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Research Article

The Second Third of the Canine Parvovirus VP2 Gene as a Target for Detection and Comparison with the Vaccine Virus

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Abstract

Canine Parvovirus Type 2 (CVP-2) is a virus that primarily affects young dogs, although it can also affect adults. It causes severe gastrointestinal hemorrhagic symptoms, making it a disease of high clinical importance. The virus was first identified in 1978 in Texas, United States, and emerged as a pandemic due to the lack of prior immunity in dogs, which facilitated its rapid global spread. In Chile, the first cases of parvovirus were recorded between 1980 and 1981.

Canine parvovirus is a single-stranded, negative-sense DNA virus composed of approximately 5,000 nucleotides encoding three structural proteins (VP1, VP2, and VP3) and two nonstructural proteins (NS1 and NS2). Of these, VP2 is the key protein that allows the virus to enter cells through its interaction with the cellular transferrin receptor. This protein exhibits antigenic epitopes that trigger the host's immune response and is encoded by the VP2 gene, composed of approximately 1,750 nucleotides.

Alterations in three essential amino acids of VP2, particularly in the antigenic region of the capsid, which interacts with the cellular transferrin receptor, have given rise to three variants of PVC-2: PVC-2a, PVC-2b and PVC-2c.

Puppies' immunity to PVC-2 during the first weeks of life is provided by antibodies present in maternal colostrum. However, antibody titers decline over time, making it necessary to actively immunize puppies from the sixth to eighth week of age. It is crucial not to vaccinate before this period to avoid neutralization of the vaccine by maternal antibodies. The octuple or sextuple vaccines should be administered until 16 weeks of age, with intervals of 2 to 4 weeks between each dose. A booster dose should be given thereafter, as the antibodies generated by the last vaccine decline over time. Furthermore, revaccination is recommended at 6 to 12 months of age, and booster doses every 3 years.

Vaccination remains the most effective method for preventing the disease. However, in Chile, cases have been reported of dogs vaccinated with at least one dose that, despite being immunized, contracted PVC-2 infection. Mutations in the VP2 gene have also been documented in regions previously described in the literature (CPV-2b: 297 and 324; CPV-2c: 440), as well as new mutations not previously reported (CPV-2b: 514; CPV-2c: 188, 322, 379, 427, and 463).

These mutations could have implications for the protective immunity generated by vaccines. This phenomenon could be explained by the virus's genetic diversity, which allows it to evade the immune system. There is also the possibility that vaccination procedures were inadequate (e.g., booster doses were not administered in a timely manner), or that high levels of maternal IgG antibodies interfered with the effectiveness of the initial vaccination.

In view of the above, and with the aim of comparing the percentage of nucleotide identity between the second third of the VP2 gene present in a clinical sample from a vaccinated, diseased dog and the sequence of the vaccine used in that dog, this study focused on comparing nucleotide sequences. In silico primers for Polymerase Chain Reaction (PCR) were designed, and various open-access software programs were used to compare the obtained sequences.

Background

Currently, two types of canine parvovirus have been identified worldwide: PVC-1 and PVC-2. PVC-1 causes infections without clinical signs, while PVC-2 was first detected in 1978 in Texas, United States, and emerged as a pandemic due to the lack of prior immunity in dogs, which favored its rapid spread. In Chile, the first cases of parvovirus were recorded between 1980 and 1981 [1,2].

PVC-2 is classified as a subspecies within the *Parvoviridae* family, *Parvovirinae* subfamily, *Protoparvovirus* genus, and carnivore parvovirus type 1 species, along with *feline panleukopenia* virus [3].

This virus is small, approximately 25 nanometers (nm) in size, and lacks a lipid envelope (it is naked), allowing it to survive in the environment for 5 months to 1 year. It is highly resistant to disinfectants and proteolytic enzymes and can tolerate a wide pH range [4,5].

PVC-2 has a single-stranded, negative-sense DNA composed of approximately 5,000 nucleotides that encode three structural proteins (VP1, VP2, VP3) and two non-structural proteins (NS1 and NS2) [6,7]. The structural proteins form an icosahedral capsid composed of 60 protein subunits, known as capsomeres, which protect the viral genome from the action of proteases. VP2 constitutes 90% of these subunits, while VP1 represents only 10% [3,4].

