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Detection of environmental contamination with feline and canine parvoviruses: new perspectives and challenges

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Abstract

Aims: To develop a protocol for environmental sampling to detect parvoviruses of dogs and cats in the environment.
Methods and Results: Environmental contamination was carried out using different dilutions of parvovirus-contaminated materials; further field samplings were performed in areas in which clinical cases of parvovirus infections were present. Sterile cotton swabs and sponges for microbial surface sampling were used. Viruses were detected in these samples with different methods: conventional PCR, nested PCR and real-time PCR, detecting viral DNA; virus isolation, detecting infectious virus; and a commercial rapid enzyme immunoassay, detecting viral antigen. No substantial differences were observed in the two sampling methods, although the sponge was more convenient for sampling rough surfaces. Molecular assays were the most sensitive methods, identifying even very low amounts of viral DNA (up to 10 copies of viral DNA/10 μl of sample). Virus isolation and the rapid test detected the viruses only at the highest viral concentrations, both in the experimental setting and field conditions.
Conclusions: Environmental sampling and molecular protocols were effective in detecting environmental contamination with parvoviruses.
Significance and Impact of the Study: The protocol will be useful to identify possible sources of infection and to assess the efficacy of disinfection protocols in the environment.

Introduction

Canine parvovirus type 2 (CPV-2, including the variants CPV-2a, -2b and -2c) and Feline panleukopaenia virus (FPLV) are small non-enveloped DNA viruses, endemic in dogs and cats. They are responsible for outbreaks throughout the world, with high morbidity and mortality (Turk *et al*. 1990; Decaro and Buonavoglia 2012; Sarpong *et al*. 2017).

Despite the widespread use of vaccines, the frequency of parvovirus infection is still very high because the efficacy of vaccination is reduced by maternally derived antibodies (Decaro *et al*. 2020), and the high resistance of the virus in the environment allows it to remain infectious and to be transmitted indirectly for >1 year (Greene and Decaro 2012). Parvoviruses are recognized as some of the most resistant viruses and are therefore often used

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to evaluate the efficacy of disinfection protocols (Eterpi *et al.* 2009).

Typically, clinical cases of infection in dogs affect puppies aged between 6 weeks and 6 months, with symptoms of fever, vomiting, mucoid to haemorrhagic diarrhoea and leukopaenia (Kruse *et al.* 2010; Schoeman *et al.* 2013). The clinical signs in cats are less specific, with greater involvement of the bone marrow. When atypical clinical presentations appear in dogs and cats, which range from hyperacute forms to cases with few clinical signs, misdiagnosis or no diagnosis is possible (Marenzoni *et al.* in press; Kruse *et al.* 2010; Faz *et al.* 2017).

Previous studies have shown that infected dogs shed considerable amounts of the virus into the environment, even when they lack marked clinical signs (Appel *et al.* 1979; Pollock 1982; Decaro *et al.* 2005a). Therefore, environmental contamination with parvovirus can be very high, and 1 g of faeces from an infected animal can contain 1 billion copies of viral DNA (Decaro *et al.* 2005b). Vaccinated animals can also shed the virus even when they show no clinical signs (Decaro and Buonavoglia 2017). Freisl *et al.* (2017) showed that sub-clinically infected adult dogs shed amounts of virus ranging from $3{\cdot}87 \times 10^5$ to $8{\cdot}39 \times 10^8$ viral copies per mg of faeces. The faecal shedding of field FPLV by vaccinated cats has also been observed (Bergmann *et al.* 2019).

All these traits, including the delay in diagnosis, favour viral spread, environmental contamination and transmission of parvovirus infection.

The presence of contaminated faeces has also been reported on urban surfaces and in public areas (Gogone *et al.* 2020). Recurrent outbreaks are described in the environments where previous cases have been reported (Sarpong *et al.* 2017; Cavalli *et al.* 2018; Porporato *et al.* 2018). Contaminated environments, including clinics and shelters, can be sources of infection. Nosocomial infections have financial, social and environmental effects on both the clients and staff of a clinic, and many nosocomial infections occur unnoticed (Marenzoni *et al.* in press; Stull *et al.* 2018; Marenzoni *et al.* 2019). Estimates of the economic impact of clinical cases of parvovirus infection are significant, with a median cost for a single case of about €600–900 in central Italy and A$1500 (nearly €930) in Australia (Kelman *et al.* 2019; Arronenzi *et al.* 2020).

Therefore, the prompt identification of infected animals and of the environmental sources of the virus is required. However, to the authors' knowledge, no study has evaluated environmental contamination with parvoviruses from dogs or cats.

The aim of the present study was to develop an easy, effective, rapid and inexpensive diagnostic protocol to detect and identify environmental parvovirus contamination and possible sources of infection. To do this, different tests (conventional PCR, nested PCR, real-time PCR, virus isolation and an in-clinic immunoassay) were evaluated.

Materials and Methods

Study design

Experimental environmental contamination was established in a controlled area using different dilutions of parvovirus, to assess the reliability of the protocols used for environmental sampling. The virus was detected in the collected samples with different methods, comprising conventional and nested PCR, real-time PCR, virus isolation and a rapid commercial in-clinic enzyme immunoassay (SNAP Canine Parvovirus Antigen Test, IDEXX Laboratories, Germany) that was also validated for cats with feline panleukopaenia (Esfandiari and Klingeborn 2000; Abd-Eldaim *et al.* 2009).

The effectiveness of the protocol (its capacity to detect the presence of parvovirus) was assessed with real-time PCR, which is considered the gold standard technique (Desario *et al.* 2005). Other approaches were tested to reduce the cost and/or accelerate the process using in-clinic testing and/or nested PCR. The virus isolation technique was used to assess the infectivity of the virus collected by sampling the environment.

Field samples, that were obtained in different settings in which clinical cases of parvovirus infection were present, were tested to assess and validate the protocol under field conditions.

