Comparison of diagnostic performances of different serological tests for SARS-CoV-2 antibody detection in cats and dogs

Running head: SARS-CoV-2 serological tests in pets

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Summary

Serosurveillance among animals, including pets, plays an important role in the current coronavirus disease 2019 (COVID-19) pandemic, because severe acute respiratory coronavirus 2 (SARS-CoV-2) infections in animal populations could result in the establishment of new virus reservoirs. Serological assays that offer the required sensitivity and specificity are essential. In this study we evaluated the diagnostic performance of three different commercially available immunoassays for the detection of SARS-CoV-2 antibodies in pets, namely two ELISA tests for the detection of antibodies against SARS-CoV-2 nucleocapsid [ID Screen SARS CoV-2 double antigen multispecies (Double antigen) and ID Screen® SARS-CoV-2-N IgG indirect ELISA (Indirect)] and one test for the detection of neutralizing antibodies against SARS-CoV-2 receptor-binding-domain [surrogate virus neutralization test (sVNT)]. The obtained results were compared with those of conventional virus neutralization test (VNT), which was regarded as reference method. A total of 191 serum samples were analyzed. Thirteen (6.8%) samples showed VNT positive results. The overall sensitivity was higher for sVNT (100%) compared to nucleocapsid-based ELISA assays (23% for Double antigen and 60% for Indirect). The specificity was 100% for Indirect ELISA and sVNT, when a higher cut-off (>30%) was used compared to the one previously defined by the manufacturer (>20%), whereas the other test showed lower value (99%). The sVNT test showed the highest accuracy and agreement with VNT, with a perfect agreement when the higher cut-off was applied. The agreement between each nucleocapsid-based ELISA test and VNT was 96% for Indirect and 94% for Double antigen. Our findings showed that some commercially available serological tests may lead to a high rate of false negative results, highlighting the importance of assays validation for the detection of SARS-CoV-2 antibodies in domestic animals.

Keywords: Serosurveillance; immunological assay; diagnostic tests accuracy; severe acute respiratory coronavirus 2; cats; dogs

1. INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to date over 6 million of deaths worldwide (WHO, 2021a) with devastating effects on global health and society. Since the beginning of the pandemic, the susceptibility of different animal species to SARS-CoV-2 has been investigated (Meekins et al., 2021). Given their close contact with humans, susceptibility of dogs and cats has been explored, according to the One Health approach. Under experimental setting, cats were highly susceptible to infection and capable to transmit the virus to other cats, whereas dogs displayed a lower susceptibility. Seroconversion after SARS-CoV-2 experimental infection has been observed in both dogs and cats (Bosco-Lauth et al., 2020; Shi et al., 2020). In addition, natural SARS-CoV-2 infection has been reported worldwide in dogs and cats, often associated with the exposure to COVID-19 affected owners, supporting reverse zoonotic transmission events (Patterson et al., 2020; Goryoka et al., 2021; Hamer et al., 2021). Following natural or experimental infection, dogs and cats usually shed virus only

for few days making infection surveillance in pets challenging when using molecular methods (Bosco-Lauth et al., 2020; Hamer et al., 2021). Antibody levels in naturally infected cats have been shown to decrease below the detection limit within 110 days (Zhang et al., 2020), even if recent and more complete studies showed that neutralizing antibodies in pets display relatively stable or increasing titers with no evidence of seroreversion (Hamer et al., 2021) and can persist for up to 10 months (Decaro et al., 2021), making serological assays a useful tool to investigate SARS-CoV-2 infections in pets.

