Research Article

Development and Optimization of a Triplex qRT-PCR Assay for Simultaneous Detection of Feline Herpesvirus-1, Feline Calicivirus, and SARS-CoV-2

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Abstract | Rapid and accurate detection of feline respiratory viruses remains a diagnostic challenge, particularly when multiple pathogens coexist. In addition to feline calicivirus (FCV) and feline herpesvirus-1 (FHV-1), the inclusion of SARS-CoV-2 is important due to its zoonotic potential and documented cross-species transmission. In this study, a triplex TaqMan quantitative RT-PCR (qRT-PCR) assay was developed and optimized for the simultaneous detection of FCV, FHV-1, and SARS-CoV-2. Primer and probe concentrations were systematically optimized to enhance assay performance in a multiplex format involving both DNA and RNA viruses. Analytical sensitivity was evaluated using tenfold serial dilutions of vaccine-derived nucleic acids, and performance was compared with a commercial SARS-CoV-2 detection kit. The optimized assay demonstrated high analytical sensitivity with limits of detection (LoD) of approximately 2×10^0 copies/4 µL for FHV-1, 6.3×10^1 copies/4 µL for FCV, and 10 copies/2 µL for SARS-CoV-2. The multiplex assay showed improved sensitivity for FCV compared to the uniplex format, while maintaining comparable performance for FHV-1. For SARS-CoV-2, the developed assay achieved a LoD that was only onefold higher than that of the commercial kit. Application of the assay to 67 clinical samples revealed positivity rates of 22.4% for FCV and 23.8% for FHV-1, with no detection of SARS-CoV-2. The developed multiplex qRT-PCR assay was highly suitable for rapid detection and accurate diagnosis of feline respiratory viruses (FCV, FHV-1) and SARS-CoV-2 worldwide, with the highest specificity and sensitivity. However, rapid and accurate diagnosis is critical for controlling infection in cat populations.

Keywords | *FHV-1, FCV, SARS-CoV-2*, Multiplex qRT-PCR

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INTRODUCTION

The most prevalent diseases in stressful, crowded feline populations are feline upper respiratory tract diseases (URTD). The five most common etiological agents are *Mycoplasma felis*, *Bordetella bronchiseptica*, *Chlamydophila felis*, feline calicivirus (FCV), and feline herpesvirus-1 (FHV-1) (Litster and Leutenegger, 2015) and recently SARS-CoV-2 (Sharun *et al.*, 2021; Hamdy *et al.*, 2023). The FHV-1 genome is DNA, and this virus causes

rhinotracheitis in felid species worldwide (Cohn, 2011). Since the virus stays dormant in the trigeminal ganglia after recovery, infected cats are lifetime carriers (Najafi *et al*., 2014; Ward and Kelman, 2011). *FCV* is an RNA virus that characteristically causes oral ulcerations (Chandler *et al*., 2008). In contrast to *FHV-1*, cats may continue to excrete the virus through their oral, respiratory, and ocular secretions for weeks to years after infection (Ward and Kelman, 2011).

An outbreak of the coronavirus disease, COVID-19, was reported in Wuhan, China, at the end of 2019. Severe acute respiratory syndrome coronavirus 2 *(SARS-CoV-2)* was the cause of this outbreak (Wu *et al*., 2020). This virus is a spherical pleomorphic virus and belongs to the Nidovirales order, the Coronaviridae family, and the *Betacoronavirus* genus of the *Coronavirinae* subfamily.

Furthermore, numerous studies have confirmed infection of cats with SARS-CoV-2, with mild-to-severe clinical signs, detected by detection of viral nucleic acid or specific antibodies worldwide (Sharun *et al*., 2021) and in Egypt (Hamdy *et al*., 2023). In the molecular diagnosis of feline respiratory infections, multiplex PCR is a valuable tool for simultaneous detection of major viral pathogens. Although there is an article describing an assay for the detection of viral and nonviral respiratory infections in cats (Thieulent *et al*., 2024), Although bacterial respiratory pathogens such as *Mycoplasma felis*, *Chlamydia felis*, and *Bordetella bronchiseptica* are clinically relevant, the primary objective of the present study was to develop a molecular assay targeting the major viral respiratory pathogens of cats.

The specificity, sensitivity, and efficiency of PCR technology have made it popular for diagnosing a wide range of infections. By amplifying numerous templates in a single reaction, multiplex PCR significantly increases detection efficiency as compared to uniplex PCR (Ali *et al*., 2014; Chamberlain and Chamberlain, 1994). Because of its high specificity, sensitivity, and time-saving capabilities, quantitative real-time PCR (qRT-PCR), a second-generation PCR technology, is now widely employed in clinical diagnostics and scientific research. Despite the availability of multiplex PCR assays, optimizing assay performance when combining DNA and RNA viruses in a single reaction remains a significant challenge. Differences in genome type, replication mechanisms, and amplification efficiency may lead to competition among targets, ultimately affecting assay sensitivity and reliability. In particular, RNA viruses such as FCV and SARS-CoV-2 often require higher primer and probe concentrations compared to DNA viruses like FHV-1, especially in multiplex formats (Bustin *et al*., 2009; Mackay *et al*., 2002). In this study, we designed a multiplex (triplex) qRT-PCR assay for the detection of FHV-1, FCV, and SARS-CoV-2 with high analytical performance. Therefore, the present study aims to develop and optimize a triplex TaqMan quantitative RT-PCR (qRT-PCR) assay for the simultaneous detection of FCV, FHV-1, and SARS-CoV-2. Specifically, this study focuses on optimizing primer–probe concentrations to enhance assay sensitivity and performance in a multiplex system involving both DNA and RNA viral targets. The developed assay was further evaluated for analytical sensitivity, specificity, and applicability to clinical samples.

MATERIALS AND METHODS

Unless otherwise specified, the uniplex and multiplex assays were performed using identical thermal cycling conditions and reaction chemistry. The only experimental variable evaluated during optimization was the primer/probe concentration, allowing direct comparison between assay formats. The reported LoD values represent estimated endpoint limits of detection based on repeated endpoint assessment and were not derived from probit analysis.

Positive controls and clinical samples

FCV (attenuated vaccine strain F9) and *FHV-1* (attenuated vaccine strain FVRm) were obtained from a feline Fellocell 4® vaccine purchased from Zoetis. The virus titers were $10^{5.5}$ $CCID_{50}$/1 mL for *FCV* and 10^{5} $CCID_{50}$/1 mL for *FHV-1*. SARS-CoV-2 (Sinovac inactivated vaccine strain) is a vaccine from Sinovac Company, obtained from the Human VACSERA Institute, Agouza, Giza, Egypt, with a concentration of 1200 SU/mL.

A total of 67 clinical nasal and oropharyngeal samples were collected from different shelters in Giza governorate. Samples were collected from randomly selected cats of both sexes, different breeds, and ages exhibiting respiratory signs. Methods for sample collection and storage were as described. Swabs were collected from infected cats by firmly and vigorously swabbing the mucosal surface(s) of the nose and/or the oropharynx of cats using a dry swab that had been moistened with saline. Specimens were stored at - 20°C before being sent to AHRI, where they were stored at - 80°C before being processed and assayed.

Primer and probe designs

The published sequences of the ORF2 gene of *FCV* and the TK gene of *FHV-1* were retrieved from GenBank and aligned using MEGA7 software. The most conserved regions were selected to design two pairs of primers and two probes for fluorescent detection of both *FCV* and *FHV-1*. In addition, two pairs of primers for conventional PCR for both *FCV* and *FHV-1*. The specificity of the designed primers was verified through BLAST analysis in NCBI before use. For *SARS-CoV-2*, previously published primer sets targeting the N gene were used for qPCR screening (Chu *et al*., 2020). Naturally infected cats are infected with

SARS-CoV-2 rather than a feline-specific coronavirus variant. Therefore, the published primer/probe set targeting the highly conserved N gene remains applicable to both human and feline SARS-CoV-2 isolates. The three probes were labeled with FAM/TAMRA (*SARS-CoV-2*), VIC/BHQ1 (*FCV*), and CY5/BHQ2 (*FHV-1*) at their 5′ and 3′ ends, respectively. Two degenerate nucleotide positions were incorporated into the FCV forward primer (W= A/T and Y= C/T) to accommodate sequence variability identified among conserved FCV isolates during multiple sequence alignment. All primers and probes were synthesized by Metabion International AG (Plenegg, Germany). The detailed sequences of these primers are presented in Table 1.

Viral RNA and DNA extraction

Total genomic DNA and RNA were extracted from the vaccine-derived positive-control materials using the All-Prep DNA/RNA Mini Kit according to the manufacturer's instructions (QIAGEN, 2020a). Viral RNA from the FCV and SARS-CoV-2 vaccine materials was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, 2020b), whereas DNA from the FHV-1 vaccine material was extracted using the QIAamp DNA Mini Kit (QIAGEN, 2020c). The purified nucleic acids were subsequently used for downstream qRT-PCR analysis and estimated copy-number determination.

Quantification of RNA copy number

The developed assay demonstrated analytical performance comparable to the evaluated commercial SARS-CoV-2 assay while simultaneously detecting FCV and FHV-1 within a single reaction. It is known that $CCID_{50}$ cannot be directly converted into genome copy number using a universal conversion factor. Therefore, for the purpose of preparing serial dilution panels and estimating the initial template concentration used in analytical sensitivity experiments, genome copy numbers were approximated from the manufacturer-reported vaccine titers using following equations.

SARS-CoV-2 RNA

The inactivated SARS-CoV-2 vaccine was used solely as a source of viral RNA for analytical assay optimization and comparison with the commercial RT-qPCR kit. The assay targets a short conserved genomic region, and therefore successful amplification depends primarily on the integrity of the target RNA fragment rather than viral infectivity. Although chemical inactivation may affect viral infectivity, it is not expected to substantially impair amplification of intact target sequences. Tenfold serial dilutions of SARS-CoV-2 vaccine RNA were run with both SARS-CoV-2 Nucleic Acid Detection Kit was produced by TransGen Biotech Company (Madrid, Spain), which has a known LoD (500 copies/1 mL) and our Multiplex PCR design using TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG, by quantities and thermal profiles as shown in Tables 2 and 3, respectively.

FHV-1 and FCV titre by $CCID_{50}$ and conversion to estimated copy number

Plaque assay and $CCID_{50}$ are cell culture-based methods used to estimate the titers of viruses that cause cytopathogenic damage in infected cell cultures; therefore, we calculated estimated copy number based on the $CCID_{50}$ titers. It is known that $CCID_{50}$ and viral genome copies are not equivalent, so in our study, we ran tenfold serially diluted vaccine nucleic acid on twofold serially diluted probes of FHV-1 and FCV to obtained best diluted probe gave lowest LoD, as shown in Figures 1 and 5, and considered that 1 $CCID_{50} \approx 1{,}000 - 10{,}000$ copies (based on viral type and replication efficiency). We calculated the copy no. in the next Equations 1 and 2 based on

Table 1: Data of real time primers and conventional primers used in our study.

