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Surveillance of SARS-CoV-2 in Companion Animals in Izmir, Turkey: Development and Analytical Evaluation of a Multiplex RT-qPCR Assay

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Simple Summary

The COVID-19 pandemic has shown that diseases shared between humans and animals need to be monitored from both human and veterinary perspectives. Cats and dogs live in close contact with people and can become infected with the virus responsible for COVID-19, although their role in maintaining or spreading the virus remains uncertain. This study investigated SARS-CoV-2 RNA positivity in companion animals in Izmir, Turkey, and evaluated a newly developed laboratory test designed for veterinary samples. Between March 2021 and March 2024, samples from 796 animals, including 318 cats and 478 dogs, were examined. SARS-CoV-2 RNA was detected in 42 cats and 40 dogs, corresponding to 13.2% and 8.4% of the sampled animals, respectively. Many RT-qPCR-positive animals showed mild signs such as sneezing, nasal discharge, breathing difficulties, or low-grade fever, although positive results were also obtained from animals without obvious illness. The newly developed test showed favorable analytical performance and was able to detect low concentrations of viral RNA, while antibody testing provided complementary information on previous exposure. These results demonstrate the value of including companion animals in infectious disease surveillance. Reliable veterinary molecular tools may help improve early detection, strengthen coordination between human and animal health services, and contribute to preparedness for future outbreaks involving diseases that can cross species boundaries.

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Abstract

The COVID-19 pandemic, caused by SARS-CoV-2, has necessitated a One Health approach to monitor infection across human and animal populations. While human diagnostics have been prioritized, evidence increasingly suggests that companion animals, particularly cats and dogs, may act as susceptible or incidental hosts. This study aimed to assess SARS-CoV-2 RNA positivity among domestic pets in Izmir, Turkey, and to develop and analytically evaluate a multiplex real-time RT-qPCR assay tailored for veterinary

application. Between March 2021 and March 2024, a cross-sectional surveillance study was conducted analyzing oropharyngeal/nasal swabs and serum from 796 animals (478 dogs and 318 cats). Following the design of primers and probes targeting conserved regions of the ORF1ab and RNase P genes, the assay was optimized and analytically evaluated using an ORF1ab-positive lentiviral particle-derived reference material and clinical samples. Molecular analysis identified SARS-CoV-2 RNA in 42 cats (13.2%) and 40 dogs (8.4%) within the sampled population. Notably, 81.0% of RT-qPCR-positive cats and 57.5% of RT-qPCR-positive dogs displayed mild respiratory symptoms and low-grade fever. Serological testing provided complementary information on previous exposure but was not used as an independent molecular reference standard. The assay demonstrated a Limit of Detection (LoD) of 200 copies/mL and an optimal amplification efficiency of 99.6%. Further studies incorporating cross-reactivity testing against other relevant companion-animal coronaviruses and independent molecular confirmation by sequencing or an additional viral target would further strengthen assay validation. Given the selected surveillance population, the observed proportions should be interpreted as SARS-CoV-2 RNA positivity within the sampled animals rather than population-level prevalence. Overall, these findings support the value of molecular surveillance of companion animals and the integration of veterinary data within a One Health framework.

Keywords: cat; COVID-19; dog; multiplex RT-qPCR; SARS-CoV-2; One Health

1. Introduction

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in December 2019 precipitated a global public health crisis, characterized by rapid transmission and significant morbidity. Within months, the virus spread over 200 countries, prompting the World Health Organization (WHO) to declare a global pandemic by March 2020. As of 2023, the cumulative toll exceeds 750 million confirmed cases and 6 million deaths globally [1]. SARS-CoV-2 is a positive-sense single-stranded RNA virus belonging to the *Betacoronavirus* genus [2]. Its genome encodes critical structural proteins, most notably the Spike (S) protein [3], which mediates host cell entry via the angiotensin-converting enzyme 2 (ACE2) receptor [4–6]. Since the ACE2 receptor is evolutionarily conserved across various mammalian species, the potential for interspecies transmission remains a significant concern, emphasizing the need to examine viral dynamics beyond the human population [7,8].

Although phylogenetic analyses suggest that SARS-CoV-2 likely originated from bats (*Rhinolophus affinis*) [9,10], with pangolins proposed as potential intermediate hosts [11–13], its rapid human-to-human transmission has been the primary focus of mitigation strategies. The capacity of the virus for interspecies transmission emphasizes the need to examine its dynamics not only in humans but also in animals that may serve as susceptible or incidental hosts [14,15]. Despite the pandemic's primary focus on human health, increasing attention has been directed toward animal infections, particularly among companion animals. Characterizing SARS-CoV-2 infection in pets is relevant to the One Health approach, a framework that integrates human, animal, and environmental health for infectious disease surveillance and pandemic preparedness [15,16]. Since the onset of the pandemic, numerous reports have elucidated the susceptibility of domestic animals to SARS-CoV-2 [17]. Systematic evaluation of the available evidence has further demonstrated natural SARS-CoV-2 infection in household cats and dogs, particularly in animals living in close contact with infected people [18]. Notably, the majority of documented animal cases, including the first confirmed canine case [19,20], are associated with reverse

zoonosis (anthroponosis), where infection occurs following exposure to COVID-19-positive owners [17,21]. These observations support the importance of monitoring human-to-animal transmission and SARS-CoV-2 infection in companion animals within a One Health framework.

