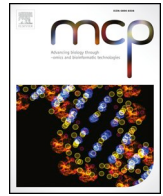




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One-step multiplex *TaqMan* probe-based method for real-time PCR detection of four canine diarrhea viruses

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ARTICLE INFO

Keywords:

Canine diarrhea
Multiplex real-time PCR
TaqMan probe

ABSTRACT

Viral canine diarrhea has high morbidity and mortality and is prevalent worldwide, resulting in severe economic and spiritual losses to pet owners. However, diarrhea pathogens have similar clinical symptoms and are difficult to diagnose clinically. Thus, fast and accurate diagnostic methods are of great significance for prevention and accurate treatment. In this study, we developed a one-step multiplex *TaqMan* probe-based real-time PCR for the differential diagnosis of four viruses causing canine diarrhea including, CPV (Canine Parvovirus), CCoV (Canine Coronavirus), CAstV (Canine Astrovirus), and CaKoV (Canine Kobuviruses). The limit of detection was up to 10^2 copies/ μ L and performed well with high sensitivity and specificity. This assay was optimized and used to identify possible antagonistic relationships between viruses. From this, artificial pre-experiments were performed for mixed infections, and a total of 82 canine diarrhea field samples were collected from different animal hospitals in Zhejiang, China to assess the method. The virus prevalence was significantly higher than what previously reported based on RT-PCR (Reverse Transcription-Polymerase Chain Reaction). Taken together, these results suggest that the method can be used as a preferred tool for monitoring laboratory epidemics, timely prevention, and effective monitoring of disease progression.

1. Introduction

Viral canine diarrhea is usually highly contagious, and even more likely to cause mixed infections, leading to fatal diarrhea [39]. The long treatment cycle and slow healing of puppies can cause mental injury to pet owners. Canine Parvovirus (CPV), Canine Coronavirus (CCoV), Canine Astrovirus (CAstV), Canine Kobuviruses (CaKoV), Rotavirus and Canine Adenovirus (CAV) are causative agents of diarrhea. With the exception of CAstV and CaKoV, the research of detection method for other diarrheal viruses are common [7,12]. CPV, one of the most serious causes of diarrhea, can have a mortality rate of 70% in puppies and lead to hemorrhagic enteritis [1,2] of rapid transmission [4]. CCoV mainly causes gastroenteritis and clinical signs include vomit and mild to severe diarrhea [3], with the high positivity rate next to CPV [23,30]. CAstV was first discovered in 1980 [39] and a series of documents successively confirmed the existence of this pathogen in diarrheal feces by molecular biology, consist of electron microscopy, RT-PCR and then sequencing identification [36]. CaKoV was first detected in canines with acute gastroenteritis in the United States [14,18]. In China, there are only two recent reports on CaKoV and a handful of reports have discussed the prevalence of CAstV [16,17]. Thus, CAstV and CaKoV are

controversial in terms of prevalence and clinical symptoms, and supported by few relevant studies which was especially needed to monitor virus in real time. Differential diagnosis must combine specific antigen detection assays. However, there is no such method for the simultaneous and rapid identification of CPV, CCoV, CaKoV, and CAstV.

Until now, it is common laboratory practice the use of conventional polymerase chain reaction (PCR) methods for single or multiplex detection. RT-PCR is a powerful genetic analysis method. However, its limitations are low efficiency and the requirement of sufficient concentration of virus [11,32]. In addition, it is time-consuming, laborious, and not suitable for high throughput. Since 1993, Roche and scientist Higuchi et al. first revealed real time-PCR [13,15]. Real-time PCR is a quantitative and more sensitive PCR [24,38] that uses target-specific dual fluorescently labeled DNA oligonucleotides, referred to as *TaqMan* probes [24,25] which are labeled with a quencher and a reporter. During amplification, the quencher is separated from the reporter gene releasing fluorescence signal [35]. MGB, a small tripeptide quencher, increases the annealing temperature and leads to great stability, thereby improving the specificity and sensitivity of the amplification process [26].

In this study, four diarrhea viruses with similar clinical presentation

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were selected for a comprehensive epidemiological study using a multiplex real-time PCR designed based on highly conserved genome areas. The objective of this study was to develop a more rapid and accurate method for diagnosing canine viral diarrhea diseases than conventional molecular detection assays, which has been verified by specificity, sensitivity and repeatability assay, and is well established [19,34].