	Real time (Quantitative) PCR primers and probes	Length (bp)	Conventional PCR primers	Length (bp)
Feline calicivirus /ORF2 (Capsid)	CCTGATTCCTTTGCWGTYTA	189	CCTGATGGWTGGCCAGACAC	950
	TCCTAATGTTGGAGGCAAGCCG		GTACCCTTTGCTCAAGAATTTTG	
	Hex-GCCTCCTACATGGGAAT-TCAATTGG-BHQ-1			
Feline herpes virus/ Thymine Kinase gene	TTGTATGTGAGGAACACCCCGACG	155	TTGTATGTGAGGAACACCCCGACG	590
	GAGGTTCTCGTGGAAGTGTTGC		CCATATCTTGTCTCAGTGCTCCC	
	Cy5-GTTTCCCACTCGCAAGATAT-TTTGTG-BHQ-2			
SARS-CoV2 *	TAATCAGACAAGGAACTGATTA	105	CCCTCAGGGTTTTTCGGCTT	1092
	CGAAGGTGTGACTTCCATG		CTGTGGATCACGGACAGCAT	
	FAM–GCAAATTGTGCAATTTGCGG-TAM			

* = Nucleoprotein Gene of SARS-CoV2 for Real time (Quantitative) PCR and Spike Gene for Conventional PCR.

Table 2: Reaction Mix of different Real time kits used in the study (TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG, TransScript® Probe One-Step qRT-PCR SuperMix and SARS-CoV-2 Nucleic Acid Detection Kit).

Reaction Mix of different Real time kits used in the study	SARS-CoV2 Transcript 1x Reaction®	1x Uniplex Transcript Reaction®	1x Multiplex Transcript reaction®
2x Multiplex Probe One Step Reaction Mix	15 ul	10 ul	10 ul
Multiplex Probe one step Enzyme Mix	1 ul	0.5 ul	0.8 ul
Passive Reference dye 50x	---	---	---
Nuclease free H2O	---	4.5 ul	0.2 ul
Calici Pr./pb Mix (HEX Probe)	4 ul of closed kit Primer/pb mix	1 ul of only one Primer / probe mix	1 ul
Herpes Pr./pb Mix (CY5 Probe)			1 ul
SARS-CoV2 Pr./pb Mix (FAM Probe)			1 ul
Total Mix Volume	20 ul	16 ul	14 ul
Template	5 ul	4 ul	6 ul*
Total Volume	25 ul	20 ul	20 ul

* = In case positive control put 2 µl SARS-CoV2 RNA with 4 ul Zoetis vaccine nucleic acid. Master mix volume refers to the reaction mixture before template addition. Final reaction volumes were 25 µL for the commercial SARS-CoV-2 kit and 20 µL for the developed uniplex and multiplex assays. Water volume was adjusted according to the number of primer/probe mixes and template volume required for each format.

Table 3: Thermal Profiles of different PCR Kits used in the study.

qRT-PCR Transcript Thermal Profile (TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG, TransScript® Probe One-Step qRT-PCR SuperMix and SARS-CoV-2 Nucleic Acid Detection Kit)						
Steps	RT- Activation	DNA Polymerase Activation		Cycling (40x)		
Temp/Time	50 C/5 mints	94 C/30 sec		94 C/05 sec	C/60 sec, data col.	
Conventional RT-PCR Thermal Profile (EasyScript® One-Step RT-PCR SuperMix)						
Steps	RT- Activation and DNA Polymerase Activation		Denaturation	Annealing	Extension	Final Extension
Temp/Time	45 C/30 mints	94 C/5 m	94 C/30 sec	60 C/45 sec	72 C/1 m	72 C/10 m
Cycling	1x	1x	35x			1x

Equation No. 1 for FCV	Volume	$CCID_{50}$	Estimated copies (Total)	Estimated copies (in 4 µL template)
FCV Origin (1 mL Zoetis vaccine vial)	1,000 µL	$10^{5.5}$ (≈ 316227.8)	≈ 316227.8×10^3 copies in 195 µL (30 µL*6.5 times) elution buffer	[(47434.16×10^3) /30] x 4 µL = 6.3×10^6 copy
Extraction Volume from FCV Origin	150 µL (Acc. to kit protocol)	≈ 47434.16	≈ 47434.16×10^3 copies in 30 µL elution buffer	

Equation No. 2 for FHV-1	Volume	$CCID_{50}$	Estimated copies (Total)	Estimated copies (in 4 µL template)
FHV-1 Origin (1 mL Zoetis vaccine vial)	1000 µL	10^5 (≈ 100000)	≈ $10^5 \times 10^3$ copies in 195 µL(30 µL*6.5 times) elution buffer	[(15000×10^3) / 30] x 4 µL = 2×10^6 copy
Extraction Volume from FHV-1 Origin	150 µL (Acc. to kit protocol)	≈ 15000	≈ 15000×10^3 copies in 30 µLelution buffer	

the standard assumption that 1 $CCID_{50}$ = 1,000 copies, which is conservative also, nucleic acid extraction efficiency was not experimentally determined and that the calculated values represent theoretical estimates derived from the manufacturer's reported infectious titers, extraction volumes, elution volume, and template volume per reaction.

The one-step real-time TaqMan RT-PCR method was established using a feline Felocell 4 vaccine nucleic acid (FCV titre was 105.5 $CCID_{50}$/1 mL vaccine, while FHV-1 titre was 105 $CCID_{50}$/1 mL). Notably, $CCID_{50}$ and viral genome copies are not equivalent. In our study, we estimated that 1 $CCID_{50}$ ≈ 1,000–10,000 copies (depending on viral type and replication efficiency). Serial dilution in Figure 1 and (Supplementary Tables 1-3) shows tenfold dilutions, starting from "Origin" down to Origin × 10^{-6}, with qPCR input of 4 µL from each dilution.

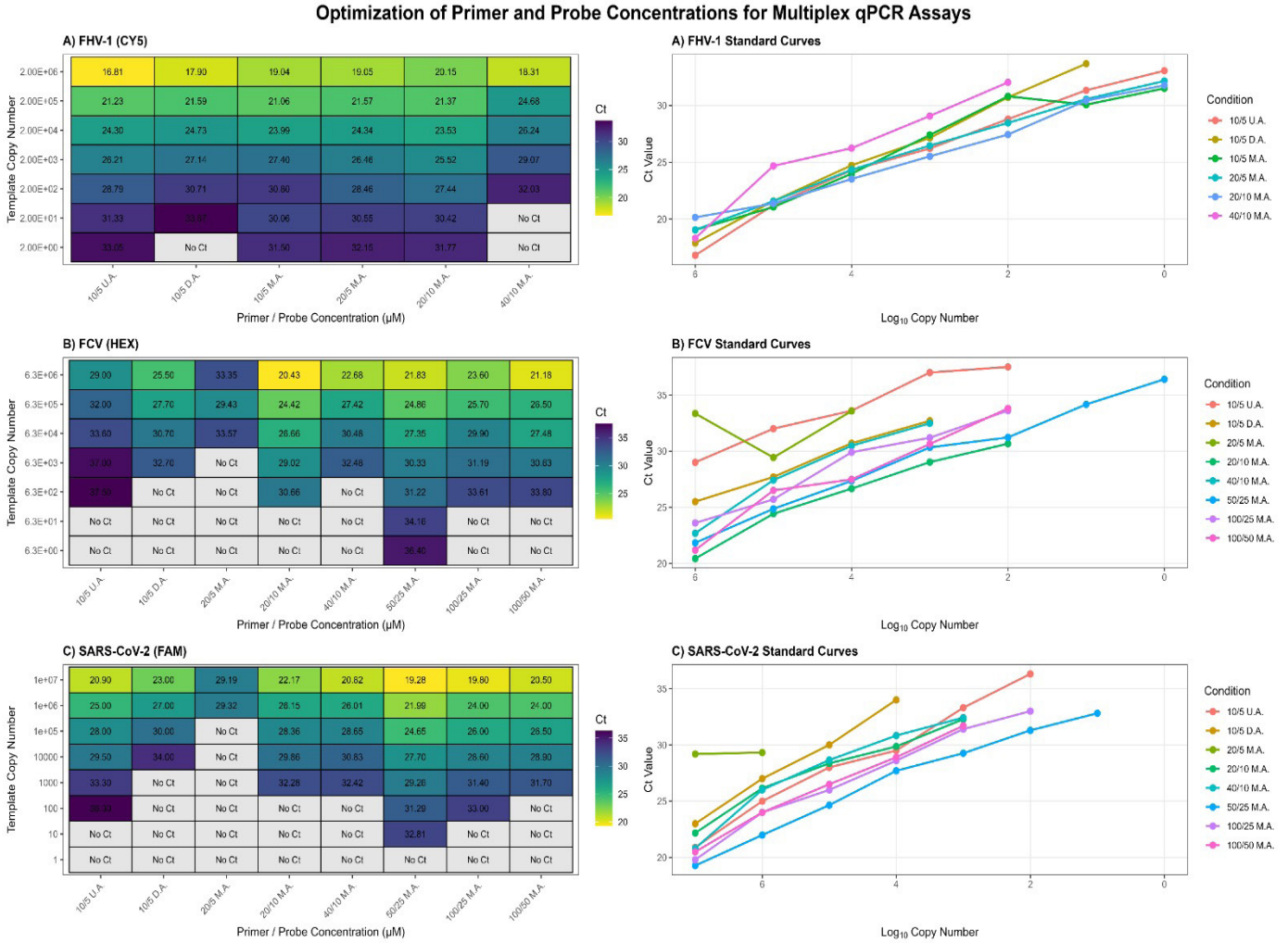

Figure 1: Optimization of primer/probe concentrations and performance evaluation across serial dilutions for the triplex qRT-PCR assay. (A–C) Heatmaps illustrating Ct values obtained across different primer/probe concentrations for FHV-1 (CY5), FCV (HEX), and SARS-CoV-2 (FAM) targets, respectively, using 10-fold serial dilutions. (D–F) Line plots comparing Ct values across all tested primer/probe combinations for each viral target. (G–I) Standard curves generated using the optimized primer/probe concentrations, demonstrating strong linearity between Ct values and $\log_{10}$ copy number for all targets. The optimal conditions yielding the lowest Ct values and best amplification efficiency were identified as 20/5 µM for FHV-1 and 50/25 µM for both FCV and SARS-CoV-2.

Establishment of the one-step real-time TaqMan RT-PCR method

Several twofold serial dilutions were performed for each primer/probe set corresponding to each virus. These were analyzed in a uniplex format using one-step real-time TaqMan RT-PCR with TransScript® Probe One-Step qRT-PCR SuperMix. The quantities and thermal profiles used are detailed in Tables 2 and 3. The annealing/extension step was extended from 30 s to 60 s to improve amplification efficiency and fluorescent signal acquisition in the multiplex assay containing both DNA and RNA viral targets. This adjustment aimed to achieve the highest dilution with the lowest detection limit and optimal correlation coefficient (R^2) values, as presented in Supplementary Tables 1-3 and summarized in Supplementery Table 8.

