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Research article

Establishment of a chip digital PCR detection method for canine circovirus

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ABSTRACT

Canine circovirus (CanineCV), which is a new mammalian circovirus first reported in the United States in 2012, mainly causes diarrhea and vomiting in dogs. As CanineCV evolves and new subtypes emerge, there is an urgent need for new detection technologies to improve the sensitivity and detection rates of viruses in complex scenarios. A chip digital PCR(cdPCR) assay was established for the detection of CanineCV in this study. The results showed good reproducibility, specificity and a linear relationship; the minimum detection limit of CanineCV by cdPCR was 6.62 copies/μL, which is 10 times more sensitive than quantitative real-time PCR (qPCR). The qPCR-positive detection rate was 1 %, while CanineCV cdPCR (2.1 %) exhibited a greater positive detection rate. Fifteen complete genomes were sequenced and subdivided into CanineCV-1 and CanineCV-3. In conclusion, we developed a rapid, reliable, and specific cdPCR method for screening and monitoring canine CV.

1. Introduction

Canine circovirus (CanineCV) was first detected in canine serum samples from the United States in 2012 and was named canine circovirus genotype 1 (CanineCV-1) [1]. Li et al. identified a CanineCV in the liver of a dog that had necrotizing vasculitis and granulomatous lymphadenitis and named it the dog circovirus (DogCV) [2]. According to the International Committee on Taxonomy of Viruses (ICTV) in 2017, CanineCV was classified into the genus Canine circovirus in the family *Circoviridae* [3]. In 2014, CanineCV was isolated from a 5-to 6-month-old dog that died of acute gastroenteritis in Italy, and the complete genome sequence of Bari/411-13 was obtained by sequencing. Sequence alignment results revealed a high degree of homology between the Italian strain and the American strain [4]. In 2016, CanineCV was detected in China for the first time and the whole genome sequence of strain JZ98/2014 was

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obtained [5]. Genetic evolution analysis ervealed that the Chinese strain and the European and American strains were in different branches, which was speculated to be the reason for geographical isolation during the evolutionary process [5]. Subsequently, CanineCVs were reported in South America, Germany, Taiwan, Vietnam and other places.

In the clinical detection of CanineCV and epidemiological studies, the traditional PCR method is still the main detection method, but it has poor sensitivity. Han et al. reported that the detection limit of SYBR Green real-time PCR under optimized conditions was 6.18×10^1 copies/μL, and the lower limit of detection of common PCR was 6.18×10^4 copies/μL [6]. Chip digital PCR (cdPCR), a newly emerging PCR technique, was designed to enclose nanoliters of liquid in high-throughput microcells or microchannels for subsequent PCR amplification and direct fluorescence interpretation of amplified results; this technique can quantify nucleic acids without reliance on external standards, standard curves, or Ct values [7,8].

Therefore, we established and validated a cdPCR assay that can rapidly and accurately detect CanineCV in clinical samples. This accurate and reliable technical tool can be used for the detection and epidemiological investigation of CanineCV.

2. Materials and methods

2.1. Viral strains and test samples

Canine CV, rabies virus (RV), canine parvovirus (CPV), canine distemper virus (CDV), canine parainfluenza virus (CPIV), and canine adenovirus (CAV) were all stored in our laboratory. Fifty-one canine serum samples were collected from Jilin in 2018; 45 canine serum samples were collected from Beijing in 2020. Five hundred canine serum samples were collected from the Guangxi Zhuang Autonomous Region by the Guangxi Centre for Animal Disease Control and Prevention in 2022. A total of 596 canine serum samples were immediately at $\leq$4 °C to our laboratory and stored at −80 °C until use.

2.2. Primer and probe design

Replication associated protein (Rep) is a more conservative region in CanineCV. CanineCV whole-genome sequences were downloaded from the GenBank database (JZ98/2014 GenBank accession NC_KT946839), and the most conserved sequence (Rep gene) was obtained by DNAMAN 7.0 software and Primer Premier 5.0 software for primer and probe design. One pair of probe primers (CanineCV-qF and CanineCV-qR) was used for the detection of test samples. The probes were labeled with a 5′-fluorophore (FAM) and 3′- fluorescence quenching (BHQ) agent. One pair of full-length amplification primers (CanineCV-1CF, CanineCV-1CR) was used for the amplification of CannieCV-positive samples. All primers and probes used were synthesized by a commercial corporation (Biotech Bioengineering [Shanghai]). The details of the primers and probes used are listed in Table 1.

2.3. Nucleic acid extraction and reverse transcription

The collected canine serum samples were centrifuged at 5000 r/min for 5 min, and then, 200 μL of the supernatant was taken to extract nucleic acids according to the instructions of the nucleic acid extraction kit. Viral DNA/RNA from CanineCV, RV, CDV, CPIV, CAV, and CPV and 96 canine serum samples were harvested using the UNIQ-10 Columnar Viral Genomic DNA/TRIzol Total RNA Extraction Kit (Biotech Bioengineering [Shanghai]). RV, CDV and CPIV were subsequently reverse-transcribed to cDNA using a reverse transcription recording kit (Biotech Bioengineering [Shanghai]).

2.4. Preparation of the cloned plasmid standard

The full-length sequence of the CanineCV Rep gene was obtained by PCR amplification using CanineCV genomic DNA (JZ98/2014 GenBank accession NC_ KT946839) as a template. The CanineCV Rep gene was subsequently cloned and inserted into the pMD-19T vector and subsequently transformed into *Escherichia coli* FAST-T1 cells (TransGen Biotech). According to the instructions of the Axy Prep Plasmid Small Volume Extraction Kit (Corning[wujiang]), recombinant plasmids were extracted and sequenced by Sangon Biotech. A NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Dreieich, Germany) was used to determine concentration of the positive plasmid. Copy numbers were calculated according to the following formula: (plasmid concentration [ng] $\times$ 6.02×10^{23})/(genome length $\times$ 10^9 $\times$ 660 Da/bp). The plasmid was diluted tenfold consecutively and stored at −20 °C until use.

