Detailed epitope mapping of SARS-CoV-2 nucleoprotein reveals specific immunoresponse in cats and dogs housed with COVID-19 patients

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Detailed epitope mapping of SARS-CoV-2 nucleoprotein reveals specific immunoresponse in cats and dogs housed with COVID-19 patients

Barbara Colitti,[a,*] Francesco Bonfante,[b] Santina Grazioli,[c] Laura Anfossi,[d] Chiara Nogarol,[e] Daniela Scalas,[a] Luigi Bertolotti,[a] Alessio Bortolami,[b] Matteo Pagliari,[b] Giulia Pezzoni,[c] Ana Moreno,[f] Davide Lelli,[f] Maria Silvia Gennero,[g] Alessandro Dondo,[g] Emiliana Brocchi,[c] and Sergio Rosati[a]

Abstract

Since the initial emergence in December 2019, the novel Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has been reported in over 200 countries, representing an unprecedented challenge related to disease control worldwide. In this context, cases of human to animal transmission have been reported, raising concern about the potential role of companion animals in the pandemic and stressing the need for reliable animal testing. In the study, a detailed epitope mapping of SARS-CoV-2 nucleoprotein, using both human and pet sera, allowed the identification of the most antigenic region in the C-terminus domain of the protein, which was used to develop an experimental double antigen-based ELISA. A panel of pre-pandemic sera and sera of animals immunized against (or naturally infected with) related coronaviruses was used to assess assay specificity at 99.5%. Positive sera belonging to animals housed with COVID-19 patients were confirmed with the experimental double-antigen ELISA using Plaque Reduction Neutralization test (PRNT) test as gold standard. The availability of a serological assay that targets a highly specific viral antigen represents a valuable tool for multispecies monitoring of Coronavirus Disease 2019 (COVID-19) infection in susceptible animals.

Keywords: SARS-CoV-2, COVID19, Epitope mapping, Double antigen ELISA, SARS-CoV-2 nucleoprotein

1. Introduction

The new coronavirus disease 2019 (COVID-19), firstly reported in Wuhan, China in December 2019, has now spread to over 200 countries causing a global pandemic (WHO, 2021.; Zhou et al., 2020). Since its initial spread, a few cases of human-to-animal transmission were reported and several animal species have been found to be susceptible to SARS-CoV-2 after experimental and natural infections (Abdel-Moneim and Abdelwhab, 2020; Bosco-Lauth et al., 2020; de Morais et al., 2020; Decaro et al., 2021a, Decaro et al., 2021c), raising concern about animal role in the COVID-19 pandemic. These include ferrets, hamsters, minks, wild felids (tiger and lion), cats and dogs. In this context, OIE has defined COVID-19 as an Emerging Disease in animals promoting surveys on the prevalence of infection in animals (OIE, 2020). Moreover, serological studies are important tools for rapid and accurate screening of animal population. The

development of specific serological tests for companion animals should take into account potential cross-reaction, being dog and cat susceptible to their own coronaviruses. In a previous cross-sectional study (Colitti et al., 2021) we developed a novel immunoassay based on paramagnetic beads (xMAP; Luminex Corp., Austin, TX) coated with recombinant SARS-CoV-2 nucleoprotein (Np) and a flow cytometry-based system. The assay was applied to screen different cohorts of cats and dogs samples including pre-pandemic and pandemic sera, suggesting the susceptibility of companion animals housed with SARS-CoV-2 infected human beings under natural exposure. Besides a few limited serological surveys, a large-scale application of widely used immunoenzymatic techniques, such as ELISA, would be an important tool to evaluate potential animal reservoirs of SARS-CoV-2 after the introduction of human mass vaccination strategy. This would require a more accurate evaluation of tests in terms of sensitivity and specificity. The antibody response against the viral Spike (S) and Nucleoprotein (Np), the most immunogenic proteins of SARS-CoV-2, have been largely studied in human population in both acute and post-infection phases (Dan et al., 2021; Fenwick et al., 2021; Hartley et al., 2020). The results suggested the use of S and N proteins as equally sensitive in particular in the early phase of infection and the use of neutralization assays, such as PRNT, as reference standards for the confirmation of positive results for both N and S based serological assays (Bauer et al., 2021; Fenwick et al., 2021; Liu et al., 2020; Post et al., 2020; Valcourt et al., 2021; Van Elslande et al., 2020).

