and other dogs. None of the environmental samples collected from the dog cells tested positive for the presence of CCoV, but this can be simply caused by the small number of cages sampled.

Eight of the positive animals in our study also tested positive for CPV, which is in agreement with the involvement of CCoV in mixed infections. Although this finding can be due to an important environmental presence of CPV, none of these animals had clinical history of diarrhoea, supporting the idea that CCoVs aren't usually related to clinical disease in adult dogs. The role of this agent in the epidemiology of gastroenteritis is still poorly understood, but it is clear that co-infections with other agents, such as CPV, can be responsible for mortality and efforts must be made to reduce its prevalence in kennel populations.

There has been renewed interest in the genotyping and molecular characterisation of CCoVs, because of its close relationship with the feline coronavirus, which can be responsible for fatal disease in cats, and also because of the recent discovery of a group 2 canine coronavirus (CRCoV) which is closely related to the SARS-CCoV affecting humans. Recent studies identified different CCoV genotypes, whose genetic and pathogenic characteristics are not yet fully understood, but which already seem to be widespread in the dog population. Pratelli et al. (2003a) first described the genetic diversity found among CCoV isolates from dogs with diarrhoea in Italy by sequencing and analysis of the S gene, showing that there were samples which were highly divergent from the reference strains and more closely related to FCoV type I. In a subsequent study, Pratelli et al. (2004b) designed an RT-PCR assay which allows the differentiation of both genotypes. They confirmed the wide diffusion of both genetic lineages as well as the occurrence of simultaneous infection with both genotypes and reported prevalences of 14.5% for CCoV type I, 8.7% for CCoV type II and 76.8% for both. In Sweden, Escutenaire et al. (2007) performed a phylogenetic analysis on both the M and the S gene amplified from samples of diarrhoeic dogs with intriguing results. Most samples clustered closer to the CCoV type II identified in Italy based on the M gene sequences, but two samples showed contradictory clustering based on the S gene, supporting the idea that co-infection with genetically different viruses favours the occurrence of recombination events, and that viral populations in the same environment may be complex and represented by distinct genetic variants.

The distribution of CCoV genotypes found in our study was very different than previously reported, since the prevalence of both genotypes was almost the same (43.8% to 56.% for types I and II, respectively) and none of the animals was positive for both genotypes. The kennel environment could provide these viruses with extensive opportunities for recombination events because of the high turnover and density of animals, including opportunities of infection by feline and canine coronaviruses in the same animal. Unfortunately, these results would only be further clarified by sequencing and extensive phylogenetic analysis of the collected samples, which was not feasible at the time.

The evolution of parvoviruses and their diagnostic has changed in the last 30 years, and the emergence of new viral variants have caused this disease which was once controlled by vaccination in domestic animals, to be the target renewed research efforts. This is one case where most of the current scientific literature focuses on molecular methods and phylogenetic analyses and most of the studies on the disease prevalence are geared toward wildlife and wildlife conservation. In our work we detected a prevalence of 51% in dog samples and 68% in cat samples from the municipal kennel analysed by PCR. One of the earliest studies on seroprevalence of CPV was undertaken in Ontario, Canada, by Carman and Povey (1984), during the years after CPV-2 was first isolated. Canine sera from dogs presented to the Ontario Veterinary College between 1976 and 1980, were assayed for CPV antibodies using a hemagglutination inhibition (HI) test. The first positive sera are from 1978, the same year as the first isolation of the virus in the USA, and by 1980 there were 31.2% of dogs with antibodies to this new virus. Dezengrini et al. (2007) submitted sera from unvaccinated dogs in Santa Maria, Brazil, to an HI assay which resulted in a prevalence of 68.7%. According to the authors this number was lower than previously reported in Brazil, but the sampling method used tested only animals which hadn't been vaccinated against CPV. In the Galapagos, dogs and cats submitted to a neutering campaign on Isabela Island, were tested for the presence of antibodies to CPV by HI and to FPV by ELISA by Levy et al. (2008a). In spite of the isolation of these animals the prevalence for CPV was 100% and for FPV 67%. Blanco et al. (2009) assayed the sera of 97 domestic cats in Costa Rica for antibodies to FPV, resulting in 92.8% positives, of which 17.8% had been previously vaccinated with a modified live vaccine.

The prevalence for parvovirus in both cats and dogs at the municipal kennel was particularly high, but not unexpected when dealing with a virus which is shed in such high quantities and is highly resistant to environmental conditions. As expected, there was a strong environmental contamination and only one of the six cages from both dogs and cats had no positive results during our sampling. It was interesting to note that most infections with parvovirus in dogs were sub-clinical and veterinarians at the kennel registered no signs of haemorrhagic diarrhoea for most of the animals. On the contrary, there was a large mortality of cats which they attributed to feline panleukopenia, though no confirmatory diagnosis was available. The contaminated environment precludes any possible hypothesis of previous exposure to the virus and the only way to remedy this situation would have been a different sampling methodology, testing animals upon entry in the kennel, for example. Parvovirus is one of the most difficult pathogens to eradicate in a shelter environment and only concerted efforts combining, testing, vaccination, and sanitization can hope to succeed.

The characteristics of parvoviruses and their recent evolution are still a current topic of interest among virologists and efforts are still made to understand the antigenic differences

and phylogenetic relations between different genotypes. The characterisation of CPV type 2c near the turn of the millennium spurred a new wave of publications which attempted to describe the epidemiology of CPV and its newest genotype, as well as its genetic characteristics. Ikeda et al. (2000), reported that 80% of isolates from cats in Vietnam and Taiwan were of the CPV type, and sequence analysis described a new antigenic type present in the Asian cat population. A genotype with a different mutation was identified in Italy by Buonavoglia et al. (2001) and went on to spread throughout the continent in a couple of years. The new type 2c variant was detected by 2006 in most of the European countries, with high prevalences in Italy, Portugal and Germany (Decaro et al., 2007a; João Vieira et al., 2008), and had also crossed the Atlantic to North America (Hong et al., 2007; Kapil et al., 2007) and South America (Calderon et al.; 2009; Pérez et al., 2007). A recent report from Clegg, Coyne, Gaskell, Dawson & Radford (2009) found evidence of geographic clustering within different cities in the UK when sequencing the full VP2 gene, indicating that the virus suffers continuous mutations in sites other than the key amino acid sites used to identify different types, which may have important ramifications for disease control. Even if the full significance of these mutations and geographic clustering is unknown it is clear that the continuous evolution of parvovirus will eventually allow the emergence of new CPV types which could possibly break through the antibody barrier, meaning that surveillance is of the utmost importance, especially in shelter environments.

Feline coronaviruses are considered ubiquitous pathogens of the cats and the prevalence of FCoV varies considerably between different populations, depending on several factors. Recent studies have focused, therefore, more on the interactions between virus and host and virulence factors causing the fatal feline infectious peritonitis. Seroprevalence has been reported in several studies to be between 80 and 90% in catteries and between 10 and 50% in single cat-households (Herrewegh et al., 1995). There were 70% of cats in the municipal kennel in Lisbon shedding the virus in faeces, as determined by RT-PCR assay. Kiss, Kecskeméti, Tanyi, Klingeborn & Belák (2000) evaluated 113 cats living in urban areas in eastern Hungary which presented at private veterinary practices for routine examination with the objective of determining the prevalence of asymptomatic FCoV carriers. Samples were submitted to an RT-PCR assay targeting a highly conserved region of the N gene, resulting in 31.8% of positive animals, of which 45.2% were pure-bred and 23.9% mixed breed. Luria et al. (2004) evaluated feral cats in Florida for the presence of antibodies to FCoV using immunofluorescence antibody assay and 18.3% of the tested cats had antibodies, most of which had low titres. Based on immunofluorescence tests, Kummrow et al. (2005) reported a 50% prevalence among healthy cats in Switzerland (with no clinical signs associated with disease or FCoV infection) with antibodies against FCoV, representing a more than twofold

increase in prevalence since the previous study (Lutz et al., 1990), which could be caused by differences in sample selection, serologic tests, or reflect a true increase in seroprevalence. In Bursa, Turkey, 100 sera belonging to catteries, community shelters and households were tested by Pratelli et al. (2008) for the presence of antibodies using a virus neutralization assay and the reported prevalence was 21%. Holst, Englund, Palacios, Renström and Berndtsson (2006) analysed 209 serum samples from healthy cats visiting veterinary clinics in Sweden, with the objective of determining the prevalence of antibodies to FCoV in cats without signs of infectious disease. The overall seroprevalence, determined by indirect immunofluorescent tests, was 31% and was significantly higher among pure-bred cats (65% versus 17%) and among cats living in groups of at least five (79% versus 29%). One previous study in Portugal by Duarte et al. (2009 submitted) evaluated samples collected from stray and feral cats captured for a neutering program in the metropolitan area of Lisbon. Stool samples and rectal swabs from 127 cats were evaluated by RT-nPCR for FCoV RNA with 7.1% positives. The authors suggest that the lower prevalences found in this study could be explained by the fact that stray cats aren't subject to confinement, which would reduce the odds of infection when compared to shelters and multi-cat environments.

Regarding FCoV, the prevalence found was higher than reported in other countries and significantly higher than previously found in stray cat population in Portugal (Duarte et al., 2009, submitted). Ever since the discovery of the relationship between feline coronavirus and the fatal disease feline infectious peritonitis, there have been increasing efforts to control the viral spread by segregation and sometimes vaccination, but the single most important measure in the fight against this virus is strict hygiene. Ideally, cats should be housed separately, but the large number and heavy rotation of animals in the municipal kennel makes it difficult to implement an efficient sanitization procedure. Most of these stray cats may have never experienced FCoV infection before entering the kennel premises and there is a heavy presence of viral nucleic acid in the environment, but since the adoption rate of these animals is lower than that of dogs, most of them are euthanized before presenting any possible clinical signs of FIP. Still, efforts should be made to improve the hygiene in the cat rooms at the kennel in order to lower the prevalence of this infection.

There is evidence that both genotypes described for FCoV are able to develop FIP, but contradictory numbers have been reported by researchers worldwide on the prevalence of the two different genotypes, as well as their association with the development of FIP. In the municipal kennel there were 25.7% of cats infected with type I, 17% infected with type II and 34.3% were infected with both types. In Japan, Hohdatsu et al. (1992) determined by competitive ELISA using monoclonal antibodies against FCoV that type I was the most common serotype, with 69% among natural cases of FIP and 100% among asymptomatic animals. However, it was still the largest prevalence of FCoV type II reported to date. As part

of a larger study of FCoV endemic infection in households throughout the UK, Addie et al. (2003) developed an RT-nPCR assay to differentiate the two FCoV genotypes and reported a prevalence of 97% of samples positive for type I. Upon further analysis of the samples by nucleotide sequencing the authors reported mutations which support the idea that there are many geographical differences among FCoV strains and also concluded that superinfection with more than one strain at the same time is uncommon, although possible. Benetka et al. (2004) retrospectively tested samples from cats which had FIP verified by histopathological lesions in Austria. Out of 74 samples, 86% were positive for type I, 7% for type II and 7% were positive for co-infection with both types, suggesting the involvement of both FCoV genotypes in FIP and postulating that contact with dogs excreting CCoV is necessary for the emergence of type II in the field. In Switzerland, Kummrow et al. (2005) confirmed by immunofluorescent assay that the prevalent serotype for FCoV was type I, with 35.1% prevalence among healthy cats and 83% among animals with a confirmed FIP diagnosis. Interestingly, while 4.4% of the healthy cats had higher antibody titres to FCoV type II, 10.5% of the cats in this group and 17% of the animals diagnosed with FIP had identical titres for both serotypes. Unfortunately, the serological tests used in the study did not allow the differentiation between animals infected by both serotypes at the same time or consecutively, or even rule out the the presence of different strains with similar immunological properties. Among diseased animals, antibodies against type I were overrepresented, but not at a statistically significant level. Another retrospective study in Taiwan on clinical specimens collected between 2003 and 2007 from both asymptomatic as well as clinically confirmed FIP cases analysed by RT-nPCR the distribution of FCoV genotypes. Type I was prevalent in 54.3%, type II in 3.6% and both genotypes were detected in 3.3% of the samples, but surprisingly there was a significant correlation between infection with type II and development of FIP (Lin et al., 2008). In Portugal, Duarte et al. (2009) evaluated the molecular epidemiology of FCoV for the first time in the country using samples collected for laboratory diagnosis at the Veterinary Hospital of the Faculty of Veterinary Medicine. Among 57 samples positive for viral RNA, 79% were classified as type I and only 3.5% as type II, while no amplification was obtained from 17.5%. These samples were further analysed by heteroduplex mobility assay and nucleotide sequencing, which revealed that 21% of the samples were positive for viral quasispecies, found within the same animal, but also in samples collected from multi-cat households. While no conclusions could be drawn regarding the association of any particular FCoV genotype with disease, an interesting fact was the confirmation that nucleotide sequences from the type II samples showed a more homogeneous distribution of sequences than type I, including a high similarity between geographically distant isolates from previous studies.