Experimental contamination of the environment

A homogenate (1 ml, 1 g ml^{-1} phosphate-buffered saline, [PBS]) of pooled tissues, containing lung, liver, spleen and gut, from a 3-month-old cat that had died from FPLV infection, and 2 ml samples of the 10-fold-diluted homogenate (from 10^{-1} to 10^{-10}) were used to contaminate two square areas of 10×10 cm^2 marked on a 25×25 cm^2 tile. These were used to simulate commonly used surfaces, such as the floors in clinics, kennels or houses. The tiles were contaminated under a level II biological risk laminar-flow hood to avoid the spread of the pathogen into the environment. The two contaminated surfaces were identical and were sampled with the two tools, cotton swabs or sponges (Sani-Sponge kit, VWR, Milan, Italy), in parallel (Fig. 1).

In case of difficult interpretation or to further characterize the differences in the sensitivity between the two sampling methods, the experiment was repeated using additional serial twofold dilutions of the homogenates,

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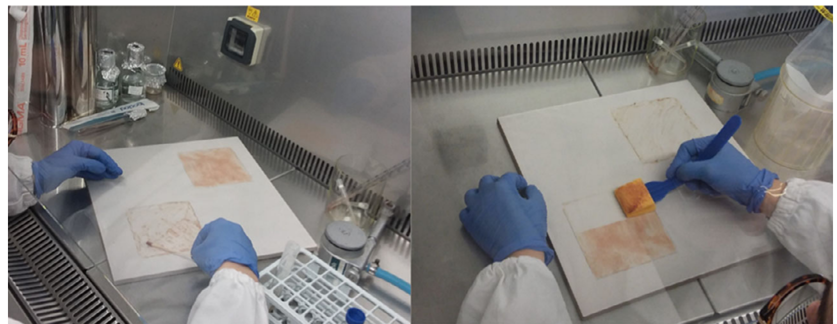

Figure 1 Setting of the experimental infection, carried out with parvovirus infected materials at different dilutions, using cotton swab (on the left) and sponge (on the right), under a level II biological risk laminar flow hood. [Colour figure can be viewed at wileyonlinelibrary.com]

testing the 10-fold dilutions resulted at the limit of the detection (the lowest positive dilution and the immediately following negative one, which in the specific case, occurred in the study, was 10^{-6} and 10^{-7}).

The surfaces were first contaminated with the homogenate and then with each serial dilution of virus, each in a separate set of experiments. The contaminated liquid (1 ml) was left to dry (from 30 min to 1 h) in the square areas, and the material was then collected with a cotton swab or sponge.

The wood stick cotton swab was humidified using sterile PBS. Sampling started from one corner of the drawn square, proceeded to the opposite corner; the swab was then rolled over the area to the right and left, to cover the entire surface. The swab was then immersed in PBS and the procedure was repeated starting from each of the other three corners.

The sponge was immersed in 10 ml of PBS, as recommended by the manufacturer. The entire surface of the contaminated area (10×10 cm^2) was sampled, passing the sponge horizontally, first on one side, then on the other, and then vertically, first on one side and then on the other.

The swabs and sponges were kept in 3 and 10 ml of PBS, respectively, for about 30 min, as described above, to allow the material taken into the liquid to be released. The final eluted material was about 3 ml for the cotton swab and 4 ml for the sponge, and the samples were divided into four aliquots for the subsequent diagnostic tests: 200 μl for conventional and nested PCR, 200 μl for real-time PCR, 600 μl for the rapid enzyme immunoassay and at least 1 ml for virus isolation. The aliquots were frozen at −20°C for later analysis.

Field samples

To confirm the feasibility and reliability of the sampling protocol under field conditions, environmental swabs were collected at locations in which at least one clinical case of canine or feline parvovirus infection was recorded in the days preceding sampling. Samples were collected from different sites at a public cattery (A, n = 4), a private kennel (B, n = 11) and a university veterinary teaching hospital (D, n = 9). When possible, biological samples from infected animals or animals suspected of being infected were collected to better define the context of the environment and the status of the infection of the animals.

Under field conditions, the samples were initially collected with sponges and cotton swabs, but in response to the comparison of the sensitivity of the two methods, the field samples were later only collected with sponges because they were more practical and resistant on rough and more extensive surfaces. At each site, three areas of 10×10 cm^2 (total 300 cm^2) were swabbed for each sample. Briefly, the sponge was humidified with sterile PBS and was then streaked across the surface three times, in different directions, on three square areas of 10×10 cm^2. The sponge was soaked in 10 ml of PBS. The ~4 ml of eluate final obtained was divided into four aliquots for the diagnostic tests (200 μl for conventional and nested PCR, 200 μl for real-time PCR, 600 μl for the rapid enzyme immunoassay and at least 1 ml for virus isolation). The aliquots were frozen at −20°C until analysis.

The public cattery (A) consisted of a feline colony of almost 100 cats, about 20 m from a public veterinary clinic, which also had annexed structures for the hospitalization of the animals. The facility also had several cages inside dedicated to the isolation of infectious animals. No separated and specific area was reserved for animals with infectious diseases. The cattery was chosen for the sampling because 6 days before the sampling, a cat that lived there died of FPLV infection. For this reason, the walls of the cat carrier where the cat that died was placed (A1), the sheets of paper used as flooring in the cat carrier (A2), the walls of cages used for hospitalization in the

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adjacent veterinary clinic (A3), and the floors of an outdoor box for cats, repeatedly washed and disinfected (A4), were sampled.

