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Effect of sampling site on the diagnosis of canine parvovirus infection in dogs using polymerase chain reaction

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Abstract

Background: Accurate diagnosis is imperative in dogs with clinical signs of parvovirus infection (CPV-2).

Objectives: To assess quantitative real-time PCR (qRT-PCR) for the diagnosis of CPV-2 infection, and determine the optimal sampling site. Secondarily, to compare qRT-PCR with a point-of-care PCR kit (PCRun), and to assess sensitivity of serology for CPV diagnosis.

Animals: Sixty dogs with naturally acquired parvovirus infection, 44 unvaccinated puppies, of which 16 were followed after first and second vaccination, 15 adult dogs, of which 10 were followed also after a booster vaccine, and 9 dogs with distemper virus infection.

Methods: Prospective study. Samples from the rectum, blood, and pharynx were obtained for PCR.

Results: All dogs with a clinical diagnosis of parvovirus infection were positive by qRT-PCR in at least 1 sampling site (ie, rectum, blood, pharynx), and 50 (83%) of 60 were positive in all sites. qRT-PCR was negative in 67 (99%) of 68 healthy puppies (before-vaccination), puppies with distemper, and healthy adult dogs. Ten days after initial vaccination of puppies, 62% (fecal), 31% (blood), and 12% (pharyngeal) of samples were positive for CPV-2 on qRT-PCR. The proportion of positive pharyngeal samples decreased 20 days after vaccination and all sites were negative 12-28 days after second vaccination. Vaccinated adults were negative before and after booster vaccination.

Conclusions and Clinical Importance: Molecular detection of CPV is sensitive, but specificity is hampered temporarily during the vaccination period. Blood, feces, and pharynx are suitable sampling sites. Fecal samples had the lowest sensitivity in sick dogs and highest positivity in puppies after vaccination.

KEYWORDS
diagnosis, polymerase chain reaction, puppy, serology, viral, vomiting

ABBREVIATIONS: CPV, canine parvovirus; MGB, minor groove binder; MLV, modified live virus; qRT-PCR, quantitative real-time PCR; TTP, time to peak.

1 | INTRODUCTION

Canine parvovirus (CPV-2) was recognized in 1978 as the cause of 2 diseases in dogs, myocarditis, and enteritis.[1,2] CPV has particular tropism for tissues containing rapidly dividing cells. Viral replication occurs initially in the oropharynx and local lymphoid tissue, followed by viremia, 1-5 days after infection. The virus localizes predominantly in the epithelial lining of the gastrointestinal tract, bone marrow, and lymphoid tissues,[3] resulting in intestinal crypt epithelium destruction and necrosis, villous atrophy, impaired absorptive capacity, and disrupted gut barrier function, allowing for bacterial translocation and bacteremia.

CPV-2 spreads rapidly among dogs by direct transmission (dog to dog) via the fecal-oral route or indirectly from the environment through oronasal exposure.[4] The virus is shed in the feces typically for several days, although shedding has been detected up to 6 weeks.[4] Weaned puppies are at increased risk for infection because of decreased serum concentration of maternal antibodies and changes in the gastrointestinal bacterial flora.[5]

Preliminary diagnosis is made based on the vaccination history, signalment, clinical signs, and leukopenia, while confirmation rests on laboratory tests such as antibody or antigen detection and molecular tests (eg, PCR, ELISA).[6,7] Electron microscopy and virus isolation are additional diagnostic methods[6]; however, these are not available in the clinical setting. Accurate diagnosis is important as positive dogs need to be hospitalized in isolation and are at high risk of becoming infected in the isolation ward if they are incorrectly diagnosed. Diagnostic methods have varying degrees of sensitivity and specificity.[8] Molecular methods based on various types of PCR protocols are considered 1 of the most precise and sensitive methods for CPV detection.[9,10]

The CPV genome contains 2 open-reading-frames. The first encodes 2 non-structural proteins (NS1 and NS2), while the second encodes 2 structural proteins, VP1 and VP2.[11] VP2, which is a major capsid protein composing approximately 90% of the capsid, plays an important role in virus pathogenicity and the host immune response.[12] Due to its easily accessible location, this capsid protein has been thoroughly investigated and several diagnostic antibody and antigen tests have been developed using VP2 as a target. Its sequence is also the most useful marker for epidemiologic studies of the virus.[13] To date, the most accurate method to classify the CPV-2 variants is by DNA sequencing of VP2 gene after PCR amplification.