Global surveillance efforts have employed various diagnostic modalities, including RT-PCR, histopathology, and neutralization assays, to monitor infection in pet populations [22]. Notable molecular surveillance efforts in Europe have adapted human diagnostic protocols targeting genes such as Envelope (E), Nucleocapsid (N), and RdRp for use in companion animals [23,24]. More recently, advanced multiplex RT-qPCR panels have been developed to simultaneously detect and differentiate SARS-CoV-2 from other common feline and canine viral pathogens, highlighting the evolving capability of veterinary molecular diagnostics [25]. Veterinary-tailored multiplex RT-qPCR assays incorporating species-specific endogenous internal controls have also been developed for canine and feline clinical samples [26]. In the specific context of Turkey, however, prior research has predominantly relied on serological assays to assess exposure risks [27–29]. While serology provides valuable retrospective data, it does not directly detect viral RNA and therefore provides different information from molecular testing. This highlights the value of molecular surveillance for characterizing SARS-CoV-2 RNA positivity in companion animals.

To address this gap, this study had two primary objectives: (1) to conduct molecular surveillance for SARS-CoV-2 RNA in domestic cats and dogs in Izmir, Turkey, and (2) to develop and analytically evaluate a multiplex real-time RT-PCR (RT-qPCR) assay for use with veterinary samples. Given the limited accessibility of validated veterinary-specific molecular kits in the region, the assay was designed to target the conserved ORF1ab gene region together with the host RNase P gene as an endogenous internal control. This study reports SARS-CoV-2 RNA positivity within the sampled surveillance population of cats and dogs and evaluates the analytical performance of the developed multiplex RT-qPCR assay.

2. Material and Methods

2.1. Study Design and Ethical Compliance

This cross-sectional surveillance study was conducted between March 2021 and March 2024 across veterinary clinics and animal shelters in Izmir Province, Turkey. Participation was voluntary, and informed consent was obtained from all pet owners and shelter managers prior to enrollment. The study protocol was approved by the Animal Experiments Local Ethical Committee of Dokuz Eylul University (Protocol No. 06/2020).

2.2. Animal Cohort and Sampling Strategy

A total of 796 animals, comprising 478 dogs and 318 cats (aged 1–3 years), were enrolled. Animals were recruited from 20 private veterinary clinics and four municipal shelters in Izmir Province. Approximately 20% of the animals originated from private veterinary clinics and 80% from municipal shelters characterized by high-density housing conditions. Each animal was included only once in the study, and repeated sampling of the same animal was not performed. Priority was given to animals presenting with clinical signs compatible with COVID-19 (e.g., fever and respiratory distress), while asymptomatic animals residing in the same environments were also sampled to assess subclinical viral circulation. Approximately 70% of the samples were collected during 2021–2022, while the remaining samples were collected between 2023 and March 2024.

Biological specimens were collected using standardized protocols. Oropharyngeal and nasal swabs were obtained using sterile flocked swabs, placed immediately into 1.5 mL microtubes containing 300 µL of DNA/RNA stabilization solution, and sealed to prevent contamination. Blood samples were collected via venipuncture into 6 mL vacutainer

tubes; serum was separated by centrifugation at 1500× *g* for 10 min within two hours of collection. All samples were transported under cold chain conditions (2–8 °C) and stored at −80 °C until analysis.

2.3. Primer Design and Multiplex RT-qPCR Optimization

A multiplex RT-qPCR assay was developed targeting conserved regions of the SARS-CoV-2 ORF1ab gene and the host RNase P gene (Internal Control). Specifically, the primers were designed to amplify the exon region of the RPP30 (Ribonuclease P/MRP subunit p30) gene. The RNase P gene was selected as the endogenous internal control to monitor extraction efficiency. To assess its suitability for veterinary applications, in silico analysis was performed using the NCBI Basic Local Alignment Search Tool (BLASTn, web-based tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi). This analysis showed that the selected primer and probe sequences shared 100% homology with the RPP30 gene in both canine (Canis lupus familiaris; GenBank: NC_051832.1) and feline (Felis catus; GenBank: NC_058378.1) reference genomes, supporting its use as an endogenous internal control in both species. Primers and probes were designed using Primer3 (Primer3Web, version 4.1.0) software to ensure thermodynamic compatibility. All primers and probes were synthesized by Atlas Biyoteknoloji, Turkey. The assay was optimized for a single-reaction duplex format: FAM channel for ORF1ab and JOE/HEX/VIC channel for RNase P. The reaction utilized a one-step RT-qPCR master mix (A.B.T.™, Ankara, Turkey, Cat. No. Q05-02-05) containing high-fidelity DNA polymerase, reverse transcriptase, and a ribonuclease inhibitor. Oligonucleotide sequences and reaction concentrations are detailed in Tables 1 and 2.