Table 1
Primers for the detection of CanineCV.

Primer name	Sequence (5′-3′)	Product size/bp
CanineCV RepqF	AGTAAGCAAGGCAAACGAAATG	120
CanineCV RepqR	CGAGAATTTGACGTAGGTCTCC	
CanineCV Probe	FAM-AACTGAAAGAGACGAAGAGCCTTGCC-BHQ	
CanineCV-1CF	GTGATATGCTGAGCCCATTC	2064
CanineCV-1CR	CTGAGACAAATGAAAGATATGAGGC	

2.5. cdPCR instrument operation procedure

1. For the preparation of the reaction system: a microtiter plate was prepared (QIAcuity Nanoplate 26k 24-well; QIAGEN Germany), and the system was configured in a PCR tube according to the instructions of the QIAcuity Probe PCR Kit (QIAGEN Germany) and mixed well.
2. The reaction mixture was then transferred to round sample wells on a microtiter plate. When the microtiter plate was removed from the box, only the side was removed. To avoid the bottom from being scratched or dirty and affecting the photo, the microtiter plate was placed in the white tray.
3. The sealing film was removed from the microplate box. The sealing film was blue, with a layer of transparent protective plastic on top and a layer of white protective plastic underneath. The blue sealing film was added to the microplate, and the left side of the microplate and the lower side were narrower.
4. Microplate sealing: After the sealing film was aligned with the plate, the film was carefully removed. First, a roller was used to roll the transparent protective plastic in the horizontal and vertical directions several times so that the blue sealing film is more stable and able to stick to the microporous plate. Then, the lower left corner of the blue sealing film was pressed horizontally and vertically back and forth at least three times more than during rolling. After adhering, the edge of the roller is taken along the board around the sealing edge.
5. After the microplate was on the machine, the ejector tray button on the upper right side of the front of the instrument was pushed, the instrument on the sample tray was launched, and the microplate from the white tray was carefully removed. The microplate was placed into the sample tray with the barcode facing the inside of the instrument, the eject tray was pressed again on the upper right side of the instrument, and the microplate entered the instrument. The search icon was clicked, the corresponding plate in the pop-up dialog box was selected, the apply button was clicked, the experiments were associated with the barcode of the microplate, the triangle run button was clicked, and the microplate started running.
6. Results analysis: After the test was complete, the results were analyzed with Software Suite software. The plate to be analyzed was selected, the three-dot icon was clicked on, and the analysis was performed. In the Absolute Quantification interface, the holes to be analyzed were selected, the imaging step on the right side of the software was selected for imaging analysis, and the choice according to the target (Target) or channel (Channel) was selected for analysis. The target or channel to be analyzed was selected, and the results were subsequently shown. After the target or channel to be analyzed was selected, the results were shown, and all the results were displayed at the bottom, including the results list, signal map, heatmap, histogram, 1D scatterplot, 2D scatterplot, and concentration diagram.

2.6. Optimization of cdPCR

The reaction system and reaction conditions were initially established according to the AceQ qRT−PCR Probe Master Mix (Vazyme Nanjing) reagent instructions. The 20 μL PCR system consisted of 2 × AceQ qPCR Probe Master Mix (10 μL), primers, probes, DNA/cDNA templates and distilled water. The reaction conditions, including the probe concentrations (0.2, 0.3, 0.4, 0.5 μmol/L), primer concentrations (0.6, 0.8, 1.0 μmol/L), and annealing temperatures (52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C), were optimized by the gradient method and matrix method. The concentration with the lowest Ct value according to the amplification curve was determined as the optimal primer concentration and probe concentration. Repeat experiments were performed for each sample and combination. No template control (NTC) was used as a negative control.

2.7. Standard curve and sensitivity assay of the cdPCR and qPCR results

To obtain the standard curve of qPCR, the prepared standard plasmid was serially diluted tenfold and amplified by qPCR (10^9~10^1 copies/μL) under the optimized conditions. The standard curve was plotted and the formula and correlation coefficient were calculated by using the logarithmic value of the copy number of plasmid standards as the X-axis and the mean value of the corresponding detected concentrations as the Y-axis. The sensitivities of the two detection methods were compared by determining their detection limits. Each concentration was tested three times, and nuclease-free water was used as a negative control.

2.8. Specificity and repeatability of the cdPCR and qPCR

Specificity tests were performed for the developed CanineCV cdPCR and qPCR methods using CanineCV, RV, CDV, CPIV, CAV and CPV as templates. We performed a series of tenfold serial dilutions of the p-Rep plasmid in series, ranging from 10^5 to 10^3 copies/μL, and interassay and intra-assay repeatability tests were performed in triplicate for each respective sample to assess variability in cdPCR and qPCR.

2.9. Evaluating cdPCR and qPCR assays with test samples

The cdPCR and qPCR assays were performed on 96 samples collected from Beijing and Jilin Provinces. The positive detection rate of the two methods was calculated to evaluate their sensitivity. Each reaction included a negative control, and the qPCR method with an additional setting of CanineCV-positive samples serving as a template positive control.