The private kennel (B) was chosen for the sampling because a 3-month-old puppy, living in the kennel, was diagnosed with haemorrhagic gastroenteritis by parvovirus. The kennel contained a population of pedigree hunting epagneul Breton dogs, consisting of five adult females (4–10 years of age), and the puppy. The animals lived in adjacent covered outdoor boxes, four dogs in pairs and two alone (the puppy, identified as dog n. 1, and the oldest adult dog, identified as dog n. 4). The breeder applied a regime that restricted entrance to the kennel. The adult dogs were all regularly and repeatedly vaccinated, whereas the puppy had not yet been vaccinated and had never gone outside the kennel. The adult dogs left the kennel once a week to go hunting. The boxes were built on a slope to facilitate cleaning; they were washed 1–2 times daily with running water and the waste flowed into a sewer. Specific disinfection measures were occasionally taken under normal conditions, and disinfection with sodium hypochlorite (5% w/v) was performed during the occurrence of the clinical case. For the characteristics of the kennel, biological samples (rectal swabs, identified as B_RS) were collected from the puppy, both in the symptomatic (B_RS1, B_RS7) and convalescent phases repeated at different times (B_RS8-B_RS10), and from adult dogs (B_RS2-B_RS6) to better define the epidemiological context of the infection. Overall, 11 environmental samples (floor of the dog boxes B1–B6, B10; floor of the owner home B7; contaminated and cleaned shoes of the owner B8, B9 and B11) and 10 biological samples (five from the puppy, B_RS1,B_RS7, B_RS8-B_RS10 and five from adult dogs, B-RS2-B_RS6) were collected for the study. Moreover, serums of the puppy and adult dogs were collected at the convalescent phase to assess the presence of the antibodies against parvovirus using a haemagglutination inhibition (HI) test (Decaro *et al.* 2005a). The details of the biological samples, technical comments and their results are reported in the Table S1.

The university veterinary teaching hospital (D) was chosen for the sampling because it is used to hospitalize cases of parvovirus infection; moreover, it had hospitalized three infected puppies (dead 2 days before the sampling), and further cases of infected cats when the sampling was performed. The hospital reported nearly 60–70 daily admissions of dogs and cats. The structure was characterized by a frequent movements of animals, people and goods, due to the presence of medical staff, auxiliary staff and students. The rules of conduct and access to the various structures were well established. Isolation areas for infectious animals (separately for dogs and cats) were present. Each room reserved for infectious animals had an adjacent room (ante-room) at the entrance in which the workers prepared and changed their clothes to enter the infectious room. Access was restricted to authorized personnel, who were required to wear disposable boots, gloves, caps, masks, gowns and glasses, according with a procedure indicated on the premises. Cleaning and disinfection procedures were defined in the hospital and differed between the standard rooms or common areas and the restricted areas for infectious animals. Briefly, the standard hospital rooms and common areas were washed and cleaned with a floor-cleaning machine by a professional company at least twice a day. The cleaning and disinfection procedures for the restricted areas in which the infectious subjects were hospitalized were only performed by authorized staff and involved the washing of objects, utensils, cages, walls and floors with abundant water to remove any solid dirt. The wastewater was collected in a biohazard tank for disposal. These utensils and areas were then cleaned again with detergent. A commercial biocide, based on hydroxyacetic acid (1–5%, Fumagri HA), was then applied by fumigation, according to the manufacturer's instructions. Considering the characteristics of the hospital, nine environmental samples were obtained from walls or floors of rooms having different use and different level of cleaning (room reserved for isolation of infectious dogs or cats, D1, D3, D8; ante-room of the rooms reserved for isolation, D2, D4, D9; acceptance and entrance area of the hospital, D5; room of standard hospitalization, D6, D7).

Two other areas (C and E) were used as the negative controls in this study: the laboratory counter of a molecular veterinary diagnostics laboratory for infectious diseases, after it was cleaned and disinfected (C1); and three sites (E1: corridor, laboratory and entrance) in the animal anatomy section, attended by students in the first year of a university course in veterinary medicine who did not have access to the veterinary teaching hospital.

DNA extraction and assessment of the amplificability

DNA was extracted with a commercial kit (QIAamp DNA Blood Mini kit, Qiagen, Milan, Italy) from the two aliquots of 200 μl (one for conventional and nested PCR and the other for real-time PCR) of samples collected during the experimental and field sampling. The final elution of the DNA was carried out on the same volume of the starting aliquots (200 μl) to maintain compliance with the volumes and to permit a direct comparison of the methods. The concentration and purity of the extracted nucleic acids were quantified with a NanoDrop® spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan, Italy).

A screening PCR targeting the 16S ribosomial RNA gene was performed to verify DNA amplificability and to

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rule out possible PCR inhibitors in biological samples (Kitano *et al.* 2007).

Conventional and nested PCR

The protocol for the conventional PCR, targeting 583-bp fragments of the VP2-capsid-protein-encoding genes of FPLV and CPV, has been described by Buonavoglia *et al.* (2001). The nested PCR protocol was developed from the conventional PCR protocol and amplified a fragment of 211 bp (Marenzoni *et al.* 2018).

The extracted DNA (10 μl, corresponding to an equivalent volume of the sample) was tested in duplicate in the PCR assays (Microtech, Naples, Italy). The reaction mixture (25 μl) contained 10× buffer, 3 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} each deoxyribonucleotide triphosphate, 1 μmol l^{-1} each primer (Sigma-Genosys, Milan, Italy), 0·5 U of Taq DNA polymerase (Microtech, Italy) and DNA (as described above). The DNA (1 μl) from the first test was used for the nested step. The cycling conditions have been described by Marenzoni *et al.* (2018). In each set of reactions, a positive control (CPV Cornell strain or FPLV strain), a negative control (no-DNA sample) and a negative reaction-mix control were included.

