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Journal of Virological Methods 285 (2020) 113944

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development of an SYBR Green I-based real-time PCR assay for the rapid detection of canine kobuvirus

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ARTICLE INFO

Keywords:
Detection
Canine kobuvirus
Real-time PCR
SYBR Green I

ABSTRACT

Canine kobuvirus (CaKoV) is a causative agent of gastroenteritis in dogs. Rapid detection of CaKoV is important for preventing and controlling this condition. In this study, an SYBR Green I-based quantitative real-time PCR assay was established for CaKoV detection. Specific primers targeting a highly conserved region of the CaKoV 3D gene were developed. After optimization, the method detected a minimum of 1×10^1 copies/μL with high specificity, stability, and repeatability. Moreover, the entire process only required approximately 1.5 h for completion. Our results were supported by those obtained for clinical samples, in which our developed method was successfully applied. The newly established real-time PCR is a rapid, sensitive, specific, and repeatable method for the quantitative detection of CaKoV and can, therefore, be used in epidemiological studies.

1. Introduction

Canine kobuvirus (CaKoV) is a member of *Aichivirus A* and a single-stranded positive-sense RNA virus with a genome of approximately 8.1–8.2 kb (Ambert-Balay et al., 2008; Kapoor et al., 2011). CaKoV infections have been reported in many countries, including China, the United States, the United Kingdom, Italy, South Korea, Tanzania, Kenya, and Japan (Carmona-Vicente et al., 2013; Di Martino et al., 2013; Choi et al., 2014; Olarte-Castillo et al., 2015; Li et al., 2016). This prevalent pathogen can lead to serious diarrhea in dogs; however, the infection may also be asymptomatic. CaKoV can be detected in the cerebellum, lungs, tonsils, and liver, indicating that it can cause serious systemic infection (Ribeiro et al., 2017).

Recent studies involving CaKoV have focused on its prevalence and evolutionary relationships. However, an effective detection method, particularly to differentiate CaKoV from other pathogens, such as canine parvovirus (CPV), has not been established. Therefore, it is necessary to develop a rapid, sensitive, and specific method for CaKoV detection. SYBR Green I-based quantitative real-time PCR assays are rapid, sensitive, and specific, and have been successfully used to detect many viruses (Rao and Sun, 2015; Chen et al., 2018, 2019; Gao et al., 2020).

In this study, an SYBR Green I-based quantitative real-time PCR assay was successfully developed to detect CaKoV. This method is suitable to test clinical samples and useful in epidemiological research.

2. Materials and methods

2.1. Virus strains and clinical samples

The CaKoV virus strain CaKoV AH-1/CHN/2019 (accession number: MN449341), maintained in our laboratory, was isolated from an animal hospital in Anhui province and has been verified using sequencing. In total, 57 fecal samples were collected between January 2019 and January 2020 from different animal hospitals located in Anhui province, eastern China. Samples were obtained from dogs with diarrhea and healthy dogs.

2.2. Nucleic acid extraction

The fecal samples were resuspended by vortexing in phosphate-buffered saline at approximately 0.1–0.2 g/mL and centrifugation at 12000 ×*g* for 10 min. The suspension was collected in a new RNase-free centrifuge tube. Viral RNA was extracted using the TIANamp Virus

