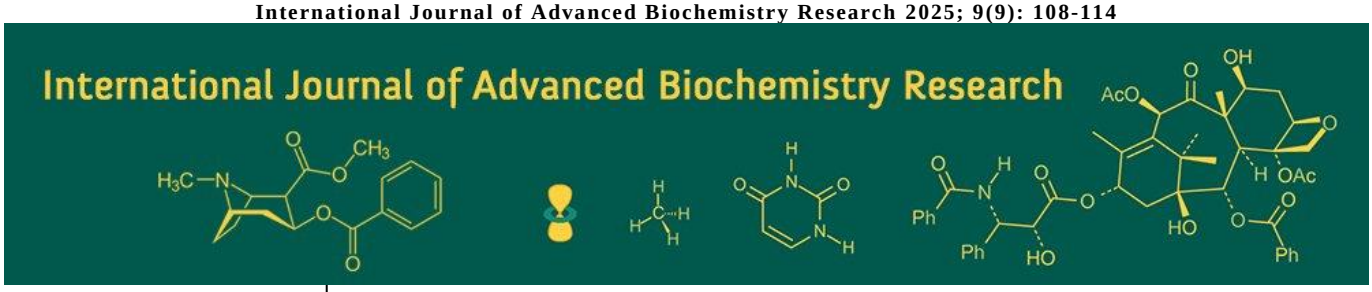

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(9): 108-114
www.biochemjournal.com
Received: 02-07-2025
Accepted: 05-08-2025

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SARS-CoV-2 infection in companion dogs: An analytical study

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i9b.5517

Abstract
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has continued to circulate among humans and caused numerous COVID-19 outbreaks since its emergence in late 2019. In addition to humans, the virus has caused spillover infection events in various animal species, including both companion and wild animals. Since dogs are highly susceptible to SARS-CoV-2 infection and are in direct contact with their owners, it is important to assess the presence of SARS-CoV-2 in dogs. Several studies have been conducted worldwide since 2019 to investigate COVID-19 exposure in companion animals. In this study, we estimated the infectivity of SARS-CoV-2 in dogs presented at a veterinary clinic between May 2021 and February 2022. To perform serological and molecular detection of SARS-CoV-2, serum and swab samples (nasal and rectal) were collected from 83 dogs. Neutralizing antibodies against the virus were detected in 25 out of 83 serum samples (30.12%). All the positive serum samples were subjected to a plaque reduction neutralization test and were found to be positive. However, all collected swab samples tested negative by RT-qPCR. This study confirmed the circulation of neutralizing antibodies against SARS-CoV-2 in dogs. Furthermore, seropositivity to SARS-CoV-2 in dogs was found to be comparable to the number of human cases at a given point in time. The present study suggests a probable spillover of the virus from humans to dogs during the COVID-19 pandemic in India.

Keywords: SARS-CoV-2; dogs; seropositivity; plaque reduction neutralization test.

1. Introduction
In late December 2019, an outbreak of atypical pneumonia of unknown etiology was reported for the first time in Wuhan Province, China. The outbreak was caused by a novel Betacoronavirus strain (β-CoV), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which is responsible for the coronavirus disease 2019 (COVID-19) pandemic in humans (Zhu *et al*., 2020; CSG-ICTV, 2020) [27]. Within a short period, the virus spread worldwide, causing millions of confirmed cases and deaths. The actual origin of this novel coronavirus remains uncertain; however, molecular studies have revealed that the SARS-CoV-2 strain shares 96.2% nucleotide identity with a bat-derived coronavirus (Bat-CoV RaTG13 strain) and shows amino acid similarity with the Malayan Pangolin-CoV strain. Thus, the probable origin of the virus may be attributed to bats, with Malayan pangolins (*Manis javanica*) acting as an intermediate host for possible spillover to humans. Additionally, the virus has a high mutation and recombination rate, which allows for genomic variability and enables it to infect a wide range of animal species (Michelitsch *et al*., 2021) [28].
According to data provided by WOAH (2023), more than 774 outbreaks of SARS-CoV-2 have been documented in various animal species across different continents. These include ferrets, cats, minks, dogs, non-human primates, and wild felines such as lions and tigers. Moreover, certain species such as felines, minks, and ferrets have been reported to be more susceptible to SARS-CoV-2 infection (Hammer *et al*., 2021) [17]. While species such as pigs, chickens, and ducks are comparatively less susceptible to the virus (Shi *et al*., 2020) [36].
Companion animals such as dogs and cats are more prone to acquiring SARS-CoV-2 infection. The first case of SARS-CoV-2 infection in dogs was reported in Hong Kong,

