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Original article

Virus neutralization test for confirmation of ELISA-positive SARS-CoV-2 antibodies in dogs

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Abstract

Constant antigenic changes, new variants and easy transmission of SARS-CoV-2 virus should acquire greater zoonotic attention and need to remain alert. In this retrospective study the aim was to analyze seropositivity to SARS-CoV-2 in dogs by commercial ELISA. The Virus neutralization test (VNT) was modified for the purpose of confirmation of SARS-CoV-2 antibodies in ELISA-positive dog sera. The sera were collected from 204 dogs from different veterinary clinics across Vojvodina Province, Serbia, during COVID-19 pandemic. For the screening of antibodies a commercial double multi-species antigen ELISA was used, followed by the VNT modified with SARS-CoV-2 as a confirmatory test. VNT was modified as “one step” test using local isolate of SARS-CoV-2 and the results were checked by cytopathic effect in cell culture on the 96-well microtiter plate. Obtained data have shown that 9 out of 204 dogs were positive by ELISA (4.4%), while 2 (0.97%) sera were doubtful. VNT confirmed 9 positive dogs, but 2 doubtful samples were negative, exhibiting the seroconversion to SARS-CoV-2 in 4.4% dogs from Vojvodina region during pandemic of COVID-19. VNT with SARS-CoV-2 helped to elucidate ELISA ambiguous results. The occurrence of antibodies to SARS-CoV-2 in dogs in this study during COVID-19 pandemic suggested the possibility of viral transmission to dogs, implicating the potential for zoonotic transmission. This was the first research on seroconversion to SARS-CoV-2 in dogs from the Province of Vojvodina, the northernmost part of Serbia.

Keywords: dogs, virus neutralization test, SARS-CoV-2, Serbia, seroconversion



Introduction

Severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) was identified as an emerging coronavirus in humans in December 2019, causing new infectious disease Coronavirus disease 2019 (COVID-19). After the emergence of the first human cases in Wuhan, People's Republic of China, the virus spread rapidly throughout the world (Zhou et al. 2020). Up to date, the zoonotic origin of SARS-CoV-2 is not yet fully resolved (Lawrence et Raquet 2022). Animal infections mostly result from human-to-animal transmission ("spillback" or "spillover") (Sparrer et al. 2023) and can lead to epizootic circulation of the virus, e.g. in minks (Oude Munnink et al. 2021), tigers (Grome et al. 2022), hamsters (Sia et al. 2020) or white-tailed deer (Kuchipudi et al. 2022). Moreover, transmission is possible between species, as reported from mink to cat (van Aart et al. 2021). Transmissions from animal-to-human were observed from farmed minks (Oude Munnink et al. 2021), pet hamsters (Yen et al. 2022), cats (Corukoglu et al. 2021, Sila et al. 2022), and probably raccoon dogs (Wilson 2023). These secondary spillovers resulted in the mass culling of minks (Frutos and Devaux 2020). Hence, SARS-CoV-2 is not only a threat for public health, but also poses a threat for animal health and preservation of wild animals (Mahajan et al. 2022). Currently, human to human transmission is in decline, but animal cases and the number of infected animal species continue to rise (Nederlof et al. 2024). The World Organization for Animal Health (WOAH 2023) reports 775 outbreaks in animals globally, including 29 species in 36 countries. It is important to highlight that detection of the disease in animals is very dependent on the level of surveillance implemented in each country, and consequently, these numbers are most likely underestimated (WOAH 2023). Although many countries discontinue COVID-19-specific reporting, World Health Organization (WHO) indicates that COVID-19 is still a major threat and recommends surveillance and virus variant tracking. The WHO developed COVID-19 Global Strategic Preparedness, Readiness and Response Plan (SPRP) for 2023-2025. This plan is designed to guide countries on managing the virus over the next two years as it transits from an emergency phase to a long-term sustained response (WHO 2024). During the pandemic period, formally from March 11th, 2020 to May 5th, 2023 (WHO 2024), animal infections were also registered in dogs, cats, minks and zoo animals (WOAH 2023), since they have ACE2 protein receptor similar to humans (Frazzini et al. 2022). Therefore, WOAHA recommends implementation of animal infections' surveillance, especially in pets, which are in close contact with their owners

