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A Quadruplex Reverse Transcription Quantitative Polymerase Chain Reaction for Detecting Canine Coronavirus, Canine Rotavirus, Canine Parvovirus, and Canine Distemper Virus

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Abstract: Background: Canine coronavirus (CCoV), canine rotavirus (CRV), canine parvovirus (CPV), and canine distemper virus (CDV) cause gastroenteritis in dogs, and co-infections of these pathogens are common in China. In particular, CCoV and CRV are confirmed to have important zoonotic potential and cause public health issues. It is difficult to diagnose these diseases based only on clinical manifestations and pathological damage. Methods: In this study, four pairs of specific primers and probes targeting the CCoV M, CRV VP7, CPV VP2, and CDV N genes were designed. The reaction conditions, including the primer and probe concentrations, annealing temperatures, and reaction cycles, were optimized for the development of a quadruplex RT-qPCR for the detection of CCoV, CRV, CPV, and CDV. The assay was used to test 1028 clinical samples to validate its application. Results: A quadruplex RT-qPCR was successfully established for the differential detection of CCoV, CRV, CPV, and CDV, with good specificity, high sensitivity, and excellent repeatability. The assay could specifically detect CCoV, CRV, CPV, and CDV without cross-reactivity with the other canine viruses tested. It showed high sensitivity with limits of detection (LOD) of 1.1×10^2 copies/reaction for all four plasmid constructs. It showed excellent repeatability, with 0.05–0.90% intra-assay variation and 0.02–0.94% inter-assay variation. The 1028 clinical samples were tested using the quadruplex RT-qPCR and a reported reference RT-qPCR. The positivity rates of CCoV, CRV, CPV, and CDV were 9.53%, 0.97%, 25.68%, and 5.06% using the developed assay, and 9.05%, 0.88%, 25.68%, and 4.86% using the reference assay, with agreements higher than 99.32%. Conclusion: The results indicated that a rapid and accurate quadruplex RT-qPCR was developed for the detection and differentiation of CCoV, CRV, CPV, and CDV.

Keywords: canine coronavirus (CCoV); canine rotavirus (CRV); canine parvovirus (CPV); canine distemper virus (CDV); multiplex RT-qPCR

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1. Introduction

Dogs are important companion animals of humans, and dog populations are continuously increasing worldwide. Numerous factors cause canine diseases, of which canine coronavirus (CCoV), canine rotavirus (CRV), canine parvovirus (CPV), and canine distemper virus (CDV) are four important viruses that cause gastroenteritis. CCoV is an enveloped, single-stranded, positive-sense RNA virus with 27–32 kb genome, which belongs to the *Alphacoronavirus* genus in the *Coronaviridae* family [1]. CCoV was first identified in 1971, and two genotypes of CCoV (type I, type IIa and IIb) are prevalent in many countries worldwide [2]. CRV is a non-enveloped, double-stranded RNA virus with a genome size of approximately 18.5 kb and it belongs to the *Rotavirus* genus in the *Reoviridae* family [3].

The most frequent genotypes of CRV are G3P[3], and G3P[9] [4]. CPV is a non-enveloped, single-stranded negative-sense DNA virus with a genome of approximately 5.2 kb and it belongs to the *Protoparvovirus* genus in the *Parvoviridae* family [5]. CPV can be categorized into genotypes CPV-2, CPV-2a, CPV-2b, and CPV-2c [6]. CDV is an enveloped, single-stranded, non-segmented negative-sense RNA virus with a genome size of approximately 15.6 kb and it belongs to the *Morbillivirus* genus in the *Paramyxoviridae* family [7]. CCoV, CRV, CPV, and CDV cause similar symptoms in infected dogs, including gastrointestinal abnormalities such as diarrhea, bloody stools, vomiting, constipation, and loss of appetite, respiratory abnormalities such as coughing, runny nose, sneezing, shortness of breath, and other symptoms such as fever, eye inflammation, and depression. Of these pathogens, CPV is usually considered the most frequent and important clinical pathogen in canine gastroenteritis, with a mortality rate of up to 70% in puppies once infected [8]. Furthermore, CPV shows high genetic variation, and variants of CPV can cause serious harm to dogs [9,10]; some variants of CPV can even infect felines besides canines [11]. The G3 group CRV's inter-species transmission to children has been reported in Italy and China [12,13], and its zoonotic potential is of special interest to public health. In addition, co-infection with these viruses exacerbates clinical signs and pathological damage to infected dogs [5,14,15]. Currently, CCoV, CRV, CPV, and CDV are prevalent around the world and are highly hazardous to dogs.

Usually, the symptoms of gastrointestinal abnormalities associated with these four viruses are similar, such as diarrhea and vomiting. Co-infections of these viruses are common and can exacerbate manifestations and pathological changes, with increased morbidity and mortality [14,15]. This leads to difficulty in the differentiation and diagnosis of these four diseases based only on clinical symptoms. Therefore, the rapid detection of these viruses and accurate diagnosis of these diseases are essential to prevent and control these diseases. Real-time quantitative RT-PCR (RT-qPCR) has the advantages of sensitivity, accuracy, high throughput, and reduced risk of contamination, and it has been widely used to detect viral nucleic acids in different veterinary laboratories [16]. To date, singleplex RT-qPCR [17–19] and multiplex RT-qPCR [20–22] have been developed to detect one or more pathogens of CCoV, CRV, CPV, and/or CDV. Unfortunately, no multiplex RT-qPCR has been reported for the simultaneous detection of these four viruses. It would be useful to establish a multiplex RT-qPCR for the detection and differentiation of CCoV, CRV, CPV, and CDV in order to rapidly and accurately diagnose and survey these pathogens.

In this study, we have developed and evaluated a quadruplex RT-qPCR for the sensitive, rapid, and accurate detection and differentiation of CCoV, CRV, CPV, and CDV. The assay can detect four viral pathogens in one reaction within two hours, and it can be used for the detection and surveillance of diarrheic canine viruses. To the best of our knowledge, this is the first report on the development of quadruplex RT-qPCR for the detection of CCoV, CRV, CPV, and CDV.

2. Materials and Methods

2.1. Ethical Approval

This study did not involve any experiments on live animals. All feces and anal and nasal swabs were collected from pet hospitals. Ethical approval was not necessary for our study, as per the guidelines of the Guangxi Center for Animal Disease Control and Prevention (CADC), Nanning, China.

2.2. Vaccine Strains

The vaccine strains of canine adenovirus type 2 (CAV-2, Manhattan strain), CCoV (NL-18 strain), CPV (NL-35-D strain), CDV (Synder Hill strain) and canine parainfluenza virus (CPIV, NL-CPI-5 strain) were purchased from Zoetis Inc. (ZTS) (Lincoln, NE, USA). The vaccine strain of canine adenovirus type 1 (CAV-1, YCA18 strain) was purchased from Zhengzhou Muxin Vaccine Co., Ltd. (Zhengzhou, China), and the vaccine strain of rabies

virus (RABV, Pasteur RIV strain) was purchased from Intervet International B.V. (Boxmeer, The Netherlands). All vaccine strains were stored at −80 °C until use.

