A Duplex SYBR Green I-based Real-time Polymerase Chain Reaction Assay for Rapid Detection of Canine Kobuvirus and Canine Circovirus

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Abstract

Background: Canine Kobuvirus (CaKoV) and Canine Circovirus (CaCV) are viruses that infect dogs causing diarrheal symptoms that are very similar. However, there is no clinical method to detect a co-infection of these two viruses.

Results: In this study, a duplex SYBR Green I-based quantitative real-time polymerase chain reaction (PCR) assay for the rapid and simultaneous detection of CaKoV and CaCV was established. CaKoV and CaCV were distinguished by their different melting temperature which was 86°C for CaKoV and 78°C for CaCV. The assay was highly specific, with no cross-reactivity with other common canine viruses and demonstrated high sensitivity. The detection limits of CaKoV and CaCV were $8.924 \times 10^1$ copies/μL and $3.841 \times 10^1$ copies/μL, respectively. The highest intra- and inter-assay Ct value variation coefficients (CV) of CaKoV were 0.40% and 0.96%, respectively. For CaCV, the highest intra- and inter-assay Ct value variation coefficients were 0.26% and 0.70%, respectively. In 57 clinical samples, positive detection rates of CaKoV and CaCV were 8.77% (7/57) and 15.79% (9/57), respectively. The co-infection rate was 7.02% (4/57).

Conclusions: The duplex SYBR Green I-based real-time PCR assay established in this study is a fast, efficient, and sensitive method for the simultaneous detection of the two viruses and provides a powerful tool for the rapid detection of CaKoV and CaCV in clinical practice.

Highlights

1. The study establishes a novel assay for simultaneous detection of canine kobuvirus and canine circovirus.

2. SYBR Green I-based duplex real-time qPCR method was established with high sensitivity, specificity and reliability.

3. The study provides a tool that could be beneficial for clinical diagnostics of canine kobuvirus and canine circovirus infection as well as future research on the virus.

1 Background

Canine Kobuvirus (CaKoV) is a novel single-strand positive-sense RNA virus belonging to the Picornaviridae virus family[1], with a genome of 8.1–8.2 Kb. It contains one 7,332-7,341 nucleotides (NT) open read frame (ORF) that encodes 2,442-2,475 amino acids (AA)[2, 3]. CaKoV was identified in a feces sample of a diarrhea dog brought in from the United States in 2011[4], and has since been identified in the United Kingdom, Italy, South Korea, Tanzania and Japan[1, 5–7]. CaKoV is widespread in China, it was first detected in 2015 in northeast China[8], and has been subsequently identified in southwest and estern China[2, 9]. CaKoV exists in a wide range of body parts, including the digestive system, cerebellum, amygdala and liver[10]. However, CaKoV is currently considered a pathogen associated with intestinal
diseases; epidemiological investigations have shown that it has been detected both in dogs with diarrhea and in healthy dogs[11], suggesting that there may be a hidden infection of the virus, giving it a large transmission advantage.

Canine Circovirus (CaCV) is a single-stranded, non-capsule DNA virus belonging to the circovirus family[12]. It has two ORFs that encode replicase proteins and capsid proteins, respectively[13]. It was first reported in the United States in 2012[14], and has also been reported in China[15]. CaCV infects dogs of different ages, especially young dogs, with high infection rates, and also infects carnivores, such as wolves and badgers[16]. CaCV causes hemorrhagic enteritis in dogs, though this conclusion is controversial[17]. Notably, CaCV may play a role as a co-infectious factor in the development of diarrheal diseases[17].

CaKoV and CaCV are viruses that infect dogs causing diarrheal symptoms that are very similar. However, there is no clinical method to detect a co-infection of these two viruses. Therefore, it is necessary to establish a simple, sensitive and rapid method for simultaneous detection of these two viruses. Fluorescent quantitative polymerase chain reaction (PCR) technology has the advantages of high specificity, high sensitivity and good reproducibility, which results in reduced reaction time and intuitive results. Dual real-time quantitative fluorescence PCR is a method of rapid diagnosis that may be used to identify two kinds of viruses based on a difference in the melting temperature (Tm) value caused by the difference in GC content of the amplified fragments.