(WOAH 2020). At the beginning of pandemic, there were only a few sporadic cases of infection in animals across the world. The first case of infection in dogs was identified in Hong Kong, when the virus was found in the nasal swabs, followed by detection of SARS-CoV-2 antibodies a few days later (Sit et al. 2020). During COVID-19 pandemic, only one published paper about serological status of SARS-CoV-2 in dogs and cats in Serbia was found. The study that included samples from a small district in South-east Serbia reported only one ELISA positive sample out of 68 tested dogs, with a relative frequency of 1.45% (Stanojevic et al. 2022). Up to date, seroepidemiological studies of SARS-CoV-2 in dogs conducted worldwide showed different prevalence rates ranging from 3.14% in Thai dogs (Jairak et al. 2022), to 26.2% in Ecuador (Alberto-Orlando et al. 2022). In the light of the re-spreading, re-emerging and growing number of infected people at the end of 2023 across the world, and also in Serbia, it is necessary to monitor and examine the role of animals, especially dogs and cats, in the epidemiology of COVID-19 disease. Although cats are more susceptible to the infection by SARS-CoV-2 than dogs (Gaudreault et al. 2020, Shi et al. 2020, Barroso et al. 2022), and while both are mostly asymptomatic or paucisymptomatic, dogs represent more significant reservoir of the virus due to the closer contact with infected owners, as previously described (Barua et al. 2021). In this study, the serosurvey of dogs in the northern part of Serbia was conducted. A total of 2,583,470 human cases of COVID-19 have been registered and 18,057 deaths were confirmed in Serbia from the beginning of COVID-19 pandemic (<https://www.who.int/countries/>).

As COVID-19 is a new disease, the main goal was to confirm the detection of antibodies in dogs obtained by a commercial ELISA with VNT using live SARS-CoV-2 local virus isolate.

Materials and Methods

Animal sampling

In accordance with national legislative, the serum samples were collected from 204 owned dogs from veterinary practitioners across the whole territory of Vojvodina Province, where the first human case was registered on March 20th, 2020. Since the sampling was performed in the context of a diagnostic test, no ethical approval was needed from the University Ethics Committee. Written informed consent was obtained from each pet owner before obtaining blood samples. The samples were collected four times during a 3-year period, starting from October/November 2021. The second sampling was performed in February 2022 during

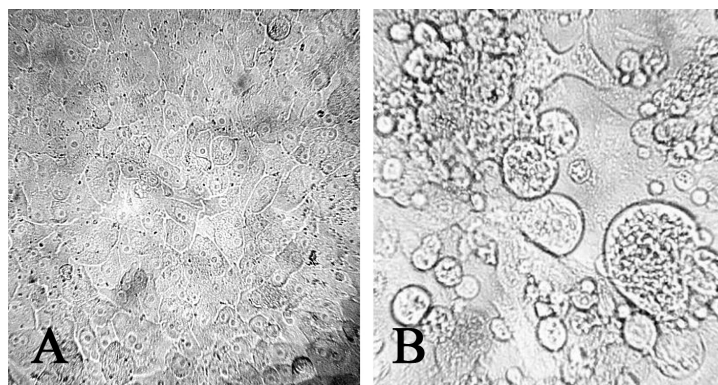


Fig. 1. Intact Vero cells (A) and cytopathic effect of Omicron variant of SARS-CoV-2 on the Vero cells after 48-hour incubation (B); x400.

the domination of Omicron variant of SARS-CoV-2. After that, the sample collection was carried out in June 2022, when the number of new SARS-CoV-2 positive patients was low, as well as those with an active COVID-19 clinical presentation. The most recent serum samples were collected in May 2023, when the number of COVID-19 positive patients started to rise again. The samples were accompanied by dogs' anamnestic data (date of sampling, health status – healthy or with any symptoms, gender, age, and breed). Serum samples were stored at -20°C until analysis. All serum samples were inactivated for 30 minutes at 56°C in thermal block before testing.

ELISA

To screen antibodies against SARS-CoV-2 we used ID Screen SARS-CoV-2 Double Multi-species antigen ELISA kit (IDvet, Grabels, France) following the manufacturers' instructions. The ELISA was based on the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein. Optical density of each sample was used to calculate the S/P ratio score expressed as percentage, and the samples were positive when the S/P ratio score was $\geq 60\%$. Ratios from 50-60% were considered as doubtful, and those below 50% as negative. To determine optical density at 450 nm we used ELISA Reader Bio Tek.Gen 5v3, Bio Tek, USA.