2.3. Positive Samples

Positive samples of CRV, canine circovirus (Canine CV), canine norovirus (CNV), and canine respiratory coronavirus (CRCoV), which were obtained from clinical samples and verified by genetic sequencing, were provided by the Guangxi CADC, Nanning, China. The positive samples were used to generate recombinant standard plasmid constructs, and were used as positive controls in this study. All positive samples were stored at −80 °C until use.

2.4. Clinical Samples

A total of 1028 clinical samples (the samples of feces, and anal and nasal swabs from each dog were mixed together in one tube for test of viruses, so the mixture of the feces, and anal and nasal swabs from the same dog was considered as a sample) were obtained from 1028 sick dogs with different signs of gastroenteritis. They were collected from 25 different pet hospitals in the Guangxi province, China, from December 2021 to June 2023. All samples were placed in a specialized medical ice box and transported to our laboratory at ≤4 °C within 12 h of collection. The total nucleic acids were extracted from clinical samples immediately using the Viral DNA/RNA Extraction Kit (Ver4.0) (Tianlong Technology, Xi'an, China), and used for the detection of CCoV, CRV, CPV, and CDV, or stored at −80 °C until use.

2.5. Primers and Probes

Thirty-six CCoV M gene sequences (including 7 strains of CCoV-I, 21 strains of CCoV-IIa, and 8 strains of CCoV-IIb), 22 CRV VP7 gene sequences (including 12 strains of G3, and 10 strains of G3P[3]), 37 CPV VP2 gene sequences (including 15 strains of CPV-2a, 12 strains of CPV-2b, and 10 strains of CPV-2c), and 43 CDV N gene sequences were downloaded from the NCBI GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/ (accession on 6 December 2021). These strain sequences are the typical strains from China and other countries around the world, and the information of these strains is shown in Supplementary Tables S1–S4. The nucleotide sequences were compared to find conserved regions using the MEGA-X 10.2.6 software (https://www.megasoftware.net/dload_win_gui/ (accessed on 20 July 2023)). These were selected as targeted regions to design the four pairs of specific primers and corresponding probes (Table 1) using the Oligo 7.0 software (https://www.oligo.net/doenlods.html (accessed on 20 July 2023)) for the detection of CCoV, CRV, CPV, and CDV. The principle of designing a primer and probe is to select the conserved region of the viral genome as targeting regions, and design primers and probes that are complementary to the template gene sequences. The lengths of the primer and probe are set between 20 and 30 bp, and the energy at the 5′ end of the primer and probe is higher than that at the 3′ end. Strive to ensure as much as possible that no hairpin loop formation is formed inside the primer or probe, and that no primer dimer is formed between the primer and probe. After the determination of the locations and sequences of the primers and probes, the Tm values are obtained automatically using the Oligo 7.0 software (https://www.oligo.net/doenlods.html (accessed on 20 July 2023)). The multiple nucleotide alignments of the reference sequences using the MEGA-X 10.2.6 software (https://www.megasoftware.net/dload_win_gui (accessed on 20 July 2023)) and the locations of the designed primers and probes are shown in Figure 1.

Table 1. The used primers and probes.

Name	Sequence (5′–3′)	Tm/°C	Genotype	Product/bp
CCoV(M)-F	GGTGGTATGAACATCGACAATT	57.3	CCoV-I, CCoV-II	134
CCoV(M)-R	TTAGATTTTACATAGTAAGCCCATCC	56.0		
CCoV(M)-P	FAM-CGTAATGGTTGCATTACCTAGCAGGACCAT-BHQ1	65.6		
CRV(VP7)-F	GCKGATCCAACTACAGC	52.2	CRV-G3P[3]	128
CRV(VP7)-R	CGTGATCTTTTGGACATTG	53.6		
CRV(VP7)-P	Texas red-GATGCGTGTTAATTGGAAGAAATGGTGGC-BHQ2	64.0		
CPV(VP2)-F	CCATTTACTCCAGCAGCTATG	56.4	CPV-2, CPV-2a, CPV-2b, CPV-2c	131
CPV(VP2)-R	CCACTAGTTCCAGTATGAGATGGTATT	58.7		
CPV(VP2)-P	CY5-ATGGAAACCAACCATACCAACTCCATGG-BHQ3	64.9		
CDV(N)-F	TTCATGGTGGCACTCATCTTGG	53.6	CDV	143
CDV(N)-R	GTTTCAATGCCAAATTTGATAGT	54.4		
CDV(N)-P	VIC-CAGGGAACAAGCCTAGAATTGCTGAAAT-BHQ1	63.4		

Note: K = G/T. The reference strains for designing the specific primers and probes were as follows: CCoV (Genbank accession number MZ420153.1), CRV (MT364840.1), CPV (AB054214.1), and CDV (JN896331.1).