resulting from close contact with a confirmed human case of COVID-19 (Sit *et al*., 2020) [38]. Hence, some studies have revealed a higher binding affinity between the dog angiotensin-converting enzyme 2 (dACE2) and the receptor-binding domain (RBD) of the SARS-CoV-2 spike (S) protein (Hayashi *et al*., 2020; Zhang *et al*., 2021) [18, 46]. Subsequently, to determine the prevalence of SARS-CoV-2 in dogs, many studies were conducted worldwide using both molecular and serological approaches (Temmam *et al*., 2020; Patterson *et al*., 2020; Barua *et al*., 2021; Colitti *et al*., 2021; Smith *et al*., 2021; Laidoudi *et al*., 2021; Bessiere *et al*., 2022; Jairak *et al*., 2022; Zambrano-Mila *et al*., 2022) [41, 31, 4, 9, 26, 24, 5, 19, 2]. According to a meta-analysis of various sero-surveillance studies, the serological prevalence of SARS-CoV-2 in dogs was found to be less than 1%, while it increased to as much as 10% when COVID-19 positive patients were present in the household (Guo *et al*., 2023) [15]. Recently, cases of SARS-CoV-2 infection have been reported in wild felines (lions and leopards) (Karikalan *et al*., 2022) [6] and other non-human hosts such as dogs, cattle, and buffaloes in India (Kumar *et al*., 2022) [23]. Additionally, a former study in India reported a high seroprevalence of antibodies specific to SARS-CoV-2 in wild felines (Borkakoti *et al*., 2023). However, there are no significant studies available in India regarding the seropositivity of SARS-CoV-2 in dogs. Hence, we conducted the current study during the active COVID-19 pandemic to determine the role of household dogs in SARS-CoV-2 transmission.

2. Materials and Methods

2.1 Ethical approval

The study has been approved by the IBSC (Institutional Bio-Safety Committee) and the Review Committee on Genetic Manipulation (RCGM) (IBKO ID: TAI:C100346). Verbal consent was obtained from all pet owners after the objectives and benefits of the study were explained during sample collection.

2.2 Sample collection

The investigation involved randomly contacting 114 pet owners from Bareilly District (28.3905°N, 79.4358°E), Uttar Pradesh (Province), India, who brought their dogs to the ICAR-IVRI Referral Veterinary Polyclinic for treatment. Out of 114 pet owners, 83 consented to include samples from their pets in the study. For the present study, serum (n=83), nasal (n=83), and rectal (n=83) swab samples were aseptically collected from 83 dogs. All collected samples were transported to the laboratory according to OIE standard protocols and stored in a -80ºC refrigerator. Sample collection was performed during the active COVID-19 pandemic period, ranging from May 2021 to February 2022. The B.1.617.2 (Delta) variant, Delta Plus, and Omicron variants (subvariants BA.1 and BA.2) of SARS-CoV-2 were prevalent in the country during this period. The dogs were clinically examined prior to sample collection, and a detailed history of any signs of health-related discomfort was obtained through a questionnaire. Furthermore, the present study included a brief history of SARS-CoV-2 infection among dog owners during the peak of the COVID-19 outbreak.

2.3 Molecular detection of SARS-CoV-2

Swab samples (nasal and rectal) were processed for the molecular detection of SARS-CoV-2 genome, as described previously (Sit *et al*., 2020; Karikalan *et al*., 2022) [6, 38]. In brief, RNA was extracted from the samples using the commercially available QIAamp Viral RNA Mini Kit (QIAGEN, Germany), following the manufacturer's instructions. Subsequently, the extracted RNA was quantified and subjected to a real-time RT-qPCR assay. The assay was performed using the COVISure RT-PCR Test Kit (Genetix, India) according to the manufacturer's instructions.