2.10. Sample detection, full-length amplification and sequencing of virulent strains

We collected five hundred canine serum samples for detection via the established cdPCR assay. Based on the whole-genome sequence of CanineCV published in the GenBank database on NCBI (JZ98/2014 GenBank accession NC_ KT946839), Primer Premier 5.0 software was used to design one pair of full-length amplification primers (CanineCV-1CF and CanineCV-1CR) (Table 1). The samples that were initially identified as positive by the test primers were pooled, and the full-length amplification primers were used to amplify the whole-genome sequence. Finally, 15 whole-genome sequence strains of CanineCV were successfully obtained.

2.11. Comparative analysis of sequences and construction of genetic evolutionary trees

SeqMan software of DNASTAR was used to assemble the sequencing results to obtain the whole-genome sequence of CanineCV. The sequences obtained and the published sequences of several CanineCV strains in the GenBank database were matched and compared for homology using DNASTAR's MegAlign software. A genetic evolutionary tree was constructed using the neighbor–joining method with Mega 7.0 software, and the span was set to help clarify the intended meaning, please consider replacing the selected text with 1000 bootstrap replicates.

3. Results

3.1. Plasmid standard optimization of the parameters of cdPCR and qPCR

CanineCV standard plasmids were successfully sequenced by Sangon Biotech and named p-Rep. According to the standard plasmid concentration, the copy number was 6.62×10^{10} copies/μL. Standard plasmids were used to optimize the annealing temperature, primer concentration, and probe concentration for cdPCR and qPCR. The final reaction system and conditions of the cdPCR and qPCR assays of CanineCV established in our study were based on the results of qPCR optimization. The plasmid was used as the template for qPCR, with annealing temperatures ranging from 52 °C to 62 °C. The results showed that the optimal annealing temperature was 56 °C, which generated the optimal Ct value. In addition, the probe concentrations and primer concentrations were optimized, and the results showed that the probe concentration (0.4 μmol/L) and the primer concentration (1 μmol/L) were the optimal primer concentration and probe concentration, respectively (Table 2).

After optimization of the reaction conditions, cdPCR and qPCR were successfully developed. The 20 μL qPCR mixture contained 10 μL of 2 × AceQ qPCR Probe Master Mix, 2 μL each of the CanineCV qF/R primers (10 μmol/L), 0.8 μL of the CanineCV Probe (10 μmol/L), 1 μL of the DNA/cDNA template, and 4.2 μL of RNase-free water. The qPCR amplifications were carried out as follows: 95 °C for 5 min; 40 cycles of 95 °C for 15 s and 58 °C for 30 s. A total volume of 40 μL of each cdPCR mixture contained 10 μL of 4× Probe PCR Master Mix, 4 μL each of the primers CanineCV qF/R (10 μmol/L), 1.6 μL of CanineCV dProbe (10 μmol/L), 1 μL of DNA/cDNA template, and 19.4 μL of RNase-free water. The qPCR amplifications were carried out as follows: 95 °C for 2 min; 40 cycles of 95 °C for 15 s and 58 °C for 30 s; green was chosen as the fluorescence channel; the exposure time was set to 500 ms; and the signal gain was set to 6.

3.2. Comparison of the sensitivity of the cdPCR and qPCR

The results showed that the limit of detection (LOD) of p-Rep was $6.62 \times 10^{\circ}$ copies/μL according to cdPCR (Fig. 1) and 6.62×10^{1} copies/μL according to qPCR (Fig. 2), indicating that cdPCR was ten times more sensitive than qPCR. In this study, plasmids ranging from $6.62 \times 10^{\circ}$ to 6.62×10^{9} copies/μL were used to generate standard curves. qPCR ($R^2 = 0.996$) (Fig. 3) exhibited excellent linearity according to the regression analysis.

3.3. Comparison of the specificity and repeatability of cdPCR and qPCR

The specificity of cdPCR and qPCR was evaluated using the DNA/cDNA of CanineCV, RV, CDV, CPIV, CAV, and CPV and a negative control as templates. The results showed that signals were obtained only from CanineCV, indicating that both assays were specific for the detection of CanineCV (Figs. 4 and 5). The established cdPCR detection method, which has strong specificity, did not result in cross-reactions. In the repeatability tests of the cdPCR and qPCR systems, three concentrations, 6.62×10^{5}, 6.62×10^{4}, and 6.62×10^{3} copies/μL CanineCV plasmids (p-Rep), were simultaneously tested in triplicate. The coefficients of variation (CVs) were calculated. These experiments revealed that the intraassay CV for concentration was 0.16–2.5 % and that the CV of the interassay was 1.18–3.82 %

Table 2
The selection of the optimal reaction concentrations for the primers and probe.

Concentration of primers(μmol/L)	Concentration of probe(μmol/L)			
	0.2	0.3	0.4	0.5
0.6	25.91 ± 0.08	25.72 ± 0.22	25.57 ± 0.10	25.4 ± 0.04
0.8	25.84 ± 0.32	26.25 ± 0.14	25.68 ± 0.09	25.77 ± 0.13
1	25.66 ± 0.09	26.07 ± 0.15	25.28 ± 0.21	25.77 ± 0.19