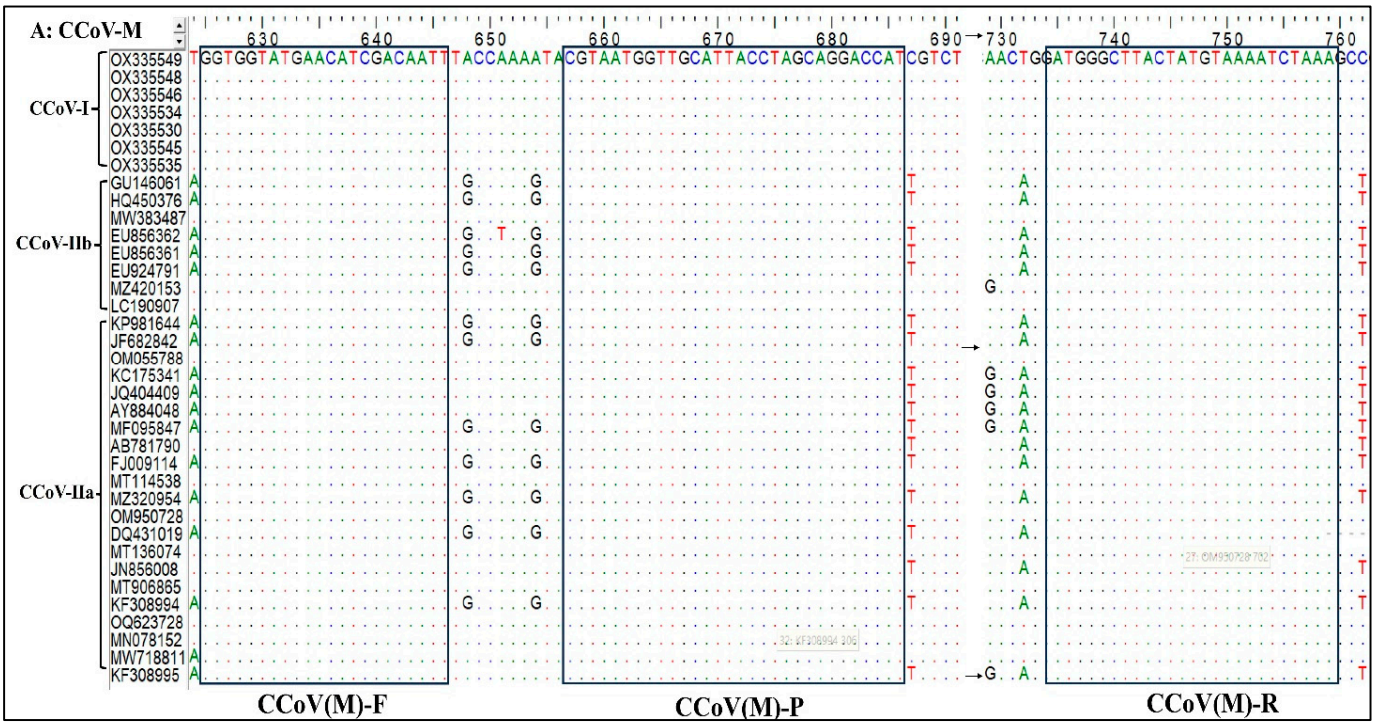

Figure 1. *Cont.*

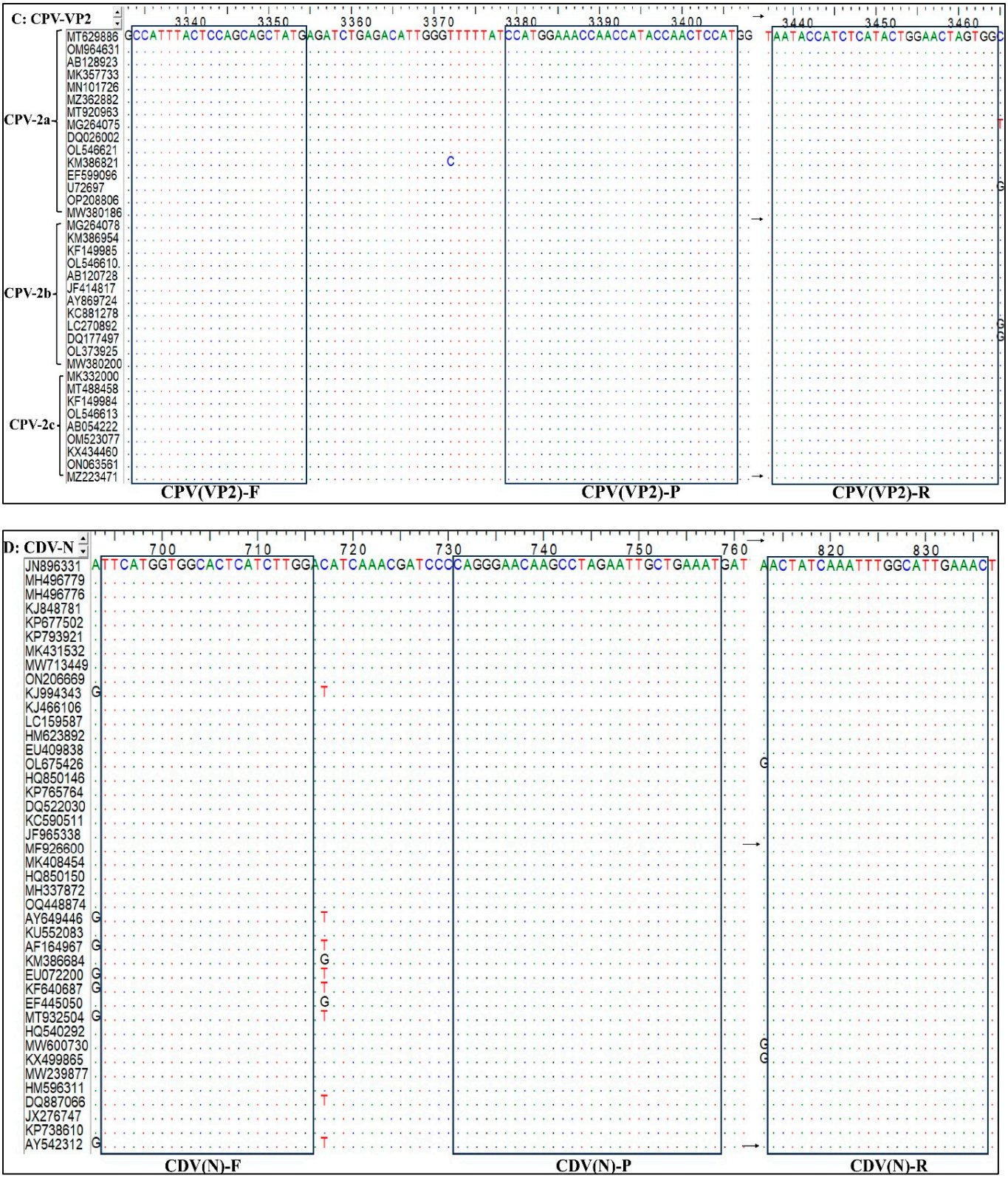

Figure 1. The locations of the specific primers and probes designed for the quadruplex RT-qPCR. The nucleotide sequence alignments of the partial CCoV M gene (**A**), CRV VP7 gene (**B**), CPV VP2 gene (**C**), and CDV N gene (**D**) show the locations of the primers and probes. F, P, and R indicate the forward primer, TaqMan probe, and reverse primer, respectively.

2.6. Extraction of Nucleic Acids

A total of 0.5 mL of phosphate-buffered saline (PBS, pH7.2) was added to the tube containing the feces, anal swabs, and nasal swabs, vortexed for 5 min, and then centrifuged at 12,000 rpm for 10 min (at 4 °C), and a 200 μL supernatant was used for nucleic acid extraction. Total DNA/RNA was extracted on a GeneRotex96 Nucleic Acid Extractor using the Viral DNA/RNA Extraction Kit (Ver4.0) (Tianlong Technology, Xi'an, China) according to the manufacturer's instructions and stored at −80 °C until use.

