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Design of a protocol for the detection of parvovirus interspecies through Polymerase Chain Reaction

Mora-Cornejo AN [1], Navarro C [2*]
[1-2] Department of Preventive Animal Medicine, FAVET, Universidad de Chile, Chile

Corresponding Author: **Navarro C. *canavarr@uchile.cl**

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Abstract
Parvoviruses infect a wide variety of hosts, from insects to primates. Human, canine, feline, porcine, and others hosts stand out. Therefore, it is important to develop a diagnostic method that can demonstrate the presence of the viruses belonging to this family in any host species. For this work, *Canine Parvovirus* type 2 (CPV-2) and *Carnivore protoparvovirus* 1 (FPLV) were used as interspecies models, as they are prevalent diseases in the clinic of small animals.
One of the leading causes of hemorrhagic enteritis in dogs worldwide is caused by CPV-2 and FPLV. Feline panleukopenia produces a case like *Canine Parvovirus* but also generates a significant decrease in leukocytes. Therefore, Veterinarians need to have a highly sensitive diagnostic technique for both clinical forms. In the present work, the foundations were laid for implementing a protocol that uses conventional PCR to detect a DNA fragment of CPV-2 and FPLV from feces of dogs or cats with symptoms corresponding to parvovirus.
For the generation of the primers capable of detecting the newly named viruses, the GenBank database was first searched for nucleotide sequences of the complete CPV-2 and FPLV genome. With this information, 15 complete genome nucleotide sequences of both viruses were chosen and aligned using the Clustal Omega Software to obtain the consensus sequence of the complete genome of both viruses. Subsequently, the percentage of conservation of the genes coding for the canine and feline Parvovirus proteins was obtained. It was found that the genes that code for the NS1 protein are the most conserved, so they were used to generate the primers to be used in the PCR for diagnosis.

Keywords: *Parvoviruses*, Diagnosis, Primers, PCR, Sequence, Alignment

Introduction
In 1967 the first parvovirus infecting canines was detected (Goddard and Leisewitz, 2010) [14]. *Canine Parvovirus* type 2 was later detected in 1978, after emerging as a pandemic due to the lack of previous immunity in dogs, which allowed the rapid spread of the virus (Sykes, 2013) [35]. The origin of the virus is unclear, but it is postulated that it arose as a variant of the *Feline Panleukopenia Virus* (FPLV). Among other hypotheses about the emergence of this virus, it is postulated that it would be a mutation of the FPLV present in the vaccine or an adaptation of parvoviruses that affect wild carnivores such as foxes and minks (Truyen, 2006) [37]. The detected CPV-2 continued to evolve, and in 1980 its first variant was identified, called CPV-2a. This variant presented substitutions in some amino acids of the sequence that forms the VP2 protein of the viral capsid. In 1984 a new variant was detected from CPV-2a and was named CPV-2b. Both spread rapidly around the world and completely displaced the original virus (CPV-2) in a short time. In 2000, a new variant from CPV-2b was described in Italy, which was called CPV-2c, and which was also rapidly detected in other parts of the world. CPV-2, known mainly to be the causative agent of acute hemorrhagic enteritis, is one of the most important viruses of veterinary interest worldwide.

Since its first characterization in the 70s, it has been established as a pathogen that mainly affects canines, both domestic and wild, globally, with high morbidity (100%) and an estimated mortality rate of 10% in adult dogs. and over 90% in puppies (Nandi and Kumar, 2010) [27]. The current study highlights the CPV-2 and FPLV potential methods for improvement of molecular diagnosis