The distribution of FCoV genotypes in our study is unique because of the high prevalence of type II virus and even higher prevalence of co-infections with both types. The majority of FCoV infections reported worldwide are due to type I viruses and it's not known whether type II is transmitted naturally among cats (Addie et al. 2003). While there is evidence that the appearance of FCoV type II is the result of a double recombination event between FCoV I and CCoV II (Herrewegh et al., 1998), some authors are of the opinion that animals have to be infected by both parental viruses in order for type II to be detected (Petter J.M. Rottier, personal communication, 24th August, 2009). It is a known fact that several group 1 coronaviruses cross-react in virus neutralization tests, to the point that Horzinek, Lutz and Pedersen (1982) suggested that TGEV, FCoV and CCoV be regarded as host range mutants. Recently, Le Poder et al. (2009) were able to identify CCoV type I in a naturally infected cat living with a dog, infected with the same strain. On the other hand, reports of cats with no known contact with dogs infected with type II viruses exist, and the high degree of similarity between the sequences of isolates from geographically distinct locations lead us to believe that this newer genotype has spread into the cat population and occurs naturally. The implications for our study are important, because these animals are living in a very closed environment where there are plenty of chances of infection by both viruses, in both hosts. Further studies should be conducted to analyse the strains present in this population, but there is sufficient evidence that superinfection of cats with multiple strains of the virus is possible in a heavily contaminated environment. The nucleotide sequencing would be especially useful in cases where the RT-nPCR assay could not amplify either genotypes, which could be due to the presence of quasispecies which the primers do not detect. It is probable that the evolution of these viruses will reach a point in the future where it is no longer possible to separate them into neatly arranged genotypes, but only in clusters with varying degrees of phylogenetic relationship. It would have also been interesting to know more about the role of double infections in the development of FIP, but unfortunately we had no clinical data available on these animals to reach any conclusion.

FIV has been demonstrated to be endemic throughout cat populations worldwide by various serological surveys since its discovery in 1986, and seroprevalences are highly variable between regions. While a vaccine against this disease is available, it is actually not recommended by the most recent vaccination guidelines from the World Small Animal Veterinary Association (WSAVA), because of its interference with the most common diagnostic tests (Day et al., 2007). Cat sera in our study were assayed for the presence of antibodies to FIV by ELISA which resulted in a seroprevalence of 18%. In Madrid, Arjona et al. (2000) evaluated one group of healthy cats and another group of cats which presented typical signs of retroviral infection for the presence of antibodies against FIV. Among the cats

in the healthy group, 8.3% were positive, while 13.9% of the sick cats were FIV positive, which proved that an important percentage of cats infected with FIV have no clinical signs. Almost two thousand free-roaming cats admitted to neutering programs in the USA were tested by ELISA for antibodies against FIV. In spite of the fact that these cats were considered a high-risk population, the overall prevalence of 3.5% is similar to infection rates reported for owned cats (Lee, Levy, Gorman, Crawford & Slater, 2002). Owned cats referred to veterinary hospitals in Japan were assayed for FIV antibodies using a commercial kit and among 1088 animals, 9.8% tested positive. The same study showed a significantly higher seroprevalence in males and cats with outdoor access (Maruyama et al., 2003). In Florida, USA, Luria et al. (2004) sampled 553 feral cats as part of a TNR program and evaluated the presence of antibodies to FIV, with a prevalence of 5.2%. They concluded from the similar prevalences that the cats assayed in the study did not present a higher risk to domestic cats than pet cats. Little (2005) tested a total of 246 cats from several different groups in Ottawa, Canada, for antibodies to FIV using an in-hospital ELISA kit, resulting in 11% positives and a similar prevalence for both the feral and client-owned group. One of the largest seroprevalence studies ever performed on retroviral infections in cats was undertaken by Levy et al. (2006), where 18038 samples from 345 veterinary clinics and 145 animal shelters were tested using a commercial ELISA kit and risk factors were analysed. The seroprevalence for FIV was 2.5% and the risk factors identified are in agreement with previous studies which indicate male cats, with outdoor access as the highest risk population. However, cats in all categories were considered at risk and current guidelines to test all cats with unknown status and again in the event of sickness should be followed whenever possible. Very different prevalences were reported by Norris et al. (2007) in Australia, where feral cats from two colonies showed a seroprevalence of 21 and 25%, while the prevalence in cattery-confined pedigree cats was nil and the prevalence in the pet population sampled from veterinary clinics was 8%. The authors cite population density as one explanation for the higher prevalence found in the feral cats in this study, and the efficacy of FIV screening and an indoor lifestyle as preventive measures against the spread of this disease. During the year of 2004, Murray, Roberts, Skillings, Morrow and Gruffydd-Jones (2008) collected test results regarding FIV prevalence from 7098 cats which entered adoption centres in the UK, with an overall value of 3.1% positives. In the previously mentioned study by Blanco et al. (2009) in Costa Rica, the seroprevalence for FIV was 8.8% with a higher prevalence in male cats. Gleich, Krieger and Hartmann (2009) recently published the results of 10 years worth of FIV testing in Germany, which they used to conclude that the FIV prevalence of 3.2% did not change significantly during the study years and identified once again the most important risk factors as male cats with aggressive behaviour and outdoor living style. Furthermore, they determined from interviews with the

owners of infected cats that the median survival time for FIV positive animals was not significantly different compared to non-infected cats. In the recent survey in Portugal by Duarte et al. (2009, submitted), the prevalence of stray cats from the Lisbon urban area with FIV antibodies was 13.8%.

The prevalence of FIV in cat populations seems to depend greatly on the characteristics of the study population, as well as their past exposure to the agent. Lifestyle, gender and health status of the animals affect the infection rate and there seems to be a noticeable difference in prevalences between Northern Europe and Southern Europe, where higher prevalences are reported. The infection rate in our study was higher than previously reported in Madrid among healthy owned cats (Arjona et al., 2000), but similar to the prevalence reported recently in stray cats in Lisbon (Duarte et al., 2009 submitted), the same area where the animals in the kennel were captured from. Since the virus survives only minutes outside the host and is susceptible to all disinfectants we didn't test for its presence in the environment and judging from the average time these animals spend at the kennel, it's reasonable to conclude that the positive samples came from cats infected prior to capture. Portuguese FIV isolates display a unique viral genetic diversity where most samples group within a subcluster of the subtype B and a few sequences seem to cluster within a newly proposed subtype F (Duarte & Tavares, 2006). The sequence analysis of the samples from stray and feral cats at the municipal kennel could provide a better understanding of the molecular epidemiology of FIV in the country and perhaps support the assignment of a new subtype.

The improved understanding of FeLV pathogenesis, specially of "carrier" states, along with the introduction of reliable vaccines and diagnostic procedures aimed at identifying infected animals and preventing disease spread, have contributed to the decrease in the prevalence of this disease over the last 25 years. Recent advances in molecular techniques have added a whole new dimension to the diagnostic of this disease and its latent infections, but in practical terms the most popular techniques are the ELISA and immunochromatography assays for detection of p27 antigen. Due to the importance of this disease in both in domestic as well as in wild felines, and its close relation to FIV, they are usually tested together and most of the surveys referred next have been mentioned previously. The proportion of cats from the municipal kennel in Lisbon which tested positive to the presence of FeLV antigen by ELISA was 10%. Previous studies have reported prevalences of 15.6% in healthy cats and 30.4% in sick cats in Madrid (Arjona et al., 2000), while in the USA this value was 4.3% (Lee et al., 2002). In Japan, this prevalence was 2.9% in owned cats (Maruyama et al., 2003), 3.3% in feral cats in Florida (Luria et al., 2004) and 2.3% in a large sample including owned and shelter cats from all over the USA (Levy et al., 2006). Blanco et al. (2009) reported a prevalence of 16.7% among owned cats in Costa Rica at a time when the vaccine still wasn't

available in the country, while Gleich et al. (2009) reported a significant decrease in the prevalence of FeLV in Germany over a 10 year study, from 6% to 1% between 1993 and 2002, with an overall prevalence of 3.6%. The latest survey in Portugal among stray and feral cats reported a prevalence of 7.7% positive cats, with a larger proportion of infected animals younger than one year (Duarte et al., 2009, submitted).

The number of infected cats at the municipal kennel reflects the fact that these are stray animals, which aren't tested, vaccinated or segregated to keep prevalences down, but they are also similar to previous reports from the Iberian Peninsula. Just like FIV, the virus doesn't survive long outside the host and it's easily inactivated, which is why we didn't test for it in the environment. Also like FIV, it takes a few weeks for viraemia to develop, leading us to conclude that these animals were probably infected prior to capture and not in the kennel premises. FeLV infection has typically been classified as either regressive or progressive, and later also latent infections, but recent advances in molecular techniques have refined the spectrum of possible host response categories (Hofmann-Lehmann et al., 2008). Techniques like real time and quantitative PCR are used to investigate viral plasma RNA and provirus DNA loads even in the absence of antigenaemia, providing important insights into the pathogenesis of this disease. However, until the clinical relevance of this provirus-positive status is clarified, the recommended procedure is still to test for viraemic carriers, and remove them.

During the course of this study most of the methods used to assess infection were molecular, such as PCR or RT-PCR, and serology was only used for the detection of FIV antibodies and FeLV antigen. There is a growing, and in some cases worrying, tendency to forego classical virology techniques in favour of molecular techniques. Both have advantages and disadvantages, but it is only when we integrate both approaches that we can truly hope to reach new heights of understanding. For example, propagation of viruses in cell cultures is still an important step towards understanding pathogenesis and immunologic reactions, and an essential step for vaccine production. Serological techniques are only a measure of past exposure to a pathogen, but molecular techniques can sometimes detect attenuated viruses from vaccines which are not indicative of natural infection or disease. In the case of coronaviruses, it has been determined that RT-PCR is the best method for diagnostic, since the immunity conferred by previous infections doesn't last long and vaccination in both dogs and cats is still uncommon. The most currently used vaccines for both CDV and CPV/FPV are attenuated or modified live vaccines, but according to previous studies, the virus is only shed for a few days after vaccination (Kim et al., 2001; McCaw & Hoskins, 2006). Adding to that the fact that most of the animals in this study were captured directly from the street, we can assume that none of the samples had virus from a live vaccine. The only way to be

absolutely sure though, would be to sequence the samples and compare them to the vaccine strains.

All the analysed samples were from animals euthanized. One of the euthanasia criteria is the likelihood to be adopted, so there is a chance that results were biased towards a high prevalence of disease by choosing animals which demonstrated clinical signs of illness. Yet, from the information gathered from the animals clinical history, most were “apparently normal” on observation, and were chosen for euthanasia for their age or aggressive behaviour.

Since most of these animals were strays, one could argue that they should have come in contact with these agents during their early life and be immune to infection later on. This is especially true for viruses which provide life-long immunity like CPV/FPV. With other viruses like CDV and FCoV, the stress of capture could have induced immunosuppression and reactivated latent infections. Interestingly, all animals positive to CDV RNA were also infected with CPV and three of them with CCoV, which might explain viral reactivation and subsequent excretion. In these cases, it would be interesting to evaluate the titre of IgM antibodies against CDV and CPV, to find out if one of them was the result of a recent infection.