2.7. Generation of Standard Plasmid Constructs

The standard plasmid constructs were generated according to Chen et al. [23] with minor modifications. In brief, the total RNAs of CCoV, CDV, and CRV were extracted from the vaccine strains of CCoV (NL-18 strain), CDV (Synder Hill strain), and the CRV-positive sample, respectively, and then reverse-transcribed to cDNA using the PrimeScript^{TM} II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The total DNA of CPV was extracted from the vaccine strain of CPV (NL-35-D strain). The cDNA/DNA of CCoV, CRV, CPV, and CDV were used as templates to amplify the targeted fragments using the primers listed in Table 1. The PCR products of the CCoV M gene, CRV VP7 gene, CPV VP2 gene, and CDV N gene were purified using the MiniBEST DNA Fragment Purification Kit Ver.4.0 (TaKaRa, Dalian, China), ligated to the pMD18-T vector (TaKaRa, Dalian, China), and transformed into *E. coli* DH5α competent cells (TaKaRa, Dalian, China). The positive clones were cultured at 37 °C for 20–24 h, and the recombinant plasmid constructs were extracted using the MiniBEST Plasmid Purification Kit Ver.4.0 (TaKaRa, Dalian, China). The recombinant standard plasmid constructs were confirmed by sequencing and named p-CCoV, p-CRV, p-CPV, and p-CDV, respectively. Their concentrations were determined at OD260 and OD280 values using the following equation: plasmid (copies/μL) = $\frac{(6.02\times10^{23})\times(X\ ng/\mu L\times10^{-9})}{\text{plasmid length (bp)}\times660}$. The initial concentrations were finally determined to be 3.18×10^{10}, 2.96×10^{10}, 2.77×10^{10}, and 2.1×10^{10} copies/μL for p-CCoV, p-CRV, p-CPV, and p-CDV, respectively. All were diluted to 2.1×10^{10} copies/μL, and stored at −80 °C until used.

2.8. Determination of the Reaction Conditions

Experiments were performed on the ABI QuantStudio 5 qPCR system (ABI, Carlsbad, CA, USA) by adjusting the annealing temperature (55–61 °C), primer and probe concentrations (200 pmol/μL, 0.2–0.6 μL), and reaction cycles (25–45 cycles). The total volume of the 20 μL reaction system contained the following: 2 μL of the mixture of 4 standard plasmid constructs (all four standard plasmid constructs were 10^7 copies/μL), 10 μL of 2 × One-Step RT-PCR Buffer III (TaKaRa, Dalian, China), 0.4 μL of Ex Taq HS (5U/μL) (TaKaRa, Dalian, China), 0.4 μL of PrimeScript RT Enzyme Mix II (TaKaRa, Dalian, China), 0.2–0.6 μL of primers and probes (200 pmol/μL), and nuclease-free distilled water to a final volume of 20 μL. The amplification procedure used was 42 °C 5 min, 95 °C 10 s, and 40 cycles of 95 °C 5 s, 55–61 °C 34 s. The fluorescence signal was automatically obtained by the machine at the end of each cycle, and the optimal conditions were determined.

2.9. Generation of Standard Curves

The p-CCoV, p-CRV, p-CPV, and p-CDV standard plasmid constructs were mixed at an equal ratio of 1:1:1:1 and serially diluted 10-fold. The mixtures of concentrations from 2.1×10^8 to 2.1×10^2 copies/μL for each plasmid construct were used as templates to generate the standard curves.

2.10. Assessment of Analytical Specificity

The DNA or RNA of CCoV, CRV, CPV, CDV, CAV-1, CAV-2, CPIV, canine CV, CNV, CRCoV, and RABV were used as templates to assess specificity. Negative clinical samples and nuclease-free distilled water were used as negative controls.

2.11. Assessment of Analytical Sensitivity

The p-CCoV, p-CRV, p-CPV, and p-CDV standard plasmid constructs were mixed at a ratio of 1:1:1:1. The mixtures of the four plasmid constructs with 420 copies/reaction were serially diluted 2-fold to concentrations of 420, 210, 105, and 52.5 copies/reaction and then used as templates to evaluate the limit of detection (LOD). Probit regression analysis was used to determine the values of LOD.

2.12. Repeatability Analysis

Mixtures of the p-CCoV, p-CRV, p-CPV, and p-CDV standard plasmid constructs with a final reaction concentration of 2.1×10^7, 2.1×10^5, 2.1×10^3 copies/μL were used to assess repeatability. The intra-assay was repeated three times, and the inter-assay was repeated in three different weeks to calculate the coefficients of variation (CV).

2.13. Detection of Clinical Samples

The 1028 clinical samples collected from December 2021 to June 2023 were tested by multiplex RT-qPCR, as reported by Thieulent et al. [24], for CCoV, CPV, CRV, and/or CDV. The assay described by Thieulent et al. [24] used quadruplex RT-qPCR to detect the CCoV ORF5 gene, CPV VP2 gene, CDV Phosphoprotein gene, and canine adenovirus-1 (CAdV-1) Hexon gene, and it used duplex RT-qPCR to detect the CRV NSP3 gene and SARS-CoV-2 N gene. The assay had analytical sensitivity (LOD) of 6 copies/μL (150 copies/reaction) for CCoV, 14 copies/μL (350 copies/reaction) for CPV, 5 copies/μL (125 copies/reaction) for CDV, and 35 copies/μL (875 copies/reaction) for CRV. In our study, these 1028 clinical samples were tested for CCoV, CPV, CDV using triplex RT-qPCR, and were tested for CRV using singleplex RT-qPCR described in the reference assay [24].

The 1028 clinical samples were tested by the developed quadruplex RT-qPCR. The detection results of these two assays were compared, and the clinical sensitivity and specificity of the developed assay, and the agreement between them, were evaluated.

3. Results

3.1. Determination of the Reaction Conditions

After optimization, the annealing temperature, concentrations of primers and probes, and reaction cycles of the quadruplex RT-qPCR were determined. The optimal 20 μL system and reaction conditions were shown in Table 2. The amplification procedure used the following parameters: 42 °C for 5 min and 95 °C 10 s, and 40 cycles of 95 °C for 5 s and 56 °C 34 s. The Ct value of the positive sample was set at ≤36.

Table 2. The optimal reaction system.

Reagent	Volume (μL)	Final Concentration (nM)
CCoV(M)-F (200 pmol/μL)	0.4	400
CCoV(M)-R (200 pmol/μL)	0.4	400
CCoV(M)-P (200 pmol/μL)	0.3	300
CRV(VP7)-F (200 pmol/μL)	0.4	400
CRV(VP7)-R (200 pmol/μL)	0.4	400
CRV(VP7)-P (200 pmol/μL)	0.2	200
CPV(VP2)-F (200 pmol/μL)	0.3	300
CPV(VP2)-R (200 pmol/μL)	0.3	300
CPV(VP2)-P (200 pmol/μL)	0.3	300
CDV(N)-F (200 pmol/μL)	0.4	400
CDV(N)-R (200 pmol/μL)	0.4	400
CDV(N)-P (200 pmol/μL)	0.2	200
2 × One-Step RT-PCR Buffer III	10	/
Ex Taq HS (5 U/μL)	0.4	/
PrimeScript RT Enzyme Mix II	0.4	/
Nucleic acid template	2	/
Nuclease-free distilled water	3.2	/
Total	20	/

3.2. Generation of Standard Curves

Mixtures of the four standard plasmid constructs p-CCoV, p-CRV, p-CPV, and p-CDV with final reaction concentrations from 2.1×10^7 to 2.1×10^1 copies/μL were used to generate the standard curves. The results showed that the slope of the corresponding equation, correlation coefficient (R^2), and amplification efficiency (E) were −3.241, 0.999 and 103.474% for CCoV; −3.258, 0.999 and 102.721% for CRV; −3.215, 0.999 and 104.646% for CPV; and −3.246, 0.998 and 103.251% for CDV, respectively (Figure 2).