The amplification efficiency (AE) and correlation coefficient (R2) were used in the uniplex assay as described previously (Thieulent *et al.*, 2024). The optimal concentrations of probes and primers that yielded the highest sensitivity were tested using tenfold serial dilutions of each vaccine nucleic acid, repeated three times in a uniplex assay with different operators to assess reproducibility, as shown in Supplementary Table 9 (interassay tests). Cycle threshold (Ct) cutoff values were determined by submitting the average replicate values of the endpoint dilution along with 3 × standard deviation (SD) (Burd, 2010).

Determination of specificity of primers and probe sets

The specificities of the primer pairs and probes were evaluated using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). An *in silico* BLAST analysis was conducted to assess the sequence

specificity of each primer and probe and to identify potential nonspecific homologies with unrelated sequences from other respiratory virus genomes.

The specificity of the established qRT-PCR assay was confirmed using *Bordetella bronchiseptica*, *Chlamydophila felis*, and *Mycoplasma felis*, which cause similar clinical signs in infected cats. To ensure assay specificity, the nucleic acids of *Chlamydophila felis* are already present in the purchased feline vaccine. At the same time, those of *Mycoplasma felis* and *Bordetella bronchiseptica* were obtained from RLQP at AHRI, Doki, Giza, Egypt. During the preparation of the uniplex assay, we included a negative control containing 3 µL of *Mycoplasma felis* DNA and 3 µL of *Bordetella bronchiseptica* DNA.

Determination of sensitivity of primers and probe sets in multiplex assay

To evaluate the sensitivity of the method, a feline Felocell 4 vaccine nucleic acid was diluted serially starting from "Origin" down to Origin × 10^{-7} (from nearly 6.3 × 10^6 *FCV* copy/4 µL down to 6.3 × 10^0 *FCV* copy/4 µL Zoetis vaccine nucleic acid and 2.00 × 10^6 *FHV-1* copy/4 µL down to 2.00 × 10^0 *FHV-1* copy/4 µL Zoetis vaccine nucleic acid) and *SARS-CoV-2* with assumed origin concentration of 10^7 copy no./2 µL down to 10 copy/2 µL of Sinovac vaccine RNA using TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG, by quantities and thermal profiles, as shown in Tables 2 and 3, respectively.

All diluted vaccine nucleic acid subjected to the sensitivity of the multiplex assay by using primers and probe sets of the highest dilution and lowest LoD from (Supplementary Table 7) and Figure 5. The amplification efficiency (AE) and correlation coefficient (R^2) were used as parameters to evaluate the sensitivity of the triplex assay (Simmons *et al*., 2016; Töwe *et al*., 2010), as shown in Supplementary Table 6 for *FCV* and *FHV-1* and Supplementary Table 7 for *SARS-CoV-2*. The most suitable probe and primer concentrations that yielded the highest sensitivity were used with tenfold serial dilutions of each vaccine nucleic acid, 3 times, in our multiplex assay, called the intra-assay, for repeatability checks, as shown in Supplementary Table 9. Cycle thresholds (Ct) of cutoff values were calculated by submission of the average replicate values of the endpoint dilution with (3 × standard deviation (SD) (Burd, 2010). The analytical sensitivity of the optimized triplex qRT-PCR assay was evaluated using tenfold serial dilutions of positive-control nucleic acids. The final LoD assessment was repeated three times under the optimized primer/probe concentrations. The LoD was operationally defined as the lowest estimated copy number that yielded reproducible amplification at the endpoint dilution in the repeated assessment. Probit analysis was not performed; therefore, the reported LoD represents an observed endpoint LoD rather than a 95% probability-based LoD.

Conventional PCR, DNA sequencing, and homology search

Long fragments with conventional primers were run in 20 µL reaction volume and composition of the reaction mixture of PCR was 10 µL 2x EasyScript One Step Reaction Mix, 0.4 µL EasyScript One-Step Enzyme Mix, 1 µL Primer Mix (forward, 20 pm; reverse, 20 pm), 3.6 µL nuclease-free water and 5 µL template to become total volume 20 µL then run with thermal profiles (Table 3). PCR products were run at 120 volts for 20 minutes in an Agarose gel with a concentration of 1.5–2 % to allow short PCR products to appear in the gel. Gel products of the expected sizes were purified using the Thermo Scientific GeneJET Gel Extraction Kit (EU, Lithuania) following the manufacturer's instructions. Sequencing reactions were then carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) according to the manufacturer's protocol. The resulting products were purified using the CENTRI-SEP Kit (Applied Biosystems, USA) and subsequently analyzed by capillary electrophoresis on an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, USA) immediately after a heat shock treatment at 100°C for 5 min. The obtained sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify homologous nucleotide sequences in GenBank.

Statistical analysis

Data generation and collection were carried out with qTOWER Analytikjen SW. Data management and analysis were performed using Microsoft Excel 2007 (Microsoft, USA) and MODDE 12.1 software (Umetrics, Sweden). Results are presented as mean value ± standard deviation (SD). Intra- and interassay variations were calculated from the mean Ct values and expressed as coefficients of variation (CV), as shown in Supplementary Table 9. Intra-assay precision was evaluated by testing three replicate reactions of the dilution of lowest LoD within the same experimental run. Inter-assay precision was assessed by repeating the experiment independently on different runs and by different operators. Repeatability and reproducibility were evaluated using the optimized triplex qRT-PCR conditions and the tenfold serial dilution panel used for LoD confirmation. The assay was repeated three times for each viral target. Intra-assay variation was calculated from repeated Ct measurements obtained under the same assay conditions, while inter-assay variation was calculated from repeated runs performed using the same optimized protocol. SD and CV% were calculated from Ct values across the serial dilutions.

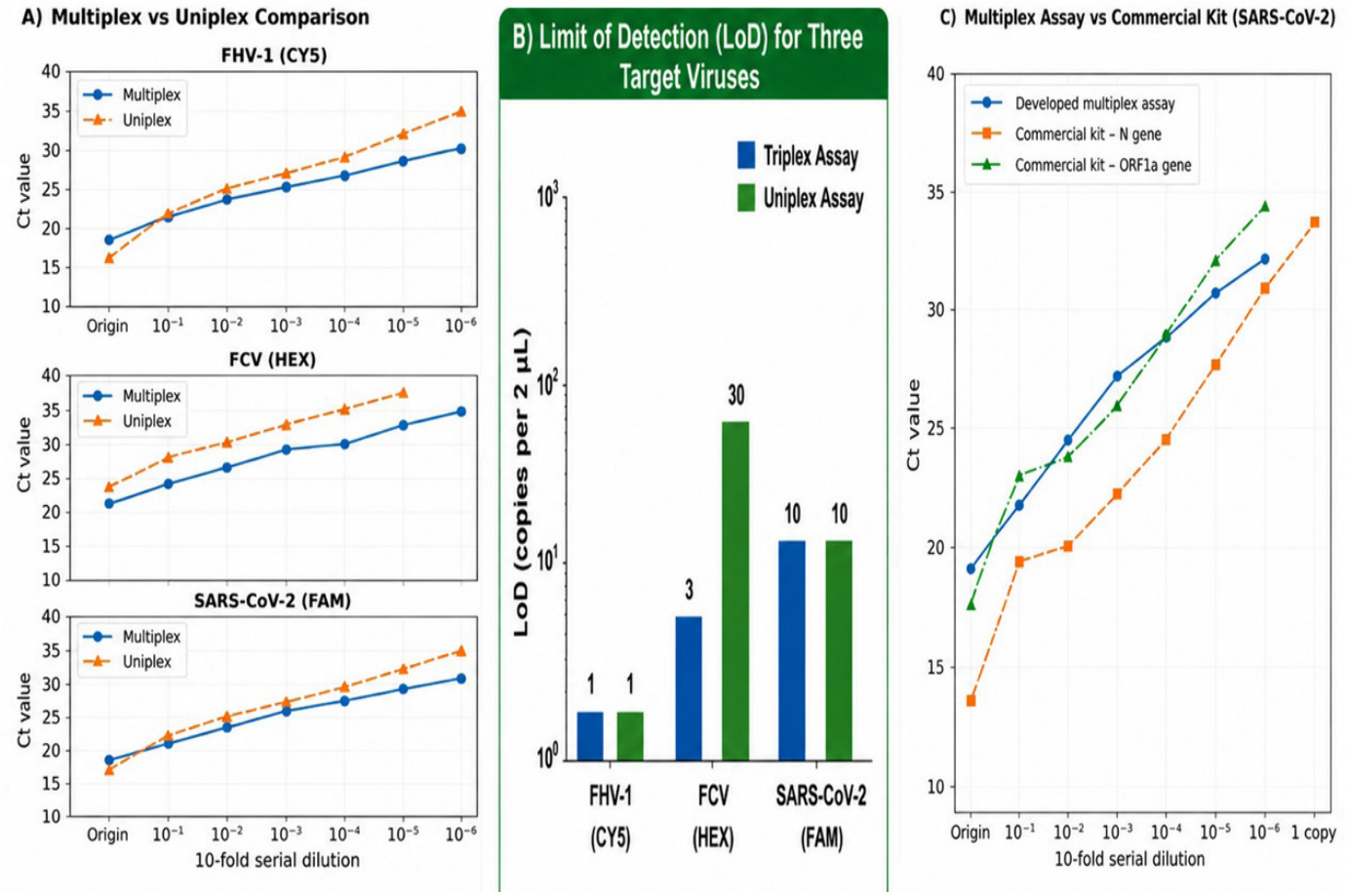

Figure 2: Comparative performance of multiplex and uniplex qRT-PCR assays and evaluation against a commercial SARS-CoV2 detection kit.

Comparative performance of multiplex and uniplex qRT-PCR assays and evaluation against a commercial SARS-CoV-2 detection kit. (A) Ct values for multiplex and uniplex assays across 10-fold serial dilutions of FHV-1, FCV, and SARS-CoV-2. These data are presented as descriptive Ct values for assay-performance comparison. (B) Limit of detection (LoD) expressed as copies/2 µL for multiplex and uniplex assays. The LoD experiment was repeated three times to confirm endpoint detection. (C) Comparison of the multiplex assay with commercial SARS-CoV-2 kits targeting the N gene and ORF1a across serial dilutions. Ct: cycle threshold; LoD: limit of detection.