Real-time PCR

Real-time PCR was used to quantify and characterize the virus. CPV or FPLV DNA was detected with real-time PCR using a conventional TaqMan probe (Decaro *et al.* 2005c), and the viruses were characterized using a panel of minor groove binder (MGB) probe assays able to identify CPV viral types 2, 2a, 2b and 2c (Decaro *et al.* 2005c, 2006a), and to discriminate between CPV vaccine strains (type 2 or 2b) and field strains (2a, 2b, 2c) (Decaro *et al.* 2006b, 2006c) and between CPV and FPLV (Decaro *et al.* 2008). Amplifications were performed using 10 μl of extracted DNA (corresponding to an equivalent volume of the sample), with IQ™ Supermix (Bio-Rad Laboratories Srl, Milan, Italy). Real-time PCR was performed with the iCycler iQ™ Real-Time Detection System (Bio-Rad Laboratories Srl), and the data were analysed with the iCycler iQ™ Real-Time Detection System software (ver. 3.0). Duplicates of CPV and FPLV standard dilutions and DNA templates were analysed simultaneously in the real-time PCR analysis.

Virus isolation

Six-well tissue culture plates were inoculated with 200 μl of the specific aliquots, and each well contained 2×10^5 A-72 cells for CPV-2 or 2×10^5 Crandell feline kidney (CrFK) cells for FPLV. The plates were incubated at 37°C for 5 days under a humidified 5% CO_2 atmosphere. The plates were then frozen and thawed three times, and the supernatant in each well was tested for CPV haemagglutination (HA) activity using 1% pig erythrocytes. The 50% end points were calculated with the Karber formula (Desario *et al.* 2005).

Rapid enzyme immunoassay

The aliquots dedicated for the rapid immunoassay were used on behalf of the faecal samples required by the kit (SNAP Canine Parvovirus Antigen Test, IDEXX Laboratories, Milan, Italy). Briefly, the swab from the kit was dipped into the aliquot, and then the manufacturer's instructions were followed. The extraction buffer and conjugate, available in the kit, were dispensed into the sample tube via swab supplied with the kit. The sample swab was then inserted into the tube containing the liquid and vortexed. The solution obtained was transferred into the test device with the swab pipette of the kit.

Before using the kit in this way, a proof of concept was performed to verify whether the rapid test worked on biological samples other than faeces, recommended by the manufacturer's instructions, and to demonstrate that sample homogenization and dilution did not compromise the reaction. Accordingly, 20 rectal swabs from dogs with clinical signs of parvovirus infection (vomiting, haemorrhagic diarrhoea and leukopaenia) and PCR positive for parvovirus, were also subjected to rapid test. The samples were retrospectively selected from those collected during routine diagnostic activity and characterized by a strong positive PCR result. The rectal swabs were obtained at the time of the clinical visit and placed in 600 μl of PBS. A 200 μl aliquot was used for the diagnostic PCR, and the remaining part was frozen at −20°C until analysis. This second aliquot (diluted rectal swab) was thawed for use with the rapid test at room temperature, as specified by the instructions for the kit. The swab in the kit was then inserted into the tube containing the liquid.

Statistical analysis

The level of agreement among conventional PCR, nested PCR, real-time PCR and the rapid enzyme immunoassay was evaluated with both Cohen's κ and the McNemar test. Values for Cohen's $\kappa < 0$ indicated no agreement; 0–0·20, slight agreement; 0·21–0·40, fair agreement; 0·41–0·60, moderate agreement; 0·61–0·80, substantial agreement and 0·81–1, almost perfect agreement (Landis and Koch, 1977). A $P < 0·05$ was considered statistically significant. The OpenEpi software was used for this analysis.

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Table 1 Results of the experimental contamination, obtained using different dilutions of parvovirus infected materials and sampling by cotton swab and sponge

Method of sampling	ID sample	Dilution of the material	Conventional PCR	Nested PCR	Real-time PCR (copies of viral DNA/10 μl)	Viral isolation	Rapid enzyme immunoassay
Sponge*	1S	Non-diluted homogenate	+	+	$1{\cdot}95 \times 10^{7}$	+	–
	2S	10^{-1}	+	+	$1{\cdot}70 \times 10^{6}$	±†	–
	3S	10^{-2}	+	+	$6{\cdot}20 \times 10^{5}$	–	–
	4S	10^{-3}	+	+	$7{\cdot}58 \times 10^{4}$	–	–
	5S	10^{-4}	+	+	$4{\cdot}82 \times 10^{3}$	–	–
	6S	10^{-5}	+	+	$8{\cdot}53 \times 10^{2}$	–	–
	7S	10^{-6}	±†	±†	$2{\cdot}53 \times 10^{1}$	–	–
	8S	10^{-7}	–	–	Negative	–	n.e.
	9S	10^{-8}	–	–	Negative	n.e.	n.e.
	10S	10^{-9}	–	–	Negative	n.e.	n.e.
	11S	10^{-10}	–	–	Negative	n.e.	n.e.
Cotton swab*	1T	Non-diluted homogenate	+	+	$1{\cdot}56 \times 10^{7}$	+	–
	2T	10^{-1}	+	+	$4{\cdot}04 \times 10^{6}$	+	–
	3T	10^{-2}	+	+	$9{\cdot}21 \times 10^{5}$	±†	–
	4T	10^{-3}	+	+	$9{\cdot}55 \times 10^{4}$	–	–
	5T	10^{-4}	+	+	$9{\cdot}70 \times 10^{3}$	–	–
	6T	10^{-5}	+	+	$1{\cdot}23 \times 10^{3}$	–	–
	7T	10^{-6}	±†	±†	$4{\cdot}40 \times 10^{1}$	–	–
	8T	10^{-7}	–	–	$1{\cdot}10 \times 10^{1}$	–	n.e.
	9T	10^{-8}	–	–	Negative	n.e.	n.e.
	10T	10^{-9}	–	–	Negative	n.e.	n.e.
	11T	10^{-10}	–	–	Negative	n.e.	n.e.

n.e., not executed.

*Final elution of samples collected with the sponge is 4 ml (obtained after soaking in 10 ml of phosphate-buffered-saline, PBS), while with cotton swab is 3 ml.

†The symbol ± means a weak signal for PCR test or the presence of a single or maximum two foci of cytopathic effect in the case of viral isolation.

