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Development of an accurate and rapid method for whole genome characterization of canine parvovirus

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Summary
Canine parvovirus is a highly contagious pathogen affecting domestic dogs and other carnivores globally. Monitoring CPV through continuous genomic surveillance is crucial for mapping variability and developing effective control measures. Here, we developed a method using multiplex-PCR-next-generation sequencing to obtain full-length CPV genomes directly from clinical samples. This approach utilizes tiling and tailed amplicons to amplify overlapping fragments of roughly 250 base pairs. This enables the creation of Illumina libraries by conducting two PCR reaction runs. We tested the assay in 10 fecal samples from dogs diagnosed with CPV and one CPV-2 vaccine strain. Furthermore, we applied it to a feline sample previously diagnosed with the feline panleukopenia virus. The assay provided 100% genome coverage and high sequencing depth across all 12 samples. It successfully provided the sequence of the coding regions and the left and right non-translated regions, including tandem and terminal repeats. The assay effectively amplified viral variants from divergent evolutionary groups, including the antigenic variants (2a, 2b, and 2c) and the ancestral CPV-2 strain included in vaccine formulations. Moreover, it successfully amplified the entire genome of the feline panleukopenia virus found in cat feces. This method is cost-effective, time-efficient, and does not require lab expertise in Illumina library preparation. The multiplex-PCR-next-generation methodology facilitates large-scale genomic sequencing, expanding the limited number of complete genomes currently available in databases and enabling real-time genomic surveillance. Furthermore, the method helps identify and track emerging CPV viral variants, facilitating molecular epidemiology and control. Adopting this approach can enhance our understanding of the evolution and genetic diversity of *Protoparvovirus carnivoran1.*

1. Introduction

Canine parvovirus (CPV) causes one of the most common and significant infectious diseases in domestic dogs, with high mortality and morbidity rates. This virus also threatens other carnivore species, with a constant viral flow observed among domestic, peridomestic, and wild animal populations (Desario et al., 2005). CPV belongs to the *Protoparvovirus carnivoran1* species (genus *Protoparvovirus,* family *Parvovirinae*), which consists of closely related viruses such as raccoon parvovirus, mink enteritis virus, and feline panleukopenia (Penzes et al., 2022; Zerbini et al., 2023). CPV causes hemorrhagic gastroenteritis and acute myocarditis, particularly in puppies, often resulting in fatal outcomes. Despite vaccination programs, CPV continues to be a significant pathogen affecting domestic dogs and other carnivores of the *Canidae* family (Decaro & Buonavoglia, 2012; Desario et al., 2005; Hoelzer et al., 2008; Robinson et al., 1980).

The CPV genome comprises a single strand of DNA, approximately 5.2 kb in length. It contains two ORFs that code for four proteins: non-structural NS1 and NS2, and structural VP1 and VP2. These proteins are produced through the process of alternative splicing. On the right end of the genome, there are two sets of unrelated repeated motifs. The first set, located near the VP2 end, consists of 60-nt monomers repeated once or twice. The second set of repeated motifs, consisting of 62 nt, is located downstream and repeated up to three times (Berns, 1990; Reed et al., 1988). The genome is also flanked by palindromic hairpins (terminal repeat sequences) involved in replication and packaging (Berns, 1990).

CPV emerged in the 1970s in Europe through a host jump from wild carnivores to dogs (Truyen et al., 1994). The original strain, CPV-2, spread globally and caused a dog pandemic (Parrish, 1999). CPV-2a replaced CPV-2 within two years and regained the ability to infect cats and dogs (Hoelzer & Parrish, 2010; Truyen et al., 1996; Parrish et al., 1988).
This lineage continues to evolve and comprises three antigenic variants known as 2a, 2b, and 2c, which differ by a few changes in the coding region of VP-1/VP-2 genes (Buonavoglia et al., 2002; Parrish et al., 1991). Differentiation among the three CPV variants occurs at a crucial antigenic site, codon 426 of the VP2 protein, resulting in Asn (2a), Asp (2b), and Glu (2c) variants. Currently, three variants of CPV-2 are circulating worldwide, each with varying prevalence and genetic variability levels. However, the traditional CPV typing based on residue 426 does not correlate with the evolutionary relationships among the circulating strains (Grecco et al. 2018).

