



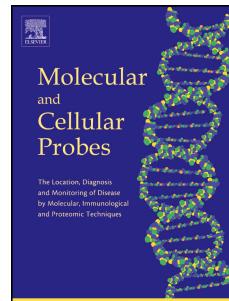
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One-step multiplex TaqMan probe-based method for real-time PCR detection of four canine diarrhea viruses

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Conflict of interest

The authors declare no conflict of interest.

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

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9

10 **Abstract**

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

26 the method can be used as a preferred tool for monitoring laboratory epidemics,
27 timely prevention, and effective monitoring of disease progression.

28 **Keywords:** Canine diarrhea; Multiplex Real-time PCR; TaqMan probe

29

30 **Introduction**

31 Viral canine diarrhea is usually highly contagious, and even more likely to cause
32 mixed infections, leading to fatal diarrhea(Williams, 1980).. The long treatment cycle
33 and slow healing of puppies can cause mental injury to pet owners. Canine Parvovirus
34 (CPV), Canine Coronavirus (CCoV), Canine Astrovirus (CAstV), Canine
35 Kobuviruses (CaKoV), Rotavirus and Canine adenovirus (CAV) are causative agents
36 of diarrhea. With the exception of CAstV and CaKoV, the research of detection
37 method for other diarrheal viruses are common(X. Deng et al., 2018; Hao et al., 2019).
38 CPV and CCoV are serious threats to canines. CPV, one of the most serious causes of
39 diarrhea, can have a mortality rate of 70% in puppies and lead to hemorrhagic
40 enteritis (Amrani et al., 2016; Appel, Scott, & Carmichael, 1979) of rapid
41 transmission (Clayton & Lindsay, 1979). CCoV mainly causes gastroenteritis and
42 clinical signs include vomit and mild to severe diarrhea (Binn et al., 1974), with the
43 high positivity rate next to CPV(Mochizuki, Sugiura, & Akuzawa, 1987; Pratelli et al.,
44 2000). CAstV was first discovered in 1980 (Williams, 1980) and a series of
45 documents successively confirmed the existence of this pathogen in diarrheal feces by
46 molecular biology, consist of electron microscopy, RT-PCR and then
47 sequencingidentification (Toffan et al., 2009). CaKoV was first detected in canines
48 with acute gastroenteritis in the United States (Kapoor et al., 2011; L. L. Li et al.,
49 2011). In China, there are only two recent reports on CaKoV and a handful of reports
50 have discussed the prevalence of CAstV (Kong et al., 2016; C. Q. Li et al., 2016).
51 Thus, CAstV and CaKoV are controversial in terms of prevalence and clinical
52 symptoms, and supported by few relevant studies which was needed to monitor virus
53 via co-infection. Differential diagnosis must combine specific antigen detection
54 assays. However, there is no such method for the simultaneous and rapid

55 identification of CPV, CCoV, CaKoV, CAstV.
56 Until now, it is common laboratory practice the use of conventional polymerase chain
57 reaction (PCR) methods for single or multiplex detection. RT-PCR is a powerful
58 genetic analysis method. However, its limitations are low efficiency and the
59 requirement of sufficient concentration of virus (Ferre, 1992; Raeymaekers, 1995). In
60 addition, it is time-consuming, laborious, and not suitable for high throughput. Since
61 1993, Roche and scientist Higuchi et al. first revealed real time-PCR(Higuchi, Fockler,
62 Dollinger, & Watson, 1993; Klein, 2002). Real-time PCR is a quantitative highly
63 sensitive PCR (Mullis, 1990; Valasek & Repa, 2005) that uses target-specific dual
64 fluorescently labeled DNA oligonucleotides, referred to as TaqMan probes (Mullis,
65 1990; Mullis & Falloona, 1987) which are labeled with a quencher and a reporter.
66 During amplification, the quencher is separated from the reporter gene releasing
67 fluorescence signal (Tan, Sun, Gonzalez-Crussi, Gonzalez-Crussi, & Hsueh, 1994).
68 MGB, a small tripeptide quencher, increases the annealing temperature and leads to
69 great stability, thereby improving the specificity and sensitivity of the amplification
70 process (Navarro, 2015).