The concept of One Health recognizes that human, animal and mental health are inextricably linked and one aspect of this initiative are the joint efforts to better understand cross-species disease transmission, as well as joint cross-species disease surveillance and control efforts in public health. The viruses chosen for this study have no proven zoonotic impact, but they are nonetheless relevant in this concept, because of their ability to cause terminal illness in multiple species of animals, both wild and domestic, meaning their presence and status among different wildlife and domestic populations should be assayed. Baseline information on potential pathogen exposure is critical for monitoring the population health of threatened wildlife species. One of the objectives of this study was to determine whether the stray and feral animals in the urban area of Lisbon could act as reservoirs for these different viruses.

Two particular studies related to domestic animals and their interaction with wildlife have proven that domestic animals represent a disease risk for wildlife. Fiorello et al. (Fiorello, Noss & Deem, 2006; Fiorello, Noss, Deem, Maffei & Dubovi 2007) studied the relationship between hunting dogs used in the Isoso region of Bolivia and the wildlife found in the contiguous Kaa-Iya Del Gran Chaco National Park. They discovered a high seroprevalence of CPV and CDV (over 95%) associated with high turnover in the domestic dog population and a high number of opportunities to encounter wildlife on hunting trips in the adjacent areas to the national park. Accordingly, several small carnivores sampled directly from the area of the national park, showed antibody titers to CDV, CPV and FPV, among others.

The effect of these pathogens on naive wildlife populations can have disastrous effects. In 1999 a suspected canine distemper epidemic killed 95% of the island fox population on Santa Catalina Island, California, USA (Timm et al., 2009). The comparison of seroprevalences between previous years and the evidence derived by PCR from a recovered carcass implicates canine distemper brought by the introduction of mainland raccoons on the island as the cause for the population decline.

Canine distemper and its related viruses have caused a number of epidemics around the world in the last 20 years. In total, over 50000 harbour seals died in two separate occasions around Europe, in 1988 and again in 2002 (Harris, Travis & Harwood, 2008). In both cases the virus identified is closely related to CDV and has also been proven to infect terrestrial carnivores, namely minks (Blixenkronne-Möller et al., 1992). The complex virus-host relationship necessary to cause such an epidemic is far from being completely understood. Another two examples have been studied in lions in Africa where two different populations, the Serengeti lions in 1994 and the Ngorongoro Crater lions in 2001, suffered severe mortality caused by CDV. Further serological analyses indicated that at least five “silent” distemper epidemics swept through the same two lion populations between 1976 and 2006 without causing clinical signs or increased mortality. Munson et al. (2008) related data from clinical and pathological findings, as well as climate information, to determine that these epidemics with high mortality happened in years after extreme droughts, when herbivore numbers were lowest and hemoparasitism by *Babesia* was highest in both lions and herbivores. The magnified effects of the climate changes and immunosuppression caused by coincident CDV infection led to the unprecedented mortality. Craft, Volz, Packer and Meyers (2009) determined by modelling a contact network based on detailed behaviour and movement data that although it is possible for Serengeti lions to sustain epidemics of CDV and similar infectious diseases, the 1994 epidemic was caused by spillovers from other carnivore species, such as jackals and hyenas.

Recently, the discovery of canine distemper in free-ranging felids in Brazil has also been linked with the presence of domestic dogs in the same area (Nava et al., 2009). All of the domestic dogs sampled in the Ivinhema State Park in Mato Grosso do Sul, Brazil, were seropositive to CDV and 60% of the total jaguar population at that park also had antibody titers to CDV. These results provide evidence of a spillover effect at the state park as the jaguar territories seasonally overlap with the nearby cattle grazing lands and provide opportunities for interaction between the two species. The finding leads once again to the eminent need for systematic health monitoring of wild carnivore populations and associated domestic carnivores.

Goldstein, Mazet and Gill (2009) have also recently found evidence of the introduction of phocine distemper in Northern Sea Otters in the Pacific Ocean close to Alaska, USA.

Sequence analysis from samples recovered between 2005 and 2008 has shown that the viral fragments amplified were identical to the isolates from the European epidemic of 2002. The authors propose that the sea ice reduction may have resulted in contact between Atlantic, Arctic and Pacific Ocean species which was not possible prior to the 2002 epidemic and additional studies are needed to determine whether PDV has played a role in the reduction of the sea otter population and what risk it represents to other susceptible species in the Pacific Ocean.

In Germany, Frölich et al. (2000) collected sera and tissues of hundreds of free-ranging carnivores such as foxes, martens and minks in order to compare the seroprevalence of CDV in different areas. They concluded that there was a significant difference in numbers of seropositive foxes between urban, suburban and rural areas, with a greater number in urban areas. Assuming a positive correlation between human population density and the density of domestic dogs this is another evidence for the association between contact with domestic animals and a higher number of seropositive reactors. A similar study was conducted by Riley, Foley and Chomel (2004) where grey foxes in two different areas of Golden Gate National Recreation Area, in California, USA, were radio-tracked and tested for the presence of antibodies to CPV. The seroprevalence to CPV was higher in foxes in the urban area (63% versus 21% in the rural area) and the only seropositive foxes in the rural area were radio-located in a town on the border of this zone.

In Africa several different species have been identified as susceptible to infection by these viruses. Free-ranging jackals in Kenya were found to be seropositive to CPV (34%) and CDV (9%) by Alexander, Kat, Wayne & Fuller (1994). In the same year, Alexander and Appel (1994) published findings of a CDV epizootic in domestic dogs near the Masai Mara National Reserve, Kenya which they believed led to the disappearance of at least two packs of African wild dogs inside the reserve. They based this decision on the fact that the seroprevalence of CDV in domestic dogs rose substantially between 1990 and 1991 (from 1% to 76%), while all African wild dogs, which were seronegative between 1989 and 1990, disappeared by the beginning of 1991. While no carcasses were recovered which could help ascertain a causal relationship between the CDV epizootic and the decline in wild dog population, the importance of monitoring populations of domestic animals which are in close contact with endangered species was established. In 2008, retrospective data collected by Alexander et al. from over a thousand animals went on to identify widespread exposure to a number of multi-host canine and feline pathogens among domestic dogs and free-ranging predators in four countries across both protected and unprotected areas in southern Africa. Wild dogs showed evidence of exposure to CDV, CPV, CCoV, among other viruses, with CPV having the lowest prevalence. Lions also showed evidence of widespread exposure to CDV, FPV, FCoV and FIV, among others, and the highest prevalence was for feline herpesvirus,

followed by FIV. While in this study spotted hyenas were found to be seropositive only to CDV (24%), Harrison et al. (2004) discovered antibodies to CDV (47%), FIV (35%), FPV/CPV (81%) and FCoV (72%) in the population of the Masai Mara National reserve. Besides rabies, CDV was found to be probably the most important pathogen affecting large predator populations in southern Africa, in spite of the fact that seropositive animals were found in areas isolated from domestic animals. This led to the conclusion that all susceptible species populations together could constitute a maintenance community irrespective of the size of a particular species component. In this scenario, even small and low density domestic dog populations could provide the pathogen flow to allow invasion into a diverse community of susceptible hosts which together reach the critical community size required for pathogen invasion and onward transmission. Further research is needed to better understand the potential role of host population structure on divergent patterns of mortality seen with CDV outbreaks in predator populations in Africa, possibly explaining why the lion population seems to have remained remarkably stable during the past 25 years, while others did not. Outside of Africa, several surveys have focused on these viruses in wildlife, especially in felids. In Brazil, Filoni et al. reported in 2006 the first infection of free-ranging felids by FPV. Sera from pumas, ocelots and spotted cats were tested for the presence of antibodies to FPV, FeLV, FCoV and FIV, with respective prevalences of 48%, 10%, 5% and 5%. On Mauna Kea, in Hawaii, feral cats responsible for the decline of other endangered species, specially birds, were shown to be FIV antibody positive (9%) and FeLV antigen positive (16%) (Danner, Goltz, Hess & Banko, 2007). In Taiwan and Vietnam, endangered species such as the leopard cat and the Formosan gem-faced civet were shown to have high antibody titers to FPV and in spite of this it was possible to isolate the virus from peripheral blood mononuclear cells indicating the possibility of a persistent infection with FPV in feral cats in Asia (Ikeda et al., 1999). In Florida, USA, Brown et al. (2008) characterized the emergence of FeLV in a previously naïve, and intensively monitored, endangered Florida panther population, concluding from retrospective data and phylogenetic analysis that the virus was probably introduced from a single domestic cat in 2001, leading to the death of 5 adult animals until 2005.

Closer to our home, in the Iberian Peninsula, the main environmental concerns regarding endangered wildlife are the Iberian lynx, which is considered the most endangered felid species in the world, and the Iberian wolf. There is evidence of widespread exposure of wild carnivores to CDV and CPV throughout Europe. Damien et al. (2002) identified a seroprevalence of 13% in red foxes in Luxembourg and Sobrino, Arnal, Luco and Gortázar (2008) sampled wolves and foxes in Spain and concluded that CPV infection was endemic in the wolf population, with a seroprevalence of 62.2% compared to 5.1% in foxes. The total CDV seroprevalence was 18.7%. In Portugal, an unprecedented high prevalence of

antibodies to CPV was recorded in gennets (94.4%) by Santos, Almendra & Tavares (2009), along with seroprevalences to CDV and CPV in wolf (11.1% and 32.1%) and red fox (9.1% and 14.3%) similar to previously reported. Leutenegger et al. (1999) identified high prevalences of FeLV (75%) in the European wildcat, which shares the same habitat as the lynx, and in Spain, similar prevalences (77%) were reported by Millán and Rodríguez (2009). Subsequent studies have identified cases of FeLV in Iberian lynxes and identified this as well as other pathogens as important threats to the survival of this critically endangered species. Meli et al. (2009) reported the prevalence of FeLV, FPV, FCoV and CDV in the lynx population of the Doñana area as 28.9%, 29.5%, 15.9% and 25%, and one dead animal presented high viral loads of CDV, indicating that the disease may also represent a threat to this species. The authors recommended the implementation of a vaccination program of both lynxes and domestic cats and perhaps even dogs in and around lynx's habitats in order to keep the infectious pressure of these pathogens as low as possible. Recently there have been disease cases of CDV confirmed in lynxes and bobcats in North America (Daoust, McBurney, Godson, van de Bildt & Osterhaus, 2009)

Unfortunately, the lack of information regarding the health status of the animals sampled from the municipal shelter, as well as previous location or possible contacts with wild populations living in and around the Lisbon urban area makes it impossible to draw any conclusion about the role of these animals in spread and maintenance of common canine and feline viruses. An initial testing for infectious agents or a proper quarantine would be advisable to determine which animals are infected at the time of arrival in the municipal kennel.

While the animals in this survey were sampled from an area where there is little concern for environmental issues like disease spillover, the information from these examples show the importance of monitoring the health status of domestic animals which may come in contact with wild populations, specially endangered species, such as the Iberian lynx and wolf. There is a need for surveillance of stray and feral cats and dogs in areas further to the North of Portugal, where the Iberian wolf has its habitat, and around the Reserva da Malcata and the Algarve, where the Iberian lynx will be reintroduced in Portugal. The municipal kennels in these areas would be good candidates for these surveillance actions.