2. Materials and methods

2.1. Samples, primers, and probes

Positive samples were screened for CPV [6], CCoV, CAstV [21], CaKoV [27], and other related viruses, including Canine distemper virus (CDV) [10], Torque teno canis virus (TTCV) [28], and Canine influenza virus (H3N2 CIV), as previously verified by RT-PCR. CCoV was detected by RT-PCR using the following primers designed by *N* gene: Forward; 5'-GGTTTTGAACACGTTGTATTTGGAGA-3', and Reverse; 5'-ACCYTTCCTTAGTAATCCAACRACCA-3'. In addition, H3N2 CIV was detected by RT-PCR using the following primers designed by *M* gene: Forward; 5'-TTCTAACGGAGGTCGAAAC-3', and Reverse; 5'-AAGCGTCTACGCTGCAGTCC-3'. The corresponding templates were preserved at -80 °C for long-term storage.

Specific primers and probes (Table 1) were designed by the Beacon Designer 8 software based on conserved regions of all complete genome sequences, deposited in the National Center for Biotechnological Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). The *NS1*, *N*, *3D*, *ORF2* gene was chosen for CPV, CCoV, CaKoV and CAstV, respectively. The probes characteristics of CPV, CCoV, CaKoV, and CAstV contained the following reporter dye FAM, HEX, Texas Red, and Cy5, and the MGB, BHQ1, BHQ2, and BHQ2 quenchers. Primers and probes were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China).

2.2. RNA, DNA extraction, and reverse transcription

Feces or anal swabs were mixed with phosphate buffered saline (PBS). After a few minutes of vortexing, then centrifuged at 12,000 rpm at 4 °C for 10 min. Nucleic acids were extracted using the Nucleic acid extractor machine NP968-C (TianLong technology, China) following the manufacturer's instructions. The concentration of total mixture was determined by 260/280 nm uv absorption using Nano Drop (Thermo Scientific, USA) and stored in -80 °C.

First Strand cDNA was synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, China). The samples were stored at -40 °C for later usage.

Table 1
Primers and probes.

Target virus	Name	Gene	Length (bp)	Sequence (5'-3')	Position
CPV	QF	<i>NS1</i>	85	TTCGGTAAACTTAACACCAAC	675-759 ^a
	QR			CTGTATGTTAATATAGTCACCCA	
CCV	QF	<i>N</i>	74	6-FAM-CTGCAATTTCTCTGAGCTTA-MGB	27043-27116 ^b
	QR			CAGTCTAGAAATAGATCTCAATC	
CaKoV	QF	<i>3D</i>	82	GCTTGTCTACACTGTCA	6980-7061 ^c
	QR			HEX-CCTTCITGTTATTGGATTGTGCTTC-BHQ1	
CAstV	QF	<i>ORF2</i>	79	CCGGATTATGTCTACTCCA	6303-6381 ^d
	QR			CAACGATCCTGGTGAGTC	
	Probe			Texas Red-TCCTGAAAGATGAACTCCGCC-BHQ2	
	Probe			CAGAGCAATGGTCAATGA	
	Probe			CTCACTTAGTGTAGGGAGA	
	Probe			CY5-CGCTCAGCCTGGTCTCTGG-BHQ2	

^a Genbank number No. MK388674.

^b Genbank number No. KY063618.

^c Genbank number No. MN449341.

^d Genbank number No. KX599351.

2.3. Construction of standard plasmids

The standard fragments of the target viruses were amplified via RT-PCR and cloned into the pMD18-T vector (Takara, Japan). The primer used here was identical to multiplex real-time PCR method. The identity of the standard plasmids was determined by sequencing of TA linkage reaction. The nucleic acid content was quantified using a Nano Drop (Thermo Scientific, USA) and the copy number was calculated using the following formula (1):

$$\text{Copy number} = \frac{(6.02 \times 10^{23}) \times \left(\frac{\text{ng}}{\mu\text{L}} \times 10^{-9}\right)}{(\text{DNA length} \times 660)} \quad (1)$$

The plasmids were 10-fold serially diluted from 10⁷ copies/μL to 10¹ copies/μL, and standard curves and equations were prepared to verify the reliability of the dilution product.

2.4. Optimization of the reaction system of multiplex real-time PCR

Real time PCR procedure was carried out on a LightCycler96 machine (Roche, Shanghai). The annealing temperature included 54 °C, 56 °C, 58 °C, and 60 °C using 10⁷ copies/μL standard plasmid were used in multiple procedure and selected the optimal annealing temperature according to the amplification efficiency. The cycling parameters were as follows: denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s. Acquisition of fluorescence signals was recorded during the annealing-extension steps and analyzed using the LightCycler® 96 SW 1.1 software. Primers and probes concentrations for optimization ranged from 0.05 to 0.2 μM and 0.025 to 0.1 μM, respectively. The optimal primers and probes were selected based on amplification efficiency determined by the Cq (Cycle of quantification) value and the fluorescence intensity.