The specificity of PCR can be affected by vaccinations. The standard samples used for PCR are fecal samples, but DNA extraction of fecal samples is time consuming and accurate quantitative PCR results are often compromised by the presence of inhibitors. The ideal sampling site is yet to be determined.

The primary objectives of this study were: (a) to assess the sensitivity and specificity of quantitative real-time TaqMan PCR (qRT-PCR) for the diagnosis of naturally occurring parvovirus infection and (b) to assess the sensitivity and specificity of different sampling sites, namely blood, pharynx and rectum, for the diagnosis of parvovirus infection. Secondary objectives were: (a) to compare a commercial Point of Care (POC) kit, PCRun CPV DNA Detection Kit (Biogal Israel) with qRT-PCR and (b) to assess the sensitivity of IgM serology for the diagnosis of CPV infection.

2 | MATERIALS AND METHODS

2.1 | Animals

The study was approved by the Institutional Research Committee (KSVM-VTH/22_2015). Four groups were included: Group 1 (n = 60) consisted of dogs presenting to Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem and diagnosed with CPV infection based on acute onset of compatible clinical signs (anorexia, vomiting, and diarrhea) and clinicopathological abnormalities (leukopenia, <5000 leukocytes/μL). qRT-PCR testing was performed on all the sample sites. At least 1 sample site test was required to be positive for confirmation of the diagnosis. The diagnosis of CPV infection was retrospectively confirmed by qRT-PCR followed by DNA amplicon sequencing and differential Minor Groove Binder Probe Assay. No case was removed based on a negative PCR test or sequencing consistent with vaccination rather than infection. The onset of clinical signs was defined as the time owners first reported clinical signs associated with the disease.

Group 2 (n = 44) included healthy puppies before start of core vaccination protocol, of which 16 puppies underwent vaccination (Nobivac Puppy DP vaccine). Blood, pharyngeal, and fecal swabs were collected from all the puppies before vaccination. In puppies undergoing vaccination, blood, pharyngeal, and fecal samples were collected before vaccination, 10 and 20 days after the first vaccination and 12 and 28 days after the second vaccination. Group 3 (n = 15) consisted of adult dogs inhabiting a multiple dog household environment, of which 10 dogs received their annual core vaccination (Zoetis VANGUARD PLUS 5). Samples were collected before vaccination, and 14 and 28 days after vaccination. Group 4 (n = 9) included puppies presented with signs of gastrointestinal disease, not associated with CPV, and diagnosed with canine distemper virus infection by qRT-PCR of RNA extracted from blood samples.

2.2 | Sample collection

Blood samples were collected by venipuncture directly into EDTA anticoagulant vacuum tubes and stored at −20°C before DNA extraction. Rectal and pharyngeal swab samples were collected using sterile dry FLOQSwabs (COPAN Flock Technologies Srl, Brescia, Italy), which were rubbed against the inner surface of the rectum or the pharynx and maintained at −20°C pending DNA extraction. Pharyngeal samples were obtained by quickly and firmly rubbing the swab on the tonsillar area surface on both sides of the pharynx for approximately 3 seconds.

2.3 | DNA extraction

Total DNA was extracted from the blood samples employing DNeasy Blood & Tissue Extraction Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The same kit was used for DNA extraction from pharyngeal swabs. DNA was extracted from fecal swabs using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's special protocol "Isolation of DNA from Stool for Human DNA Analysis."

An internal control F-deoR endA1 relA1 gyrA96 hsdR17 (rk^-, mk^+) supE44 thi-1 phoA Δ(lacZYA-argF) U169 Φ80lacZΔM15 λ-pBR322 (ranseqb1 AmpR) (Bioline GmbH, Germany) was spiked into the lysis buffer of each sample to allow for detection of potential PCR inhibition or failure of extraction. Extracted genomic DNA was quantified by Qubit Fluorometric Quantitation (Thermofisher, Massachusetts).