Phylogenetic analyses based on the VP2 gene of *Protoparvovirus carnivoran1* classify them into two major groups: FPV-like, comprising feline and mink viruses dating back to the earliest isolates in 1960, and CPV-like, subdivided into the original CPV-2 pandemic strains from 1978–79 and the CPV-2a lineage encompassing the three antigenic variants from CPV-2a lineage (Hoelzer & Parrish, 2010). Through the analysis of complete genomes, new insights about the evolution of the CPV-2a lineage have been discovered. This has led to the creation of updated classifications based on the phylogenetic relationships of the variants (Chung et al., 2020; de Oliveira Santana et al., 2022; Grecco et al., 2018; Nguyen Manh et al., 2021). The study on the evolution of CPV, focusing on South America revealed that the CPV-2a ancestral lineage diversified into distinct monophyletic groups, regardless of antigenic classification. The diversification of these clades was driven by migratory events within and between continents, followed by subsequent local differentiation. In South America, the identified clades can be classified according to their geographic origin into Asia I, Europe I, Europe II and a unique clade exclusive to South America, South America I (Grecco et al., 2018).
Despite the rapid evolutionary rate of CPV-2, our current knowledge of viral evolution and the underlying mechanisms is incomplete due to limited genomic data availability. While approximately 30 complete genomes and 600 coding genome sequences have been submitted to the databases,

additional analysis of genomic data is crucial to gain deeper insights into the mechanisms driving viral evolution, such as recombination and selective pressures.

Next-generation sequencing has proven valuable in obtaining complete viral genomes, including the novel coronavirus, and enabling real-time genomic epidemiology. In this study, we developed an enrichment approach based on the tiling and tailed-amplicon method, providing a scalable solution for directly sequencing many genomes from global canine parvovirus strains.
This assay enhances our understanding of the viral genome characteristics, contributing to the broader knowledge of CPV evolution, genetic diversity, and potential mechanisms driving the emergence and spread of viral variants.

2. Materials and Methods

2.1. Primer design

The Primal Scheme Designer tool was used to implement the genome sequencing strategy (Quick et al., 2017) with an alignment file containing the 161-coding genome *of Protoparvovirus carnivoran1* published by Grecco et al. 2018 as input. To cover the ends of the genome, a full-length genome dataset was aligned with MAFFT v7.490 in Geneious Prime® 2023.1.2 (Katoh et al., 2002; Katoh & Standley, 2013). Initial primer pools were annotated on this dataset, followed by primer redesigning. New primers were added for the inverted repetitive 3´and 5´ends of the CPV genome (Supplementary Table 1). Illumina adapters were added at the 5´end of each primer. The adapter sequences used were TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG for the left primers and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG for the right primers.
Multiplexing tiling and tailed PCR was used to amplify overlapping fragments of approximately 250 base pairs in length that covered the full-length CPV genome.

2.2. Samples

To evaluate the system, 11 CPV-positive fecal samples were used from five South American countries. The selected samples included the three antigenic variants of the CPV-2 (2a, 2b, and 2c) from dogs and a cat sample from Argentina diagnosed with FPV (Table 1). Additionally, the system was tested using the original CPV-2 strain contained in a commercial vaccine.

2.3. Viral nucleic acid extraction

The viral nucleic acid extraction was performed using Qiacube sample prep automation equipment (Qiagen, USA) with DSP Virus Spin Kit, following the manufacturer's protocol. The nucleic acid was eluted in 40 µL of elution buffer (Buffer AVL, Qiagen).

2.4. Multiplex PCR-NGS reaction

The enrichment method based on tiling and tailed amplicon allows the preparation of the Illumina libraries through two runs of PCR reactions. The multiplex PCR reaction generated ≈250 bp amplicons corresponding to specific primers flanked by Illumina adapter sequences. This reaction was performed in a final volume of 25 µL comprising 12.5 µL of 2× Immomix (Bioline, USA), 1.75 µL of CPV primer set (each primer at a final concentration of 0.02 µM), and 2 µL of viral nucleic acid. Individual reactions were conducted for each primer set pool (pools 1 and 2). The PCR cycling conditions included an initial denaturation at 95 Cº for 10 minutes, followed by 35 cycles of denaturation at 95Cº for 30 seconds and

primer annealing and extension at 55 Cº for 5 minutes. Amplification was visualized by 0.8% agarose gel electrophoresis stained with ethidium bromide and imaged with a MultiDoc-It Imagins System transilluminator (UVP).