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https://doi.org/10.1016/j.jviromet.2020.113944
Received 24 June 2020; Received in revised form 23 July 2020; Accepted 24 July 2020
Available online 28 July 2020
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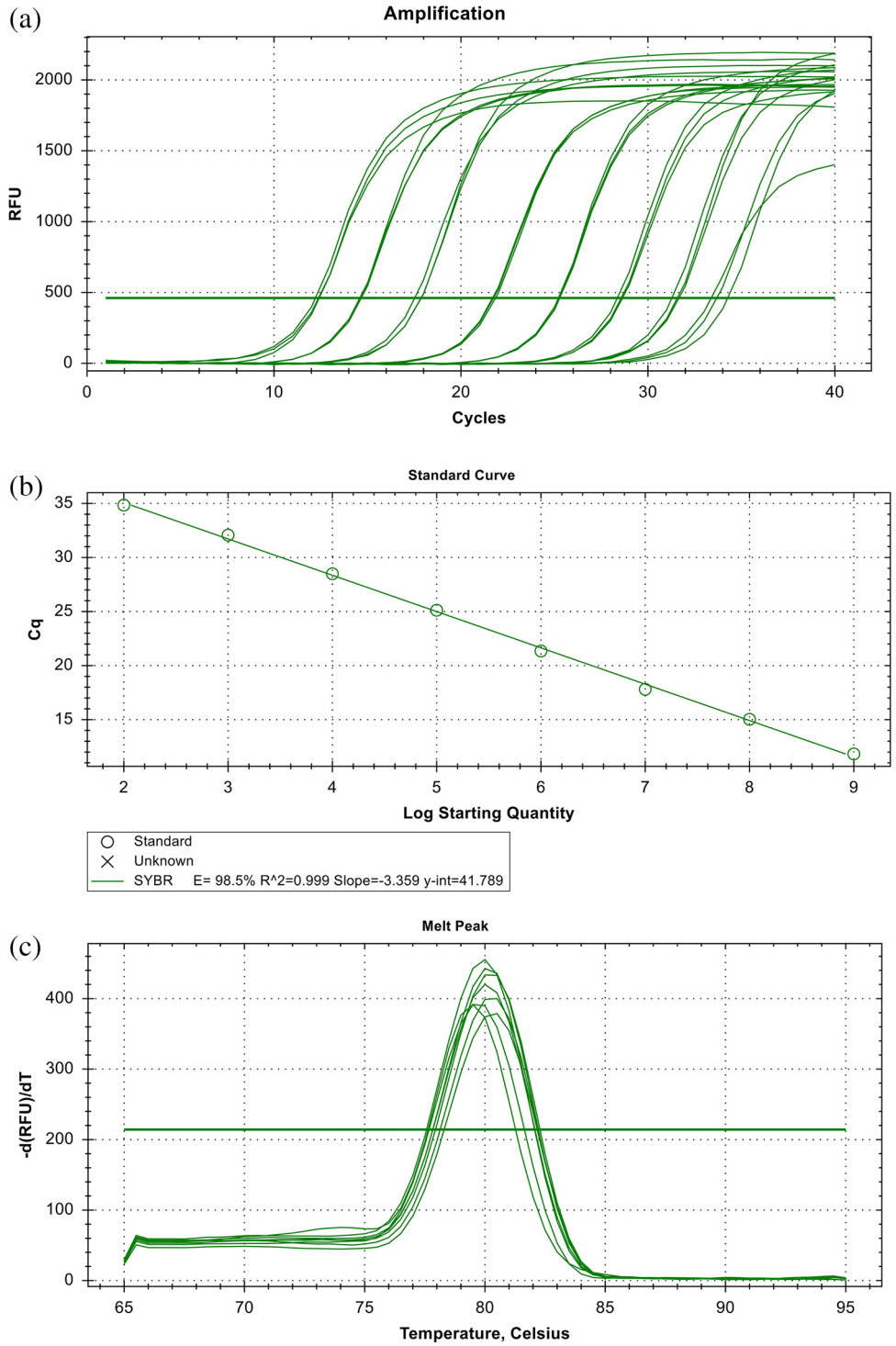

Fig. 1. a) Amplification curve of CaKoV by SYBR Green I-based real-time PCR. Ten-fold dilutions of standard template ranged from 1×10^8 to 1×10^1 copies/μL. b) Standard curve of CaKoV by SYBR Green I-based real-time PCR using a standard template diluted 1×10^8 to 1×10^1 copies/μL. The equation for the standard curve for CaKoV was $y = -3.359\ x + 41.789$, with R^2 values of 0.999. c) Melting curve. Dilutions ranged from 1×10^8 to 1×10^1 copies/μL, and the Tm value was 80 °C ± 0.5 °C (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

DNA/RNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed into cDNA using the PrimeScript 1 st Strand cDNA Synthesis kit (TaKaRa Bio, Kusatsu, Japan) according to the manufacturer's instructions. The cDNA was stored at -80 °C until use.