The VP2 protein is encoded by the VP2 gene, which contains approximately 1,750 nucleotides [8]. This protein allows the virus to enter the cell by interacting with the transferrin receptor on the host cell. In addition, VP2 presents antigenic epitopes that activate the host immune response [6,7]. VP2 antigenic epitopes originate from mutations at positions 93 (lysine to asparagine) and 323 (aspartate to asparagine) of the protein [9].

Consecutive mutations in three key amino acids of VP2, particularly in the antigenic region of the capsid that interacts with the cellular transferrin receptor, have given rise to three PVC-2 variants: PVC-2a, PVC-2b, and PVC-2c [10].

VP1 has a 143-amino acid amino acid amino acid sequence containing a nuclear localization signal that facilitates the transport of viral particles to the cell nucleus. It also has a phospholipase domain necessary during the late stages of viral entry, allowing for virus release from perinuclear vesicles or late endosomes [9]. VP3 is generated when VP2 undergoes proteolysis after protein translation [3].

Regarding non-structural proteins, NS1 is involved in viral DNA replication, as it possesses endonuclease and helicase activity, facilitating the binding and cleavage of viral DNA during replication. Furthermore, it has a cytotoxic function, as it can induce apoptosis mediated by caspase activation [11,12]. Although the exact function of NS2 is not fully defined, it is known to be involved in the control of nuclear and cytoplasmic transport by interacting with the nuclear export factor CRM1 [12]. It is also believed to contribute to the assembly of the viral capsid in a specific host [6].

PVC-2 enters the body mainly through the oronasal route, through direct contact with feces or vomit of infected dogs, people or contaminated surfaces (fomites), or even by vertical transmission from mother to fetus [5]. The virus has tropism for tissues with constantly dividing cells, and initially replicates in lymphoid tissues associated with the pharynx, mesenteric lymph nodes, thymus, and Peyer's patches in the intestine. It then spreads through the blood to the intestinal mucosa, especially to the intestinal crypts located in the microvilli of the small intestine. It can also spread to the bone marrow, kidney, and other tissues [13-15].

The viral incubation period is 4 to 14 days, from infection to the onset of clinical signs. Viral shedding begins in the animal's feces and vomit between the third and fourth day post-infection and can last 7 to 10 days [15,16].

The clinical picture of the infection is called parvovirus and mainly affects puppies under one year of age, although it can also infect unvaccinated adult dogs [17]. Parvovirus occurs in two clinical forms: enteric and myocardial. The enteric form affects the cells of the intestinal crypts, causing the destruction of intestinal villi. This leads to severe losses of proteins, fluids (such as blood), nutrients, and ions into the intestinal lumen, causing severe diarrhea (often hemorrhagic) and vomiting (also hemorrhagic), which can lead to severe dehydration and hypovolemic shock. Other clinical signs include loss of appetite, lethargy, abdominal pain, and high fever. In some cases, bacterial translocation and/or endotoxins into the circulation may occur due to alterations in the intestinal barrier [9,13,18].

The less common myocardial form occurs when the virus infects the heart muscle cells, causing myocarditis. This form of the disease is more common in puppies that have not received enough colostrum or that acquired the virus transplacentally, or during the first weeks of life, when myocardial cells have a high rate of cell division. Pups that survive this form are left with heart failure and edema, often leading to early death [19,20].

Treatment for PVC-2 is palliative and generally requires hospitalization due to the severity of clinical signs. Management includes fluid therapy to restore fluid and electrolyte balance, administration of antibiotics for possible bacterial translocation, antiemetics, gastric mucosal protectants, and parenteral or enteral nutrition. Antidiarrheal agents may also be administered [15,19].

Although a presumptive diagnosis can be made based on clinical signs, confirmatory testing is required. There are several diagnostic tests for PVC-2 that use stool, vomitus, or blood samples, such as the hemagglutination and hemagglutination inhibition test, ELISA, and PCR [21].

Rapid diagnostic kits for PVC-2 are designed to detect viral antigens in feces using membrane immunochromatography. These tests have high sensitivity (97%) and specificity (100%) [22],

but can produce false negatives if used outside the viral shedding period, which is 7 to 10 days after infection. False positives can also occur in vaccinated dogs that shed the virus in the first few days after vaccination [19].