Virus isolation

During COVID-19 pandemic three human coronavirus strains were isolated, the first two were the "British" from April 2021 (Lalosevic et al. 2021), while in February 2022 one isolate was "Omicron" type, the latter being used for the purpose of this investigation. Our Omicron strain of SARS-CoV-2 named hCoV-19/Serbia/Novi_Sad_VSS_1402/2022, was isolated from a 63-year old male patient from Novi Sad, the capital of Vojvodina Province, and was identified as the Omicron 21K (Pango BA.1.1), accession ID: EPI_ISL_18846325 GISAID. Vero cells, provided by the Department of

Histology and Embryology (University of Novi Sad, Faculty of Medicine), served to produce SARS-CoV-2 stocks. The virus titers were determined via a 50% TCID₅₀ assay.

Neutralization assay

A modified one-step VNT was prepared to confirm presence of neutralizing antibodies to SARS-CoV-2 in doubtful and positive samples screened by ELISA. VNT was modified with our Omicron variant of SARS-CoV-2, since at the time of dogs' sera sampling, this strain was dominant in Serbia. The principle of the neutralization test is adding a constant concentration of the SARS-CoV-2 virus to each of fivefold serial dilution of serum samples. Fivefold serial dilutions of serum samples (1/5, 1/25, 1/125, 1/625) were prepared in a 96 well microtiter plate by adding 25 μl of serum in 100 μl DMEM with 10% FBS, glutamine, Hepes buffer and antibiotic/antimycotic solution (all ingredients were ready to use, Himedia, India). Each sample was tested in quadruplicate. Previously, Omicron SARS-CoV-2 was harvested from Vero cell culture as virus stock with a titer of 6.0 log TCID₅₀/mL, then aliquoted (1 mL) and stored at -70°C . The working dilution of virus in DMEM was made from the stock, and calculated to yield about 100 TCID₅₀/50 μL . Subsequently, 12 mL of working solution of virus per plate was prepared, and 50 μL were added to each well. The microtiter plate was placed in incubator at 37°C during one hour for neutralization. Two-day-old Vero cells previously prepared in T75 sterile plastic flask were trypsinized, and 100 μL of the cell suspension was added per well. After this step, the microtiter plate was incubated at 37°C and 5% CO₂ for 5 days, followed by examination by inverted microscope. The absence of CPE in particular serial dilution served as a proof of the presence of corresponding antibodies. Microscopy (x400) was performed to easily observe cytopathic effect (CPE) on the Vero cells without antibodies from dogs' sera (Fig. 1). The results corresponded to the titer

Table 1. Number of seropositive dogs in comparison to human COVID-19 cases in Vojvodina Province.

Date of sampling	No. of dogs	ELISA positive dogs	Sex	Breed	Age y/m	VNT titer	New human cases per day	Active human cases
20.10.2021.	17	2	M	Golden retriever	1/5	$\geq 1/625$	2113	22980
			F	Bulldog	2/0	1/125		
03.11.2021.	31	1	F	American akita	6/0	1/125	205	2821
08.11.2021.	31	4	M	Cocker spaniel	1/0	1/125	184	2905
			F	Beagle	0/11	1/25		
			F	Shi tsu	13/0	1/5		
			F	Labrador	1/0	1/25		
21.02.2022.	38	1	M	Golden retriever	5/0	1/25	320	23737
20.06.2022.	4	1	M	White Swiss shepherd	7/0	$\geq 1/625$	3	440

of sample reciprocal of the highest serum dilution that provided 100% neutralization of our reference virus, as determined by the visualization of CPE absence.

Results

This study included 204 dogs' serum samples collected from 2021 to 2023 (Table 1). Out of that, 135 (66.2%) were pure breed and 69 (33.8%) were mixed breed, while 109 were female (53.4%) and 95 were male dogs (46.6%). Age range was from 6 months to 14.5 years. The most frequently reported breeds were German Shepherd (8.8%), French Bulldog (5.9%) and Cane Corso (3.7%). The majority of participating animals were reported to have been asymptomatic. Only 6 dogs (2.94%), exhibited clinical signs: nasal discharge, coughing and labored breathing, but all symptomatic dogs were serologically negative on SARS-CoV-2 antibodies. Commercial multispecies ELISA showed that 9 out of 204 dogs were positive (4.4%), while 2 (0.97%) were doubtful. SARS-CoV-2 VNT confirmed 9 positive dogs but 2 doubtful samples were negative. All dogs seropositive on SARS-CoV-2 were asymptomatic. In only two cases the highest dilution (1/625) of sera completely neutralized the virus, but the end-point was not examined, because it had no influence on the results (Table 1).