71 In this study, four diarrhea viruses with similar clinical presentation were selected for
72 a comprehensive epidemiological study using a muliplex real-time PCR designed
73 based on highly conserved genome areas. The objective of this study was to develop a
74 more rapid and accurate method for diagnosing canine viral diarrhea diseases than
75 conventional molecular detection assays, which has been verified by specificity,
76 sensitivity and repeatability, and is well established(Liu et al., 2018; Sultana et al.,
77 2020).

78

79 Materials and methods

80 Samples, primers, and probes

81 Positive samples were screened for CPV(Decaro et al., 2005), CCoV, CAstV(Martella
82 et al., 2011), CaKoV(Oem, Choi, Lee, Lee, & Choi, 2014), and other related viruses,
83 including Canine distemper virus(CDV)(Elia et al., 2006), Torque teno canis virus

84 (TTCV)(Okamoto et al., 2001), and Canine influenza virus (H3N2 CIV), as
 85 previously verified by RT-PCR. CCoV was detected by RT-PCR using the following
 86 primers designed by N gene: Forward; 5'-
 87 GGTTTGAAACACGTTGTATTGGAGA-3', and Reverse;
 88 5'-ACCYTTCCCTTAGTAATCCAACRACCA-3'. In addition, H3N2 CIV was
 89 detected by RT-PCR using the following primers designed by M gene: Forward; 5'-
 90 TTCTAACCGAGGTCGAAAC-3', and Reverse; 5'-
 91 AAGCGTCTACGCTGCAGTCC-3'. The corresponding templates were preserved at
 92 -80°C for long-term storage.

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

101 RNA, DNA extraction, and reverse transcription

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

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

111 Construction of standard plasmids

112 The standard fragments of the target viruses were amplified via RT-PCR and cloned into

113 the pMD18-T vector (Takara, Japan). The primer used here was identical to multiplex
 114 real-time PCR method. The identity of the standard plasmids was determined by
 115 sequencing of TA linkage reaction. The nucleic acid content was quantified using a Nano
 116 Drop (Thermo Scientific, USA) and the copy number was calculated using the following
 117 formula (1):

$$118 \quad \text{Copy number} = \frac{(6.02 \times 10^{23}) \times (\frac{ng}{\mu L} \times 10^{-9})}{(DNA \ length \times 660)} \quad (1)$$

119 The plasmids were 10-fold serially diluted from 10^7 copies/ μL to 10^1 copies/ μL , and
 120 standard curves and equations were prepared to verify the reliability of the dilution
 121 product.

122 Optimization of the reaction system of multiplex real-time PCR

123 Real time PCR procedure was carried out on a LightCycler96 machine (Roche,
 124 Shanghai). The annealing temperature included 54°C, 56°C, 58°C, and 60°C using
 125 10^7 copies/ μL standard plasmid were used in multiple procedure and selected the
 126 optimal annealing temperature according to the amplification efficiency. The cycling
 127 parameters were as follows: denaturation at 95°C for 2 minutes, 40 cycles of
 128 denaturation at 95°C for 10s, annealing at 56°C for 20s, and extension at 72°C for
 129 30s. Acquisition of fluorescence signals was recorded during the annealing-extension
 130 steps and analyzed using the LightCycler® 96 SW 1.1 software. Primers and probes
 131 concentrations for optimization raged from 0.05-0.2 μM and 0.025-0.1 μM ,
 132 respectively. The optimal primers and probes were selected based on amplification
 133 efficiency determined by the Cq(Cycle of quantification) value and the fluorescence
 134 intensity

135 Uniplex and multiplex real-time PCR reactions

136 Uniplex and multiplex real-time PCR were carried out in a 20 μL reaction, consisting
 137 of 10 μL qPCR probe master mix (Vazyme, China); 0.4 μL primer pair and 0.1 μL
 138 probe added with corresponding target fragment; 1.5 μL target standard plasmid, and

139 the rest were supplemented with ddH₂O. In the multiplex real-time PCR reactions, the
140 four primer pairs, probes, and the template of the four mixed standard plasmids were
141 added. The cycling conditions were the same as described above.