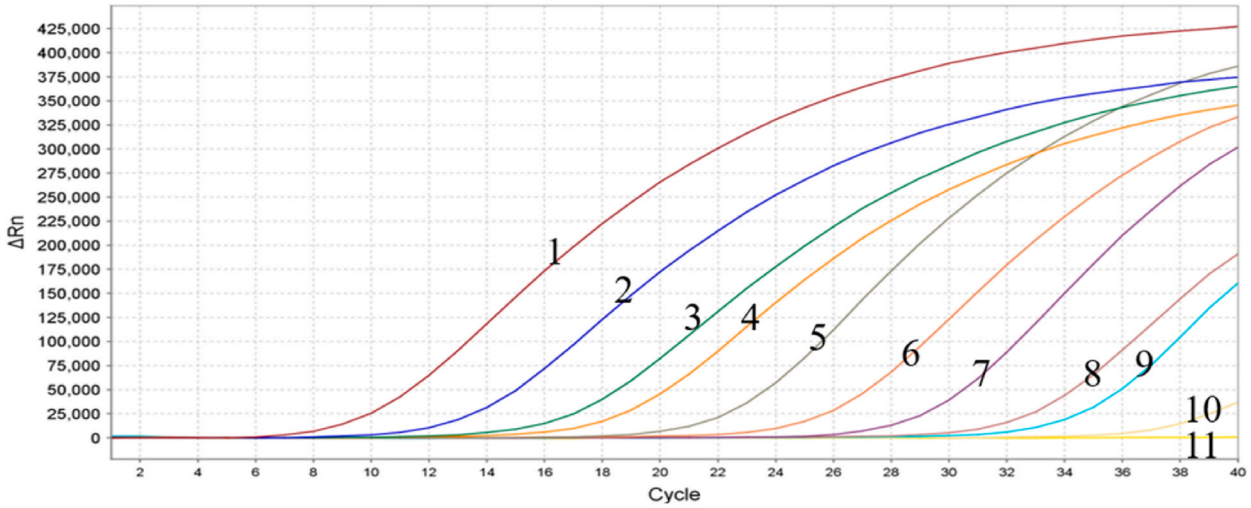

Fig. 1. The sensitivity of quantitative real-time PCR for CanineCV was 6.62×10^{1} copies/μL. 1–10: The concentrations of the standard plasmids were 6.62×10^{9}~$6.62 \times 10^{\circ}$ copies/μL; 11: Negative control.

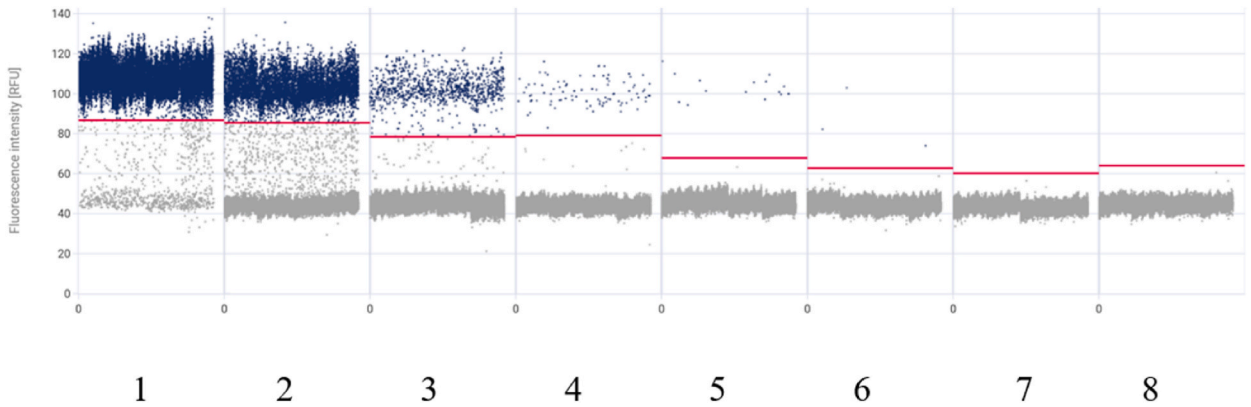

Fig. 2. The sensitivity test of chip digital PCR for CanineCV was $6.62 \times 10^{\circ}$ copies/μL 1–7: the concentrations of the standard plasmids were 6.62×10^{5}~6.62×10^{-2} copies/μL; 8: Negative control. X = Plasmid copy number Y = fluorescence intensity.

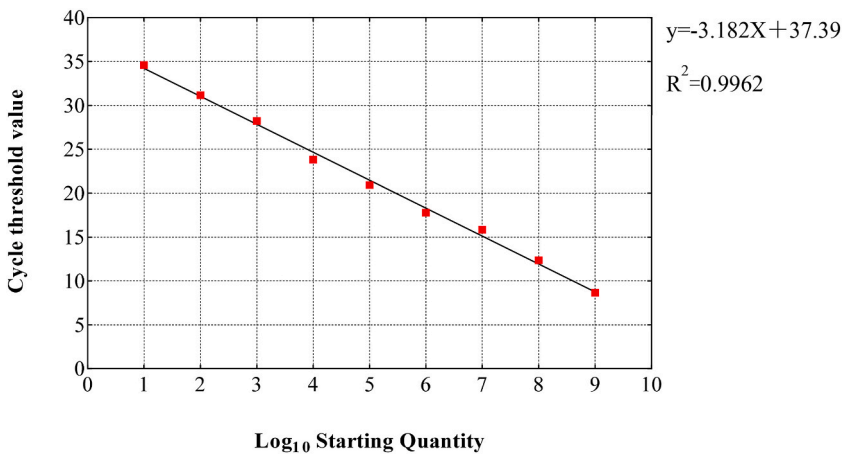

Fig. 3. Standard curve of the quantitative real-time PCR X = Plasmid copy number Y = threshold cycle.