2.5. Uniplex and multiplex real-time PCR reactions

Uniplex and multiplex real-time PCR were carried out in a 20 μL reaction, consisting of 10 μL qPCR probe master mix (Vazyme, China); 0.4 μL primer pair and 0.1 μL probe added with corresponding target fragment; 1.5 μL target standard plasmid, and the rest were supplemented with ddH₂O. In the multiplex real-time PCR reactions, the four primer pairs, probes, and the template of the four mixed standard plasmids were added. The cycling conditions were the same as described above.

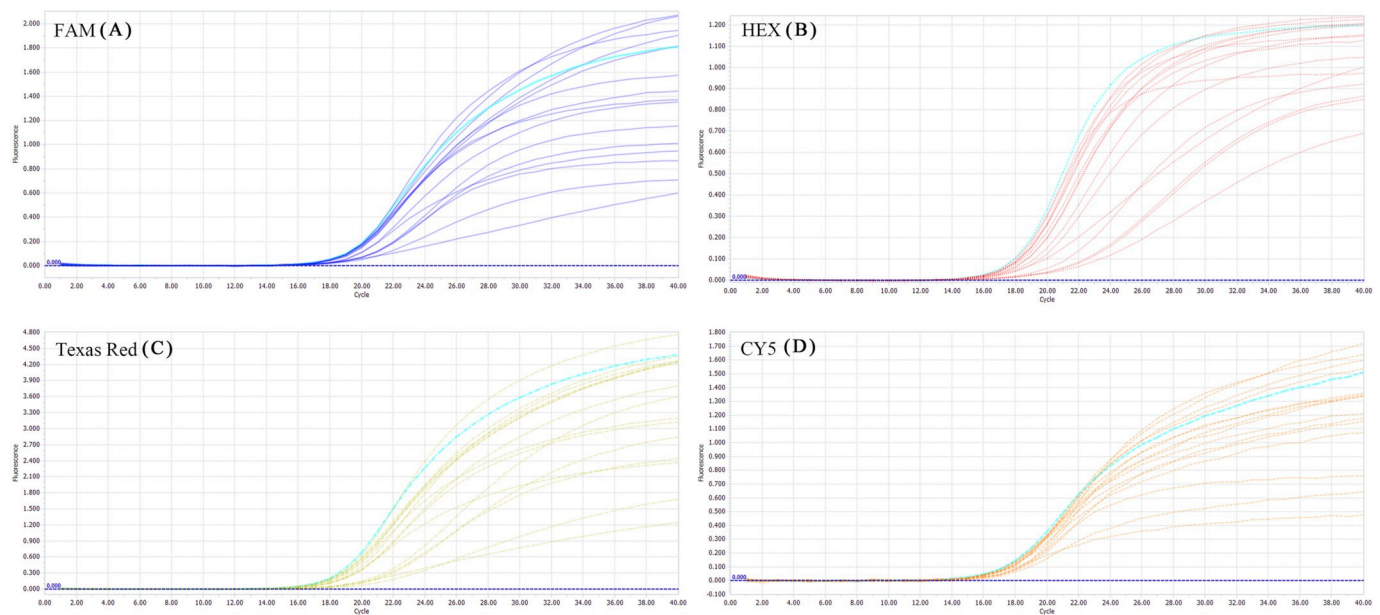


Fig. 1. Optimization of primers and probes. The blue, red, yellow and orange amplification curves represent CPV(A), CCoV(B), CaCoV(C) and CAstV(D), respectively. The pale blue curve represents primer and probe concentrations of 0.2 μM and 0.05 μM . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.6. Specificity

To prove that the experiment did not react with other non-targeted viruses, specificity was determined by addition of target and non-target virus, including viral DNA or cDNA templates of Torque teno canine virus (TTCV), Canine influenza virus (H3N2 CIV), Canine distemper virus (CDV) and CPV, CCoV, CAstV, CaCoV and negative control to the reaction mix.

2.7. Sensitivity

The standard plasmid constructed according to the material method were diluted from 10^3 to 10^1 copies/ μL to determine the limit of detection. Each concentration tested three times as well as negative control. The average and standard deviation (SD) were calculated. It is considered that the minimum copy concentration at which a Cq value is the limit of copy concentration that can be detected.