RESULTS

Optimization and establishment of the uniplex Assay

By using a 5 µM probe with 10 µM primers of each target as usual in our lab (2 concentration primers: 1 concentration probe as shown in Figure 1 with taking 1 µL of primer/probe mix (0.33 µL forward primer +0.33 µL reverse primer + 0.33 µl probe). The highest *FHV-1* primer/probe dilution gave the lowest estimated LoD, and an excellent R^2 was observed with 5 µM probe and 20 µM primers, or 5 µM probe and 10 µM primers. In contrast, for FCV and SARS-CoV-2, lower estimated LoD was obtained in the uniplex and duplex assays than Multiplex assay. However, the highest FCV and SARS-CoV-2 primer/probe dilution yielded the lowest estimated LoD, and the best R^2 was observed with 25 µM probes and 50 µM primers (Figure 1) and Supplementery Tables 1-3 and summarized in Supplementery Table 7.

Specificity of the one-step real-time TaqMan RT-PCR method

SARS-CoV-2, *FCV*, *FHV-1*, and other feline respiratory pathogens, including *Bordetella bronchiseptica*, *Chlamydophila felis*, and *Mycoplasma felis*, were used to evaluate assay specificity. The results demonstrated that only *FCV*, *FHV-1*, and *SARS-CoV-2* produced specific amplification curves. A nucleotide BLAST analysis of each primer and probe revealed sequence homology exclusively with the corresponding target regions of the intended viral genomes. No amplification was detected in the negative controls of the uniplex assays, confirming the triplex assay's high specificity.

Sensitivity of the Multiplex (Triplex) One-Step Real-Time TaqMan RT-PCR Method

The estimated LoD was established through serial dilutions of the positive control to identify the lowest estimated copy number capable of yielding a detectable PCR

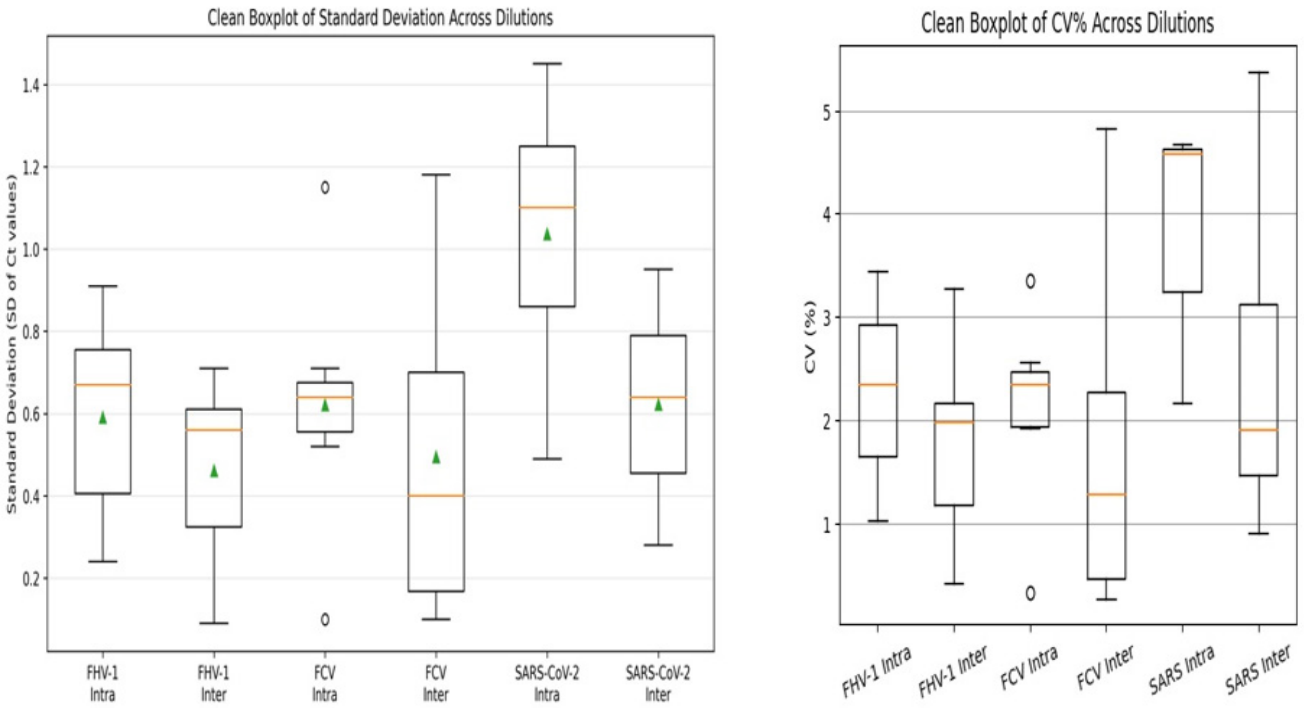

Figure 3: Precision analysis of the triplex qRT-PCR assay across dilution ranges.
(A) Boxplot representation of standard deviation (SD) of Ct values across the tested tenfold serial dilution range for intra-assay and inter-assay variability of FHV-1, FCV, and SARS-CoV-2 targets.(B) Boxplot representation of coefficient of variation (CV%) across the same conditions. Precision analysis was performed using the optimized primer/probe concentrations and the final tenfold serial dilution panel used for endpoint LoD confirmation. The optimized dilution panel was repeated three times for each target under the same assay conditions. SD and CV% values were calculated from the available Ct values obtained during these repeated LoD/precision runs. Median values are indicated by horizontal lines within boxes, while mean values are represented by triangular markers. Whiskers denote the range of observed values. The reported LoD values represent observed endpoint analytical sensitivity estimates and were not derived from probit analysis.

amplification signal (Angen *et al*., 2001; Burns and Valdivia, 2008). As shown in Supplementery Table 7, when using 1/16 *FHV-1* (5 µM) with final concentration 0.08 µM in the reaction probe dilution, we obtained the best estimated LoD (2.00×10^0), while the 20 µM primer concentration was slightly better than the 10 µM primer concentration with final concentration 0.16 µM in the reaction of the triplex assay. In case of *FCV* and *SARS-CoV-2* when we used (1/4) probe dilution (25 µM)) with final concentration 0.4 µM in the reaction and primers concentration were 50 µM with final concentration 0.8 µM in the reaction, we obtained the best R^2 value (0.97 for *SARS-CoV-2*, 0.98 for *FCV*) and best estimated LoD (10 copy/2 µl for *SARS-CoV-2* and 6.3×10^1 copy/4 µL for *FCV*) in comparison with other probe concentrations in Supplementery Table 7 and Figure 5. The standard curves for the triplex assay for these viruses are generated (Figure 2) using a tenfold serial dilution of the positive control by multiplex qRT-PCR.

Our SARS-CoV-2 primers/probe set in multiplex assay gave a higher estimated LoD by only onefold than the commercial SARS-CoV-2 that has a known estimated LoD (500 copies/1 mL or 1 copy/2 µL) using N-gene detection (Figure 2), so we assumed that LoD of our SARS-CoV-2 primers/probe set in multiplex assay was 10 copy/2 µL; hence, the original concentration was 10^7 copy no./2 µL Sinovac vaccine RNA.

Repeatability and reproducibility of the triplex assay

Tenfold serial dilutions of the positive controls for the study viruses were used to evaluate the repeatability and reproducibility of the established qRT-PCR assay. As presented in Supplementery Table 9 and Figure 3, the intra- and inter-assay coefficients of variation (CVs) for Ct values ranged between 0.4% and 6%, demonstrating the high reliability and accuracy of the triplex assay. While SD values were less than 1 and near zero, indicating very low deviated CT values. Using this endpoint-based approach, the observed estimated LoD values of the optimized triplex qRT-PCR assay were approximately (10 copy/2 µl for *SARS-CoV-2* and 6.3×10^1 copy/4 µL for *FCV)*. These values represent observed endpoint estimated LoD estimates based on repeated estimated LoD assessment and were not derived

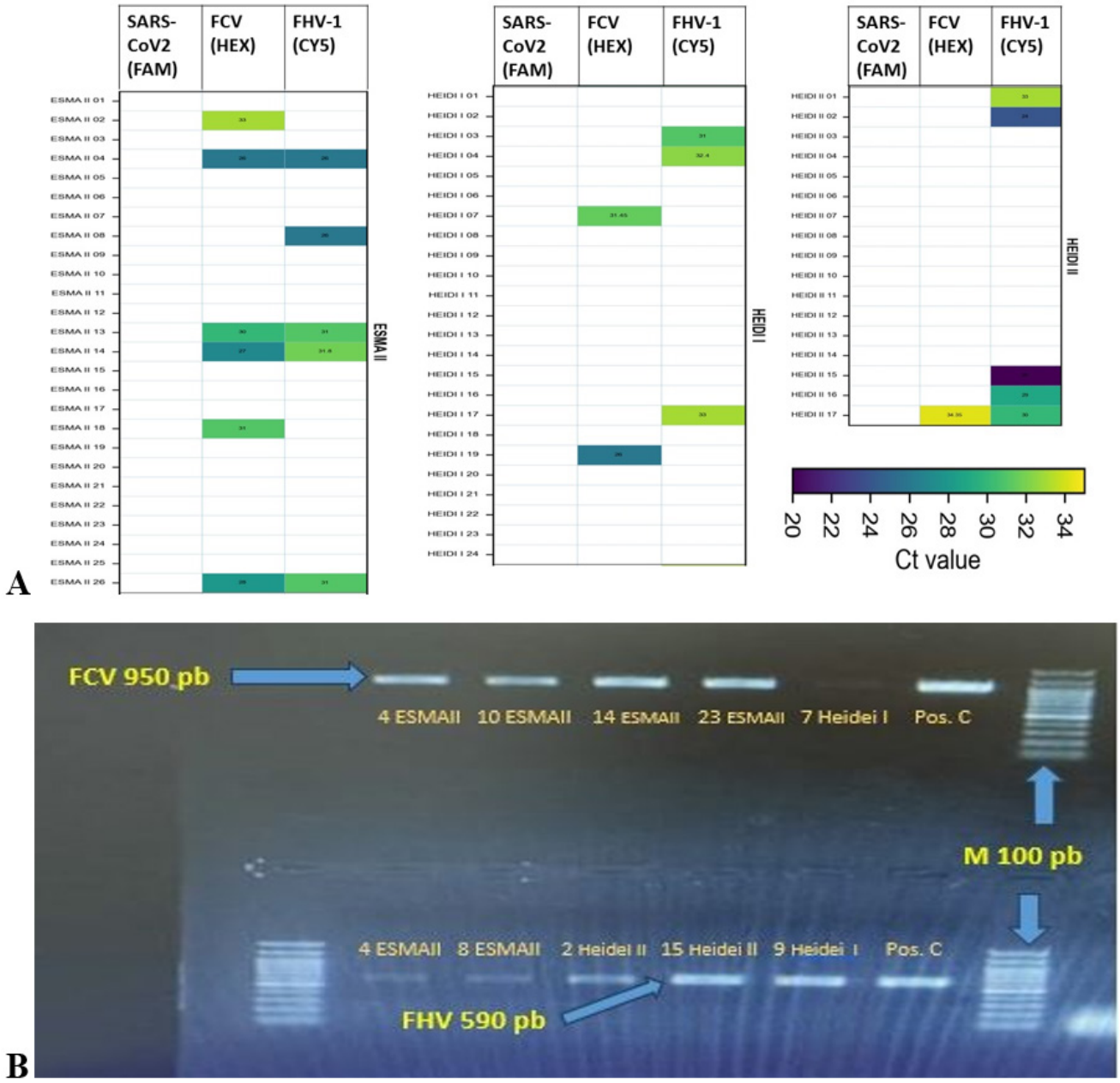

Figure 4: Clinical performance and validation of the developed triplex qRT-PCR assay.
(A) Heatmap representation of Ct values obtained from clinical samples (n = 67) across three viral targets: FHV-1 (CY5), FCV (HEX), and SARS-CoV-2 (FAM). Samples are displayed on the horizontal axis, and Ct values are color-coded according to amplification intensity. Gray cells indicate no detectable amplification (No Ct). No SARS-CoV-2-positive samples were detected. (B) Gel electrophoresis confirmation of selected positive samples, showing specific amplification bands for FCV (950 bp) and FHV-1 (590 bp), with a 100 bp molecular ladder used as reference.

from probit analysis. Repeatability and reproducibility were evaluated using the optimized triplex qRT-PCR conditions and the tenfold serial dilution panel used for estimated LoD confirmation. The assay was repeated three times for each viral target. Intra-assay variation was calculated from repeated Ct measurements obtained under the same assay conditions, while inter-assay variation was calculated from repeated runs performed using the same optimized protocol. SD and CV% were calculated from Ct values across the serial dilutions.