The PCR products from pools 1 and 2 for each sample were combined and purified by 1.8× concentration of AMPure XP beads (Beckman Coulter, Indianapolis, USA). The purified PCR product (100 ng) was used as a template in a second PCR round to add dual indexes for multiplexing the samples (sample-specific libraries). The PCR was performed using Q5 DNA Polymerase (New England Biolabs) and followed the Illumina DNA prep protocol conditions to add Index 1 (i7) and Index 2 (i5) adapters (Illumina, San Diego, CA). The specific libraries were pooled and purified together before sequencing. The PCR reactions were carried out using an Arktik Thermal Cycler (Thermo Scientific, USA).

2.5. Illumina Sequencing and Bioinformatics workflow

The enriched purified library pool, including a negative sample, was subjected to sequencing for 151 cycles at paired-end reads (2×150) on the Illumina MiniSeq platform.
The obtained reads were processed using Geneious Prime (https://www.geneious.com). First, the BBDuk tool removed Illumina adapters, primer sets, and low-quality reads (Phred quality scores <30). The resulting reads were aligned to the reference CPV-2 genome (M38245.1) using Minimap 2.17 (Li, 2018). Finally, the majority consensus sequence was generated (threshold 0%).

2.6. Genomic Characterization

The consensus sequences were annotated to identify the non-structural (NS) and structural (VP) ORFs and the repeat regions at the ends of the genome. Amino acid 426 of VP2 protein was determined to identify antigenic variants.
Sequence alignment was performed using the dataset of 161 coding genomes published by Grecco et al. 2018 and the new sequences obtained using MAFFT (Katoh et al., 2002; Katoh & Standley, 2013). Maximum-likelihood trees were inferred in Geneious using PhyML (Guindon et al., 2010) and visualized with FigTree.

3. Results

3.1. *Multiplex PCR-NGS*

The developed workflow based on multiplex- PCR-NGS for full-length CPV genome sequencing is schematized in Figure 1. The CPV primer set designed consisted of 70 left and right primers with 35 primers in each pool (Supplementary Table 1). The multiplex-PCR-NGS approach was tested on 11 clinical samples (10 dogs and 1 cat) and one vaccine strain (Table 1).
In the first round of PCR, which included the separate reactions multiplex pool 1 and 2, amplicons of the expected size of 250 bp were obtained for all samples; therefore, the purified overlapping amplicons were subjected to the second round of indexing PCR.

The bioinformatics workflow resulted in a 100% breadth of genome coverage for all samples. The mean coverage depth ranged from 4000× to 19000× (Table 1). Although all primer sets products provided excellent coverage depth, certain regions displayed lower coverage, a phenomenon observed in most CPV variants (Figure 2). This decrease was in the 3' and 5' UTR regions, where the repeats are located. Additionally, a short region of ≈9 nt in NS1 (corresponding to amino acid residues 175-178) and a coding region of VP1 downstream of the A homopolymer coding for 4 lysines at position 127-130 also showed decreased mapped reads.

Repeating the workflow using the consensus sequence produced in the initial mapped round as a new reference sequence increased the genomic coverage significantly.

3.2. Genomic Characterization

Consensus sequences obtained through reference mapping were annotated and deposited in the GenBank (Table 1). The full-length genomes ranged from 5059 to 5216 nts. All strains contained the two ORFs with the same extension, 2007 nts for NSs and 2184 nts for the VPs.
CPV field, vaccine strains and FPV could be differentiated based on the number of monomers in the repeat motifs at the right end of the genome. CPV field strains have a single 60-nt monomer, while cat and vaccine have two repeat monomers. In the second set of repeat motifs, CPV and FPV field strains have a single 62-nt monomer, whereas the vaccine CPV strain has three repeat monomers (58-nt, 62-nt, and 62-nt).

According to the amino acid 426 of the VP2 protein (Table 1), it was determined that three field strains were classified as CPV-2a due to the presence of the Asn residue (two Uruguayan and one Peruvian strains). Five strains from Argentina, Ecuador, Peru, and Uruguay have Glu at residue 426, corresponding to CPV-2c. Two strains from Peru and Argentina were classified as CPV-2b by Asp 426. The FPV strain from the cat (Arg-9) and the vaccine displayed residue Asn426.

Among the characterized strains, only two had been previously obtained by traditional Sanger sequencing (CPV-Arg-22 and CPV-Uy-6). The consensus sequence of the Arg-22 strain was identical with both methods (Sanger and NGS). The CPV-Uy-6 differs on one A/G non-synonymous substitution with the Sanger sequence (MF177281) at codon N140D of VP1. This single nucleotide polymorphism occurs near the low coverage region of VP1.