SARS-CoV-2 nucleoprotein is highly used in immunoassays since it is overexpressed during infection and highly immunogenic in infected patients (Rikhtegaran Tehrani et al., 2020; Zeng et al., 2020) thus representing an ideal antigen to develop a COVID-19 antibody test. In this study a detailed epitope mapping of Nucleoprotein was carried out and an experimental double antigen-based ELISA was developed using HRP-conjugate C-terminal subunit. A subset of sera from cats and dogs housed with COVID-19 patients were also reactive with the novel double antigen ELISA and PRNT, confirming a specific immunoresponse under natural conditions.

2. Material and methods

2.1. Sequence analysis

Nucleotide sequences for N protein of SARS-CoV-2 (GenBank Acc. Num. MN908947) and other coronaviruses, Feline Coronavirus FCoV (GenBank Acc. Num. EU186072), Canine Coronavirus CCoV (GenBank Acc. Num. EF056485), Canine Respiratory Coronavirus CRCoV (GenBank Acc. Num. KT852998), Bovine Coronavirus BCoV (GenBank Acc. Num. MK095170), Human Coronavirus HCoV strain OC43 (GenBank Acc. Num. KF572713), Porcine hemagglutinating encephalomyelitis virus PHEV (GenBank Acc. Num. FJ009234), SARS-CoV-1 (GenBank Acc. Num. AY278488) and Middle East Respiratory Syndrome Coronavirus MERS-CoV (GenBank Acc. Num. NC_019843), were obtained from GenBank for sequence alignments using ClustalW software (Thompson et al., 2003). Protein sequence alignments were done using MAFFT program (ver. 7) (Katoh et al., 2002).

Sequence alignment and predicted antigens profile was visualized using Genious software.

2.2. Human sera

Human serum samples were collected from 23 patients with COVID-19 (13 males and 10.

females) between March 2020 and November 2020. All patients were diagnosed with COVID-19 by RT-PCR test on nasopharyngeal specimens and tested serologically positive to SARS-CoV-2 ELISA (In3diagnostic Eradikit COVID-19). Three pre-pandemic sera were also tested as negative controls.

2.3. Animal sera

A total of 506 animal sera was used for this study.

A set of pandemic sera consisted of 199 serum samples from animals from different regions of Italy that were collected during the period March–December 2020.

Among them 15 animals showed positive results to SARS-CoV-2 serology in a previous study (Colitti et al., 2021) and were then further characterized.

All pandemic sera were tested with the double antigen ELISA and PRNT tests.

A set of pre-pandemic (negative) sera (234 dogs and 35 cats) consisted of sera sampled before January 2019 and was used to assess specificity of the Np double antigen assay.

Specificity was also assessed by using rabbits and guinea pigs immune sera produced against three different Beta coronaviruses (Bovine Beta-CoV (BCov) 9WBL77 strain, Porcine hemagglutinating encephalomyelitis virus (PHEV) ATCC VR-741 and Human CoV OC43 ATCC VR-1558). Briefly, for each virus, six animals (3 rabbits and 3 guinea pigs), were inoculated subcutaneously with partially purified inactivated antigen in complete Freund's adjuvant and boosted via the same route with the same antigen once a intervals of 21–30 days. Their positive serological reactivity with the homologous antigens was demonstrated by virus neutralization test (VNT). Two-fold serial dilutions (starting from 1:4) of the experimental sera were incubated with 100 $TCID_{50}$ of the homologous virus. Serum virus neutralization titer (NT) was defined as the reciprocal value of the sample dilution that showed 100% protection of virus growth. Neutralization titers for each serum are shown in Supplementary table 2.

Finally, three sera from cats previously shown to be positive for Feline Coronavirus, three dog sera positive for Canine Respiratory Coronavirus, five dog sera positive for Canine Enteric Coronavirus and eight bovine sera positive for Bovine Coronavirus were also tested (Supplementary Fig. 4).

A hyperimmune serum was generated in a goat repeatedly immunized with the whole recombinant Nucleoprotein expressed in *E. coli* and served as positive control in all assays (Colitti et al., 2021). A summary of samples included in the study is shown in Supplementary table 1.

