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Research Article

Design of Primers for the Detection of Parvovirus in Dogs and Cats Using the Same Polymerase Chain Reaction

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Introduction

Parvoviruses infect a wide variety of hosts, from insects to primates. Human, canine, feline, and porcine hosts stand out, among others. Therefore, it is interesting to develop a diagnostic method that can demonstrate the presence of viruses belonging to this family in any host species. For this work, *Canine Parvovirus* type 2 (CPV-2) and *Carnivorous Protoparvovirus* 1 (FPLV) will be used as an interspecies model, since they cause prevalent diseases in the clinic of small animals (dogs and cats). One of the leading causes of hemorrhagic enteritis in pets worldwide is caused by CPV-2 and FPLV. In *feline Panleukopenia*, a case similar to that caused by *Canine Parvovirus* occurs, but it generates a significant decrease in leukocytes. Therefore, veterinary doctors need to have a highly sensitive diagnostic technique for both clinical forms. In this project, the design of in silico primers is proposed as the basis for a diagnostic proposal using PCR for the molecular detection of parvovirus that affects dogs and cats. Thus, the generation of primers capable of detecting the aforementioned viruses involves the search for at least 1000 official nucleotide sequences stored in GenBank® for each of the genes that encode VP1, VP2, NS1 and NS2, both of CPV2 and FPLV. The chosen sequences involve both individual sequences and those incorporated within the genome sequence, partial or complete. With this information, the sequences obtained for each gene will be aligned using Clustal Omega software or another similar software to choose the gene that presents the highest percentage of nucleotide identity. This alignment will also allow the construction of a sequence that will be incorporated into the OligoPerfect Design® or similar program, to obtain the optimal starters and the recommended conditions for their use in PCR.

Background

Canine Parvovirus

In 1967, the first parvovirus was detected in canines [1]. *Canine Parvovirus* type 2 was detected in 1978 (*Sykes*, 2013). CPV-2 continued to evolve and in 1980 its first variant was identified, called CPV-2a, which presented substitutions in some amino acids of the sequence that forms the VP2 protein of the viral capsid. In 1984, another new variant was detected: CPV-2b, which spread and completely displaced the original virus (CPV-2). CPV-2, known to be the causal agent of acute hemorrhagic enteritis, is one of the viruses of greatest medical interest and it has been established that it mainly affects canines, both domestic and wild, with high morbidity (100%) and a rate mortality of 10% in adult dogs and more than 90% in puppies [2]. CPV-2 is classified within the family *Parvoviridae*, subfamily *Parvovirinae* [3], recently included in the genus *Protoparvovirus* (*Tinky et al.,* 2015). This etiological agent was characterized in 1978 from fecal samples and tissues of affected individuals [4]. The virus is small, 18 to 26 nm in diameter, non-enveloped, with an icosahedral capsid composed of three structural proteins (VP1, VP2 and VP3) and a single-stranded DNA genome, which encodes two structural proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2) [3]. The viral capsid comprises 60 protein subunits (capsomers), which are composed of 90% of the VP2 protein and 10% of the VP1 protein. VP2 is the most numerous [3] and highly conserved protein (*Tu et al.,* 2015), which participates in host recognition and nuclear translocation of the viral particle [5]. VP1 plays a role in virus infectivity (*Tu et al.,* 2015). The NS1 and NS2 proteins have essential functions for virus invasion and replication. NS1 is the major non-structural protein

and fulfills functions as a promoter of viral DNA replication, a regulator of its transcription and as a cytotoxic component, since it can induce apoptosis through caspases and has helicase and ATPase properties (*Saxena et al.,* 2013). The functions and properties of NS2 have been less studied, but its main role is believed to regulate the transport of the viral particle from the cytoplasm to the nucleus [6]. The route of entry is oropharyngeal [7], through contact with feces of infected animals or contaminated surfaces [2]. The virus has an incubation period of 3 to 10 days [8], initially it replicates in the lymphoid tissue of the region [9], in the mesenteric lymphoid tissue and the thymus; it then spreads through the blood to the crypt epithelium in the mucosa of the small intestine [1]. CPV-2 affects cells in the intestinal crypts, destroying and shortening the intestinal villi, which prevents the absorption of nutrients and causes diarrhea. Furthermore, the deterioration of the intestinal mucosa allows blood to leak into the intestinal lumen and bacteria from the intestine to enter the blood [2]. Lymphoid tissue is affected and the destruction of lymphocytes will cause immunosuppression and predispose to secondary infections [9].

