BMC Veterinary Research

RESEARCH

Open Access

Development and validation of nanoplate-based RT-dPCR assay for canine respiratory coronavirus detection in various clinical samples

Panida Poonsin[1,2], Vorapun Wiwatvisawakorn[3], Chutchai Piewbang[1,2] and Somporn Techangamsuwan[1,2*]

Abstract

Background Canine respiratory coronavirus (CRCoV) is a major contributor to the canine infectious respiratory disease complex (CIRDC). Despite its widespread prevalence, molecular assays for CRCoV detection remain limited. Additionally, the efficiency and accuracy of detection can vary depending on the type of clinical sample used, such as nasal swabs (NS), oropharyngeal swabs (OS), and rectal swabs (RS). To address these challenges, we developed a nanoplate-based reverse transcription digital polymerase chain reaction (RT-dPCR) method for detecting the spike gene of CRCoV in various clinical samples.

Results The RT-dPCR assay demonstrated consistent repeatability and reproducibility, ensuring reliable results. With a detection limit of 1.83 copies/µL, the RT-dPCR assay exhibited 100-fold greater sensitivity than probe-based reverse transcription quantitative polymerase chain reaction (RT-qPCR). It showed no cross-reactivity with other common CIRDC-associated viruses or coronaviruses, confirming its high specificity for CRCoV. The assay was further validated using 162 clinical swab samples (NS, OS, and RS) collected from both healthy dogs and those with respiratory distress. The RT-dPCR assay showed a higher overall positivity rate for CRCoV compared to RT-qPCR, with the most notable difference observed in rectal swabs ($P < 0.05$), where RT-dPCR detected CRCoV in 53.7% of samples compared to 22.22% by RT-qPCR.

Conclusions This study demonstrated that the RT-dPCR assay provided high sensitivity for detecting low viral loads across various sample types, making it a valuable tool for precise CRCoV detection. In contrast, RT-qPCR remains valuable for its broader detection range and suitability in initial screening. Both techniques proved to be versatile tools that can contribute to advancing CRCoV research and improving clinical diagnostics.

Keywords Canine respiratory coronavirus, Clinical samples, Diagnosis, RT-dPCR, RT-qPCR

*Correspondence:
Somporn Techangamsuwan
somporn62@hotmail.com
[1]Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[2]Animal Virome and Diagnostic Development Research Unit, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[3]SLV Pet Hospital, Bangkok, Thailand

Background

Canine respiratory coronavirus (CRCoV) is a betacoronavirus that affects dogs' respiratory systems, primarily targeting the upper respiratory tract and causing mild respiratory distress [1]. CRCoV is considered a one of the most important pathogens to the canine infectious respiratory disease complex (CIRDC). It initially compromises the host's innate immune response, often leading to secondary infections by other pathogens or vice versa, which can exacerbate respiratory symptoms [2, 3]. Due to the transmissibility of respiratory viruses, CRCoV infection is particularly concerning in crowded environments such as shelters, rehoming centers, and animal hospitals [4]. Additionally, CRCoV has been identified in dogs across numerous countries worldwide, underscoring its widespread prevalence and potential risk to global dog population [4–13].

CRCoV infection in dogs can lead to a range of non-specific respiratory symptoms, making diagnosis based solely on clinical signs challenging and necessitating additional laboratory investigations [1, 2, 14]. Although CRCoV typically causes mild respiratory symptoms, it can spread rapidly in both field conditions and experimental settings [2, 4]. This high contagious rate, combined with non-specific symptoms and its broad geographical distribution, underscores the need for effective, reliable, and rapid detection methods to facilitate global monitoring and control of CRCoV infections.

An effective diagnostic tool is essential not only for accurate diagnosis but also for supporting preventive strategies against CRCoV. Current diagnostic methods include virus isolation through cell culture and viral RNA detection using reverse transcription polymerase chain reaction (RT-PCR) techniques [12, 15, 16]. Although effective, viral isolation is time-consuming and labor-intensive. RT-PCR offers rapidity, reproducibility, and flexibility; however, it cannot quantify viral load. To address this limitation, the RT-quantitative PCR (RT-qPCR) method was developed, allowing both detection and quantification of CRCoV viral loads [11, 17–19].

However, RT-qPCR has some limitations, such as the requirement for a standard curve for absolute viral quantification and potential interference from complex sample backgrounds. RT-digital PCR (RT-dPCR), an endpoint PCR, addresses these challenges. It enables the detection and absolute quantification of nucleic acid targets, including viral loads, without requiring references or a standard curve. By applying Poisson statistics, RT-dPCR determines the absolute number of nucleic acid molecules in a sample, offering enhanced sensitivity and superior precision compared to RT-qPCR. This makes RT-dPCR suitable for analyzing low-concentration samples or those with complex backgrounds that might interfere with other methods such as RT-PCR and RT-qPCR [20–22]. The increased sensitivity and precision of RT-dPCR over RT-qPCR further reinforces its effectiveness across a broad range of applications [23–25]. This highlights the robustness and versatility of RT-dPCR in pathogen detection and quantification, demonstrating its potential to significantly improve diagnostic capabilities.

The variety of RT-dPCR approaches depends on the partitioning method, resulting in different RT-dPCR platforms such as droplet-based, chip-based, and nanoplate-based RT-dPCR. Notably, nanoplate-based RT-dPCR offers a workflow similar to RT-qPCR, allowing conditions to be adapted directly from RT-qPCR protocols. This adaptability is a key advantage of the nanoplate RT-dPCR platform, enabling a smooth and efficient transition with minimal adjustments [23, 26]. Additionally, nanoplate RT-dPCR provides a shorter processing time compared to RT-digital droplet PCR (RT-ddPCR) [27]. RT-ddPCR, in contrast, involves droplet-based partitioning that requires more extensive optimization to ensure droplets are uniform and stable, making it less straightforward to transition from RT-qPCR. Nanoplate RT-dPCR, by using preset well partitions, minimizes the need for such optimization and allows the use of conditions similar to those in RT-qPCR. Although RT-dPCR technology has been employed for various viral pathogens, including respiratory and enteric viruses [20, 28–31], no study to date has developed or validated a nanoplate-based RT-dPCR assay for canine respiratory coronavirus (CRCoV). Additionally, this study is among the first to apply and compare this platform across multiple clinical sample types, including nasal, oropharyngeal, and rectal swabs, highlighting both the analytical performance and its application to samples with varying biological complexity.