In humans, virus neutralization test (VNT) is considered the gold standard for the detection of serum neutralizing antibodies, that are primarily against the S1, S2, and RBD domains of the SARS-CoV-2 spike protein (Brouwer et al., 2020), represent only a small subset of the total polyclonal immune response (Girl et al., 2022) and are fundamental for the evaluation of convalescent plasma and efficacy of vaccination (Yamamoto et al., 2022). VNT is considered the gold standard also for SARS-CoV-2 antibody detection in pets (Embregts et al., 2021; Perera et al., 2021). However, a limitation of VNT is the requirement of a biosafety level 3 (BSL-3) laboratory (WHO, 2021b), which is not always available in diagnostic laboratories. VNT has been used as standalone or confirmation method for SARS-CoV-2 antibody detection by different studies (Patterson et al., 2020; Zhang et al., 2020; Calvet et al., 2021; Goryoka et al., 2021; Hamer et al., 2021; Krafft et al., 2021). To date, several serological tests are commercially available for the detection of SARS-CoV-2 antibodies in animals, including pets, that are directed against the spike or nucleocapsid protein. The use of a surrogate VNT test (sVNT) detecting neutralizing antibodies, that can be performed in BSL-2 laboratories (Tan et al., 2020), has been recently reported in animals, showing high sensitivity and specificity in comparison to the VNT assay, without cross-reactivity to other animal coronaviruses, such as feline coronavirus (FCoV) and canine coronavirus (CCoV) (Embregts et al., 2021; Perera et al., 2021). A commercially available enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibodies against the nucleocapsid antigen (N) of SARS-CoV-2 (ID Screen SARS CoV-2 double antigen multispecies; ID.Vet, France) has been used for antibody detection in animals (Decaro et al., 2021; Jemeršić et al., 2021; Stranieri et al., 2021; Udom et al., 2021). Despite the nucleoprotein does not elicit neutralizing antibodies, a good correlation between antibody responses to this protein and the neutralizing antibody titer has been described in humans (To et al., 2020). However, discordant results among different serological assays have been often reported (Michelitsch et al., 2020; Zhang et al., 2020; Decaro et al., 2021; Stranieri et al., 2021; Jemeršić et al., 2021; Klaus et al., 2021; Udom et al., 2021). The discrepancy between ELISA and VNT or among different commercial ELISA tests can be due to the lack of antibodies with neutralizing activity (Michelitsch et al., 2020; Zhang et al., 2020; Udom et al., 2021) or to the different kinetics between the antibody responses against different viral antigens (Decaro et al., 2021). An evaluation of different serological assays is needed to define reliable methodologies for SARS-CoV-2 antibody detection in pets that may be used for the surveillance of the infection, also in the light of the emerging of new viral variants that may adapt to new hosts (Meekins et al., 2021).

Therefore, the aim of the present study was to evaluate the diagnostic performance of three different commercially available serological tests for the detection of SARS-CoV-2 antibodies in dogs and cats, in comparison with the gold standard VNT assay.

2. MATERIALS AND METHODS

2.1. Sample collection

Cats and dogs from Italy were sampled between April 2nd 2020 and September 12th 2021. Samples were collected for the purpose of this study (approval n. 31/20 of the Institutional Animal Care and Use Committee and n. 43/20 of the Institutional Ethical Committee of the University of Milan) or were collected following diagnostic procedures performed within the Veterinary Teaching Hospital (VTH) of Lodi after obtaining written consent from the pet owner. According to the Ethical Committee of the University of Milan decision 29 Oct 2012, renewed with the protocol no. 02-2016, the use for research purposes of residual aliquots of samples collected for diagnostic purposes at the VTH under informed consent of the owners is allowed without any additional formal request of authorization. Complete information regarding animal signalment, including breed, sex, age, localization and timing of exposure to COVID-19 infected humans was collected when available.

Blood samples were collected by jugular or cephalic venipuncture and placed immediately in serum-separating tubes. After collection, blood samples were centrifuged at 2500 × *g* for 10 min and serum was stored at -20°C until serological analysis.
In cats, rectal swabs were also collected and stored at -80 °C until RNA extraction for feline coronavirus (FCoV) detection.

2.2. Serological tests

Serum samples were tested by VNT and three commercial serological tests.

2.2.1 Virus neutralization test

the virus neutralization assay was performed as described by Rijkers et al., 2020 with few modifications. Briefly, sera were previously heat-inactivated (30 min, 56°C) and tested in duplicate. Two-fold serial dilutions (starting at 1:5) of the sera were incubated with 100 TCID50 of the SARS-CoV-2 HCoV-19/Italy/310904/46/2020 strain (EPI_ISL_9011947) at 37°C and 5% CO2, for 1 hour at 37°C in 96-well plates. Vero-E6 cells were added at a concentration of 2×10^4 cells per well and incubated for 72 hours at 37°C with 5% CO2. Serum virus neutralization titer (VNT50) was defined as the reciprocal value of the sample

dilution that showed 50% protection of virus growth. Sera with titers ≥1/10 were considered positive for SARS-CoV-2 antibodies. The analysis is considered valid when there is a difference of less than 1 log2 between the two replicates. For each serum, the mean between the titers of the two replicates is reported. Samples reactive in VNT with a titer of 5 were further classified as positive in case of positive results obtained using the commercial serological assays.