Discussion

The study has demonstrated the seroconversion to SARS-CoV-2 in 4.4% owned dogs from Vojvodina region, especially at the peak of COVID-19 infection in humans. The Table 1 displays the data of daily reports on registered SARS-CoV-2 positive patients and the number of active COVID-19 human infections at the moment of dogs' blood sampling (according

to the publicly available data at the web page of the regional Institute of Public Health of Vojvodina, [www.https://izjzv.org.rs/](https://izjzv.org.rs/), daily reports). The peak of the particular corona wave was during the last week of October 2021. At the beginning of November the number of cases dropped slightly. At that moment, Delta and Omicron strains of the SARS-CoV-2 virus were circulating in Serbia (Institute of Public Health "Milan Jovanović Batut", <https://covid19.data.gov.rs/>). By the end of 2021, the number of patients with COVID-19 suddenly dropped in Vojvodina, so the number of reported human cases was 286.

SARS-CoV-2 has the ability to transmit between humans, but also transmit from humans to animals, between animals and from animals back to humans (EFSA 2023). Studies on SARS-CoV-2 infection in dogs show that these animals are susceptible to infection and that virus transmission via contact with the owners with proven COVID-19, is possible (Goletic et al. 2022). Dogs are able to acquire infection, although a lower risk is associated with this species when compared to cats. This is mostly because dogs have low number of ACE2 receptors in lung and poor virus replication, all contributing to their asymptomatic or mild clinical manifestations (Kamel et al. 2023). In European countries (Italy, France, Spain, Croatia, Germany, the Netherlands, UK, Poland), three Asian countries (Iran, Japan, China) and the USA, SARS-CoV-2 seroprevalence in the general pet population was usually below 5%, but exceeded 10% when COVID-19 positive people were known to be present in the household (Guo et al. 2023). Two reports from Thailand singled out this possibility, revealing SARS-CoV-2 transmission from humans to animals and from animals to animals and reversed transmission from animals to humans (Piewbang et al. 2022, Sila et al. 2022). In one earlier study from Serbia, authors found only 1 out of 69 (1.45%) dogs tested positive for SARS-CoV-2 specific

antibodies (Stanojević et al. 2022), which is lower than the percentage of seropositive dogs confirmed in our study. In comparison, in a similar study conducted in France the seroprevalence among dogs was much higher, 15.4% (Fritz et al. 2020) or in Croatia with 14.69% ELISA positive dogs in the general population (Stevanovic et al. 2021a).

Seropositivity among dogs in our study was proven during the presence of Delta and Omicron variants in Serbia but, from the end of 2021, Omicron SARS-CoV-2 strain was dominant in Serbia. Cross-reactivity between Delta and Omicron antibodies probably exists, but since Omicron strain was predominant at the time of sampling dogs' sera, it was not further examined. Previous study has not observed cross-reactivity between SARS-CoV-2 and other canine's corona viruses (Stevanovic et al. 2021b).

Although VNT test is not routinely performed as diagnostic test for dogs since it is time-consuming and requires isolated virus, it can help in resolving doubtful results obtained by commercial ELISA tests due to its higher specificity for particular antibodies. However, our one-step modified VNT on COVID-19 antibodies is simpler than other newly described methods, such as plaque reduction neutralization antibody test (Onen et Demirci 2024). The spike protein-based ELISA test used in our research may show different results than nucleocapsid protein-based ELISA tests (Wang et al. 2023) in animals, but recent comparative study showed that spike protein-based ELISAs are the most accurate tests for serodiagnosing SARS-CoV-2 infections in cats and dogs (Diezma-Díaz et al. 2023).

Conclusion

This study has determined the seroprevalence of SARS-CoV-2 in 4.4% dogs without any clinical signs by two tests – commercial ELISA and VNT. Our study demonstrated that VNT test could be used for confirming positive and resolving ambiguous results of SARS-CoV-2 antibodies obtained by ELISA in dogs.

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