2.4 Serological detection of antibodies against SARS-CoV-2

2.4.1 Virus neutralization test

To detect neutralizing antibodies against SARS-CoV-2, serum samples were subjected to a virus neutralization test (VNT) (Cerutti *et al*., 2022) [8]. A wild-type SARS-CoV-2 virus isolate (SARS-CoV-2/human/IND/CAD1339/2020; GenBank Acc. No. MZ203529) was used to conduct the test. Furthermore, the presence of the virus was verified by immunofluorescence assay (IFA) using fluorescein isothiocyanate (FITC)-conjugated anti-human antibody (Thermo Fisher Scientific, US) (Figure 1). The test was performed as described earlier (Bessiere *et al*., 2022; Cerutti *et al*., 2022) [5, 8], with minor modifications. Briefly, the test serum samples were heat-inactivated at 56 °C for 30 minutes and then serially two-fold diluted (starting from 1:16) in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA), supplemented with 2% fetal bovine serum (FBS) (Gibco, USA) and 100X antibiotics (HiMedia, India). Subsequently, 100 $TCID_{50}$ of SARS-CoV-2 virus was mixed with the diluted serum samples and incubated at 37 °C for 1 hour. Post incubation, 100 µL of the serum–virus mixture was transferred to 96-well cell culture plates (Falcon®, USA) containing Vero cell monolayers and incubated at 37 °C with 5% CO_2 for 72 hours. After 3 days of incubation, plates were analysed for observation of cytopathic effect (CPE), and the endpoint titre was calculated using Reed and Muench method. The titre was expressed as the logarithm of the reciprocal of the dilution, falling within the ranges of <1.5, 1.8, 2.1, and >2.4. Furthermore, to assess cross-reactivity, we tested dog serum samples (n = 55) collected during the pre-COVID-19 period using the above-mentioned procedure.

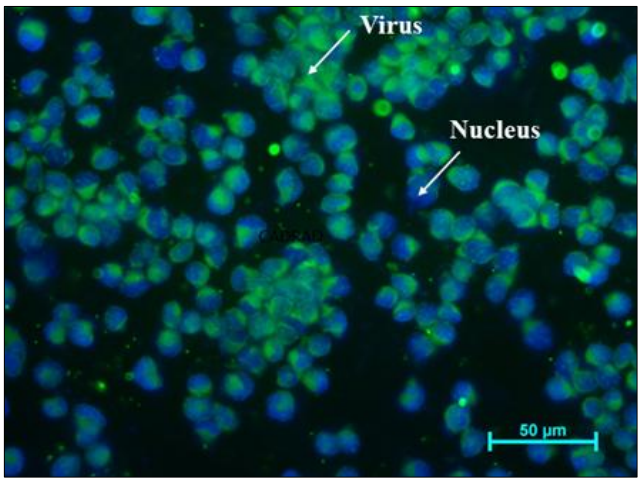

Fig 1: Immuno-fluorescence assay (IFA) of SARS-CoV-2 virus-infected Vero cells.

Infected cells showed fluorescent green colour; DAPI-stained nucleus of cells emitted blue colour.

2.4.2 Plaque reduction neutralization test

All the serum samples that tested positive by VNT were re-tested using the plaque reduction neutralization test (PRNT) to confirm the specificity of antibodies against SARS-CoV-2 (Dolscheid-Pommerich *et al*., 2022) [14]. The PRNT was performed as described earlier by Lau *et al*. (2022). In brief, heat-inactivated positive test serum samples were serially two-fold diluted in DMEM (Sigma-Aldrich, USA) supplemented with 2% fetal bovine serum (FBS) (Gibco, USA) and 100X antibiotics (HiMedia, India). Subsequently, 40 plaque-forming units (PFU) of SARS-CoV-2 were mixed with the diluted serum samples and incubated at 37 °C for 1 hour. After incubation, 0.1 mL of the serum–virus mixture was added to 24-well cell culture plates (Falcon®, USA) containing Vero cell monolayers and incubated for an additional hour at 37 °C. Following this, an overlay of 1% agar was added to each well, and the plates were further incubated at 37 °C for 72 hours with 5% CO_2. After the 3-day incubation period, the plates were stained to visualize plaque reduction. Samples were considered positive when the highest serum dilution resulted in ≥90% ($PRNT_{50}$) reduction in the number of virus plaques. All experiments involving live virus handling were conducted in the Biosafety Level-3 (BSL-3) facility at ICAR-IVRI, Izatnagar, Bareilly.