2.4 | Quantitative real-time PCR

CPV DNA was detected by the TaqMan qRT-PCR using HPLC grade primers and probes (Syntezza Bioscience Ltd, Jerusalem, Israel), designed to target a 112 bp segment of the VP2 CPV capsid protein gene. The selected primers and probe were challenged using online NCBI Nucleotide BLAST before use. Each 20 μL reaction consisted of 10 μL 2X Lightcycler Probes Master Mix (Roche), 700 nM of each primer (CPV all 2F 5′-ACTTATGGTCCTTTAACTGCAT-3′; CPV all 2R 5′-GTGCATTTACATGAAGTCTTGG-3′), 150 nM probe (CPV all 2prb 56FAM/TGTACCACC/ZEN/AGTTTATCCAAATGGTCA/3IABkFQ), 0.8 μL of internal control mix and 5 μL of extracted DNA. Amplification was initiated with a denaturing step (10 minutes, 95°C) followed by 40 cycles (95°C, 10 seconds; 47°C, 40 seconds; and 72°C for 1 second) and a final cooling step (10 seconds, 40°C). Data were acquired employing channels FAM (carboxyfluorescein) for the sample and Red 610 for the internal control during the extension step. Each sample was analyzed in triplicate. Ct values were calculated for each sample by determining the point of the fluorescence value exceeding the threshold limit and in comparison with the negative control, molecular grade water.

2.5 | PCRun

PCRun, (Biogal Galed Labs ACS, Galed Israel) was performed according to the manufacturer's instructions. Fifteen microliter PCRun buffer and 5 μL DNA sample were added to the PCRun Reaction Tubes, which were then incubated at 60°C for 60 minutes in the PCRun Reader. Positive and negative controls were included in each amplification series. Results were observed on the bio-illuminator touch screen of the PCRun Reader and recorded as time to peak (TTP) in minutes.

2.6 | RT Quantification of viral load—Standard Curve

The Ct values of the test samples were compared to the values derived from a standard curve (10^6-10^2 copies of the target gene) to determine the viral titers in each analyzed sample. qRT-TaqMan PCR amplifications were performed on a Roche Light cycler 96 System, as were all of the qRT-PCR reactions performed in the study. All qPCR and PCRun samples were run in triplicate.

2.7 | Differential minor groove binder (MGB) probe assay

CPV types 2a/2b- and 2b/2c-specific real-time PCR assays based on differential MGB technology were performed.[14,15] The MGB 2b-specific probes were labeled with FAM in both the 2a/2b and 2b/2c assays. The MGB type 2a (type 2a/2b assay) and type 2c (type 2b/2c assay) probes were labeled with VIC (2′-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein; Table 1). Amplification for all reactions: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles (92°C for 10 seconds, 60°C for 60 seconds) and a final cooling step of 30 seconds at 37°C. Data were acquired in the FAM and VIC channels during extension. Samples that were positive for type 2b in the type 2a/2b assay were also tested with the type 2b/2c assay.

2.8 | Proofreading PCR and sequencing of the PCR product

Sequencing of amplified VP2 genes was performed either directly on purified PCR amplicons or using PCR products ligated into pJet

TABLE 1 Sequence of real-time PCR primers and MGB probes

Assay	Primer	Sequence 5′-3′
Type 2a/2b MGB probe assay	CPV2a-b_F	AGGAAGATATCCAGAAGGAGATTGGA
	CPV2a-b_R	CAATTGGATCTGTTGGTAGCAATACA
Type 2a	CPV2a-b_V	VIC—CTTCCTGTAACAAATGATA—NFQ
Type 2b	CPV2a-b_M	FAM—CTTCCTGTAACAGATGATA—NFQ
Type 2b/2c MGB probe assay	CPV2b-c_F	GAAGATATCCAGAAGGAGATTGGATTC
	CPV2b-c_R	TTACCTCCAATTGGATCTGTTGGTA
Type 2c	CPV2b-c_V	VIC—CCTGTAACAGAAGATAAT—NFQ
Type 2b	CPV2b-c_M	FAM—CCTGTAACAGATGATAAT—NFQ

plasmids (Thermo Scientific, Vilnius, Lithuania). PCR primers VP2 F and VP2 R (VP2 F 5′- GTGATGGAGCAGTTCAACCA-3′; VP2 R 5′-TGGATTCCAAGTATGAGAGGCT-3′) were designed (http://primer3.ut.ee/) to amplify 1637 bp of the VP2 gene encompassing all the genetic variant-defining nucleotides. Two internal primers CPV seq F (5′GGGTGTGGGGATTTCTAC-3′) and CPV seq R (5′-GGTGCATTTACATGAAGTCTTGG-3′) were used for sequencing of the VP2 variant section. One microliter of the PCR amplified product was resolved on a 1.5% agarose gel (Amresco, Ohio) in Tris-Acetate-EDTA buffer (Biological Industries, Beit Haemek, Israel) containing 0.01% of GelRed Nucleic Acid Gel Stain (Biotium, California) and 100 bp Ladder. Following size verification, the remaining PCR products were column-purified (Qiagen MinElute Reaction Cleanup Kit; GmbH, Hilden, Germany).

