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Short communication

An updated TaqMan real-time PCR for canine and feline parvoviruses

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A B S T R A C T

Canine parvovirus type 2 (CPV-2) emerged in late 1970s from the feline panleukopenia virus (FPLV) and developed, since then, into novel genetic and antigenic variants (CPV-2a, -2b and -2c). Canine and feline parvoviruses cause an acute enteric disease in their hosts, with high level of viral shedding. In this study, a quantitative TaqMan PCR for detection and quantitation of canine and feline parvoviruses in serum and fecal samples was developed. The primers were designed based upon the entire GenBank content for CPV and FPLV. A standard curve was generated, and validation tests were performed using 10-fold serial dilutions of CPV-2 virus in CPV/FPLV-negative feces and CPV/FPLV-negative serum samples. As a result, the 100% detection limit of the PCR was 18 copies of the viral genome per μl of serum and fecal sample. All canine parvovirus types as well as FPLV were detected. In conclusion, the real-time PCR represents an upgraded and useful tool to identify and quantify canine and feline parvoviruses in different sample matrices.

Canine parvovirus type 2 (CPV-2) and feline panleukopenia virus (FPLV) are members of the autonomous replicating viruses of the Parvoviridae family. They have a single-stranded DNA genome which encodes two capsid proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2). The CPV-2 emerged from a FPLV or a closely related virus as a novel pathogen in the late 1970s (Shackelton et al., 2005) and rapidly spread worldwide. Within a few years, the virus underwent a rapid evolution and, new antigenic types, termed CPV-2a, CPV-2b and CPV-2c (Buonavoglia et al., 2001; Parrish et al., 1985, 1988), replaced the original type CPV-2 (Truyen, 2006).

Clinical diagnosis of CPV-2 infection can be inconclusive, since several other pathogens may cause diarrhea in dogs. For this reason, a clinical diagnosis should always be confirmed by laboratory tests. For FPLV, which can cause anorexia, weight loss, vomiting and diarrhea, even the finding of severe leucopenia with compatible clinical signs is presumptive without laboratorial confirmation tests. Several methods have been developed for the laboratory diagnosis of CPV-2 or FPLV infection. Usually, feces from diarrheic cats and dogs are screened using ELISA or hemagglutination (HA) tests, but these techniques are affected by relatively low sensitivity (Mochizuki et al., 1993; Uwatoko et al., 1995). Additionally, the HA method depends upon the antigenic structures in the virus capsid. Virus mutants with amino acid changes in regions determining the antigenicity may exhibit altered hemagglutination capacity (Strassheim et al., 1993). Virus isolation in cell lines can also be used; however the technique requires a time period of at least 3–4 days, and, some strains do not produce clear cytopathic effects in the cell culture (Decaro et al., 2005). Molecular methods to detect CPV-2 and FPLV have been described, mostly based upon the polymerase chain reaction for the detection of the parvovirus in feces of dogs and cats (Buonavoglia et al., 2001; Hirasawa et al., 1996; Schunck et al., 1995). In the last ten years, several methods to detect CPV-2 were reported applying the real-time PCR technology. The comparison of the conventional PCR with the real-time PCR demonstrated a greater sensitivity for the latter one (Decaro et al., 2005; Kumar and Nandi, 2010), progressively replacing the virus isolation and HA as the standard procedure for virus surveillance and virus diagnosis. However, a technique able to detect the virus in feces of clinical compromised animals and to quantify the virus in body fluids from sick and healthy animals was not described. Additionally, with the increased number of novel strains (containing new nucleotide substitution), an upgrade in the used primers becomes important. Based upon this, the aim of the present study was to develop a qPCR method applying the TaqMan technology for the detection and quantitation of CPV-2 and FPLV in serum and fecal samples.

To design the primers, all VP gene sequences deposited in DNA databases (up to November 2011) were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) using “canine parvovirus” and “feline panleukopenia virus” as keywords. In total 503 sequences of the VP2 gene were obtained for both viruses. The dataset was aligned by applying the ClustalW method in the MEGA 4 software (Tamura et al., 2007). Several primers and probes were designed by using the software Primer 3 v. 0.4.0 (http://primer3.sourceforge.net/). The chosen

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primers and probes were selected by matching them with the alignment. The nucleotide sequences were: Primer Forward 5′-TGGAACTAGTGGCACACCAA (nucleotide position number 3456–3473, according the reference canine parvovirus strains CPV-N, GenBank accession number: M19296), Primer Reverse 5′-AAATGGTGGTAAGCCCAATG (3636–3655) and the Probe 5′-CAGGTGATGAATTTGCTACAGG (3555–3576). Potential hairpins and self dimerization were calculated using OligoCalc software (Kibbe, 2007). Primers and probe were obtained from TIB MOLBIOL Syntheselabor (Berlin, Germany). The TaqMan probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and with the BlackBerry Quencher (BBQ) at the 3′ end.

