

# When the diagnosis of parvovirus in dogs and cats becomes challenging

Maria Luisa Marenzoni<sup>1\*</sup>, Monica Momesso<sup>1</sup>, Maria Chiara Marchesi<sup>1</sup>, Elisabetta Manuali<sup>2</sup>, Silvia Pavone<sup>2</sup>, Elisa Sgariglia<sup>1,2</sup>, Elena Tordo<sup>1,3</sup>, Francesco Vescera<sup>3</sup>, Giuseppe De Nicola<sup>3</sup>, Valentina Stefanetti<sup>1</sup> and Chiara Brachelente<sup>1</sup>

<sup>1</sup>Department of Veterinary Medicine, University of Perugia, via S. Costanzo 4, 06126 Perugia, Italy.

<sup>2</sup>Istituto Zooprofilattico Sperimentale of Umbria and Marche, via Salvemini 1, 06126 Perugia, Italy.

<sup>3</sup>Private Practitioner, Central Italy, via S. Costanzo 4, 06126 Perugia, Italy.

\*Corresponding author at: Department of Veterinary Medicine, University of Perugia, via S. Costanzo 4, 06126 Perugia, Italy.

Tel.:+39 0755857720, e-mail: marialuisa.marenzoni@unipg.it.

*Veterinaria Italiana* 2020, **56** (2), 67-76. doi: 10.12834/VetIt.1415.7682.1

Accepted: 08.04.2018 | Available on line: 31.12.2020

## Keywords

Cat,  
Diagnosis,  
Dog,  
Misdiagnosis,  
Parvovirus infection,  
PCR.

## Summary

Parvoviruses (PV) can cause outbreaks with high morbidity and mortality in dogs and cats. Even if typical cases exist in puppies and kittens, PV infection (PVI) can have many different clinical presentations, making the laboratory support necessary. The aim of this work was to evaluate retrospectively the frequency of misdiagnoses, particularly missed diagnoses, of PVI in 144 suspected cases (88 clinical cases and 56 necropsies) involving 96 dogs and 48 cats. A nested PCR test was chosen as the gold standard. An index of diagnostic suspicion (IDS) for PVI, based on parameters reported upon submittal of the samples, was introduced to classify the initial diagnoses issued by veterinarians. The agreement between the IDS of PVI and PCR results was calculated. The effect of species, age and clinical versus necroscopic presentation was evaluated by logistic regression. In 63.6% of the cases, the IDS was confirmed by the PCR, whereas in 36.4% there was a missed diagnosis or a diagnosis wrongly attributed to PVI. More accurate results were obtained for dogs, animals aged < 1 year, and necropsies. Parvovirus infection should be better investigated in patients with atypical or few clinical signs, in particular in cats and animals over 1 year old.

## Introduction

Parvoviruses (PV), including Feline panleukopenia virus (FPV) and variants of Canine parvovirus (CPV), are small DNA viruses, highly resistant in the environment. They are able to cause worldwide outbreaks with high morbidity and mortality. The genetic evolution of these viruses, especially CPV, has given rise to the progressive emergence of CPV-2 antigenic variants, which include the type 2 (currently available only as vaccinal strain for dogs), 2a, 2b, and 2c (Decaro and Buonavoglia 2012, Decaro *et al.* 2013a).

A recent study found PV infection (PVI) to be the main cause of mortality in dogs according to post mortem diagnoses in Italy (Eleni *et al.* 2014). Typical cases, consisting of puppies and kittens aged from 6 weeks to 6 months, with fever, vomiting, mucoid to haemorrhagic diarrhoea and leukopenia, are generally easy to diagnose, although with a variable prognosis (Kruse *et al.* 2010, Schoeman *et al.*

2013). Some useful risk and prognostic factors for predicting and diagnosing PVI have been previously investigated, especially in symptomatic animals (Houston *et al.* 1996, Kruse *et al.* 2010, Miranda *et al.* 2015). However, PV can have extremely variable clinical presentations, ranging from hyperacute emergencies requiring intensive care to cases characterised by the presence of few clinical signs. In the latter case, diagnosis can become challenging especially if some of the typical clinical signs are vague, or not present, or in presence of a vaccination history of the animal that suggests the existence of some level of immunity and immunoprotection (Kruse *et al.* 2010, Faz *et al.* 2016). For these reasons, laboratory support is necessary to reach a correct diagnosis (Kruse *et al.* 2010). Parvoviruses are often characterised by an acute course thus, only direct tests, that are able to detect the etiological agent, are appropriate for diagnosis. Several tests with different sensitivities, specificities and prices are available. These include antigenic (often in-clinic) and

biomolecular tests, such as traditional and real-time polymerase chain reaction (PCR) (Desario *et al.* 2005, Decaro and Buonavoglia 2012, Decaro *et al.* 2013b, Proksch *et al.* 2015, Faz *et al.* 2016). However, these tests are performed only when veterinarians include PVI in the list of differential diagnoses, or when owners are willing to pay for them. This may limit the possibility of performing a correct diagnosis. Therefore, considering all these aspects, misdiagnosis is possible. The consequences of a missed diagnosis can include the underestimation of the prevalence of PVI and the dissemination of the virus through a susceptible population in a clinic or animal shelter, resulting in a costly outbreak. On the contrary, a correct diagnosis is a keystone for controlling the spread of the infection and reducing PVI mortality rates.

The aim of this work was to evaluate, retrospectively, the frequency of PVI misdiagnoses, particularly missed diagnoses, in 144 suspected cases (88 clinical cases and 56 necropsies) involving 96 dogs and 48 cats. A nested PCR test was chosen as the gold standard. An index of diagnostic suspicion (IDS) for PVI, based on parameters reported upon submittal of the samples, was introduced to classify the initial diagnoses issued by veterinarians. A qualitative description of host factors or details of the history of the most misleading cases is given.

## Materials and methods

### Data collection

A retrospective study was conducted on cases observed between October 2008 and December 2014. They included at least one PVI clinical sign. Collected samples were subjected to biomolecular investigation for PVI.