The VP2-PCR products were ligated into pJet1.2/blunt vectors according to the manufacturer's instructions. Heat shock transformation of competent *E. coli* strain NEB 5-alpha F'I^q (New England BioLabs, Massachusetts) was performed according to manufacturer instructions. Transformed cells were plated onto carbenicillin Luria Broth (LB) Agar plates and incubated overnight (ON) at 37°C. Single colonies were selected and cultured ON at 37°C in 5 mL LB carbenicillin broth. Plasmid DNA was extracted from the cultures using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Hamburg, Germany). Sanger sequencing of DNA was performed at the Weizmann Institute of Science. DNA sequences obtained were analyzed using the free software BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.9 | Proofreading PCR and direct sequence of commercial vaccines

To evaluate the post-vaccinal virus shedding, VP2 F/VP2 R -PCR was carried out as described above from DNA material extracted from 6 commonly used vaccines. The contents of the parvovirus entity in each vaccine were as follows: Intervet Nobivac Puppy DP (live attenuated CPVstrain C 154); Merial Primodog (non-adjuvanted modified-live strain Cornell 780 916-115); Merial Eurocan (attenuated CPV); Virbac Canigen (modified live CPV-2 strain 780 916); MSD Quantum (modified live CPV strain SAH 2b), and Zoetis Vanguard (live attenuated CPV-2 strain NL-35-D).

2.10 | Serology

Serological tests were performed using Biogal's semi-quantitative Solid Phase ELISA kits (Biogal Galed Labs ACS, Galed Israel). The antibody titers are determined according to the color intensity. Canine VacciCheck was used for measurement of anti-CPV IgG antibody titers and the Canine Parvovirus & Distemper IgM antibody test kit was employed for anti-CPV IgM antibody titers.[16] The results of IgG and IgM tests were expressed in S Units (S) ranging from S0 to S6 relative to a positive control cutoff (S3). The S scale for anti-CPV IgG antibody titers is calibrated against the Hemagglutination Inhibition Test: S1 ≤ 1 : 4, S2 = 1 : 40, S3 = 1 : 80, S4 = 1 : 160, S5 = 1 : 320, and S6 = ≥1 : 640 HI units. The S scale for anti-CPV IgM is calibrated against an Immunofluorescence Assay test with the following values: S1 = 1 : 10, S2 = 1 : 50, S3 = 1 : 250, S4 = 1 : 1250, S5 = 1 : 6250, S6 > 1 : 6250 IF units.

2.11 | Statistical analysis

Continuous variables are described as mean and SD. One-way analysis of variance was used to evaluate the average log viral load among the different sample types. Pearson correlation was used to assess the relationship between the number of days from onset of clinical signs and log viral load. Sensitivity was defined as the proportion of animals with CPV infection that were identified as such by the test. Specificity was defined as the proportion of animals without CPV infection that tested negative. All healthy dogs (with the exclusion of puppies undergoing vaccinations) and dogs with distemper were used to calculate specificity. These PCR results are reported both for each group separately as well as for all the dogs combined (excluding dogs that are undergoing vaccinations). $P < .05$ was considered statistically significant. Analyses were performed using a statistical software (SPSS 22.0 for Windows, IBM Corp., Armonk, New York).

2.12 | Calibration of TTP and Ct cut-off values

The PCR calibration process involved the results of the study group (Group 1) and the control groups (Groups 2, 3, and 4). The linear operating range of the 2 PCR methods was determined during development of the assays. The experimental analytical sensitivity was derived from estimation of the lowest and highest limit of detection, which would separate positive results of dogs diagnosed with CPV cases from passive carriers associated with post vaccination shedding. This included the definition of cut-off values for Ct (≤23 cycles) and TTP (20 minutes). The conclusive results noted in this study were defined using the above conditions unless otherwise indicated. Information relating to the process are presented in the Tables S1–S3.

3 | RESULTS

3.1 | Dogs with parvovirus infection (Group 1 n = 60)

Sixty dogs were included in this group (31 females and 29 males). Median age was 2 months (range, 1-19 months). Nineteen dogs had been vaccinated for parvovirus once, 3 were vaccinated twice, 4 dogs were vaccinated 3 times, and 19 had never been vaccinated. Vaccination history was unknown for 15 dogs. Median time between onset of clinical signs and sampling was 5 days (range, 2-15). All dogs were

hospitalized with a median hospitalization time of 7 days (range, 1-17 days). Eighteen percent (11/60) of dogs did not survive.