Table 1. Oligonucleotide sequences.

Primer Name	Primer Sequence	Channel/Gene	Amplicon Length (bp)
Forward Primer	5′-TTTACGCCCTGACACACGTT-3′	FAM/ORF1ab	131
Reverse Primer	5′-TTTCACAAGTGCCGTGCCTA-3′		
Marked Probe	5′-FAM-CCTAACACCTACCTTGAA-BHQ1-3′		
Forward Primer	5′-AGCTTTTGCTCACCATTTCCACA-3′	JOE-HEX-VIC/RNase P	300
Reverse Primer	5′-TAATAGAGTCCCTGAGTTTTGGCT-3′		
Marked Probe	5′-JOE-CTGTTTTCTTACCTGTGGAT-BHQ1-3′		

Table 2. Reagents and their concentrations constituting the kit content.

Component Name	Volume (100 rxn)	Composition & Concentration	Function/Aim of Use
Oligo Mix	1 × 250 µL	ORF1ab Primers (F/R): 500 nM ORF1ab Probe (FAM): 150 nM RNase P Primers (F/R): 500 nM RNase P Probe (HEX/VIC): 150 nM	Simultaneous detection of SARS-CoV-2 target and Internal Control.
2X Master Mix	1 × 1000 µL	High-fidelity DNA polymerase, Reverse Transcriptase, RNase Inhibitor, dNTPs, Reaction Buffer	One-Step Real-Time RT-PCR reaction core components.
Negative Control (NTC)	1 × 1000 µL	DEPC-Treated Nuclease-Free Water (Molecular Biology Grade)	PCR Negative Control: Monitoring for contamination in extraction or reaction setup.
Positive Control (PC)	1 × 250 µL	Target 1: Synthetic SARS-CoV-2 RNA (10^3 copies/mL) Target 2: Synthetic/Plasmid Nucleic Acid (10 ng/µL) for *RNase P*	PCR Positive Control: Validation of reaction performance and reagent integrity.

2.4. Viral RNA Extraction and Amplification

Total viral RNA was extracted from swab samples using the High Pure Viral RNA Extraction Kit (Roche, Mannheim, Germany, Cat. No. 11858882001) in a Biosafety Level 2 (BSL-2) facility. For each extraction, 200 µL of sample was processed and RNA was eluted in 50 µL of elution buffer. The RT-qPCR reaction was prepared in a final volume of 20 µL, containing 10 µL of 2X Master Mix, optimized primer/probe concentrations (0.5 µM primers, 0.15 µM probes), and 5 µL of RNA template.

Thermal cycling was performed using the LightCycler® 96 (Roche Diagnostics, Mannheim, Germany), CFX96 Touch™ (Bio-Rad, Hercules, CA, USA), Rotor-Gene® 5 Plex Platform (QIAGEN, Hilden, Germany), QuantStudio™ 5 qPCR System (Applied Biosystems, Foster City, CA, USA), and StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: reverse transcription at 52 °C for 15 min, initial denaturation at 95 °C for 45 s, followed by 45 cycles of denaturation (95 °C, 5 s) and annealing/extension (55 °C, 45 s). For the purposes of this study, a Ct < 38 for the ORF1ab target (FAM), accompanied by a valid RNase P signal, was used as the operational threshold for RT-qPCR positivity. Samples showing no signal in both channels were classified as invalid and subjected to re-extraction.

2.5. Analytical Evaluation and Efficiency Analysis

To evaluate assay kinetics and analytical performance, a plasmid construct containing a synthetic ORF1ab gene fragment (Bioligo Biotechnology, Ankara, Turkey; Cat. No. 152-20260121-2) was used to generate lentiviral particles in HEK293T cells using the ABP Lentiviral Packaging System (ABP Biosciences, Rockville, MD, USA, Cat. No. D020-01). The resulting lentiviral particle preparation provided an ORF1ab-positive encapsulated reference material for assessment of the extraction and RT-qPCR workflow. Standard curves were generated using 4-fold serial dilutions of RNA extracted from this reference preparation. For the amplification-efficiency analysis, target quantities were expressed relative to the highest concentration tested (1.0000), followed by sequential 4-fold dilutions corresponding to relative quantities of 0.2500, 0.0625, 0.0156, and 0.0039. While 10-fold dilutions are conventional, a 4-fold dilution factor was specifically selected to increase the resolution of data points within the evaluated concentration range. Efficiency was calculated from the slope of the regression line of Ct values versus the log of relative concentration (Table 3).