The analyzed dataset of the 172 coding genomes showed that the strains could be grouped into two main clades, FPV-like and CPV-like. FPV-Arg9 was found to be clustered with the FPV-like clade. In the CPV-like clade, two groups were identified: the original CPV-2 variant, which also included the amplified vaccine strain and the CPV-2a lineage. The remaining 10 obtained sequences fell within the CPV-2a lineage, comprising the clades of South America 1 (CPV6_2a_UY), Asia 1 (CPV252_2a_UY), Europe I (all Argentinian and Ecuadorian strains and CPV86_2c_UY) and Europe II (all Peruvian strains) (Figure 3).

4. Discussion

Pathogen surveillance is aided by genomic epidemiology, which provides insight into their genetic variations, spread, source, and evolutionary patterns. As sequencing methodologies become more advanced and affordable and computational analysis becomes more straightforward, the use of whole genome sequencing has dramatically increased. This expansion was particularly evident during the COVID-19 Pandemic, where financial contributions from governmental and private sectors enabled widespread sequencing applications to track the SARS-CoV-2 evolution in real time and inform health control plans.

Applying NGS to viruses can be challenging and requires viral enrichment steps beforehand. Enrichment methods based on amplicon sequencing are widely used to identify microbial communities through 16S ribosomal RNA amplification and fungi through internal transcribed spacer amplification (Knight et al., 2018). Whole genome sequencing by viral enrichment PCR has been chiefly applied to new emerging and re-emerging viruses such as SARS-CoV-2 and Zika (Itokawa et al., 2020; Quick et al., 2017).

We have applied these methods to detect cases of co-infection and recombination between CPV variants by amplifying the complete coding genome using two PCR amplicons (Pérez et al., 2014). This system was recently employed to characterize the complete coding region of Peruvian CPV strains (Luna Espinoza et al., 2022). Moreover, a new preprint report described a system for obtaining the complete genome using multiplex-PCR-NGS with Nanopore technology that amplified the genome in overlapping fragments of 400 pb or 1000 pb and was tested explicitly on two antigenic variants (2a and 2c) (Oliveira et al., 2023).

Here, we developed a fast, simple, and cost-effective method to obtain the complete genome of CPV. This study employed a multiplex PCR approach combined with NGS based on the tiling and tailed-amplicon method to obtain full-length CPV genomes from clinical samples. This strategy employed two PCR reactions to directly generate specific Illumina libraries for sequencing each sample (Figure 1). The tiling and tailing approach used is widely employed for 16S sequencing in bacteria (Amos et al., 2020; Bjerre et al., 2019), amplification of eukaryotic marker genes (Cruaud et al., 2017) and recently developed for SARS-Cov-2 (Gohl et al., 2020). However, there was no such method for the CPV virus.

This method showed robustness as the designed primer set successfully amplified all samples with 100% breadth coverage for the current circulating CPV-2 antigenic variants (2a, 2b, and 2c) and the original CPV-2 variant. The three antigenic variants also span several evolutionary groups (ASI, EUI, EUII and SAI) defined by Grecco et al. (2018) (Figure 3). In addition, the system was successfully applied to another member of the *Protoparvovirus carnivoran1* species, the feline panleukopenia virus.

Obtaining full-length genomic sequences from diverse evolutionary groups demonstrates the assay has high sensitivity and efficiency in capturing globally circulating variants. Although CPV has a high substitution rate (Shackelton et al., 2005), different CPV variants differ by relatively few nucleotide changes (Parrish, 1999). Accordingly, the assay can be easily adapted to target new variants that may emerge and other members of *Protoparvovirus carnivoran1* (raccoon parvovirus and mink enteritis virus) by slightly modifying or adding individual primers to variable regions.

The amplicons produced high coverage depth, although some genome regions showed decreased coverage (Figure 2). This low coverage seems intrinsic to the type of sequences and their sequencing since it is repeated in most samples. The low coverage in the contiguous polyA region has already been reported for CPV with Sanger and Illumina sequencing (Pérez et al., 2014; Voorhees et al., 2019). Only two of the sequenced strains were previously obtained by traditional Sanger sequencing (CPV-Uy-6 and CPV-Arg-22). The comparison revealed that CPV-Uy-6 has one different position concerning the submitted sequence (MF177281) in the 140 codon of VP1 (AAT to GAT). This nonsynonymous change (N to D) corresponds to the surroundings of the polyA region. The variation may be caused by homopolymers, which affect the polymerase and make it harder to detect misincorporation, especially at a 2× coverage usually employed in Sanger sequencing. NGS techniques can enhance sequencing depth and improve genome sequence consensus despite low coverage in specific regions.

