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Short communication

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

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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...
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 1st 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 AAGGCGCT-3' and reverse primer R1 was 5'-TCTTGTGTCATAGAT GTGGTG-3'. The 504-base pair (bp) product was cloned into the pMD-19 T vector (TaKaRa). The recombinant plasmid was named pMD-19T-
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 × 10²³ × 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’-AATTCTTAGCATAAGT-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 ddH₂O. 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 × 10⁸-1 × 10⁵ 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 × 10⁷ copies/μL; ddH₂O 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 × 10⁸-1 × 10⁵ copies/μL. The equation for the standard curve was y = -3.359x + 41.789 and had an R² value of 0.999 (Fig. 1a and b). Melting curve analysis revealed a single peak for the template ranging from 1 × 10⁸ to 1 × 10⁵ 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.
3.2. Sensitivity and specificity of real-time PCR assay

The minimum detection limits of the real-time PCR and conventional PCR assays were $10^7$ 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 0.20. The intra- and inter-assay CVs ranged from 0.11 % to 0.30 %, 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-based quantitative real-time PCR assay was developed to detect CaKoV. The lower detection limit of the assay was $1 \times 10^3$ 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

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**Table 1**

| category | DNA standard (copies/μL) | Mean(Ct) | SD  | CV (%) |
|----------|-------------------------|----------|-----|--------|
| Intra-assay | $1 \times 10^7$ | 14.64 | 0.017 | 0.12 |
|          | $1 \times 10^5$ | 21.72 | 0.025 | 0.11 |
|          | $1 \times 10^3$ | 28.58 | 0.045 | 0.16 |
|          | $1 \times 10^1$ | 34.25 | 0.060 | 0.19 |
| Inter-assay | $1 \times 10^7$ | 14.73 | 0.029 | 0.20 |
|          | $1 \times 10^5$ | 21.84 | 0.042 | 0.19 |
|          | $1 \times 10^3$ | 28.66 | 0.070 | 0.24 |
|          | $1 \times 10^1$ | 34.43 | 0.110 | 0.30 |

**Table 2**

| 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 | CPV                  |
| 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 | CPV                  |
| 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 Guanguing 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.

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