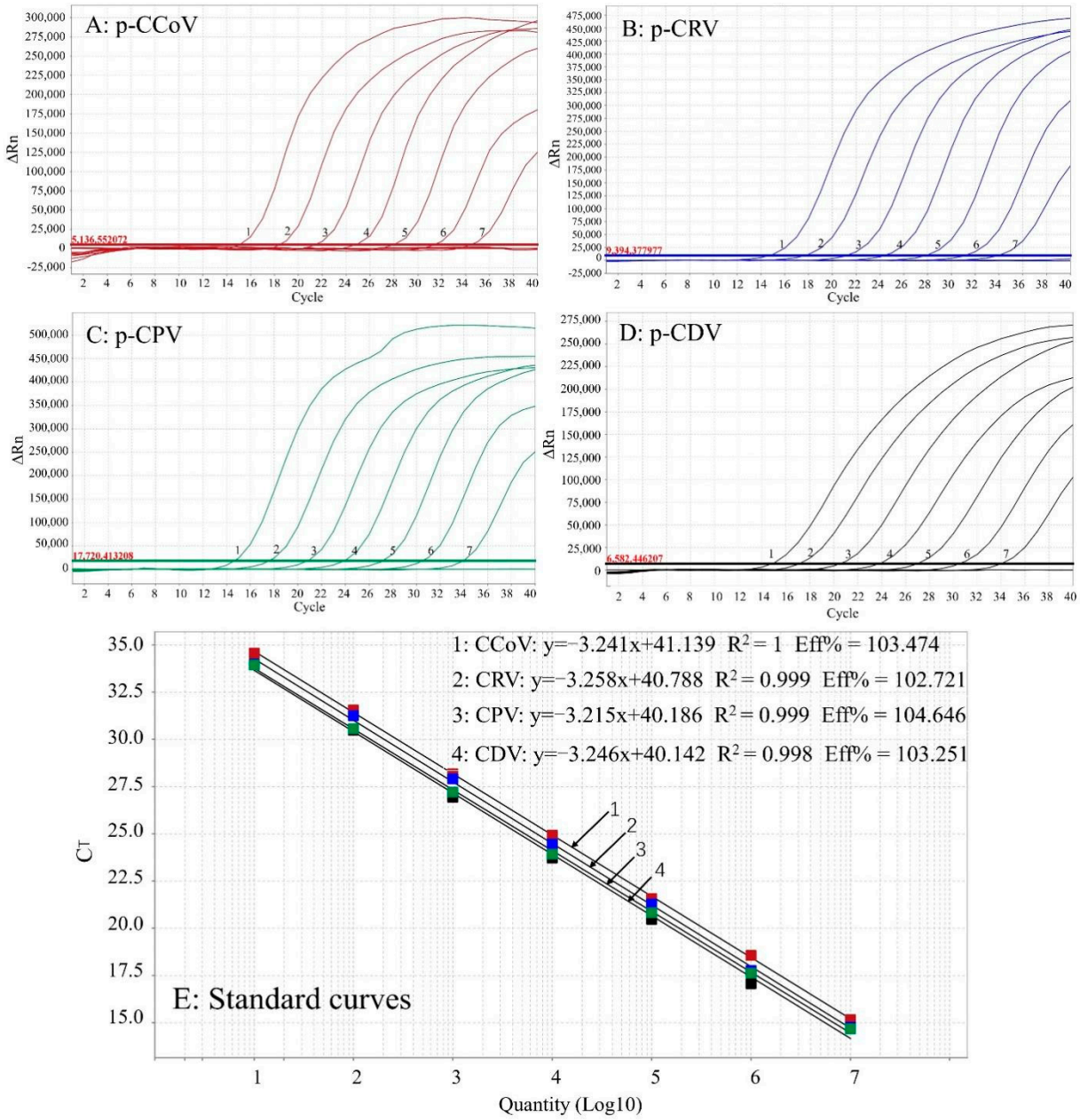

Figure 2. Generation of the standard curves. The amplification curves of p-CCoV (**A**), p-CRV (**B**), p-CPV (**C**), and p-CDV (**D**), and their standard curves (**E**) are shown. In (**A**–**D**), the *x*-axis shows the reaction cycle, and the *y*-axis shows the derivative reporter signal (ΔRn); 1–7, the final reaction concentrations of the standard plasmid constructs ranged from 2.1×10^7 to 2.1×10^1 copies/μL. In (**E**), the *x*-axis shows the logarithmic value of the template, and the *y*-axis shows the Ct value.

3.3. Specificity Analysis

Total DNA/RNA of CCoV, CRV, CPV, CDV, CAV-1, CAV-2, CPIV, canine CV, CNV, CRCoV, and RABV were used as templates to assess specificity. The results showed that quadruplex RT-qPCR could detect only CCoV, CRV, CPV, and CDV (with amplification curves), without cross-reactivity with CAV-1, CAV-2, CPIV, canine CV, CNV, CRCoV, and RABV (no amplification curve) (Figure 3).