142 **Specificity**

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

148 **Sensitivity**

149 The standard plasmid constructed according to the material method were diluted from
150 10³ to 10¹ copies/µL to determine the limit of detection. Each concentration tested
151 three times as well as negative control. The average and standard deviation (SD) were
152 calculated. It is considered that the minimum copy concentration at which a Cq value
153 is the limit copy concentration that can be detected.

154 **Reproducibility**

155 To evaluate the stability of the experiment, four different concentrations of standards
156 including 10⁷, 10⁶, 10⁴, and 10³ copies/µL were repeated in triplicate on two separate
157 occasions with a week apart in both intra- and inter-assay as well as negative control.
158 The final coefficient of variation (CV) was calculated. The more stable the data, the
159 smaller the CV value.

160 **Co-infection simulation and clinical testing experiments**

161 Standard plasmids with a concentration of 10³ and 10² copies/µL were used to
162 simulate co-infection of duplex and triplex viruses. In addition, we tested 82 clinical
163 diarrhea samples collected from different hospitals in Zhejiang, China and compared
164 them with the results of RT-PCR in the first part of material and methods, with
165 positive and negative controls throughout the process.**RT-PCR assay**

166 The RT-PCR was incubated in a PCR machine (Eppendorf, Germany) with a 20µL
167 reaction system, including, 10µL Taq master mix; 1µL Forward and Reverse primer;
168 7µL ddH₂O; 1µL template. The cycling conditions included denaturation at 95°C for 5

169 minutes, followed by 35 cycles of denaturation at 95 $^{\circ}$ C for 30 seconds, annealing at 55 $^{\circ}$ C
170 for 30 seconds, and extension at 72 $^{\circ}$ C for 1 minute, followed by a final extension at 72 $^{\circ}$ C
171 for 10 minutes. Fragments were observed in a 1.5% agarose gel.

172 **Statistical analysis**

173 Figure data was generated using the LightCycler® 96 SW 1.1 software and Graphpad
174 prism 6 (GraphPad Software Inc., San Diego, CA, USA). The mean value is the
175 quantity indicating the central tendency of the data sets. Standard deviation(SD) refers
176 to a standard that measures the degree of dispersion of data distribution and is used to
177 measure the degree to which the data value deviates from the arithmetic mean. The
178 CV(Coefficient of variation), is the ratio of standard deviation to the average. The
179 SE(standard error) refers to the error between the sample rate and the overall rate due
180 to sampling. All the above statistics were conducted using Microsoft Excel
181 2007(Microsoft, USA).

182

183 **Results**

184 **System optimization**

185 The annealing temperature optimization was carried out over a range including 54 $^{\circ}$ C,
186 56 $^{\circ}$ C, 58 $^{\circ}$ C, and 60 $^{\circ}$ C. The optimal efficiency was at 56 $^{\circ}$ C (data not shown). The
187 optimal reaction conditions for the different concentrations of primers and probes is
188 shown in Figure 1. The highest amplification efficiency was achieved in all four
189 fluorescence channels along with a low Cq value for concentrations of 0.2 μ M of
190 primer and 0.05 μ M of probe.

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

194 **Establishment of the standard curve for the multiplex real-time PCR**

195 The standard curves for the four viruses were prepared by 10-fold serial dilution of
196 standard plasmids ranging from 10^7 to 10^1 copies/ μ L as templates. The corresponding
197 slope of the equation, correlation coefficient (R^2), and amplification efficiency (E)

198 were as follows: -3.1611, 1.00, and 107% for CPV; -3.2261, 1.00, and 104% for
 199 CCoV; -3.3264, 1.00, and 100% for CaKoV; and -3.40540, 1.00, and 97% for CAstV
 200 (Figure 2), indicating an excellent linear equation and the standards were qualified.

201 **Sensitivity of the multiplex real-time PCR**

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

209 **Specificity of the multiplex real-time PCR**

210 The method was performed to detect canine distemper (CDV), torque teno canis virus
 211 (TTCV) and canine influenza virus (H3N2 CIV), which are common viral diseases in
 212 pet hospitals, as templates for amplification. Relevant fluorescent signals could only
 213 detect the corresponding targeted viruses while none of the three non-targeted viruses
 214 were detected (Figure 3).