At the risk of straying from the objectives proposed at the beginning of this study, and leaving the field of virology for a moment, it is this author's belief that there are important observations to be made regarding animal shelters and their role in our society. Animal shelters, and especially municipal kennels have gone from a place where unowned pets, rounded up from the streets for bounty, were deposited to be clubbed or drown to death, to the second main source of adoption for dogs (Zawistowski, Morris, Salman & Ruch-Gallie, 1998; Scarlett, 2008). Ironically, 90% of a surveyed population in Italy identifies free roaming

dogs and cats as a problem and cite personal safety and animal welfare as their main concern (Slater et al., 2008). However, we do seem to be taking new steps every day in our quest to regard animals, especially companion animals, as our equals. The Five Freedoms are a reflection of this, but we must also understand that the notion of animal welfare is different depending on our cultural background (Houpt et al., 2007). The issue is complicated and the municipal kennels are in the thick of it, but one thing researchers worldwide agree on is the need to collect more information, and better, regarding pet population and its dynamics. This lack of information became known in the early 1990s as Rowan's "statistical black hole", based on papers by Dr Andrew Rowan, who questioned the expenditure of millions of dollars without data to address even the basic questions of this issue (Scarlett, 2008). While there are still no definitive answers regarding basic issues like the number of pets owned and abandoned, other questions are being resolved. The American Society for the Prevention of Cruelty to Animals (ASPCA) managed the New York municipal kennel for a hundred years, until 1994, and was the first to report a drastic reduction in the number of euthanasias per human population after the introduction of a spay-neuter program in 1972 (Zawistowski et al., 1998). In Taiwan, a recent survey suggested that preventing owners with a history of unsuccessful dog ownership from acquiring dogs was predicted to yield the largest reduction of risk of unsuccessful dog ownership, and that >30% of dog owners felt a male dog would feel shame after being neutered (Weng, Kass, Hart & Chomel, 2006). Several surveys have reported that behavioural problems are one of the main causes of abandonment and euthanasia, and cost and lack of proper counseling are issues preventing owners from having animals neutered (Houpt et al., 2007; Scarlett, 2008). Sterilization has proven to be more useful than euthanasia in the control of pet population, but Natoli et al. (2006) proved that >10 years after the implementation of a "no-kill" policy in Rome, Italy, the success of TNR programmes depend on education of the population to stop abandonment and control reproduction of owned cats in order to achieve control of feral-cat population. In Barcelona, management of the municipal kennel by an animal protection organization which discontinued the routine use of euthanasia was actually followed by a drastic increase in abandonment rates in the region, and unwillingness to perform euthanasia caused CDV to become endemic at the kennel (García-Rodríguez et al. 2008). As we can see, a difficult balance has to be maintained and we must not forget that the welfare of dogs and cats cannot be considered separately from the human social and cultural contexts in which they live. There are suggestions of a reduction in number of euthanasias, which cannot be proven scientifically and counseling at the time of adoption is essential to make a difference (Scarlett, 2008).

The situation in Portugal is quite similar to what has been described, and there are estimates of 1.9 million dogs and 1.5 million cats in the country, but no official statistics to support it.

News reports claim an increase in abandonment rates since 2006, which some speculate is related to the economical situation, but a lot more data is needed on pet population and municipal kennels to help determine the global picture and trends. Epidemiological surveys are an important tool in this aspect, and should help estimate the number of shelters/kennels, reasons for abandonment and euthanasia, as well as identify high-risk populations for unsuccessful pet ownership. More effort should be spent on educating Portuguese pet owners and caretakers than on capturing feral cats, with the sole purpose of euthanasia. International committees, like the European Advisory Board on Cat Diseases (ABCD), the American Association of Feline Practitioners, and the World Small Animal Veterinary Association (WSAVA) have established specific guidelines for the management of infectious diseases in shelter environment. Special attention should be paid to separate housing of untested animals and vaccination of cats against FPV and dogs against CPV and CDV.

With the rapid accumulation of new data comes the task of disseminating results to the appropriate people, explaining their strengths and limitations, and assisting policy makers translate appropriate results into reasonable policy. The term “consequential epidemiology” has been used to describe applied research that has the potential to improve public health policy (Scarlett, 2008), but some question whether this task should fall on the hands of veterinary epidemiologists. In the relatively small circle of people interested in pet issues, if epidemiologists do not assist in interpretation of the results of their studies, it is questionable who can.

For every question answered during our study, more were raised. There is still work to be done involving the samples already collected, but further studies would also require the collection of new samples from more specimens. Hopefully the previous discussion was able to underline the importance of phylogenetic analysis in a world of growing viral genetic diversity. Further characterisation of CDV samples could be attempted by hemi-nested multiplex PCR designed to genotype the five major CDV lineages, differentiated by specific primers targeted to the H gene (Martella et al., 2007). Initial work had begun using this method, but was unsuccessful and more time would be needed to adapt the assay to our laboratory and samples. Another possibility would be to sequence the complete H gene, providing more in-depth knowledge of the characteristics of CDV isolates from the kennel.

The epidemiology of parvoviruses in Europe is slowly beginning to form a picture showing different geographical clustering of samples from different countries, and even different cities within the same countries. The presence of CPV-2c has already been reported in Portugal, but it would be interesting to take advantage of the large number of positive samples from the municipal kennel to learn more about the evolution of this virus. The heavily contaminated environment combined with the high density of cats and dogs creates chances

for cross-species infection, and even recombination between the two host-range variants has been reported (Ohshima & Mochizuki, 2009). The amplification of the full VP2 gene by PCR followed by sequencing would be needed to perform further analysis.

Much of the same is true for coronavirus infections in both dogs and cats. The kennel environment is conducive to recombination events, as seen by the prevalence of co-infection with both FCoV types. Further genotyping and sequencing of the kennel samples would allow us to differentiate between true feline or canine viruses and their recombinants, and provide information on their evolution and how frequently these events occur. Another possibility would be to test dogs for CRCoV, as the prevalence for this recently identified agent hasn't been reported in the Iberian Peninsula yet.

The information gathered from this study will hopefully pave the way for future collaborations with the municipal kennel in Lisbon and other areas. One of the most important points to consider in future collaborations would be the possibility of sampling live animals, in order to evaluate health status upon entry at the kennel and possibly correlate test results with clinical signs observed.

There is a lot of useful information to be gathered from surveys like this, not only regarding virological data, but also epidemiology and animal welfare. Of paramount importance is also the information that can be gathered at kennels and shelters about the reasons for relinquishing and abandoning pet animals, which will help prevent unnecessary culling and lead us to a better understanding and relationship with our four-footed friends.

The municipal kennel of Lisbon is a fertile ground for a variety of different viruses at the moment. Our main priority should be to control and eradicate these agents, where possible, but in the process we should take advantage of this unique environment to better study the interaction between viruses, or virus strains, and their hosts. The results found on this study, particularly on parvovirus and FCoV brought to our attention the need for a continued and more precise evaluation of the health status of free-roaming stray or feral animals in the municipal kennel, to correctly evaluate their role as viral reservoirs within and without the kennel premises. The high prevalence of coronavirus infection found in both dogs and cats in the Lisbon Municipal Kennel allowed some viral genetic characterization, showing a high genetic diversity exemplified by the rate of co-infection with both genotypes of FCoV and presence of both types of CCoV. However, the implications of this high exposure to a virus with the ability to recombine and jump hosts easily is yet to be determined and further investigation is needed in order to maintain a molecular epidemiological surveillance and help identify further CoV strains, as well as understand the pathogenic potential of these viruses.

Outside of urban areas this information is also important for wildlife conservation, specially in the case of the critically endangered Iberian lynx and its reintroduction project. It is this author's opinion that besides typical feline viruses, CDV will also become a very important pathogen to monitor for in carnivores sharing the same habitat as the lynx.

In conclusion, our work is just beginning and a lot more research is needed to improve our knowledge of the dynamics of pet and stray population and how they interact with their viruses both in and out of our homes and animal shelters. Our ultimate goal should be to uphold the principles set out by the One Health Initiative, seeking to prevent epidemic and epizootic diseases, while maintaining the integrity of ecosystems, for the benefit of mankind, domestic animals and biodiversity.

Bibliography

- Addie, D. D., Schaap, I. A. T., Nicolson, L., & Jarrett, O. (2003). Persistence and transmission of natural type I feline coronavirus infection. *J Gen Virol*, *84*(10), 2735-2744. doi: 10.1099/vir.0.19129-0.
- Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., et al. (2009). Feline infectious peritonitis. ABCD guidelines on prevention and management. *Journal of Feline Medicine & Surgery*, *11*(7), 594-604. doi: 10.1016/j.jfms.2009.05.008.
- Addie, D. D., & Jarrett, O. (2006). Feline Coronavirus Infections. In *Infectious Diseases of the Dog and Cat , Revised Reprint* (3rd ed., pp. 88-103). St Louis, Missouri: Saunders.
- Agbandje, M., Parrish, C. R., & Rossmann, M. G. (1995). The structure of parvoviruses. *Seminars in Virology*, *6*(5), 299-309. doi: 10.1006/smv.1995.0036.
- Alexander, K., & Appel, M. (1994). African wild dogs (*Lycaon pictus*) endangered by a canine distemper epizootic among domestic dogs near the Masai Mara National Reserve, Kenya. *J Wildl Dis*, *30*(4), 481-485.
- Alexander, K., Kat, P., Wayne, R., & Fuller, T. (1994). Serologic survey of selected canine pathogens among free-ranging jackals in Kenya. *J Wildl Dis*, *30*(4), 486-491.
- Alexander, K., McNutt, J., Briggs, M., Standers, P., Funston, P., Hemson, G., et al. (2008). Multi-host pathogens and carnivore management in southern Africa. *Comparative Immunology, Microbiology and Infectious Diseases, In Press, Corrected Proof*. doi: 10.1016/j.cimid.2008.10.005.
- Arjona, A., Escolar, E., Soto, I., Barquero, N., Martin, D., & Gomez-Lucia, E. (2000). Seroepidemiological survey of infection by feline leukemia virus and immunodeficiency virus in Madrid and correlation with some clinical aspects. *Journal of Clinical Microbiology*, *38*(9), 3448-3449.
- Avizeh, R., Shapouri, M. S., & Akhlaghi, N. (2007). Antibody Titers Against Canine Distemper Virus in Unvaccinated Rural Dogs from Ahvaz, Iran. *Pakistan Journal of Biological Sciences*, *10*(21), 3970-3972. doi: 10.3923/pjbs.2007.3970.3972.
- Barrett, T. (1999). Morbillivirus infections, with special emphasis on morbilliviruses of carnivores. *Veterinary Microbiology*, *69*(1-2), 3-13. doi: 10.1016/S0378-1135(99)00080-2.
- Beineke, A., Puff, C., Seehusen, F., & Baumgärtner, W. (2009). Pathogenesis and immunopathology of systemic and nervous canine distemper. *Veterinary Immunology and Immunopathology*, *127*(1-2), 1-18. doi: 10.1016/j.vetimm.2008.09.023.
- Bendinelli, M., Pistello, M., Lombardi, S., Poli, A., Garzelli, C., Matteucci, D., et al. (1995). Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin. Microbiol. Rev.*, *8*(1), 87-112.
- Benetka, V., Kübber-Heiss, A., Kolodziejek, J., Nowotny, N., Hofmann-Parisot, M., & Möstl, K. (2004). Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. *Veterinary Microbiology*, *99*(1), 31-42. doi: 10.1016/j.vetmic.2003.07.010.
- Blanco, K., Prendas, J., Cortes, R., Jimenez, C., & Dolz, G. (2009). Seroprevalence of viral infections in domestic cats in costa rice. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, *71*(5), 661-663.