Regarding hemagglutination, one disadvantage is that some CPV-2 variants lack hemagglutinating activity, which limits their effectiveness in certain cases [18,21]. On the other hand, the hemagglutination inhibition test is more specific, as it uses antibodies directed against the viral antigen, improving diagnostic accuracy [23].

PCR (Polymerase Chain Reaction) is another highly sensitive diagnostic technique that allows for the detection of the virus from a few molecules of viral DNA in stool or tissue samples. This technique is highly specific because it uses specific primers that hybridize with complementary sequences in viral DNA, making it an effective tool for confirming the presence of PVC-2 [21].

Regarding PVC-2 immunization, during the first weeks of life, puppies receive maternal antibodies through colostrum, which provides temporary protection. However, over time, these maternal antibodies diminish, making it necessary to initiate active immunization. This is achieved by administering octuple or sextuple vaccines between 6 and 8 weeks of age to avoid interference with maternal passive immunity [24].

These vaccines, in addition to offering protection against PVC-2, contain antigens for other diseases such as canine distemper. It is recommended to administer booster doses up to 16 weeks of age (or later), with an interval of 2–4 weeks between each dose, to ensure antibody titers do not decline prematurely. Then, after a period of 4 weeks between doses, a new booster dose should be administered, as the antibodies generated by the last vaccine may have disappeared. Subsequently, boosters are administered every 6–12 months and then every 3 years [24].

Although vaccination is the most effective method for preventing PVC-2 infection, cases of dogs immunized with at least one dose that still contracted the disease have been reported in Chile [25]. Furthermore, mutations have been identified in previously undocumented regions of the VP2 gene, such as the CPV-2b (514) and CPV-2c (188, 322, 379, 427, and 463) variants. These mutations could have implications for vaccine-induced protective immunity, highlighting the need for further research into the relationship between these variants and immunization effectiveness [26].

Based on this information, this work aims to study and compare the percentage of nucleotide identity in the second third of the VP2 gene (nucleotides 3273 to 3858 of the PVC-2 genome), which is crucial for antibody generation. The VP2 sequence of a clinical sample obtained from a vaccinated dog that presented the disease was compared with the VP2 gene sequence present in the vaccine used to immunize the animal.

A possible hypothesis of this work was that the variability that explains the cases of immunized dogs that presented parvovirus is found in the second third of the VP2 gene of canine parvovirus

Materials and Methods

This work was developed in the Virology Laboratory of the Department of Animal Preventive Medicine of the Faculty of Veterinary and Animal Sciences at the University of Chile.

Samples and Controls

The samples required for this study were fecal samples from dogs of any age vaccinated with one or more doses of parvovirus vaccine that presented clinical signs compatible with parvovirus. In the case of the study dogs, the vaccine inoculated in them was VANGUARD® PLUS 5/L4.

Furthermore, the samples had to be collected at least thirteen days after inoculation of the last vaccine dose, since the parvovirus present in the vaccine can be shed in feces for up to twelve days.

Ten samples were obtained from patients attending various veterinary clinics, registered, and stored in sterile containers frozen at -18°C until DNA extraction.

As a positive control, the vaccine inoculated in the patients was used, along with one sample of VANGUARD® PLUS 5/L4, which contains the NL-35-D strain of canine parvovirus.

DNA from three different sources was used as a negative control: *canine herpesvirus, Brucella suis, and Salmonella Enteritidis*. Nuclease-free water was used as a reagent control.

Primers: The design of optimal primers for amplifying the second third of the VP2 gene included access to the Genbank® database to identify the official sequences of 12 sequences from the second third of the canine parvovirus VP2 gene. These sequences were aligned using the online program Clustal Omega to obtain a consensus sequence, which was used for primer design according to Invitrogen's Oligoperfect Design® program. These primers, which amplified a fragment of approximately 500 bp, were sent to IDT® for synthesis.

DNA Extraction: Extraction columns (Roche® High Pure PCR Template Preparation Kit) were used according to the manufacturer's instructions. A total of 200 µL of each sample (vaccine or feces) was obtained and incubated in a solution of 200 µL of Binding buffer plus 40 µL of Proteinase K for 10 minutes at 72°C. 100 µL of Binding buffer was then added and transferred to the column where it was centrifuged at 13,000 rpm for 1 minute. 500 µL of Inhibitor Removal buffer was then added to the column and centrifuged once more at 13,000 rpm for 1 minute. Two washes were then performed with 450 µL of Wash buffer. Finally, 50 µL of Elution buffer was added to the column to recover the purified DNA.