2.4. Ethical statement

This study is part of the SIRIT project, which has been approved by the Committee on Bioethics of the University of Torino (31 March 2020), Ethics coordinator committee (AOU City of health and science of Turin; Prot. N ° 0035599 of 07/04/2020) and satellite ethics committees.

Enrolled patients have signed regular Consent to the participation and processing of their personal data in accordance with Regulation (EU) 2016/679 (GDPR). Written consent, as well as a data collection questionnaire, was obtained from all pet owners, at the time of blood collection. The study was carried out in compliance with the national legislation upon authorization by the competent authority (Italian Ministry of Health D.lgs 26/2014 Aut. N. 694/2020-PR). Polyclonal immune sera in rabbits and guinea pigs were obtained in compliance with the national legislation upon authorization by the competent authority (Italian Ministry of Health D.lgs 26/2014 Aut. N. 307/2020-PR).

2.5. Epitope mapping of SARS-CoV-2 Nucleoprotein

The full open reading frame encoding the N protein was RT-PCR amplified from a nasal swab of SARS-CoV-2 infected human donor and cloned into a mammalian expression vector in frame with monomeric streptavidin. Further five N protein subunits, encompassing the hydrophilic domains as detected by Kite and Doolittle analysis (Kyte and Doolittle, 1982) were PCR amplified and cloned into pGEX-2 T prokaryotic expression vector. Additional 4 overlapping subunits were generated spanning from two adjacent hydrophilic picks to cover (if any) overlapping epitope and cloned into the same vector. Schematic representation of each subunit, including hydrophilic profile, location and length is shown in Fig. 1 . PCR primers designed on SARS-CoV-2 N gene (GenBank Acc. Num. MN908947) are shown in Supplementary Table 3.

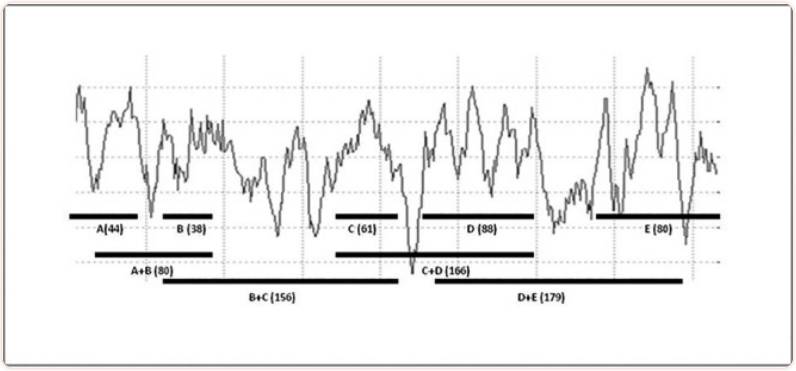

Fig. 1

Hydrophilic profile (upper half) of SARS-CoV-2 Nucleoprotein (Kyte and Doolittle, 1982). Letters (A-E) and numbers identify location and length of each subunit expressed in *E.coli*.

All gene subunits were expressed, upon induction, in chemically competent *E. coli* BL21 (DE3) cells, in frame with GST carrier moiety and purified from cell lysate using ELISA plates coated with bovine beta-casein linked with reduced glutathione via Sulfo-SMPB chemistry.

After a wash step, plates were probed with the aforementioned serum panel.

Briefly, sera were diluted 1/20 in PBS-Tween buffer and incubated (100 µl/well) for 1 h. Plates were washed three times and incubated for 45 min at room temperature with 100 µl of HRP labeled secondary antibody (HRP protein G for human, dog or goat sera; HRP protein A for cat sera). After a wash step, the reaction was developed using 3,3′,5,5′-tetramethylbenzidine as

substrate and stopped after 10 min by adding 100 µl of 1 M sulfuric acid/well. Plates were analyzed in a Biorad Microplate Reader at an optical density (O.D.) of 450 nm. Net absorbance for each serum against each subunit was obtained by subtracting the OD value of GST moiety, as negative control, to the OD value of each fusion protein.

2.6. Double antigen Np-ELISA

Once the immunodominant region was correctly identified, an experimental ELISA was developed for multi-species immunoglobulins detection. The ELISA plates coated with eukaryotically expressed rNp and provided with the Covid19 IgG Eradikit ELISA (In3diagnostic, Italy) were used. Briefly, 40 µl of each serum sample and control sample were diluted in 60 µl of dilution buffer and incubated for 1 h at room temperature. Plates were washed three times and incubated with 100 µl of the second, HRP labeled, detection antigen in conjugate dilution buffer. The latter corresponded to the subunit D + E, expressed in *E. coli* in fusion with GST, affinity purified, coupled with HRP via standard periodate chemistry and purified by ConA sepharose.