In this study, we developed a nanoplate-based RT-dPCR assay for detecting CRCoV in various clinical samples. We optimized the RT-dPCR assay conditions based on an established RT-qPCR assay, then evaluated the sensitivity, specificity, repeatability, and reproducibility of both assays. Additionally, we compared their sensitivities using three types of clinical samples from dogs including nasal swabs (NS), oropharyngeal swabs (OS), and rectal swabs (RS).

Methods

Positive standard control preparation

To prepare the standard control, a partial spike gene from the CRCoV strain BJ232 (Accession number: KX432213.1) was commercially synthesized as string DNA (GeneArt™ Strings™ DNA Fragments, Thermo Fisher Scientific, Regensburg, Germany), resulting in a 500-bp fragment. The copy number of the positive control was calculated using a previously reported method [17].

Virus and clinical samples

The modified live vaccine Vanguard® HTLP 5/CV-L (Zoetis, Lincoln, NE, U.S.A.), containing canine distemper virus (CDV) ($10^{2.5}$ $TCID_{50}$/mL), canine adenovirus type 2 (CAV-2) ($10^{2.9}$ $TCID_{50}$/mL), canine parainfluenza virus (CPIV) ($10^{5.0}$ $TCID_{50}$/mL), canine parvovirus (CPV) ($10^{7.0}$ $TCID_{50}$/mL), and inactivated canine enteric coronavirus (CCoV) (≥ 1.0 relative potency), was employed to determine the assay's analytical specificity. Additional virus strains used for specificity testing included canine influenza virus (CIV) and canid herpesvirus-1 (CaHV-1), which were obtained from naturally infected dogs and confirmed by nucleic acid sequencing in a previous study [13]. Feline coronavirus (FCoV), from naturally infected cats, and transmissible gastroenteritis virus (TGEV), from naturally infected pigs, were also obtained and characterized by sequencing. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) strain *Canis lupus*/THA/BKK-K1/2020 (Accession number: ON966106.1) was included as well.

A total of 162 clinical samples were collected from 54 dogs, including both clinically inapparent and those with respiratory problems such as nasal discharge, cough, and/or evidence of bronchopneumonia. Dogs with respiratory problems associated with underlying cardiopulmonary disease, functional or anatomical airway abnormalities, or neoplasia, as determined through physical examination and radiographic investigations, were excluded from the study. These samples were collected between 2021 and 2023 at multi-centered animal hospitals across Thailand. For each dog, three types of swab samples were collected: NS, OS, and RS, totaling 54 samples of each type. Among the 54 dogs, 42 were clinically apparent and 12 were clinically inapparent. Sample were collected using sterile swabs (Puritan®, Puritan Medical Product, ME, U.S.A.) following a standardized protocol that included consistent swab type, collection technique, and immediate transfer into1 × phosphate-buffered saline (PBS) solution. All samples were stored at − 80 °C under uniform conditions until analysis. All clinical samples were tested for CRCoV using a conventional RT-PCR assay based on a previous study [12]. A total of 53 samples tested positive for CRCoV by RT-PCR, comprising 21 NS, 20 OS, and 12 RS samples.

RNA extraction of clinical samples

RNA was extracted from fresh-frozen clinical samples using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) on the QIAcube Connect automated extractor (Qiagen, Hilden, Germany), following the manufacturer's protocol. The quality and concentration of the extracted RNA were assessed with spectrophotometric analysis using a Nanodrop Lite (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Extracted RNA samples were then stored at − 80 °C until further analysis.

Design of primers and probe

Spike gene sequences of various CRCoV strains available in the GenBank database were retrieved and aligned using MAFFT version 7 [32, 33]. A conserved region of the CRCoV spike gene was selected based on the alignments and visualized with BioEdit v7.0.5.3. The selected region met criteria for primer and probe design [34, 35]. The primers and probe were validated in silico for melting temperature, hairpin structure, self-dimer, and hetero-dimer formation using the OligoAnalyzer Tool (https://sg.idtdna.com/calc/analyzer). They were synthesized by Bionics (Seoul, South Korea), with the hydrolysis probe labeled with 6-Carboxyfluorescein (6-FAM) at the 5′ ends and the non-fluorescent quencher, Black Hole Quencher-1 (BHQ-1), at the 3′ ends. The nucleotide sequences of the primers and probe used in this study are shown in Table S1.

RT-qPCR assay and optimization

The QuantiNova Probe RT-PCR Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. The master mix was prepared in a 0.1 mL strip tube (Qiagen, Hilden, Germany) and consisted of 10 µL of 2× QuantiNova Probe RT-PCR Master Mix, 0.2 µL of QN Probe RT-Mix, varying concentrations of each forward and reverse primer (0.3, 0.4, and 0.5 µM) and hydrolysis probes (0.15 and 0.2 µM).

Assays were conducted on the Rotor-Gene Q 5plex platform (Qiagen, Hilden, Germany) using Rotor-Disc 72. The cycling conditions included an RT step at 45 °C for 10 min, followed by initial PCR activation at 95 °C for 5 min. Forty-five two-step cycles were then performed with denaturation at 95 °C for 5 s, and combined annealing/extension at varying annealing temperatures (51 °C, 53 °C, and 55 °C) for 30 s. Fluorescence was detected at the end of each cycle. Optimal RT-qPCR conditions were determined based on achieving lower Cq values and minimal background noise. Results were analyzed with Q-Rex software version 2.0.

RT-dPCR assay and optimization

Given the similar workflow between RT-qPCR and RT-dPCR on a microfluidic nanoplate, RT-dPCR conditions were optimized based on those established for RT-qPCR. The optimized RT-qPCR assay parameters were then applied to the RT-dPCR platform and tested on the QIAcuity One platform.