The Limit of Detection (LoD) was established by spiking negative feline and canine clinical matrices with serial dilutions of the ORF1ab-positive reference material to final concentrations ranging from 200 to 66 copies/mL prior to RNA extraction. The LoD was defined as the lowest concentration, yielding a ≥95% detection rate across 20 replicates.

2.6. Serological Analysis

Serum samples from all 796 enrolled animals (318 cats and 478 dogs) were analyzed for SARS-CoV-2 antibodies to provide complementary serological information. Serological status was assessed using the SARS-CoV-2 Neutralization Antibody ELISA Kit (Elabscience®, Houston, TX, USA, Cat. No. E-EL-E606) targeting the Receptor-Binding Domain (RBD) to assess antibody reactivity associated with previous exposure. Absorbance was measured at 450 nm, with results interpreted according to manufacturer criteria.

2.7. Reproducibility Assessment

Assay precision was evaluated through intra-assay repeatability and inter-assay reproducibility. Intra-assay repeatability was assessed using 10 replicate reactions at the concentrations reported in Table 4, whereas inter-assay reproducibility was evaluated

across 10 separate days. Mean Ct values and standard deviations were used to characterize variability across replicate measurements.

2.8. Statistical Analysis

Statistical analyses were primarily descriptive. Species-specific SARS-CoV-2 RNA positivity proportions were calculated as the number of RT-qPCR-positive animals divided by the total number tested for each species. Corresponding 95% confidence intervals (CIs) were calculated using the Wilson score method. Clinical findings among RT-qPCR-positive animals were summarized as counts and percentages. Ct values were summarized as mean ± standard deviation (SD) and range. Serological findings were summarized using counts and percentages.

3. Results

3.1. Analytical Performance of the Multiplex RT-qPCR Assay

The optimized multiplex RT-qPCR assay demonstrated consistent analytical performance under the evaluated conditions. To evaluate amplification kinetics with high resolution, 4-fold serial dilutions of the ORF1ab-positive reference material were analyzed. The assay exhibited a linear relationship between target concentration and Ct values, with $R^2 = 0.9998$ (Figure 1). Regression analysis of the 4-fold dilution series yielded a slope of −3.33, corresponding to a PCR efficiency of 99.66% (Figure 1; Table 3). Reproducibility assessment showed low variability in Ct values across the evaluated concentrations. Intra-assay variability (repeatability) and inter-assay variability (reproducibility) showed standard deviations (SDs) below 0.3 Ct across all tested concentrations (Table 4). No amplification was observed in the no-template controls (NTCs), indicating the absence of detectable contamination during the evaluated runs.

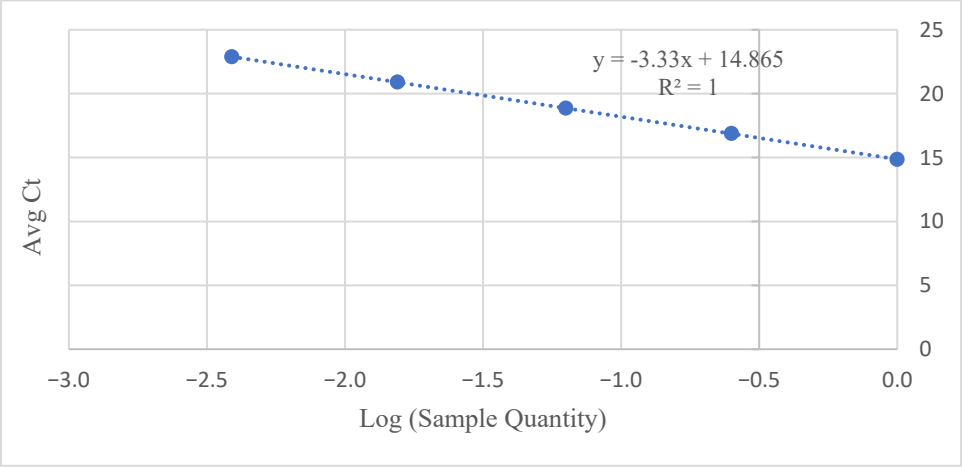


Figure 1. PCR efficiency standard curve. Linear regression analysis of 4-fold serial dilutions of the ORF1ab-positive reference demonstrated an amplification efficiency of 99.66% and a coefficient of determination of $R^2 = 0.9998$. X-axis: Log of Sample Quantity (log dilution series); Y-axis: average Ct values.

Table 3. PCR Efficiency Calculation from Serial Dilutions (4-Fold).