For each case, when possible, the following data were collected: specimen (number and type), identification of the animal, species, age, gender, origin (kennel, ownership, pet shop, or other), veterinary practice requiring the test, list of clinical signs, treatment, history, vaccinal status, date of vaccination (to evaluate possible vaccinal interference with maternal antibodies, in case of recent vaccination in puppies and kittens), laboratory test results (when available) and outcome.

The referral clinician for clinical cases was a general practitioner or an internal medicine specialist. The referral veterinarian for necropsies was a pathologist, generally appointed by the clinician.

The classification of the diagnostic suspicion of the cases subjected to biomolecular diagnosis of PVI was retrospectively summarised based on

the IDS of PVI. This represents the result of the diagnostic evaluation issued by the veterinarian (clinician or pathologist), based on clinical signs, clinicopathological parameters, patient history and/or gross examination. The cases were classified into weak or strong IDS of PVI. A strong IDS was defined when there were at least two of the typical clinical signs or clinicopathological biomarkers of PVI (presence of acute or hyperacute gastrointestinal signs: vomiting; haematemesis; mucoid diarrhea; haemorrhagic diarrhoea; melena; fever; leukopenia or neutropenia) or when there was a history consistent with PVI or typical gross or histological lesions (necrohaemorrhagic gastroenteritis with crypt necrosis and villous blunting; intranuclear basophilic inclusion bodies in the gastrointestinal tract; necrosis and depletion of lymphoid tissues) (Decaro and Buonavoglia 2012, Schoeman *et al.* 2013, Maxie 2015). In these cases, PVI was generally considered the first, or among the first three diseases in the list of differential diagnoses. A weak IDS was defined when the veterinarian considered PVI an unlikely cause of the disease and the criteria of the strong index were not satisfied (only one of the clinical signs mentioned above was present). The specimens with weak IDS were generally subjected, at least initially, to other investigations.

When possible, especially after a negative PCR result, the referral veterinarian was contacted in order to discuss the case and try to reach a definitive diagnosis.

### Necroscopic examination

When available, the dead animals were subjected to a thorough gross examination. Tissues were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin and stained with haematoxylin and eosin for histopathological examination. The results of this last examination generally followed those of the biomolecular test.

### Sampling

Specimens consisting of rectal swabs (RS) from live animals or tissues from necropsies (the gut in case of intestinal lesions, or a pool of organs including gut, liver, spleen and lung when no gross lesions were evident) were collected. Rectal swabs were obtained using sterile cotton swabs, kept in tubes with 0.5 mL of phosphate buffered saline (PBS).

Specimens of each case were processed upon arrival and this limited the risk of contamination between samples.

### Biomolecular investigations

Two hundred µl of RS or 20 mg of tissues were used

to extract DNA using a commercial kit (DNeasy Tissue kit, Qiagen, Milan, Italy). The concentration and purity of the extracted DNA was quantified using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan, Italy).

A conventional PCR, targeting a fragment of 583 bp of the VP2 capsid protein-encoding gene of PV, was used to detect PV DNA, including both FPV and the variants of CPV (Table I, Buonavoglia *et al.* 2001). This protocol was able to amplify the DNA of all the PV strains, including the MbolI restriction site, which is able to recognise the mutation at position 426, which characterises the type 2c and some 2a variants of CPV (Buonavoglia *et al.* 2001, Demeter *et al.* 2010). This allowed to recognise the presence of vaccinal interference, in case the DNA of vaccinal (variant 2 or 2b) and wild (variants 2a, 2b, and 2c) strains were present simultaneously.

Moreover, a nested PCR protocol was developed to increase the sensitivity of the test: two internal primers inside the fragment amplified by the previously published primer pair (Buonavoglia *et al.* 2001) were designed using the Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) (Table I). However, this nested protocol was not able to distinguish PV strains, as it does not amplify the variant-specific site.

An aliquot of 10 µL of RS DNA or 100 ng of DNA from tissues was tested in duplicate in a PCR assay (Microtech, Italy). Twenty-five µL of reaction mixture contained 10x buffer, 3 mM MgCl<sub>2</sub>, 200 µM each deoxyribonucleotide triphosphate, 1 µM each primer (Sigma-Genosys), 0.5 U Taq DNA polymerase (Microtech, Italy), and DNA as described above. One µL of DNA from the first test was used for the nested protocol developed in this study. Cycling conditions are given in Table I. In each set of reactions a positive (CPV Cornell strain) and a negative control (negative DNA sample), as well as a negative reaction mix control, were included.

The DNA of a feline cell culture (Crandell feline kidney), infected with 100 TCID<sub>50</sub>/100 µL of the Cornell strain, was subjected to serial 10-fold dilutions, ranging from 100 ng to 0.01 fg, to determine the difference

in the sensitivity of both single and nested PCR protocols in the biological samples.

PCR products of the expected size, originating from four samples only at the nested step, were purified using an appropriate extraction kit (Qiaquick PCR purification kit, Qiagen) and directly sequenced on both strands with the same primers previously described, using a DNA analyser (ABI 3730, Applied Biosystems) capillary sequencer (BioFab Research srl). The sequences were assembled and aligned using BioEdit (2009). The sequence similarity was checked against sequences deposited in GenBank using the BLAST (Basic Local Alignment Search Tool) software (2009) to confirm the specificity of the PCR.

Further procedures to validate the nested protocol were performed (data not shown).