Results

Experimental contamination of the environment

The results for experimental contamination are reported in Table 1. Conventional and nested PCR gave the same limit of detection (10^{-6}) with both swab and sponge sampling, despite different volumes of sample diluents used. Based on the additional serial twofold dilutions of the homogenates, from 10^{-6} to 10^{-7}, the cotton swab was fourfold more sensitive than the sponge using these tests, with no difference between conventional and nested PCR protocols.

Using both real-time PCR and virus isolation, virus was detected in the cotton swab at one higher dilution (10^{-7} for real-time PCR and 10^{-2} for virus isolation) than in the sponge sample (10^{-6} for real-time PCR and 10^{-1} for virus isolation), whereas the rapid test failed to detect virus in any sample tested.

Field samples

The results for the field samples are reported in Table 2. Overall, parvovirus DNA was detected in environmental samples when cases of parvovirus infection were present. Discordant results between nested PCR and real-time PCR were limited to some samples from site B (three negative on real-time PCR and positive on nested PCR and five positive on real-time PCR and negative on nested PCR). The five samples positive on real-time PCR and negative on nested PCR occurred when the concentrations of viral DNA were low (10^{1}–10^{2} viral copies/10 μl of sample). Unexpectedly, sites B9 (shoes of the owner worn before the outbreak) and B10 and B11 (environmental samples obtained almost 1 year after the clinical case at B_RS1) were positive for parvovirus on real-time PCR, whereas they were negative on nested PCR. In the case of the three samples negative on real-time PCR and

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Table 2 Results obtained from sampling in field conditions

ID site	ID sample	Description of the site of sampling	Application of cleaning (C) and disinfection (D) before sampling	Conventional PCR	Nested PCR	Real-time PCR (copies of viral DNA/10 μl)	Parvovirus characterization by real-time PCR*	Viral isolation	Rapid enzyme immunoassay
Public cattery (A)	A1	Walls of a cat carrier where an infected cat was placed in isolation and died	No	–	+	$8{\cdot}73 \times 10^4$	FPLV	–	n.e.
	A2	Sheets of paper used as flooring in cat carrier A1	No	+	+	$2{\cdot}93 \times 10^6$	FPLV	n.e.	–
	A3	Walls of the cages used for hospitalization of cats in the veterinary clinic	C	+	+	$4{\cdot}54 \times 10^4$	FPLV	–	–
	A4	Floors of the outdoor cattery	C, D	–	+	$3{\cdot}71 \times 10^1$	n.a.	–	–
Private kennel (B)	B1	Floor of the box of the puppy n. 1	C, D	–	+	$2{\cdot}01 \times 10^8$	CPV-2b	+	+
	B2	Floor of the box of dogs nos 2–3	C	–	+	$6{\cdot}94 \times 10^1$	n.a.	–	n.e.
	B3	Floor of the box of the dog n. 4	C	–	+	$1{\cdot}23 \times 10^3$	CPV-2b	–	–
	B4	Floor of the box of dogs nos 5 and 6	C	–	+	$4{\cdot}56 \times 10^4$	CPV-2b	–	–
	B5	Floor of the corridor connecting boxes	C	–	+	Negative	n.a.	–	–
	B6	Floor of the hut for dog feed (10 m away from the boxes)	C	–	+	Negative	n.a.	–	–
	B7	Floor of the home of the owner where the puppy n. 1 was hosted when ill	C, D	–	+	$2{\cdot}07 \times 10^3$	CPV-2b	–	–
	B8	Shoes of the owner that he wore when he went to the kennel	No	–	+	$1{\cdot}09 \times 10^2$	n.a.	–	–
	B9	Shoes of the owner that he had worn before the clinical case	C	-	–	$4{\cdot}46 \times 10^1$	n.a.	n.e.	n.e.
	B_RS1	Rectal swab of the puppy n. 1 with haemorrhagic diarrhoea	n.a.	+	+	$5{\cdot}62 \times 10^8$	CPV-2b	+	+
	B_RS2	Rectal swab of the asymptomatic adult dog n. 2	n.a.	–	+	Negative	n.a.	–	–
	B_RS3	Rectal swab of the asymptomatic adult dog n. 3	n.a.	–	–	Negative	n.a.	n.e.	–
	B_RS4	Rectal swab of the asymptomatic adult dog n. 4	n.a.	+	+	$5{\cdot}47 \times 10^3$	CPV-2b	–	–
	B_RS5	Rectal swab of the asymptomatic adult dog n. 5	n.a.	+	+	$2{\cdot}17 \times 10^2$	n.a.	–	–
	B_RS6	Rectal swab of the asymptomatic adult dog n. 6	n.a.	+	+	$5{\cdot}50 \times 10^2$	n.a.	–	–
	B_RS7	Rectal swab of the puppy n. 1 in the acute phase (after 4 days from B1)	n.a.	+	+	$8{\cdot}55 \times 10^5$	CPV-2b	–	–
	B10	Floor of the four boxes (after 10 months from the clinical case)	C	–	–	$6{\cdot}19 \times 10^1$	n.a.	n.e.	n.e.
	B11	Shoes of the owner (after 10 months from the clinical case)	C	–	–	$2{\cdot}13 \times 10^2$	n.a.	n.e.	n.e.