Canine Parvovirus type 2. CPV-2 is classified within the *Parvoviridae* family, *Parvovirinae* subfamily (Decaro and Buonavoglia, 2012) [7]. It and was recently included in the *Protoparvovirus* genus *Protoparvovirus* 1 species, a group among FPLV (Tinky *et al*., 2015) [36]. The etiological agent was characterized for the first time in 1978 from fecal samples and tissues of affected individuals (Appel *et al*., 1979) [1]. The virus is small, 18 to 26 nm in diameter, without an envelope, with an icosahedral capsid composed of three structural proteins (VP1, VP2, and VP3), and a single-stranded DNA genome of approximately 5,000 nucleotides. This strand encodes two structural proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2) (Decaro and Buonavoglia, 2012) [7]. The viral capsid comprises 60 protein subunits (capsomeres), which made up of 90% of the VP2 protein and 10% of the VP1 protein. VP2 is the most numerous (Decaro and Buonavoglia, 2012) [7] and highly conserved protein (Tu *et al*., 2015) [38] that participates in the recognition of the host and the nuclear translocation of the viral particle (Li *et al*., 2017) [22].

VP1 plays a role in the infectivity of the virus (Tu *et al*., 2015) [38]. A third structural protein can be found, VP3, which is not detected in all viruses, and which originates from the action of a host protease on VP2, which undergoes a cleavage process and becomes VP3, which allows the interaction of the viral particle with cell membranes (Li *et al*., 2017) [22]. The NS1 and NS2 proteins have essential roles for virus invasion and replication. NS1 is the majority non-structural protein and performs functions as a promoter of viral DNA replication, the regulator of its transcription, and as a cytotoxic component as it can induce apoptosis through caspases and has helicase and ATPase properties (Saxena *et al*., 2013) [33]. The functions and properties of NS2 have been less studied, but it is believed that its primary role is to regulate the transport of the viral particle from the cytoplasm into the nucleus (Grecco, 2014) [15]. Because the genome of parvoviruses is small and only encodes a few proteins, these viruses are highly dependent on the host cell for their replication. The proteins necessary for viral replication are found only in the S phase of the cell cycle (Carter and Saunders, 2013) [3], so replication occurs in the nucleus of constantly dividing cells, such as fetal, newborn, or newborn cells. and intestinal tissue from young or adult animals (Decaro and Buonavoglia, 2012) [7]. By the action of DNA polymerase, the single strand of virus DNA is converted into a double strand. Utilizing cellular RNA polymerase II, two classes of messenger RNA (mRNA) are generated. One of greater length encodes for non-structural proteins and another of shorter length that codes for capsid proteins (Carter and Saunders, 2013) [3].

The most common entry route is through the oropharyngeal region (Parrish, 2011) [29], through contact with feces of infected animals or contaminated surfaces (Nandi and Kumar, 2010) [27]. The virus has an incubation period of 3 to 10 days (Diaz *et al*., 2008) [11], initially replicates in the lymphoid tissue of the region (Li and Humm, 2015) [23], in the mesenteric lymph nodes and the thymus, then it spreads through the blood to the crypt epithelium in the mucosa of the small intestine (Goddard and Leisewitz, 2010) [14]. CPV-2 directly affects the cells of the intestinal crypts, leading to the destruction and shortening of the intestinal villi, which prevents the absorption of nutrients, resulting in diarrhea. In addition, the deterioration of the intestinal mucosa allows blood to escape into the intestinal lumen and the passage of bacteria from the intestine to the blood (Nandi and Kumar, 2010) [27]. Lymphoid tissue is also affected, and the destruction of lymphocytes will produce immunosuppression, predisposing to secondary infections (Li and Humm, 2015) [23]. The virus rarely affects the myocardium when the mother does not have antibodies (Humm and Hughes, 2009) [18] or when the puppy acquires the infection within the first week of life, during the rapid division of myocardial cells (Parrish, 2011) [29].

Clinical features of CVP-2: Enteric infection presents with anorexia, depression, vomiting, abdominal pain, and eventually fever. Diarrhea can be severe and hemorrhagic, especially in puppies. Due to diarrhea and vomiting, dehydration occurs quickly. Symptoms appear three to five days after the virus enters the body, and the death of the puppy can occur three days after the appearance of clinical signs (Li and Humm, 2015) [23]. When CVP-2 infects the myocardium, clinical signs are usually evident throughout the litter (Cohn and Langdon, 2008) [5] and death occurs from congestive heart failure. Most puppies (approximately 70%) may die within the first eight weeks, and about 30% will undergo pathological changes in the organ, which will cause death weeks or months later (Li and Humm, 2015) [23].