### Statistical analysis

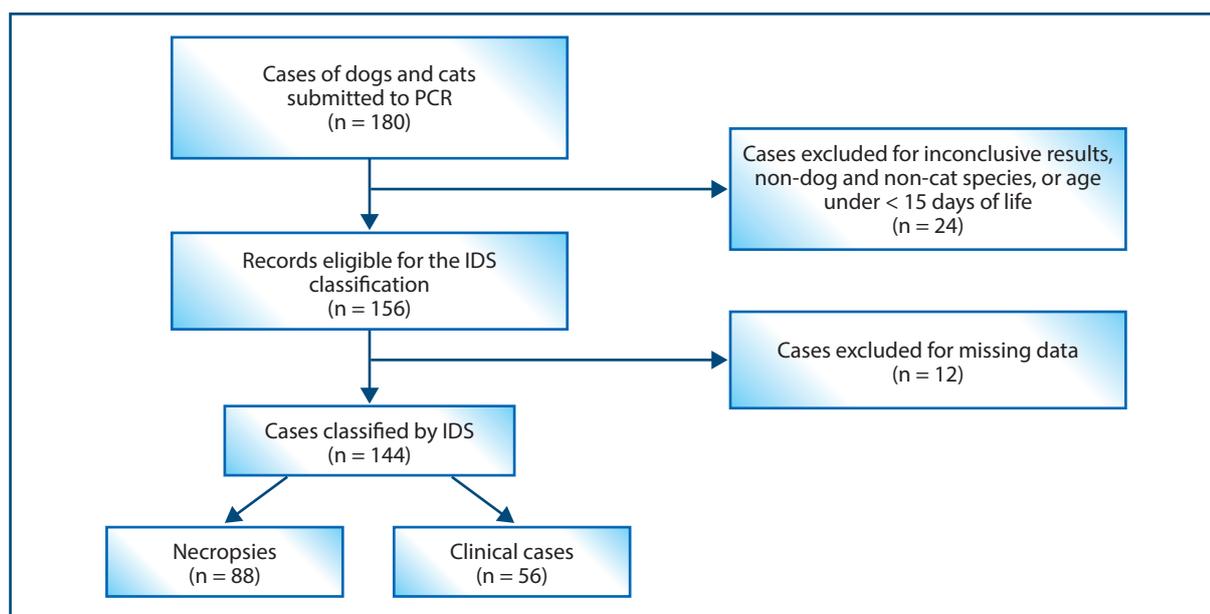
The main diagnostic parameters of the IDS compared with PCR results were estimated (World Organisation for Animal Health 2016). Cohen's kappa test was calculated to assess the agreement between strong or weak IDS of the referral veterinarian and PCR, which was considered as the gold standard. Values greater than 0.8 represented an excellent agreement, values between 0.61-0.8 represented a substantial agreement, between 0.41-0.6 a moderate agreement, between 0.21-0.4 a fair agreement and below 0.20 a slight agreement (Landis and Koch 1977). A two-sided p value (*P*) of ≤ 0.05 was considered statistically significant. Statistical analyses were performed using StatsDirect software, version 2.7.9, and OpenEpi software.

Logistic regression was used to weight the overall effect of species, age and type of diagnosis (clinical vs post mortem examination) of the case on performing a correct diagnosis. The variables were examined separately for their association with the missed diagnoses (cases with weak IDS and positive PCR, considered false negative) or with incorrect PVI diagnoses (cases with strong IDS and negative PCR, considered false positive) evaluated as the outcomes. The effect of age was analysed separately, grouping

**Table I.** PCR test protocol used for the biomolecular detection of Parvovirus infection.

PCR assay	Target gene	Primer sequence (5'-3')	Amplification profile					Product size (bp)	PCR type	References
			Initial denaturation	Denaturation	Annealing	Extension	Final extension			
555 F	VP2	CAGGAAGATATCCAGAAGGA	94 °C,	94 °C,	40 cycles	72 °C,	72 °C,	583	Single	Buonavoglia <i>et al.</i> 2001
555 R		GGTGCTAGTTGATATGTAATAACA	5 min	15 sec	53 °C, 30 sec	45 sec	5 min			
Parvo- FN	VP2	CACCAGTTTATCCAAATGGTCA	94 °C,	94 °C,	35 cycles	72 °C,	72 °C,	211	Nested	The current study
Parvo- RN		CCTTCCACCAAAAATCTGAG	5 min	30 sec	60 °C, 30 sec	45 sec	5 min			

F = Forward primer of the first round of PCR; R = Reverse primer of the first round of PCR; FN = Forward primer of the second round of PCR; RN = Reverse primer of the second round of PCR.



**Figure 1.** Flow chart of the cases analysed.

it into three classes: animals younger than 6 months, from 6 to 1 year and older than 1 year. The list of the single clinical signs was not statistically evaluated. Variables scoring  $P \leq 0.20$  in an early univariate analysis, or considered to be biologically relevant, were included in the regression model. Odds Ratios (ORs) and corresponding 95% Confidence Intervals (95% CI) were obtained by means of logistic regression. Data were analysed by commercial software R, version 2.8.1 (R, Development Core Team 2007). A value of  $P \leq 0.05$  was considered statistically significant for the analysis.

## Results

### Data collection

Between October 2008 and December 2014, 180 samples were tested by PCR for PVI regardless of the IDS. However, cases from members of the *Canidae* family (wolf and fox,  $n = 2$ ), puppies aged

$\leq 15$  days ( $n = 16$ ), cases where identification of PV strains was not possible due to the risk of vaccinal interference ( $n = 3$ ), and cases with non-repeatable PCR results ( $n = 3$ ) were excluded (Figure 1). Thus, 156 cases, 51 from cats (32.7%) and 105 (67.3%) from dogs, were eventually counted in the study. The flow chart of the included cases is reported in Figure 1.

PCR was requested by 15 different veterinary practices (clinical cases) and 2 diagnostic laboratories (necropsies). Due to a special pricing arrangement during the study period, one veterinary practice sent to the laboratory not only the regular PVI suspected cases ( $n = 32$ ), but also those presenting only a single clinical sign ( $n = 12$ ).

Of the 156 cases included in the study, 93 (59.6%) were from clinical cases whereas 63 (40.4%) from post mortem examination. Table II reports the age and species of the animals examined. Unfortunately, data were not available for all cases. Complete blood or biochemical analyses were not always available, as well vaccinal status was unknown in 59 cases (37.8%, 36 cats and 23 dogs). Forty-three animals (28.8%,

**Table II.** Distribution of positive (+) and negative (-) results of the cases, based on species and age, submitted to biomolecular diagnosis for Parvovirus infection.