The optimized RT-dPCR assay was performed using the QIAcuity One-Step Viral RT-PCR Kit (Qiagen, Hilden, Germany). Briefly, the reaction mix consisted of 10 µL of 4× One-Step Viral RT-PCR Master Mix, 0.4 µL

of 100× Multiplex Reverse Transcription Mix, 0.3 µM of each forward and reverse primer, 0.15 µM of the hydrolysis probe, 5 µL of template RNA, and RNase-free water for a total volume of 40 µL. The reaction mixtures were transferred to a QIAcuity Nanoplate 26k 24-well (Qiagen, Hilden, Germany), allowing 24 samples with 26,000 partitions per well. The nanoplate was sealed, loaded onto the QIAcuity One dPCR 5plex Device (Qiagen, Hilden, Germany), and subjected to an automated workflow. This workflow included a priming step, followed by thermocycling with an RT step at 50 °C for 40 min, inactivation of the RT enzyme at 95 °C for 2 min, then a 45-cycle, two-step process of denaturation at 95 °C for 5 s and annealing/extension at 53 °C for 30 s. After PCR amplification, an imaging step was performed in the green channel with exposure duration of 500 milliseconds and a gain of 6 to capture fluorescence signals in all positive partitions. This gain setting is the default for the green fluorescence channel in this assay and, together with the selected exposure time, produced clear and balanced signal intensities. Results were analyzed using QIAcuity Software Suite version 2.2.0.26.

Analytical performance of RT-qPCR and RT-dPCR assays

Analytical sensitivity, repeatability, and reproducibility

A 10-fold serial dilution of the CRCoV synthetic oligonucleotide, ranging from 3.65×10^8 to 3.65×10^{-1} copies/µL, was prepared to assess assay's sensitivity. This dilution series served as the template for both RT-qPCR and RT-dPCR assays, enabling the determination of the limit of detection (LOD) for each PCR platform. Repeatability and reproducibility of both assays were evaluated across three independent experiments, with each dilution tested in triplicate.

Analytical specificity

The assay's specificity was assessed against various common canine respiratory viruses, including CDV, CAV-2, CPIV, CIV, and CaHV-1, as well as other coronaviruses such as SARS-CoV-2, CCoV, FCoV, and TGEV. Nuclease-free water was used as a negative control in place of the sample.

Dynamic range of detection

The dynamic range of detection for both RT-qPCR and RT-dPCR assays was determined using CRCoV synthetic oligonucleotide concentrations from 3.65×10^8 to 3.65×10^{-1} copies/µL. This dynamic range represents the spectrum from the lowest to the highest concentrations that each assay could detect reliably.

Assay validation using clinical samples

To validate the RT-dPCR assay, the optimized RT-dPCR assay was performed in parallel on each clinical sample (162 swabs) with the established RT-qPCR assay. Assay performance was evaluated by comparing detection rates. For RT-qPCR, samples with a Cq value greater than 45 were considered negative. In RT-dPCR, samples without any positive partitions were deemed negative.

Statistical analysis

The Chi-square test was employed to compare detection rates between the two assays across the three sample types, assessing any significant differences in positive detections for each sample type. A $P < 0.05$ was considered statistically significant. All analyses were conducted using SAS® Studio software (2022, SAS Institute Inc., Cary, NC, U.S.A).

Results

Optimization of RT-qPCR and RT-dPCR assays

The optimized conditions for the RT-qPCR assay included an annealing temperature of 53 °C and primer and probe concentrations of 0.3 µM and 0.15 µM, respectively (Fig. S1). These conditions were successfully applied to the RT-dPCR assay.

For RT-dPCR, the same annealing temperature of 53 °C and primer and probe concentrations of 0.3 and 0.15 µM, respectively, were used. The RT-dPCR assay effectively amplified the target, producing a clear positive signal after 45 cycles with a 500-milliseconds exposure and a gain of 6 for imaging. A 1D scatterplot displayed distinct separation between positive and negative partitions, confirming successful optimization and a seamless transition from RT-qPCR to RT-dPCR (Fig. S1).

Analytical sensitivity, repeatability, and reproducibility

Serial dilutions of the CRCoV synthetic oligonucleotide were used to assess both RT-qPCR and RT-dPCR assays. The RT-qPCR standard curve displayed strong linearity, with a coefficient of determination (R^2) value of 0.9993 and an amplification efficiency of 89% (Fig. 1a), and the LOD was determined to be 1.83×10^2 copies/µL (Fig. 2). The amplification curve corresponding to Fig. 1a and the standard curve associated with Fig. 2 are provided in the Figs. S2 and S3, respectively. Additionally, RT-qPCR showed high repeatability and reproducibility, with coefficients of variation (CV) consistently below 3% including intra-assay CVs of less than 2.25% and inter-assay CVs of less than 2.23% (Table 1).

In parallel, the RT-dPCR assay also displayed robust linearity, with an R^2 value of 0.9991 (Fig. 1b). The LOD for RT-dPCR was significantly lower at 1.83 copies/µL, making it 100 times more sensitive than RT-qPCR (Fig. 3). The CVs were less than 6% for intra-assay variability and did not exceed 20% for inter-assay variability, based on three independent runs conducted on different days, indicating good repeatability and reproducibility