The integrity of the DNA extracted from each sample was controlled by electrophoresis on a 2% agarose gel in TAE (Tris-acetate-EDTA) buffer at 90 volts for 40 minutes, using a molecular weight marker. This gel was incubated in a GelRed solution for 30 minutes at room temperature, then observed under an ultraviolet

light transilluminator, and a photographic record was obtained.

PCR Mix: The PCR mix included DNA (viral, positive control (vaccine), negative controls) or reagent controls, the designed primers, and a MasterMix solution (*Taq* polymerase, nucleotides (A, T, C, G), and Mg+2) according to the manufacturer's instructions.

PCR Reaction: The PCR reaction was carried out using a protocol compatible with the design of the optimal primers and included three steps: denaturation at 94°C for 30 seconds; annealing at the average temperature of both primers used minus 5 degrees Celsius (55°C) for 30 seconds; and the polymerization process at 72°C for 1 minute. Thirty cycles were used, followed by an extension step at 72°C for 10 minutes.

Visualization of the Amplified Product: Once the PCR was completed for each sample, the product was visualized through electrophoresis on a 2% agarose gel in TAE (Tris-Acetate-EDTA) buffer. This electrophoresis was performed at 90 Volts for 40 minutes; a molecular weight marker was used. The agarose gel was then incubated in a GelRed solution for 30 minutes at room temperature. Finally, the gel was observed in an ultraviolet light transilluminator and a photographic record was obtained.

Determination of Amplified Product Identity: To determine the percentage of nucleotide identity, the positive PCR fragments (including the vaccine sample) were sent to the Genytec Ltda. Sequencing Center for sequencing in duplicate. The nucleotide sequences obtained were aligned using Clustal Omega, and a consensus sequence was obtained for each sample submitted. These sequences were then aligned using Clustal Omega, and the percentage of nucleotide identity between them was obtained.

Biosafety Measures. All laboratory work was carried out in accordance with the biosafety standards established for the Microbiology and Virology laboratories at FAVET, which include the use of clean materials, proper waste disposal, a closed apron, and gloves during laboratory work. Visualization of the amplified product involved a UV light transilluminator. Therefore, an acrylic plate and UV-filtering glasses were used when viewing the gel.

Resultados

To determine the usefulness of a pair of primers specific to the second third of the canine parvovirus VP2 gene sequence.

The 12 sequences obtained from Genbank® corresponding to the second third of VP2 were aligned using the online program Clustal Omega to obtain a consensus sequence (Appendix 1), which was used for primer design according to Invitrogen's Oligoperfect Design® program. The characteristics of each primer are shown in Figure 1.

Available Primers

Amplicon Siz...	Primer Name	Sequence	Length ...	%GC	Tm(°C) ...	Start	Stop
525	ITALO_F_1	CCAACCATACCAACTCCATGGA	22	50.00	60.03	4	25
	ITALO_R_1	TGGATCACCATCTGCTGCTT	20	50.00	59.38	509	528
526	ITALO_F_2	CCAACCATACCAACTCCATGGA	22	50.00	60.03	4	25
	ITALO_R_2	TTGGATCACCATCTGCTGCT	20	50.00	59.38	510	529
502	ITALO_F_3	CCAACCATACCAACTCCATGGA	22	50.00	60.03	4	25
	ITALO_R_3	TTTCATCTGTTTGCGCTCCC	20	50.00	59.12	486	505
526	ITALO_F_4	ACCAACCATACCAACTCCATGG	22	50.00	60.29	3	24
	ITALO_R_4	TGGATCACCATCTGCTGCTT	20	50.00	59.38	509	528
527	ITALO_F_5	ACCAACCATACCAACTCCATGG	22	50.00	60.29	3	24
	ITALO_R_5	TTGGATCACCATCTGCTGCT	20	50.00	59.38	510	529