Cases	PCR results						Total
	$\leq 6$ months		$> 6$ months-1 year		$> 1$ year		
	+	-	+	-	+	-	
Dogs	63 (41.4%)	8 (5.3%)	13 (8.6%)	2 (1.3%)	10 (6.6%)	8 (5.3%)	104 (68.4%)
Cats	18 (11.8%)	5 (3.3%)	9 (5.9%)	0 (0%)	13 (8.6%)	3 (1.9%)	48 (31.6%)
Total	81 (53.2%)	13 (8.6%)	22 (14.5%)	2 (1.3%)	23 (15.2%)	11 (7.2%)	152* (100%)

\*The age was not recorded in 4 cases (1 positive PCR dog, 1 positive PCR cat, and two negative PCR cats).

39 dogs and 3 cats) just begun the vaccine protocol while 54 (37.5%, 26 cats and 28 dogs) were not vaccinated. Vaccinal interference was considered as probable in 17 cases; however, the presence of CPV 2c or 2a variants was subsequently identified for 14 of them by enzymatic digestion. In 74 (47.4%) cases, it was possible to know the PVI clinical outcome. Sixty four animals died while 10 survived.

Of the 144 cases for which it was possible to express an IDS, 98 were characterised by strong and 46 by weak IDS (Table II).

### Biomolecular investigations

Conventional PCR was able to detect 0.1 ng extracted from DNA of the cell culture infected with 100 TCID<sub>50</sub>/100 µL of Cornell strain, while nested PCR 0.01 ng. The new developed nested PCR also proved to be highly specific as the amplified PCR product sequences showed 100% similarity with FPV or CPV.

When the 156 cases were tested by conventional and nested PCR assays, 111 (71.2%) resulted positive to both tests, whereas 17 cases (10.9%) were positive to nested PCR only.

### IDS classification

Results of the classification of the 144 cases based on IDS and PCR are reported in Table III. As shown, it was possible to reach a definitive diagnosis in 124 out of 144 cases (86.1%). One hundred and

nineteen (82.6%) samples were positive on PCR testing for PV; in 10 cases the presence of PV was associated with comorbidities. They consisted of 2 dogs with a history of chronic diarrhoea, 2 dogs with canine distemper virus coinfection, 1 dog with megasophagus, 1 cat with respiratory signs, 1 cat with pyometra, 1 cat with an obstructive lower urinary tract disease, 1 cat with a perforated gastric ulcer, and 1 cat with severe cardiac hypertrophy. In other 20 cases which were negative to PV PCR and without a diagnostic suspicion issued by the veterinarian, it was not possible to reach a definitive diagnosis because either the owner did not allow further testing or the animal died and necropsy was not possible. In other 5 PCR negative cases, diagnosis of coccidiosis (3 kittens and 1 puppy) and severe nephropathy (1 dog) was made.

In 20 cases (including 11 dogs, 2 of them with weak IDS, and 9 cats, 5 of them with weak IDS, all confirmed by PCR positive results), case history reported the death within a few days of another animal belonging either to the same owner or to the same shelter. However, there was also a case of a PV PCR positive puppy with weak IDS which came from a litter in which the other puppies were healthy.

Cases with weak IDS and positive PV PCR, also included 3 dogs with intestinal stasis and one dog subjected to surgery for acute abdomen. Eighteen cases (10 dogs and 8 cats) with weak IDS and PCR positive results presented vomiting episodes or non-haemorrhagic diarrhoea. Two kittens, one with fever and another with leukopenia, were

**Table III.** Performances of the index of diagnostic suspicion (IDS) of Parvovirus infection (PVI) and corresponding 95% Confidence Interval (95% CI) on total cases, and the subgroups of clinical cases and necropsies, subjected to biomolecular diagnosis for PVI.

Parameter	Number of cases (%) Estimate (95% CI)		
	Total cases	Clinical cases	Necropsies
Strong IDS and positive PCR	83 (57.6%)	51 (57.9%)	32 (57.1%)
Weak IDS and negative PCR	10 (6.9%)	3 (3.4%)	7 (12.5%)
Strong IDS and negative PCR	15 (10.5%)	13 (14.8%)	2 (3.6%)
Weak IDS and positive PCR	36 (25%)	21 (23.9%)	15 (26.8%)
Sensitivity	69.75% (60.98 - 77.28)	70.83% (59.49 - 80.06)	68.09% (53.83 - 79.6)
Specificity	40% (23.4 - 59.26)	18.75% (6.6 - 43.01)	77.78% (45.26 - 93.68)
Positive Predictive Value	84.69% (76.27 - 90.5)	79.69% (68.29 - 87.73)	94.12% (80.91 - 98.37)
Negative Predictive Value	21.74% (12.26 - 35.57)	12.5% (4.34 - 31)	31.82% (16.36 - 52.68)
Accuracy	64.58% (56.49 - 71.92)	61.36% (50.92 - 70.86)	69.64% (56.66 - 80.1)
Positive Likelihood Ratio	1.162 (1.01 - 1.34)	0.8718 (0.74 - 1.03)	3.064 (1.12 - 8.4)
Negative Likelihood Ratio	0.76 (0.53 - 1.07)	1.556(0.08 - 28.97)	0.4103 (0.33 - 0.51)
Diagnostic Odds	1.54 (0.63 - 3.74)	0.56 (0.14 - 2.17)	7.467 (1.38 - 40.34)
Cohen's kappa	0.07 (-0.08 - 0.22)	-0.09 (-0.29 - 0.11)	0.29 (0.07 - 0.51)

Sensitivity = The proportion of positives that are correctly identified as such; Specificity = The proportion of negatives that are correctly identified as such; Positive Predictive Value = The proportion of true positive tests out of the overall positive tests; Negative Predictive Value = The proportion of negative positive tests out of the overall negative tests; Accuracy = The proportion of correctly classified subjects among all the results; Positive Likelihood Ratio (LR+) = Sensitivity / (1 - Specificity); Negative Likelihood Ratio (LR-) = (1 - Sensitivity) / Specificity; Diagnostic Odds = LR+/LR-

initially suspected to be affected by feline infectious peritonitis.