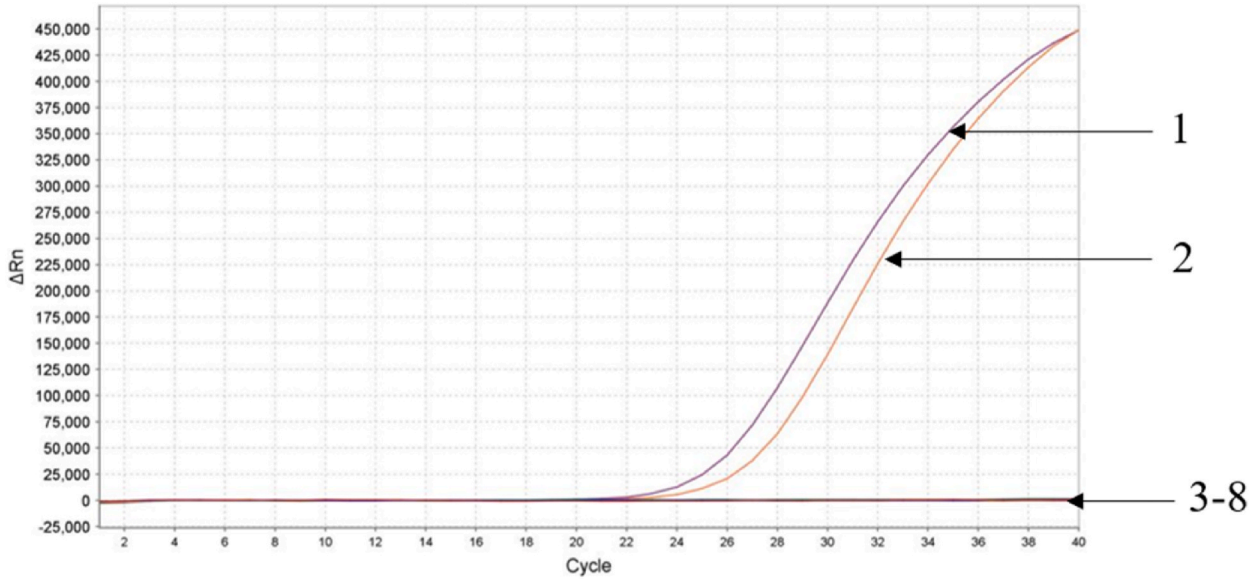

Fig. 4. Specificity test of quantitative real-time PCR for CanineCV 1: CanineCV; 2: p-Rep 3–8: rabies virus (RV), canine parvovirus (CPV), canine distemper virus (CDV), canine parainfluenza virus (CPIV), canine adenovirus (CAV) and negative control.

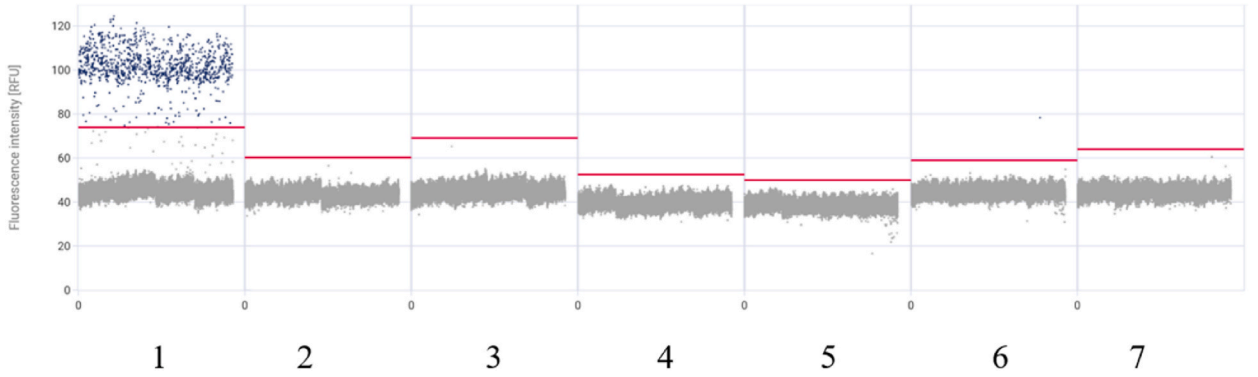

Fig. 5. Specificity test of chip digital PCR for CanineCV 1: CanineCV; 2–7:rabies virus (RV), canine parvovirus (CPV), canine distemper virus (CDV), canine parainfluenza virus (CPIV), and canine adenovirus (CAV) and negative control X = Plasmid copy number Y = fluorescence intensity.

(cdPCR), the intraassay CV for concentration was 0.05–0.35 % and that the CV of the interassay was 0.42–0.97 % (qPCR), which indicated that the repeatability of the dPCR and qPCR systems were high (Tables 3 and 4).

3.4. Clinical sample testing of the cdPCR and qPCR

The 96 clinical canine serum samples were tested simultaneously using cdPCR and qPCR to compare the sensitivities of the assays. The results showed that the positive rate of the cdPCR assay was 2.1 % (2/96), whereas that of the qPCR method was 1 % (1/96). This finding indicates that the cdPCR assay is more efficient at detecting CanineCV infection.

Table 3
Robustness and repeatability of the chip digital PCR assay for CanineCV.

Concentration of standard plasmids(copies/μL)	Intra-assay variability			Inter-assay variability		
	mean	SD	CV(%)	mean	SD	CV(%)
6.62×10^3	57.64 ± 1.45	1.45	2.53	56.00 ± 2.14	2.14	3.82
6.62×10^4	453.50 ± 0.73	0.73	0.16	446.07 ± 10.07	10.07	2.26
6.62×10^5	3854.53 ± 38.31	38.31	0.99	3822.37 ± 45.03	45.03	1.18

Table 4
Robustness and repeatability of the quantitative real-time PCR assay for CanineCV.