2.8. Reproducibility

To evaluate the stability of the experiment, four different concentrations of standards including 10^7 , 10^6 , 10^4 , and 10^3 copies/ μL were repeated in triplicate on two separate occasions with a week apart in both intra- and inter-assay as well as negative control. The final coefficient of variation (CV) was calculated. The more stable the data, the smaller the CV value.

2.9. Co-infection simulation and clinical testing experiments

Standard plasmids with a concentration of 10^3 and 10^2 copies/ μL were used to simulate co-infection of duplex and triplex viruses. In addition, we tested 82 clinical diarrhea samples collected from different hospitals in Zhejiang, China and compared them with the results of RT-PCR in the first part of material and methods, with positive and negative controls throughout the process.

2.10. RT-PCR assay

The RT-PCR was incubated in a PCR machine (Eppendorf, Germany)

with a 20 μL reaction system, including, 10 μL Taq master mix; 1 μL Forward and Reverse primer; 7 μL ddH₂O; 1 μL template. The cycling conditions included denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Fragments were observed in a 1.5% agarose gel.

2.11. Statistical analysis

Figure data was generated using the LightCycler® 96 SW 1.1 software and Graphpad prism 6 (GraphPad Software Inc., San Diego, CA, USA). The mean value is the quantity indicating the central tendency of the data sets. Standard deviation (SD) refers to a standard that measures the degree of dispersion of data distribution and is used to measure the degree to which the data value deviates from the arithmetic mean. The CV (Coefficient of variation), is the ratio of standard deviation to the average. The SE (standard error) refers to the error between the sample rate and the overall rate due to sampling. All the above statistics were conducted using Microsoft Excel 2007 (Microsoft, USA).

3. Results

3.1. System optimization

The annealing temperature optimization was carried out over a range including 54 °C, 56 °C, 58 °C, and 60 °C. The optimal efficiency was at 56 °C (data not shown). The optimal reaction conditions for the different concentrations of primers and probes is shown in Fig. 1. The highest amplification curve was achieved in all four fluorescence channels along with a low Cq value for concentrations of 0.2 μM of primer and 0.05 μM of probe.

All the Cq value of negative wells was no fluorescence signal, or Cq value greater than 35, thus we defined that a Cq value less than 35 would be considered positive. When a Cq value was between 35 and 40, the sample needed to be repeated.