215 **Reproducibility of the multiplex real-time PCR**

216 Concentrations of 10^7 , 10^6 , 10^4 , and 10^3 copies/ μ L of standard plasmids were chosen
 217 to perform three runs and measure intra- and inter-assay variation in the form of %CV.
 218 The CV values were almost lower than 1% with a few value ranging from 1% to
 219 3%(Table 3) indicating good repeatability and high accuracy.

220 **Co-infection simulation and clinical sample detection**

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

226 In clinic, 82 fecal samples were tested by both multiplex real-time PCR and RT-PCR
 227 (Table 4) to validate its usability. The results for the multiplex real-time PCR were as

228 follows: 29.3% positive samples for CPV, 7.3% positive for CCoV, 3.7% positive for
229 CaKoV, and 6.1% positive for CAstV. Co-infection of two viruses included two
230 samples of CPV and CCoV; one positive of CPV and CaKoV; and one of CCoV and
231 CAstV. Co-infections of three viruses included one positive of CCoV, CaKoV, and
232 CAstV. Furthermore, there was one sample co-infected with four viruses. For
233 RT-PCR, 29.3% samples were positive for CPV, 2.4% positive for CCoV, 1.22%
234 positive for CaKoV, and 2.44% positive for CAstV. However, only one co-infection of
235 CPV and CCoV and one of CCoV, CaKoV and CAstV was detected. Furthermore, the
236 standard error of the prevalence is close to 0, indicating that the results are
237 representative and reliable. In general, the results showed that the number of positives
238 detected by real-time PCR was exceed than that of RT-PCR.

239

240 Discussion

241 Nowadays, the of cross-species transmission of viruses poses a significant threat to
242 public health. The risk of host range transmission is real leading to expansion renewal
243 (Davidson, Appel, Doster, Baker, & Brown, 1992; Parrish, 1999; Truyen,
244 1999)(Madeley & Cosgrove, 1975; Yamashita et al., 1991). In addition,
245 newly-emerged SARS-CoV-2 has also been found to infect canines and was
246 successfully isolated from nasal and oral swabs. The evidence shows that these are
247 instances of human-to-animal transmission(Sit et al., 2020). Can infected dogs spread
248 the virus to other animals or to humans? As companion animals for humans, the four
249 virus-infected canines selected here for research have particularly extensive host
250 characteristics, so real-time monitoring is of public health significance.

251 Over the past few years, molecular diagnostics methods have been greatly improved.
252 (Dowgier et al., 2016) Several reports have been published on detection methods
253 focusing on a single canine diarrhea-related disease (Mizak & Rzezutka, 1999;
254 Pratelli et al., 1999).Here, a multiplex Taqman probe-based real-time PCR method
255 was developed for the first time and optimized to better monitor CPV, CAstV, CaKoV,
256 and CCoV epidemiology. The limitation of this method can reach to 10^2 (copies/ μ L),

257 while multiplex RT-PCR shows no noticeable advantage in terms of sensitivity (X.
258 Y. Deng et al., 2018). A multiplex RT-PCR assay for CPV, CCoV and CAV, in which
259 the detection limit of the method was 1×10^4 viral copies/ μL (X. Deng et al., 2018)
260 and a multiplex PCR detection developed for canine respiratory and enteric diseases.
261 The limit of detection of this method was also 1×10^4 copies/ μL (Hao et al., 2019).
262 Furthermore, based on the analysis of 82 clinical samples, we found this method is
263 able to detect positive samples previously considered negative by RT-PCR, especially
264 in the case of co-infection, were more sensitive than the results of RT-PCR tests,
265 which can effectively detect the virus epidemic situation. Moreover, CPV was found
266 to be the most prevalent virus in diarrhea samples in Chinese canines, as previously
267 shown (Wu, Li, Wang, Liu, & Tian, 2018). The inaccuracy of RT-PCR usually leads to
268 identity confirmation via sequencing which normally results in undetermined
269 sequences. Real-time PCR can effectively prevent false positives by digitizing the
270 results(Higuchi et al., 1993). This method can be used in the early diagnosis of viral
271 infection at a low titer. Thus, our detection method has a powerful monitoring ability,
272 so as to achieve a rapid cure for canines.