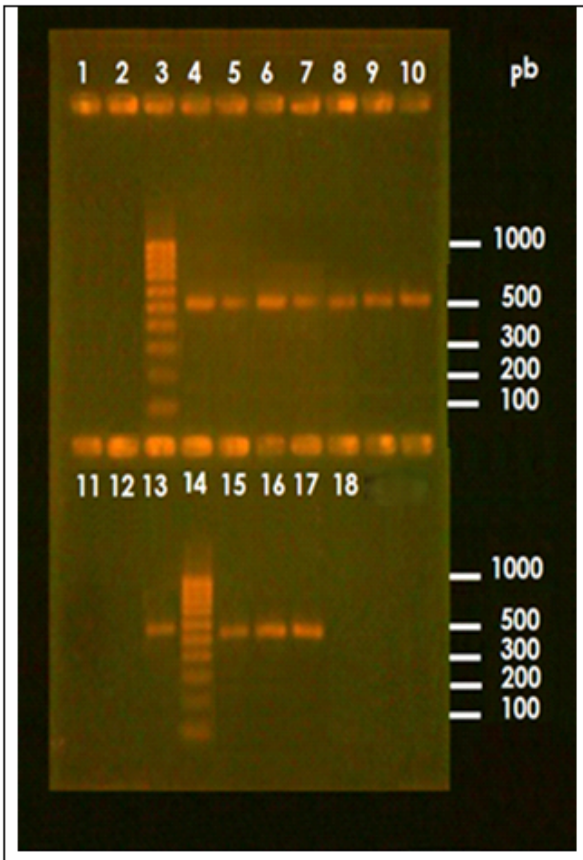
Figure 1: No. 1. Characteristics of the starters according to Oligoperfect Design®.

Subsequently, using the selected primers and the aforementioned conventional PCR protocol, the PCR products were applied to the proposed electrophoresis system, and observation of the 2% agarose gel under the UV transilluminator revealed the presence of clear, single bands, approximately 500 bp in size, in only the 10 samples analyzed and in the positive control Figure 2.

To determine the percentage of nucleotide identity (PNI) of the second third of the VP2 gene between the sequences from the commercial vaccine and clinical samples

All samples that tested positive for the PCR described above were sent for sequencing in duplicate, and an alignment was performed using Clustal Omega, which yielded a consensus sequence for each of the PCR products.

These consensus sequences were compared using Clustal Omega with the sequence derived from the commercial vaccine in duplicate, yielding nucleotide identity percentages greater than 90% for each sample (Table 1)



Lane	Content
1	Negative control 1 (Canine herpes virus DNA).
2	Reagent control
3	Molecular size standard (100 bp to 1,000 bp).
4	Sample 1
5	Sample 2.
6	Sample 3.
7	Sample 4
8	Sample 5
9	Sample 6
10	Sample 7
11	Reagent control.
12	Negative control 2 (*Salmonella Enteritidis* DNA)
13	Positive control (VANGUARD® PLUS 5/L4 Vaccine).
14	Molecular size standard (100 bp to 1,000 bp).
15	Sample 8
16	Sample 9.
17	Sample 10
18	Negative Control 3 (*Brucella suis* DNA).

Figure No. 2. 2% agarose gel PCR second third VP2 gene

Figure 2: No. 2. 2% agarose gel PCR second third VP2 gene.

In parallel, these consensus sequences were entered into the online BLAST program, confirming that they belong to the parvovirus genome and specifically to the VP2 gene (Appendix 2).

Discussion

Canine parvovirus is a disease of high clinical importance due to the symptoms it causes and because various vaccines have been developed throughout history to prevent this disease. Despite this, cases of parvovirus have occurred in Chile and around the world in dogs that are up to date with their vaccination schedule, which at least allows us to ask a question such as, *"Do these vaccinated and reinfected individuals have a canine parvovirus gene other than the one contained in the vaccine*?" This seeks an explanation that includes possible genetic variability in the virus that could evade the individual's immune system.

Considering the above and that one of the genes mainly involved in the immune response of individuals is VP2 [6,7], a gene of approximately 4700 nucleotides, in this work the second third of the VP2 gene of canine parvovirus was used as a study target, as part of a study that covers each third of VP2 separately, that is, involving two additional works (first and third third respectively).

First, the methodology involved in primer design using Invitrogen's Oligoperfect® indicates that the use of online biotools is highly useful for initiating the search for a response such as the one proposed, as unique, clear PCR products of the expected size were observed in all the samples considered and in the positive control. The absence of DNA products in the negative control or the reagent control reflects another important point for the detection of canine parvovirus in general.