Clinical Characteristics of CVP-2 Infection: Enteric infection presents with anorexia, depression, vomiting, abdominal pain, and eventually fever. Diarrhea can be severe and hemorrhagic in puppies. Symptoms appear three to five days after the virus enters the body and the death of the puppy can occur three days later [9].

Immunization: Puppies acquire antibodies through colostrum, which protects them against the virus in the first weeks of life. Vaccination shows an antibody response in 90% of puppies by twelve weeks of age, after the decline of maternal immunity. Polyvalent vaccines are generally used [2]. In Chile, the Sextuple vaccine is used, an antigenic preparation that includes canine distemper virus, canine parvovirus, adenovirus type I and II, and canine parainfluenza virus as a modified live virus; and Leptospira interrogans serovars canicola e icterohaemorragiae as bacterin [10]. Vaccines in Chile are registered by the Agricultural and Livestock Service [11].

Diagnosis: ELISA test and Immunochromatography (IC) are the routine methods used in medicine because they are simple, rapid and inexpensive [2]. The ELISA test is quick and simple, and despite having found a high variability in its sensitivity, it can detect the three variants of CPV-2 [12]. CI delivers the result quickly, but its sensitivity does not exceed 50% [3]. Conventional PCR has proven to be the most sensitive test for the detection of Canine Parvovirus with a sensitivity of 93.15% [13].

Feline Parvovirus

Feline panleukopenia is the clinical disease syndrome caused by infection with *carnivorous protoparvovirus* 1 (FPLV). Feline parvovirus causes 95% of cases, while 5% are caused by canine parvovirus variants, specifically CPV-2a, CPV-2b, and CPV-2c [14].

Taxonomy, Structure and Viral Genome: FPLV belongs to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Protoparvovirus* [15]. Feline panleukopenia was first identified in 1928 (*Verge and Cristoforoni*, 1928). In 1964, FPLV was isolated from tissue cultures of infected cats, allowing the development of inactivated tissue culture vaccines and modified live virus vaccines [16]. With the progressive adoption of cat vaccines, FPLV became a rare disease in companion animals in several countries [17].

Pathogeny: *Carnivorous protoparvovirus* 1 is a highly contagious and resistant virus, capable of persisting in infected facilities for a year. The virus is shed in large quantities in all excretions of infected cats, including saliva, urine, feces, and vomit. Viral replication in oropharyngeal lymphoid tissue occurs 18 to 24 hours after infection and viremia can be detected within 2 to 7 days thereafter. Clinical disease occurs in cats after 2 to 10 days of incubation. Low-level virus shedding can persist for more than 6 weeks [18].

Clinical Signs: The disease can be acute, resulting in sudden death from septic shock without prior signs, especially in kittens less than 2 months of age. Presentation is characterized by an acute course of the disease with high fever of 40ºC, lethargy, anorexia, vomiting, diarrhea and severe dehydration. Only some of these signs may be present, vomiting usually precedes diarrhea, and unlike dogs with CPV-2 enteritis, hemorrhagic diarrhea is much less common in cats [19].

Immunization: The World Small Animal Veterinary Association (WSAVA) vaccination guidelines recommend a vaccination schedule beginning at 6 to 8 weeks of age [14]. In Chile, the registered vaccine [11], which is applied to cats from puppies annually, contains feline Parvovirus, feline Herpesvirus and Calicivirus as modified live viruses [10].

Diagnosis: Diagnostic tests are used to identify antigens in stool, such as PCR or viral isolation. Additionally, ELISA kits can be used to detect antibodies against CPV in dogs. In this way, both the CPV-2a-c and FPLV antigens are diagnosed in canine and feline feces [20]. Both diseases, since they exceed 90% mortality in patients, have special importance in small animal veterinary medicine in places where there is a high concentration of unvaccinated individuals. Both are worldwide distributed [21], their presence in Chile directly affects the health of domestic and wild animals [22], in addition to being a threat to public health. Based on this background, this work proposes the design of in *silico primers* as a basis for a diagnostic proposal using PCR for the molecular detection of parvovirus in dogs and cats. The proposed method could have an interesting diagnostic value for interspecies parvovirus considering both the detection time and the epidemiology involved.