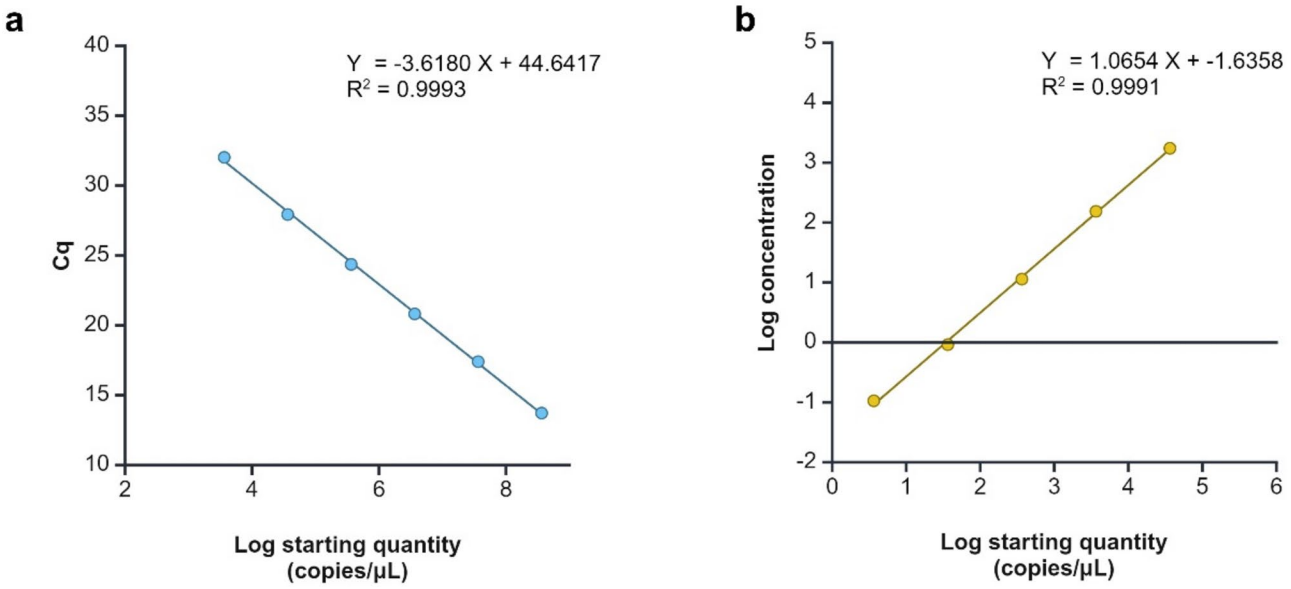

Fig. 1 Linearity assessment of serially diluted CRCoV synthetic oligonucleotide detection using RT-qPCR and RT-dPCR. (**a**) RT-qPCR standard curve for CRCoV synthetic oligonucleotide, displaying the correlation between the quantification cycle (Cq) values (Y axis) against the logarithm of the CRCoV synthetic oligonucleotide concentration (X axis). (**b**) RT-dPCR standard curve for CRCoV synthetic oligonucleotide, showing the correlation by plotting the logarithm of the absolute concentration (Y axis) against the logarithm of the CRCoV synthetic oligonucleotide concentration (X axis)

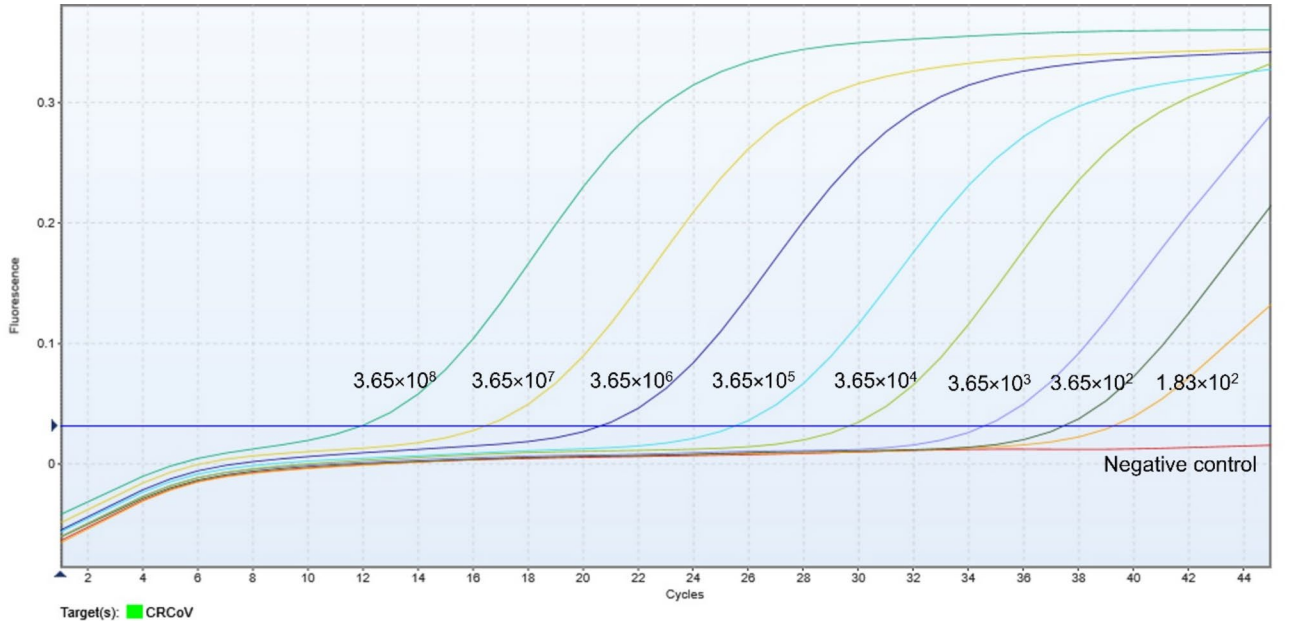

Fig. 2 Analytical sensitivity of the RT-qPCR assay for CRCoV detection. The graph displays the amplification curves for the CRCoV standard positive control across a dilution range from 3.65×10^8 to 3.65×10^2 copies/µL, with the detection limit established at 1.83×10^2 copies/µL

Table 1 Repeatability and reproducibility analysis of RT-qPCR

Calculated concentration of CRCoV synthetic oligonucleotide (copies/µL)	Intra-assay variation (repeatability)			Inter-assay variation (reproducibility)		
	Mean (Cq)	SD	CV (%)	Mean (Cq)	SD	CV (%)
3.65×10^6	20.48	0.46	2.25	21.07	0.47	2.23
3.65×10^5	24.90	0.30	1.20	25.09	0.31	1.23
3.65×10^4	29.87	0.09	0.30	29.39	0.42	1.43

Cq = Quantification cycle; SD = Standard deviation; CV = Coefficient of variation

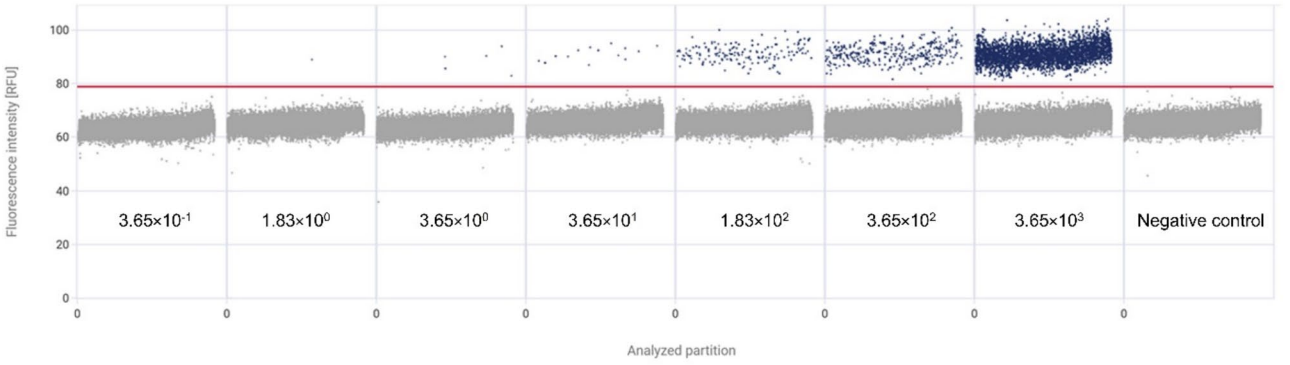

Fig. 3 Analytical sensitivity of the RT-dPCR assay for CRCoV detection. The 1D scatterplot illustrates amplification of the CRCoV standard positive control across a concentration range from 3.65×10^3 to 3.65×10^{-1} copies/µL, with the detection limit identified at 1.83 copies/µL