Concentration of standard plasmids(copies/μL)	Intra-assay variability			Intera-assay variability		
	mean	SD	CV(%)	mean	SD	CV(%)
6.62×10^3	27.59	0.04	0.15	27.97	0.2	0.73
6.62×10^4	24.78	0.01	0.05	24.43	0.1	0.42
6.62×10^5	21.87	0.08	0.35	21.39	0.21	0.97

3.5. Genotyping and genetic evolutionary analysis

The ORF2 nucleotide sequences of the sequenced CanineCV strains and the reference strains are shown in Fig. 6. CanineCV-1 can be further subdivided into three subtypes: CanineCV-1a, CanineCV-1b and CanineCV-1c. The CanineCV-1a subtype has the largest geographical range of strains, including one German strain, one Argentinean strain, one American strain, three Colombian strains, four Italian strains and three Iranian strains. CanineCV-1b included the Shandong strain SD16, the Jilin strain JL9 and two Guangxi strains; CanineCV-1c included the Shandong strain SD8, two Beijing strains, two Heilongjiang strains and three Thailand strains; and three strains were obtained from Hechi, Guangxi and Qinbei City, China. CanineCV-2 consists of European and American strains, including two Canadian strains, one Italian strain and six Norwegian strains; CanineCV-3 consists mainly of Guangxi strains, including five Guangxi strains, two Chongqing strains, the Jilin strain JL21 and ten Guangxi strains obtained by sequencing in this study.

4. Discussion

CanineCV is often associated with hemorrhagic gastroenteritis and granulomatous lymphadenitis, and infected dogs often present vomiting, acute diarrhea, digestive disorders and other symptoms [9]. CanineCV usually appears in the form of mixed infection with other pathogens such as canine parvovirus and canine distemper virus [10,11]. CanineCV alone or in combination with other enteroviruses can cause diarrheal disease in dogs [12]. The clinical manifestations of these viruses are similar to those of other enteroviruses. Acute gastroenteritis is the most common disease in kennel dogs and is caused by a wide range of pathogens, including viruses, bacteria, protozoa and parasites [9]. Han-Siang et al. investigated the prevalence of CanineCV in Taiwan and explored the correlation between CanineCV and canine diarrhea. The chi-square test showed that CanineCV was significantly correlated with canine diarrhea ($P = 0.00028$) [13]. Haiwong et al. investigated two outbreaks of severe bloody diarrhea in papillon breeding farms in March 2013 and February 2014. It was found that canine parvovirus and CanineCV were present in the infected dogs by PCR detection [14]. CanineCV acts synergistically with other viruses during viral coinfection. CanineCV may damage the host immune system and cause immunosuppression, thus causing coinfection with other pathogens and worsening of clinical disease. Or after other pathogens damage host intestinal epithelial cells and lymphocytes, the pathogenicity of CanineCV changes, thus exacerbating the disease [15]. Therefore, the detection of CanineCV in clinical samples is urgently needed. Although various methods for detecting canine circovirus have been developed over the past two years, they still have drawbacks, such as low sensitivity and easy contamination. Digital PCR is a new generation of PCR detection technology that does not depend on the establishment of standards or standard curves and is suitable for the detection of low-abundance products or samples with complex backgrounds. To better understand the prevalence of CanineCV in dogs, it is necessary to establish an efficient and accurate laboratory diagnostic method.

In 2017, we reported the first CanineCV SYBR Green I fluorescent PCR assay for the CanineCV ORF2 gene in China; the sensitivity was 4.67×10^1 copies/μL, and the percentage of positive canine serum samples was 12.5 % [16]. Sun et al. developed a hydrolysis probe-based CanineCV fluorescence quantitative PCR assay with a minimum detection limit of 8.42×10^1 copies/μL, and the sensitivity was a thousand times that of the conventional PCR method [17]. Wang et al. established an ELISA based on the CanineCV recombinant capsid protein for the first time and used it for the detection of canine serum. A kappa of 0.796 was statistically obtained between the iELISA and Western blot results, indicating good agreement between these assays [18]. However, conventional tests such as PCR and ELISA are not suitable for the current complex situation of widespread CanineCV transmission in China, foreign CanineCV importation, or the emergence of regional epidemic strains because of the complexity of the operational procedures and low sensitivity of these methods. The cdPCR method is a third-generation PCR technique. Compared with other detection methods for viruses, cdPCR has the advantages of absolute quantitation, good sensitivity and specificity, good precision, high tolerance, and a high clinical detection rate [19–22]. Thus, the cdPCR for the detection of CanineCV established in this study provides a rapid, convenient, and reliable technical tool for the laboratory diagnosis and epidemiological investigation of CanineCV.

In this study, a sensitive and feasible CanineCV cdPCR assay based on specific primers was developed. The standard curve showed a good linear relationship, indicating that the established method can be used to evaluate clinical samples. Moreover, this method is highly specific and does not exhibit cross-reaction with other pathogens. The qPCR method was used to detect CanineCV with a minimum detection limit of 6.62×10^1 copies/μL, whereas our cdPCR assay detected 6.62 copies/μL, and the sensitivity of cdPCR was approximately 10-fold greater than that of qPCR. CanineCV cdPCR exhibited greater sensitivity than qPCR in samples with low virus content, which showed that cdPCR is more conducive to diagnosing viral disease. For the same 96 clinical canine serum samples from Beijing and Jilin Provinces, the positive detection rate of cdPCR was 2.1 times greater than that of qPCR. Moreover, cdPCR showed high repeatability, with low intra-assay and inter-assay CVs. Moreover, five hundred serum samples from the Guangxi region were tested via the cdPCR method established in this study, and 15 positive samples were identified. The sequences of the positive samples obtained were analyzed for genetic evolution with other CanineCV sequences. Evolutionary tree analysis revealed that some of the