Analysis of the clinical samples using the one-step real-time TaqMan RT-PCR method

We examined 67 clinical samples using our designed triplex assay. The results showed that 31 cats were infected with FHV-1 or FCV, as shown in Supplementery Table 8 and Figure 4A. The positive rates for FCV and FHV-1 were 22.4% (15/67) and 23.8% (16/67), respectively. And there weren't samples positive for SARS-CoV-2. These strongly positive samples (CT < 30) were run by conventional PCR with specific primers and loaded onto an agarose gel to detect the specific band for each virus (Figure 4B).

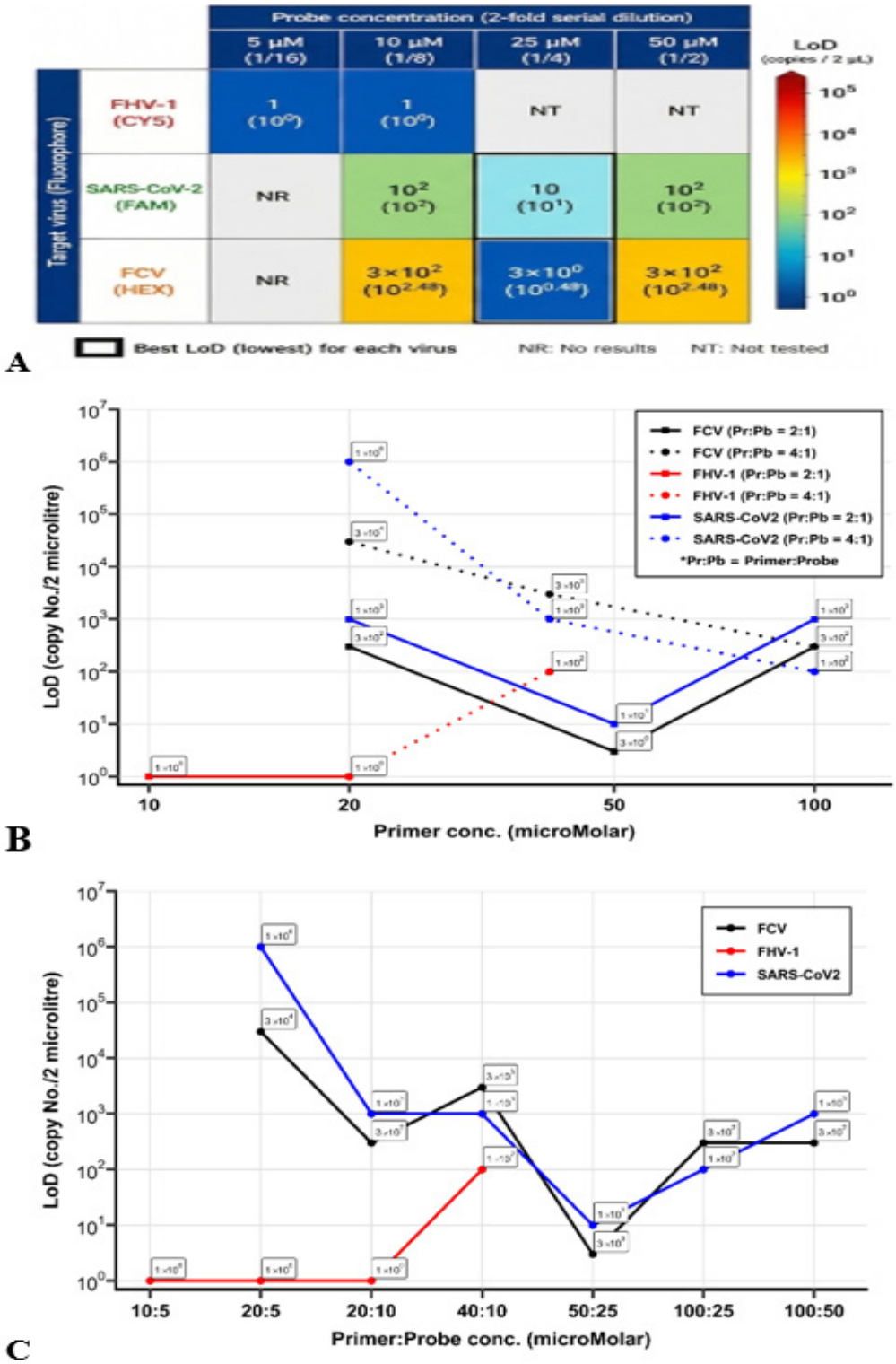

Figure 5: Analytical sensitivity and limit of detection (LoD) performance of the developed triplex qRT-PCR assay. (A) Comparison of the limit of detection (LoD) for two fold serially diluted Probes across the three viral targets (FHV-1, FCV, and SARS-CoV-2). (B) LoD comparison across the three viral targets (FHV-1, FCV, and SARS-CoV-2) by different Probes: Primers concentration either Our Trials (primary conc.) concentration = 1:2 or Probes: Primers concentration= 1: 4 according to TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG kit instruction. (C) Chart show optimized primer/probe concentrations , Two-fold serial dilution of the study primers and probes were run on tenfold serially diluted positive controls of the study viruses for Establishment of most suitable One-Step Real-Time TaqMan RT-PCR Method for the three viruses of the study. The LoD values shown represent observed endpoint LoD estimates obtained during primer/probe optimization. Differences between primer/probe concentrations were interpreted descriptively, and no statistical significance testing was performed.

DISCUSSION

The prevalence of infectious feline diseases can be reduced in developed countries through vaccination and disinfection practices, but remains higher in developing countries. Among these diseases, *FCV* and *FHV-1* are prevalent worldwide and pose a serious threat to feline health. This work aimed to establish a triplex test for the simultaneous detection of these three viruses. There is no rapid PCR-based detection system for respiratory viral diseases in cats in Egypt; in addition, no previous studies have documented endemic strains of these feline respiratory viruses, especially FCV, which is highly mutated. In this study, we established a triplex assay for the rapid and accurate detection of these three viruses, accommodating diverse worldwide viral strains and No marked reduction in analytical performance was observed after optimization of the multiplex assay compared with the corresponding uniplex assays, suggesting that competitive inhibition, if present, was minimal under the optimized experimental conditions.

Notably, two degenerate nucleotides were added to the forward primers of the *FCV* assay to ensure optimal binding to *FCV* strains retrieved from the *FCV* Research Database. However, the inclusion of more than four degenerate nucleotides is known to reduce amplification sensitivity by up to tenfold (Nam *et al*., 2015; Gaby and Buckley, 2017). Introducing a two-degenerate nucleotide per primer in this assay did not compromise sensitivity, as the estimated LoD for *FCV* remained 6.3 copies/4 µL under both uniplex and multiplex conditions. To evaluate the potential global applicability of the assay, the FCV primer and probe sequences were further assessed *in silico* against representative FCV strains deposited in the NCBI database. Sequence alignment demonstrated excellent conservation of the primer-binding regions across geographically diverse isolates. The only consistent sequence variability corresponded to the two intentionally introduced degenerate nucleotide positions (W and Y) within the forward primer, which were specifically incorporated to accommodate naturally occurring polymorphisms among FCV strains. Occasional additional mismatches were limited to the 5′ region of the primer, whereas no critical mismatches were observed near the 3′ terminus, suggesting that primer binding and amplification efficiency are expected to be maintained for the vast majority of currently available global FCV sequences.

In this study, we used previously published *SARS-CoV-2* primer sets for the detection of *SARS-CoV-2* in cats, as some studies showed that the N-gene RT-PCR assay is more sensitive for detecting *SARS-CoV-2* infection. After all, the clinical samples contain infected cells that express subgenomic mRNA, thereby increasing the N-gene copy number in the samples (Simons *et al*., 2005). Except for *SARS-CoV-2* and SARS coronavirus, none of the sarbecoviruses have been previously detected in humans. The last reported human SARS case was detected in 2004 (Chu *et al*., 2020).

Cats whose samples test positive for SARS-CoV-2 or related animal coronaviruses in these RT-PCR tests should be regarded as infected. The Orf1b assay is suggested as a confirmatory test, while the N gene RT-PCR is suggested as a screening test based on their detection capabilities. An N gene-positive/Orf1b-negative result should be considered suspected positive, and the patient should be referred to a WHO reference lab for additional testing regarding MERS. Sequence analysis of PCR-amplified positive amplicons can be used to validate the result and differentiate SARS-CoV-2 from other genetically similar coronaviruses. (e.g., SARS coronavirus) (Chu *et al*., 2020). *The SARS-CoV-2* virus strain used in our study was an inactivated *SARS-CoV-2* strain of Sinovac vaccine without known virus titre or estimated LoD, so we tested tenfold serially diluted Sinovac vaccine RNA triplicate by both commercial *SARS-CoV-2* closed kit, which has known estimated LoD (1 copy/ 2 µL), and our multiplex qPCR design as detailed in Figure 2 and Supplementary Table 7. Our multiplex qPCR showed a onefold lower estimated LoD than the commercial SARS-CoV-2 assay using N-gen detection, so we assumed that the estimated LoD of our SARS-CoV-2 primers/probe set in the multiplex assay was 10 copies/2 µL. The inactivated SARS-CoV-2 vaccine used as a source of viral RNA for analytical assay optimization for The assay which targets a short conserved genomic region, and therefore successful amplification depends primarily on the integrity of the target RNA fragment rather than viral infectivity. Although chemical inactivation may affect viral infectivity, it is not expected to substantially impair amplification of intact target sequences but it may explain the onefold difference in estimated LoD compared to the commercial kit.