Each dog was sampled from all 3 sample sites. qRT-PCR was positive in 95%, 98%, and 88% for the blood, pharyngeal, and fecal samples, respectively, with no difference in viral load (as determined by qRT-PCR) between the blood, pharynx, and fecal sample ($P = .15$; Figure 1). There was a significant negative correlation between days from onset of clinical signs and the viral load in the blood, pharynx, and fecal samples ($r = -.79$, $r = -.91$, and $r = -.78$, respectively, $P < .05$ for all).

There was an excellent agreement between the qRT-PCR and the PCRun amplifications for all sample types. Agreement between the 2 tests for blood, feces, and pharyngeal samples was present in 59 of 60, 59 of 60, and 58 of 60 of the dogs, respectively. When the results of all sample types are combined, there was 98% agreement between qRT-PCR and the PCRun.

All of the qRT-PCR positive samples were characterized using the MGB Probe technology. Consequently, 31 dogs (52%) carried CPV type 2a, 28 dogs (47%) type 2b, while CPV type 2c was not found in any of the samples. One dog harbored CPV-2a in blood and pharyngeal samples and CPV-2b in its rectal sample.

Amplicons were further sequenced to verify MGB results and to determine if additional mutations were present. Initially all 3 sample types from 30 dogs were sent for sequencing. Analysis of these sequences revealed complete homology in the targeted sequences, therefore only blood samples were sequenced in the remaining 30 dogs. The results acquired from MGB characterization and classification of the sample by sequencing were identical. All of the sequenced positive CPV samples belonged to known CPV field strains (CPV-2a or 2b). The vaccine strain (CPV-2) was not identified in any of the amplicons.

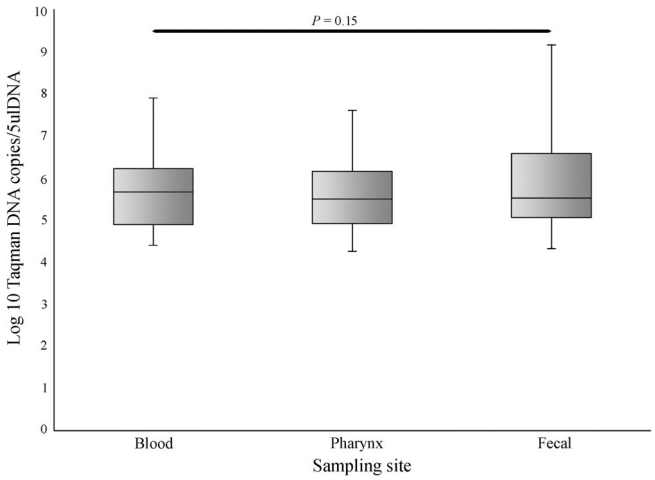

FIGURE 1 Viral load from 3 sampling sites of 60 dogs with parvovirus infection (DNA copies per 5 μL DNA) in blood, pharyngeal, and fecal samples. The box represents the interquartile range, the horizontal line represents that median and the whiskers represent the range

Anti-CPV IgM antibody titers during the hospitalization period were positive (≥S2) in 92% of the dogs, of which 55% were in the S6 range (1 : 6250 IF; Table 2). Five (8%) of the dogs had IgM titer <S2. Median anti-IgG titer was S5 (range, S0-S6).

3.2 | Healthy puppies and puppies before and after vaccination (Group 2)

This group included 44, 6-week-old unvaccinated puppies, of which 16 puppies were also followed after the first and second vaccination. All samples of unvaccinated dogs were negative with the exception of 1 positive pharyngeal sample by qRT-PCR and 2 positive pharyngeal samples with PCRun. None of the fecal or the blood samples were positive.