3. Results and Discussion

According to the history provided by dog owners regarding SARS-CoV-2 infection in their families, it was found that in twenty-one households, one or more family members were actively infected with COVID-19. In contrast, fifty-three owners reported past infections in their families, while the remaining nine reported risky interactions between their pet dogs and stray animals, such as licking, sniffing, and fighting, which potentially exposed the dogs to infection. However, companion animals live in close contact with humans and are more prone to acquiring SARS-CoV-2 infection from them (Sit *et al*., 2020; Halfmann *et al*., 2020) [38, 16]. Recently, Alberto-Orlando *et al*. (2022) [2] reported the possible transmission of SARS-CoV-2 from infected owners to their household dogs.

During the molecular detection of SARS-CoV-2, all collected swab samples tested negative by RT-qPCR, indicating no active infection in the sampled dogs. Similar findings have also been reported in other studies (Dias *et al*., 2021; de Carvalho *et al*., 2021; Akhtardanesh *et al*., 2023) [13, 11, 1]. In a study by Bosco-Lauth *et al*. (2020), dogs that were intranasally infected with SARS-CoV-2 seroconverted and developed neutralizing antibodies, but did not shed the virus following infection. These findings suggest that the viral shedding window in dogs is not fully understood and warrants further investigation.

However, through serological detection using the virus neutralization test (VNT), neutralizing antibodies against SARS-CoV-2 were detected in 25 out of 83 serum samples (30.12%, with a 95% confidence interval of 20.1%–39.8%), with titres ranging from 1.5 to >2.4 (log_{10}) (Figure 2). Among the positive samples, 17 showed antibody titres greater than 2.4, while 8 had titres between 1.5 and 2.4 (Table 1). Out of the 25 positive serum samples, 23 were collected between May 2021 and July 2021, while the remaining two were collected in January 2022. All pre-COVID-19 dog serum samples (n = 55) tested negative for neutralizing antibodies against SARS-CoV-2 in the virus neutralization test. Additionally, the positive serum samples (n = 25) showed >90% plaque reduction at a 1:128 dilution in the PRNT assay, confirming the specificity of the neutralizing antibodies against SARS-CoV-2. Similar studies conducted in Poland, the United Kingdom, and Croatia reported seropositivity rates of less than 3% in dogs against SARS-CoV-2 (Pomorska-Mol *et al*., 2021; Smith *et al*., 2021; Stevanovic *et al*., 2021) [26], while studies from Italy and France reported seropositivity rates of approximately 5.8% and 5.4%, respectively (Patterson *et al*., 2020; Bessiere *et al*., 2022) [31, 5]. However, the present study has revealed a comparatively higher seropositivity rate of SARS-CoV-2 infection in dogs. Several factors could contribute to this variation, including geographical location, the number of human cases, and most importantly, the sample size of animals included in the study. Recently, Decaro *et al*. (2022) [12] reported that neutralizing antibodies against SARS-CoV-2 can persist for more than six months in dogs, similar to humans.

The number of COVID-19 cases per day was plotted on the X-axis, and the number of seropositive cases per month was plotted on the Y-axis, which suggests a linear correlation between seropositivity to SARS-CoV-2 in dogs and COVID-19 cases in humans in India. A marked rise in dog seropositivity was observed during peaks of COVID-19 cases, and vice versa. The number of active COVID-19 cases in humans was higher during the second wave compared to the first and third waves in the country, with a corresponding increase in seropositivity to SARS-CoV-2 in dogs during the same period. This may have been due to the widespread circulation of the highly virulent Delta variant (B.1.617.2) of SARS-CoV-2 in the country during the second wave. Therefore, the time point of positive serum samples was proportional to the number of human cases of COVID-19 in that locality (Figure 3).