3.2. Establishment of the standard curve for the multiplex real-time PCR

The standard curves for the four viruses were prepared by 10-fold

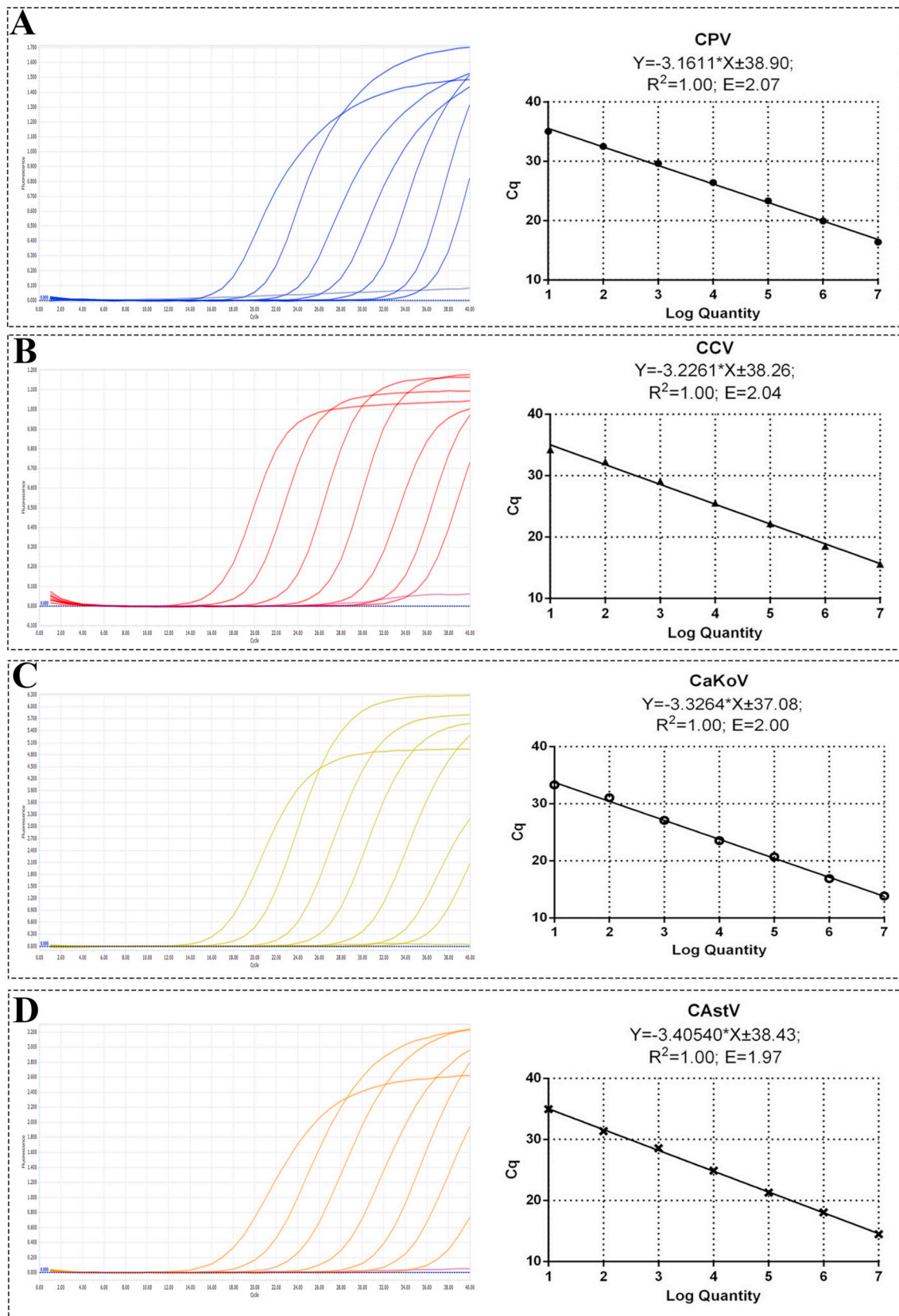


Fig. 2. Amplification and standard curves of (A) CPV, (B) CCoV, (C) CaKoV, and (D) CAstV. The standard curve was evaluated using standards containing 10^7 to 10^1 copies/ μ L. (E) Equation, correlation coefficient (R^2) and amplification efficiency.

Table 2
Multiplex real-time PCR limit of detection test of 10^1 to 10^3 copies/ μ L.

	10^3 copies/ μ L			Mean	SD
CPV	31.24	30.37	30.66	30.76	0.44
CCoV	30.17	29.66	30.14	29.99	0.29
CaKoV	28.17	28.27	29.39	28.61	0.68
CAstV	29.30	28.99	29.30	29.20	0.18
	10^2 copies/ μ L			Mean	SD
CPV	33.29	33.19	33.32	33.27	0.07
CCoV	32.97	33.00	33.06	33.01	0.05
CaKoV	32.09	31.92	32.45	32.15	0.27
CAstV	31.58	31.34	31.22	31.38	0.18
	10^1 copies/ μ L			Mean	SD
CPV	37.09	–	37.44	37.27	0.25
CCoV	–	–	37.78	37.78	–
CaKoV	35.04	34.50	35.22	34.92	0.37
CAstV	–	–	35.31	35.31	–

serial dilution of standard plasmids ranging from 10^7 to 10^1 copies/ μ L as templates. The corresponding slope of the equation, correlation coefficient (R^2), and amplification efficiency (E) were as follows: -3.1611 , 1.00 , and 107% for CPV; -3.2261 , 1.00 , and 104% for CCoV; -3.3264 , 1.00 , and 100% for CaKoV; and -3.4054 , 1.00 , and 97% for CAstV (Fig. 2), indicating an excellent linear equation and the standards were qualified.

3.3. Sensitivity of the multiplex real-time PCR

To identify the detection limits, reactions were prepared containing 10^3 to 10^1 copies/ μ L in triplicate. Since we defined Cq values ≥ 35 as the critical point of negative, the reliable detection limit was 10^2 copies/ μ L (Table 2). However, the average Cq value for CaKoV at 10^1 copies/ μ L was less than 35, there were two values greater than 35 among the three replicates. Therefore, we used a conservative approach and defined that the lowest detectable concentration of the virus in this experiment was 10^2 copies/ μ L. Subsequent experiments also relies on these criteria.