None of the 16 puppies that were followed after the first and second vaccination developed clinical signs during the vaccination period; therefore, any positive test result was attributed to the administered modified live virus (MLV) vaccine. Samples were taken before vaccination, 10 and 20 days after first vaccination, and 12 and 28 days after the second vaccination. The highest rate of positive qRT-PCR and PCRun results were detected at days 10 and 20 after the 1st vaccination. Ten days after first vaccination 31% (blood), 12% (pharyngeal) and 62% (fecal) samples were positive by qRT-PCR while 37% (blood), 12% (pharyngeal), and 44% (fecal) were positive by PCRun. Before the second vaccination (20 days after 1st vaccination), CPV was detected in the blood (19% by both qRT-PCR and PCRun) and fecal samples (62% by qRT-PCR and 81% by PCRun), while the number of positive pharyngeal samples was very low (0% by qRT-PCR and 6% by PCRun). Twelve and 28 days after the second vaccination (32 and 48-day after 1st vaccination, respectively), all samples were negative, except for 1 dog (6%: qRT-PCR) and 2 dogs (12%; PCRun), which had positive blood samples and 1 dog (6%; PCRun) with a positive fecal sample at 48 days.

Seven random positive DNA amplicons generated from the vaccinated puppies were sequenced, revealing that the CPV strain of 6 dogs was identical to the Nobivac Puppy DP vaccine strain sequence. In 1 dog, the sequence was similar to that of the PRIMODOG vaccine.

Anti-CPV IgM antibody tests results were all negative before vaccination and increased after vaccination (Table 3). Eleven dogs reached level S5 by day 20 after vaccination, 1 dog had titer level of S4, and the remaining 4 dogs reached level S5 by day 32.

Anti-CPV IgG antibodies titers before vaccination varied between negative and low positive (median S3, range, S1-S3). At day 32, IgG titers reached a level of S6 in all puppies.

3.3 | Multi household adult vaccinated dogs (Group 3)

Fifteen vaccinated dogs were included in this group, of which 10 were revaccinated with an annual core booster vaccine 1 year after the previous vaccination. Before vaccination, whole blood, pharyngeal, and

TABLE 2 Dogs with CPV infection

"S" Units (IF)	S0 (-)	S1 (1 : 10)	S2 (1 : 50)	S3 (1 : 250)	S4 (1 : 1250)	S5 (1 : 6250)	S6 (>1 : 6250)
Number of positive puppies (%)	0 (0%)	5 (8%)	3 (5%)	2 (3%)	0 (0%)	17 (28%)	33 (55%)

Note: Distribution of the Anti-CPV IgM antibody titers ("S" Units). The S scale for anti-CPV IgM is calibrated against an Immunofluorescence Assay test with the following values: S1 = 1 : 10, S2 = 1 : 50, S3 = 1 : 250, S4 = 1 : 1250, S5 = 1 : 6250, S6 > 6250 IF units.

TABLE 3 Canine parvovirus antibody titers of 16 puppies evaluated by Immunocomb® "S" Units over a period of 48 days after vaccination with an attenuated live vaccine at days 0 and 20

	IgG					IgM				
	Day									
	0	10	20	32	48	0	10	20	32	48
Median (range)	S3 (1-3)	S3.5 (1-6)	S5.5 (2-6)	S6 (6)	S6 (6)	S0 (0-1)	S0 (0-6)	S5 (0-5)	S3 (1-5)	S1 (0-3)

Note: The puppies were vaccinated with an attenuated live vaccine at days 0 and 20. The S scale for anti-CPV IgG antibody titers is calibrated against the Hemagglutination Inhibition Test. According to Biogal S1 ≤ 1 : 4, S2 = 1 : 40, S3 = 1 : 80, S4 = 1 : 160, S5 = 1 : 320, and S6 = ≥1 : 640 HI units. The S scale for anti-CPV IgM is calibrated against an Immunofluorescence Assay test with the following values: S1 = 1 : 10, S2 = 1 : 50, S3 = 1 : 250, S4 = 1 : 1250, S5 = 1 : 6250, S6 > 1 : 6250 IF units.

fecal samples were collected from all the dogs and additional samples were collected at 14 and 28 days after vaccination. All dogs were negative for CPV by qRT-PCR and PCRun in all of the sample types, before and after vaccination at all time periods.

The serological tests revealed that all of the dogs had positive CPV IgG titers (average S5.4 ± 0.7) before the booster vaccination and remained positive at 14 (S5.7 ± 0.2) and 28 days (S5.9 ± 0.1) after vaccination. IgM titers were undetectable before vaccination and remained undetectable at 14- and 28-days after vaccination.

3.4 | Dogs with signs of gastrointestinal disease and canine distemper infection (Group 4)

This group included 9 dogs with signs of gastrointestinal disease and diagnosed with infection by canine distemper virus by PCR. None tested positive for CPV in any of the sampling sites by both molecular methods.