We performed a twofold serial dilution of the study primers and probes and ran them on tenfold serially diluted positive controls of the study viruses, as shown in Supplementary Table 7. This was done to determine the most suitable probe concentration for our designed primers and probes for each virus. The primer concentrations were tested according to the multiplex qPCR kit instructions (4 primer concentrations to 1 probe concentration) or based on our trials (2 primer concentrations to 1 probe concentration). When the probe concentration of FHV-1 was set to 5 µM, the estimated LoD was 2.00 copies per 4 µL template, regardless of primer concentration. The most

suitable primer and probe concentrations (highest dilutions that yielded LoD and the highest R^2 value) for FHV-1 were either 20 µM primers plus 5 µM probe or 10 µM primers plus 5 µM probe, as FHV-1 is a relatively stable DNA virus with low mutations. In contrast, the primer and probe concentrations for FCV and SARS-CoV-2 were 50 µM primers plus 25 µM probe, as these viruses are highly mutable. Additionally, the FCV primers and probes contained degenerate nucleotides to accommodate the various FCV genotypes worldwide.

FHV-1 was a DNA virus with a stable genome and used DNA polymerase directly in PCR reactions. Hence, the lowest probe dilution (5 µM) gave the best results in both uniplex and multiplex PCR reactions with other RNA viruses. While in case of *FCV-1* and *SARS-CoV-2* were RNA viruses with highly mutated genome and used reverse transcriptase firstly for conversion to cDNA then competition occurred with *FHV-1* on DNA polymerase for PCR reaction so primer/probe concentration of these RNA viruses should have been increased to reach the primer/probe concentration gave lowest estimated LoD (50 µM primers plus 25 µM probe) as shown in Supplementary Table 7 and Figures 1 and 5. The standard curves for the triplex assay for these viruses are generated (Figure 2) using a tenfold serial dilution of the Positive control by multiplex qRT-PCR. According to Figures 1 and 5, *FCV* 50 µM primer conc./25 µM probe concentration gave a higher estimated estimated LoD in the Multiplex qPCR assay than in the Uniplex qPCR assay for FCV. In contrast, for FHV-1, both Multiplex and Uniplex assays had the same sensitivity with 20 µM *FHV-1* primer concentration/ 5 µM probe concentration.

A total of 67 clinical nasal and oropharyngeal samples were collected from different shelters in Giza governorate, as these areas were highly suitable for the transmission of respiratory diseases. The samples were used to verify the accuracy of the established multiplex one-step real-time TaqMan RT-PCR assay. Positive qPCR samples with lower CT were run by conventional PCR and PCR Products were loaded in agarose gel electrophoresis for confirmation of the results of our multiplex qRT-PCR assay as shown in Figure 4 then DNA sequencing was done, the BLAST analysis of the obtained *Feline calicivirus* (FCV) sequence revealed significant nucleotide identity with multiple *FCV* strains available in the NCBI database, displaying high percentages of sequence coverage and varying degrees of identity across different isolates but *FHV-1* were analyzed by BLAST against sequences available in the NCBI database showed high coverage and strong nucleotide identity, reaching values of approximately 90%–98% when compared with other *FHV-1* strains. These results indicate that *FHV-1* exhibits a relatively stable genome with limited mutation rates, in contrast to the higher genetic variability typically observed in *Feline calicivirus* (FCV). These findings confirm that the primers used in the conventional PCR assay were highly specific to *Feline calicivirus* and did not cross-react with nontarget sequences. Moreover, the samples analyzed by real-time PCR demonstrated consistent amplification patterns specific to FCV and FHV-1, supporting the assay's strong specificity and reliability. Overall, the BLAST results provide robust molecular evidence that the designed primers and probes efficiently detect *Feline calicivirus* with high precision.

Analysis and testing of 67 field samples from disease cats with respiratory signs showed only 22.4% (15/67) and 23.8% (16/67) for FCV and FHV-1, respectively, with different CT values as detailed in Supplementary Table 8, indicating higher sensitivity for these detected viruses. While 52% of tested samples showed negative qPCR results with our designed multiplex qPCR, indicating the highest specificity of our design by detecting no other pathogen causing respiratory signs in these samples, such as *Mycoplasma felis*, *Chlamydia felis*, or *SARS-CoV-2*. None of the 67 tested samples were positive for SARS-CoV-2. So, the sensitivity of our designed multiplex qPCR for the detection of *SARS-CoV-2* was confirmed by testing of tenfold serially diluted Sinovac SARS-CoV-2 vaccine RNA by both our design and the Transcript *SARS-CoV-2* closed kit, as detailed in Supplementary Table 7 and Figure 2, which showed that our multiplex qPCR gave a lower estimated LoD by only onefold than commercial *SARS-CoV-2* using N-gene detection.

Although the reported prevalence of SARS-CoV-2 infection in domestic cats is generally lower than that of FCV and FHV-1, sporadic natural infections have been documented worldwide, including Egypt. Because infected cats may exhibit respiratory signs overlapping with those caused by common feline respiratory viruses, incorporation of SARS-CoV-2 into a multiplex molecular assay provides a practical differential diagnostic approach while simultaneously supporting One Health surveillance programs. Although no SARS-CoV-2-positive cats were detected in the present study, inclusion of this target remains valuable because viral prevalence may vary geographically and temporally, and surveillance of companion animals continues to represent an important component of One Health monitoring.

Analysis of naturally coinfected clinical samples (5 samples (7.5%) showed coinfection with both viruses) demonstrated that the developed multiplex qPCR assay could simultaneously detect FCV and FHV-1 across a wide range of viral loads. Coinfected samples contained

different combinations of Ct values, representing both high- and low-abundance viral targets (Figure 4) and Supplementary Tables 8. Yet, the assay accurately identified each pathogen without apparent competitive inhibition. The successful detection of both viruses in mixed clinical specimens indicates that variations in target concentration did not adversely affect assay performance. This finding provides further evidence of the assay's high analytical sensitivity and its applicability for routine diagnosis of FCV/FHV-1 coinfections in field samples.

Finally, our study limitations include the use of vaccine-derived nucleic acid as a positive control, the lack of comparison between the multiplex assay and commercial FCV or FHV-1 kits (with known estimated LoD) across serial dilutions, and limited sample size, expansion of the assay to include bacterial pathogens represents an important direction for future studies also additional validation using FCV field isolates from multiple geographical regions.

CONCLUSIONS

Multiplex qRT-PCR assay of our study was very suitable for rapid detection and accurate diagnosis of the feline respiratory viruses (*FCV*, *FHV-1)* and *SARS-CoV-2* present in all over the world with highest specificity and sensitivity (in comparison to other used primers and probes concentrations in the study) using probe concentration of 5 µM for *FHV-1* and 25 µM for *FCV* and *SARS-CoV-2* with primers concentrations were double concentration of corresponding probes (primer concentration of 10 or 20 µM for *FHV-1* and 50 µM for other viruses).

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NOVELTY STATEMENT

This study reports the development and optimization of a triplex TaqMan qRT-PCR assay for the simultaneous detection of FHV-1, FCV, and SARS-CoV-2 in a single reaction involving both DNA and RNA viral targets. The novelty lies in the target-specific optimization of primer and probe concentrations to reduce multiplex competition, combined with analytical sensitivity, specificity, repeatability, reproducibility, and clinical-sample evaluation. In addition, commercially available vaccine-derived viral materials were successfully utilized as accessible positive-control sources for assay optimization and analytical evaluation. This practical approach may reduce dependence on expensive cultured viral isolates or specialized reference materials, offering a more economical and resource-efficient strategy for laboratories with limited facilities. The assay therefore provides a rapid, practical, and potentially cost-effective platform for the simultaneous molecular screening of major feline respiratory viruses.

AUTHOR'S CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Osman N.O. Mansour and Naglaa Hagag. The first draft of the manuscript was written by Osman N. O. Mansour, and all authors commented on previous versions. All authors read and approved the final manuscript.

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Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of IACUC, Faculty of Veterinary Medicine, Cairo University.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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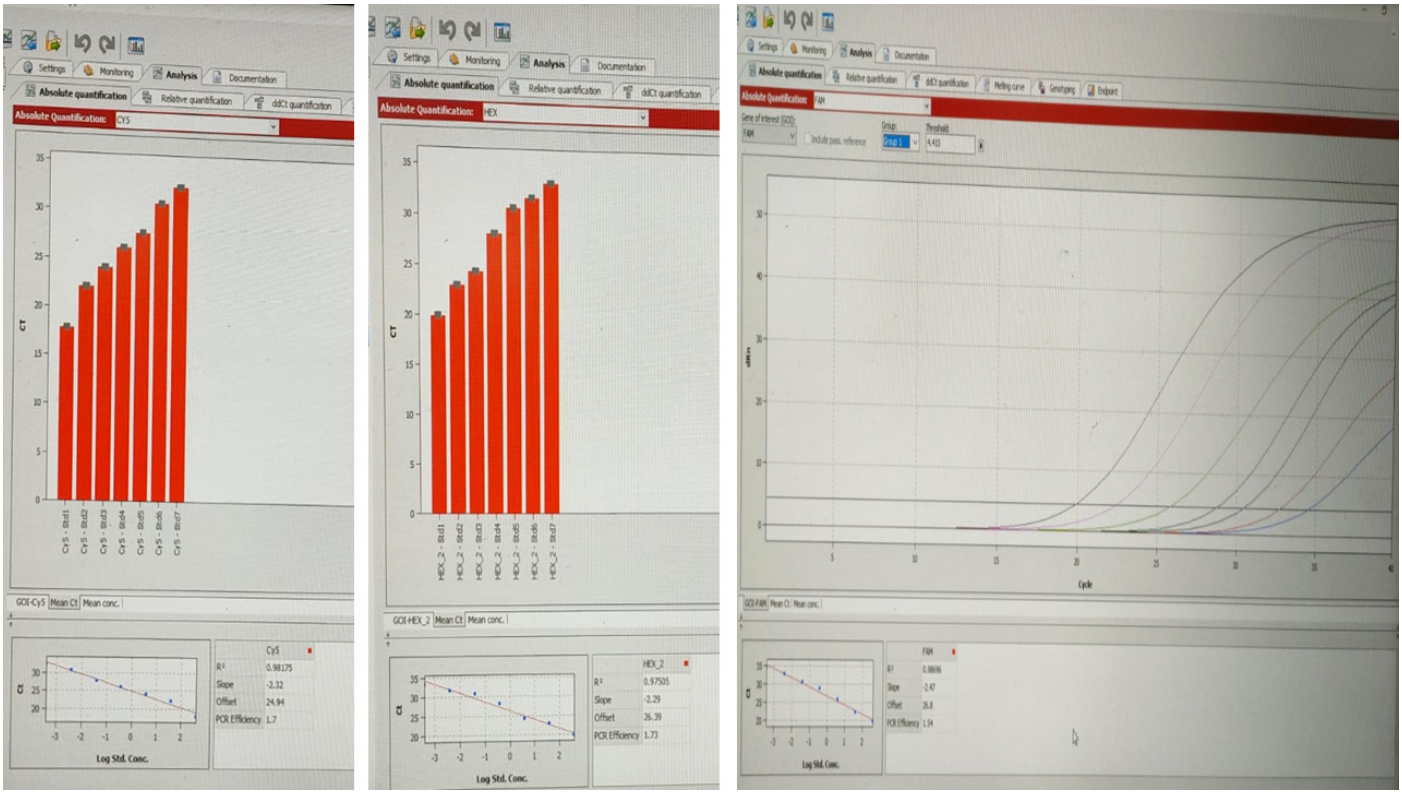

Supplementary Figure 1: Standard curves generated from estimated vaccine-derived copy numbers of multiplex real-time PCR assay targeting FHV (Cy5), FCV (HEX), and SARS-CoV-2 (FAM). Representative amplification plots show typical sigmoidal curves, and corresponding standard curves exhibit strong linearity between Ct values and log standard concentrations, supporting the accuracy and reproducibility of the assay.