In this study, to effectively detect CaKoV and CaCV in dogs, a duplex SYBR Green I-based quantitative real-time PCR assay for CaKoV and CaCV was established.

2 Materials And Methods

2.1 Viruses and nucleic acid extraction

The CaKoV RNA and CaCV DNA genomes were extracted from positive fecal samples using the TIANamp Virus DNA/RNA kit (Tiangen, Beijing, China), according to the manufacturer’s instructions. Then, the CaKoV RNA genome was converted to cDNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Kusatsu, Japan). The following viral strains were used in this study: CaKoV, CaCV, canine astrovirus (CaAstV), canine distemper virus (CDV), canine coronavirus (CCV), and canine parvovirus (CPV).

2.2 Preparation of standard plasmids

The 3D of CaKoV and the Rep of CaCV were used as template DNA. Target fragments have been PCR-amplified using primers shown in Table 1 (CaKoV-F/CaKoV-R and CaCV-F/CaCV-R). Products were cloned into the pMD-19T vector in accordance with the manufacturer’s protocol and transformed into Escherichia coli DH5α cells. Recombinant plasmids were extracted using the EasyPure Plasmid MiniPrep kit (TransGen Biotech, Beijing, China) and named pMD-19T-CaKoV and pMD-19T-CaCV, respectively.
General Biosystems (Chuzhou, China) sequenced the recombinant plasmids. The concentrations of the recombinant plasmids were determined using an ND-2000 spectrophotometer (Thermo Scientific, Dreieich, Germany) and the copy number was calculated according to the formula: (plasmid concentration [ng] × 6.02 × 10^{23})/(genome length × 10^9 × 660 Da/bp). Next, the standard plasmids were serially diluted 10-fold and stored at -20°C for further use.

Table 1

| Primer name       | Sequences (5’-3’) | Product size (bp) |
|-------------------|-------------------|-------------------|
| CaKoV-F           | CCCTGGAACACCCAAGGCGCT | 504               |
| CaKoV-R           | TCTGGTTGCCCATAGATGTGGT |                   |
| CaCV-F            | ATAGTCTACACAAATGGACCAGC | 912               |
| CaCV-R            | TCAGTAGTTATACATGTGGAAAC |                   |
| CaKoV-SYBR-F      | TGGTCTCATTGACTACAT | 137               |
| CaKoV-SYBR-R      | AACACTTGTATCCAGAT |                   |
| CaCV-SYBR-F       | GTCCTGAGTGAACATTGG | 84                |
| CaCV-SYBR-R       | CAAGACAGATCATCATCAAGA |                   |

2.3 Primers design

MN449341 was used as a reference sequence for CaKoV and MN863535 was used as a reference sequence for CaCV. Two specific primers were designed using Primer Premier 5 for the 3D and Rep genes of CaKoV (CaKoV-SYBR-F and CaKoV-SYBR-R) and CaCV (CaCV-SYBR-F and CaCV-SYBR-R), respectively. The primers for conventional PCR were designed using the same conserved region. All primer sequences used in this study are listed in Table 1. General Biosystems Company (Chuzhou, China) synthesized all primers.

2.4 Optimization of the duplex real-time PCR assay

Standard plasmids of CaKoV and CaCV were used as templates to optimize the duplex real-time PCR assay. The reaction system including the parameters of PCR reaction, concentrations of primers and reagent was optimized, in order to get the best detection results.