3.4. Specificity of the multiplex real-time PCR

The method was performed to detect canine distemper (CDV), torque teno canis virus (TTCV) and canine influenza virus (H3N2 CIV), which are common viral diseases in pet hospitals, as templates for amplification. Relevant fluorescent signals could only detect the

Table 3
Intra- and inter-assay reproducibility of multiplex real-time PCR.

Assay	DNA (copies/ μ L)	Intra-assay			Inter-assay		
		Mean Cq	SD	CV (%)	Mean Cq	SD	CV (%)
CPV	10^7	16.66	0.01	0.07	16.83	0.15	0.88
	10^6	19.94	0.02	0.09	20.11	0.15	0.73
	10^4	27.06	0.18	0.68	27.21	0.16	0.59
CCoV	10^3	30.52	0.19	0.62	30.52	0.21	0.67
	10^7	15.81	0.01	0.06	15.89	0.07	0.45
	10^6	19.23	0.02	0.11	19.41	0.16	0.82
CaKoV	10^4	26.09	0.25	0.98	26.26	0.15	0.58
	10^3	30.66	0.18	0.60	30.71	0.23	0.74
	10^7	13.46	0.02	0.13	13.58	0.14	1.00
CAstV	10^6	17.28	0.01	0.03	17.36	0.07	0.42
	10^4	24.44	0.09	0.36	24.55	0.29	1.20
	10^3	28.14	0.28	0.98	27.95	0.62	2.22
CAstV	10^7	14.02	0.04	0.25	14.20	0.18	1.29
	10^6	17.61	0.03	0.14	17.70	0.08	0.44
	10^4	24.00	0.19	0.77	24.20	0.18	0.74
	10^3	28.05	0.20	0.70	28.24	0.17	0.61

corresponding targeted viruses while none of the three non-targeted viruses were detected (Fig. 3).

3.5. Reproducibility of the multiplex real-time PCR

Concentrations of 10^7 , 10^6 , 10^4 , and 10^3 copies/ μ L of standard plasmids were chosen to perform three runs and measure intra- and inter-assay variation in the form of %CV. The CV values were almost lower than 1% with a few value ranging from 1% to 3% (Table 3) indicating good repeatability and high accuracy.

3.6. Co-infection simulation and clinical sample detection

One of the most important aspects of multiplex PCR detection methods is primer interaction-primer dimers. We used standard plasmids to simulate duplex and triplex co-infection at low concentrations using standard plasmids of 10^3 and 10^2 copies/ μ L (Fig. 4). We found that the fluorescence channels corresponding to the targeted virus could be accurately detected, even regardless of the pairing combination.

In clinic, 82 fecal samples were tested by both multiplex real-time PCR and RT-PCR (Table 4) to validate its usability. The results for the multiplex real-time PCR were as follows: 29.3% positive samples for CPV, 7.3% positive for CCoV, 3.7% positive for CaKoV, and 6.1% positive for CAstV. Co-infection of two viruses included two samples of CPV and CCoV; one positive of CPV and CaKoV; and one of CCoV and