Tube	Ct 1	Ct 2	Avg Ct	Sample Quantity	Log (Sample Quantity)
A	14.82	14.88	14.85	1.0000	0.00
B	16.85	16.91	16.88	0.2500	−0.60
C	18.84	18.88	18.86	0.0625	−1.20
D	20.88	20.92	20.90	0.0156	−1.81
E	22.85	22.91	22.88	0.0039	−2.41

	Dilution Factor	4
	Slope	−3.3302
	R Squared	0.9998
	Efficiency (%)	99.66

Note: The average Ct values derived from duplicate measurements for each dilution were used to construct the PCR efficiency standard curve. PCR efficiency calculated from these 4-fold dilutions was 99.66%, with an R^2 value of 0.9998 (Figure 1).

Table 4. Intra- and Inter-Assay Reproducibility of RT-qPCR Assay.

RNA Concentration (Copies/mL)	Mean Intra-Assay Ct (*n* = 10)	Intra-Assay SD	Mean Inter-Assay Ct (*n* = 10 Independent Assays)	Inter-Assay SD
200	36.52	0.04	36.58	0.07
133	37.06	0.23	37.10	0.29
100	37.55	0.03	37.59	0.06
66	37.88	0.03	37.92	0.05
Negative control	–	–	–	–

Ct: Cycle threshold; SD: Standard deviation. Intra-assay reproducibility was evaluated using 10 replicate reactions within a single assay. Inter-assay reproducibility represents mean Ct values calculated across replicates run on 10 separate days.

3.2. Limit of Detection (LoD)

The analytical sensitivity of the assay was assessed using serial dilutions of the ORF1ab-positive reference. The Limit of Detection (LoD) was determined as 200 copies/mL in the spiked clinical matrix prior to RNA extraction, the lowest concentration at which 100% of replicates (20/20) yielded a positive ORF1ab signal. During the LoD assessment, both 10 µL and 20 µL reaction formats were evaluated; the finalized routine RT-qPCR protocol used a 20 µL reaction volume as described in Section 2.4. At lower concentrations, detection rates decreased as expected due to stochastic effects near the detection limit: 90% detection at 133 copies/mL, 50–55% at 100 copies/mL, and 20% at 66 copies/mL (Table 5).

Table 5. Results of the limit of detection study.

Reaction Volume	Viral RNA Concentration (Copies/ml)	Number of Tests	SARS-CoV-2 *(ORF1ab)*			Internal Control *(RNase P)*		
			Positive	Ct (Avg)	SD	Positive	Ct (Avg)	SD
10 µL	200	20	20 (100.0%)	36.53	0.03	20 (100.0%)	33.78	0.08
	133	20	18 (90.0%)	37.05	0.24	20 (100.0%)	33.49	0.04
	100	20	11 (55.0%)	37.54	0.04	20 (100.0%)	34.07	0.04
	66	20	4 (20.0%)	37.91	0.03	20 (100.0%)	33.66	0.04
	0	10	0 (0.0%)	-	-	10 (100.0%)	33.73	0.03
20 µL	200	20	20 (100.0%)	36.52	0.04	20 (100.0%)	33.71	0.09
	133	20	18 (90.0%)	37.06	0.23	20 (100.0%)	33.51	0.05
	100	20	10 (50.0%)	37.55	0.03	20 (100.0%)	34.02	0.03
	66	20	4 (20.0%)	37.88	0.03	20 (100.0%)	33.67	0.06
	0	10	0 (0.0%)	-	-	10 (100.0%)	33.63	0.05

Ct = Cycle threshold; SD = Standard deviation. Ct and SD values were calculated from amplification-positive replicates.

3.3. SARS-CoV-2 Positivity and Clinical Presentation

Molecular surveillance conducted between March 2021 and March 2024 detected SARS-CoV-2 RNA in both feline and canine samples collected in Izmir. Of the 318 cats

tested, 42 (13.2%; 95% CI: 9.9–17.4%) were positive for SARS-CoV-2 RNA, while 40 out of 478 dogs (8.4%; 95% CI: 6.2–11.2%) tested positive. Positive samples were characterized by distinct amplification curves on the FAM channel (ORF1ab). RNase P amplification was observed as the endogenous internal control in valid reactions (Figure 2).