Plates were incubated 45′ at room temperature and washed as before. The reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) and stopped with 0.2 M H_2SO_4. Results were expressed as percentage of reactivity versus positive control included in each plate.

2.7. Plaque reduction neutralization test (PRNT)

PRNT assays were performed in a Biosafety Level 3 laboratory using a SARS-CoV-2 isolate as previously described (Padoan et al., 2020). Briefly, two-fold dilutions of heat-inactivated sera (56 °C for 30 min) were mixed with an equal volume (1:1) of a virus solution containing approximately 25 focus-forming units (FFUs) of SARS-CoV-2 and incubated for 1 h at 37 °C. Fifty microliters of the virus-serum mixtures were added to confluent monolayers of Vero E6 cells, in 96-wells plates and incubated for 1 h at 37 °C, in a 5% CO_2 incubator. 100 µl of an overlay solution was then added to each well after inoculum removal. After 26 h of incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution. Visualization of plaques was obtained with an immunocytochemical staining method using an anti-dsRNA monoclonal antibody (J2, 1:10,000; Sci- cons) for 1 h, followed by 1 h incubation with peroxidase-labeled goat anti-mouse antibodies (1:1000; DAKO) and a 7 min incubation with the True Blue (KPL) peroxidase substrate. The neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% (PRNT50). Samples recording titers equal to or above 1:10 were considered as positive.

2.8. Statistical analyses

The best antigen subunit was identified as the one showing an equal or significantly higher reactivity compared to the whole Np recombinant antigen using two-tailed Wilcoxon signed rank exact test embedded in R Core software. The 95% confidence intervals were calculated with binomial test embedded in R Core software.

3. Results

A total of 9 subunits were expressed in prokaryotic system (five corresponding to hydrophilic peaks and four overlapping fragments as shown in Fig. 1 and Supplementary Fig. 1). Since all subunits were generated as soluble GST fusion proteins, they were purified on plate using affinity chromatography. Goat hyperimmune serum, raised against the whole recombinant Np, recognized all subunits and served as positive control in epitope mapping assay. Serum panel from human patients was reactive mainly against the C-terminal half of Np with lower degree of reactivity versus individual fragments D and E compared to higher reactivity versus the overlapping fragment D + E, suggesting that immunodominant region of Np is located between D and E fragment, potentially overlapping with few residues upstream (fragment D) and downstream (fragment E). Spurious reactivity was also observed against other subunits (Fig. 2 A). Statistical analyses confirmed this result, showing a significant higher reactivity of D + E fragment compared to the whole Np antigen (Wilcoxon signed rank exact test $p < 0.001$). All the other fragments showed significant lower reactivities (Wilcoxon signed rank exact test $p < 0.01$).