Second, the sequencing and alignment of the proposed PCR product sequences allow for the yield of nucleotide identity percentages—which, in relation to the question posed, do not indicate a close relationship with a positive answer, as there would be no significant variability to explain the cases identified in Chile and around the world. This is considering the high percentage of nucleotide identity (over 90%) when comparing the different stool

samples with the vaccine. In addition, it is worth noting that no amino acid mutations were found in the present study, as there are no changes in three consecutive nucleotides when comparing the sequences obtained with the vaccine.

Thus, this high value would indicate that there would be no considerable genetic variability between the sequence of the second third of the VP2 gene obtained from the samples compared to that obtained from the vaccine.

On the other hand, the available information regarding vaccination protocols shows discrepancies between the World Small Animal Veterinary Association WSAVA (24) and the instructions of the manufacturer of the vaccine used in the study, which is VANGUARD sextuple/octuple from Zoetis [27], which translate into WSAVA indicating that primary vaccination against parvovirus should be given every three or four weeks from six weeks to sixteen or more weeks of age, then revaccination should be given at six to twelve months of age, and then every three years. In contrast, Zoetis indicates that doses of octuple/sextuple vaccines (which contain other agents apart from canine parvovirus) should be inoculated at six weeks of age and then at 9 and finally at 12 weeks of age, finally a booster every 1 year.

The differences between protocols relate to the issue of "*until which week of primary vaccination should be given*" and also to boosters, where there is no booster at six or twelve months of age, according to the vaccine manufacturers. Furthermore, the manufacturer states that annual boosters are given every year, not every three years. All of this information creates confusion for veterinarians when it comes to vaccinating, leading to different protocols among professionals.

Conclusions

The study conducted does not confirm the proposed hypothesis, as it does not confirm that variability in the second third of VP2 is part of the cause of cases of parvovirus in dogs with an up-to-date vaccination schedule.

Thus, in this thesis, none of the samples obtained from dogs vaccinated with parvovirus had a significantly lower percentage of nucleotide identity compared to the vaccine, which suggests that no patient exhibits significant genetic variability that would explain the finding of cases of parvovirus in vaccinated dogs.

The above could be corroborated or not, considering the sequencing of a PCR product of similar size to the second third of VP2 (585nt), since in this study, the primers used allow obtaining sequences compatible with the VP2 gene, but of a smaller size (525 nt), suggesting considering the overlap with the other two thirds for the design of other primers.

Thus, we will have to await the results that include the other two-thirds of the developing VP2 gene to determine whether or not there is a potential significant genetic variation in the canine parvovirus VP2 gene that could lead to changes in the immune response.

Finally, in the future, the discrepancies between the different vaccination protocols will need to be considered to define the correct one or to create a combination of them. This could be achieved through a registry of practicing veterinarians in Chile to determine the characteristics of their vaccination protocols, such as the weeks considered during primary vaccination, whether or not a booster is administered at six or twelve months of age, and, finally, how frequently they revaccinate with booster doses.

Acknowledgment

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Conflict of Interest

None.

Appendices

Appendix 1.

Alignment using Clustal Omega, PNI, and consensus sequences for 12 sequences from the second third of the canine parvovirus VP2 gene.

1 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

2 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

3 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

4 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

5 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

6 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAAACATTGGGTTTTTATCCATGG 600

7 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

8 AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600

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9  AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
10  AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
11  AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
12  AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG 600
    ************************************ ********************

1  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
2  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
3  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
4  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
5  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
6  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
7  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGATCATTAATACCA 660
8  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
9  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
10  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
11  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
12  AAACCAACCATACCAACTCCATGGAGATATTATTTTCAATGGGATAGAACATTAATACCA 660
    ********************************************** ***********

1  TCTCATACTGGAACTAGTGGCACACCAACAAACATATACCATGGTACAGATCCAGATGAT 720
2  TCTCATACTGGAACTAGTGGCACACCAACAAACATATACCATGGTACAGATCCAGATGAT 720
3  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
4  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
5  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
6  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
7  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
8  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
9  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
10  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
11  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
12  TCTCATACTGGAACTAGTGGCACACCAACAAATATATACCATGGTACAGATCCAGATGAT 720
    ******************************** ***************************

1  GTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
2  GTTCAATTTTATACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
3  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
4  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
5  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
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 6  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
 7  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
 8  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
 9  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
10  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
11  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
12  GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA 780
    ********** **************************************************

 1  TTTGCTACAGGAACATTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
 2  TTTGCTACAGGAACATTTTTTTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
 3  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACATACATGGCAA 840
 4  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
 5  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
 6  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
 7  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
 8  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
 9  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
10  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
11  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
12  TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA 840
    ****************** ****************************** *********

 1  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
 2  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
 3  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
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 8  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
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11  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
12  ACAAATAGAGCATTGGGCTTACCACCATTTCTAAATTCTTTGCCTCAAGCTGAAGGAGGT 900
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1  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
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3  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
4  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
5  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
6  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
7  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
8  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
9  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
10  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
11  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
12  ACTAACTTTGGTTATATAGGAGTTCAACAAGATAAAAGACGTGGTGTAACTCAAATGGGA 960
    ************************************************************

1  AATTCAAACTATATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
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4  AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
5  AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
6  AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
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11  AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
12  AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA 1020
    *** ***** **************************************************

1  CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
2  CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
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8  CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
9  CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080
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10 CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080

11 CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080

12 CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA 1080

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1 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

2 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

3 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

4 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

5 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

6 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

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9 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

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11 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

12 CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT 1140

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1 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

2 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

3 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

4 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

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6 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

7 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

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9 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

10 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

11 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

12 GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT 1200

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Secuencia Consenso: PIN: (1190/1200) % = 92.2 %

AATACTATGCCATTTACTCCAGCAGCTATGAGATCTGAGACATTGGGTTTTTATCCATGG

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GTTCAATTTTACACTATTGAAAATTCTGTGCCAGTACACTTACTAAGAACAGGTGATGAA

TTTGCTACAGGAACATTTTATTTTGATTGTAAACCATGTAGACTAACACACACATGGCAA

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AATACAAACATTATTACTGAAGCTACTATTATGAGACCAGCTGAGGTTGGTTATAGTGCA

CCATATTATTCTTTTGAGGCGTCTACACAAGGGCCATTTAAAACACCTATTGCAGCAGGA

CGGGGGGGAGCGCAAACAGATGAAAATCAAGCAGCAGATGGTGATCCAAGATATGCATTT

GGTAGACAACATGGTCAAAAAACTACCACAACAGGAGAAACACCTGAGAGATTTACATAT

Appendix 2.

Identity verification of the obtained sequences. Online BLAST program.

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Canine parvovirus isolate AH2005 VP2 gene, complete cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	OQ868526.1
Canine parvovirus isolate HB2005 capsid protein gene, complete cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	OR724752.1
Canine parvovirus isolate MWA8 VP2 protein gene, partial cds	Canine parvovirus	920	920	100%	0.0	98.29%	736	OP966003.1
Canine parvovirus isolate MWA10 VP2 protein gene, partial cds	Canine parvovirus	920	920	100%	0.0	98.29%	723	OP966000.1
Canine parvovirus isolate CN/HN1715 capsid protein (VP2) gene, partial cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	MK517980.1
Canine parvovirus isolate QN-8 VP2 (VP2) gene, complete cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	PQ442330.1
Canine parvovirus isolate CN/HN1709 capsid protein (VP2) gene, partial cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	MK517974.1
Canine parvovirus isolate HN2102 VP2 gene, complete cds	Canine parvovirus	920	920	100%	0.0	98.29%	1755	OQ868529.1
Canine parvovirus strain CPV-zj12 VP2 (VP2) gene, partial cds	Canine parvovirus	920	920	100%	0.0	98.29%	1270	KM386942.1
Canine parvovirus isolate 20160810-BJ-59 capsid protein (VP2) gene, partial cds	Canine parvovirus	915	915	100%	0.0	98.10%	1752	MF347729.1
Canine parvovirus isolate Sbzv1 capsid protein (VP2) gene, complete cds	Canine parvovirus	915	915	100%	0.0	98.10%	1755	MT860096.1
Canine parvovirus strain GuangZhou/P1-2/2016 VP2 protein (VP2) gene, complete cds	Canine parvovirus	915	915	100%	0.0	98.10%	1755	KY937669.1
Canine parvovirus strain CPV-js62 capsid protein (VP2) gene, partial cds	Canine parvovirus	915	915	100%	0.0	98.10%	1270	MK076920.1
Canine parvovirus strain CPV-ZXY VP2 protein gene, complete cds	Canine parvovirus	915	915	100%	0.0	98.10%	1755	KP686093.1

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