2.3. Preparation of standard plasmids

To amplify the 3D gene of CaKoV, a pair of primers was designed using Primer Premier using GenBank accession no. MN449341 as a reference sequence. The forward primer F1 was 5′-CCCTGGAACACCC AAGGCCGCT-3′ and reverse primer R1 was 5′-TCTGGTTGCCATAGAT GTGGTG-3′. The 504-base pair (bp) product was cloned into the pMD-19 T vector (TaKaRa). The recombinant plasmid was named pMD-19T-

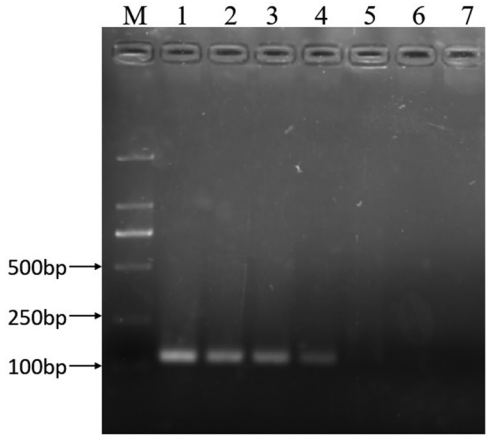

Fig. 2. Sensitivity analysis. The detection limit of the real-time PCR assay was 1 × 10^1 copies/μL. In contrast, conventional PCR had a detection limit of 1 × 10^3 copies/μL. The conventional PCR product was about 152bp. Template amounts for curves were 1 × 10^6–1 × 10^1 copies/μL and templates for lanes 1–6 were 1 × 10^6–1 × 10^1 copies/μL, and lanes 7 was negative control.

3D-CaKoV and verified using sequencing by Sangon (Shanghai, China). The plasmid was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA copy number was calculated using the following formula: DNA copy number (copy number/μL) = [6.02 × 10^{23} × plasmid concentration (ng/μL) × 10^{-9}]/[DNA length in nucleotides × 660]. The plasmid was serially diluted 10-fold and stored at −20 °C until use.

2.4. Primer design for SYBR green real-time PCR

Specific primers (F2 and R2) for CaKoV were designed to target the 3D gene using Primer Premier software. The forward primer F2 was 5′-TATGATCTGGATTACAAG-3′ and reverse primer R2 was 5′-AATTC TTAGCCATAGATG-3′. The primer was designed to amplify a 152-bp region of the 3D gene of CaKoV AH-1/CHN/2019 (GenBank accession no. MN449341). All primers were synthesized by Sangon Biotech.

2.5. SYBR Green I-based quantitative real-time PCR assay

The real-time PCR assay was performed in a 20-μL reaction volume using SuperReal PreMix Plus (Tiangen) according to the manufacturer's instructions. The reaction mixture contained 10 μL of SYBR mix, 0.6 μL each of F2 and R2, 1 μL of plasmid, and 7.8 μL of ddH_2O. The reaction was performed using a CFX 96 Real-time System (Bio-Rad, Hercules, CA, USA) under the following conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Each process was performed three times.

2.6. Sensitivity and specificity of real-time PCR assay

To determine the minimum detectable CaKoV, we used recombinant plasmids (1 × 10^8–1 × 10^1 copies/μL) as positive controls. Conventional RT-PCR was performed using primers F2 and R2. The results were analyzed using agarose gel electrophoresis. Each experiment was performed three times.

The specificity of the established real-time PCR assay was examined using canine distemper virus (CDV), canine astrovirus (CaAstV), canine coronavirus (CCV), canine circovirus (CaCV), and CPV nucleic acid as templates, with all templates used at concentrations of 1 × 10^7 copies/μL; ddH_2O used as a negative control.