In order to create a standard curve for viral quantitation, a plasmid harboring the target sequence of the real-time PCR was generated. Briefly, DNA of the CPV-2 strain vBI 265 was amplified using the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) together with the forward and reverse primers. The amplicon was purified with the NucleoSpin purification kit (Macherey-Nagel, Düren, Germany) and cloned into the pDRIVE vector (Qiagen, Hilden, Germany) using One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen, Darmstadt, Germany). Plasmid was extracted using the PeqGOLD Plasmid Miniprep Kit I (Peqlab, Erlangen, Germany) and quantified by UV spectroscopy. The number of copies of the plasmid was calculated using the equation described by Whelan et al. (2003).

The standard curve was generated by applying successive 10-fold dilutions of the plasmid. The real-time PCR reaction was set up in a total volume of 25 μl containing 12.5 μl of HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany), 10 pmol from the forward and reverse primers, 5 pmol of the Probe, 5 pmol of Rox reference dye and 3 μl of the target DNA (Plasmid DNA). The final volume was adjusted with deionized water. The cycling condition consisted of the initial denaturation at 95 °C for 15 min to allow the activation of the polymerase, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The PCR was performed on the Mx3000P platform (Stratagene, La Jolla, USA). Amplification efficiency (E) was determined using the relative standard curve. The threshold cycle (Ct) value was determined by the real-time PCR Mx3000P settings (method as follows).

$$Ct = -\frac{1}{\log(E+1)} \times \frac{\log Ro + \log R}{\log(E+1)}$$

$$E = 10^{-1/\text{Slope}} - 1; \quad \text{slope} = -\frac{1}{\log[E+1]}$$

Based upon the real-time PCR standard curve, the correlation coefficient (0.998) and PCR efficiency (105.2%) were obtained. The specificity of the real-time PCR assay was assessed by testing several enteric pathogens, including viruses [coronavirus (identification: KS-20), canine distemper virus (111207) and canine herpesvirus (568/11)], and bacteria [*Salmonella* Typhimurium (DT104), *E. coli* (DSM682) and *Campylobacter jejuni* (02/07)]. No specific amplification was detected for any of these samples while the feline panleukopenia virus (292), canine parvovirus 2 (vBI 265), 2a (140211), 2b (825/98) and 2c (7/97) were detected. Since parvoviruses are DNA viruses with a capsid that provides high stability, added to the fact that our extraction method provides high efficiency (260/260 ratio >1.7 and 260/280 ratio >1.8), an internal control was not performed. However, this may be indicated if DNA extraction methods which are not as efficient in removing amplification inhibitors.

To evaluate the sensitivity of the real-time PCR we artificially contaminated and tested two commonly used matrices (serum und feces). Briefly, fecal samples were taken from a clinically healthy dog. The total DNA of the feces was extracted with the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) and tested negative against CPV-2 with a nested PCR (Schunck et al., 1995). Negative serum was obtained from a SPF dog. A hemagglutination inhibition (HI) test (Carmichael et al., 1980) was performed with the serum to verify the absence of CPV-2 antibodies. The CPV-2 strain vBI 265 was initially quantified, serially diluted (10-fold) in sterile phosphate-buffered saline solution (PBS, pH 7.2) and mixed with the feces or the serum (the final solutions had a predetermined range of 1.8 copies/μl to 1.8×10^6 copies/μl). DNA from the resulting mixture of serum was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). For the feces, DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). The extracted DNA of each matrix was tested with the real-time PCR in five times on distinct days.

Additionally, feces and serum samples from dogs and cats submitted to our laboratory for diagnostic purposes were evaluated and compared with other tests (Table 1). Initially, the feces samples

Table 1
IF, conventional PCR and real-time PCR results for the canine and feline diagnostic samples from feces and serum.