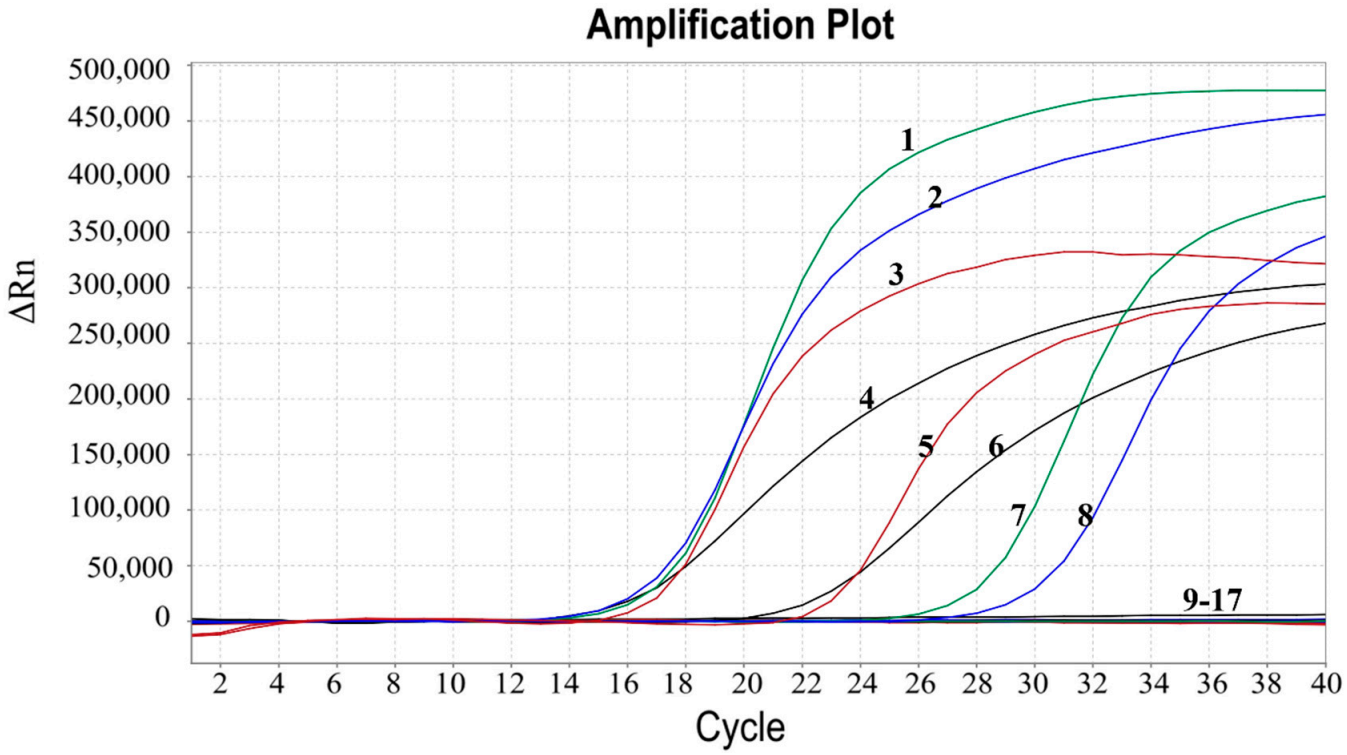

Figure 3. Specificity analysis of the quadruplex RT–qPCR. 1, p–CPV; 2, P–CRV; 3, p–CCoV; 4, p–CDV; 5, CCoV; 6, CDV; 7, CPV; 8, CRV; 9–15, CAV–1, CAV–2, CPIV, canine CV, CNV, CRCoV, and RABV, respectively; 16, negative clinical sample; 17, nuclease-free distilled water.

3.4. Sensitivity Analysis

Mixtures of p-CCoV, p-CRV, p-CPV, and p-CDV standard plasmid constructs with final reaction concentrations of 420, 210, 105, 52.5 copies/reaction were used to evaluate the LOD of the developed assay. The threshold cycle (Ct) values and hit rates were analyzed using PROBIT regression (Table 3). The LOD and their 95% confidence interval (CI) for p-CCoV, p-CRV, p-CPV and p-CDV were determined to be 111 (95% CI = 101–129), 109 (98–127), 111 (101–129), and 114 (104–133) copies/reaction, respectively (Figure 4), which was about 1.1×10^2 copies/reaction for all plasmid constructs.

3.5. Repeatability Analysis

Mixtures of the p-CCoV, p-CRV, p-CPV and p-CDV standard plasmid constructs with final reaction concentration of 2.1×10^7, 2.1×10^5, 2.1×10^3 copies/μL were used to evaluate the repeatability of the developed assay. The results showed that the intra-assay CVs were 0.05–0.90%, and the inter-assay CVs were 0.02–0.94% (Table 4).

Table 3. Threshold cycle (Ct) values and hit rates of serially 2-fold diluted plasmid constructs.

Standard Plasmid	Concentration (Copies/Reaction)	Number of Samples	Multiplex qRT-PCR	
			Ct (Average)	Hit Rate (%)
p-CCoV	420	26	34.91	100
	210	26	35.43	100
	105	26	35.98	92.31
	52.5	26	ND	0
P-CRV	420	26	34.71	100
	210	26	35.35	100
	105	26	35.96	96.15
	52.5	26	ND	0
P-CPV	420	26	34.19	100
	210	26	34.82	100
	105	26	35.50	92.31
	52.5	26	ND	0
P-CDV	420	26	34.45	100
	210	26	35.13	100
	105	26	35.78	88.46
	52.5	26	ND	0