(Continued)

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Table 2 (Continued)

ID site	ID sample	Description of the site of sampling	Application of cleaning (C) and disinfection (D) before sampling	Conventional PCR	Nested PCR	Real-time PCR (copies of viral DNA/10 μl)	Parvovirus characterization by real-time PCR*	Viral isolation	Rapid enzyme immunoassay
	B_RS8	Rectal swab of the puppy n. 1 in the convalescent phase (after 1, 5 months)	n.a.	–	+	$1{\cdot}08 \times 10^2$	n.a.	–	n.e.
	B_RS9	Rectal swab of the puppy n. 1 in the convalescent phase (after 3 months)	n.a.	–	–	$1{\cdot}14 \times 10^1$	n.a.	n.e.	n.e.
	B_RS10	Rectal swab of the puppy n. 1 in the convalescent phase (after 10 months)	n.a.	–	–	$6{\cdot}80 \times 10^1$	n.a.	n.e.	n.e.
Laboratory (C)	C1	Counter of the laboratory of veterinary infectious diseases	C, D	–	–	Negative	n.e.	n.e.	n.e.
Veterinary teaching hospital (D)	D1	Swab on the walls of the room reserved for isolation of infectious dogs	C, D	–	+	$4{\cdot}45 \times 10^3$	CPV-2C	–	–
	D2	Swab on the floor of the anteroom adjacent to D1	C, D	+	+	$1{\cdot}69 \times 10^3$	CPV-2C	–	–
	D3	Swab on the floor of the room reserved for isolation of infectious cats (hosting three cats suspected of parvovirus infection)	No	+	+	$5{\cdot}08 \times 10^5$	FPLV	–	–
	D4	Swab on the floor of the anteroom adjacent to D3	No	–	+	$4{\cdot}38 \times 10^3$	FPLV	–	n.e.
	D5	Swab on the floor of the acceptance and entrance area of the teaching hospital	C	–	+	$1{\cdot}43 \times 10^3$	FPLV	–	–
	D6	Swab on the floor of the room of standard hospitalization of dogs	C	–	+	$1{\cdot}17 \times 10^4$	CPV-2b	–	n.e.
	D7	Swab on the floor of the room of standard hospitalization of cats	C	–	+	$1{\cdot}23 \times 10^4$	FPLV	–	n.e.
	D8	Swab on the floor of the room reserved for isolation of infectious dogs	No	+	+	$1{\cdot}03 \times 10^4$	CPV-2c	±	–
	D9	Swab on the floor of the anteroom adjacent to D8	C	–	+	$1{\cdot}06 \times 10^5$	CPV-2c	–	–
Non-clinic area of the Veterinary Department (E)	E1	Swab on the floor of the Anatomy section	C	–	–	Negative	n.e.	n.e.	n.e.

n.e., not executed; n.a., not applicable.

*The characterization by real-time PCR was able to differentiate vaccines, field strains of canine parvovirus (CPV 2a, 2b and 2c) and feline panleukopaenia (FPLV).

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positive on nested PCR, viral load was not appreciable.

Virus was only isolated in three cases (B_RS1, the rectal swab of a symptomatic puppy; B1, the floor where this puppy lived; D8, the floor of the room reserved for the isolation of infectious dogs), whereas the rapid test detected it in only two cases (B_RS1, the same rectal swab of the symptomatic puppy; and B1, the floor where it lived), that were the same cases in which the virus was isolated. The doubt about the inefficiency of the rapid test, using samples other than faecal ones, was resolved by the observation that 8 of the 20 diluted PCR-positive rectal swabs were positive for parvovirus antigen according to the rapid test, achieving a sensitivity of 40%, consistent with that obtained in studies comparing rapid antigen tests with PCR performed with faecal samples (Desario *et al.* 2005; Proksch *et al.* 2015; Faz *et al.* 2017), and confirming the possibility of further use of the test with other types of samples than suggested by the manufacturer's instructions.

The agreement between the nested and real-time PCR results for the environmental samples was fair (0·29), and the result of the McNemar test was not significant, indicating general agreement. The results were discordant for eight samples, which all came from kennel B, showing a low level of positivity on real-time PCR in five cases with negative nested PCR and three cases of negativity on real-time with positive nested PCR. The agreement was slight between conventional and nested PCR (0·08) and between conventional and real-time PCR (0·14). The agreement between real-time PCR and virus isolation was also slight (0·028), as it was between real-time PCR and the rapid test (0·03). The agreement between virus isolation and the rapid test was substantial (0·77), and the result of the McNemar test was also not significant in this case, indicating general agreement.

Discussion

The sampling protocol developed in this study using either cotton swabs or sponges detected parvovirus in different environments (cages, floors of different types of structures, shoes), especially when molecular methods were used. On the contrary, the virus isolation technique detected the virus in only limited cases, generally only at the highest concentrations in the experimental setting and in a very few samples under field conditions. The rapid enzyme immunoassay also identified the virus in only a limited number of environmental samples, and usually in those with the highest levels of virus.

As observed in previous studies, the choice of sampling method is important in detecting viral environmental contamination because it influences the number of viral particles recovered (Spiri *et al.* 2019). The cotton swab method was four orders of magnitude more sensitive than the sponge method, probably because different quantities of diluent were used for the final elution (3 ml of PBS for cotton swabs versus 4 ml for sponges, which resulted from the previous soaking in 10 ml of PBS). However, when environmental samples from the field were analysed, no difference was observed, probably because this difference of four dilutions was too low to be important under field conditions (data not shown). The cotton swab is a device that is usually available in veterinary clinics and is easy to use, but it is suitable for sampling small surfaces and is not convenient for sampling rough surfaces. The sponge can be used to sample an area of up to 300 cm^2 and rough surfaces. Therefore, the cotton swab could be recommended for occasional or unscheduled sampling, whereas the sponge is appropriate for extensive and systematic sampling.