2.5 Standard curves for CaKoV and CaCV

The 10-fold serially diluted recombinant plasmid standards of CaKoV and CaCV were used as templates with each concentration repeated thrice. According to the optimized reaction system, duplex SYBR Green I-based real-time PCR assay was performed to obtain standard curves for CaKoV and CaCV.
2.6 Sensitivity analysis

Standard plasmid concentrations for CaKoV and CaCV ranging from $10^1$ to $10^8$ copies/µL were applied to the optimized duplex real-time PCR assay to determine the minimum detection limit of this method.

2.7 Specificity analysis

The specificity of the duplex qPCR assay was evaluated using several viruses which are common in dogs. The DNA concentrations of CaCV and CPV and the cDNA concentrations of CaKoV, CaAstV, CDV, and CCV were $1 \times 10^7$ copies/µL. A negative control (ddH$_2$O) was also included. After each amplification, 2% agarose gel electrophoresis was performed to verify whether the expected product was obtained.

2.8 Reproducibility analysis

Three different concentrations of $10^7$, $10^5$ and $10^3$ were elected to assess the reproducibility. Each dilution was performed three parallel tests under the same conditions at equal intervals. The Ct value variation coefficients (CV) were calculated to determine intra- and inter-assay variation.

2.9 Detection of clinical samples

A total of 57 unknown clinical samples from diarrheic dogs were collected and then nucleic acid of the virus was extracted using the DNA/RNA kit. After all products were reverse transcribed, the duplex SYBR Green I-based real-time PCR assay was used to detect the target viruses. And the same samples were also tested using conventional PCR.

3 Results

3.1 Optimization of the duplex SYBR Green I-based real-time PCR assay

The total volume of the optimized duplex real-time PCR system was 40 µL, including 20 µL of SuperReal PreMix Plus, 0.6 µL of forward and reserve primes for CaKoV, 0.4 µL of forward and reserve primes for CaCV, 1 µL of each template, and ddH$_2$O. The conditions were $95^\circ C$ for 15 min, followed by 40 cycles of $95^\circ C$ for 10 s and $60^\circ C$ for 30 s. As shown in Fig. 1a-b, the Tms were 86 $^\circ C$ for CaKoV and 78 $^\circ C$ for CaCV. And as shown in Fig. 2, the duplex melting curve showed the same $T_m$ as the single experiment.

3.2 Establishment of CaKoV and CaCV standard curves

After calculation, the copy numbers of CaKoV and CaCV were $8.924 \times 10^{10}$ copies/µL and $3.841 \times 10^{10}$ copies/µL, respectively. The standard curves equations of $y=-3.329x+32.947$ and $y=-3.560x+34.821$, and the amplification efficiencies were 99.7% and 90.9%, for CaKoV and CaCV, respectively. The correlation coefficients ($R^2$) were all 0.999 for CaKoV and CaCV, respectively (Fig. 3a-b).

3.3 Sensitivity analysis
As shown in Fig. 4a-b, 10 folded dilution of CaKoV and CaCV were used as templates from $10^8$ to $10^1$. The amplification curve showed that the lowest sensitivity detected by the duplex real-time PCR assay was $10^1$ copies/µL.

### 3.4 Specificity analysis

As shown in Fig. 5, CaKoV, CaCV, CaAstV, CDV, CCV and CPV templates were amplified using the newly developed duplex real-time PCR assay, and ddH$_2$O was used as a negative control. There were no specific melting peaks for other viruses and negative control as well as the target viruses could be distinguished.

### 3.5 Repeatability analysis

As shown in Table 2 and Table 3, the intra- and inter-assay CVs for CaKoV and CaCV were small. The intra-assay CV values for CaKoV and CaCV ranged from 0.21–0.34% and 0.04–0.26%, respectively and the inter-assay CV values for CaKoV and CaCV ranged from 0.31–0.96% and 0.16–0.70%, respectively.