Material and Methods

This work It can be developed in any virology laboratory with basic equipment, such as the presence of a thermocycler and electrophoresis equipment. In this sense, the idea is what is important, since it constitutes a model for other animal or human viruses.

Design Primers for The Detection of *Carnivorous Protoparvovirus* 1 Present in Canines and Felines by The Polymerase Chain Reaction

At least 50 Canine Parvovirus sequences and 50 FPLV sequences existing in Genbank® will be considered, for the NS1, NS2, VP1 and VP2 genes. The sequences obtained for each gene in each animal species will be aligned using the Clustal Omega program, which will allow obtaining the gene that presents the highest percentage

of nucleotide identity (PIN; for example: VP2). Then, the sequence with the highest PIN value will be used to know the nucleotide sequence of the best pair of starters proposed, using the OligoPerfect Desig® program from Invitrogen or another similar program.

Propose an Appropriate Diagnostic Methodology

The generation of the primers is closely linked to the Polymerase Chain Reaction (PCR) and this will be the relevant methodology proposed. The best primers proposed according to the program involve the determination of the optimal alignment temperature, a key parameter in the PCR technique. This temperature can be determined from the Tm (melting temperature) of each proposed primer. Thus, the final proposal for conventional PCR conditions or protocol will consider the typical ones described [23] that involve at least 30 cycles, with a denaturation step at 94-95°C for 1 minute, followed by l alignment step at the temperature suggested by the Tm of the proposed starters and finally an extension step at 72°C. A final stage of 10 minutes at 72°C will be suggested as well as the use of a temperature gradient thermocycler for optimal alignment temperature selection. Finally, the establishment of a detection system based on the electrophoresis of nucleic acids in 1% agarose gel will be suggested [24].

Results

When aligning the sequences of the NS1, NS2, VP1 and VP2 genes of canine parvovirus and feline panleukopenia, the following Percentages of Nucleotide Identity (PNI) were observed: (Table 1).

Table 1:

Gen	NS1	NS2	VP1	VP2
PNI	87.6	88	66.9	80.9

In this way we can choose the NS2 gene or the NS1 gene because it has the highest PNI. Mathematically we choose NS2 for the design of the optimal primers. By entering the NS2 sequence into the Invitrogen® Oligoperfect program, the following ranking of primers is obtained: (Table 2).

Table 2:

Available Primers							
Amplicon Siz.	**Primer Name**	**Sequence**	**Length -**	**%GC**	**Tm(*C)-**	**Start**	**Stop**
460	consenso_F_1 consenso_R_1	AAACCACAGTGACGACAGCA GCTTGTGCTATGGCTTGAGC	20 20	50.00 55.00	60.11 59.90	790 1230	809 1249
451	consenso_F_2 consenso_R_2	TGACGACAGCACAGGAAACA GCTTGTGCTATGGCTTGAGC	20 20	50.00 55.00	59.82 59.90	799 1230	818 1249
454	consenso_F_3 consenso_R_3	CAGTGACGACAGCACAGGAA GCTTGTGCTATGGCTTGAGC	20 20	55.00 55.00	60.25 59.90	796 1230	815 1249
466	consenso_F_4 consenso_R_4	CCGTTGAAACCACAGTGACG GCTTGTGCTATGGCTTGAGC	20 20	55.00 55.00	59.70 59.90	784 1230	803 1249

With the use of a temperature gradient thermocycler and considering the Tm proposed in each case, it is possible to obtain a fragment of around 460 bp, as previously stipulated as input to the program.

Conclusion

In any laboratory in the third world, it is possible today to simultaneously detect one or more pathogens that affect humans and/or animals. Kary Mullis' fantastic idea, together with the program that aligns sequences and the primer designer, allow the aforementioned.

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Conflict of Interest

None.

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