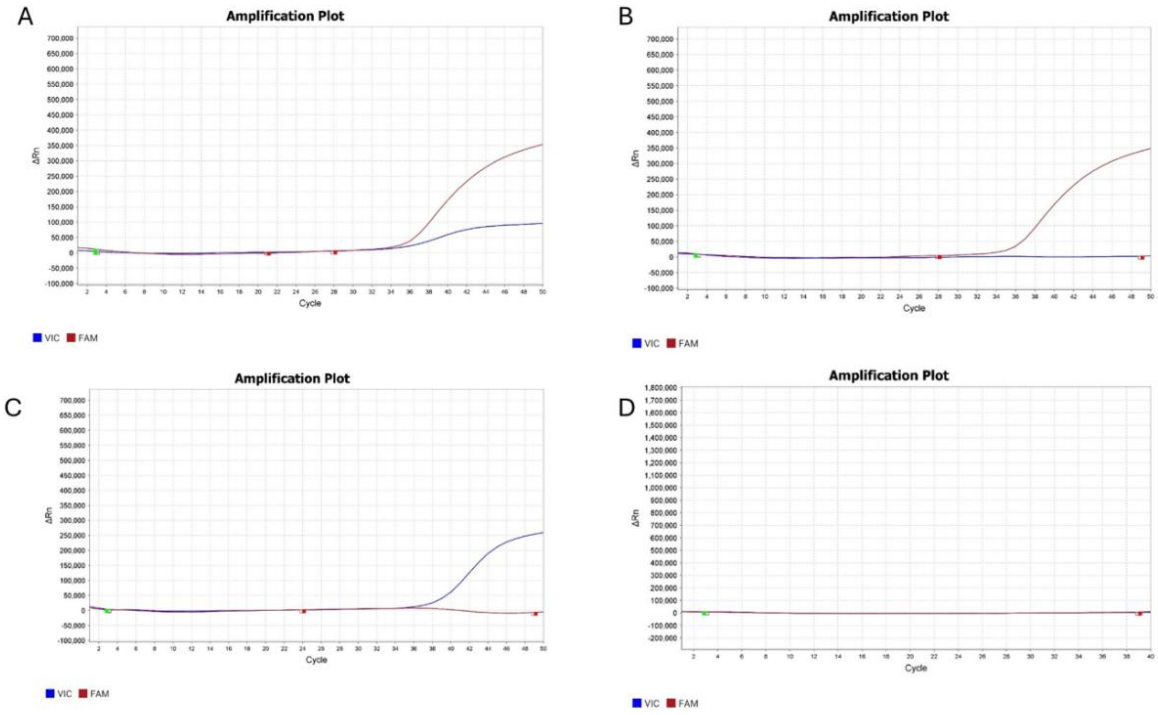


Figure 2. Representative amplification patterns for positive, negative, and invalid clinical samples. Panels (**A**,**B**) represent valid RT-qPCR-positive samples, showing amplification of both ORF1ab target (red line) and internal control (RNase P gene, blue line); Panel (**C**) illustrates a valid negative sample, with amplification of the internal control only (RNase P gene, blue line), confirming sample integrity; Panel (**D**) depicts an invalid result, with no amplification observed for either target, suggesting possible sampling, extraction, or PCR inhibition issues requiring retesting. X-axis: PCR Cycle Number; Y-axis: fluorescence signal (ΔRn).

Clinical signs were observed in 81.0% (34/42) of RT-qPCR-positive cats and 57.5% (23/40) of RT-qPCR-positive dogs, predominantly including mild respiratory distress, sneezing, nasal discharge, and low-grade fever. The remaining RT-qPCR-positive animals showed no obvious clinical signs at the time of sampling.

Among RT-qPCR-positive animals, the mean ORF1ab Ct value was 24.29 ± 3.86 in cats and 26.78 ± 4.20 in dogs. The Ct range was 12.82–33.90 in cats and 17.44–37.46 in dogs. Ct values were reported descriptively and were not interpreted as direct measures of infectious viral shedding.

3.4. Serological Findings

Serological testing provided complementary information to the molecular findings. In total, 44 feline serum samples were ELISA-positive. All 42 RT-qPCR-positive cats (100%) were also seropositive, while two additional ELISA-positive cats were RT-qPCR-negative. Among the 40 RT-qPCR-positive dogs, 38 (95%) were seropositive and two were ELISA-negative. A summary of the molecular, clinical, and serological findings is provided in Table 6.

Table 6. Summary of molecular, clinical, and serological findings in cats and dogs.

Parameter	Cats	Dogs
Animals tested, *n*	318	478
RT-qPCR-positive, *n* (%)	42 (13.2%)	40 (8.4%)
95% CI	9.9–17.4%	6.2–11.2%
Symptomatic among RT-qPCR-positive animals, *n* (%)	34/42 (81.0%)	23/40 (57.5%)
Asymptomatic among RT-qPCR-positive animals, *n* (%)	8/42 (19.0%)	17/40 (42.5%)
Mean ORF1ab Ct ± SD	24.29 ± 3.86	26.78 ± 4.20
Ct range	12.82–33.90	17.44–37.46
ELISA-positive among RT-qPCR-positive animals, *n* (%)	42/42 (100%)	38/40 (95.0%)
RT-qPCR-positive/ELISA-negative, *n*	0	2

4. Discussion

The present study combined molecular surveillance of SARS-CoV-2 RNA in cats and dogs in Izmir, Turkey, with the development and analytical evaluation of a duplex RT-qPCR assay for veterinary samples. The assay showed a strong linear relationship between target concentration and Ct values (R^2 = 0.9998), an amplification efficiency of 99.66%, an LoD of 200 copies/mL, and low intra- and inter-assay variability. In parallel, SARS-CoV-2 RNA was detected in 42 of 318 cats (13.2%) and 40 of 478 dogs (8.4%). These findings extend the available molecular surveillance data for companion animals in Turkey while providing analytical performance data for an assay incorporating a viral target and an endogenous host control within the same reaction.