2.7. Reproducibility of real-time PCR

Standard plasmids were chosen to evaluate the reproducibility of the real-time PCR assay. Every dilution was evaluated with regard to intra-assay and inter-assay variation. Every process was subjected to three parallel tests under similar amplification conditions. Real-time PCR was used to detect the coefficient of variation (CV) and determine the repeatability of inter-batch detection.

2.8. Evaluation of real-time PCR assay using clinical samples

In total, 57 fecal samples from different animal hospitals located in Anhui province were analyzed using real-time PCR assay; the details of all samples are listed in Table 2. All positive samples were confirmed using sequencing.

3. Results

3.1. Standard curve for real-time PCR

The 504-bp partial CaKoV 3D gene was amplified and cloned into the pMD-19 T vector, and the recombinant plasmids were used as standard templates. A linear standard curve was constructed using standard plasmids with dilutions of 1 × 10^8–1 × 10^1 copies/μL. The equation for the standard curve was $y = -3.359x + 41.789$ and had an R^2 value of 0.999 (Fig. 1a and b). Melting curve analysis revealed a single peak for the template ranging from 1 × 10^8 to 1 × 10^1 copies/μL, and the melting temperature (Tm) was 80 °C ± 0.5 °C (Fig. 1c).

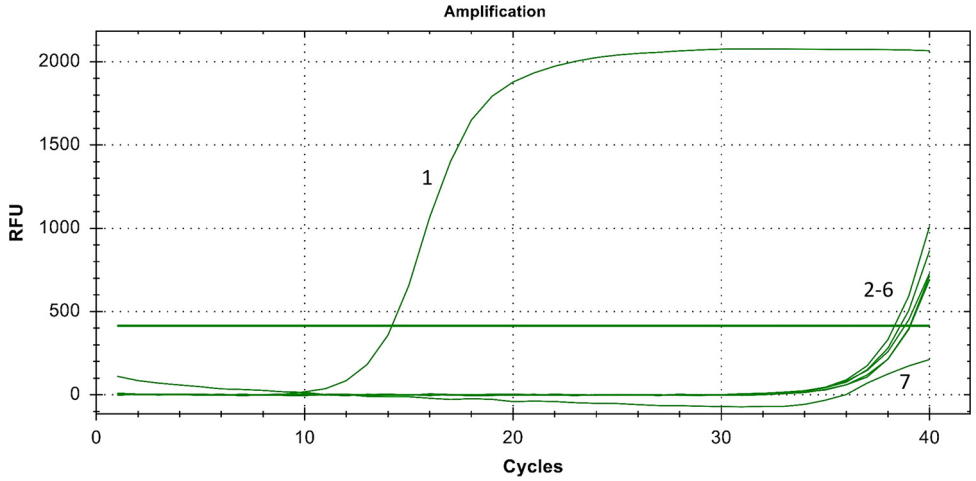

Fig. 3. Analysis of specificity. Sample 1 represents CaKoV virus, samples 2–6 represent CCV, CPV, CCoV, CDV, and CaAstV, and sample 7 is the negative control.

Table 1
Intra- and inter-reproducibility assay.

category	DNA standard (copies/uL)	Mean(Ct)	SD	CV (%)
Intra-assay	1×10^7	14.64	0.017	0.12
	1×10^5	21.72	0.025	0.11
	1×10^3	28.58	0.045	0.16
	1×10^1	34.25	0.066	0.19
Inter-assay	1×10^7	14.73	0.029	0.20
	1×10^5	21.84	0.042	0.19
	1×10^3	28.66	0.070	0.24
	1×10^1	34.43	0.110	0.30

3.2. Sensitivity and specificity of real-time PCR assay

The minimum detection limits of the real-time PCR and conventional PCR assays were 10^1 and 10^3 copies/μL, respectively. These results showed that the sensitivity of the real-time PCR assay was approximately 100-fold higher than that of conventional PCR (Fig. 2).