Sample Id.	Animal	Description	IF	PCR	Real-time PCR[a]
9P	Feline	Serum	–	–	–
67P	Feline	Serum	–	–	–
90P	Feline	Serum	–	–	–
140P	Feline	Serum	–	–	–
187P	Feline	Serum	–	–	4.99E+00
225P	Feline	Serum	–	–	–
233P	Feline	Serum	–	–	–
242P	Feline	Serum	–	–	–
245P	Feline	Serum	–	–	–
262P	Feline	Serum	–	–	–
86Pc	Canine	Serum	–	–	–
87Pc	Canine	Serum	–	–	–
88Pc	Canine	Serum	–	–	–
89Pc	Canine	Serum	–	–	–
90Pc	Canine	Serum	–	–	–
91Pc	Canine	Serum	–	–	–
92Pc	Canine	Serum	–	–	2.43E+00
93Pc	Canine	Serum	–	–	–
94Pc	Canine	Serum	–	+	5.17E+05
95Pc	Canine	Serum	–	–	–
96Pc	Canine	Serum	–	–	–
97Pc	Canine	Serum	–	–	–
98Pc	Canine	Serum	–	–	–
99Pc	Canine	Serum	–	–	5.52E+00
100Pc	Canine	Serum	–	+	7.25E+04
120/12	Canine	Feces	–	+	2.03E+03
121/12	Canine	Feces	–	–	9.17E+00
122/12	Canine	Feces	–	–	–
123/12	Canine	Feces		–	1.30E+02
124/12	Canine	Feces	–	+	6.44E+06
125/12	Canine	Feces	–	–	7.26E+00
126/12	Canine	Feces	–	+	4.57E+06
127/12	Canine	Feces	–	–	4.80E+01
128/12	Canine	Feces	–	+	1.66E+02
129/12	Canine	Feces	–	–	2.11E+01
130/12	Canine	Feces	–	+	1.98E+01
131/12	Canine	Feces	+	+	1.24E+09
132/12	Canine	Feces	–	+	4.79E+04
133/12	Canine	Feces	+	+	1.28E+09
134/12	Canine	Feces	+	+	1.38E+04
135/12	Canine	Feces	–	+	8.00E+03
136/12	Canine	Feces	–	+	1.37E+02
137/12	Canine	Feces	–	+	3.43E+02
138/12	Canine	Feces	+	+	5.02E+07
139/12	Canine	Feces	–	+	4.88E+02
140/12	Canine	Feces	+	+	2.64E+08
124/11	Feline	Feces	–	+	3.26E+02
125/11	Feline	Feces	–	+	5.11E+04
126/12b	Feline	Feces		+	3.36E+04
127/12b	Feline	Feces	–	+	5.10E+01
Total			6	16	29

[a] Number of DNA copies.

were homogenized with PBS, pH 7.2 (1 g:1 mL). Thereafter, the feces and serum samples were filtered and added in Crandell Rees feline kidney (CRFK) cells maintained in Dulbecco's Modified Eagle's Medium with 5% Fetal Calf Serum (FCS) at 37 °C. After 72 h, the cells were fixed with acetone/methanol (1:1, v/v), adsorbed with FCS 3% and incubated with monoclonal antibodies against CPV-2 and FPLV. Positive cells were visualized after being incubated with anti-mouse antibodies conjugated with fluorescein. For the PCR reactions, DNA from the feces and serum were extracted (as described above). Conventional PCR was performed according to Schunck et al. (1995) and real-time PCR was performed as previously described.

As a result for the sensitivity using distinct matrices, we observed that the assay had an 80% limit of detection (four from five repetitions) of 1.8 copies/μl and a 100% limit of detection of 1.8×10^1 copies/μl for the serum matrix. In the feces, the limit of detection was 20% detecting 1.8 copies/μl and 100% detecting 1.8×10^1 copies/μl. Comparing the methods usually applied for diagnostic purpose with the real-time PCR, we found that the IF technique could detect only 12% of the samples (submitted for diagnostic purpose) as positive. In contrast to that, 32% of the samples were tested positive using the conventional PCR, whereas the real-time could detect 58% of the samples as positive (Table 1).

Real-time PCR is an attractive alternative to conventional PCR for the study of the virus load with low inter-assay and intra-assay variability (Mackay et al., 2002). The analytical sensitivity of the real-time PCR is equivalent or greater compared with traditional virus culture, immunofluorescence, conventional single-round PCR and nested-PCR (Mackay et al., 2002). Such methods may demand tissue culture to isolate the virus or serological methods to determine the identity of the isolate, are known to be highly time and labor consumptive. In contrast, the real-time PCR method facilitates a large increase in the throughput, enables simultaneous processing of several samples and provides the possibility of computer based data analysis of the results.

The real-time PCR approach described above has high sensitivity in both matrices tested (feces and sera) and was able to detect as few as 18 copies of DNA per microliter in every test round. This is in accordance with other quantitative methods for the detection of CPV-2 based upon the real-time PCR (Decaro et al., 2005; Kumar and Nandi, 2010), highlighting the excellent suitability of the real-time PCR method for canine and feline parvovirosis diagnosis. When the method was used for samples collected for clinical purposes, a higher sensitivity compared to the IF technique and conventional PCR could clearly be observed. This sensitivity may permit insights in the time course of viral loads in serum samples, even in the complete absence of clinical symptoms. This can represent an advantage for vaccine development tests or vaccine efficacy tests, when even low amounts of DNA are important to be measured. As additional advantage, this method was designed to detect both canine and feline parvoviruses. Therefore in cats, that may be infected by both viruses, only one diagnostic test is needed to confirm the diagnosis. Notably, it has been observed that canine and feline parvoviruses displays high evolutionary rates (Shackelton et al., 2005), and for this reason, nucleotide substitutions are a frequent finding. In the present study, the high number of sequences used to select the primers provides an upgraded method for the detection of those viruses and reduces the probability of false-negative detection due to new viral mutations.

In conclusion, the real-time PCR developed in this study proved to be a useful tool in the detection of both, canine and feline parvovirus infection, as demonstrated through the sensitivity test in feces and serum the compared tests (IF and conventional PCR). This indicates that the method can be widely used for the clinical diagnostic. In addition, the high sensitivity ensures the detection at an initial stage in the infection, as well as the identification of carrier animals.

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