Veterinary-tailored molecular assays for SARS-CoV-2 have previously been developed using different target and internal-control strategies. Jeon et al. [26] described multiplex RT-qPCR assays targeting the viral RdRp and N genes together with canine- or feline-specific 16S rRNA endogenous controls. More recently, Thieulent et al. [30] developed and validated multiplex one-step qPCR/RT-qPCR assays incorporating SARS-CoV-2 together with other feline respiratory pathogens, demonstrating high linearity, analytical sensitivity, repeatability, and reproducibility. The present assay uses ORF1ab as the viral target and RPP30 as the endogenous internal control. The selected RPP30 primer/probe sequences showed 100% in silico homology with the corresponding canine and feline reference sequences, enabling the same internal-control system to be used for both species. Endogenous controls are particularly useful in veterinary swab samples because they provide information on the presence of host-derived nucleic acid throughout the extraction and amplification workflow. The use of a common RPP30 control therefore offers a relatively simple duplex configuration for parallel analysis of feline and canine samples without requiring separate species-specific internal-control assays.

The RNA-positivity proportions observed in the present cohort fall within a highly heterogeneous body of companion-animal SARS-CoV-2 literature. Guo et al. [18] found that molecular and serological positivity in general pet-screening studies was usually below 5%, whereas estimates exceeding 10% were more frequently reported when animals had known exposure to COVID-19-positive owners. A multi-year surveillance study by Kimmerlein et al. [31] further showed temporal variation in SARS-CoV-2 seropositivity in companion animals in relation to human infection dynamics, emphasizing the influence of epidemic phase and exposure context on observed infection patterns. Sampling strategy itself can markedly influence the observed frequency of detection. In the Spanish survey by Barroso-Arévalo et al. [24], SARS-CoV-2 RNA was detected in 2.59% of selectively sampled dogs compared with none of the randomly sampled dogs, and in 1.63% of selectively sampled cats compared with 0.17% of randomly sampled cats. Thus, differences among surveillance studies reflect not only geographical variation in viral circulation but also

exposure history, clinical selection, epidemic period, specimen type, and diagnostic methodology. In the present study, the predominance of shelter animals and prioritization of animals with compatible clinical signs likely contributed to the relatively high RNA-positivity proportions compared with general population-screening studies.

Experimental studies have reported species-related differences in susceptibility to SARS-CoV-2. Shi et al. [32] demonstrated efficient replication of SARS-CoV-2 in cats, whereas replication in dogs was substantially more limited. Bosco-Lauth et al. [33] similarly showed productive infection, prolonged nasal and oral viral detection, and direct-contact transmission in cats, while experimentally exposed dogs seroconverted but showed more restricted viral replication. Natural infections in both species have subsequently been documented. Hamer et al. [34], for example, identified SARS-CoV-2 infection in cats and dogs living with infected owners using RT-PCR and sequencing and recovered infectious virus from selected animals. These experimental and field observations indicate that biological differences between feline and canine hosts may influence infection dynamics, although detection frequencies under natural conditions are also shaped by exposure intensity, human–animal contact patterns, sampling strategy, and the epidemiological context.

Clinical presentation was heterogeneous among RT-qPCR-positive animals. Clinical signs were recorded in 81.0% of positive cats and 57.5% of positive dogs, predominantly including mild respiratory distress, sneezing, nasal discharge, and low-grade fever, whereas other positive animals were clinically asymptomatic at the time of sampling. Natural-infection studies have frequently reported mild or absent clinical disease; notably, Hamer et al. [34] found that 82.4% of infected pets in SARS-CoV-2-positive households were asymptomatic. The comparatively high proportion of symptomatic animals in the present study is consistent with the sampling strategy, which preferentially included animals presenting with compatible clinical signs. Respiratory signs in companion animals are also etiologically nonspecific and may occur in association with a range of viral and bacterial respiratory infections, particularly in high-density environments such as shelters. SARS-CoV-2 RNA detection should therefore be considered alongside the broader clinical and epidemiological context of each animal.

The ORF1ab Ct distributions provide an additional perspective on the molecular findings. Mean Ct values were lower in cats than in dogs, indicating a greater abundance of detectable target RNA in the feline specimens under the conditions of the present assay. Ct values, however, reflect both biological and pre-analytical factors, including the amount of material collected, anatomical sampling site, cellular content, RNA integrity, extraction efficiency, and amplification conditions. They are therefore most informative as semiquantitative measures within a standardized sampling and analytical framework. Studies combining RT-qPCR with sequencing and virus isolation, such as those of Hamer et al. [34] and Barroso-Arévalo et al. [24], further demonstrate that molecular detection, genomic characterization, and viral viability provide complementary levels of information when investigating SARS-CoV-2 infection in animals. Venkat et al. [35] similarly combined RT-PCR, neutralizing-antibody testing, and whole-genome sequencing in companion animals from households with confirmed human COVID-19, illustrating the additional epidemiological information obtained when molecular detection is integrated with serological and genomic data.