2.2.2 Surrogate virus neutralization assay (sVNT)

sVNT kits were obtained from GenScript, Inc., NJ, USA, and performed following the manufacturer's instructions. This assay is based on the binding inhibition between SARS-CoV-2 receptor binding domain (RBD) and the human angiotensin-converting enzyme 2 (hACE2) by the neutralizing antibodies present in the sera. Briefly, serum samples were diluted 1:10 and mixed with an equal volume of horseradish peroxidase (HRP) +conjugated to SARS-CoV-2 RBD and then incubated for 30 min at 37°C. One-hundred µl were transferred to each well coated with hACE2 receptor and incubated for 15 min at 37°C. Mixture was removed, and plates were washed with wash solution. One-hundred µl of tetramethylbenzidine (TMB) substrate were added to each well and incubated in dark at room temperature for 15 min. Reaction was stopped by adding 50 µl of stop solution to each well. The optical densities (OD) of each sample were read at 450 nm in an ELISA microplate reader (Biosan SIA, Latvia). As reported in the manufacturer's instructions, percentage of inhibition was calculated with the following formula: (1- OD sample value / OD negative control) x 100. Samples with a percentage of inhibition value >20% (low cut-off, as previously established by the manufacturer) and samples with >30% inhibition (cut-off value defined recently by the manufacturer) were considered positive for SARS-CoV-2 antibody. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

2.2.3 Double antigen ELISA

A commercial double antigen multispecies ELISA (ID Screen SARS CoV-2 double antigen multispecies; ID.Vet, France) was used for the detection of specific antibodies against SARS-CoV-2 N antigen, following the manufacturer's instructions. Briefly, 25 µl of dilution buffer and 25 µl of each sample were added to each well and incubated for 45 min at 37°C. Wells were then washed five times with wash solution. One-hundred µl of HRP conjugate N protein recombinant antigen was added to each well and incubated for 30 min at 21°C. Wells were washed five times and 100 µl of substrate solution (TMB) was added, subsequently plates were incubated for 20 min at 21°C in dark. Reaction was stopped by adding 100 µl of stop solution to each well. The OD values of each sample were read at 450 nm in an ELISA microplate reader. Sample to positive ratio (S/P) was calculated with the following formula: (OD sample value-OD negative control) / (OD positive control-OD negative control). Samples with S/P >0.60 were considered positive. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

2.2.4 Indirect ELISA

A commercially available indirect ELISA (ID Screen® SARS-CoV-2-N IgG indirect ELISA; ID, Vet, France) was used to detect specific antibodies against SARS-CoV-2 N antigen with protocol modification for the detection of dog and cat antibodies. Briefly, 10 µl controls and samples were diluted in 200 µl of dilution buffer, 100 µl of diluted samples and controls were added to each well and incubated for 45 min at room temperature. Wells were then washed three times with wash solution. One hundred µl of anti-multispecies IgG HRP-conjugate were added to each well and incubated for 30 min at room temperature. Wells were then washed three times with wash solution. One-hundred µl of substrate solution (TMB) were added to each well and incubated for 20 min kept in a dark place at room temperature. Reaction was stopped by adding 100 µl of stop solution to each well. The OD of each sample were read at 450 nm in an ELISA microplate reader. S/P was calculated with the following formula: (OD sample value-OD negative control) / (OD positive control-OD negative control). Samples with S/P $\geq$ 0.40 were considered positive. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