To the best of our knowledge, this is the first study to report seropositivity against SARS-CoV-2 infection in dogs in India. During the peak of the active COVID-19 outbreak, due to strictly imposed restrictions in disease containment zones and government guidelines, samples were not collected from stray dogs. As a result, a large-scale study could not be conducted, and no specialized analysis could be performed. However, due to strict government guidelines, no further interactions with animal owners were carried out, and more detailed information could not be obtained. It should be noted that most animals remain asymptomatic during SARS-CoV-2 infection and, therefore, the infection often goes undetected (Barroso-Arevalo *et al*., 2021; Jairak *et al*., 2022) [3, 19]. Moreover, the primary aim of the present study was to determine the presence of SARS-CoV-2 infection in dogs, with particular emphasis on its prevalence during the pandemic period. A comprehensive investigation of risk factors and their associations was not included in this study. The data obtained from the current study indicate that dogs can become naturally infected with SARS-CoV-2, either through transmission from humans or from the environment, as mentioned in previous studies (Alberto-Orlando *et al*., 2022; Zambrano-Mila *et al*., 2022) [2]. According to the WHO, India has experienced massive losses due to COVID-19, and novel variants of the virus continue to emerge (Kaku *et al*., 2024) [20]. Several instances of SARS-CoV-2 spillback infections from animals to humans, resulting in novel variants, have also been reported (Sila *et al*., 2022) [37]. Therefore, it is necessary to monitor viral circulation in both pet and wild dogs to prevent the future emergence of new variants.

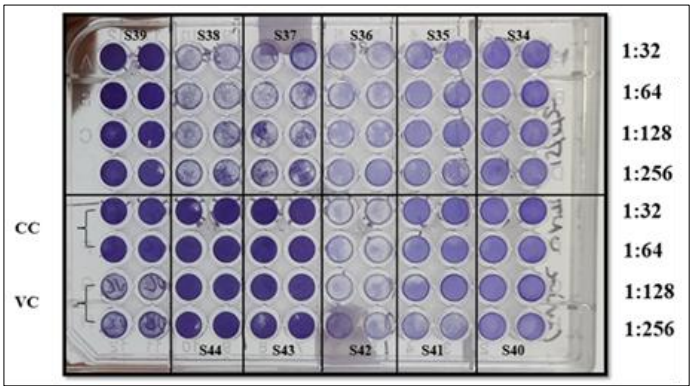

Fig 2: Virus neutralization test result.

Sample numbers 34,35,39,40,41,43,44 are positive, sample numbers 36,37,38,42 are negative, CC: Cell Control (-CPE), VC: Virus Control (+CPE). Titre of positive serum varies from 1:32 to 1:256 (log_{10}: 1.5 to >2.4).

Table 1. Status of SARS-CoV-2 in pet dogs

Sr. No	Number of Serum samples	Numbers of positive serum samples	Sero-positivity rate	Time of collection	Titre of positive samples (log_{10})	CI
1.	25	10	40%	May 2021	>2.4	20.8% - 59.2%
2.	21	08	38.09%	June 2021	1.5 -2.4	18.2% - 59.8%
3.	17	05	29.41%	July 2021	>2.4	7.2% - 50.8%
4.	20	02	10%	January 2022	>2.4	3.1% - 23.1%
Total	83	25				20.1% - 39.8%