As reported by the veterinarians, when a blood test was performed, cases with weak IDS generally had all the parameters within the normal range.

Interestingly, some clinical cases subjected to necropsy included cases in which sudden death occurred (n = 8, 4 dogs and 4 cats) and cases in which the initial suspicion was poisoning (n = 5, 3 cats and 2 dogs). Sudden deaths included 3 cases showing haemorrhagic gastro-enteritis at gross examination, 4 cases in which the histological examination supported the strong IDS, which was assigned after necropsy, and one case without specific clinical signs. In this case, the gross examination revealed an intestinal stasis with haemorrhagic exudate in the gut. The poisoning suspected cases consisted of 3 stray cats from colonies with repeated deaths, one hunting dog and a puppy, which had eaten rotten food.

A veterinary practice with which a special price was arranged, wanted to process 44 cases, 32 with strong and 12 with weak IDS (with vomiting or non-haemorrhagic diarrhoea or a low increase in body temperature). Thirty one cases with strong IDS and all cases with weak IDS were positive to PV PCR. The veterinarian of this practice reported that these weak IDS cases would not have been sent for laboratory testing without having the incentive of the special price offer that was specifically arranged during this project.

Three veterinary practices (3 cats, 2 with strong IDS

and 1 with weak IDS) reported the possibility that the PVI originated in their structures, as various animals with different owners, which came to the practice for routine procedures, had PVI clinical signs just few days after their admission to the clinic.

## Statistical analysis

Overall, in 93 out of 144 cases (64.6%) the referral veterinarian correctly identified PVI cases, as confirmed by PCR, but failed to diagnose PVI in the remaining 36.4% of the cases. As in some cases data could not be obtained, some final subgroups used for statistical analysis were inconsistent. Even if the analyses were performed with both, the conventional and the nested PCR assays, and the results obtained from both tests did not significantly differ, data used for statistical analyses were from nested PCR.

The agreement between IDS and nested PCR assay was moderate. Necropsies appeared to have a better diagnostic prediction (69.6%) than clinical cases (61.4%) (Table III). The PV infection was more easily diagnosed in animals aged  $\leq 6$  months (72.9%) compared to animals aged  $> 6$  months-1 year (69.6%) and in animals over 1 year (36.4%) (Table IV). The diagnostic accuracy of PVI in dogs was higher than in cats (71.9% vs. 50%, respectively, Table V).

The final model of the logistic regression based on cases with missed diagnoses (false negative, n = 36) found that cats had an OR = 2.37 (95% CI 0.99-5.68,  $P = 0.05$ ), if the model was adjusted with a cutoff of  $\leq 6$  months of age. On the other hand, the effect of

**Table IV.** Performances of the index of diagnostic suspicion (IDS) of Parvovirus infection (PVI) and corresponding 95% Confidence Interval (95% CI) on the subgroups of different ages, subjected to biomolecular diagnosis for PVI. The sum of the subgroups is under 144, as the age was not recorded in 3 cases.

Parameter	Number of cases (%) Estimate (95% CI)		
	Aged $\leq 6$ months	Aged $> 6$ months-1 year	Aged $> 1$ year
Strong IDS and positive PCR	60 (70.5%)	15 (65.2%)	7 (21.2%)
Weak IDS and negative PCR	2 (2.4%)	1 (4.3%)	5 (15.15%)
Strong IDS and negative PCR	9 (10.6%)	1 (4.3%)	5 (15.15%)
Weak IDS and positive PCR	14 (16.5%)	6 (26.2%)	16 (48.5%)
Sensitivity	81.08% (70.71 - 88.38)	71.43% (50.04 - 86.19)	30.43% (15.6 - 50.87)
Specificity	18.18% (5.137- 47.7)	50% (9.453 - 90.55)	50% (23.66 - 76.34)
Positive Predictive Value	86.96% (77.03 - 92.98)	93.75% (71.67 - 98.89)	58.33% (31.95 - 80.67)
Negative Predictive Value	12.5% (3.5 - 36.02)	14.29% (2.6 - 51.31)	23.81% (10.63 - 45.09)
Accuracy	72.94% (62.66 - 81.24)	69.57% (49.13 - 84.4)	36.36% (22.19 - 53.38)
Positive Likelihood Ratio	0.99 (0.79 - 1.24)	1.43 (0.19 - 10.69)	0.6087 (0.22 - 1.70)
Negative Likelihood Ratio	1.04 (0.01 - 98.47)	0.57 (0.06 - 5.62)	1.391 (0.83 - 2.33)
Diagnostic Odds	0.96 (0.18 - 4.9)	2.5 (0.13 - 46.78)	0.4375 (0.1 - 2.01)
Cohen's kappa	-0.01 (-0.21 - 0.2)	0.1006 (-0.21 - 0.41)	-0.15 (-0.42 - 0.12)

Sensitivity = The proportion of positives that are correctly identified as such; Specificity = The proportion of negatives that are correctly identified as such; Positive Predictive Value = The proportion of true positive tests out of the overall positive tests; Negative Predictive Value = The proportion of negative positive tests out of the overall negative tests; Accuracy = The proportion of correctly classified subjects among all the results; Positive Likelihood Ratio (LR+) = Sensitivity / (1 - Specificity); Negative Likelihood Ratio (LR-) = (1 - Sensitivity) / Specificity; Diagnostic Odds = LR+/LR-

**Table V.** Performances of the index of diagnostic suspicion (IDS) of Parvovirus infection (PVI) and corresponding 95% Confidence Interval (95% CI) on subgroups of species, subjected to biomolecular diagnosis for PVI.