2.3. FCoV real-time reverse transcriptase PCR (real-time RT-PCR)

RNA extraction from rectal swabs was performed using commercial NucleoSpin viral RNA isolation kit (Macherey-Nagel, Bethlehem, PA) following manufacturer's instructions. RNA quality control targeting vertebrate 12S rRNA locus (Kitano et al., 2007) was performed on randomly selected samples (results not shown). Real-time RT-PCR based on the amplification of the 7b gene of FCoV was performed on extracted RNA, according to a previously described protocol (Gut et al., 1999) with minor modifications. The real time RT-PCR reaction was performed using a commercial kit (TaqMan Fast Virus 1step master mix, Applied Biosystems) in a final volume of 25 µl: 5 µl master mix, 600 nM of primers FCoV1128f (GATTTGATTTGGCAATGCTAgATTT) and FCoV1229r (AACAATCACTAGATCCAGACGTTAGCT), 200 nM of probe FCoV1200p (FAM-TCCATTGTTGGCTCGTCATAGCGG-TAMRA) and 5 µl of template RNA. Reactions were performed using a QS3 instrument (Applied Biosystems). As a positive control, an FCoV-positive cat sample was used, while the negative control consisted of an FCoV-negative sample from a domestic cat. A sample was considered positive in the presence of an amplification curve and a value of threshold cycle (Ct) <40, as previously reported (Felten et al., 2020). For absolute quantitation, a pCR4 plasmid (Invitrogen, Carlsbad, California, USA) containing the FCoV 7b target sequence produced according to previously published protocols (Balboni et al., 2012) and kindly provided by Professor Mara Battilani, was used. Serial $\log_{10}$ dilutions of the recombinant plasmid with a known copy number (10^1–10^7 copies/µl) were amplified with the samples in order to obtain a standard curve.

2.4. Data analysis

For each of the three commercial serological assays, sensitivity and specificity were calculated using VNT as the reference method (Embregts et al., 2021; Perera et al., 2021). Concordance among the assays was calculated using the Cohen's Kappa coefficient. Kappa value <0.00 indicates a poor concordance, 0.00-0.20 a slight concordance, 0.21-0.40 a fair concordance, 0.41–0.60 a moderate concordance, 0.61–0.80 a substantial concordance, and

≥0.81 represents almost perfect concordance (Landis & Koch, 1977). Spearman's correlation coefficient was used to evaluate the correlation between VNT titers and the results obtained using the commercial tests (OD and percentage of inhibition). A Spearman's rho value between 0.81 and 1 indicated a very strong correlation, r= 0.61 to 0.80 strong, 0.41 to 0.60 moderate, 0.21 to 0.40 weak and 0 to 0.20 negligible correlation (Prion & Haerling, 2014). All statistical analyses were performed using Epitools (https://epitools.ausvet.com.au) and Analyse-it v5.66 software (Analyse-it software, Ltd, Leeds, United Kingdom). The significance was set at p value < 0.05.

3. RESULTS

In total, 191 serum samples from dogs (n = 66) and cats (n = 125) were included in this study. Thirty-nine animals belonged to COVID-19 positive owners, 42 belonged to COVID-19 negative owners. Information on owner's disease status was not available for 11 privately-owned animals, whereas the other 99 animals were stray cats. Regarding time of sampling from owners' positivity, samples collection ranged from 16 to 251 days, with a median of 93 days (Table 1).

All serum samples were analyzed using VNT and sVNT assays. Out of these samples, 189 (65 dogs and 124 cats) and 123 (18 dogs and 105 cats) sera were analyzed with Double antigen and Indirect ELISA, respectively. Overall results showed that 17 (8.9%) serum samples were positive to at least one test (Table 1), while all the others serum samples tested negative for all the applied tests. Thirteen (6.8%) samples tested seropositive with the VNT assay, including 11 (16.7%) sera from dogs and 2 (1.6%) sera from cats. Nine VNT-positive animals belonged to COVID-19 owners, whereas information on owners' COVID-19 status was not available for 4 animals. Available information on timing of samples from owner's positivity showed that samples from positive animals belonging to COVID-19 positive owners were collected between 17 and 251 days from owner's diagnosis (Table 1). Results of commercial assays showed that false negative results were observed using Double antigen ELISA and Indirect ELISA. The false negative results obtained in our study using the N-based ELISA tests were observed regardless of the VNT titer, since the absence of antibodies against the N protein was observed in animals showing both high and low neutralization antibody titers (Table 1). The 8 false negative results using Double antigen ELISA were obtained in four samples collected less than two months after owner's COVID-19-positivity, whereas this information was not available for the remaining four animals. The four false negative results obtained using the Indirect ELISA were from 3 samples collected more than 2 months after owners' diagnosis of SARS-CoV-2 infection, whereas for one sample this information was not available. Two false positive results were obtained using sVNT with cut off of >20% and Double antigen ELISA.