Table 2 Repeatability and reproducibility analysis of RT-dPCR

Calculated concentration of CRCoV synthetic oligonucleotide (copies/µL)	Intra-assay variation (repeatability)			Inter-assay variation (reproducibility)		
	Mean (copies/µL)	SD	CV (%)	Mean (copies/µL)	SD	CV (%)
3.65×10^4	13883.73	92.75	0.67	12021.87	2399.04	19.46
3.65×10^3	1203.20	24.60	2.00	1129.07	221.51	19.62
3.65×10^2	91.12	5.43	5.95	74.59	9.54	12.79

SD = Standard deviation; CV = Coefficient of variation

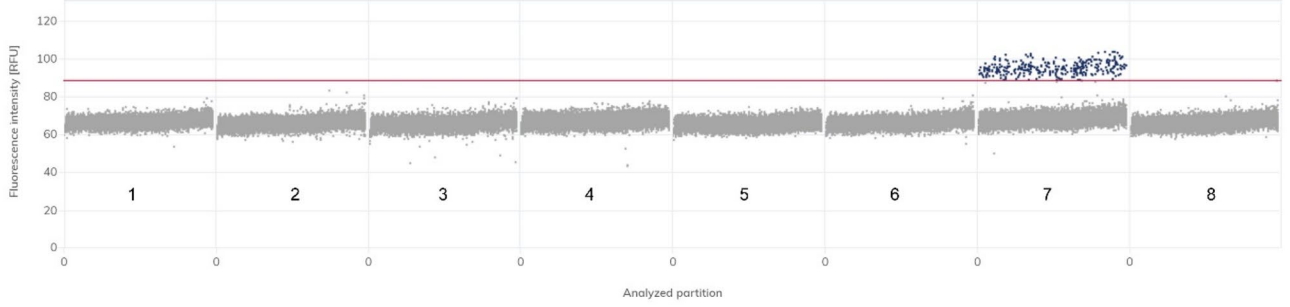

Fig. 4 Analytical specificity of the RT-dPCR assay for CRCoV detection. The 1D scatterplot depicts the results of specificity testing for the RT-dPCR assay, including the following samples: (1) Vanguard® HTLP5/CV-L vaccine (containing CDV, CAV-2, CPIV, CPV, and CCoV), (2) CIV, (3) CaHV-1, (4) SARS-CoV-2, (5) FCoV, (6) TGEV, (7) CRCoV, and (8) negative control

even at lower concentrations (Table 2). The running time for RT-qPCR was approximately 1 h and 29 min, whereas RT-dPCR required approximately 2 hours and 45 min.

Analytical specificity

The specificity of the RT-qPCR and RT-dPCR assays was evaluated against a range of common canine respiratory viruses and various coronaviruses. Neither assay showed cross-reactivity with CDV, CPIV, CIV, CAV-2, or CaHV-1. Additionally, no cross-reactivity was observed with other coronaviruses, including SARS-CoV-2, CCoV, FCoV, or TGEV. These findings indicated that both the RT-qPCR and RT-dPCR assays possessed high specificity for CRCoV detection (Fig. 4 and Fig. S4).

Dynamic range of detection

The RT-dPCR exhibited a dynamic detection range for CRCoV spanning from 3.65×10^4 to 1.83 copies/µL, suitable for detecting lower concentrations of the virus. In comparison, RT-qPCR displayed a broader detection range, detecting higher concentrations from 3.65×10^8 to 1.83×10^2 copies/µL (Table 3).

Assay validation using clinical samples

The RT-qPCR and RT-dPCR assays were validated with 162 clinical samples, comprising NS, OS, and RS samples. The RT-dPCR assay demonstrated a higher overall CRCoV detection rate than RT-qPCR (Table 4). Specifically, RT-qPCR showed CRCoV positivity rates of 50% (27/54) for NS, 42.59% (23/54) for OS, and 22.22% (12/54) for RS samples. In contrast, RT-dPCR revealed

Table 3 Comparison of detection range between RT-qPCR and RT-dPCR using serial dilutions of CRCoV standard positive control

Calculated concentration of CRCoV synthetic oligonucleotide (copies/μL)	RT-qPCR	RT-dPCR
3.65×10^{8}	+	NA
3.65×10^{7}	+	NA
3.65×10^{6}	+	NA
3.65×10^{5}	+	Overload
3.65×10^{4}	+	+
3.65×10^{3}	+	+
3.65×10^{2}	+	+
1.83×10^{2}	+	+
3.65×10^{1}	-	+
3.65×10^{0}	-	+
1.83×10^{0}	-	+
3.65×10^{-1}	-	-

+ = Positive; - = Negative; NA = Not available; Overload = The number of template molecules in a partition exceeds the optimal limit, compromising quantification accuracy

CRCoV positivity rates of 66.67% (36/54) for NS, 62.96% (34/54) for OS, and 53.70% (29/54) for RS samples.

Both assays agreed on 45 NS samples (27 positive, 18 negative), 43 OS samples (23 positive, 20 negative), and 37 RS samples (12 positive, 25 negative), with 125 samples (62 positive, 63 negative) showing consistent results across both methods. The overall positivity rate across the three types of samples indicated that NS samples exhibited the highest rate of positivity, followed by OS samples. RS samples yielded the lowest detection rates in both assays, although RT-dPCR detected more positives than RT-qPCR in all sample types (Fig. S5). Statistically significant differences in positivity rates between RT-qPCR and RT-dPCR were observed for OS ($P = 0.03$) and RS samples ($P = 0.00075$). However, no significant difference was noted for NS samples ($P > 0.05$).