In this study, molecular tools were the most reliable way to detect the presence of parvovirus. They detected very limited quantities of virus (as few as 10 viral copies/10 μl of sample). The different PCR protocols, especially nested (qualitative, but cheaper) and real-time PCR (quantitative but also qualitative for parvovirus characterization, and more expensive), gave similar results in almost all cases, considering the specific laboratory setting used in this study. However, cost containment for real-time PCR could be obtained modifying some aspects (use of dye-based method, increase in the number of samples to be processed). Few cases of discordant results were observed in the field samples, all from kennel B; a part of them were the samples with the lowest viral loads (10^1–10^2 viral DNA copies/10 μl of sample), at which the virus is probably not infectious. In two of these cases, real-time PCR detected the virus in environmental samples (B10 and B11) collected 1 year after the last outbreak in kennel B, with low viral loads (10^1–10^2 viral DNA copies/10 μl of sample), whereas nested PCR was negative for these samples. Another part of the discordant results could be explained by a possible different analytical sensitivity of the tests, with the presence of the viral DNA at threshold values of positivity. Unfortunately, virus isolation was negative for all the samples collected from the kennel, so it was impossible to assess the infectivity of the virus. Moreover, no susceptible dogs were present in the kennel after the initial case of the infected puppy (dog n. 1) because all the adult dogs displayed high CPV antibody titres (ranging from 1 : 160 to 1 : 1280, data reported in the Table S1) during the occurrence of the clinical case. In the samples from kennel B that unexpectedly tested positive, it is possible that the traces of CPV-2 DNA detected on shoes not used during the ongoing clinical case (B9, B11) or in the environment (B10) were

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transported from outside the kennel or, alternatively for B10 and B11, that the virus persisted in the contaminated environment for 1 year after the outbreak.

A limitation of molecular methods is that they cannot evaluate the infectivity of the virus. Virus isolation was used for this purpose in this study, but it was probably impaired by other factors, maybe the presence of antibodies in the samples analysed or a decrease in the viral amount due to the evolution of the infection. In the same way, in a recent study of Feline calicivirus, no virus was isolated from the contaminated environment. A possible explanation proposed in that case was that only a small part of the environment was sampled, rather than the overall surface, and the environmental contaminant could have been distributed irregularly, even when the sampled area was considered at high risk of contamination (Spiri *et al.* 2019).

A substantial agreement was between the results for virus isolation and those for the rapid test (Desario *et al.* 2005; Decaro *et al.* 2005a). The rapid in-clinic enzyme immunoassay was tested because it is inexpensive, rapid and easy to use. However, it recognizes a limit in sensitivity (Desario *et al.* 2005; Decaro *et al.* 2010, 2013). The sensitivity of these in-clinic assays is also affected by the viral titre, which must be greater than 10^5 DNA copies per mg of faeces (Decaro *et al.* 2010). The presence of gut antibodies or a progressive decrease in viral amount, following the evolution of the infection, could have reduced the rate of virus detection. In two cases, a difference of a few days or even 24 h in the time of sampling completely changed the results of the rapid test from positive to negative (samples B_RS1 and B_RS7, rectal swabs of the puppy in kennel D collected after 4 days; rectal swabs of a puppy hospitalized in D1, the day before the environmental sampling and the rectal swab of the same dog on the following day after, data not shown). Unfortunately, but as expected, the rapid test was insufficiently sensitive to detect environmental parvovirus contamination. Therefore, despite its low cost, rapidity and ease of use, these tests are not currently suitable for the detection of environmental contamination with parvovirus. A marked improvement in the analytical sensitivity of this test is required before it is ready for such use.

A pure viral isolate could have been used to test experimental contamination to better compare the four tests used in this study. This would have prevented at least a part of the problems caused by interfering factors in the samples (i.e. sequestration of the viral particles by antibodies), that may have caused the negative results observed when virus isolation or the rapid test was used. However, the aim of this study was to develop a protocol for detecting parvoviruses under field conditions, and the presence of these factors always can limit the use of these methods in this context.

For the same reason, the environmental samples were analysed with the rapid test even in the presence of animals that were negative on this assay, to verify whether results agree. However, only a single environmental sample (B1) was positive on the rapid test, and this was the site where the puppy (dog n. 1 with B_RS1) tested positive with this assay was living.

Since only viral isolation can be used to assess the infectivity of a virus present in the environment, but failed in field conditions, quantitative real-time PCR is the best surrogate to test for environmental contamination with parvoviruses, based on the assumption that when a high load of virus is present, it is probably infectious.

In this study, parvovirus DNA was detected in each situation when a clinical case of parvovirus infection was present. Testing field conditions is very useful because the presence of dust, litter and food can influence the capacity to detect the virus, for example, by modifying the efficiency of viral nucleic acid extraction (Spiri *et al.* 2019). Therefore, in the present study, we tested different field conditions in which different groups of animals with natural infections were represented. The protocol used in the present study identified parvovirus-contaminated environments on tables, cages, pet carriers, visitor rooms, clinics, kennels, floors and shoes, and was therefore able to identify the possible sources of infection. At sites A and D, other clinical cases of parvovirus infection were also present after sampling. The origins of infections were not generally investigated to distinguish whether they arise outside a site or have a nosocomial origin.

However, parvovirus characterization with real-time PCR when parvovirus DNA is present at a sufficient titre (usually > 10^2 viral DNA copies/10 μl) contributed epidemiological information about these cases. FPLV was present in the cats and CPV in the dogs. In hospital D, both CPV-2b and CPV-2c were detected simultaneously in different areas (CPV-2b contamination at D6, the room for the standard hospitalization of dogs; CPV-2c at D8, an anteroom reserved for the isolation of infectious dogs, and at D9, a room reserved for the isolation of infectious dogs). Parvovirus characterization also suggested the wide circulation of parvovirus in D: FPLV was detected in the areas reserved for cats but also at the entrance (D5). This approach could be important in environments with constant or extensive movements of dogs and cats, such as veterinary hospitals, clinics and shelters, to better define the epidemiological links underlying parvovirus infections or outbreaks.

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When the environmental viral loads were examined, those detected in the boxes of the adult dogs at kennel B (especially B3 with $1{\cdot}23 \times 10^3$ viral DNA copies/10 μl and B4 with $4{\cdot}46 \times 10^4$ viral DNA copies/10 μl) were higher than those detected with rectal swabs of the other single dogs living in the same boxes (B_RS4, B_RS5, and B_RS6, with 10^2–10^3 viral DNA copies/10 μl), despite daily cleaning and disinfection by the owner. In this situation, it is possible that the virus had accumulated during the massive viral shedding, perhaps also in the preceding days so that the environment posed an even higher risk for infection than a single dog. Parvovirus DNA was present in their rectal swabs of the adult dogs at site B and they had high antibody titres (up to 1 : 1280 in three of the five dogs). Viral shedding in animals with antibodies has already been reported (Decaro *et al.* 2005a; Decaro and Buonavoglia 2017; Freisl *et al.* 2017; Cavalli *et al.* 2020).