Sensitivity and specificity of each of the assays for overall samples and samples collected from dogs and from cats are reported in Table 2. Due to the low number of positive cat samples, sensitivity for the two ELISA assays was not calculated for feline samples.

The overall concordance between each of the assays and VNT is reported in Table 3.

Comparison between VNT titers and results of the different assays is reported in Figure 1. Spearman's rho value showed a very strong correlation (r=0.935) between the VNT titer and

the sVNT percentage of inhibition value, a strong correlation (r=0.753) between the VNT titer and Indirect ELISA S/P value and a weak correlation (r=0.379) between the VNT titer and Double antigen ELISA S/P value, always with a statistical significance level of $p < 0.001$. The best correlation was observed between VNT and sVN with the use of cut-off value of >30%.

Regarding samples from cats, real-time RT-PCR for FCoV detection was performed on a subset of 106 available feline rectal swabs. FCoV RNA was detected in 57 (53.8%) of the tested cats, with Ct values ranging from 15.6 to 39.8, corresponding to 5.6×10^7 and 1.8×10^0 copy numbers/µl, respectively. Two (1.9%) out of the 106 FCoV tested cats showed VNT-positive results. More precisely, among the 57 FCoV-positive cats, one (1.8%) cat was VNT-positive (VNT titer 80). This VNT positive sample, collected 59 days after owner's positivity, was correctly identified by sVNT but resulted negative by the N-antigen based ELISA tests. The Double antigen ELISA identified as seropositive a FCoV-positive stray cat with VNT-negative result. Among the 49 FCoV-negative cats, one (2%) cat showed neutralization antibodies. This VNT positive sample, collected 251 days after owner's positivity, was correctly identified by sVNT but not by the Indirect ELISA test, whereas it was not tested using the Double Antigen test.

4. DISCUSSION

Reliable methods for antibody detection are essential to understand susceptibility and immune-response to SARS-CoV-2 in animals and assays with high sensitivity should be used for epidemiological surveillance (Yamamoto et al., 2022). However, the gold standard VNT execution requires BSL-3 laboratories and trained personnel, making it inaccessible for a wider community of diagnostic and research laboratories. Therefore, in this study we investigated the diagnostic accuracy of widely accessible and easy-to-perform assays. More precisely, we evaluated sensitivity, specificity and correlation with neutralizing antibodies, considered as the gold standard for antibody detection, of three different commercially available immunoassays for the detection of SARS-CoV-2 antibody in pets that can be performed in BSL-2 laboratories.

The VNT cut-off for positive samples was set to antibody titer 10, however considering that low antibody titers have been observed in SARS-CoV-2 infected domestic dogs following experimental infection (Bosco-Lauth et al., 2020) samples reactive in VNT with a titer of 5 were subsequently classified as positive in cases where positive results were obtained using the commercial serological assays. Neutralizing antibodies were identified in animals enrolled in this study at different timing of sample collection from the owner's COVID-19 positivity. Indeed, exposure to COVID-19-positive owners was considered as the likely source of infection in the animals (Patterson et al., 2020). As previously mentioned, neutralizing antibodies in pets can persist for up to 10 months (Decaro et al., 2021). This finding is in accordance with our results that showed VNT positivity in animals after more than 8 months from owner's COVID-19 positive status.

Our results confirmed the best performances of the sVNT when using the higher cut-off value recommended by other authors (Tan et al., 2020; Embregts et al., 2021) and recently also by the manufacturer, compared with the lower cut-off and with the other two ELISA commercial

tests. Indeed, the higher cut-off value (>30%) allowed the correct negative identification of two samples from dogs that showed false positive results when using the lower cut-off value (> 20%). Moreover, even if the sVNT is not meant to be quantitative, the strong correlation between sVNT percentage of inhibition and VNT titers confirms previous results (Perera et al., 2021) and further confirms the high performance of this test.