-
- Blancou, J. (2004). Dog distemper: imported into Europe from South America? *Historia Medicinae Veterinariae*, 29(2), 35-41.
- Blixenkroner-Möller, M., Svansson, V., Appel, M., Krogsrud, J., Have, P., & Örvell, C. (1992). Antigenic relationships between field isolates of morbilliviruses from different carnivores. *Archives of Virology*, 123(3), 279-294. doi: 10.1007/BF01317264.
- Brown, M. A., Cunningham, M. W., Roca, A. L., Troyer, J. L., Johnson, W. E., & O'Brien, S. J. (2008). Genetic Characterization of Feline Leukemia Virus from Florida Panthers. *Emerging Infectious Diseases*, 14(2), 252-259. doi: 10.3201/eid1402.070981.
- Buonavoglia, C., Decaro, N., Martella, V., Elia, G., Campolo, M., Desario, C., et al. (2006). Canine coronavirus highly pathogenic for dogs. *Emerging Infectious Diseases*, 12(3), 492-494.
- Buonavoglia, C., Martella, V., Pratelli, A., Tempesta, M., Cavalli, A., Buonavoglia, D., et al. (2001). Evidence for evolution of canine parvovirus type 2 in Italy. *J Gen Virol*, 82(12), 3021-3025.
- Calderon, M. G., Mattion, N., Bucafusco, D., Fogel, F., Remorini, P., & La Torre, J. (2009). Molecular characterization of canine parvovirus strains in Argentina: Detection of the pathogenic variant CPV2c in vaccinated dogs. *Journal of Virological Methods, In Press, Corrected Proof*. doi: 10.1016/j.jviromet.2009.03.013.
- Calderon, M. G., Remorini, P., Periolo, O., Iglesias, M., Mattion, N., & La Torre, J. (2007). Detection by RT-PCR and genetic characterization of canine distemper virus from vaccinated and non-vaccinated dogs in Argentina. *Veterinary Microbiology*, 125(3-4), 341-349. doi: 10.1016/j.vetmic.2007.05.020.
- Carman, P. S., & Povey, R. C. (1984). The Seroprevalence of Canine Parvovirus-2 in a Selected Sample of the Canine Population in Ontario. *The Canadian Veterinary Journal*, 25(6), 259-262.
- Clegg, S. R., Coyne, K. P., Gaskell, R. M., Dawson, S., & Radford, A. D. (2009). Evidence of within-country clustering of canine parvoviruses from clinically-ill animals in mainland UK [abstract]. In *Proceedings of the 8th International Congress of Veterinary Virology: 20 years of ESVV: Integrating classical and molecular virology, Budapest, Hungary, 23-26 August 2009* (p. 106).
- Cornelissen, E., Dewerchin, H., Van Hamme, E., & Nauwynck, H. (2007). Absence of surface expression of feline infectious peritonitis virus (FIPV) antigens on infected cells isolated from cats with FIP. *Veterinary Microbiology*, 121(1-2), 131-137. doi: 10.1016/j.vetmic.2006.11.026.
- Craft, M. E., Volz, E., Packer, C., & Meyers, L. A. (2009). Distinguishing epidemic waves from disease spillover in a wildlife population. *Proceedings. Biological Sciences / The Royal Society*, 276(1663), 1777-1785. doi: 10.1098/rspb.2008.1636.
- Damien, B., Martina, B., Losch, S., Mossong, J., Osterhaus, A., & Muller, C. (2002). Prevalence of antibodies against canine distemper virus among red foxes in Luxembourg. *J Wildl Dis*, 38(4), 856-859.
- Danner, R. M., Goltz, D. M., Hess, S. C., & Banko, P. C. (2007). Evidence of Feline Immunodeficiency Virus, Feline Leukemia Virus, and *Toxoplasma gondii* in Feral Cats on Mauna Kea, Hawaii. *J Wildl Dis*, 43(2), 315-318.
- Daoust, P., McBurney, S. R., Godson, D. L., van de Bildt, M. W. G., & Osterhaus, A. D. M. E. (2009). Canine distemper virus-associated encephalitis in free-living lynx (*Lynx*

canadensis) and bobcats (*Lynx rufus*) of eastern Canada. *Journal of Wildlife Diseases*, 45(3), 611-624.

- Day, M. J., Horzinek, M. C., & Schultz, R. D. (2007). Guidelines for the vaccination of dogs and cats. Compiled by the Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA). *The Journal of Small Animal Practice*, 48(9), 528-541. doi: 10.1111/j.1748-5827.2007.00462.x.
- DeBlois, R. W., & Wesley, R. K. (1977). Sizes and concentrations of several type C oncornaviruses and bacteriophage T2 by the resistive-pulse technique. *Journal of Virology*, 23(2), 227-233.
- Decaro, N., Desario, C., Campolo, M., Elia, G., Martella, V., Ricci, D., et al. (2005). Clinical and virological findings in pups naturally infected by canine parvovirus type 2 Glu-426 mutant. *J Vet Diagn Invest*, 17(2), 133-138.
- Decaro, N., & Buonavoglia, C. (2008). An update on canine coronaviruses: Viral evolution and pathobiology. *Veterinary Microbiology*, 132(3-4), 221-234. doi: 10.1016/j.vetmic.2008.06.007.
- Decaro, N., Desario, C., Addie, D. D., Martella, V., Vieira, M. J., Elia, G., et al. (2007a). Molecular Epidemiology of Canine Parvovirus, Europe. *Emerging Infectious Diseases*, 13(8).
- Decaro, N., Desario, C., Elia, G., Martella, V., Mari, V., Lavazza, A., et al. (2008). Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. *The New Microbiologica: Official Journal of the Italian Society for Medical, Odontoiatric, and Clinical Microbiology (SIMMOC)*, 31(1), 125-130.
- Decaro, N., Martella, V., Elia, G., Campolo, M., et al. (2007b). Molecular characterisation of the virulent canine coronavirus CB/05 strain. *Virus Research*, 125(1), 54-60. doi: 10.1016/j.virusres.2006.12.006.
- Decaro, N., Martella, V., Elia, G., Desario, C., et al. (2007c). Tissue distribution of the antigenic variants of canine parvovirus type 2 in dogs. *Veterinary Microbiology*, 121(1-2), 39-44. doi: 10.1016/j.vetmic.2006.11.005.
- Deem, S. L., Spelman, L. H., Yates, R. A., & Montali, R. J. (2000). Canine distemper in terrestrial carnivores: a review. *Journal of Zoo and Wildlife Medicine*, 31(4), 441-451.
- De Groot, R. J., Ter Haar, R. J., Horzinek, M. C., & Van Der Zeijst, B. A. M. (1987). Intracellular RNAs of the Feline Infectious Peritonitis Coronavirus Strain 79-1146. *J Gen Virol*, 68(4), 995-1002. doi: 10.1099/0022-1317-68-4-995.
- Demeter, Z., Lakatos, B., Palade, E. A., Kozma, T., Forgách, P., & Rusvai, M. (2007). Genetic diversity of Hungarian canine distemper virus strains. *Veterinary Microbiology*, 122(3-4), 258-269. doi: 10.1016/j.vetmic.2007.02.001.
- Desario, C., Decaro, N., Campolo, M., Cavalli, A., Cirone, F., Elia, G., et al. (2005). Canine parvovirus infection: Which diagnostic test for virus? *Journal of Virological Methods*, 126(1-2), 179-185. doi: 10.1016/j.jviromet.2005.02.006.
- Dewerchin, H. L., Cornelissen, E., & Nauwynck, H. J. (2005). Replication of feline coronaviruses in peripheral blood monocytes. *Archives of Virology*, 150(12), 2483-2500. doi: 10.1007/s00705-005-0598-6.
- Dezengrini, R., Weiblen, R., & Flores, E. F. (2007). Soroprevalência das infecções por parvovírus, adenovírus, coronavírus canino e pelo vírus da cinomose em cães de Santa Maria, Rio Grande do Sul, Brasil. *Ciência Rural*, 37(1). doi: 10.1590/S0103-84782007000100029.

-
- Donahue, P. R., Hoover, E. A., Beltz, G. A., Riedel, N., Hirsch, V. M., Overbaugh, J., et al. (1988). Strong sequence conservation among horizontally transmissible, minimally pathogenic feline leukemia viruses. *Journal of Virology*, 62(3), 722–731.
- Duarte, A., Pereira da Fonseca, I., Almeida, V., Madeira de Carvalho, L., Meireles, J., Fazendeiro, M., et al. (2009). Survey of infectious disease agents in stray cats in the metropolitan area of Lisbon, Portugal. *submitted*.
- Duarte, A., & Tavares, L. (2006). Phylogenetic analysis of Portuguese Feline Immunodeficiency Virus sequences reveals high genetic diversity. *Veterinary Microbiology*, 114(1-2), 25-33. doi: 10.1016/j.vetmic.2005.11.056.
- Dunham, S. P., Bruce, J., MacKay, S., Golder, M., Jarrett, O., & Neil, J. C. (2006). Limited efficacy of an inactivated feline immunodeficiency virus vaccine. *The Veterinary Record*, 158(16), 561-562.
- Dunham, S. P., & Graham, E. (2008). Retroviral Infections of Small Animals. *Veterinary Clinics of North America: Small Animal Practice*, 38(4), 879-901. doi: 10.1016/j.cvsm.2008.03.005.
- Dye, C., & Siddell, S. G. (2007). Genomic RNA sequence of feline coronavirus strain FCoV C1Je. *Journal of Feline Medicine & Surgery*, 9(3), 202-213. doi: 10.1016/j.jfms.2006.12.002.
- Elder, J. H., Lerner, D. L., Hasselkus-Light, C. S., Fontenot, D. J., Hunter, E., Luciw, P. A., et al. (1992). Distinct subsets of retroviruses encode dUTPase. *J. Virol.*, 66(3), 1791-1794.
- Elia, G., Decaro, N., Martella, V., Cirone, F., Lucente, M. S., Lorusso, E., et al. (2006). Detection of canine distemper virus in dogs by real-time RT-PCR. *Journal of Virological Methods*, 136(1-2), 171-176. doi: 10.1016/j.jviromet.2006.05.004.
- Escutenaire, S., Isaksson, M., Renström, L. H. M., Klingeborn, B., Buonavoglia, C., Berg, M., et al. (2007). Characterization of divergent and atypical canine coronaviruses from Sweden. *Archives of Virology*, 152(8), 1507-1514. doi: 10.1007/s00705-007-0986-1.
- Filoni, C., Catao-Dias, J. L., Bay, G., Durigon, E. L., Jorge, R. S. P., Lutz, H., et al. (2006). First Evidence of Feline Herpesvirus, Calicivirus, Parvovirus, and Ehrlichia Exposure in Brazilian Free-ranging Felids. *J Wildl Dis*, 42(2), 470-477.
- Fiorello, C. V., Noss, A. J., & Deem, S. L. (2006). Demography, Hunting Ecology, and Pathogen Exposure of Domestic Dogs in the Isozo of Bolivia. *Conservation Biology*, 20(3), 762-771. doi: 10.1111/j.1523-1739.2006.00466.x.
- Fiorello, C. V., Noss, A. J., Deem, S. L., Maffei, L., & Dubovi, E. J. (2007). Serosurvey of small carnivores in the Bolivian Chaco. *Journal of Wildlife Diseases*, 43(3), 551.
- Frisk, A. L., König, M., Moritz, A., & Baumgartner, W. (1999). Detection of Canine Distemper Virus Nucleoprotein RNA by Reverse Transcription-PCR Using Serum, Whole Blood, and Cerebrospinal Fluid from Dogs with Distemper. *J. Clin. Microbiol.*, 37(11), 3634-3643.
- Frölich, K., Czupalla, O., Haas, L., Hentschke, J., Dedek, J., & Fickel, J. (2000). Epizootiological investigations of canine distemper virus in free-ranging carnivores from Germany. *Veterinary Microbiology*, 74(4), 283-292. doi: 10.1016/S0378-1135(00)00192-9.