Our approach is advantageous as it allows us to obtain complete genome coverage, including the 3´ and 5´terminal repeats (UTRs). UTRs regions are little explored in CPV but play essential roles in viral replication, transcription, and encapsidation (Burnett et al., 2006; Han et al., 2015; Rhode & Klaassen, 1982; Yu et al., 2019). These regions also serve as targets for differentiation between variants (Buonavoglia et al., 2002; Pérez et al., 2012) due to changes in the number of repetitions of the different monomers that compose the repeats. There are few approaches in CPV to obtain and annotate the

UTRs, and most of them are laborious with a combination of previous steps involving cell culture, cloning steps, or primer walking method (Chung et al., 2020; Voorhees et al., 2019).

Although CPV has a small genome size, the NGS cost limits the large-scale genomic sequencing. The process of preparing the library is a significant obstacle in NGS sequencing in terms of cost, time, and the labor-intensive nature of the procedure. Our workflow is designed to minimize costs, save time, and reduce the need for specialized lab expertise, regardless of the library preparation kit used. The developed method based on a two-step PCR reaction will increase the accuracy of CPV genome sequences and the number of samples, allowing multiplexing hundreds of samples per sequencing run, reaching a coverage mean greater than 2000x. Our viral enrichment approach provides a scalable solution for directly sequencing many genomes from the global *Protoparvovirus carnivoran1* strains.

In conclusion, we developed a robust, rapid, and cost-effective method based on multiplex-PCR-NGS able to amplify the whole- genome of all CPV variants circulating worldwide. This approach is easy to apply and particularly valuable in regions with limited genomic data, such as Latin America, to increase epidemiological knowledge of this emerging virus. Continued surveillance and genomic studies are essential to monitor the emergence of new CPV variants and inform effective control strategies to mitigate the impact of this highly infectious virus.