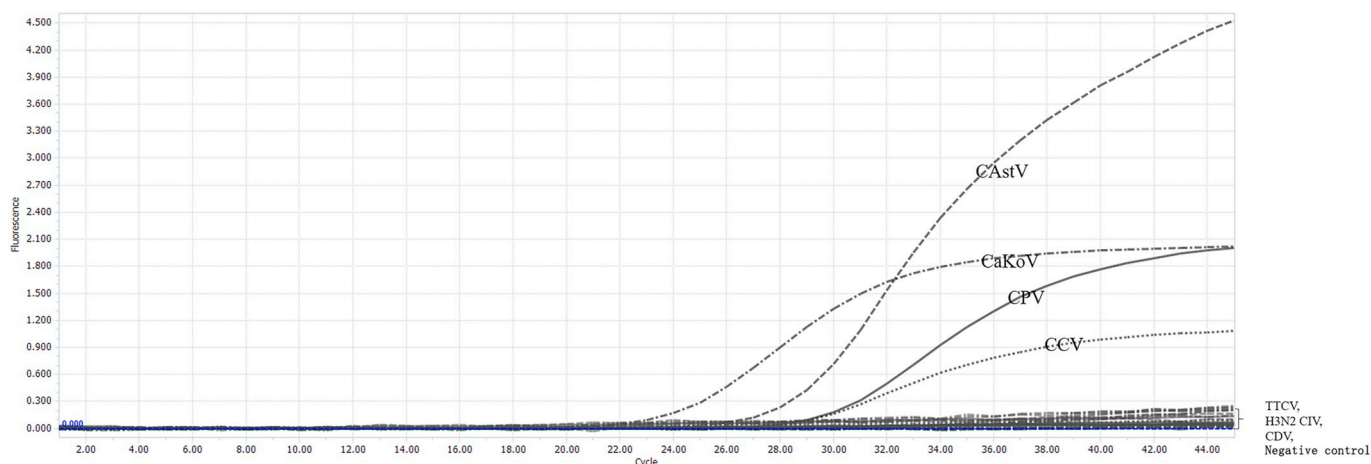


Fig. 3. Multiplex real-time PCR specificity. The X axis and Y axis represent the number of cycles and the fluorescence intensity, respectively.

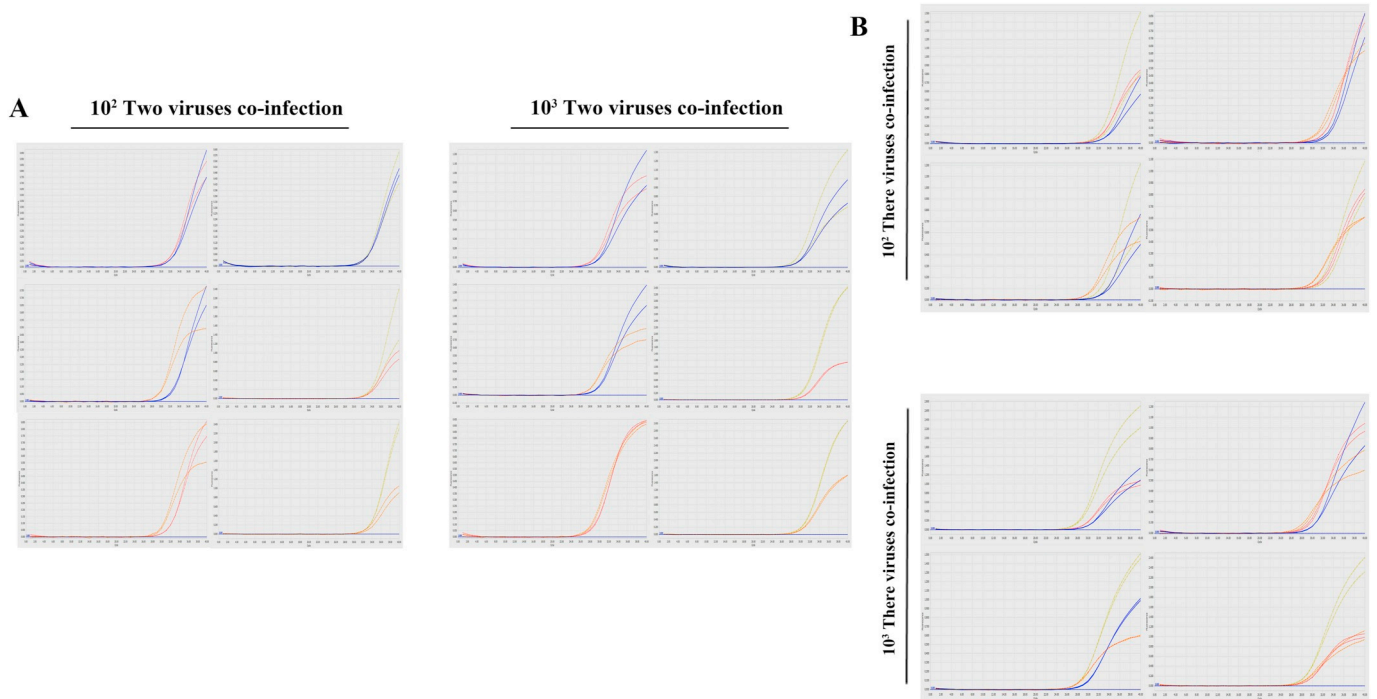


Fig. 4. Simulation of virus co-infection. (A) 6 cases of double virus infection that was verified using 10^3 and 10^2 copies/ μL respectively. (B) 4 cases of three virus infections verified using 10^3 and 10^2 copies/ μL . The color was the same as Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CAstV. Co-infections of three viruses included one positive of CCoV, CaKoV, and CAstV. Furthermore, there was one sample co-infected with four viruses. For RT-PCR, 29.3% samples were positive for CPV, 2.4% positive for CCoV, 1.22% positive for CaKoV, and 2.44% positive for CAstV. However, only one co-infection of CPV and CCoV and one of CCoV, CaKoV and CAstV was detected. Furthermore, the standard error of the prevalence is close to 0, indicating that the results are representative and reliable. In general, the results showed that the number of positives detected by real-time PCR was exceed than that of RT-PCR.