-
- García-Rodríguez, A., Peracho, V., Villalbí, J. R., Bouis, S., Duràn, J., & Guix, J. (2008). [Advances in the management of an animal shelter]. *Gaceta Sanitaria / S.E.S.P.A.S*, 22(1), 76-78.
- Gemeniano, M. C., Sawai, E. T., Leutenegger, C. M., & Sparger, E. E. (2003). Feline Immunodeficiency Virus Orf-A Is Required for Virus Particle Formation and Virus Infectivity. *J. Virol.*, 77(16), 8819-8830. doi: 10.1128/JVI.77.16.8819-8830.2003.
- Gencay, A., Oncel, T., Karaoglu, T., Sancak, A., Demir, A., & Ozkul, A. (2004). Antibody Prevalence to Canine Distemper Virus (CDV) in Stray Dogs in Turkey. *Revue de Médecine Vétérinaire*, 8-9(155), 432-434.
- Gleich, S. E., Krieger, S., & Hartmann, K. (2009). Prevalence of feline immunodeficiency virus and feline leukaemia virus among client-owned cats and risk factors for infection in Germany. *Journal of Feline Medicine & Surgery, In Press, Corrected Proof*. doi: 10.1016/j.jfms.2009.05.019.
- Goldstein, T., Mazet, J. A. K., & Gill, V. A. (2009). Phocine distemper virus in northern sea otters in the Pacific Ocean, Alaska, USA. *Emerging Infectious Diseases*, 15(6), 925-927.
- Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J., & Snijder, E. J. (2006). Nidovirales: Evolving the largest RNA virus genome. *Virus Research*, 117(1), 17-37. doi: 10.1016/j.virusres.2006.01.017.
- Greene, C. E., & Addie, D. D. (2006). Feline Parvovirus Infection. In *Infectious Diseases of the Dog and Cat, Revised Reprint* (3rd ed., pp. 78-88). St Louis, Missouri: Saunders.
- Greene, C. E., & Appel, M. J. (2006). Canine Distemper. In *Infectious Diseases of the Dog and Cat, Revised Reprint* (3rd ed., pp. 25-40). St Louis, Missouri: Saunders.
- Hajjema, B. J., Schellen, P., Egberink, H., & Rottier, P. J. (2009). A live attenuated vaccine against feline infectious peritonitis (FIP) protecting by cellular rather than virus-neutralizing immunity [abstract]. In *Proceedings of the 8th International Congress of Veterinary Virology: 20 years of ESVV: Integrating classical and molecular virology, Budapest, Hungary, 23-26 August 2009* (p. 26).
- Hajjema, B. J., Volders, H., & Rottier, P. J. M. (2004). Live, Attenuated Coronavirus Vaccines through the Directed Deletion of Group-Specific Genes Provide Protection against Feline Infectious Peritonitis. *J. Virol.*, 78(8), 3863-3871. doi: 10.1128/JVI.78.8.3863-3871.2004.
- Harris, C. M., Travis, J. M. J., & Harwood, J. (2008). Evaluating the Influence of Epidemiological Parameters and Host Ecology on the Spread of Phocine Distemper Virus through Populations of Harbour Seals. *PLoS ONE*, 3(7), e2710. doi: 10.1371/journal.pone.0002710.
- Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., et al. (2004). Antibodies to canine and feline viruses in spotted hyenas (*crocuta crocuta*) in the masai mara national reserve. *J Wildl Dis*, 40(1), 1-10.
- Hartmann, K. (1998). Feline immunodeficiency virus infection: an overview. *The Veterinary Journal*, 155(2), 123-137. doi: 10.1016/S1090-0233(98)80008-7.
- Hartmann, K. (2005). Feline infectious peritonitis. *Veterinary Clinics of North America: Small Animal Practice*, 35(1), 39-79. doi: 10.1016/j.cvsm.2004.10.011.
- Hartmann, K. (2006). Feline Leukemia Virus Infection. In *Infectious Diseases of the Dog and Cat, Revised Reprint* (3rd ed., pp. 105-131). St Louis, Missouri: Saunders.
-

-
- Hartmann, K., Binder, C., Hirschberger, J., Cole, D., Reinacher, M., Schroo, S., et al. (2003). Comparison of different tests to diagnose feline infectious peritonitis. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine*, 17(6), 781-790.
- Herrewegh, A., de Groot, R., Cepica, A., Egberink, H., Horzinek, M., & Rottier, P. (1995). Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. *J. Clin. Microbiol.*, 33(3), 684-689.
- Herrewegh, A. A., Smeenk, I., Horzinek, M. C., Rottier, P. J., & de Groot, R. J. (1998). Feline Coronavirus Type II Strains 79-1683 and 79-1146 Originate from a Double Recombination between Feline Coronavirus Type I and Canine Coronavirus. *J. Virol.*, 72(5), 4508-4514.
- Hofmann-Lehmann, R., Cattori, V., Tandon, R., Boretti, F. S., Meli, M. L., Riond, B., et al. (2008). How molecular methods change our views of FeLV infection and vaccination. *Veterinary Immunology and Immunopathology*, 123(1-2), 119-123. doi: 10.1016/j.vetimm.2008.01.017.
- Hofmann-Lehmann, R., Cattori, V., Tandon, R., Boretti, F. S., Meli, M. L., Riond, B., et al. (2007). Vaccination against the feline leukaemia virus: Outcome and response categories and long-term follow-up. *Vaccine*, 25(30), 5531-5539. doi: 10.1016/j.vaccine.2006.12.022.
- Hofmann-Lehmann, R., Huder, J. B., Gruber, S., Boretti, F., Sigrist, B., & Lutz, H. (2001). Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats. *J Gen Virol*, 82(7), 1589-1596.
- Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., & Koyama, H. (1992). The prevalence of types I and II feline coronavirus infections in cats. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, 54(3), 557-562.
- Holst, B. S., Englund, L., Palacios, S., Renström, L., & Berndtsson, L. T. (2006). Prevalence of antibodies against feline coronavirus and *Chlamydia felis* in Swedish cats. *Journal of Feline Medicine & Surgery*, 8(3), 207-211. doi: 10.1016/j.jfms.2005.12.004.
- Hong, C., Decaro, N., Desario, C., Tanner, P., Pardo, M. C., Sanchez, S., et al. (2007). Occurrence of canine parvovirus type 2c in the United States. *J Vet Diagn Invest*, 19(5), 535-539.
- Horzinek, M. C., Lutz, H., & Pedersen, N. C. (1982). Antigenic relationships among homologous structural polypeptides of porcine, feline, and canine coronaviruses. *Infect. Immun.*, 37(3), 1148-1155.
- Hosie, M. J., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline immunodeficiency. ABCD guidelines on prevention and management. *Journal of Feline Medicine & Surgery*, 11(7), 575-584. doi: 10.1016/j.jfms.2009.05.006.
- Haupt, K. A., Goodwin, D., Uchida, Y., Baranyiová, E., Fatjó, J., & Kakuma, Y. (2007). Proceedings of a workshop to identify dog welfare issues in the US, Japan, Czech Republic, Spain and the UK. *Applied Animal Behaviour Science*, 106(4), 221-233. doi: DOI: 10.1016/j.applanim.2007.01.005.
- Hueffer, K., Parker, J. S. L., Weichert, W. S., Geisel, R. E., Sgro, J., & Parrish, C. R. (2003). The Natural Host Range Shift and Subsequent Evolution of Canine Parvovirus Resulted from Virus-Specific Binding to the Canine Transferrin Receptor. *Journal of Virology*, 77(3), 1718-1726. doi: 10.1128/JVI.77.3.1718-1726.2003.
-

-
- Hueffer, K., & Parrish, C. R. (2003). Parvovirus host range, cell tropism and evolution. *Current Opinion in Microbiology*, 6(4), 392-398. doi: 10.1016/S1369-5274(03)00083-3.
- Ikeda, Y., Miyazawa, T., Nakamura, K., Naito, R., Inoshima, Y., Tung, K., et al. (1999). Serosurvey for selected virus infections of wild carnivores in Taiwan and Vietnam. *J Wildl Dis*, 35(3), 578-581.
- Ikeda, Y., Mochizuki, M., Naito, R., Nakamura, K., Miyazawa, T., Mikami, T., et al. (2000). Predominance of Canine Parvovirus (CPV) in Unvaccinated Cat Populations and Emergence of New Antigenic Types of CPVs in Cats. *Virology*, 278(1), 13-19. doi: 10.1006/viro.2000.0653.
- Inoshima, Y., Kohmoto, M., Ikeda, Y., Yamada, H., Kawaguchi, Y., Tomonaga, K., et al. (1996). Roles of the auxiliary genes and AP-1 binding site in the long terminal repeat of feline immunodeficiency virus in the early stage of infection in cats. *Journal of Virology*, 70(12), 8518-8526.
- Inoshima, Y., Miyazawa, T., & Mikami, T. (1998). In vivo functions of the auxiliary genes and regulatory elements of feline immunodeficiency virus. *Veterinary Microbiology*, 60(2-4), 141-153. doi: 10.1016/S0378-1135(98)00157-6.
- João Vieira, M., Silva, E., Oliveira, J., Luisa Vieira, A., Decaro, N., Desario, C., et al. (2008). Canine parvovirus 2c infection in central Portugal. *J Vet Diagn Invest*, 20(4), 488-491.
- Kapil, S., Cooper, E., Lamm, C., Murray, B., Rezabek, G., Johnston, L., et al. (2007). Canine Parvovirus Types 2c and 2b Circulating in North American Dogs in 2006 and 2007. *Journal of Clinical Microbiology*, 45(12), 4044-4047. doi: 10.1128/JCM.01300-07.
- Kapil, S., Allison, R. W., III, L. J., Murray, B. L., Holland, S., Meinkoth, J., et al. (2008). Canine Distemper Virus Strains Circulating among North American Dogs. *Clinical and Vaccine Immunology : CVI*, 15(4), 707-712. doi: 10.1128/CVI.00005-08.
- Kelly, P. J., Musuka, G., Eoghain, G. N., Tebje-Kelly, J. B. U., & Carter, S. (2005). Serosurvey for canine distemper virus exposure in dogs in communal lands in Zimbabwe. *Journal of the South African Veterinary Association*, 76(2), 104-106.
- Kim, Y. H., Cho, K. W., Youn, H. Y., Yoo, H. S., & Han, H. R. (2001). Detection of canine distemper virus (CDV) through one step RT-PCR combined with nested PCR. *Journal of Veterinary Science*, 2(1), 59-63.
- Kipar, A., Meli, M. L., Failing, K., Euler, T., Gomes-Keller, M. A., Schwartz, D., et al. (2006). Natural feline coronavirus infection: Differences in cytokine patterns in association with the outcome of infection. *Veterinary Immunology and Immunopathology*, 112(3-4), 141-155. doi: 10.1016/j.vetimm.2006.02.004.
- Kiss, I., Kecskeméti, S., Tanyi, J., Klingeborn, B., & Belák, S. (2000). Prevalence and Genetic Pattern of Feline Coronaviruses in Urban Cat Populations. *The Veterinary Journal*, 159(1), 64-70. doi: 10.1053/tvj.1999.0402.
- Kuehn, B. M. (2004). Multidisciplinary task force tackles Chicago distemper outbreak. Questions raised about vaccinations. *Journal of the American Veterinary Medical Association*, 225(9), 1315-1317.
- Kummrow, M., Meli, M. L., Haessig, M., Goenczi, E., Poland, A., Pedersen, N. C., et al. (2005). Feline Coronavirus Serotypes 1 and 2: Seroprevalence and Association with Disease in Switzerland. *Clin. Diagn. Lab. Immunol.*, 12(10), 1209-1215. doi: 10.1128/CDLI.12.10.1209-1215.2005.
-

-
- Lamm, C. G., & Rezabek, G. B. (2008). Parvovirus Infection in Domestic Companion Animals. *Veterinary Clinics of North America: Small Animal Practice*, 38(4), 837-850. doi: 10.1016/j.cvsm.2008.03.008.
- Lan, N., Yamaguchi, R., Furuya, Y., Inomata, A., Ngamkala, S., Naganobu, K., et al. (2005). Pathogenesis and phylogenetic analyses of canine distemper virus strain 007Lm, a new isolate in dogs. *Veterinary Microbiology*, 110(3-4), 197-207. doi: 10.1016/j.vetmic.2005.07.016.
- Lednicky, J. A., Dubach, J., Kinsel, M. J., Meehan, T. P., Bocchetta, M., Hungerford, L. L., et al. (2004). Genetically distant American Canine distemper virus lineages have recently caused epizootics with somewhat different characteristics in raccoons living around a large suburban zoo in the USA. *Virology Journal*, 1, 2. doi: 10.1186/1743-422X-1-2.
- Lee, I. T., Levy, J. K., Gorman, S. P., Crawford, P. C., & Slater, M. R. (2002). Prevalence of feline leukemia virus infection and serum antibodies against feline immunodeficiency virus in unowned free-roaming cats. *Journal of the American Veterinary Medical Association*, 220(5), 620-622.
- Le Poder, S., Duarte, L., Fournier, A., Rebega, C., Pinhas, C., & Eloit, M. (2009). Identification of canine coronavirus type I in a naturally infected cat [abstract]. In *Proceedings of the 8th International Congress of Veterinary Virology: 20 years of ESVV: Integrating classical and molecular virology, Budapest, Hungary, 23-26 August 2009* (p. 66).
- Leutenegger, C., Hofmann-Lehmann, R., Riols, C., Liberek, M., Worel, G., Lups, P., et al. (1999). Viral infections in free-living populations of the European wildcat. *J Wildl Dis*, 35(4), 678-686.
- Levy, J. K., Crawford, P. C., Lappin, M. R., Dubovi, E. J., Levy, M. G., Alleman, R., et al. (2008a). Infectious diseases of dogs and cats on Isabela Island, Galapagos. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine*, 22(1), 60-65. doi: 10.1111/j.1939-1676.2007.0034.x.
- Levy, J. K., Scott, H. M., Lachtara, J. L., & Crawford, P. C. (2006). Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in North America and risk factors for seropositivity. *Journal of the American Veterinary Medical Association*, 228(3), 371-376.
- Levy, J., Crawford, C., Hartmann, K., Hofmann-Lehmann, R., Little, S., Sundahl, E., et al. (2008b). 2008 American Association of Feline Practitioners' feline retrovirus management guidelines. *Journal of Feline Medicine & Surgery*, 10(3), 300-316. doi: 10.1016/j.jfms.2008.03.002.
- Levy, L. S. (2008). Advances in understanding molecular determinants in FeLV pathology. *Veterinary Immunology and Immunopathology*, 123(1-2), 14-22. doi: 10.1016/j.vetimm.2008.01.008.
- Lin, C., Su, B., Huang, H., Lee, J., Hsieh, M., & Chueh, L. (2009). Field strain feline coronaviruses with small deletions in ORF7b associated with both enteric infection and feline infectious peritonitis. *Journal of Feline Medicine & Surgery*, 11(6), 413-419. doi: 10.1016/j.jfms.2008.09.004.
- Lin, C., Su, B., Wang, C., Hsieh, M., Chueh, T., & Chueh, L. (2008). Genetic diversity and correlation with feline infectious peritonitis of feline coronavirus type I and II: A 5-year study in Taiwan. *Veterinary Microbiology*, *In Press, Corrected Proof*. doi: 10.1016/j.vetmic.2008.11.010.
-