Discussion

Since the discovery of CRCoV in 2003, the virus has continued to spread globally due to its contagious nature [3]. Despite its wide distribution, testing for CRCoV remains constrained by the scarcity of molecular assays. While RT-PCR, the traditional method, is a straightforward and reproducible molecular technique, it only provides qualitative data, limiting the evaluation of viral loads and its effectiveness at detecting low viral quantities in clinical samples. RT-qPCR and RT-dPCR, the second- and the third-generation of PCR technologies, respectively, have enhanced detection efficiency and provide quantitative data, making them powerful tools for molecular diagnostics [17, 20]. Although RT-qPCR can quantify viral loads, it requires a standard curve, which can lead to inconsistent results across the tests and potential inaccuracies, particularly with complex samples.

In this study, we developed a nanoplate-based RT-dPCR assay for CRCoV detection and compared its performance to an established probe-based RT-qPCR assay. Our findings indicated that the RT-dPCR assay is highly sensitive for detecting CRCoV. When comparing RT-dPCR with RT-qPCR, each technique demonstrated unique strengths and limitations. RT-dPCR proved to be significantly more sensitive than RT-qPCR, especially in challenging samples with low viral loads, demonstrating sensitivity over 100 times greater than that of RT-qPCR. Both assays showed strong repeatability and reproducibility. However, RT-dPCR displayed higher inter-assay variability, which can be attributed to the differences in quantification approaches. Specifically, RT-dPCR achieves absolute quantification by partitioning samples into numerous individual reactions, enabling precise counting of nucleic acid targets. This partitioning introduces stochastic effects, as target molecules are randomly distributed across partitions, which can lead to increased variability between runs, especially at low target concentrations. In contrast, RT-qPCR quantifies targets based on Cq values derived from the exponential amplification phase of the reaction, analyzed relative to a standard curve or reference sample. The limitation of high inter-assay variability observed in RT-dPCR, although acceptable, could be addressed by increasing the number of replicates in future studies to reduce this variation and improve reproducibility. Both RT-qPCR and RT-dPCR assays demonstrated high specificity, with

Table 4 Comparison of analytical results from 162 clinical samples using RT-qPCR and RT-dPCR

Sample type			RT-qPCR		
			Positive	Negative	
Nasal swab (NS)	**RT-dPCR**	**Positive**	27	9	36 (66.67%)
		Negative	0	18	18 (33.33%)
			27 (50%)	27 (50%)	54
Oropharyngeal swab (OS)	**RT-dPCR**	**Positive**	23	11	34 (62.96%)
		Negative	0	20	20 (37.04%)
			23 (45.59%)	31 (57.41%)	54
Rectal swab (RS)	**RT-dPCR**	**Positive**	12	17	29 (53.7%)
		Negative	0	25	25 (46.3%)
			12 (22.22%)	42 (77.78%)	54

no cross-reactivity observed with other common canine respiratory viruses or coronaviruses.

To assess assay performance, we applied both RT-qPCR and RT-dPCR to analyze clinical samples, highlighting the versatility of these methods in clinical practice. Our findings revealed a higher CRCoV positivity rate in RT-dPCR compared to RT-qPCR, underscoring the superior sensitivity of RT-dPCR over RT-qPCR.

We also compared the positivity rates across different sample types, finding that sample types significantly impacted the detection rate. RT-dPCR detected more positives than RT-qPCR in all types of samples. The most notable differences in positivity rates between the two tests were observed in RS, OS, and NS samples, respectively. RS samples posed challenges for detection due to their complex biological background and potential PCR inhibitors [36]. Moreover, as CRCoV is primarily a respiratory pathogen, RS samples are likely to contain lower viral loads than NS and OS samples. These factors contribute to the limitations of RT-qPCR in detecting CRCoV in RS samples. The ability of RT-dPCR to detect CRCoV in RS samples suggests the presence of CRCoV in the feces of naturally infected dogs [8, 37]. These results indicated that CRCoV, typically a respiratory virus in dogs, might not be confined to the respiratory tract. However, experimental studies on CRCoV infection in dogs have shown minimal evidence of rectal shedding, our finding raise the possibility of gastrointestinal involvement and highlight the need for further investigation [2]. In light of these findings, RT-dPCR proved to be the preferred method for sensitive detection, a conclusion supported by numerous studies that have successfully utilized the RT-dPCR platform on diverse biological samples, animal residues, and environmental samples [23, 38, 39]. Furthermore, RT-dPCR's capacity to detect CRCoV underscores its potential as a tool for investigating the CRCoV shedding route, thus broadening our knowledge of CRCoV pathogenesis for future research.

The RT-dPCR assay effectively detects low viral loads across various clinical samples, a capability that is crucial for monitoring CRCoV transmission, especially in crowded settings where the virus can spread readily through respiratory secretions [40]. Monitoring low viral loads in alternative sample types, such as oral and rectal swabs, may support early intervention strategies and could be valuable in immunocompromised dogs or puppies, who are more susceptible to severe disease or prolonged viral shedding [41]. Early detection is crucial in such cases to prevent complications and improve outcomes. The assay may also be useful in asymptomatic or subclinical cases. By providing more comprehensive testing options, it can enable more accurate diagnoses and better-informed clinical decisions. As CRCoV is a key pathogen in CIRDC, infected dogs require not only appropriate treatment but also stringent management and preventive strategies. The assay's high sensitivity is therefore invaluable for informing effective control and prevention measures.

While the high sensitivity of RT-dPCR in detecting low CRCoV viral loads holds significant value for epidemiological studies and transmission control, its clinical relevance may be limited. CRCoV infections generally cause only mild symptoms in dogs, except when co-infections with other respiratory pathogens occur [1, 13]. To date, no studies have investigated the relationship between viral loads and clinical symptoms in naturally infected CRCoV dogs. The only existing study, involving intranasal inoculation of CRCoV in specific-pathogen-free dogs, reported mild upper respiratory symptoms [2]. Further research is necessary to better understand the association between viral loads and clinical manifestations in naturally infected dogs.