4. Discussion

Nowadays, the cross-species transmission of viruses poses a significant threat to public health. The risk of host range transmission is real leading to expansion renewal [5,20,29,37,41]. In addition, newly-emerged SARS-CoV-2 has also been found to infect canines and was

successfully isolated from nasal and oral swabs. The evidence shows that these are instances of human-to-animal transmission [33]. Can infected dogs spread the virus to other animals or to humans? As companion animals for humans, the four virus-infected canines selected here for research have particularly extensive host characteristics, so real-time monitoring is of public health significance.

Over the past few years, molecular diagnostics methods have been greatly improved [9]. Several reports have been published on detection methods focusing on a single canine diarrhea-related disease [22,31]. Here, a multiplex Taqman probe-based real-time PCR method was developed for the first time and optimized to better monitor CPV, CAstV, CaKoV, and CCoV epidemiology. The limitation of this method can reach to 10^2 copies/ μL , while multiplex RT-PCR shows no noticeable advantage in terms of sensitivity [8]. A multiplex RT-PCR assay for CPV, CCoV and CAV, in which the detection limit of the method was 1×10^4 viral copies/ μL [7] and a multiplex PCR detection developed for canine respiratory and enteric diseases. The limit of this method was

Table 4
Positivity rate of multiplex real-time and RT-PCR tests for 82 samples.

Pathogen	Real-time PCR	Standard Error	RT-PCR	Standard Error
CPV	24/82 (29.3%)	0.050	24/82 (29.3%)	0.050
CCoV	6/82 (7.3%)	0.029	2/82 (2.4%)	0.017
CaKoV	3/82 (3.7%)	0.021	1/82 (1.22%)	0.012
CAstV	5/82 (6.10%)	0.026	2/82 (2.44%)	0.017
CPV + CCoV	2/82 (2.44%)	0.017	1/82 (1.22%)	0.012
CPV + CaKoV	1/82 (1.22%)	0.012	0/82	0
CPV + CAstV	0/82	0	0/82	0
CCoV + CaKoV	0/82	0	0/82	0
CCoV + CAstV	1/82 (1.22%)	0.012	0/82	0
CaKoV + CAstV	0/82	0	0/82	0
CPV + CCoV + CaKoV	0/82	0	0/82	0
CPV + CCoV + CAstV	0/82	0	0/82	0
CPV + CaKoV + CAstV	0/82	0	0/82	0
CCoV + CaKoV + CAstV	1/82 (1.22%)	0.012	1/82 (1.22%)	0.012
CPV + CCoV + CaKoV + CAstV	1/82 (1.22%)	0.012	0/82	0
Total	44	0.055	31	0.054

also 1×10^4 copies/ μ L [12]. Furthermore, based on the analysis of 82 clinical samples, we found this method is able to detect positive samples previously considered negative by RT-PCR. Especially in the case of coinfection, it was more sensitive than the results of RT-PCR tests, which can effectively detect the virus epidemic situation. Moreover, CPV was found to be the most prevalent virus in diarrhea samples in Chinese canines, as previously shown [40]. The inaccuracy of RT-PCR usually leads to identity confirmation via sequencing which normally results in undetermined sequences. Real-time PCR can effectively prevent false positives by digitizing the results [13]. This method can be used in the early diagnosis of viral infection at a low titer. Thus, our detection method has a powerful monitoring ability, so as to achieve a rapid cure for canines.

In general, the method established in this study overcomes the disadvantages of the prior art mentioned above and validated by comparison with RT-PCR method. This is the first report detailing the establishment of a real-time PCR method that can accurately detect CPV, CCoV, CaCoV, and CaStV simultaneously, providing a more favorable tool for mass diagnosis and prevalence investigations as well as significant savings in time and laboratory materials.

Ethical statement

This article is an article on the establishment of detection methods, and the samples were collected from feces in the pet hospital in Zhejiang, Jinhua.

Data availability statement

The data used to support the findings of this study are included within the article.

Funding

This work was financially supported by the National Key Research and Development Program of China (Grant no. 2017YFD0500101), the Fundamental Research Funds for the Central Universities (Grant no. Y0201900459) and the Natural Science Foundation of Jiangsu Province (Grant no. BK20170721).

Declaration of competing interest

The authors declare no conflict of interest.

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