Immunization: Puppies acquire antibodies through colostrum, which protects them against the virus in the first weeks of life. The highest rates of infection are seen in puppies older than six weeks. These maternal antibodies interfere with the immunization of the puppies through vaccination. Maternal antibody titers equal to or greater than 1:80 confer immunity to puppies. A titer of 1:40 does not confer immunity, but it can interfere with the active immunization of puppies (Pratelli *et al*., 2000) [31]. Immunization via vaccination show antibody response in 90% of puppies at twelve weeks of age, after the decline of maternal immunity. Generally, polyvalent vaccines are used (Nandi and Kumar, 2010) [27]. In Chile, the Sextuple vaccine is used, an antigenic preparation including the *Canine Distemper virus, Canine Parvovirus, Adenovirus* type I and II, and *Canine parainfluenza virus* as live virus modified; and *Leptospira interrogans* serovars *canicola* and *icterohaemorragiae* as a bacterin (El Roble, 2011) [12]. The vaccines marketed in Chile are registered by the Agricultural and Livestock Service (SAG, 2019) [32].

Diagnosis: The clinical diagnosis of parvovirus tends to be complicated occasionally due to the similarity of the clinical signs to other pathologies such as that caused by coronavirus and coccidiosis, particularly in the case of the enteric manifestation (Decaro and Buonavoglia, 2012) [7]. Therefore, the diagnosis through clinical signs is only presumptive and must be confirmed by a diagnostic test. The ELISA test and IC immunochromatography are the routine methods used in the veterinary clinic because they are simple, fast, cheap, and usually have an acceptable sensitivity (Nandi and Kumar, 2010) [27]. The ELISA test is quick and simple, but a great variability in its sensitivity has been found in various studies, 81.8% (Markovich *et al*., 2012) [25], 56.2% (Desario *et al*., 2005) [10] and 18.4% (Schmitz *et al*., 2009) [34]. However, it can detect the three circulating CPV-2 variants (Decaro *et al*., 2010) [8]. The IC immunochromatography technique delivers

the result quickly, but its sensitivity does not exceed 50% (Decaro and Buonavoglia, 2012) [7]. The hemagglutination test is a quick and simple test that detects the parvovirus in feces using porcine, feline, or Rhesus monkey erythrocytes (Nandi and Kumar, 2010) [27], however, it is less sensitive than viral isolation or PCR (Desario *et al*., 2005) [10], in addition to the fact that some variants of CPV-2 lack hemagglutinating activity (Cavalli *et al*., 2001) [4]. A variation of this test is hemagglutination inhibition, which is more specific since specific antibodies against the viral antigen are used (Cavalli *et al*., 2001) [4]. It is also possible to perform electron microscopy for diagnosis, but CPV-1 and CPV-2 are morphologically identical (Li and Humm, 2015) [23]. Conventional PCR has proven to be the most sensitive test for detecting *Canine Parvovirus* with a sensitivity of 93.15% (Desario *et al*., 2005) [10]. This test has become the technique of choice in cases of dogs with clinical signs that are negative in other diagnostic tests (Humm and Hughes, 2009) [18].

***Feline parvovirus*:** Feline panleukopenia is the syndrome of clinical disease caused by infection with *Carnivore protoparvovirus* 1 (FPLV). Both *Feline parvovirus* (FPLV) and *Canine Parvovirus* (CPV) can cause feline panleukopenia, although *Canine Parvovirus* infections in cats are rare. Feline parvovirus causes 95% of cases, while 5% is caused by variants of *Canine Parvovirus*, specifically CPV-2a, CPV-2b, and CPV-2c (Barrs, 2019) [2].