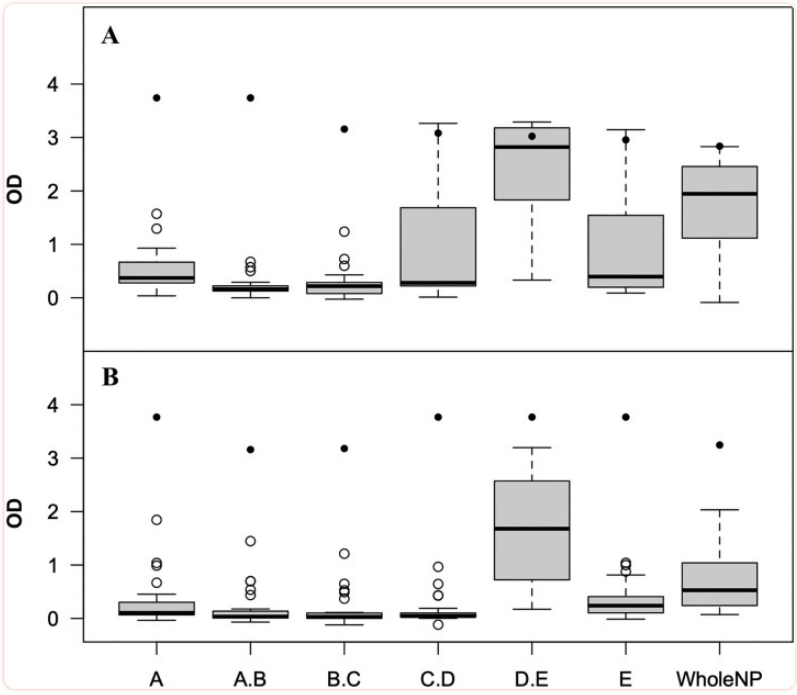

Fig. 2

Box-plots showing reactivity (given as absorbance) of human (A) and animal (B) sera against the NP subunits and the whole Np. Black dot identifies the Positive Control (Hyperimmune serum of goat immunized against the whole Np).

Then, a set of sera from pet animals housed with COVID-19 patients and from a dog hospitalized for interstitial pneumonia, were tested against the same 9 subunits ELISA and found to be highly reactive versus the same overlapping fragment (D + E) recognized by human sera (Fig. 2 B). All sera were also confirmed to be positive versus SARS-CoV-2 by PRNT with titer ranging from 640 to 2560 (Fig. 3 B). We therefore conclude that the fragment D + E represents the immunodominant region of SARS-CoV-2 nucleoprotein in both human and susceptible animal species. To assess the specificity of this immunodominant region, a double antigen ELISA was

developed in which the whole Np was immobilized on solid-phase and the D + E fragment, expressed as GST fusion protein, served as HRP conjugate, enabling detection of all class of immunoglobulin in a multispecies serological assay. As expected, all sera that recognized the fragment D + E in the epitope mapping indirect ELISA, were also reactive against double antigen ELISA. A preliminary test performance evaluation was conducted considering PRNT as the reference gold standard. An assay cutoff of 30% of the positive control reactivity was used.

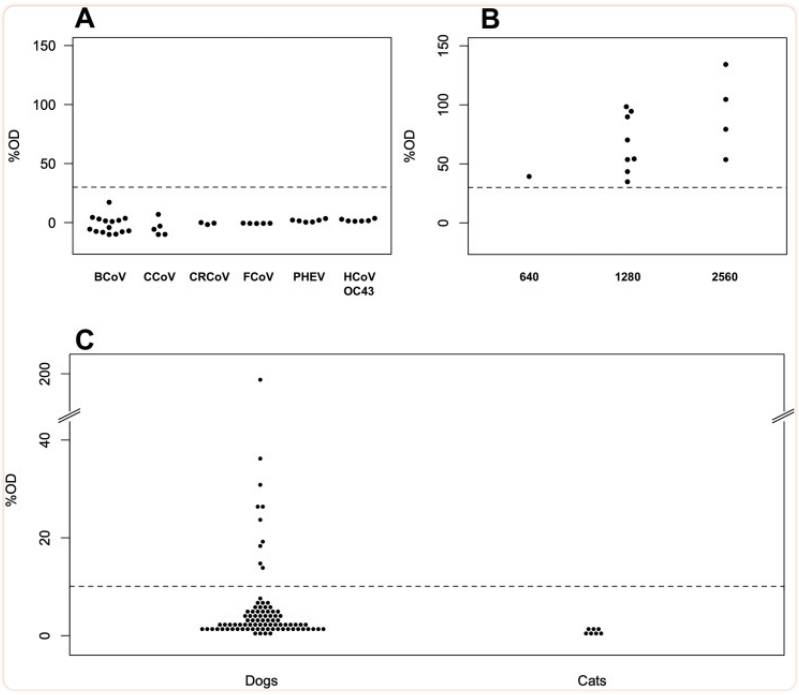

Fig. 3

Distribution of S/P results obtained from rabbit and guinea pig immune sera produced against three different Beta coronaviruses (BCoV, HCoV OC43 and PHEV) and animals naturally infected with three related coronaviruses (FCoV positive cat sera, BCoV positive bovine sera, CRCoV and CCoV positive dog sera) (A); correlation analysis between double antigen Np ELISA and PRNT assays obtained from post-pandemic positive dog and cat sera (B); and distribution of OD results from pre-pandemic sera in the double antigen Np ELISA (C). Horizontal dashed line represents the positive-negative discriminatory cut-off.