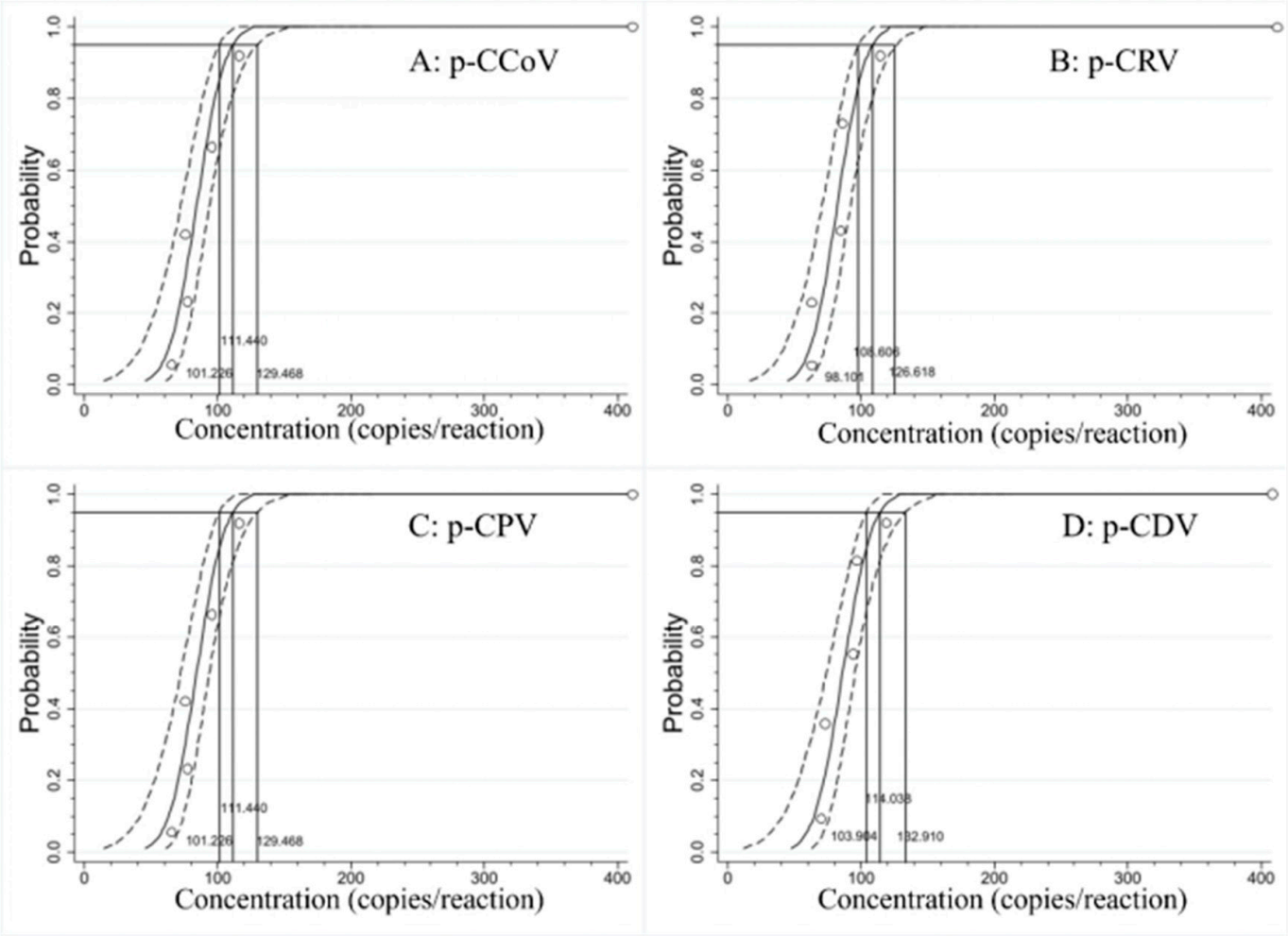

Figure 4. Sensitivity analysis using PROBIT regression at 95% CI. The limits of detection (LOD) for p-CCoV (**A**), p-CRV (**B**), p-CPV (**C**), and p-CDV (**D**) were all about 1.1×10^2 copies/reaction.

Table 4. Repeatability analysis of the quadruplex RT-qPCR.

Plasmid Construct	Concentration (Copies/μL)	Concentration (Copies/Reaction)	Ct Value of Intra-Assay			Ct Value of Inter-Assay		
			$\overline{X}$	SD	CV (%)	$\overline{X}$	SD	CV (%)
p-CCoV	2.1×10^7	4.2×10^8	14.66 (14.49–14.81)	0.13	0.90	14.74 (14.58–14.83)	0.11	0.75
	2.1×10^5	4.2×10^6	21.97 (21.77–22.21)	0.18	0.82	21.66 (21.43–21.93)	0.20	0.94
	2.1×10^5	4.2×10^4	28.60 (28.49–28.82)	0.15	0.54	28.32 (28.12–28.59)	0.20	0.71
P-CRV	2.1×10^7	4.2×10^8	14.49 (14.43–14.59)	0.08	0.53	14.82 (14.82–14.83)	0.01	0.02
	2.1×10^5	4.2×10^6	21.60 (21.51–21.68)	0.07	0.32	21.43 (21.42–21.45)	0.01	0.06
	2.1×10^3	4.2×10^4	28.47 (28.27–28.59	0.15	0.51	28.39 (28.28–28.48)	0.08	0.29
p-CPV	2.1×10^7	4.2×10^8	15.49 (15.48–15.50)	0.01	0.05	15.89 (15.86–15.93)	0.03	0.19
	2.1×10^5	4.2×10^6	20.79 (20.73–20.85)	0.05	0.23	20.18 (20.14–20.20)	0.03	0.16
	2.1×10^3	4.2×10^4	27.72 (27.60–27.84)	0.10	0.37	27.17 (27.00–27.43)	0.18	0.68
p-CDV	2.1×10^7	4.2×10^8	14.54 (14.47–14.59)	0.05	0.34	14.36 (14.25–14.44)	0.08	0.56
	2.1×10^5	4.2×10^6	20.97 (20.87–21.05)	0.08	0.36	20.69 (20.53–20.81)	0.12	0.57
	2.1×10^3	4.2×10^4	28.57 (28.44–28.77)	0.14	0.50	28.07 (27.99–28.13)	0.06	0.19

Note: SD, standard deviation; CV, coefficient of variation.

3.6. Detection Results of Clinical Samples

The positivity rates of CCoV, CRV, CPV, and CDV in the 1028 clinical samples with the established assay were 9.53% (98/1028), 0.97% (10/1028), 25.68% (264/1028), and 5.06% (52/1028), respectively (Table 5). The co-infection positivity rates of CCoV+CPV, CCoV+CDV, CRV+CPV, CPV+CDV, and CCoV+CPV+CDV were 4.38% (45/1028), 0.68% (7/1028), 0.49% (5/1028), 1.17% (12/1028), and 0.68% (7/1028), respectively (Table 5). These positive samples for CCoV, CRV, CPV and CDV had Ct values of 7.68–35.83, 15.34–35.22, 8.46–35.06, and 9.25–35.11, respectively.

Table 5. The positivity rates of the clinical samples tested by the developed quadruplex RT-qPCR.

Pathogen	Sample	Established RT-qPCR		Reference RT-qPCR	
		Positive Sample	Percentage (%)	Positive Sample	Percentage (%)
Single Infection					
CCoV	1028	39	3.79%	36	3.50%
CRV	1028	5	0.49%	5	0.49%
CPV	1028	195	18.97%	199	19.36%
CDV	1028	26	2.53%	27	2.63%
Co-Infection					
CCoV+CPV	1028	45	4.38%	44	4.28%
CCoV+CDV	1028	7	0.68%	6	0.58%
CRV+CPV	1028	5	0.49%	4	0.39%
CPV+CDV	1028	12	1.17%	10	0.97%
CCoV+CPV+CDV	1028	7	0.68%	7	0.68%
Total (Single+Co-Infection)					
CCoV	1028	98	9.53%	93	9.05%
CRV	1028	10	0.97%	9	0.88%
CPV	1028	264	25.68%	264	25.68%
CDV	1028	52	5.06%	50	4.86%

The positivity rates of CCoV, CRV, CPV, and CDV in the 1028 clinical samples with the reference assay [24] were 9.05% (93/1028), 0.88% (9/1028), 25.68% (264/1028), and 4.86% (50/1028), respectively.

The detection results of the established assay and the reference assay showed that the clinical sensitivity and specificity of the established assay were determined to be 98.00–100% and 99.36–100%, respectively (Table 6), and the agreements of CCoV, CRV, CPV, and CDV between these methods were 99.32%, 99.42%, 100%, and 99.61%, respectively (Table 7).

Table 6. The clinical sensitivity and specificity of the developed multiplex RT-qPCR.

The Current RT-qPCR		The Reference RT-qPCR		Total	Clinical Sensitivity (95% CI)	Clinical Specificity (95% CI)
		Positive	Negative			
CCoV	Positive	92	6	98	98.92% (94.16–99.81%)	99.36% (98.61–99.71%)
	Negative	1	929	930		
	Total	93	935	1028		
CRV	Positive	9	1	10	100% (70.09–100%)	99.80% (99.45–99.98%)
	Negative	0	1018	1018		
	Total	9	1019	1028		
CPV	Positive	264	0	264	100% (98.57–100%)	100% (99.50–100%)
	Negative	0	764	764		
	Total	264	764	1028		
CDV	Positive	49	3	52	98.00% (89.51–99.65%)	99.69% (99.10–99.91%)
	Negative	1	975	976		
	Total	50	978	1028		

Table 7. Agreements of the detection results between two assays.