Taxonomy, structure and viral genome: FPLV belongs to the *Parvoviridae* family, *Parvovirinae* subfamily, *Protoparvovirus* genus (Cotmore *et al*., 2014) [6]. Feline Panleukopenia was first identified as a viral cause in 1928 (Verge and Cristoforoni, 1928) [39]. Cats were successfully vaccinated against FPLV in 1934 using formalin-inactivated tissue extracts from infected cats (Leasure *et al*., 1934) [21]. In 1964 FPLV was isolated from infected cat tissue culture, allowing the development of inactivated tissue culture vaccines and modified live virus vaccines (Johnson, 1964) [19]. With the progressive adoption of cat vaccines by pet owners, FPLV became a rare disease in companion animals in several countries (PDSA, 2017) [30].
Carnivore protoparvovirus 1 is a small, non-enveloped, single-stranded DNA virus with a 5.1 kb genome encoding 2 main genes, non-structural (NS) and structural protein. The NS gene encodes the NS1 and NS2 proteins involved in DNA replication, capsid assembly, and intracellular transport, while the structural gene encodes the VP1 and VP2 virus capsid proteins. The viral capsid comprises 60 protein subunit molecules (approximately 10% VP1 and 90% VP2) arranged in icosahedral symmetry (Barrs, 2019) [2].

Pathogeny: *Carnivore protoparvovirus* 1 is a highly contagious and resistant virus, capable of persisting in infected facilities for one year. The virus is shed in large amounts in all excretions of infected cats, including saliva, urine, feces, and vomit. The main portals of infection are the gastrointestinal (GI) tract through orofecal transmission and, less frequently, via the respiratory tract through inhalation of aerosolized viruses. In the field, transmission is predominantly indirect by fomites (Barrs, 2019) [2]. After infection, *Carnivore protoparvovirus* 1 binds to its cellular receptor (Transferrin receptor; TfR), a transmembrane protein expressed in many tissues (Hueffer *et al*., 2003) [16]. Virions enter cells through clathrin-mediated endocytosis and colocalize with transferrin in endosomes before entering the cytoplasm to allow viral DNA to access the nucleus (Hueffer *et al*., 2004) [17]. Viral DNA is released from the capsid and replicates through double-stranded RNA intermediaries in the nucleus of the cell. The virus does not have its DNA polymerase and must "sequester" the host polymerase for replication to occur. Since the virus can only replicate in S-phase cells, it has a tropism for lymphatic tissue, bone marrow, intestinal crypt epithelium, and newborn tissues that are still actively replicating, FPLV can replicate in Purkinje cells of the cerebellum in neonates less than 10 days old (Barrs, 2019) [2]. Viral replication in lymphoid tissue oropharyngeal occurs 18 to 24 hours after infection, and viremia can be detected within 2 to 7 days post-infection. Clinical disease occurs in cats after 2 to 10 days of incubation. Excretion of the virus in feces can occur in the absence of clinical signs (subclinical infections), or before clinical signs of the disease are detected. Low-level virus shedding can persist for more than 6 weeks. Transplacental infection can also occur, resulting in miscarriage, mummified fetuses, stillborn kittens (early gestation), or kittens born with central nervous system deficit (late gestation) (Barrs, 2019) [2].

Clinical signs: FPLV or CPV-2 infection can be clinical or subclinical. In some unvaccinated adult cat populations, high seroprevalence rates suggest that subclinical infections are common in young adult cats. The determinants of the clinical disease include age, immune status, and co-infections with intestinal parasites, viruses, and bacteria (Moschidou *et al*., 2011) [26]. The disease can be acute, resulting in sudden death from septic shock without prior signs, especially in kittens less than 2 months of age. The most common presentation is characterized by an acute course of the disease over several days with a high fever of 40ºC, lethargy, anorexia, vomiting, diarrhea, and severe dehydration. Only a few of these signs may be present, vomiting generally precedes diarrhea, and unlike dogs with CPV-2 enteritis, hemorrhagic diarrhea is much less common in cats (Kruse *et al*., 2011) [20]. There may be hypersalivation due to nausea. Abdominal palpation can be painful and reveal thickened intestinal segments and/or enlarged mesenteric lymph nodes (Litster and Benjanirut, 2014) [24]. Myocarditis is a recognized complication of CPV-2 infection in puppies, but convincing evidence is lacking to support the role of parvovirus infection in cats with myocarditis. Depending on the stage of pregnancy in which infection occurs, infected females may abort or give birth to kittens with the central nervous system and eye defects, including cerebellar hypoplasia, hydrocephalus, hydranencephaly, retinal dysplasia, and optic nerve hypoplasia. Common complications that generally result in the death of the animal include circulatory shock, septicemia, and disseminated intravascular coagulation. Cats with FPLV are also susceptible to co-infections due to severe immunosuppression (Barrs, 2019) [2].