-
- Little, S. E. (2005). Feline immunodeficiency virus testing in stray, feral, and client-owned cats of Ottawa. *The Canadian Veterinary Journal*, 46(10), 898.
- Lockridge, K. M., Himathongkham, S., Sawai, E. T., Chienand, M., & Sparger, E. E. (1999). The Feline Immunodeficiency Virus vif Gene Is Required for Productive Infection of Feline Peripheral Blood Mononuclear Cells and Monocyte-Derived Macrophages. *Virology*, 261(1), 25-30. doi: 10.1006/viro.1999.9831.
- Lorusso, A., Decaro, N., Schellen, P., Rottier, P. J. M., Buonavoglia, C., Haijema, B., et al. (2008). Gain, Preservation, and Loss of a Group 1a Coronavirus Accessory Glycoprotein. *Journal of Virology*, 82(20), 10312–10317. doi: 10.1128/JVI.01031-08.
- Lukashov, V. V., & Goudsmit, J. (2001). Evolutionary Relationships among Parvoviruses: Virus-Host Coevolution among Autonomous Primate Parvoviruses and Links between Adeno-Associated and Avian Parvoviruses. *Journal of Virology*, 75(6), 2729–2740. doi: 10.1128/JVI.75.6.2729-2740.2001.
- Luria, B. J., Levy, J. K., Lappin, M. R., Breitschwerdt, E. B., Legendre, A. M., Hernandez, J. A., et al. (2004). Prevalence of infectious diseases in feral cats in Northern Florida. *Journal of Feline Medicine & Surgery*, 6(5), 287-296. doi: 10.1016/j.jfms.2003.11.005.
- Lutz, H., Lehmann, R., Winkler, G., Kottwitz, B., Dittmer, A., Wolfensberger, C., et al. (1990). [Feline immunodeficiency virus in Switzerland: clinical aspects and epidemiology in comparison with feline leukemia virus and coronaviruses]. *Schweizer Archiv Für Tierheilkunde*, 132(5), 217-225.
- Lutz, H., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline leukaemia. ABCD guidelines on prevention and management. *Journal of Feline Medicine & Surgery*, 11(7), 565-574. doi: 10.1016/j.jfms.2009.05.005.
- Martella, V., Cirone, F., Elia, G., Lorusso, E., Decaro, N., Campolo, M., et al. (2006). Heterogeneity within the hemagglutinin genes of canine distemper virus (CDV) strains detected in Italy. *Veterinary Microbiology*, 116(4), 301-309. doi: 10.1016/j.vetmic.2006.04.019.
- Martella, V., Elia, G., Lucente, M. S., Decaro, N., Lorusso, E., Banyai, K., et al. (2007). Genotyping canine distemper virus (CDV) by a hemi-nested multiplex PCR provides a rapid approach for investigation of CDV outbreaks. *Veterinary Microbiology*, 122(1-2), 32-42. doi: 10.1016/j.vetmic.2007.01.005.
- Martella, V., Elia, G., & Buonavoglia, C. (2008). Canine Distemper Virus. *Veterinary Clinics of North America: Small Animal Practice*, 38(4), 787-797. doi: 10.1016/j.cvsm.2008.02.007.
- Maruyama, S., Kabeya, H., Nakao, R., Tanaka, S., Sakai, T., Xuan, X., et al. (2003). Seroprevalence of Bartonella henselae, Toxoplasma gondii, FIV and FeLV Infections in Domestic Cats in Japan. *MICROBIOLOGY and IMMUNOLOGY*, 47(2), 147-153.
- McCaw, D. L., & Hoskins, J. D. (2006). Canine Viral Enteritis. In *Infectious Diseases of the Dog and Cat, Revised Reprint* (3rd ed., pp. 63-73). St Louis, Missouri: Saunders.
- Meli, M., Kipar, A., Müller, C., Jenal, K., Gönczi, E., Borel, N., et al. (2004). High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. *Journal of Feline Medicine & Surgery*, 6(2), 69-81. doi: 10.1016/j.jfms.2003.08.007.
- Meli, M. L., Cattori, V., Martínez, F., López, G., Vargas, A., Simón, M. A., et al. (2009). Feline Leukemia Virus and Other Pathogens as Important Threats to the Survival of the

-
- Critically Endangered Iberian Lynx (*Lynx pardinus*). *PLoS ONE*, 4(3), e4744. doi: 10.1371/journal.pone.0004744.
- Mendoza, R., Anderson, M. M., & Overbaugh, J. (2006). A Putative Thiamine Transport Protein Is a Receptor for Feline Leukemia Virus Subgroup A. *Journal of Virology*, 80(7), 3378–3385. doi: 10.1128/JVI.80.7.3378-3385.2006.
- Millán, J., & Rodríguez, A. (2009). A serological survey of common feline pathogens in free-living European wildcats (*Felis silvestris*) in central Spain. *European Journal of Wildlife Research*, 55(3), 285-291.
- Munson, L., Terio, K. A., Kock, R., Mlengeya, T., Roelke, M. E., Dubovi, E., et al. (2008). Climate Extremes Promote Fatal Co-Infections during Canine Distemper Epidemics in African Lions. *PLoS ONE*, 3(6), e2545. doi: 10.1371/journal.pone.0002545.
- Murray, J. K., Roberts, M. A., Skillings, E., Morrow, L. D., & Gruffydd-Jones, T. J. (2008). Risk factors for feline immunodeficiency virus antibody test status in Cats Protection adoption centres (2004). *Journal of Feline Medicine & Surgery, In Press, Corrected Proof*. doi: 10.1016/j.jfms.2008.11.001.
- Nakamura, M., Tohya, Y., Miyazawa, T., Mochizuki, M., Phung, H. T. T., Nguyen, N. H., et al. (2004). A novel antigenic variant of canine parvovirus from a Vietnamese dog. *Archives of virology*, 149(11), 2261-2269.
- Natoli, E., Maragliano, L., Cariola, G., Faini, A., Bonanni, R., Cafazzo, S., et al. (2006). Management of feral domestic cats in the urban environment of Rome (Italy). *Preventive Veterinary Medicine*, 77(3-4), 180-185. doi: 10.1016/j.prevetmed.2006.06.005.
- Nava, A., Cullen Jr, L., Sana, D., Nardi, M., Ramos Filho, J., Lima, T., et al. (2009). First Evidence of Canine Distemper in Brazilian Free-Ranging Felids. *EcoHealth*. doi: 10.1007/s10393-008-0207-8.
- Naylor, M., Monckton, R., Lehrbach, P., & Deane, E. (2001). Canine coronavirus in Australian dogs. *Australian Veterinary Journal*, 79(2), 116-119. doi: 10.1111/j.1751-0813.2001.tb10718.x.
- Nishimura, Y., Goto, Y., Pang, H., Endo, Y., Mizuno, T., Momoi, Y., et al. (1998). Genetic heterogeneity of env gene of feline immunodeficiency virus obtained from multiple districts in Japan. *Virus Research*, 57(1), 101-112. doi: 10.1016/S0168-1702(98)00085-9.
- Norris, J. M., Bell, E. T., Hales, L., Toribio, J. L., White, J. D., Wigney, D. I., et al. (2007). Prevalence of feline immunodeficiency virus infection in domesticated and feral cats in eastern Australia. *Journal of Feline Medicine & Surgery*, 9(4), 300-308. doi: 10.1016/j.jfms.2007.01.007.
- Ohshima, T., & Mochizuki, M. (2009). Evidence for Recombination Between Feline Panleukopenia Virus and Canine Parvovirus Type 2. *The Journal of Veterinary Medical Science*, 71(4), 403-408.
- Pancino, G., Castelot, S., & Sonigo, P. (1995). Differences in Feline Immunodeficiency Virus Host Cell Range Correlate with Envelope Fusogenic Properties. *Virology*, 206(2), 796-806. doi: 10.1006/viro.1995.1002.
- Pardo, I. D. R., Johnson, G. C., & Kleiboeker, S. B. (2005). Phylogenetic Characterization of Canine Distemper Viruses Detected in Naturally Infected Dogs in North America. *Journal of Clinical Microbiology*, 43(10), 5009–5017. doi: 10.1128/JCM.43.10.5009-5017.2005.
-

-
- Parrish, C. R. (1995). Pathogenesis of feline panleukopenia virus and canine parvovirus. *Baillière's Clinical Haematology*, 8(1), 57-71. doi: 10.1016/S0950-3536(05)80232-X.
- Parrish, C. R. (1999). Host range relationships and the evolution of canine parvovirus. *Veterinary Microbiology*, 69(1-2), 29-40. doi: 10.1016/S0378-1135(99)00084-X.
- Patel, J., & Heldens, J. (2009). Review of companion animal viral diseases and immunoprophylaxis. *Vaccine*, 27(4), 491-504. doi: 10.1016/j.vaccine.2008.11.027.
- Pecoraro, M. R., Tomonaga, K., Miyazawa, T., Kawaguchi, Y., Sugita, S., Tohya, Y., et al. (1996). Genetic Diversity of Argentine Isolates of Feline Immunodeficiency Virus. *J Gen Virol*, 77(9), 2031-2035. doi: 10.1099/0022-1317-77-9-2031.
- Pedersen, N. C., Black, J. W., Boyle, J. F., Evermann, J. F., McKeirnan, A. J., & Ott, R. L. (1984). Pathogenic differences between various feline coronavirus isolates. *Advances in Experimental Medicine and Biology*, 173, 365-380.
- Pedersen, N. C., Sato, R., Foley, J. E., & Poland, A. M. (2004). Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus. *Journal of Feline Medicine & Surgery*, 6(2), 83-88. doi: 10.1016/j.jfms.2003.08.008.
- Pedersen, N. C., Allen, C. E., & Lyons, L. A. (2008). Pathogenesis of feline enteric coronavirus infection. *Journal of Feline Medicine & Surgery*, 10(6), 529-541. doi: 10.1016/j.jfms.2008.02.006.
- Pérez, R., Francia, L., Romero, V., Maya, L., López, I., & Hernández, M. (2007). First detection of canine parvovirus type 2c in South America. *Veterinary Microbiology*, 124(1-2), 147-152. doi: 10.1016/j.vetmic.2007.04.028.
- Phillips, T. R., Lamont, C., Konings, D. A., Shacklett, B. L., Hamson, C. A., Luciw, P. A., et al. (1992). Identification of the Rev transactivation and Rev-responsive elements of feline immunodeficiency virus. *Journal of Virology*, 66(9), 5464-5471.
- Pratelli, A., Tinelli, A., Decaro, N., Martella, V., Camero, M., Tempesta, M., et al. (2004a). Safety and efficacy of a modified-live canine coronavirus vaccine in dogs. *Veterinary Microbiology*, 99(1), 43-49. doi: 10.1016/j.vetmic.2003.07.009.
- Pratelli, A., Yesilbag, K., Yalçın, E., & Yılmaz, Z. (2008). Prevalence of Feline Coronavirus Antibodies in Cats in Bursa (Turkey) by An Enzyme-Linked Immunosorbent Assay. *International Journal of Infectious Diseases*, 12(Supplement 1), e132-e133. doi: 10.1016/j.ijid.2008.05.330.
- Pratelli, A., Decaro, N., Tinelli, A., Martella, V., Elia, G., Tempesta, M., et al. (2004b). Two Genotypes of Canine Coronavirus Simultaneously Detected in the Fecal Samples of Dogs with Diarrhea. *J. Clin. Microbiol.*, 42(4), 1797-1799. doi: 10.1128/JCM.42.4.1797-1799.2004.
- Pratelli, A., Martella, V., Decaro, N., et al. (2003a). Genetic diversity of a canine coronavirus detected in pups with diarrhoea in Italy. *Journal of Virological Methods*, 110(1), 9-17. doi: 10.1016/S0166-0934(03)00081-8.
- Pratelli, A., Martella, V., Pistello, M., et al. (2003b). Identification of coronaviruses in dogs that segregate separately from the canine coronavirus genotype. *Journal of Virological Methods*, 107(2), 213-222. doi: 10.1016/S0166-0934(02)00246-X.
- Pratelli, A., Tinelli, A., Decaro, N., Camero, M., Elia, G., Gentile, A., et al. (2002). PCR assay for the detection and the identification of atypical canine coronavirus in dogs. *Journal of Virological Methods*, 106(2), 209-213. doi: 10.1016/S0166-0934(02)00165-9.
-