Regarding the two N-antigen-based ELISA assays, the discrepancies between these ELISA tests and the neutralization assay for both positive and negative results observed in our study is consistent with previous reports that have frequently performed SARS-CoV-2 serological investigations on animal samples based on a screening test using commercial assays and subsequent confirmation of results with neutralization assays (Michelitsch et al., 2020; Barua et al., 2021; Decaro et al., 2021; Jemeršić et al., 2021; Klaus et al., 2021; Stranieri et al., 2021; Udom et al., 2021; Adler et al., 2022). Concerning the low sensitivity values of the two N-antigen-based ELISA assays from our study, it should be reminded that the false negative results detected in samples from pets were based on the confirmation by VNT of all serum samples regardless of their positive ELISA results. Previous reports may have underestimated false negative results of N-antigen-based ELISA because only ELISA positive samples or randomly selected ELISA negative samples were confirmed by VNT (Jemeršić et al., 2021; Udom et al., 2021). False negative results using the N-based-antigen ELISA tests evaluated in this study may be due to the absence or lower presence of antibodies against the viral nucleoprotein compared to the gold standard assay detecting neutralizing antibodies. Indeed, a lower persistence of anti-nucleocapsid compared to anti-spike antibodies has been reported in humans (Van Elslande et al., 2022) and this may explain why ELISA tests based on the spike (S) antigen have shown a higher sensitivity and a better correlation with the presence of neutralizing antibody in humans compared to the N antigen-based ELISA tests (Kontou et al., 2020; Ni et al., 2020; Mohit et al., 2021; Rathe et al., 2021). The different kinetics between the antibody responses raised against the viral nucleoprotein and the one directed against the spike protein has also been suggested as a possible cause of the lower sensitivity of ELISA N-based assays compared to VNT in domestic animals (Decaro et al., 2021). In this respect it is intriguing that two samples with neutralizing antibodies collected from animals after 251 days from owner's COVID-19 positivity were both negative in the Indirect ELISA. Further investigations are needed to define the kinetics between the antibody responses against different SARS-CoV-2 antigens in pets as well as the possible explanations of the lower sensitivity of N-based ELISA assays also considering that other studies have shown similar diagnostic performances between S- and N-based commercially available assays (Folegatti et al., 2020; Ni et al., 2020; Okba et al., 2020) or higher specificity and sensitivity for in-house N antigen-based ELISA in comparison with RBD antigen-based ELISA for the detection of SARS-CoV-2 antibody in pets (Dileepan et al., 2021).

Given that both the ELISA assays investigated in the present study are N-antigen-based, the higher sensitivity and the better correlation of the indirect ELISA with the presence of neutralizing antibodies compared with the double antigen ELISA, could also be ascribed to the different type of N protein used and assay procedure for antibody detection that may have influenced the assay performance (Rikhtegaran Tehrani et al., 2020; Adler et al., 2022).

The conserved structure of the N protein has raised concerns on a possible cross-reactivity with antibodies against other animal coronaviruses when using N-based ELISA (Udom et al., 2021). This aspect was apparently not observed in our study and serological cross-reactivity between SARS-CoV-2 and other animals coronaviruses was likely ruled out by our results, confirming previous reports (Michelitsch et al., 2020; Zhang et al., 2020; Decaro et al., 2021; Dileepan et al., 2021; Embregts et al., 2021; Perera et al., 2021). Indeed, our results did not show different prevalences of SARS-CoV-2 seropositive cats among cats with and without FCoV. Furthermore our results showed very high specificity values for the N-based-antigen immunoassays, especially considering the high prevalence of FCoV positive cats, thus confirming the widespread presence of FCoV in cat population (Addie et al., 2009) and the consideration that canine coronaviruses are known to be widespread in dog populations (Priestnall et al., 2007). However, recent reports have observed a significantly higher number of SARS-CoV-2 seropositive cats in FCoV-infected groups (Adler et al., 2022). For the only sample showing N antigen-based ELISA positivity in the absence of neutralizing activity, we cannot definitively rule out that cross-reactivity may have caused the false positive result, also considering that the cat was shedding FCoV RNA in the feces, but recognition of non-neutralizing epitopes or different antibody kinetics could also explain this result, as previously reported (Michelitsch et al., 2020; Zhang et al., 2020; Decaro et al., 2021; Udom et al., 2021). Therefore, further studies are needed to definitively rule out cross-reactivity with antibodies against endemic carnivore coronaviruses when using N-based ELISA.

This study has some limitations, which should be considered. First, the low number of domestic animals with neutralizing antibodies, due to the sporadic frequency of infection among pets, may have impacted the accuracy of the diagnostic tests (Leeflang et al., 2013), especially for cats in this study. Second, the results of our study are related to SARS-CoV-2 variants that circulated from 2020 to 2021 and diagnostic accuracy is unknown for SARS-CoV-2 variants that have circulated after 2021. Therefore, further studies, with a higher number of SARS-CoV-2 positive pet samples and with samples collected during 2022 are needed to confirm tests accuracy. Finally, the antibody kinetics in pets was not evaluated and further studies are needed to investigate the development of antibody responses against different SARS-CoV-2 antigens in cat and dog.