Supplementary Table 1: CT values of standard curve of tenfold serially diluted zoetis vaccine nucleic acid tested by several dilutions of primers and probes sets of *FHV-1*, table showed lowest *FHV* primer/probe concentration gave lowest LOD was 20 µM primer conc./ 5 µM probe conc or 10 µM primer conc./5 µM probe conc.

	FHV-1 primers and probe concentration (CY5 labeled probe)								
	U. A.	**D. A.**	**Triplex or Multiplex assay (M. A.)**						
Zoetis Vaccine nucleic acid 10-fold serial dilution	**10 µM primer /5 µM probe**	**10 µM primer /5 µM probe**	**10 µM primer /5 µM probe**	**20 µM primer conc./ 5 µM probe conc.**				**10 µM probe conc.**	**40 µM primer conc./10 µM probe conc.**
				1 st	**2 nd**	**3 rd**	**Mean**		
Origin (2.00×10^6)	16.81	17.90	19.04	18.56	17.80	20.80	19.05	20.15	18.31
2.00×10^5	21.23	21.59	21.06	21.43	22.11	21.16	21.57	21.37	24.68
2.00×10^4	24.30	24.73	23.99	24.50	24.07	24.45	24.34	23.53	26.24
2.00×10^3	26.21	27.14	27.40	27.37	26.16	25.86	26.46	25.52	29.07
2.00×10^2	28.79	30.71	30.80	29.46	27.67	28.25	28.46	27.44	32.03
2.00×10^1	31.33	33.67	30.06	29.80	30.75	31.09	30.55	30.42	No CT
2.00×10^0	33.05	No CT	31.50	32.08	32.49	31.87	32.15	31.77	No CT
Negative control*	No CT	No CT	No CT	No CT	No CT	No CT	---	No CT	No CT
R^2 of St. Curve	0.95	0.99	**0.93**	0.97	0.98	0.98	0.98	0.99	**0.95**
CT Cut-off	NC	NC	NC	**33.09**				NC	**NC**
LOD/ 4 µl template	2.00×10^0	2.00×10^1	2.00×10^0	2.00×10^0 copies				2.00×10^0	2.00×10^2

* = Negative control used in Uniplex assay run by addition of 3 ul Mycoplasma felis DNA with 3 ul Bordetella bronchiseptica DNA. NC= Not Calculated.

Supplementary Table 2: CT values of standard curve of tenfold serially diluted zoetis vaccine nucleic acid tested by several dilutions of primers and probes sets of *FCV*, table showed lowest *FCV* primer/probe concentration gave lowest LOD was 50 µM primer conc./ 25 µM probe conc.

	FCV primers and probe concentration (HEX labeled probe)										
	U. A	D. A	Triplex or multiplex assay (M. A.)								
Zoetis Vaccine nucleic acid 10-fold serial dilution	**10 µM primer conc./ 5 µM probe conc.**	**10 µM primer conc./ 5 µM probe conc.**	**20 µM primer conc./**		**40 µM primer conc./**	**50 µM primer conc./**				**100 µM primer conc./**	
			5 µM probe conc.	**10 µM probe conc.**	**10 µM probe conc.**	**25 µM probe conc.**				**25 µM probe conc.**	**50 µM probe conc.**
						1 st	**2 nd**	**3 rd**	**Mean**		
Origin (6.3×10^6)	29.00	25.50	33.35	20.43	22.68	21.27	20.85	23.37	21.83	23.60	21.18
6.3×10^5	32.00	27.70	29.43	24.42	27.42	24.40	25.42	24.75	24.86	25.70	26.50
6.3×10^4	33.60	30.70	33.57	26.66	30.48	26.65	27.52	27.89	27.35	29.90	27.48
6.3×10^3	37.00	32.70	No CT	29.02	32.48	30.71	29.59	30.70	30.33	31.19	30.63
6.3×10^2	37.50	No CT	No CT	30.66	No CT	31.13	30.69	31.85	31.22	33.61	33.80
6.3×10^1	No CT	No CT	No CT	No CT	No CT	34.23	34.04	34.20	34.16	No CT	No CT
6.3×10^0	No CT	No CT	No CT	No CT	No CT	35.50	No CT	37.69	36.40	No CT	No CT
Negative control*	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	---	No CT	No CT
R^2 of St. Curve	0.97	0.96	0.97	0.97	0.966	0.97	0.958	0.99	0.98	0.98	0.96
CT Cut-off	NC	NC	NC	NC	NC	**39.84**				NC	NC
LOD/ 4 µl template	6.3×10^2	6.3×10^3	6.3×10^4	6.3×10^2	6.3×10^3	6.3×10^0				6.3×10^2	6.3×10^2

* = Negative control used in Uniplex assay run by addition of 3 ul Mycoplasma felis DNA with 3 ul Bordetella bronchiseptica DNA instead of diluted vaccine. NC= Not Calculated.

Supplementary Table 3: CT values of standard curve of tenfold serially diluted Sinovac Vaccine nucleic acid tested by several dilutions of primers and probes sets of *SARS-CoV2*, table showed lowest SARS-CoV2 primer/probe concentration gave lowest LOD was 50 µM primer conc./ 25 µM probe conc.

	SARS-CoV2 Primers and Probe Concentration (FAM labeled probe)										
	U. A	D. A	Triplex or Multiplex assay (M. A.)								
Sinovac Vaccine RNA 10-fold serial dilution	**10 µM primer. / 5 µM probe**	**10 µM primer. / 5 µM probe**	**20 µM primer conc./**		**40 µM Primer Con.**	**50 µM primer conc./**				**100 µM primer con. /**	
			5 µM probe conc.	**10 µM probe conc.**	**10 µM probe conc.**	**25 µM probe conc.**				**25 µM probe conc.**	**50 µM probe conc.**
						1 st	**2 nd**	**3 rd**	**Mean**		
Origin (10×10^6)	20.90	23.00	29.19	22.17	20.82	18.90	19.06	19.89	19.28	19.80	20.50
10×10^5	25.00	27.00	29.32	26.15	26.01	21.44	22.19	22.35	21.99	24.00	24.00
10×10^4	28.00	30.00	No CT	28.36	28.65	23.96	24.19	25.81	24.65	26.00	26.50
10×10^3	29.50	34.00	No CT	29.86	30.83	27.85	26.48	28.77	27.70	28.60	28.90
10×10^2	33.30	No CT	No CT	32.28	32.42	29.07	28.26	30.44	29.26	31.40	31.70
10×10^1	36.30	No CT	No CT	No CT	No CT	31.16	30.00	32.70	31.29	33.00	No CT
10×10^0	No CT	No CT	No CT	No CT	No CT	31.86	32.09	34.48	32.81	No CT	No CT
Negative control*	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	---	No CT	No CT
R^2 of St. Curve	0.96	0.98	1	0.97	**0.94**	0.96	0.98	0.97	0.98	0.98	0.99
CT Cut-off	NC	NC	NC	NC	NC	**37.16**				NC	NC
LOD/ 2 µl template	10×10^1	10×10^3	10×10^5	10×10^2	10×10^2	10×10^0 copies				10×10^1	10×10^2

* = Negative control used in Uniplex assay run by addition of 3 ul Mycoplasma felis DNA with 3 ul Bordetella bronchiseptica DNA. NC= Not Calculated.

Supplementary Table 4: CT values of standard curve of Uniplex qPCR of *FCV* and *FHV-1*in comparison with Multiplex qPCR using tenfold serially diluted Zoetis Vaccine nucleic acid, table showed that study multiplex design was more sensitive in detection of *FCV* than uniplex assay while in case of *FHV-1*, both Multiplex and uniplex assay had the same sensitivity

Zoetis vaccine nucleic acid 10 fold serial dilution	**50 µM FCV primer conc./ 25 µM probe (HEX) conc.**					**20 µM FHV-1 primer conc./ 5 µM probe (CY5) conc.**				
	Multiplex qPCR	**Uniplex qPCR of FCV**				**Multiplex qPCR**	**Uniplex qPCR of FHV-1**			
	Mean	**1 st**	**2 nd**	**3 rd**	**Mean**	**Mean**	**1 st**	**2 nd**	**3 rd**	**Mean**
Origin	21.83	25.89	23.64	24.00	24.42	19.05	17.66	16.89	16.94	17.16
Origin x 10^{-1}	24.86	30.66	28.66	28.56	29.29	21.57	22.47	21.52	21.47	21.82
Origin x 10^{-2}	27.35	31.87	30.74	31.05	31.22	24.34	25.16	25.00	25.01	25.06
Origin x 10^{-3}	30.33	35.13	33.73	34.84	34.57	26.46	27.54	27.00	26.70	27.08
Origin x 10^{-4}	31.22	36.85	36.55	36.70	36.70	28.46	30.52	29.26	29.90	29.89
Origin x 10^{-5}	34.16	38.13	NO CT	38.33	38.23	30.55	33.97	33.56	32.80	33.44
Origin x 10^{-6}	36.40	NO CT	NO CT	NO CT	---	32.15	35.78	35.34	No CT	35.54
Origin x 10^{-7}	No CT	NO CT	NO CT	NO CT	---	---	No CT	No CT	No CT	---
R^2 of St. Curve	**0.98**	0.98	0.99	0.97	0.98	**0.85**	0.97	0.98	0.98	0.98
CT Cut-off	39.84	**38.53**				33.09	**36.22**			
LOD/ 4 µl template	6.3×10^0	6.3×10^1 copies				2.00×10^0	2.00×10^0 copies			