Despite these limitations in the clinical application of RT-dPCR assay, our study highlights significant differences in the dynamic detection range between RT-qPCR and RT-dPCR. Due to the partitioning within the nanoplate, RT-dPCR has a more limited detection range compared to RT-qPCR. While RT-dPCR performed effectively with low-concentration samples, it may be less suitable for samples with high nucleic acid target concentrations. Our study found that samples with concentration exceeding 3.65×10^4 copies/μL could overload the RT-dPCR, yielding only positive signals without accurate quantification of CRCoV RNA. This suggests that extremely high target concentrations can affect RT-dPCR's quantification capabilities. Therefore, RT-qPCR remains a practical option for initial screening or primary assay detection, while RT-dPCR is advantageous for clinical samples with low viral loads, allowing precise quantification and superior sensitivity. This distinction should be considered when selecting the most suitable detection method.

Conclusions

An RT-dPCR assay was developed for CRCoV detection and its performance was compared with an established RT-qPCR assay. Both techniques demonstrated effectiveness, with RT-dPCR exhibiting superior performance in various clinical samples, particularly those with low viral loads. Conversely, RT-qPCR provides a broader detection range, highlighting the complementary value of each method for different diagnostic needs. These assays establish a foundation for future diagnostic, monitoring, and prevention efforts, expanding the scope of CRCoV detection and supporting enhanced preventive measures. Future studies should include larger sample sizes across diverse clinical settings. Additionally, longitudinal studies examining viral load dynamics in relation to clinical

outcomes would provide deeper insights into the role of CRCoV in disease progression and transmission.

Abbreviations

CaHV-1	Canid herpes virus 1
CAV-2	Canine adenovirus type 2
CCoV	Canine enteric coronavirus
CDV	Canine distemper virus
CIRDC	Canine infectious respiratory disease complex
CIV	Canine influenza virus
CPIV	Canine parainfluenza virus
CPV	Canine parvovirus
CRCoV	Canine respiratory coronavirus
CV	Coefficients of variation
FCoV	Feline coronavirus
LOD	Limit of detection
NS	Nasal swab
OS	Oropharyngeal swab
RS	Rectal swab
RT-dPCR	Reverse transcription digital polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TGEV	Transmissible gastroenteritis virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04807-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Acknowledgements
Not applicable.

Author contributions
PP conducted the experiment, investigation, methodology, formal analysis, software, validation, visualization, and writing of the original draft. VW provided resources, interpretation of data, and reviewing. CP designed the conceptualization, formal analysis, methodology, supervision, validation, visualization, and writing (review and editing). ST designed the conceptualization, funding acquisition, project administration, resource provision, supervision, visualization, and writing (review and editing). All the authors read and approved the final manuscript.

Funding
This research project is supported by National Research Council of Thailand (NRCT): [NRCT5-RGJ63001-013]; The Second Century Fund (C2F), Chulalongkorn University (to C.P. and P.P.); and the Thailand Science research and Innovation Fund Chulalongkorn University [HEA_FF_68_051_3100_009].

Data availability
The datasets supporting the conclusions of this article are included within the article (and its additional files).

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Animal Care and Use Committee (No. 2231001) and the Institutional Biosafety Committee (No. 2331054) of Chulalongkorn University. Sampling from animals, including dogs, was carried out at multi-centered animal hospitals. Written informed consent was obtained from all owners prior to sample collection, ensuring compliance with ethical guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 16 January 2025 / Accepted: 5 May 2025
Published online: 17 May 2025

References

1. Erles K, Brownlie J. Canine respiratory coronavirus: an emerging pathogen in the canine infectious respiratory disease complex. Vet Clin North Am Small Anim Pract. 2008;38(4):815–25. viii.
2. Mitchell JA, Brooks HW, Szladovits B, Erles K, Gibbons R, Shields S, Brownlie J. Tropism and pathological findings associated with canine respiratory coronavirus (CRCoV). Vet Microbiol. 2013;162(2–4):582–94.
3. Mitchell JA, Cardwell JM, Leach H, Walker CA, Le Poder S, Decaro N, Rusvai M, Egberink H, Rottier P, Fernandez M, et al. European surveillance of emerging pathogens associated with canine infectious respiratory disease. Vet Microbiol. 2017;212:31–8.
4. Erles K, Toomey C, Brooks HW, Brownlie J. Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology. 2003;310(2):216–23.
5. An DJ, Jeong W, Yoon SH, Jeoung HY, Kim HJ, Park BK. Genetic analysis of canine group 2 coronavirus in Korean dogs. Vet Microbiol. 2010;141(1–2):46–52.
6. Lu S, Wang Y, Chen Y, Wu B, Qin K, Zhao J, Lou Y, Tan W. Discovery of a novel canine respiratory coronavirus support genetic recombination among betacoronavirus1. Virus Res. 2017;237:7–13.
7. Lorusso A, Desario C, Mari V, Campolo M, Lorusso E, Elia G, Martella V, Buonavoglia C, Decaro N. Molecular characterization of a canine respiratory coronavirus strain detected in Italy. Virus Res. 2009;141(1):96–100.
8. Yachi A, Mochizuki M. Survey of dogs in Japan for group 2 canine coronavirus infection. J Clin Microbiol. 2006;44(7):2615–8.
9. Sowman HR, Cave NJ, Dunowska M. A survey of canine respiratory pathogens in new Zealand dogs. N Z Vet J. 2018;66(5):236–42.
10. Wille M, Wensman JJ, Larsson S, van Damme R, Theelke AK, Hayer J, Malmberg M. Evolutionary genetics of canine respiratory coronavirus and recent introduction into Swedish dogs. Infect Genet Evol. 2020;82:104290.
11. De Luca E, Álvarez-Narváez S, Baptista RP, Maboni G, Blas-Machado U, Sanchez S. Epidemiologic investigation and genetic characterization of canine respiratory coronavirus in the southeastern united States. J Vet Diagn Invest. 2024;36(1):46–55.
12. Poonsin P, Wiwatvisawakorn V, Chansaenroj J, Poovorawan Y, Piewbang C, Techangamsuwan S. Canine respiratory coronavirus in Thailand undergoes mutation and evidences a potential putative parent for genetic recombination. Microbiol Spectr. 2023;11(5):e0226823.
13. Piewbang C, Rungsipipat A, Poovorawan Y, Techangamsuwan S. Cross-sectional investigation and risk factor analysis of community-acquired and hospital-associated canine viral infectious respiratory disease complex. Heliyon. 2019;5(11):e02726.
14. Reagan KL, Sykes JE. Canine infectious respiratory disease. Vet Clin North Am Small Anim Pract. 2020;50(2):405–18.
15. Erles K, Shiu KB, Brownlie J. Isolation and sequence analysis of canine respiratory coronavirus. Virus Res. 2007;124(1–2):78–87.
16. Piewbang C, Rungsipipat A, Poovorawan Y, Techangamsuwan S. Development and application of multiplex PCR assays for detection of virus-induced respiratory disease complex in dogs. J Vet Med Sci. 2017;78(12):1847–54.
17. Mitchell JA, Brooks H, Shiu KB, Brownlie J, Erles K. Development of a quantitative real-time PCR for the detection of canine respiratory coronavirus. J Virol Methods. 2009;155(2):136–42.
18. More GD, Cave NJ, Biggs PJ, Acke E, Dunowska M. A molecular survey of canine respiratory viruses in new Zealand. N Z Vet J. 2021;69(4):224–33.
19. Spiss S, Benetka V, Künzel F, Sommerfeld-Stur I, Walk K, Latif M, Möstl K. Enteric and respiratory coronavirus infections in Austrian dogs: serological