-
- Priestnall, S. L., Pratelli, A., Brownlie, J., & Erles, K. (2007). Serological prevalence of canine respiratory coronavirus in southern Italy and epidemiological relationship with canine enteric coronavirus. *J Vet Diagn Invest*, 19(2), 176-180.
- Reed, A. P., Jones, E. V., & Miller, T. J. (1988). Nucleotide sequence and genome organization of canine parvovirus. *Journal of Virology*, 62(1), 266-276.
- Rikula, U. K. (2008, February 29). *Canine distemper in Finland – vaccination and epidemiology*. Academic Dissertation, Faculty of Veterinary Medicine, University of Helsinki.
- Riley, S. P. D., Foley, J., & Chomel, B. (2004). Exposure to feline and canine pathogens in bobcats and gray foxes in urban and rural zones of a national park in California. *J Wildl Dis*, 40(1), 11-22.
- Rottier, P. J. M., Nakamura, K., Schellen, P., Volders, H., & Haijema, B. J. (2005). Acquisition of Macrophage Tropism during the Pathogenesis of Feline Infectious Peritonitis Is Determined by Mutations in the Feline Coronavirus Spike Protein. *J. Virol.*, 79(22), 14122-14130. doi: 10.1128/JVI.79.22.14122-14130.2005.
- Saito, T., Alfieri, A., Wosiacki, S., Negrão, F., Morais, H., & Alfieri, A. (2006). Detection of canine distemper virus by reverse transcriptase-polymerase chain reaction in the urine of dogs with clinical signs of distemper encephalitis. *Research in Veterinary Science*, 80(1), 116-119. doi: 10.1016/j.rvsc.2005.03.002.
- Santos, N., Almendra, C., & Tavares, L. (2009). Serologic Survey for Canine Distemper Virus and Canine Parvovirus in Free-ranging Wild Carnivores from Portugal. *Journal of Wildlife Diseases*, 45(1), 221.
- Sarkar, N. H., Nowinski, R. C., & Moore, D. H. (1971). Helical Nucleocapsid Structure of the Oncogenic Ribonucleic Acid Viruses (Oncornaviruses). *J. Virol.*, 8(4), 564-572.
- Scarlett, J. M. (2008). Interface of epidemiology, pet population issues and policy. *Preventive Veterinary Medicine*, 86(3-4), 188-197. doi: 10.1016/j.prevetmed.2008.02.007.
- Sellon, R. K., & Hartmann, K. (2006). Feline Immunodeficiency Virus Infection. In *Infectious Diseases of the Dog and Cat, Revised Reprint* (3rd ed., pp. 131-143). St Louis, Missouri: Saunders.
- Shackelton, L. A., Parrish, C. R., Truyen, U., & Holmes, E. C. (2005). High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proceedings of the National Academy of Sciences of the United States of America*, 102(2), 379-384. doi: 10.1073/pnas.0406765102.
- Shiba, N., Maeda, K., Kato, H., Mochizuki, M., & Iwata, H. (2007). Differentiation of feline coronavirus type I and II infections by virus neutralization test. *Veterinary Microbiology*, 124(3-4), 348-352. doi: 10.1016/j.vetmic.2007.04.031.
- Shin, Y., Mori, T., Okita, M., Gemma, T., Kai, C., & Mikami, T. (1995). Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, 57(3), 439-445.
- Slater, M. R., Di Nardo, A., Pediconi, O., Villa, P. D., Candeloro, L., Alessandrini, B., et al. (2008). Free-roaming dogs and cats in central Italy: Public perceptions of the problem. *Preventive Veterinary Medicine*, 84(1-2), 27-47. doi: 10.1016/j.prevetmed.2007.10.002.
-

-
- Sobrino, R., Arnal, M., Luco, D., & Gortázar, C. (2008). Prevalence of antibodies against canine distemper virus and canine parvovirus among foxes and wolves from Spain. *Veterinary Microbiology*, 126(1-3), 251-256. doi: 10.1016/j.vetmic.2007.06.014.
- Sodora, D. L., Shpaer, E. G., Kitchell, B. E., Dow, S. W., Hoover, E. A., & Mullins, J. I. (1994). Identification of three feline immunodeficiency virus (FIV) env gene subtypes and comparison of the FIV and human immunodeficiency virus type 1 evolutionary patterns. *J. Virol.*, 68(4), 2230-2238.
- Soe, L. H., Devi, B. G., Mullins, J. I., & Roy-Burman, P. (1983). Molecular cloning and characterization of endogenous feline leukemia virus sequences from a cat genomic library. *Journal of Virology*, 46(3), 829-840.
- Sokolow, S. H., Rand, C., Marks, S. L., Drazenovich, N. L., Kather, E. J., & Foley, J. E. (2005). Epidemiologic evaluation of diarrhea in dogs in an animal shelter. *American Journal of Veterinary Research*, 66(6), 1018-1024.
- Spibey, N., Greenwood, N., Sutton, D., Chalmers, W., & Tarpey, I. (2008). Canine parvovirus type 2 vaccine protects against virulent challenge with type 2c virus. *Veterinary Microbiology*, 128(1-2), 48-55. doi: 10.1016/j.vetmic.2007.09.015.
- Stavisky, J., Pinchbeck, G., German, A., Dawson, S., Gaskell, R., Ryvar, R., et al. (2009). Prevalence of canine enteric coronavirus in a cross-sectional survey of dogs presenting at veterinary practices. *Veterinary Microbiology*, *In Press, Corrected Proof*. doi: 10.1016/j.vetmic.2009.07.012.
- Tennant, B. J., Gaskell, R. M., Jones, R. C., & Gaskell, C. J. (1993). Studies on the epizootiology of canine coronavirus. *The Veterinary Record*, 132(1), 7-11.
- Tennant, B. J., Gaskell, R. M., Kelly, D. F., Carter, S. D., & Gaskell, C. J. (1991). Canine coronavirus infection in the dog following oronasal inoculation. *Research in Veterinary Science*, 51(1), 11-18.
- Tennant, B., Gaskell, R., & Gaskell, C. (1994). Studies on the survival of canine coronavirus under different environmental conditions. *Veterinary Microbiology*, 42(2-3), 255-259. doi: 10.1016/0378-1135(94)90024-8.
- Timm, S. F., Munson, L., Summers, B. A., Terio, K. A., Dubovi, E. J., Rupprecht, C. E., et al. (2009). A suspected canine distemper epidemic as the cause of a catastrophic decline in Santa Catalina Island foxes (*Urocyon littoralis catalinae*). *Journal of Wildlife Diseases*, 45(2), 333-343.
- Tomonaga, K., Shin, Y., Fukasawa, M., Miyazawa, T., Adachi, A., & Mikami, T. (1993). Feline immunodeficiency virus gene expression: analysis of the RNA splicing pattern and the monocistronic rev mRNA. *J Gen Virol*, 74(11), 2409-2417. doi: 10.1099/0022-1317-74-11-2409.
- Truyen, U., Gruenberg, A., Chang, S., Obermaier, B., Veijalainen, P., & Parrish, C. (1995). Evolution of the feline-subgroup parvoviruses and the control of canine host range in vivo. *J. Virol.*, 69(8), 4702-4710.
- Truyen, U., Müller, T., Heidrich, R., Tackmann, K., & Carmichael, L. E. (1998). Survey on viral pathogens in wild red foxes (*Vulpes vulpes*) in Germany with emphasis on parvoviruses and analysis of a DNA sequence from a red fox parvovirus. *Epidemiology and Infection*, 121(2), 433-440.
- Truyen, U. (1999). Emergence and recent evolution of canine parvovirus. *Veterinary Microbiology*, 69(1-2), 47-50. doi: 10.1016/S0378-1135(99)00086-3.
-

-
- Truyen, U. (2006). Evolution of canine parvovirus--A need for new vaccines? *Veterinary Microbiology*, 117(1), 9-13. doi: 10.1016/j.vetmic.2006.04.003.
- Truyen, U., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline panleukopenia. ABCD guidelines on prevention and management. *Journal of Feline Medicine & Surgery*, 11(7), 538-546. doi: DOI: 10.1016/j.jfms.2009.05.002.
- Truyen, U., Evermann, J. F., Vieler, E., & Parrish, C. R. (1996). Evolution of Canine Parvovirus Involved Loss and Gain of Feline Host Range. *Virology*, 215(2), 186-189. doi: 10.1006/viro.1996.0021.
- Vennema, H., Godeke, G. J., Rossen, J. W., Voorhout, W. F., Horzinek, M. C., Opstelten, D. J., et al. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. *The EMBO Journal*, 15(8), 2020-2028.
- Vennema, H. (1999). Genetic drift and genetic shift during feline coronavirus evolution. *Veterinary Microbiology*, 69(1-2), 139-141. doi: 10.1016/S0378-1135(99)00102-9.
- Verschoor, E., Boven, L., Blaak, H., van Vliet, A., Horzinek, M., & de Ronde, A. (1995). A single mutation within the V3 envelope neutralization domain of feline immunodeficiency virus determines its tropism for CRFK cells. *J. Virol.*, 69(8), 4752-4757.
- de Vries, A. A. F., Horzinek, M. C., Rottier, P. J. M., & de Groot, R. J. (1997). The Genome Organization of the Nidovirales: Similarities and Differences between Arteri-, Toro-, and Coronaviruses. *Seminars in Virology*, 8(1), 33-47. doi: 10.1006/smvy.1997.0104.
- Weng, H., Kass, P. H., Hart, L. A., & Chomel, B. B. (2006). Risk factors for unsuccessful dog ownership: An epidemiologic study in Taiwan. *Preventive Veterinary Medicine*, 77(1-2), 82-95. doi: 10.1016/j.prevetmed.2006.06.004.
- Yamanaka, M., Crisp, T., Brown, R., & Dale, B. (1998). Nucleotide Sequence of the Inter-Structural Gene Region of Feline Infectious Peritonitis Virus. *Virus Genes*, 16(3), 317-318. doi: 10.1023/A:1008099209942.
- Yeşilbağ, K., Yılmaz, Z., Torun, S., & Pratelli, A. (2004). Canine coronavirus infection in Turkish dog population. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health*, 51(7), 353-355. doi: 10.1111/j.1439-0450.2004.00773.x.
- Zarnke, R., Evermann, J., Ver Hoef, J., McNay, M., Boertje, R., Gardner, C., et al. (2001). Serologic survey for canine coronavirus in wolves from Alaska. *J Wildl Dis*, 37(4), 740-745.
- Zawistowski, S., Morris, J., Salman, M. D., & Ruch-Gallie, R. (1998). Population Dynamics, Overpopulation, and the Welfare of Companion Animals: New Insights on Old and New Data. *Journal of Applied Animal Welfare Science*, 1(3), 193-206. doi: 10.1207/s15327604jaws0103_1.