High titers of anti-canine distemper virus IgM were measured in all of the 9 dogs (median 5, range, 4-6). Median anti-canine distemper IgG titers in this group was S3 (range, S0-S5).

Median anti-CPV IgM titer was S0 (range, S0-S3) and median anti-CPV IgG titers was S6 (range, S0-S5).

When combining the results of all healthy dogs (including adult dogs receiving a booster vaccine but excluding puppies undergoing vaccination), and dogs with distemper, qRT-PCR and PCRun were negative in 67 (99%) of 68 and in 66 (97%) of 68 of the dogs, respectively.

4 | DISCUSSION

This study evaluated 2 PCR methodologies for the diagnosis of parvovirus infection. The 2 molecular techniques were employed to differentiate between disease status and passive shedding in healthy dogs. Both methods were highly sensitive compared to the clinical diagnosis of parvovirus infection. Although all sampling sites can be used for the diagnosis, blood and pharyngeal samples had higher sensitivity. Fecal and blood samples of puppies undergoing vaccination were commonly positive 10 days after first vaccination but lower when samples were obtained from the pharynx.

All dogs diagnosed with CPV infection tested positive by PCR in at least 1 of the sample types, of which 83% had positive results from all sites. The CPV viral loads were not significantly different among the various sampling sites, yet there was variability in viral load among dogs, possibly because of presentation at different times during the disease course.

The clinical behavior and pattern of fecal viral shedding using real-time PCR have been investigated in dogs naturally infected with CPV-2c.[8] Clinical signs are present 5-7 days after infection and CPV-2c DNA was detectable for a median of 46 days, as early as 4-6 days, reaching a peak at day 10 after infection and subsequently dropping toward the end of the observation.[17] In this study, dogs were hospitalized 2-15 days from the onset of clinical signs and there was a negative correlation between the number of days from onset of clinical signs and the viral load.

The present study could not delineate the minimum viral CPV load, which could support the discrimination of CPV infection from passive shedding. Because qRT-PCR detects viral nucleic acid but not infectious virus, one can only hypothesize whether CPV-2 DNA in the feces of recovering pups is associated with the shedding of infectious virus. In fact, in the late stages of the CPV-2 infection (8-10 days after infection), specific antibodies in the intestinal lumen frequently opsonize most of the CPV-2 virions, preventing parvoviral binding to cellular receptors and subsequent growth in cell cultures.[17]

CPV type 2a and type 2b were equally prevalent in sick animals (52% and 47%, respectively) and CPV type 2c was not identified. In

1 dog, both variants were detected, type 2a (blood and pharyngeal) and type 2b (fecal). This phenomenon has been reported in previous studies.[18-20] This dog had been vaccinated 3 weeks before presentation, thus the 2 variants found likely represented a combination of naturally occurring viral strain and the MLV strain that was still being shed in the feces.

The MLV vaccine contains attenuated viral particles, which induce immunity by low-level infection and replication without clinical signs of the disease. Systemic dissemination of the virus can interfere with molecular testing in cases of suspected parvovirus infections, demanding carefully calibrated protocols. The major target organ for the vaccine is the intestine, where it can replicate and intermittently be shed in the feces of the newly vaccinated puppies.[21]

Before initiating vaccination in puppies, all the blood and fecal samples were negative by both PCR platforms. One pharyngeal sample was positive by qRT-PCR and 2 by PCRun. It is possible that these pharyngeal samples presented false positive results or were positive simply because of natural licking and sniffing behavior. CPV type 2 viruses are stable in the environment for a long period of time, resulting in frequent exposure and potential positive CPV tests results even if no signs of illness are evident.[22] Puppies in breeding kennels excrete substantial viral loads of CPV-2 without any systemic signs.[22] If a puppy is tested positive in the absence of consistent clinical signs when the sample is obtained from the pharynx, it is recommended to confirm this result with additional blood test.