Parameter	Number of cases (%) Estimate (95% CI)	
	Dogs	Cats
Strong IDS and positive PCR	63 (65.7%)	20 (41.6%)
Weak IDS and negative PCR	6 (6.3%)	4 (8.4%)
Strong IDS and negative PCR	9 (9.4%)	6 (12.5%)
Weak IDS and positive PCR	18 (18.6%)	18 (37.5%)
Sensitivity	77.78% (67.58 - 85.46)	52.63% (37.26 - 67.52)
Specificity	40% (19.82 - 64.25)	40% (16.82 - 68.73)
Positive Predictive Value	87.5% (77.92 - 93.28)	76.92% (57.95 - 88.97)
Negative Predictive Value	25% (12 - 44.9)	18.18% (7.31 - 38.52)
Accuracy	71.88% (62.17 - 79.89)	50% (36.39 - 63.61)
Positive Likelihood Ratio	1.296 (1.03 - 1.63)	0.88 (0.58 - 1.33)
Negative Likelihood Ratio	0.5556 (0.73-7.43)	1.18 (0.51 - 2.75)
Diagnostic Odds	2.33 (0.73 - 7.43)	0.74 (0.18 - 3.1)
Cohen's kappa	0.14 (-0.05 - 0.3)	-0.05 (-0.29 - 0.19)

Sensitivity = The proportion of positives that are correctly identified as such; Specificity = The proportion of negatives that are correctly identified as such; Positive Predictive Value = The proportion of true positive tests out of the overall positive tests; Negative Predictive Value = The proportion of negative positive tests out of the overall negative tests; Accuracy = The proportion of correctly classified subjects among all the results; Positive Likelihood Ratio (LR+) = Sensitivity / (1 - Specificity); Negative Likelihood Ratio (LR-) = (1 - Sensitivity) / Specificity; Diagnostic Odds = LR+/LR-

species lost significance ( $P = 0.08$ ) when the age > 1 year is considered. Both dogs and cats over 1 year had a 7-fold greater probability of a missed diagnosis (OR = 7.17, 95% CI 2.53-20.29,  $P < 0.0001$ ). The type of presentation (clinical vs. necropsy cases) was left out of the model, since it did not affect it.

The logistic regression that referred to incorrect PVI diagnoses (false positive,  $n = 15$ ) found that only the type of presentation had a limited significance, with clinical presentation having a higher probability (OR = 8.92; 95% CI 1.03-77.2,  $P = 0.05$ ) to overestimate the infection.

## Discussion

This study confirms the results of a previous study (Faz *et al.* 2016) and highlights the concern that veterinarians often don't suspect parvovirus infection in dogs and cats with atypical presentation. The consequences of this can be catastrophic for kennel facilities, shelters or even veterinary clinics, because PV are highly contagious.

In this study, nested PCR was chosen to confirm the diagnostic suspect because of its high sensitivity and specificity. It reduced the rates of false negative results derived from in-clinic tests in case of low viral load or the presence of antibodies and improved the diagnostic accuracy, in the cases with few clinical signs and better prognosis (Desario *et al.* 2005, Decaro *et al.* 2013b, Decaro *et al.* 2014a, Proksch *et al.* 2015, Faz *et al.* 2016). Moreover, nested PCR was capable to identify the majority of cases with vaccinal interference, and it has a medium cost between in-clinic tests and real-time PCR. However,

we can't exclude that the sampling method used to enrol cases could have introduced a bias in this study, as a specific subpopulation of cases was selected. Considering that all cases submitted had at least one clinical sign and no healthy animals were analysed, the assay characteristics should be cautiously evaluated. Logistic regression was used to weight all these aspects.

In this study, PVI diagnoses was more accurate in dogs and young animals or following necropsy examination. Necropsies had better diagnostic prediction than clinical cases, probably because of the increased diagnostic sensitivity and specificity due to findings of gross and histological examinations. However, cases subjected to necropsy generally were those atypical. They didn't have a PVI clinical suspicion and required gross and histological examination for a definitive diagnosis. Conversely, PVI with typical clinical signs is normally diagnosed by using lower expensive tests. In this study, many necropsies with strong IDS were not initially considered as PVI because they were hyperacute or atypical cases of PVI. Accordingly, the correct diagnosis of PVI could also be overestimated in this study.

PVI diagnosis was more accurate in young rather than adult animals, as confirmed also by logistic regression. Although animals aged from 6 weeks to 6 months are the typical target of PVI (Houston *et al.* 1996, Kruse *et al.* 2010, Miranda *et al.* 2015), cases of PVI in adult dogs have also been reported (Decaro and Buonavoglia 2012). In this study, diagnosis was missed in a significant proportion of animals aged > 1 year. This was in line with the results obtained by Faz

and colleagues (Faz *et al.* 2016). On the other hand, even though in a limited number of confirmed cases, young age was also confounding. It was the case of one puppy which was referred as the result of the suspected ingestion of rotten food, a young dog which underwent surgery for acute abdomen with a suspicion of ingestion of a radiolucent foreign body or two kittens, in which the main suspect was feline infectious peritonitis, frequently observed in cats under 16 months (Pedersen 2014).

Anamnestic information was also an important diagnostic bias in this study. The death of several cats sharing the same place, or of a hunting dog was erroneously considered as a result of poisoning. In another case, a weak IDS was issued for a PV PCR positive sick puppy, because it was the only sick puppy of a healthy litter. It is likely that different titers of maternal antibodies were passively transmitted from the bitch to the puppies during lactation, giving to each puppy a variable protection (Decaro *et al.* 2005).

Vaccinal history was reported mostly for the youngest animals and dogs. Vaccinal interference was recognised in 14 out of 17 confirmed cases of PVI, whereas in the 3 non-identified cases, the more expensive real-time PCR was necessary to solve any doubt (Decaro *et al.* 2005, Decaro *et al.* 2006a). Vaccinal history was recently reported by Faz and colleagues (Faz *et al.* 2016) as a factor leading veterinarians to rule out PVI from the list of differential diagnoses. Veterinarians should instead consider that incomplete vaccine protocol doesn't protect young animals from the risk of developing PV clinical signs. To better estimate the protection level against PV in a vaccinated animal, the clinician should know how long ago the animal was vaccinated, which protocol was used, and also whether the animal had completed the protocol as indicated in the manufacturer's guidelines (Day *et al.* 2016). Moreover, our results also confirmed that the number of cats vaccinated against PV is lower than the number of dogs (Diez *et al.* 2015), suggesting that this species could be more susceptible to viral infection.