To validate the specificity of the real-time PCR assay, samples containing CaKoV and the DNA or cDNA of CDV, CPV, CCV, CaCV, and CaAstV were used as templates. Only CaKoV was amplified and no other taxa showed specific peaks in the melting curves. This indicates that the established real-time PCR assay is highly specific and does not cross-react with other pathogens (Fig. 3).

3.3. Reproducibility of real-time PCR assay

Standard plasmids were diluted (10^7, 10^5, 10^3, and 10^1 copies/μL) to assess intra- and inter-assay reproducibility. The intra-assay standard deviation (SD) ranged from 0.017 to 0.066, while inter-assay SD ranged from 0.029 to 0.110. The intra- and inter-assay CVs ranged from 0.11 % to 0.19 % and 0.2 % to 0.3 %, respectively (Table 1). These results demonstrate that the real-time PCR assay has good reproducibility.

3.4. Detection of CaKoV in clinical samples

In total, 57 clinical samples from animals with symptoms of diarrhea were evaluated using real-time PCR. The total positive detection rate was 8.77 % (5/57), whereas conventional PCR only detected 2 positive samples among the 57 samples. Importantly, all positive samples were confirmed using sequencing. These results show that the real-time PCR assay is effective for the clinical detection of CaKoV.

4. Discussion

CaKoV was first reported in 2011 in the USA and is widespread in China (Kapoor et al., 2011; (Li et al., 2016, 2018). Many studies have shown that CaKoV causes symptoms similar to those caused by some enteroviruses in dogs and may be associated with these viruses (Li et al., 2016; Miyabe et al., 2019). Thus, it is difficult to distinguish the viruses based on clinical symptoms and pathological changes alone in diseased dogs. Various approaches have been developed to detect CPV, CCV, and CDV; however, a rapid, accurate, and quantitative detection method for CaKoV is lacking. Accordingly, a method for detecting CaKoV is urgently needed.

Various methods are employed for virus detection, such as viral isolation, loop-mediated isothermal amplification, and conventional PCR. Although virus isolation is considered as the gold standard for pathogen detection, it is time-consuming and requires skilled technicians (Wang et al., 2017). Compared to these methods, the SYBR Green I real-time PCR assay is more convenient and suitable for clinical detection and can be used for quantitative analyses (Xu et al., 2016; Zhang et al., 2018).

In this study, an SYBR Green I-based quantitative real-time PCR assay was developed to detect CaKoV. The lower detection limit of the assay was 1×10^1 copies/μL and sensitivity was 100-fold higher than that of the conventional PCR. The assay showed high specificity with no cross-reactivity with other common enteroviruses. In addition, intra-assay and inter-assay CV values were low, indicating that the assay is repeatable. Furthermore, the assay was successfully applied to detect CaKoV in clinical samples. However, there are some limitations to this method. The control sample shows a peak at the beginning of 36 cycles. To overcome this problem, when we evaluated the results, if the curve was lower than the threshold throughout the experiment, the sample

Table 2
Details of all test samples.