However, it is not known whether these viral loads, determined with real-time PCR, were infectious because the virus isolation results were negative in this study, even when the viral loads were high. To the authors' knowledge, no information on this topic is available in the literature. It is not easy to define the relationship between infectious dose of parvovirus with real-time PCR because no specific studies have been performed and many variables must be considered (e.g. the presence of antibodies, infectious versus non-infectious viral particles, the units of measure used). Based on previous studies and on the assumption that dogs are infectious for up to 18–25 days (Decaro *et al.* 2005a, 2005b; Greene and Decaro 2012), it was reasonably deduced that the titres of the infectious virus exceeded 10^3–10^4 copies per mg of faeces. Even if the virus was excreted for up to 52 days, the titre did not drop below 10^2 copies per mg of faeces. The DNA detected with real-time PCR is not necessarily considered completely infectious, and the same can be assumed for environmental samples. Therefore, viral loads $<10^3$–10^4 copies per mg of faeces are not likely to be infectious and viral loads $<10^2$ may indicate only traces of parvovirus DNA. This inference is partly supported by sample D8, the one of the three samples positive on virus isolation, with the lowest viral load determined with real-time PCR ($1{\cdot}03 \times 10^4$ viral DNA copies/10 μl) in this study. Further researches are required to clarify this relationship, but if this viral load is potentially infectious, the environmental viral loads determined at some sites could indicate a risk of transmission of infection.

From another perspective, the environmental sampling protocol can be used to assess the efficacy of disinfection in an area or surface. In fact, the simple detection of parvoviral DNA, even if not infectious, can be a useful indicator of contamination because it identifies where more attention must be given to cleaning and disinfection. In the present study, environmental contamination with parvovirus was even detected in areas where the virus was not expected (A3, A4, B9, D1 and D2), and in some cases with discrete DNA viral loads, such as at A3 ($4{\cdot}54 \times 10^4$ viral DNA copies/10 μl), D1 ($4{\cdot}45 \times 10^3$ viral DNA copies/10 μl) and D2 ($1{\cdot}69 \times 10^3$ viral DNA copies/10 μl). In these cases, even if the viability and infectivity of the detected virus were unknown, and using the quantitative results of real-time PCR as a proxy for infectivity, it is possible that the protocol used for disinfection was not efficacious and that, at best, traces of non-infectious DNA were present. Looking for the virus in an environment is, in all cases, an opportunity to evaluate the effectiveness of the biosafety and disinfection protocols used, which are usually taken for granted. For example, the cages of the public cattery were considered clean, but they were positive for parvovirus on real-time PCR, with $4{\cdot}54 \times 10^4$ viral DNA copies/10 μl of sample, and this finding prompted more attention to cleaning and disinfection by the practitioner when informed. However, in that cattery, no further cases of parvovirus infection occurred in subsequent days, either because the virus was not infectious, the animals were vaccinated or convalescent, or no other susceptible animals were present.

Based on these results, the current biosafety measures applied at premises A, B and D should be carefully evaluated to achieve the most effective environmental disinfection possible. Spiri *et al.* (2019) detected no Feline calicivirus when optimal hygiene measures were applied, demonstrating the importance and efficacy of these measures. This finding was confirmed with molecular methods when no caliciviral RNA was detected (Spiri *et al.* 2019). A recent study of residual contamination on endoscopes and twitches showed that only disinfection with sodium hypochlorite completely eliminated traces of *Streptococcus equi* DNA (Svonni *et al.* 2020). Sodium hypochlorite was also shown to be one of the most effective disinfectants against CPV (Cavalli *et al.* 2018). Nosocomial parvovirus infections are often suspected by veterinarians at their facilities (Marenzoni *et al.* 2019, in press), and the accurate evaluation of the effectiveness of disinfection protocols against these viruses is important in preventing nosocomial transmission. The adoption of effective disinfection protocols that eliminate parvovirus DNA, coupled to a detector system for parvovirus DNA, offers an opportunity to better control infection and to assure clients that specific measures are applied to control infection.

The protocol described in this study detected environmental contamination with parvoviruses, especially in

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areas where the virus was not expected. Unfortunately, virus isolation (used to assess virus infectivity) and the rapid test (used to have a rapid result) failed to identify the virus in the environment. When real-time PCR was used as a surrogate for parvovirus infectivity, the detection of high viral loads approximately could predict the infectious capacity of the virus. However, the relationship between the infectious doses of the virus in dogs and cats and viral DNA load determined with real-time PCR should be studied, considering the difference for which the infectious dose decreases over time while the viral DNA load could remain essentially the same. This protocol may also be useful for assessing the correct application of biosafety measures and disinfection protocols when no parvoviral DNA is detected. The development of rapid and easy-to-use in-clinic tests remains an objective because it may facilitate the in-clinic execution of these tests.

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Ethical statement

This study was conducted using data obtained for clinical and preventive purposes and samples were collected during routine diagnostic and preventive procedures; consequently, no ethical permission was required.

Author contributions

All the authors of this study: (i) made substantial contributions to the conception and design of the work (MLM, MBC, RF, ET and ND); the acquisition of data (MLM, MBC, ER, RR, FP, SM, BF, MG, PB, OR and CD), analysis of data (MLM, MBC, CD and ND) and interpretation of data (MLM, MBC, SM, BF, CD, MPF, CB and ND); (ii) drafted the work (all the co-authors), and revised it critically for important intellectual content (MPF, CB and ND); (iii) approved the version to be published (all the co-authors) and (iv) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (all the co-authors).

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Samples, comments and results obtained from sampling in field conditions.

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