In summary, several studies have performed SARS-CoV-2 serological investigations on animal samples based on a screening test using commercial assays and confirmation of results with neutralization assays. However, assays with high sensitivity should be used for epidemiological surveillance and therefore the diagnostic perfomances of commercial test for SARS-CoV-2 should be taken into account for surveillance in pets as some methods can uncorrectly identified the presence of SARS-CoV-2 specific antibody. Overall, our results confirm that assay validation is a fundamental step for serologic studies in cats and dogs and suggest that the sVNT used with a cut-off value of 30% may be an effective method that does not require a BSL-3 laboratory for predicting serum neutralization antibodies in dogs and cats.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this study.

AUTHOR CONTRIBUTION

All authors reviewed, revised and approved the final manuscript and have contributed significantly to the work.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered. Serum samples were collected according to the diagnostic procedures and according to the Ethical Committee decision of the University of Milan, residual aliquots of samples or tissues collected for diagnostic purposes at the VTH under informed consent of the owners can be used for research purposes without any

additional formal request of authorization (EC decision 29 Oct 2012, renewed with the protocol no. 02-2016). The study on serum samples and rectal swabs was approved by the Institutional Animal Care and Use Committee and by the Institutional Ethical Committee (approval n. 31/20 and n. 43/20, respectively).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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652 **TABLE 1**. Serum samples positive for at least one assay: characteristics of samples and
653 comparison of results of three commercially available tests and gold standard VNT.

Species	No. of days after owner tested positive for COVID-19 that animal was sampled	VNT titers	sVNT (Percentage of inhibition)	Double antigen ELISA (S/P)	Indirect ELISA (S/P)
Dog	Positive (251 days)	5	Positive† (71%)	ND‡	Negative
Dog	ND	Negative	Positive (23%)	Negative	ND
Dog	Positive (78 days)	20	Positive (42%)	Negative	Positive (0.77)
Dog	Positive (16 days)	Negative	Positive (24%)	ND	Negative
Dog	Positive (47 days)	20	Positive (71%)	Negative	Positive (0.75)
Dog	Positive (17 days)	40	Positive (66%)	Negative	Positive (0.74)
Dog	Positive (ND)	10	Positive (71%)	Negative	ND
Dog	Positive (ND)	20	Positive (73%)	Positive (0.83)	ND

Dog	Positive (ND)	20	Positive (82%)	Positive (3.2)	ND
Dog	Positive (ND)	Negative	Negative	Positive (0.78)	ND
Dog	ND	5	Positive (75%)	Negative	Positive (1.22)
Dog	ND	40	Positive (67%)	Negative	Positive (1.30)
Dog	ND	20	Positive (74%)	Negative	Negative
Dog	ND	20	Positive (68%)	Positive (0.62)	Positive (1.17)
Cat	251	160	Positive (94%)	ND	Negative
Cat	ND (stray)	Negative	Negative	Positive (1.37)	ND
Cat	59	80	Positive (89%)	Negative	Negative

† Positive sample using the low cut-off of percentage of inhibition value >20% (as previously established by the manufacturer); ‡ ND = not determined

658 **TABLE 2**. Tests performance results.

	Ab Positive samples/total	Sensitivity % (95% C.I.)	Specificity % (95% C.I.)
Overall			
sVNT (cut-off >20%)	15/191	100 (75-100)	99 (96-100)
sVNT (cut-off >30%)	13/191	100 (78-100)	100 (98-100)
Double antigen ELISA	5/189	23 (5-54)	99 (96-100)
Indirect ELISA	6/123	60 (26-89)	100 (97-100)
Dog			
sVNT (cut-off >20%)	13/66	100 (71-100)	96 (87-100)
sVNT (cut-off >30%)	11/66	100 (75-100)	100 (93-100)
Double antigen ELISA	4/65	30 (6-65)	98 (90-100)
Indirect ELISA	6/18	75 (35-97)	100 (69-100)
Cat			
sVNT (cut-off >20%)	2/125	100 (15-100)	100 (97-100)
sVNT (cut-off >30%)	2/125	100 (15-100)	100 (97-100)
Double antigen ELISA	1/124	NC†	99 (95-100)
Indirect ELISA	0/105	NC	100 (96-100)