Pre-pandemic sera, as well as sera of animals naturally infected or immunized with different alpha (feline and canine sera) or beta (bovine, canine, rabbit and guinea pig sera) coronaviruses were well below the cut-off value (Fig. 3, A and Supplementary Fig. 2). Three dog sera out of 278 pre-pandemic sera, collected before January 2019, were found reactive by double antigen ELISA assessing the specificity of the test at 99.5% (95%CI: 93.1% - 98.1%) Fig. 3, C).

Among them, two sera with medium reactivity in the double antigen assay (Fig. 3, C) were tested against the Nucleoprotein of Canine Enteric and Canine Respiratory Coronaviruses. One serum showed a high reactivity against the CRCoV antigen while the other one was completely negative to both antigens (Supplementary Fig. 3).

Moreover, considering pandemic samples, 11 sera gave discordant results (4 dogs and 7 cats).

In particular, two sera out of 153 PRNT negative sera resulted positive to the experimental double antigen ELISA. On the other hand, two sera with a PRNT titer of 1:1280 resulted negative, while all the sera but one (n = 10) with a PRNT titer below 1:620 resulted negative to the double antigen ELISA (Fig. 4).

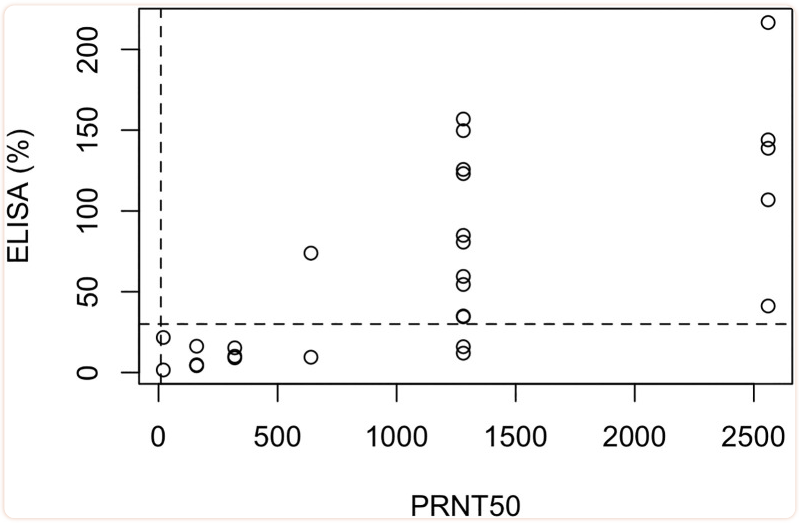

Fig. 4

Scatter plot showing the correlation between PRNT test and double antigen ELISA. Dashed lines represent the positive-negative discriminatory cut-off.

Amino acid sequence comparison between the whole Np, its immunodominant subunit and the corresponding region of related coronaviruses, confirmed that the subunit D + E displayed a lower degree of similarity (ranging from 21 to 26%) with other animal coronaviruses, compared with the whole protein (24–31%), minimizing the risk of false positive cross-reactions (Supplementary Fig. 2).

4. Discussion

In a previous study (Colitti et al., 2021) we demonstrated that a proportion of companion animals housed with COVID-19 patients produced antibodies against SARS-CoV-2. This finding was statistically supported (Colitti et al., 2021) but the initial attempt to develop a specific indirect ELISA test based on whole recombinant Np was unsatisfactory. A slight improvement was obtained in terms of specificity switching from solid-phase reaction to solution-phase kinetics, but the use of the whole Np as antigen remained questionable and required a more detailed characterization of specific immunoresponse against SARS-CoV-2 infection in animals. The choice of Np may raise concern in this context, being dog and cat susceptible to their own coronaviruses. Amino acid similarity is slightly higher between SARS-CoV-2 and the canine betacoronavirus, compared with canine and feline alphacoronaviruses and this may explain the suboptimal specificity obtained in pre-pandemic dog samples in the cited study. It is noteworthy that when potential cross-reactivity of Np protein between SARS-CoV-2 and endemic human coronaviruses was evaluated, no reactivity was shown against 229E, OC43, HKU1 and NL63 by western blot and ELISA (Guo et al., 2020), suggesting that similar results might be ex-