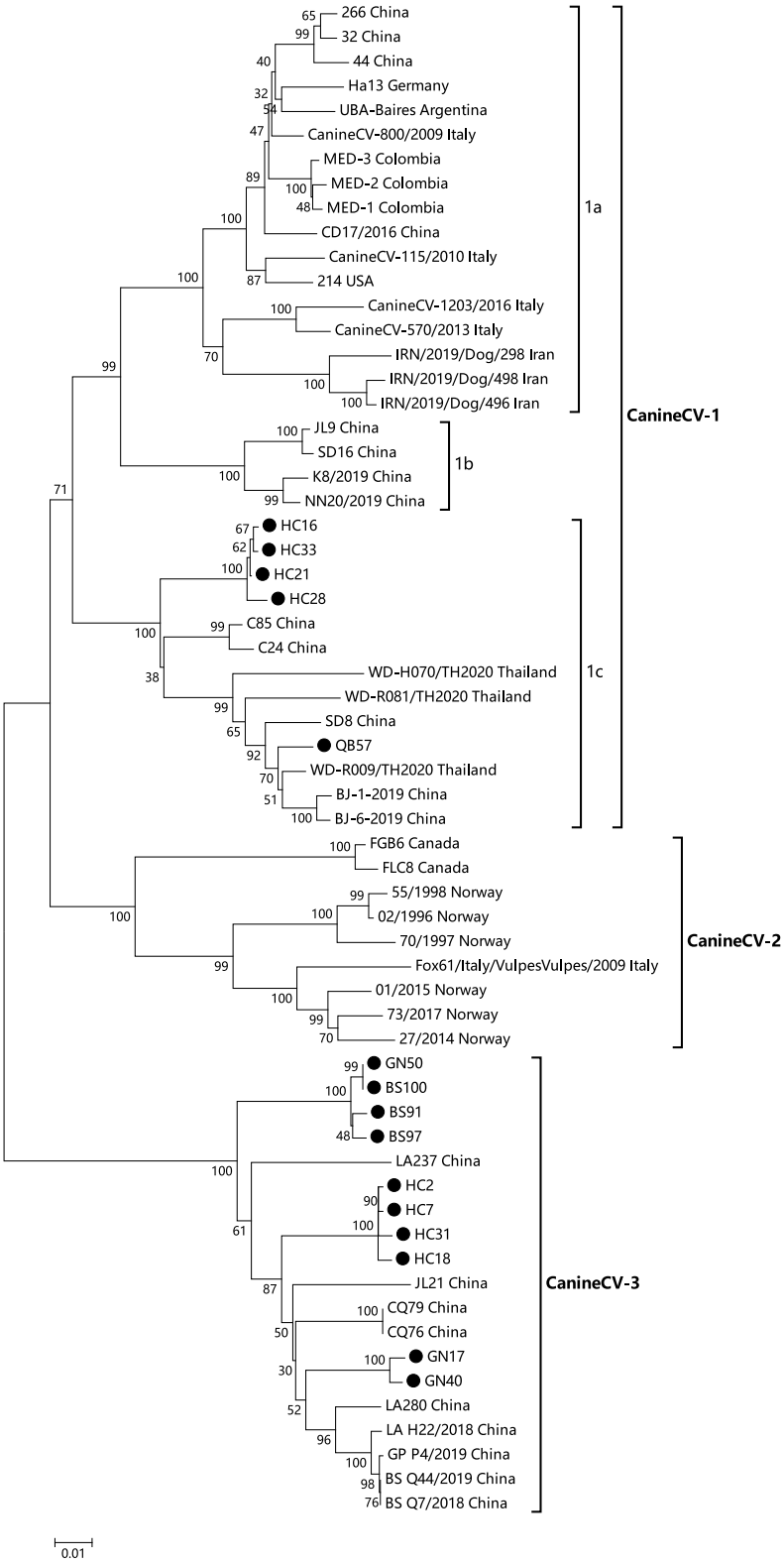

Fig. 6. Phylogenetic tree based on the ORF2 gene of CanineCV Based on the ORF2 nucleotide sequences of the CanineCV-sequenced strains and reference strains, a genetic evolutionary tree was constructed using the p-distance model of the neighbor-joining method with MEGA 7.0 software.

Guangxi strains were more closely related to CanineCV strains from the northern region, presumably because of the cross-regional movement of infected dogs.

In conclusion, a rapid, reliable, and specific cdPCR method for CanineCV detection was developed. To our knowledge, this is the first report on the development of a cdPCR for the differential detection and absolute quantification of CanineCV. In general, research on CanineCV is still in its early stage, and the results of the genome sequence analysis obtained in this study have certain scientific value for the prevention and control of CanineCV in canine populations and lay the foundation for further research on CanineCV in the future.

Consent for publication

Not applicable.

Ethics declarations

The experimental procedures were performed in strict accordance with the Guide for the Care and Use of Animals and were approved by the Animal Health Animal Care and Use Committee of Wenzhou University (wzu-2022-060). All participants provided informed consent to participate in the study. All participants provided informed consent for the publication of this article.

Data availability statement

All relevant data are provided in the present manuscript. The data associated with our study have been deposited into the NCBI repository [GN17: OR354703, GN40: OR354704, GN50: OR354705, HC2: OR354706, HC7: OR354707.HC16: OR354708, HC18: OR354709, HC21: OR354710, HC28: OR354711, HC31: OR354712, HC33: OR354713, QB57: OR354714, BS91: OR354715, BS97: OR354716 BS100 OR354717].