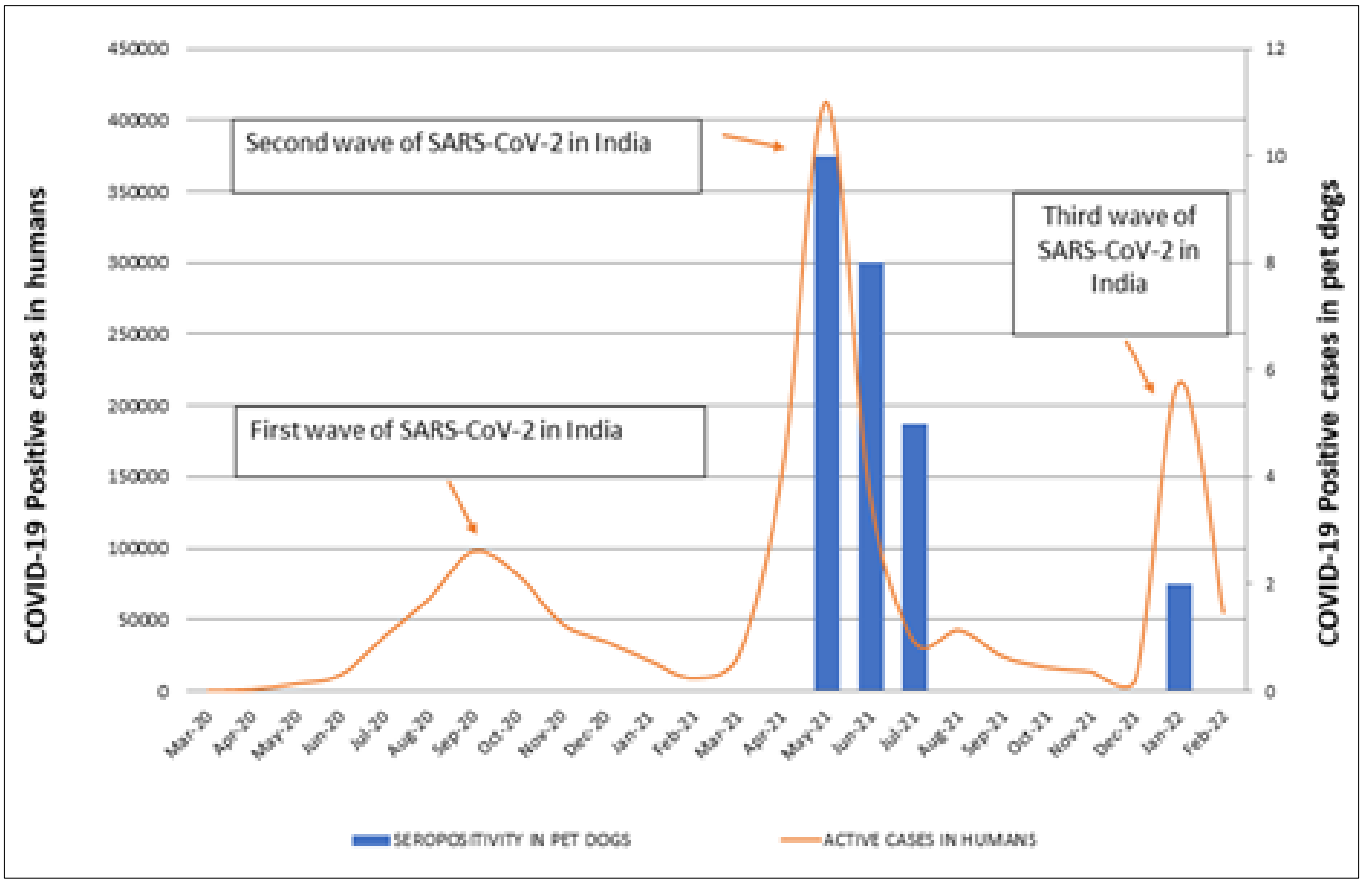

Fig 3: Temporal distribution of active cases of COVID-19 in humans as compared to the sero-positivity obtained in the pet dogs.

4. Acknowledgement
The authors are deeply grateful to the pet owners and the staff of the ICAR-IVRI Referral Veterinary Polyclinic for their cooperation during sample collection. Additionally, we would like to thank the ICAR-IVRI Deemed University for providing the BSL-3 facility to conduct this study.

5. Author contributions
HRJ, SKN, NS, RB, SM : performed molecular detection, virus revival, virus neutralization and plaque reduction neutralization assay. HRJ, VC, UKD : collected samples and prepared figures. GKS, SM, HRJ : designed the study, analysed the data and wrote the manuscript.

6. Funding
To carry out present work, funding was provided by the ICAR - National Agricultural Science Fund (NASF) and Department of Science and Technology (DST)-SERB.

7. Data availability
The primary data will be provided upon request.

8. Declaration of competing interest
The authors declare no competing interests.

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