**Table 2**

| Category   | DNA standard (copies/µL) | Mean (Ct) | SD   | CV (%) |
|------------|-------------------------|-----------|------|--------|
| Intra-assay| $1 \times 10^7$         | 11.91     | 0.04 | 0.34   |
|            | $1 \times 10^5$         | 18.83     | 0.08 | 0.40   |
|            | $1 \times 10^3$         | 25.68     | 0.06 | 0.21   |
| Inter-assay| $1 \times 10^7$         | 12.24     | 0.12 | 0.96   |
|            | $1 \times 10^5$         | 19.17     | 0.13 | 0.66   |
|            | $1 \times 10^3$         | 26.11     | 0.08 | 0.31   |
### Table 3

Intra- and inter-assay CVs of CaCV.

| Category  | DNA standard (copies/µL) | Mean (Ct) | SD  | CV (%) |
|-----------|-------------------------|-----------|-----|--------|
| Intra-assay | 1×10^7                  | 11.61     | 0.03| 0.26   |
|           | 1×10^5                  | 18.91     | 0.03| 0.16   |
|           | 1×10^3                  | 25.92     | 0.01| 0.04   |
| Inter-assay | 1×10^7                  | 12.17     | 0.09| 0.70   |
|           | 1×10^5                  | 19.12     | 0.07| 0.34   |
|           | 1×10^3                  | 26.08     | 0.04| 0.16   |

### 3.6 Clinical sample analysis

As shown in Table 4, 57 unknown clinical samples from diarrheic dogs were used in the duplex SYBR Green I-based real-time PCR and conventional PCR assays. When using the duplex real-time PCR method, the positive rates of CaKoV and CaCV were 8.77% (5/57) and 14.04% (8/57), respectively, and the co-infection rate was 7.02% (4/57). However, positive rates of CaKoV, CaCV, and co-infection by the conventional PCR method were 5.26% (3/57), 10.53% (6/57), and 3.51% (2/57), respectively.

| Virus         | Total clinical samples | Positive rate (%) | qPCR | cPCR |
|---------------|------------------------|-------------------|------|------|
| CaKoV         | 57                     | 8.77 (5/57)       | 5.26 (3/57) |
| CaCV          | 57                     | 14.04 (8/57)      | 10.53 (6/57) |
| co-infection  | 57                     | 7.02 (4/57)       | 3.51 (2/57) |

### 4 Discussion

The major cause of canine gastroenteritis is viral infection, especially in dogs under one year of age[18]. The main clinical symptom of canine gastroenteritis is diarrhea, and the common viruses that cause diarrhea in dogs are CPV, CDV and CCV[19–21]. In addition, there are two new viruses, CaKoV and CaCV, which can also cause diarrhea in dogs[8, 13, 22]. CaKoV and CaCV infection is a serious threat to the health of dogs, the mortality rate is as high as 50–100%, which has caused huge economic losses to the canine industry[23]. CaKoV has been detected in cattle, sheep, pigs, and dogs in different countries[24–
Since circovirus was first identified in the United States in 2012[13], it has been reported in Italy, Germany, China, Thailand and other countries[15, 22, 28–30]. Because the clinical symptoms of the two viruses are very similar, it is difficult to differentiate and diagnose the two viruses[17, 23]. Therefore, a test that simultaneously detects and identifies these two viruses is required.

Viruses are detected in a variety of ways, such as loop-mediated isothermal amplification, enzyme-linked immunosorbent assays, and indirect immunofluorescence assays[31–34]. Compared to these traditional detection methods, real-time fluorescence quantitative PCR has great advantages in terms of sensitivity and specificity[33, 35], and detects virus even at lower concentrations[36]. In addition, compared with TaqMan-based real-time PCR, the SYBR Green I-based real-time PCR method is cheaper and easier[12]. Compared to a single assay, a dual assay was designed with the same sensitivity, but with the ability to detect multiple viruses. Therefore, a duplex SYBR Green I-based real-time PCR was developed that simultaneously detected CaKoV and CaCV.