Immunization: The World Small Animal Veterinary Association (WSAVA) vaccination guidelines and the feline vaccination advisory panel report of the American Association of Feline Practitioners (AAFP) recommend a vaccination program for those beginning 6 to 8 weeks after age (Barrs, 2019) [2]. In Chile, this virus is part of a vaccine that is applied in a protocol way in cats from puppies and must be revaccinated annually. This vaccine is Triple feline, an antigenic preparation that contains feline Parvovirus, feline Herpes virus, and Calicivirus as modified live viruses (El Roble, 2011) [12]. Vaccine registered in the Agricultural and Livestock Service (SAG, 2019) [32].

Diagnosis: As diagnostic confirmation tests, fecal antigen tests, PCR or viral isolation are used. Fecal antigen enzyme-linked immunosorbent test kits designed to detect CPV in dogs can be used to diagnose both CPV-2a-c and FPLV antigen in the canine and feline feces (Neuerer *et al*., 2008) [28]. The diagnosis of FPLV based on a negative result of the enzyme-linked immunosorbent test with fecal antigen should never be ruled out (Freisl *et al*., 2017) [13]. PCR can be used to confirm the diagnosis of FPLV in cases that have a negative fecal antigen test result in patients whose clinical presentation suggests the disease. Commercial PCR assays are typically quantitative PCR assays that will amplify and detect DNA of C*arnivorous Protoparvovirus* 1, but may not distinguish between FPLV and CPV-2 strains. False positives can occur in recently vaccinated cats (Decaro *et al*., 2005) [9].

Study methods: Using the GenBank database, the nucleotide sequence of 15 complete genomes of CPV-2 and FPLV was obtained. The search for *Canine Parvovirus* in Genbank yielded 4514 sequences, most of them being the VP2 gene sequences, 306 VP1, and VP2 sequences. There are 145 complete genome sequences of the virus, including the NS1, NS2, VP1, and VP2 genes.

Regarding FPLV when searching GenBank for feline parvovirus, only 93 sequences were found, but when including feline panleukopenia, 702 sequences were found, of which the vast majority were VP2. Twenty-five sequences for VP1 and VP2, and 22 entire virus genome sequences.

Fifteen sequences from the whole genome of CPV-2 and 15 sequences from the entire genome of FPV were used. The GenBank access code of the used viruses are shown in Table 1. The 30 nucleotide sequences of the entire genome were aligned using Clustal Omega software to obtain the consensus sequence and locate all the common areas.

Table 1: GenBank accession number of the complete *Canine Parvovirus* and feline Parvovirus genome sequence.

Canine Parvovirus	***Feline Parvovirus***
MF510158.1; MF510157.1	MH559110.1; MG924893.1
MF134808.1; MF805798.1	KX685354.1; KX900570.1
MF805797.1; MF805796.1	KP280068.1; EU659115.1
MH476585.1; MH476584.1	EU659114.1; EU659113.1
MH476583.1; EU659121.1	EU659112.1; EU659111.1
EU659120.1; EU659119.1	KP019621.2; KX434462.1
KM457130.1; KM457129.1	KX434461.1; MG764511.1
KM457128.1	

Results

The sequence results from the alignment of the 30 nucleotide sequences in the Clustal Omega software was used to determine the percentage of conservation of each of these genes. The 30 nucleotide sequences of the genes that code for NS1, NS2, VP1, and VP2 were aligned separately (Table 2).