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

279 **Conflict of interest**

280 The authors declare no conflict of interest.

281 **Ethical statement**

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

284 **Data availability statement**

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

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446

447 Table 1. Primers and Probes

Target virus	Name	Gene	Length(bp)	Sequence (5'-3')	Position
CPV	QF	NS1	85	TTCGGTAAACTAACACCAAC	675-759 ^a
	QR			CTGTATGTTAATATAGTCACCCA	
	Probe			6-FAM-CTGCAATTCTCTGAGCTTA-MGB	
CCV	QF	N	74	CAGTCTAGAAATAGATCTCAATC	27043-27116 ^b
	QR			GCTTGTTCTACACTGTCA	
	Probe			HEX-CCTTCTGTTATTGGATTGTTGCCTTC-BHQ1	
CaKoV	QF	3D	82	CCGGATTATGTCTACTCCA	6980-7061 ^c
	QR			CAACGATCCTGGTGAGTC	
	Probe			Texas Red-TCCTGAAAGATGAACCTCCGCC-BHQ2	
CAstV	QF	ORF2	79	CAGAGCAATGGTCAATGA	6303-6381 ^d
	QR			CTCACTTAGTGTAGGGAGA	
	Probe			CY5-CGCTCAGCCTGGCCTCTGG-BHQ2	

448 ^aGenbank number No. MK388674

449 ^bGenbank number No. KY063618

450 ^cGenbank number No. MN449341

451 ^dGenbank number No. KX599351

452

453 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	-

454

455 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
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
	10^3	30.52	0.19	0.62	30.52	0.21	0.67
CCoV	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
	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
CaKoV	10^7	13.46	0.02	0.13	13.58	0.14	1.00
	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

456

457

458 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

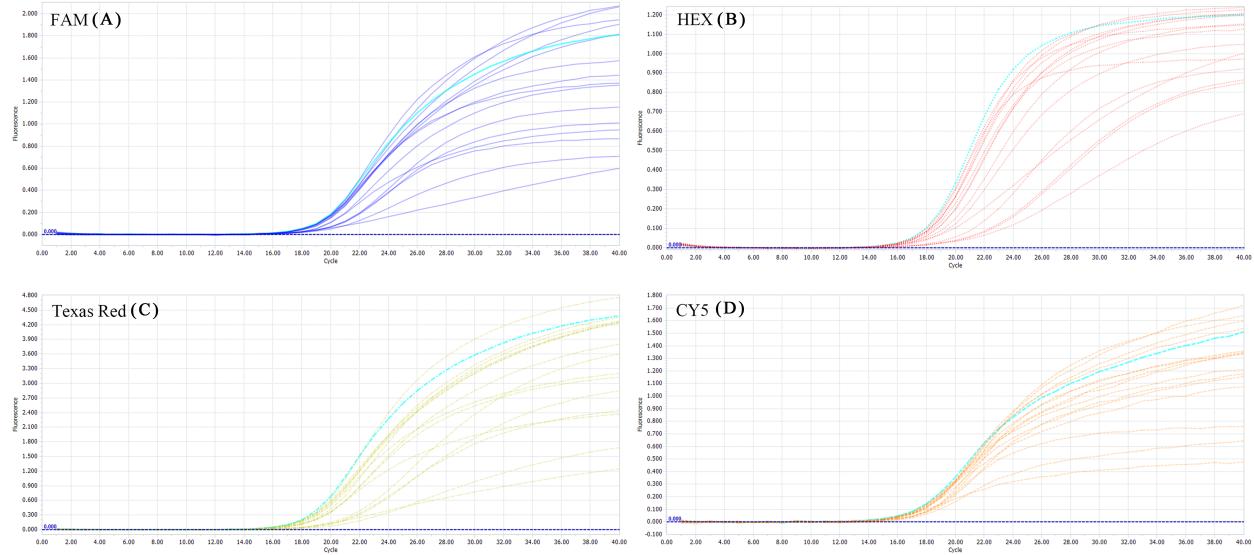
459 **Figure Legends**460 Figure 1. Optimization of primers and probes. The blue, red, yellow and orange
461 amplification curves represent CPV(A), CCoV(B), CaKoV(C) and CAstV(D),