Dogs had better diagnostic results than cats that had a significant number of missed diagnosis. The reason could be related to the fact that, generally, in dogs PVI typical clinical signs are more frequent and appear at an earlier stage than cats. Moreover, cats are often taken to the veterinarian in critical clinical conditions when they frequently develop hypothermia and lose an important sign of PVI, which is fever. Dehydration can also be more severe in cats, as well as in small dogs. This also makes it difficult to collect blood to investigate clinicopathological parameters, further reducing the possibility of reaching a correct diagnosis.

The discrepancy observed between IDS and PCR results was the most informative for understanding when veterinarians misdiagnose a case of PVI. For example, according to referred clinical signs, veterinarians often identify PVI based on haemorrhagic diarrhoea, conversely they tend to exclude it from the list of differential diagnoses in case of non-haemorrhagic diarrhoea. Logistic regression confirmed that PVI is generally overestimated by clinicians, mostly because they misdiagnosed bloody diarrhea with PVI. In this study, 4 cases (3 kittens and 1 puppy) with haemorrhagic diarrhoea were initially considered PVI, but later they were found to be Coccidiosis. This parasitic infection has already been reported as the cause of diarrhoea in puppies that were recently vaccinated for PV (Decaro *et al.* 2007a). In another case, an adult dog having haemorrhagic diarrhoea was suspected to have PVI, whereas it had a severe nephropathy. On the other hand, other infectious agents, such as canine circovirus, canine coronavirus, canine distemper, canine adenovirus, *Clostridium* spp., *Salmonella* spp. can cause clinical signs similar to PVI including haemorrhagic diarrhoea (Decaro *et al.* 2007b, Zappulli *et al.* 2008, Decaro *et al.* 2014, Merck Veterinary Manual 2016).

Moreover, PVI was also often excluded by the clinicians in cases in which vomiting was the only clinical sign present, confirming the results of a recent study (Faz *et al.* 2016). A normal blood count was another clinicopathological parameter that made PVI appear less likely to be suspected by the veterinarian, as reported by Faz and colleagues (Faz *et al.* 2016). Actually, leukopenia is a prognostic and not a diagnostic factor (Scheman *et al.* 2013, Houston *et al.* 1996, Kruse *et al.* 2010). Lastly, comorbidity, when present, can modify clinical presentations, clinicopathological parameters and, thus, complicate the diagnosis.

A factor not included in the analysis, that might have influenced the results, was the amount of money the owner wanted to spend for the diagnosis. This could have affected the choice of the test and, consequently, the performance of the diagnosis. For example, the 12 cases with weak IDS which were sent by the veterinary practice to the laboratory because of the special economic arrangement, were confirmed as cases of PVI by PCR. It is likely that, under normal conditions, these samples wouldn't be sent to the laboratory for testing as they belonged to animals with aspecific or few clinical signs that recovered within a few days. As the identification of these atypical cases is essential to limit the disease spread, costs of the laboratory tests become an important limiting factor not only for diagnosis but also for implementing surveillance and control program in the future. In such a context, any PCR positive case, even in the absence of a consistent

clinical diagnosis, becomes epidemiologically important: since many PVI cases are asymptomatic, the test results in these cases could be very useful for a better management of areas contaminated by this highly resistant virus. Without an appropriate protocol of disinfection, PV can persist in veterinary clinics, causing possible nosocomial infections. This possibility should not be underestimated considering that three veterinary practices involved in this study presumed a nosocomial PVI. To gain a better understanding of the problem, specific studies on the costs of the diagnosis and the economic losses due to PVI could be useful.

Considering the consequences that a missed diagnosis can have on virus spread, PVI should be better investigated in patients with atypical or few clinical signs, especially cats and animals over 1 year old. Animals showing only vomiting

or non-haemorrhagic diarrhoea should also be systematically tested for PVI. Some cases subjected to necropsy are probably hyperacute or atypical cases of PVI that are originally misdiagnosed at clinical presentation. The challenge given by the different clinical manifestation of the disease and overall costs to perform a diagnosis of PVI should be taken into consideration in the future, especially if a surveillance system is to be devised for this infection.

## Acknowledgements

The authors acknowledge Mr. Carlo Sanesi and Massimo Arcangeli for their skilful technical assistance. A special thanks is reserved to Dr. Mark Rishniw for his careful and considerable revision of the manuscript.