Method	Positive Sample			
	CCoV (%)	CRV (%)	CPV (%)	CDV (%)
The Current RT-qPCR	98/1028 (9.53%)	10/1028 (0.97%)	264/1028 (25.68%)	52/1028 (5.06%)
The Reference RT-qPCR [24]	93/1028 (9.05%)	9/1028 (0.88%)	264/1028 (25.68%)	50/1028 (4.86%)
Agreements (95% CI)	99.32% (98.60–99.67%)	99.90% (99.44–99.98%)	100% (99.63–100%)	99.61% (99.00–99.85%)

4. Discussion

CCoV, CRV, CPV, and CDV are common pathogens that cause gastroenteritis in domestic dogs and co-infections with these pathogens can exacerbate the severity of these diseases and increase their morbidity and mortality [5,14,15]. To date, singleplex and multiplex RT-qPCR/qPCR have been developed to detect one to three etiologies of CCoV, CRV, CPV, and/or CDV [17–22], but no multiplex RT-qPCR to simultaneously detect and differentiate these four pathogens has been reported. This may be contributed to the fact that the instruments and diagnostic reagents for qPCR are still relatively expensive, that the advantages of suitable qPCR, especially multiplex RT-qPCR, have not been recognized by most veterinary laboratories, and that the establishment of quadruplex RT-qPCR requires a certain level of technical proficiency and operational skills as well as appropriate instrumentation. However, with more and more laboratories equipped with qPCR instruments, and multiplex qPCR greatly reducing the detection cost of individual samples, multiplex qPCR will be widely used in the detection of clinical samples.

In this study, four pairs of specific primers and probes targeting the CCoV M gene, CRV VP7 gene, CPV VP2 gene, and CDV N gene, respectively, were designed to establish a quadruplex RT-qPCR for the detection and differentiation of CCoV, CRV, CPV, and CDV. The assay showed specificity for the detection of CCoV, CRV, CPV, and CDV; high sensitivity with LOD of 1.1×10^2 copies/reaction for CCoV, CRV, CPV, and CDV; excellent repeatability with intra-assay variation of 0.05–0.90% CVs and inter-assay variation of 0.02–0.94% CVs; and high clinical sensitivity of 98.00–100% and clinical specificity of

99.36–100%. In addition, the 1028 clinical samples were tested by the developed assay and the reference assays reported by Thieulent et al. [24]. The agreement of the detection results between these assays was higher than 99.32%. This validates the clinical applicability of the developed quadruple RT-qPCR. These results indicate that a quadruplex RT-qPCR for the sensitive, rapid, and accurate detection and differentiation of CCoV, CRV, CPV, and CDV were successfully developed. The assay can differentially detect these four viral pathogens in one reaction in the same tube within two hours. It is worth noting that other important canine viruses, including CAV-1, CAV-2, CPIV, canine CV, CNV, CRCoV, and RABV, were used as templates to assess specificity. However, the clinical samples may contain an enormous diversity of microorganisms, and it is impossible to prove the absence of cross-reaction for all these microorganisms. In this study, only the abovementioned viruses were used to evaluate the specificity of the developed assay. This is the limitation of the analytical specificity which needs to be considered. In addition, while exploring the sensitivity and specificity of the developed assay based on various collection approaches would certainly add value to our study, we focused on other aspects in this research.

The positivity rates of CCoV, CRV, CPV, and CDV in the Guangxi province in China during 2021–2023 were 9.53%, 0.97%, 25.68%, and 5.06%, respectively, and the co-infection rates of CPV/CCoV, CPV/CDV, CCoV/CDV, CPV/CRV, and CCoV/CPV/CDV were 3.79%, 1.36%, 0.58%, 0.78%, and 0.58%, respectively. The prevalence and coinfection of CCoV, CRV, CPV, and/or CDV have also been reported in different countries. The 109 fecal samples from Japan had positivity rates of 50.5% for CCoV and 34.9% for CPV-2, and their co-infection rate was 20.2% [25]. The 325 stool samples from Brazil had positivity rates of 54.3%, 45.1%, 30.4%, and 8.2% for CPV-2, CDV, CCoV, and CRV, respectively, and 37% were co-infected with the highest co-infection rate (52.9%, 36/68) for CDV and CPV-2 [26]. A meta-analysis of 10,609 domestic dogs and 3604 CCoV-positive cases from eight provinces in China revealed that the pooled prevalence of CCoV infection was 33%, and CCoV-II was the predominant subtype [27]. The 438 dog samples from Wuhan in China during 2019–2021 had a positivity rate of 1.60% (7/438) for species A CRV [12]. Between 2012 and 2021, 516 fecal samples from dogs in Brazil showed a positivity rate of 0.6% (3/516) for species A CRV [13]. From the 1990s to 2020, the CPV-2 morbidity and mortality rates in China varied from 3.90% to 95.8% and from 20.17% to 73.47%, respectively [28]. A systematic literature review showed that 39 studies on CPV-2 epidemiology during 2006–2019 indicated that the pooled prevalence rate of CPV-2 in China was 36% (data from 137,844 dogs) [29]. The 141 samples from domestic dogs between 2017 and 2019 in Brazil showed a 34% (48/141) positivity rate for CDV. The meta-analysis showed that 53 studies conducted in 21 different countries included 11,527 dogs, and the pooled frequency of CDV was 33% [30]. These data indicated that diarrheic canine viruses commonly circulated in domestic dogs in different countries, and co-infection was a common occurrence. In particular, CCoV and CRV were considered as the most important zoonotic potentials [31,32]. Canine-like alphacoronaviruses (α-CoVs) have been discovered in humans with acute respiratory illness in Haiti, Malaysia, and Thailand [31,33–35], and canine-origin G3P[3] rotavirus has been discovered in diarrheal children from China [36], Brazil [37], and Israel and USA [38]. Since the common prevalence and co-infections of CCoV, CRV, CPV, and/or CDV, especially the zoonotic potential of CCoV and CRV, it is urgent to monitor these pathogens and track their epidemic dynamics. The developed quadruplex RT-qPCR provides a useful and accurate method to detect and survey these viruses.

5. Conclusions

This study established quadruplex RT-qPCR and provided a sensitive, rapid, and accurate method to detect and differentiate CCoV, CRV, CPV, and CDV. The assay can simultaneously detect these four viruses in one reaction in the same tube within two hours, and can be used for the detection and surveillance of diarrheic canine viruses. To our best of knowledge, this is the first report on the development of quadruplex RT-qPCR for simultaneous detection of CCoV, CRV, CPV, and CDV.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15020049/s1. Table S1: The information on the reference strains of CCoV; Table S2: The information on the reference strains of CRV; Table S3: The information on the reference strains of CPV; Table S4: The information on the reference strains of CDV.

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