The findings are particularly relevant in the context of the limited and heterogeneous data previously reported from Turkey. Yilmaz et al. [27] detected SARS-CoV-2-reactive antibodies in domestic cats in Istanbul, with 26.45% and 28.38% positivity by spike- and RBD-based ELISAs, respectively. However, only three of the 34 sera reactive in both ELISAs showed neutralizing activity, and 27 were also positive for antibodies against feline coronavirus, illustrating the complexity of interpreting serological screening results. In contrast,

Kadi et al. [29] examined nasal swabs from 415 cats and dogs with respiratory signs in Samsun using RT-qPCR targeting two SARS-CoV-2 genomic regions and detected no RNA-positive animals. The difference between the Samsun and Izmir findings is noteworthy. The Samsun survey was conducted from July 2020 to July 2021, whereas the present study extended from March 2021 to March 2024 and therefore covered a substantially different and longer period of the pandemic. Geographic variation, temporal changes in human infection pressure and circulating viral lineages, differences in sampled populations, and assay characteristics may all have contributed to the contrasting molecular findings.

The paired molecular and serological findings in the present study further illustrate the different temporal information captured by these approaches. All 42 RT-qPCR-positive cats were ELISA-positive, while two additional ELISA-positive cats were RT-qPCR-negative; among the 40 RT-qPCR-positive dogs, 38 were ELISA-positive and two were ELISA-negative. RT-qPCR reflects viral RNA present at the sampled site, whereas serological assays reflect development and persistence of the host antibody response. Consequently, the relationship between molecular and serological results depends strongly on the stage of infection at which the animal is sampled. Patterson et al. [36] demonstrated this distinction in northern Italy, where none of the sampled animals were RT-PCR-positive despite detection of neutralizing antibodies in 3.3% of dogs and 5.8% of cats. The findings of Yilmaz et al. [27] also emphasize that serological results may vary substantially according to assay format and endpoint. More recently, Pauvolid-Corrêa et al. [37] combined RT-qPCR and neutralizing-antibody testing in active surveillance of 579 pets from households with confirmed human COVID-19, further illustrating the complementary information provided by molecular and serological approaches. Together, these observations support the value of combining molecular and serological information when characterizing SARS-CoV-2 exposure and infection dynamics in companion animals.

Several considerations should be kept in mind when interpreting the present findings. The study population was non-random and included a high proportion of animals from municipal shelters, with clinically suspicious animals preferentially enrolled. Sampling also extended over a three-year period without stratification by year or epidemiological phase, and possible clustering within sampling sites was not examined. The assay was based on a single SARS-CoV-2 target (ORF1ab), and additional molecular confirmation or experimental testing against other relevant respiratory pathogens was not included in the present study. Likewise, the serological results were interpreted according to the characteristics of the assay used. These aspects may have influenced the epidemiological estimates and should be considered when comparing the present findings with other companion-animal surveillance studies.

Overall, the present findings document SARS-CoV-2 RNA assay-positive detections in a large cohort of cats and dogs sampled in Izmir and demonstrate the analytical performance of a duplex RT-qPCR configuration combining ORF1ab detection with an endogenous RPP30 control applicable to both species. The results also reinforce the marked influence of host species, sampling strategy, exposure context, and epidemic period on SARS-CoV-2 detection in companion animals. Integration of molecular surveillance with serology, genomic characterization, and well-defined epidemiological sampling will provide a more complete understanding of SARS-CoV-2 dynamics at the human–companion animal interface and strengthen One Health surveillance of emerging respiratory viruses.

In conclusion, this study provides molecular evidence of SARS-CoV-2 RNA positivity in domestic cats and dogs in Izmir, Turkey, and describes the development and analytical evaluation of a multiplex RT-qPCR assay for veterinary samples. The detection of viral RNA in 13.2% of cats and 8.4% of dogs within the sampled surveillance population, together with complementary serological findings, provides additional molecular surveillance data on SARS-CoV-2 RNA positivity in companion animals. The analytical

performance of the assay, including an amplification efficiency of 99.66%, an LoD of 200 copies/mL, and low intra- and inter-assay variability, supports its potential use in molecular surveillance of feline and canine samples. Incorporating both viral and endogenous internal control targets within the same reaction provides a practical approach for simultaneous SARS-CoV-2 RNA detection and assessment of sample adequacy. These findings underscore the value of integrating companion-animal surveillance into a One Health framework and support continued monitoring of SARS-CoV-2 and other emerging respiratory viruses at the human–animal interface.

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