## References

- Buonavoglia C., Martella V., Pratelli A., Tempesta M., Cavalli A., Buonavoglia D., Bozzo G., Elia G., Decaro N. & Carmichael L. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. *J Gen Virol*, **82** (12), 3021-3025.
- Day M.J., Horzinek M., Schultz R.D. & Squires R.A. Vaccination Guidelines Group (VGG) of the World Small Animal Veterinary Association (WSAVA). 2016. WSAVA Guidelines for the vaccination of dogs and cats. *J Small Anim Pract*, **57** (1), E1-E45.
- Decaro N., Campolo M., Desario C., Elia G., Martella V., Lorusso E. & Buonavoglia C. 2005. Maternally-derived antibodies in pups and protection from canine parvovirus infection. *Biologicals*, **33** (4), 59-265.
- Decaro N., Elia G., Desario C., Roperto S., Martella V., Campolo M., Lorusso A., Cavalli A. & Buonavoglia C. 2006a. A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus. *J Virol Methods*, **136** (1-2), 65-70.
- Decaro N., Elia G., Martella V., Campolo M., Desario C., Camero M., Cirone F., Lorusso E., Lucente M.S., Narcisi D., Scalia P. & Buonavoglia C. 2006b. Characterisation of the canine parvovirus type 2 variants using minor groove binder probe technology. *J Virol Methods*, **133** (1), 92-99.
- Decaro N., Desario C., Elia G., Campolo M., Lorusso A., Mari V., Martella V. & Buonavoglia C. 2007a. Occurrence of severe gastroenteritis in pups after canine parvovirus vaccine administration: a clinical and laboratory diagnostic dilemma. *Vaccine*, **25** (7), 1161-1166.
- Decaro N., Campolo M., Elia G., Buonavoglia D., Colaianni M.L., Lorusso A., Mari V. & Buonavoglia C. 2007b. Infectious canine hepatitis: an "old" disease reemerging in Italy. *Res Vet Sci*, **83** (2), 269-273.
- Decaro N. & Buonavoglia C. 2012. Canine parvovirus - a review of epidemiological and diagnostic aspects, with emphasis on type 2c. *Vet Microbiol*, **155** (1), 1-12.
- Decaro N., Desario C., Amorisco F., Losurdo M., Elia G., Parisi A., Ventrella G., Martella V. & Buonavoglia C. 2013a. Detection of a canine parvovirus type 2c with a non-coding mutation and its implications for molecular characterisation. *Vet J*, **196** (3), 555-557.
- Decaro N., Desario C., Billi M., Lorusso E., Colaianni M.L., Colao V., Elia G., Ventrella G., Kusi I., Bo S. & Buonavoglia C. 2013b. Evaluation of an in-clinic assay for the diagnosis of canine parvovirus. *Vet J*, **198** (2), 504-507.
- Decaro N., Crescenzo G., Desario C., Cavalli A., Losurdo M., Colaianni M.L., Ventrella G., Rizzi S., Aulicino S., Lucente M.S. & Buonavoglia C. 2014a. Long-term viremia and fecal shedding in pups after modified-live canine parvovirus vaccination. *Vaccine*, **32** (30), 3850-3853.
- Decaro N., Martella V., Desario C., Lanave G., Circella E., Cavalli A., Elia G., Camero M. & Buonavoglia C. 2014b. Genomic characterization of a circovirus associated with fatal hemorrhagic enteritis in dog, Italy. *PLoS One*, **9** (8), e105909.
- Desario C., Decaro N., Campolo M., Cavalli A., Cirone F., Elia G., Martella V., Lorusso E., Camero M. & Buonavoglia C. 2005. Canine parvovirus infection: which diagnostic test for virus? *J Virol Methods*, **126** (1-2), 179-185.
- Demeter Z., Palade E.A., Soós T., Farsang A., Jakab C. & Rusvai M. 2010. Misleading results of the MbolI-based identification of type 2a canine parvovirus strains from Hungary reacting as type 2c strains. *Virus Genes*, **41** (1), 37-42.
- Diez M., Picavet P., Ricci R., Dequenue M., Renard M., Bongartz A. & Farnir F. 2015. Health screening to

- identify opportunities to improve preventive medicine in cats and dogs. *J Small Anim Pract*, **56** (7), 463-469.
- Eleni C., Scholl F. & Scaramozzino P. 2014. Causes of death in dogs in the province of Rome (Italy). *Vet Ital*, **50** (2), 137-143.
- Faz M., Martínez J.S., Quijano-Hernández I. & Fajardo R. 2017. Reliability of clinical diagnosis and laboratory testing techniques currently used for identification of canine parvovirus enteritis in clinical settings. *J Vet Med Sci*, **79** (1), 213-217.
- Houston D.M., Ribble C.S. & Head L.L. 1996. Risk factors associated with parvovirus enteritis in dogs: 283 cases (1982-1991). *J Am Vet Med Assoc*, **208** (4), 542-546.
- Kruse B.D., Unterer S., Horlacher K., Sauter-Louis C. & Hartmann K. 2010. Prognostic factors in cats with feline panleukopenia. *J Vet Intern Med*, **24** (6), 1271-1276.
- Landis J.R. & Koch G.G. 1977. The measurement of observer agreement for categorical data. *Biometrics*, **33** (1), 159-174.
- Maxie G. 2015. Alimentary system, infectious and parasitic diseases of the alimentary tract. In Jubb, Kennedy & Palmer's Pathology of Domestic Animals, 6<sup>th</sup> edition, Elsevier Health Sciences, Vol 2, 135-279.
- Merck Veterinary Manual. 2016. [http://www.merckvetmanual.com/mvm/digestive\\_system/diseases\\_of\\_the\\_stomach\\_and\\_intestines\\_in\\_small\\_animals/hemorrhagic\\_gastroenteritis\\_in\\_small\\_animals.html](http://www.merckvetmanual.com/mvm/digestive_system/diseases_of_the_stomach_and_intestines_in_small_animals/hemorrhagic_gastroenteritis_in_small_animals.html) (accessed on 9 December 2016).
- Miranda C., Carvalheira J., Parrish C.R. & Thompson G. 2015. Factors affecting the occurrence of canine parvovirus in dogs. *Vet Microbiol*, **180** (1-2), 59-64.
- Pedersen N.C. 2014. An update on feline infectious peritonitis: diagnostics and therapeutics. *Vet J*, **201** (2), 133-141.
- Proksch A.L., Unterer S., Speck S., Truyen U. & Hartmann K. 2015. Influence of clinical and laboratory variables on faecal antigen ELISA results in dogs with canine parvovirus infection. *Vet J*, **204** (3), 304-308.
- Schoeman J.P., Goddard A. & Leisewitz A.L. 2013. Biomarkers in canine parvovirus enteritis. *N Z Vet J*, **61** (4), 217-222.
- World Organisation for Animal Health. 2016. Chapter 3.6.5. Statistical approaches to validation. [http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/3.6.05\\_STATISTICAL\\_VALIDATION.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.6.05_STATISTICAL_VALIDATION.pdf). (accessed on 1 December 2016).
- Zappulli V., Caliarì D., Cavicchioli L., Tinelli A. & Castagnaro M. 2008. Systemic fatal type II coronavirus infection in a dog: pathological findings and immunohistochemistry. *Res Vet Sci*, **84** (2), 278-282.