Sixteen puppies belonging to 3 litters were also followed for 48 days during the core vaccination process. The number of shedders as revealed by PCR increased in all of the sample types 10 days after the first injection. Ten days later, before the second vaccination, the proportion decreased, and by day 32 all of the puppies were negative. Fecal sample type emerged as the least accurate for diagnosis of CPV in dogs undergoing vaccination. At day 10 after vaccination, 62% and 44% of the dogs tested positive by qRT-PCR and PCRun, respectively, and at day 20, 62% and 81% tested positive, respectively. In comparison, 31% and 37% of the blood samples tested positive by qRT-PCR and PCRun, respectively, and 19% at 20 days by both methods. Pharyngeal sampling was the best sampling site in dogs undergoing vaccination, with only 2 dogs (12%) testing positive 10 days after first vaccination by both PCR methods. Twenty days after vaccination PCRun detected 1 positive sample (2%) and all the samples tested by qRT-PCR were negative and remained so until the end of the study. This is likely because of the route of vaccine administration where the virus is less likely to populate the pharynx. The results of this study demonstrated that the specificity of molecular methods for CPV is affected by vaccination, especially during the first 3 weeks after vaccination, and when samples are obtained from the rectum or blood.

Fecal samples are the most common samples used for detection of CPV by commercial tests and laboratory PCRs. Although non-invasive and easily collected, this study demonstrates that this sampling method has disadvantages such as high proportion of positive results during vaccination, and lower sensitivity in sick dogs with CPV, likely because of the presence of high degree of PCR inhibitors present in feces, and relatively complex sample preps, necessary to process these sample.[23]

This study supports the findings that the CPV vaccine can be detected in vaccinated puppies, mostly during the first 20 days after the first vaccination. After the second vaccination at day 20, the number of puppies with positive CPV detection declined to almost nil in all sites, suggesting that viremia and fecal shedding are common manifestations in pups only after the first vaccination, and thereafter positive results in dogs presenting compatible clinical signs should be considered as caused by field strain infection. In this study, sequencing confirmed that the positive CPV results were from the vaccine strain and not due to field infection. The vaccine strain used in standard vaccines is made from the original CPV-2 type, which is helpful in discriminating between vaccine and field samples. A minor groove binding (MGB) probe assay for PCR discrimination between the vaccine (CPV-2) and the field strains (types 2a, 2b, 2c) was previously designed.[15] Currently, there are new vaccines produced with the CPV-2b strain, which makes it more difficult to distinguish the vaccine from field strains. In puppies recently administered vaccine, the pharynx is the preferred sampling site.

All dogs in the multi-house hold environment tested negative in all sampling sites. To further evaluate the vaccination response, 10 healthy fully vaccinated dogs were tested before and 14 and 28 days after vaccination and none of the dogs tested positive for CPV. In another study, shedding was documented up to 21 days after booster vaccination.[24] The apparent difference between these 2 studies might relate to the fact that the current study tested only 10 dogs and the follow-up time was short compared with a lager sample size and a longer follow-up period in the previous study.

The specificity of both PCR tests was further evaluated with samples collected from dogs with signs of gastrointestinal disease but finally diagnosed with canine distemper. All of the samples were negative for canine parvovirus.

Real-time PCR testing requires expensive equipment, reagents and specialized operators. The isothermal point-of-care PCRun method has been developed to simplify and decrease the time and cost of PCR testing.[25] The PCRun kit employed in this study was found to be sensitive and specific and thus can be used as a feasible alternative for the diagnosis of CPV in the clinical setting.

Serologic tests might aid in the diagnosis of CPV but are not always sufficient as a solitary test. Of the 60 sick dogs included in this study group with CPV infection, 5 had negative IgM and IgG titers despite positive qRT-PCR, suggestive of an early infection before development of a humoral response sufficient to be measured by the serological test. As IgM was undetectable in all puppies before vaccination and positive in 92% of the dogs diagnosed with CPV, IgM serology should be considered as highly sensitive and specific before vaccination and can aid in the diagnosis of CPV. Twenty days after vaccination, IgM serology was positive in all puppies thus cannot be used to establish a diagnosis of CPV.

This study has shown that qRT-PCR and PCRun are useful molecular techniques for the diagnosis of parvovirus infection. Although all the virus PCR sampling sites used were associated with high sensitivity of viral detection, fecal swabs had the lowest sensitivity compared with blood and pharyngeal sampling. Pharyngeal samples may

uncommonly be positive because of environmental exposure, however, in dogs undergoing vaccination protocol, and in particularly within the first 20 days after the first vaccine, the pharynx was found as the preferred sampling site. Finally, IgM serology was found to aid in the diagnosis of infection in unvaccinated pups.

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CONFLICT OF INTEREST DECLARATION

Tal Yaaran and Sarah Maurice are employees of Biogal Galed Labs.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Koret School of Veterinary Medicine Institutional Research Committee (KSVM-VTH/22_2015).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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