pected from phylogenetically related feline and canine coronaviruses. However antigenic sites predicted by in silico analysis show several potential cross-reacting linear epitopes that may be responsible for false positive reaction. In this context, a more detailed mapping of specific immunoresponse may improve the interpretation of serological tests applied to different animal species. Consistent with previous studies on antigenicity of Np in coronavirus family (Bussmann et al., 2006; He et al., 2004; Ignjatovic and Galli, 1994; Li et al., 2003; Liang et al., 2005; Seah et al., 2000; Stohlman et al., 1994; Wang et al., 2020), our results clearly identify a small region of Np located in the C-terminal part of the protein which is specifically recognized by both human and positive dogs and cats housed with COVID-19 patients. Higher reactivity was found in the D + E fragment. However, since both D and E hydrophilic picks were clearly less reactive than the overlapping fragment, we can identify the immunodominant region in the short fragment between D and E sequence, not excluding few residues overlapping the C-terminal of D or the N′ terminal of E segment respectively or in the epitope created by protein folding bringing in close proximity amino acid residues of D and E fragments. We decided to express the D + E fragment since both hydrophilic picks greatly increase the chance to obtain a soluble GST fusion protein, easier to express and purify by affinity chromatography. By using a double antigen ELISA, based on HRP conjugate D + E, the specificity of the assay raised from 96%, previously detected by the whole Np, (Colitti et al., 2021) to 99,5%.

In silico comparison of amino acid sequence of immunodominant region between SARS-CoV-2 and related coronaviruses support the findings that this short fragment displays the lowest degree of similarity compared to the whole Np, further supporting its use for the development of a SARS-CoV-2 specific and sensitive serological test in susceptible animals. This was further confirmed by the negative results obtained with sera immunized or naturally infected with other coronaviruses and by the positive results in PRNT of the subset of dogs and cats housed with COVID-19 patients and testing positive to the double antigen ELISA. It is noteworthy the finding that a small subset of pet samples resulted positive by PRNT and negative by ELISA. This disagreement in animal sera between PRNT and ELISA results has been already observed in a previous work (Decaro et al., 2021b). This suboptimal sensitivity of the double antigen ELISA obtained using PRNT test as reference gold standard, may be explained by a different kinetic in antibodies raised against different viral proteins (Spike vs Np), suggesting that the use of Np as antigen in SARS-CoV-2 infection in animals may be of major significance in the early phase of infection as reported previously (Decaro et al., 2021b; Diao et al., 2021; Fenwick et al., 2021; Meyer et al., 2014).

Moreover, the small number of sera that gave inconsistent results between the two tests may be also explained considering the double antigen test principle, based on a sandwich between a HRP-rNp conjugation and a rNP immobilized on a solid phase and thus the recognition by the same antibody with both binding sites. The latter test, in fact, is more affected than PRNT by a lower avidity of specific IgG, where a less efficient binding of IgG due to lower functional affinity, for example due to a incomplete avidity maturation (Bauer et al., 2021; Löfström et al., 2021), would translate in a less efficient interaction of both antibody-binding sites, a typical feature of this assay setting.

However, the finding that both human and pet sera recognized the same C-terminal domain of SARS-CoV-2 Np, supports the hypothesis that the humoral immune response may be similar in different species, confirming the importance of epidemiological investigations also in animal population.

In this context, the double antigen based ELISA developed in our study represents several advantages over the typical indirect ELISA, being applied to multispecies analysis and able to detect potentially all classes of immunoglobulins. Moreover the two expression systems used for solid-phase antigen (eukaryotic vector) and HRP conjugate antigen (prokaryotic vector) reduces potential false positive reactions due to host proteins. Finally, the excellent signal to noise ratio enable the use of less diluted serum sample, improving sensitivity of the assay. However, further studies are needed in order to validate the use of this tool in a larger set of well-characterized serum samples belonging to different animal species.

In conclusion, the epitope mapping of SARS-CoV-2 nucleoprotein identified the immunodominant region located in the C-terminal part of the protein. A double antigen based ELISA using this portion as HRP conjugate greatly improves serological test and represents a useful tool for multispecies monitoring of COVID-19 infection in susceptible animals.

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Declaration of Competing Interest

Sergio Rosati, Chiara Nogarol and Luigi Bertolotti declare to be members of In3Diagnostic srl.

The other authors report no declarations of interest.

Footnotes

Appendix A Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2021.12.020.

Appendix A. Supplementary data

Supplementary material

Click here to view.(2.8M, docx) Image 1

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