659 †NC= not calculated
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662 **TABLE 3**. Overall proportion of concordance between immunoassays. A color gradient
663 illustrates the Cohen Kappa measure (orange=fair; blue=substantial; green= perfect)

	VNT % (Kappa)	sVNT cut-off >20% % (Kappa)	sVNT cut-off >30% % (Kappa)	Double antigen ELISA % (Kappa)	Indirect ELISA % (Kappa)
VNT % (Kappa)	100% (1)				
sVNT cut-off >20% % (Kappa)	99% (0.92)	100% (1)			
sVNT cut-off >30% % (Kappa)	100% (1)	99% (0.92)	100% (1)		
Double antigen ELISA	94% (0.31)	94% (0.30)	95% (0.35)	100% (1)	
Indirect ELISA	97% (0.73)	96% (0.69)	97% (0.79)	95% (0.24)	100% (1)

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669 **FIGURE LEGENDS**

Figure 1A

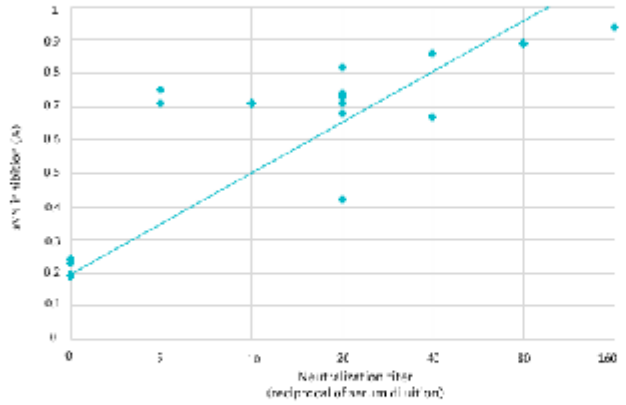

Figure 1B

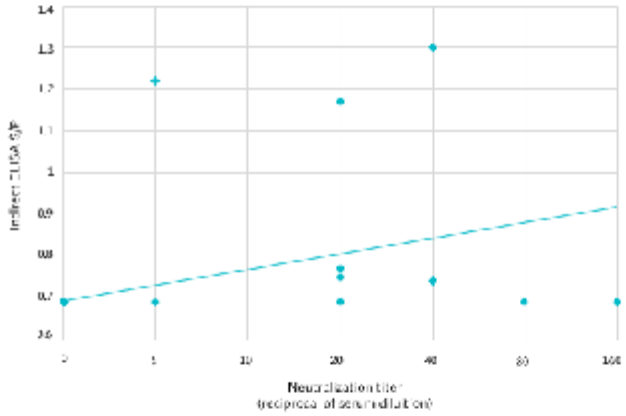

Figure 1C

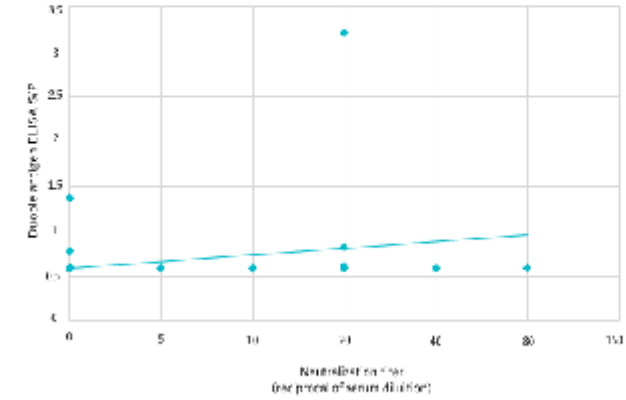

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FIGURE 1 Correlation between percent inhibition in the sVNT and VNT titers (reciprocal of serum dilution) with r= 0.935 (A), correlation between S/P values in the indirect ELISA and the VNT titers with r= 0.753 (B), correlation between S/P values in the double antigen ELISA and VNT titers with r= 0.379 (C). Spearman's test was used for correlation analysis. For clarity, the negative VNT sera that were negative in all three assays were not included in the figure.