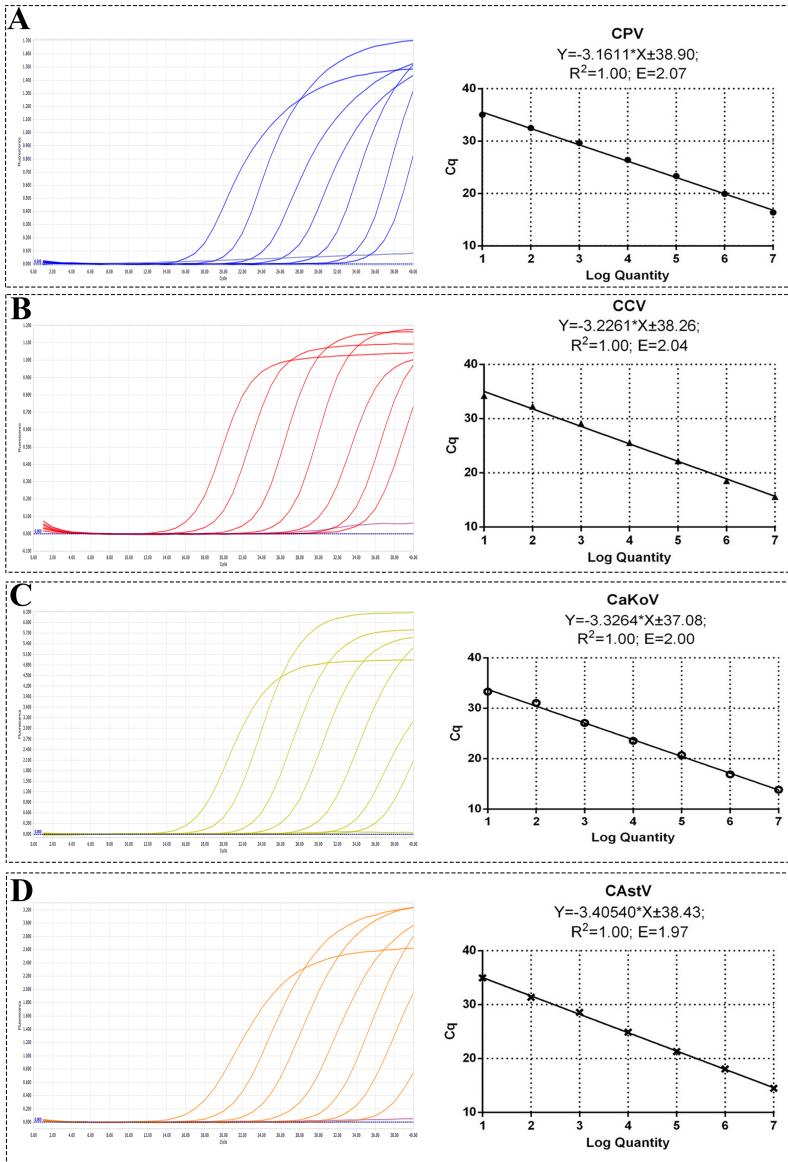
462 respectively. The pale blue curve represents primer and probe concentrations of 0.2
463 μM and 0.05 μM .