No.	Variety	Age	Virus test results
1	Teddy	One month old	CPV + CaKoV
2	Teddy	One month old	CPV
3	Teddy	One month old	CPV
4	Teddy	One month old	Negative
5	Teddy	One month old	CDV
6	Teddy	One month old	CPV
7	Teddy	One month old	Negative
8	Teddy	One month old	CPV
9	Teddy	One month old	CPV
10	Teddy	Two month old	CPV + CaCV
11	Teddy	Two month old	CPV
12	Teddy	Two month old	CPV + CaKoV
13	Teddy	Two month old	CPV
14	Teddy	Two month old	CPV
15	Teddy	Two month old	Negative
16	Teddy	Three month old	CPV
17	Pug	One month old	Negative
18	Pug	One month old	CDV
19	Pug	One month old	CPV
20	Pug	One month old	CPV
21	Pug	One month old	CPV
22	Pug	One month old	CPV
23	Pug	Three month old	Negative
24	Pug	Three month old	CPV
25	Pug	One year old	Negative
26	Bichon Frise	One month old	Negative
27	Bichon Frise	One month old	CPV
28	Bichon Frise	Three month old	CPV
29	Bichon Frise	Three month old	CPV
30	Bichon Frise	Four month old	Negative
31	Bichon Frise	Six month old	CPV + CaAstV
32	Bichon Frise	One year old	Negative
33	Bichon Frise	One and a half years old	Negative
34	Pomeranian	One month old	CPV
35	Pomeranian	One month old	CPV
36	Pomeranian	One month old	Negative
37	Pomeranian	Four month old	CPV + CaKoV
38	Pomeranian	Four month old	Negative
39	Chihuahua	Two month old	CPV
40	Chihuahua	Two month old	CPV
41	Chihuahua	Six month old	Negative
42	Chihuahua	One year old	CPV
43	Native dog	Two month old	Negative
44	Native dog	Two month old	CPV
45	Native dog	Six month old	Negative
46	Native dog	One year old	Negative
47	Native dog	One year old	CPV
48	Golden retriever	Six month old	CPV + CaKoV
49	Golden retriever	Six month old	Negative
50	Golden retriever	Seven month old	CPV
51	Golden retriever	Two years old	Negative
52	Labrador	Four month old	Negative
53	Labrador	Four month old	CPV + CaKoV
54	Labrador	One year old	Negative
55	Corgi	One month old	CCV
56	Corgi	Three month old	Negative
57	Samoyed	Four month old	Negative

Abbreviation: Canine kobuvirus (CaKoV), Canine distemper virus (CDV), Canine astrovirus (CaAstV), Canine coronavirus (CCV), Canine circovirus (CaCV), and Canine parvovirus (CPV).

was considered as negative. When the curve was higher than the threshold after 36 cycles, the result was judged as ambiguous and the test was repeated. For the second test, if the value exceeded the threshold before 36 cycles, the sample was considered as positive; otherwise, it was judged as negative.

In conclusion, we successfully established an SYBR Green I-based quantitative real-time PCR assay for CaKoV detection. The assay is rapid, sensitive, specific, and repeatable using field samples. This method can be practically implemented to prevent and control CaKoV-mediated gastroenteritis in dogs.

Author statement

Yong Wang and Yongqiu Cui conceived of the study, carried out the experiment and drafted the manuscript, contributed equally to this work. Yeqiu Li participated in the data collection and analysis. Da Zhang and Jianfei Sun involved in drafting of the manuscript. Xu Guo and Guangqing Liu participated in statistical analysis. Yongdong Li and Shudong Jiang conceived of the study, revising the manuscript critically. All authors have read and approved the final manuscript.

Compliance with ethical standards

All experiments were compliant with the ethical standards of Anhui Agricultural University.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported by the Ningbo Health Branding Subject Fund (No. ppxk2018-10), key research project of National Science and Technology (No. 2016YFD0501003), State Key Laboratory of Genetically Engineered Veterinary Vaccines (No. AGVSKL-ZD-202010) and the Science and Technology Promotating Agriculture Innovation Project of Shanghai (No. 2019 No.3-3).

References

Ambert-Balay, K., Lorrot, M., Bon, F., Giraudon, H., Kaplon, J., Wolfer, M., Lebon, P., Gendrel, D., Pothier, P., 2008. Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. J. Clin. Microbiol. 46, 1252–1258.

Carmona-Vicente, N., Buesa, J., Brown, P.A., Merga, J.Y., Darby, A.C., Stavisky, J., Sadler, L., Gaskell, R.M., Dawson, S., Radford, A.D., 2013. Phylogeny and prevalence of kobuviruses in dogs and cats in the UK. Vet. Microbiol. 164, 246–252.