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CRediT authorship contribution statement

Xiaoxiao Lei: Writing – review & editing, Writing – original draft, Formal analysis. **Qiao Lv:** Resources, Methodology, Data curation. **Yan Qin:** Methodology. **Wei Chen:** Investigation. **Yanqing Hu:** Investigation. **Chenchen Zhao:** Formal analysis, Data curation. **Xinyu Zhang:** Formal analysis, Data curation. **Haixin Huang:** Validation, Supervision. **Yuying Li:** Validation, Supervision. **Jingyi Lu:** Investigation. **Tian Lan:** Funding acquisition, Conceptualization. **Wenchao Sun:** Formal analysis, Conceptualization. **Min Zheng:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] A. Kapoor, E.J. Dubovi, J.A. Henriquez-Rivera, et al., Complete genome sequence of the first canine circovirus, J. Virol. 86 (12) (2012 Jun) 7018.
[2] L. Li, S. McGraw, K. Zhu, et al., Circovirus in tissues of dogs with vasculitis and hemorrhage, Emerg. Infect. Dis. 19 (4) (2013 Apr) 534–541.
[3] X.F. Yuan, C.M. Yu, Comparison of the International Committee on Taxonomy of viruses (ICTV) 2017 classification system with the ninth classification report and data analysis, Acta Phytopathol. Sin. (2) (2019) 145–150.
[4] N. Decaro, V. Martella, C. Desario, et al., Genomic characterization of a circovirus associated with fatal hemorrhagic enteritis in dog, Italy, PLoS One 9 (8) (2014) e105909.
[5] W.C. Sun, H.H. Cao, M. Zheng, et al., Cloning and sequence analysis of the whole genome of canine circovirus, Chin. J. Virol. 32 (4) (2016) 429–435.
[6] Z.X. Han, Q. Du, W. Wang, et al., Establishment and application of SYBR GreenIreal-time PCR method for canine circovirus Rep gene, China Veterinary Science 49 (12) (2019) 1520–1526.
[7] L.B. Pinheiro, V.A. Coleman, C.M. Hindson, J. Herrmann, B.J. Hindson, S. Bhat, et al., Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification, Anal. Chem. 84 (2012) 1003–1011, https://doi.org/10.1021/ac202578x.
[8] B.J. Hindson, K.D. Ness, D.A. Masquelier, P. Belgrader, N.J. Heredia, A.J. Makarewicz, et al., High-throughput droplet digital PCR system for absolute quantitation of DNA copy number, Anal. Chem. 83 (2011) 8604–8610, https://doi.org/10.1021/ac202028g.
[9] A. Anderson, K. Hartmann, C. Leutenegger, et al., Role of canine circovirus in dogs with acute haemorrhagic diarrhoea, Vet. Rec. 180 (22) (2017) 542.

[10] A. Balboni, A. Terrusi, L. Urbani, et al., Canine circovirus and Canine adenovirus type 1 and 2 in dogs with parvoviral enteritis, Vet. Res. Commun. 46 (1) (2022) 223–232.
[11] T. Turan, H. Işıdan, Molecular characterization of canine astrovirus, vesivirus and circovirus, isolated from diarrheic dogs in Turkey, Iran. J. Vet. Res. 21 (3) (2020) 172–179.
[12] L. Niu, Z. Wang, L. Zhao, et al., Detection and molecular characterization of canine circovirus circulating in northeastern China during 2014–2016, Arch. Virol. 165 (1) (2020) 137–143.
[13] H. Han-Siang, T.H. Lin, H.Y. Wu, et al., High detection rate of dog circovirus in diarrheal dogs [J], BMC Vet. Res. 12 (1) (2016) 116.
[14]] T. Thaiwong, A.G. Wise, R.K. Maes, et al., Canine circovirus 1(CaCV-1) and canine parvovirus 2 (CPV-2) : recurrent dual infections in a papillon breeding colony[J], Veterinary Pathology 53 (6) (2016) 1204.
[15] A. Anderson, K. Hartmann, C.M. Leutenegger, et al., Role of canine circovirus in dogs with acute haemorrhagic diarrhoea[J], Vet. Rec. 180 (22) (2017) vetrec-2016-103926.
[16] W.C. Sun, C.Z. Xie, G.Y. Zhao, et al., Establishment of a quantitative real-time PCR SYBR green I method for CannieCV, Progress in Veterinary Medicine 38 (5) (2017) 6–10.
[17] P. Sun, Y.M. Ye, Y.Q. Li, et al., Establishment of hydrolysis probe system real-time PCR assay for rapid detection of canine circovirus, 3 Biotech 11 (11) (2021) 1–6.
[18] Wang Z, Shi Y J, Wang Y, et al. Detection of Antibodies against Canine Circovirus in Naturally and Experimentally Infected Canines by Recombinant Capsid Enzyme-Linked Immunosorbent Assay..
[19] A.A. Morley, Quantification, Digital PCR: a brief history, Biomol Detect Quantif 1 (1) (2014) 1–2.
[20] C. Sun, L. Liu, H.N. Vasudevan, et al., Accurate bulk quantitation of droplet digital PCR, bioRxiv (2021).
[21] R. Kanagal-Shamanna, Digital PCR: principles and applications, Methods Mol. Biol. 1392 (2016) 43–50.
[22] A.A. Kojabad, M. Farzanehpour, H. Galeh, et al., Droplet digital PCR of viral DNA/RNA, Current progress, challenges, and future perspectives, J. Med. Virol. 93 (7) (2021) 4182–4197.