In this study, a duplex SYBR Green I-based real-time PCR assay was successfully established. Two specific primers were designed based on the conserved regions, 3D for CaKoV and Rep for CaCV. The method distinguished the two viruses by their different Tm values, which were 86°C for CaKoV and 78°C for CaCV. The detection limits of CaKoV and CaCV were $8.924 \times 10^1$ copies/µL and $3.841 \times 10^1$ copies/µL, respectively, demonstrating the high sensitivity of the method. Moreover, when this method was used to detect other viruses, such as CaAstV, CDV, CCV, and CPV, there was no obvious melting curve, indicating that the assay had good specificity. The intra-assay and inter-assay CV values were low, demonstrating that the experiment could be repeated with a high degree of reproducibility. Furthermore, the duplex SYBR Green I-based real-time PCR and conventional PCR assays were used to detect viruses within clinical samples. The positive infection rate of the former was significantly higher than that of the latter, indicating that the duplex SYBR Green I-based real-time PCR assay was more suitable for the detection of these two viruses.

**5 Conclusions**

In conclusion, a duplex SYBR Green I-based real-time PCR assay for the detection of CaKoV and CaCV was successfully established. This assay is rapid, has high sensitivity, high specificity, a simple operation, and low price. In addition, this method will facilitate the detection and differential diagnosis of these two viruses and has important significance for their study and prevention.

**Declarations**

**Ethics approval and consent to participate**

All experiments were compliant with the ethical standards of Anhui Agricultural 220 University (Permit number: SYXXK 2016-007).

**Consent for publication**
Not applicable.

**Availability of data and materials**

Availability of data and materials All data generated or analyzed in this study can be obtained within the tables and figures of the manuscript.

**Conflicts of interest/Competing interests**

The authors declare no conflict of interest.

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**Author contributions**

YP and JC conceived of the study, carried out the experiment and drafted the manuscript, contributed equally to this work. JW and YXW participated in the data collection and analysis. JZ, JY, and FX participated in statistical analysis. YW conceived of the study, revising the manuscript critically. All authors have read and approved the final manuscript.

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Figures

**Figure 1**

Melting curve analysis. (a) Melting curve analysis of canine kobuvirus (CaKoV). The single peak has good specificity (Tm value = 86 °C). (b) Melting curve analysis of canine circovirus (CaCV). The single peak has good specificity (Tm value = 78 °C).

**Figure 2**

Duplex melting curve analysis of canine kobuvirus (CaKoV) (Tm value = 86 °C) and canine circovirus (CaCV) (Tm = 76 °C). This result is highly consistent with the results of the single-curve analysis.
Figure 3

Standard curve analysis of the standard plasmids. (a) Standard curve of canine kobuvirus (CaKoV) (concentrations ranging from $8.924 \times 10^8$–$8.924 \times 10^1$ copies/μL; $y = -3.329x + 32.947$; $R^2 = 0.999$; Eff = 99.7%). (b) Standard curve of canine circovirus (CaCV) (concentrations ranging from $3.841 \times 10^8$–$3.841 \times 10^1$ copies/μL; $y = -3.560x + 34.821$; $R^2 = 0.999$; Eff = 90.9%).

Figure 4

Sensitivity analysis. Amplification curve of SYBR Green I real-time polymerase chain reaction (PCR) using the standard canine kobuvirus (CaKoV) and canine circovirus (CaCV) plasmids. (a) The recombinant plasmid standard of CaKoV is used as a template after 10-fold dilution of the concentrations ranging from $8.924 \times 10^8$–$8.924 \times 10^1$ copies/μL. (b) The recombinant plasmid standard of CaCV is used as a template after 10-fold dilution at concentrations ranging from $3.841 \times 10^8$–$3.841 \times 10^1$ copies/μL.
Figure 5

Specificity analysis. There is no cross-reactivity with canine astrovirus (CaAstV), canine distempervirus (CDV), canine coronavirus (CCV), and canine parvovirus (CPV) in the duplex melting curve except for canine kobuvirus (CaKoV) and canine circovirus (CaCV).