Chen, J., Zhao, Z., Chen, Y., Zhang, J., Yan, L., Zheng, X., Liao, M., Cao, W., 2018. Development and application of a SYBR green real-time PCR for detection of the emerging avian leukosis virus subgroup K. Poult. Sci. 97, 2568–2574.

Chen, N., Ye, M., Xiao, Y., Li, S., Huang, Y., Li, X., Tian, K., Zhu, J., 2019. Development of universal and quadruplex real-time RT-PCR assays for simultaneous detection and differentiation of porcine reproductive and respiratory syndrome viruses. Transbound. Emerg. Dis. 66, 2271–2278.

Choi, S., Lim, S.I., Kim, Y.K., Cho, Y.Y., Song, J.Y., An, D.J., 2014. Phylogenetic analysis of astrovirus and kobuvirus in Korean dogs. J. Vet. Med. Sci. 76, 1141–1145.

Di Martino, B., Di Felice, E., Ceci, C., Di Profio, F., Marsilio, F., 2013. Canine kobuviruses in diarrhoeic dogs in Italy. Vet. Microbiol. 166, 246–249.

Gao, S., Du, J., Tian, Z., Niu, Q., Huang, D., Wang, J., Luo, J., Liu, G., Yin, H., 2020. A SYBR green I-based quantitative RT-PCR assay for bovine ephemeral fever virus and its utility for evaluating viral kinetics in cattle. J. Vet. Diagn. Invest. 32, 44–50.

Kapoor, A., Simmonds, P., Dubovi, E.J., Qaisar, N., Henriquez, J.A., Medina, J., Shields, S., Lipkin, W.I., 2011. Characterization of a canine homolog of human aichivirus. J. Virol. 85, 11520–11525.

Li, C., Wei, S., Guo, D., Wang, Z., Geng, Y., Wang, E., Zhao, X., Su, M., Wang, X., Sun, D., 2016. Prevalence and phylogenetic analysis of canine kobuviruses in diarrhoetic dogs in northeast China. J. Vet. Med. Sci. 78, 7–11.

Li, M., Yan, N., Wang, M., Zhang, B., Yue, H., Tang, C., 2018. Prevalence and genomic characteristics of canine kobuvirus in southwest China. Arch. Virol. 163, 459–466.

Miyabe, F.M., Ribeiro, J., Alfieri, A.F., Alfieri, A.A., 2019. Detection of canine kobuvirus RNA in diarrheic fecal samples of dogs with parvoviruses. Braz. J. Microbiol. 50, 871–874.

Olarte-Castillo, X.A., Heeger, F., Mazzoni, C.J., Greenwood, A.D., Fyumagwa, R., Moehlman, P.D., Hofer, H., East, M.L., 2015. Molecular characterization of canine kobuvirus in wild carnivores and the domestic dog in Africa. Virology 477, 89–97.

Rao, X., Sun, J., 2015. Development of SYBR green I based real-time RT-PCR assay for specific detection of watermelon silver mottle virus. Iran. J. Biotechnol. 13, 20–24.

Ribeiro, J., Headley, S.A., Diniz, J.A., Pereira, A.H., Lorenzetti, E., Alfieri, A.A., Alfieri, A.F., 2017. Extra-intestinal detection of canine kobuvirus in a puppy from Southern Brazil. Arch. Virol. 162, 867–872.

Wang, Y., Yang, K., Bai, C., Yin, D., Li, G., Qi, K., Wang, G., Li, Y., 2017. Development of a SYBR Green I real-time PCR for the detection of the orf virus. AMB Express 7, 21.

Xu, M.Y., Liu, S.Q., Deng, C.L., Zhang, Q.Y., Zhang, B., 2016. Detection of Zika virus by SYBR green one-step real-time RT-PCR. J. Virol. Methods 236, 93–97.

Zhang, T., Chen, J., Wang, C., Zhai, X., Zhang, S., 2018. Establishment and application of real-time fluorescence-based quantitative PCR for detection of infectious laryngotracheitis virus using SYBR Green I. Poult. Sci. 97, 3854–3859.