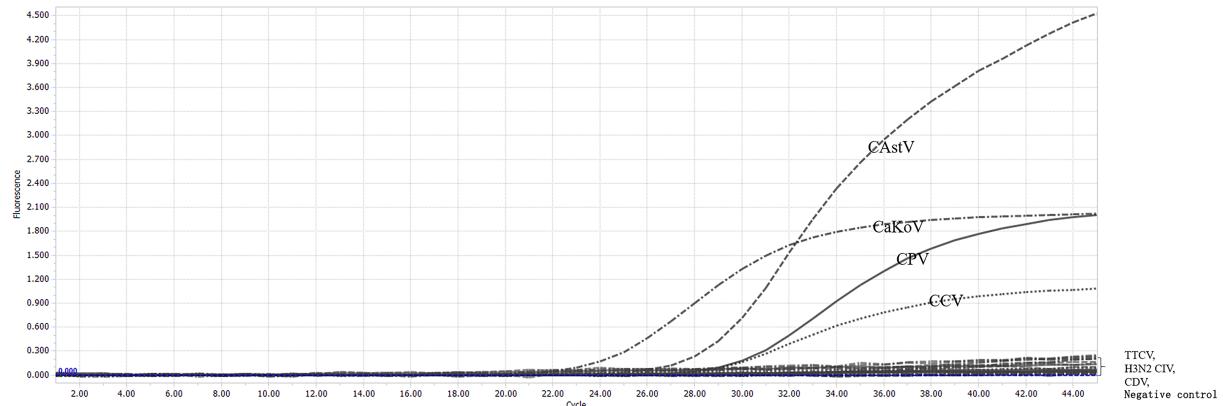
464 Figure 2. Amplification and standard curves of (A) CPV, (B) CCoV, (C) CaKoV, and
465 (D) CAstV. The standard curve was evaluated using standards containing 10^7 to 10^1
466 copies/ μL . (E) Equation, correlation coefficient (R^2) and amplification efficiency.

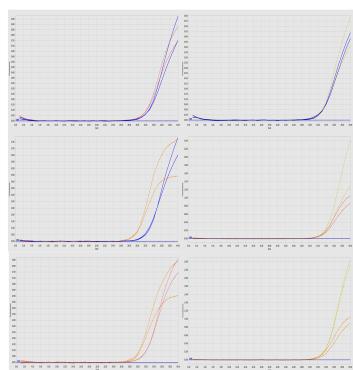
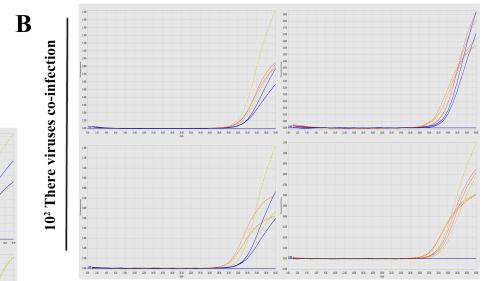
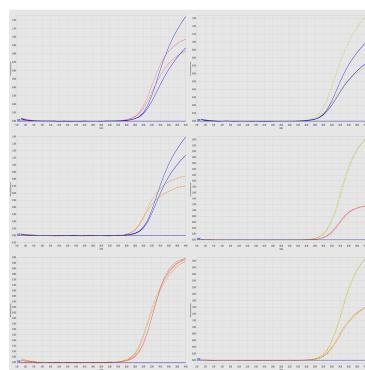
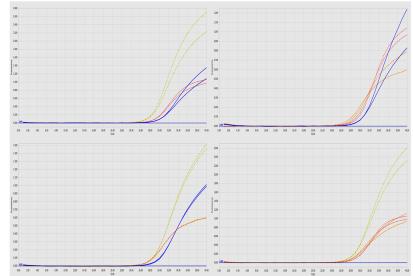
467 Figure 3. Multiplex real-time PCR specificity. The X axis and Y axis represent the
468 number of cycles and the fluorescence intensity, respectively.

469 Figure 4. Simulation of virus co-infection. (A) 6 cases of double virus infection that
470 was verified using 10^3 and 10^2 copies/ μl respectively. (B) 4 cases of three virus
471 infections verified using 10^3 and 10^2 copies/ μL . The color was the same as figure 1.







A 10^2 Two viruses co-infection 10^3 Two viruses co-infection 10^3 Three viruses co-infection

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.

The limit of detection was up to 10²copies/ μ L and performed well with high sensitivity and specificity.

Our results suggest that the method can be used as a preferred tool for monitoring laboratory epidemics, timely prevention, and effective monitoring of disease progression.