Figure legends

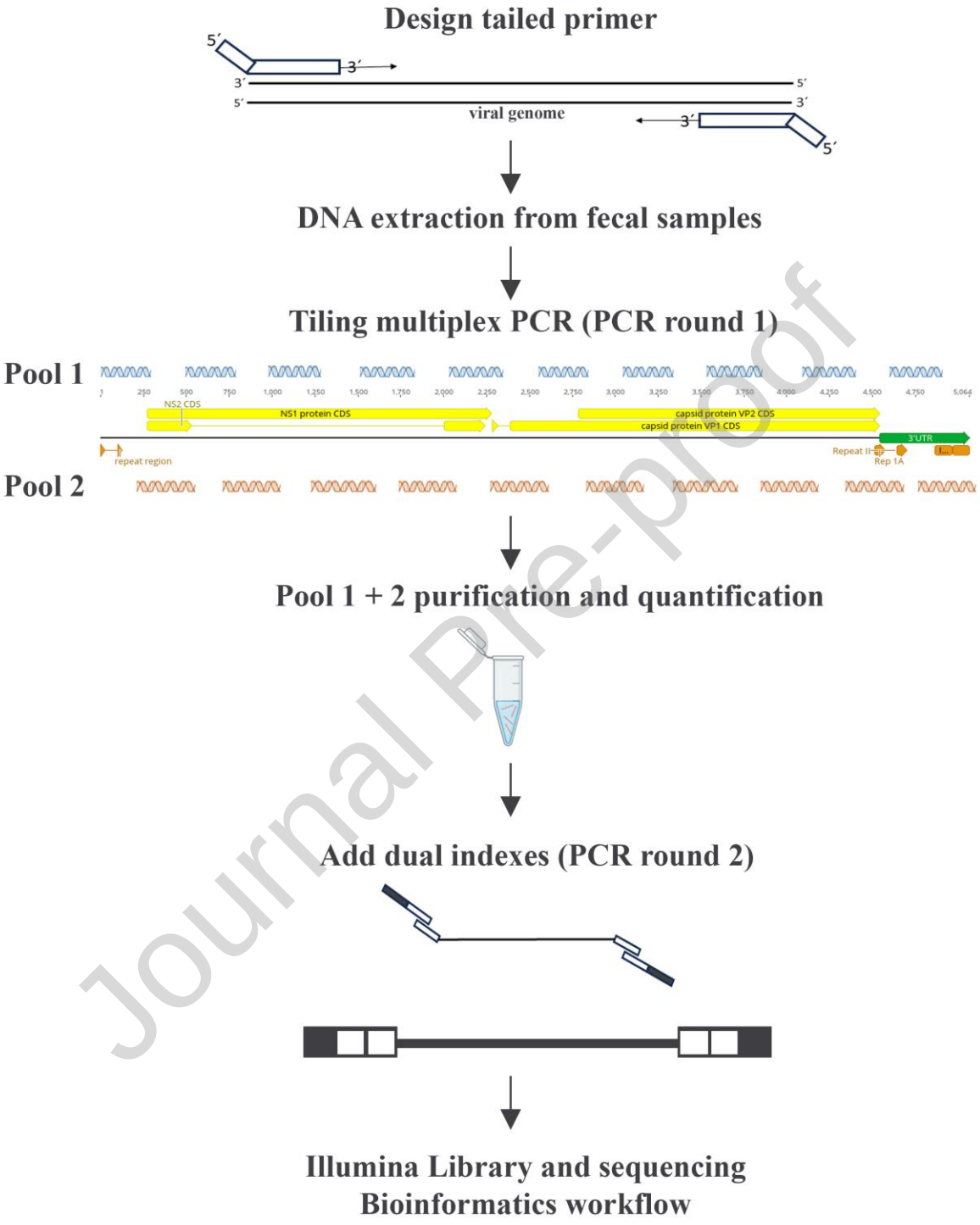

Figure 1. The tiling and tailed multiplex-PCR-NGS method workflow is depicted, with each step clearly outlined for comprehension.

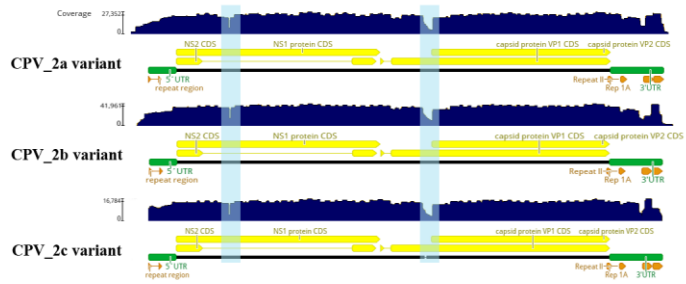

Figure 2. Coverage depth of three CPV antigenic variants. The blue boxes show the specific coding regions with a decrease in coverage. For better visualization, the coverage graph is shown on a logarithmic scale.