Table 2: Percentage of conservation of canine and feline Parvovirus genes

	NS1	**NS2**	**VP1**	**VP2**
Total bases	1937	1900	2155	1653
Communes bases	1832	1796	1976	1546
Percentage	94.57	94.53	91.69	93.52

The gene with the highest percentage of conservation corresponds to NS1 (94.57%), which makes it an optimum candidate to implement the diagnosis of parvovirus. Subsequently, the Oligo Perfect software was used to generate the primers where the NS1 consensus sequence containing 1938 nucleotides was retrieved. The regions where there was no base correspondence in the 30 sequences were excluded, and the range of the oligonucleotide primers was determined to be 450-550 nucleotide bases. The program generated 5 primers which are shown in table 3.

Table 3: Primers generated from the NS1 consensus sequence

Name	**Size (bp)**	**Sequence**	**% GC**	**Tm (ºC)**	**ΔTm (ºC)**
1	460	AAACCACAGTGACGACAGCA GCTTGTGCTATGGCTTGAGC	50.00	60.11	0.21
			55.00	59.90	
2	451	TGACGACAGCACAGGAAACA	50.00	59.82	0.08
		GCTTGTGCTATGGCTTGAGC	55.00	59.90	
3	454	CAGTGACGACAGCACAGGAA	55.00	60.25	0.35
		GCTTGTGCTATGGCTTGAGC	55.00	59.90	
4	466	CCGTTGAAACCACAGTGACG	55.00	59.70	0.20
		GCTTGTGCTATGGCTTGAGC	55.00	59.90	
5	453	AGTGACGACAGCACAGGAAA	50.00	59.54	0.36
		GCTTGTGCTATGGCTTGAGC	55.00	59.90	

To choose the optimal pair of primers, two parameters were considered, the guanine and cytosine (GC) content (%) and the melting temperature (Tm ºC). Hence, the second pair of primers are chosen for the PCR protocol (Table 3), since it has a good % GC and the temperature delta is the lowest of the generated starters. These primers must be synthesized and tested for *Canine or Feline parvovirus*. Upon obtaining the results, the fragment obtained must be sent to a commercial company to validate its identity. The sequence delivered by this company must be entered into the BLAST software.

Discussion and Conclusion

Canine Parvovirus type 2 (CPV-2) is the most studied member of the *Parvoviridae* family and of which there is a greater number of sequences of its genome in the GenBank database with respect to *Carnivore protoparvovirus* 1. The majority of the available 4514 sequences of *Canine Parvovirus* type 2 are of the VP2 gene and 306 sequenced VP1 and VP2 are available. There are only 145 sequences of the complete genome of the virus including NS1, NS2, VP1 and VP2. Regarding, *Carnivore protoparvovirus* 1, of the 702 sequences that GenBank has, the vast majority are of the genes that encode VP2. There are 25 sequences that include VP1 and VP2 and only 22 of the complete genomes of the virus. The fact that most of the sequencing is for the genes that code for VP2 due to that the literature describes that the viral capsid is made up of 90% of the VP2 protein (Decaro and Buonavoglia, 2012) [7] and is highly conserved (Tu *et al*., 2015) [38]. However, the current study shows that the genes encoding for

the NS1 protein are also conserved like the VP2. The identity percent of the NS1 between CPV-2 is 94.57% compared to 93.52% of the VP2. Although the difference in the percentage of conservation of NS1 with respect to VP2 is only 1.05%, it makes it possible to postulate a new target gene for the diagnosis of *Canine* or *Feline Parvovirus.*

Interspecies diagnosis is possible from a PCR protocol since the differences between nucleotide sequences of *Canine Parvovirus* type 2 and *Carnivore protoparvovirus* 1 are minimal. The common areas can be used for the design of specific partitions. It is important to consider the genes that code for NS1 for the interspecies diagnosis. Carrying out this tutorial represents the possibility of removing previously acquired knowledge and updating it, considering the advancement of information and technology, since the existence of these bioinformatics tools does not allow having any pretext to face the molecular detection of any pathogen of interest. Veterinarian or how to achieve its differentiation, although its genome is highly conserved between different animal species.

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