Supplementary Table 5: CT values of standard curve of serially diluted Sinovac vaccine RNA were run by both our designed multiplex qPCR and *SARS-CoV2* closed kit has known LOD (500 copies/1 ml), Our multiplex qPCR gave lower LOD by only one-fold than commercial *SARS-CoV2* using N-gen detection

Sinovac vaccine 10-fold serial dilution	**Multiplex qPCR SARS-CoV2 closed kit (has known LOD (500 copies/1 ml))**								**Our designed Multiplex qPCR**
	N-gen (HEX)				**ORF1a-gen (FAM)**				**N-gen (50 µM primer conc./ 25 µM FAM probe conc.)**
	1 st	**2 nd**	**3 rd**	**Mean**	**1 st**	**2 nd**	**3 rd**	**Mean**	**Mean CT**
Origin (10×10^6)	14.80	12.48	13.09	13.46	18.80	16.71	16.47	17.33	**19.28**
10×10^5	21.90	18.70	18.84	19.81	28.26	23.33	23.02	23.20	**21.99**
10×10^4	20.80	20.06	20.82	20.56	24.60	24.24	23.94	24.26	**24.65**
10×10^3	22.80	22.31	23.72	22.94	26.80	26.38	26.28	26.49	**27.70**
10×10^2	25.19	24.93	25.22	25.11	29.30	29.41	28.57	29.09	**29.26**
10×10^1	28.40	27.85	28.22	28.16	31.90	32.39	32.00	32.10	**31.29**
10×10^0	31.90	31.82	32.62	32.11	34.02	34.13	35.14	34.43	**32.81**
1 copy	33.70	33.37	34.31	33.79	NO CT	NO CT	NO CT	---	---
Origin x 10^{-8}	NO CT	NO CT	NO CT	NO CT	NO CT	NO CT	NO CT	---	---
R^2 of St. Curve	0.94	0.98	0.98	0.97	0.80	0.96	0.96	0.90	
CT Cut-off	**35.22**				36.28				37.16
LOD/ 2 µl template	one copy/ 2 µl				10 copy/ 2 µl				10 copy/ 2 µl

Supplementary Table 6: Comparison between LOD of both our designed multiplex qPCR for detection of *SARS-CoV2* and commercial closed *SARS-CoV2* qPCR kit has known LOD, the study multiplex qPCR and uniplex assay gave lower LOD by only one-fold than commercial *SARS-CoV2* using N-gen detection

Sinovac Vaccine RNA 10 fold serial dilution	By SARS-CoV2 closed kit		Our designed Multi-plex qPCR	Uniplex qPCR of SARS-CoV2			
	N-gen (HEX)	ORF1a-gen (FAM)	(50 µM primer conc./ 25 µM probe (FAM) conc.) for N gen	(50 µM primer conc./ 25 µM probe (FAM) conc.) for N gen			
	Mean CT	Mean CT	Mean CT	1 st	2 nd	3 rd	Mean
Origin (10×10^6)	13.46	17.33	19.28	17.50	18.50	17.00	17.70
10×10^5	19.81	23.20	21.99	22.92	23.9	22.00	22.94
10×10^4	20.56	24.26	24.65	26.44	26.99	25.28	26.24
10×10^3	22.94	26.49	27.70	28.33	28.56	27.35	28.08
10×10^2	25.11	29.09	29.26	30.38	31.12	30.73	30.74
10×10^1	28.16	32.10	31.29	33.78	33.81	33.31	33.64
10×10^0	32.11	34.43	**32.81**	37.38	36.65	37.70	37.24
1 copy	33.79	---	---	No CT	No CT	No CT	---
R^2 of St. Curve	0.97	0.90	**0.98**	0.96	0.98	0.97	0.98
LOD/ 2 µl template	1 copy / 2 µl	10 copy / 2 µl	10 copy/2 µl	**10 copy/2 µl**			

Supplementary Table 7: Summary of Two-fold serial dilution of the study primers and probes were run on tenfold serially diluted positive controls of the study viruses for Establishment of most suitable One-Step Real-Time TaqMan RT-PCR Method for the three viruses of the study.

Two-fold serial dilution of probe		Primer Conc.		Tenfold serial dilution of three viruses					
Probe dilution	Probe Conc.	Kit instr- uction * (Alternative conc.)	Our trials (primary conc.)	CY5 (Feline Herpes virus-1)		FAM (SARS-CoV2)		HEX (Feline Calicivi-rus)	
				LOD/ 4 µl template	R^2	LOD/ 2 µl template	R^2	LOD/ 4 µl template	R^2
1/16	5 µM	--	10 µM	2.00×10^0	0.94	No Results		No Results	
		20 µM	--	2.00×10^0	0.98	10×10^5	1	6.3×10^4	0.004
1/8	10 µM	--	20 µM	2.00×10^0	0.99	10×10^2	0.97	6.3×10^2	0.89
		40 µM	--	2.00×10^2	0.95	10×10^2	0.94	6.3×10^3	0.97
1/4	25 µM	--	50 µM	Not tested		10×10^0	0.97	6.3×10^0	0.98
		100 µM	--	Not tested		10×10^1	0.98	6.3×10^2	0.98
1/2	50 µM	--	100 µM	Not tested		10×10^2	0.99	6.3×10^2	0.96
Details of dilutions				Supplementry Table 1		Supplementry Table 2		Supplementry Table 3	

* Probes concentration: Primer's concentration = 1: 4 according to TransScript® II Multiplex Probe One-Step qRT-PCR SuperMix UDG kit instruction.

Supplementary Table 8: 67 clinical samples were examined using the developed triplex assay. The results demonstrated that 31 cats were infected *FCV, or FHV-1*.

S.	Sample name	CT Value of Multiplex qRT-PCR			S. N	Sample Name	CT Value of Multiplex qRT-PCR		
		CY5 (FHV-1)	HEX (FCV)	FAM (SARS-CoV2)			CY5 (FHV-1)	HEX (FCV)	FAM (SARS-CoV2)
1	ESMA II 1	N	N	N	1	ESMA II 14	32	27	N
2	ESMA II 2	N	33	N	2	ESMA II 15	N	N	N
3	ESMA II 3	N	N	N	3	ESMA II 16	N	N	N
4	ESMA II 4	26	27	N	4	ESMA II 17	N	N	N
5	ESMA II 5	N	N	N	5	ESMA II 18	N	31	N
6	ESMA II 6	N	N	N	6	ESMA II 19	N	N	N
7	ESMA II 7	N	N	N	7	ESMA II 20	N	N	N
8	ESMA II 8	26	N	N	8	ESMA II 21	N	N	N
9	ESMA II 9	N	N	N	9	ESMA II 22	N	N	N
10	ESMA II 10	N	N	N	10	ESMA II 23	N	N	N
11	ESMA II 11	N	N	N	11	ESMA II 24	N	N	N
12	ESMA II 12	N	N	N	12	ESMA II 25	N	N	N
13	ESMA II 13	32	30.5	N	13	ESMA II 26	31	28	N
14	Heidi II 1	33	N	N	14	Heidi I 1	N	N	N
15	Heidi II 2	25	N	N	15	Heidi I 2	N	N	N
16	Heidi II 3	N	N	N	16	Heidi I 3	32	N	N
17	Heidi II 4	N	N	N	17	Heidi I 4	32.4	N	N
18	Heidi II 5	N	N	N	18	Heidi I 5	N	N	N
19	Heidi II 6	N	N	N	19	Heidi I 6	N	N	N
20	Heidi II 7	N	N	N	20	Heidi I 7	N	31.5	N
21	Heidi II 8	N	N	N	21	Heidi I 8	N	N	N
22	Heidi II 9	N	N	N	22	Heidi I 9	N	N	N
23	Heidi II 10	N	N	N	23	Heidi I 10	N	N	N

Table continue on next page.....

S.	Sample name	CT Value of Multiplex qRT-PCR			S. N	Sample Name	CT Value of Multiplex qRT-PCR		
		CY5 (FHV-1)	HEX (FCV)	FAM (SARS-CoV2)			CY5 (FHV-1)	HEX (FCV)	FAM (SARS-CoV2)
24	Heidi II 11	N	N	N	24	Heidi I 11	N	N	N
25	Heidi II 12	N	N	N	25	Heidi I 12	N	N	N
26	Heidi II 13	N	N	N	26	Heidi I 13	N	N	N
27	Heidi II 14	N	N	N	27	Heidi I 14	N	N	N
28	Heidi II 15	20	N	N	28	Heidi I 15	N	N	N
29	Heidi II 16	29	N	N	29	Heidi I 16	N	N	N
30	Heidi II 17	30.5	34.35	N	30	Heidi I 17	33	N	N
					31	Heidi I 18	N	N	N
					32	Heidi I 19	N	26	N
					33	Heidi I 20	N	N	N
					34	Heidi I 21	N	N	N
					35	Heidi I 22	N	N	N
					36	Heidi I 23	N	N	N
					37	Heidi I 24	N	N	N
Total infected		8	4	0	Total infected		5	5	0

Supplementary Table 9: Coefficient of variation (CV) and standard deviation (SD) of Ct values of the intra- and inter-assay of *FHV-1*, *FCV* and *SARS-CoV2*;

	FHV-1						FCV						SARS-CoV2					
	Intra-assay			Inter-assay			Intra-assay			Inter-assay			Intra-assay			Inter-assay		
	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
Origin (O)	19.05	0.49	2.57	17.16	0.56	3.28	21.83	0.52	2.37	24.51	1.18	4.83	19.28	0.49	2.52	17.67	0.95	5.38
O x 10^{-1}	21.57	0.24	1.09	21.82	0.09	0.41	24.86	0.64	2.56	29.29	0.58	1.99	21.99	1.01	4.58	22.94	0.87	3.81
O x 10^{-2}	24.34	0.80	3.28	25.06	0.43	1.70	27.35	0.64	2.35	31.22	0.74	2.37	24.65	1.15	4.67	26.24	0.64	2.45
O x 10^{-3}	26.46	0.91	3.45	27.08	0.63	2.33	30.33	0.59	1.93	34.57	0.15	0.43	27.70	1.10	3.98	28.08	0.37	1.32
O x 10^{-4}	28.46	0.67	2.35	29.89	0.59	1.99	31.22	0.10	0.33	36.70	0.10	0.27	29.26	1.35	4.63	30.74	0.28	0.91
O x 10^{-5}	30.55	0.32	1.03	33.44	0.22	0.66	34.16	1.15	3.36	38.23	0.22	0.58	31.29	1.45	4.64	33.63	0.54	1.60
O x 10^{-6}	32.15	0.71	2.21	35.55	0.71	2.00	36.40	0.71	1.95				32.81	0.71	2.16	37.24	0.71	1.91
O x 10^{-7}																		

Table showed the intra- and inter-assay CVs of Ct values are between 0.4 – 6 %, indicating that the triplex assay is highly reliable and accurate. While SD values were less than 1 and near to zero indicating for very low deviated CT values.