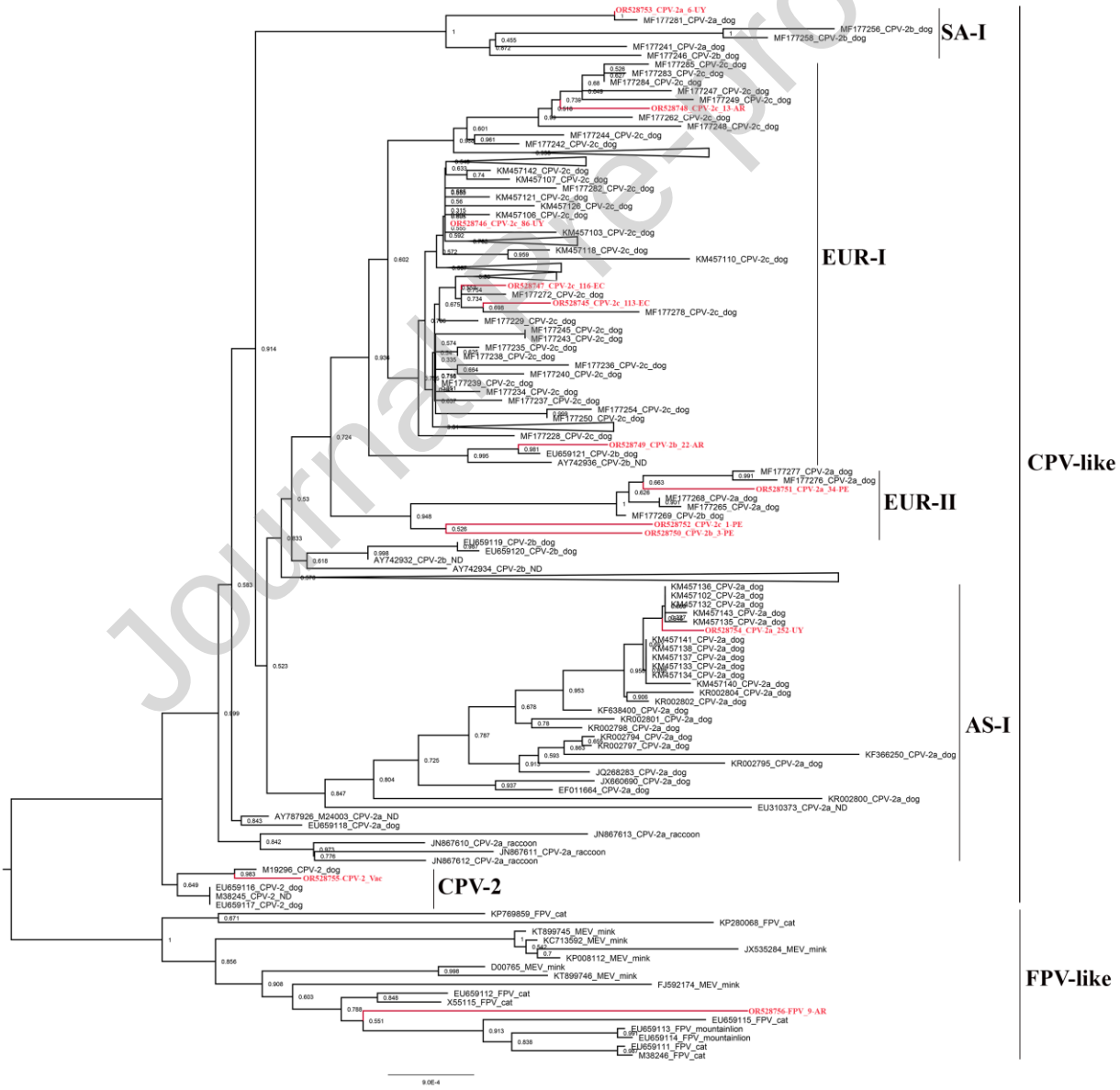

Figure 3. Maximum likelihood trees with GTR nucleotide substitution model. Evolutionary groups are indicated according to Grecco et al. 2018. Sequences here obtained are indicated in red.

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Declaration of Competing Interest
The authors declare no conflict of interest.

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Table 1. Samples used in multiplex-PCR-NGS

Sample	Evolutionary Groups*	Antigenic classification	Country/year	Host	Coverage mean	Accession number NGS
CPV-UY-6[1]	South America-I	2a	Uruguay/2006	Dog	19852	OR528753
CPV-UY-252	Asia-I	2a	Uruguay/2010	Dog	6339	OR528754
CPV-UY-86	Europe-I	2c	Uruguay/2007	Dog	11165	OR528746
CPV-PE-1	Europe-II	2c	Peru/2017	Dog	7111	OR528752
CPV-PE-3	Europe-II	2b	Peru/2017	Dog	6393	OR528750
CPV-PE-34	Europe-II	2a	Peru/2019	Dog	5394	OR528751
CPV-AR-13	Europe-I	2c	Argentina/2010	Dog	7772	OR528748
CPV-AR-22[2]	Europe-I	2b	Argentina/2013	Dog	10136	OR528749
CPV-EC-113	Europe-I	2c	Ecuador/2012	Dog	4413	OR528745
CPV-EC-116	Europe-I	2c	Ecuador/2012	Dog	7634	OR528747
CPV-vaccine (NL-35-D)		CPV-2 (2a)			4015	OR528755
FPV-AR-9	FPV-like		Argentina/2009	Cat	5342	OR528756

* Evolutionary Groups according to Grecco et al. 2018

1. Accession number Sanger, MF177281

2. Accession number Sanger, OK888554

Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- An innovative multiplex tiling and tailed PCR assay was developed for CPV sequencing.
- The assay provides complete genomes directly from clinical samples.
- CPV viral variants were obtained straightforwardly from diverse evolutionary groups, including the ancestral CPV-2 strain and feline panleukopenia virus.
- The methodology facilitates the real-time surveillance of CPV genomes.