and virological investigations of prevalence and clinical importance in respiratory and enteric disease. Wien Tierazt Monatsschr—Vet Med Austria. 2012;99:67–81.
20. Nyaruaba R, Mwaliko C, Dobnik D, Neužil P, Amoth P, Mwau M, Yu J, Yang H, Wei H. Digital PCR applications in the SARS-CoV-2/COVID-19 era: a roadmap for future outbreaks. Clin Microbiol Rev. 2022;35(3):e0016821.
21. Yu Z, Zhao Z, Chen L, Yan H, Cui Q, Ju X, Yong Y, Liu X, Ma X, Zhang G. Development of a droplet digital PCR assay to detect bovine alphaherpesvirus 1 in bovine semen. BMC Vet Res. 2022;18(1):125.
22. Ding J, Xu X, Deng Y, Zheng X, Zhang T. Comparison of RT-ddPCR and RT-qPCR platforms for SARS-CoV-2 detection: implications for future outbreaks of infectious diseases. Environ Int. 2024;183:108438.
23. Ahmed W, Smith WJM, Metcalfe S, Jackson G, Choi PM, Morrison M, Field D, Gyawali P, Bivins A, Bibby K, et al. Comparison of RT-qPCR and RT-dPCR platforms for the trace detection of SARS-CoV-2 RNA in wastewater. ACS ES T Water. 2022;2(11):1871–80.
24. Villa C, Costa J, Mafra I. First nanoplate digital PCR method to trace allergenic foods: improved sensitivity for the detection of Sesame. Food Chem. 2024;444:138650.
25. Crucitta S, Ruglioni M, Novi C, Manganiello M, Arici R, Petrini I, Pardini E, Cucchiara F, Marmorino F, Cremolini C, et al. Comparison of digital PCR systems for the analysis of liquid biopsy samples of patients affected by lung and colorectal cancer. Clin Chim Acta. 2023;541:117239.
26. Lei X, Lv Q, Qin Y, Chen W, Hu Y, Zhao C, Zhang X, Huang H, Li Y, Lu J, et al. Establishment of a chip digital PCR detection method for canine circovirus. Heliyon. 2024;10(9):e30859.
27. Specchiarello E, Carletti F, Matusali G, Abbate I, Rozera G, Minosse C, Petrivelli E, Ferraioli V, Sciamanna R, Maggi F. Development and validation of a nanoplate-based digital PCR assay for absolute MPXV quantification. J Virol Methods. 2023;321:114802.
28. Rocchigiani AM, Tilocca MG, Portanti O, Vodret B, Bechere R, Di Domenico M, Savini G, Lorusso A, Puggioni G. Development of a digital RT-PCR method for absolute quantification of bluetongue virus in field samples. Front Vet Sci. 2020;7:170.
29. Coudray-Meunier C, Fraisse A, Martin-Latil S, Guillier L, Delannoy S, Fach P, Perelle S. A comparative study of digital RT-PCR and RT-qPCR for quantification of hepatitis A virus and Norovirus in lettuce and water samples. Int J Food Microbiol. 2015;201:17–26.
30. Sedlak RH, Nguyen T, Palileo I, Jerome KR, Kuypers J. Superiority of digital reverse Transcription-PCR (RT-PCR) over Real-Time RT-PCR for quantitation of highly divergent human rhinoviruses. J Clin Microbiol. 2017;55(2):442–9.
31. Martin-Latil S, Hennechart-Collette C, Delannoy S, Guillier L, Fach P, Perelle S. Quantification of hepatitis E virus in Naturally-Contaminated pig liver products. Front Microbiol. 2016;7:1183.
32. Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform. 2019;20(4):1160–6.
33. Kuraku S, Zmasek CM, Nishimura O, Katoh K. aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Res. 2013; 41(Web Server issue):W22–28.
34. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611–22.
35. Huggett JF. The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020. Clin Chem. 2020;66(8):1012–29.
36. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors - occurrence, properties and removal. J Appl Microbiol. 2012;113(5):1014–26.
37. Decaro N, Desario C, Elia G, Mari V, Lucente MS, Cordioli P, Colaianni ML, Martella V, Buonavoglia C. Serological and molecular evidence that canine respiratory coronavirus is Circulating in Italy. Vet Microbiol. 2007;121(3–4):225–30.
38. Sedlak RH, Kuypers J, Jerome KR. A multiplexed droplet digital PCR assay performs better than qPCR on Inhibition prone samples. Diagn Microbiol Infect Dis. 2014;80(4):285–6.
39. Piewbang C, Poonsin P, Lohavicharn P, Punyathi P, Kesdangsakonwut S, Kasantikul T, Techangamsuwan S. Natural SARS-CoV-2 infection in dogs: determination of viral loads, distributions, localizations, and pathology. Acta Trop. 2024;249:107070.
40. Buonavoglia C, Sykes JE. 27 - Canine Respiratory Coronavirus Infection. In: *Greene's Infectious Diseases of the Dog and Cat (Fifth Edition)*. edn. Edited by Sykes JE. Philadelphia: W.B. Saunders; 2021: 325–331.
41. Maboni G, Seguel M, Lorton A, Berghaus R, Sanchez S. Canine infectious respiratory disease: new insights into the etiology and epidemiology of associated pathogens. PLoS ONE. 2019;14(4):e0215817.

Publisher's note