Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Novel bridge multi-species ELISA for detection of SARS-CoV-2 antibodies

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ABSTRACT

Considering the course of the current SARS-CoV-2 pandemic, it is important to have serological tests for monitoring humoral immune response against SARS-CoV-2 infection and vaccination. Herein we describe a novel bridge enzyme-linked immunosorbent assay (b-ELISA) for SARS-CoV-2 antibodies detection in human and other species, employing recombinant Spike protein as a unique antigen, which is produced at high scale in insect larvae.

Methods: Eighty two human control sera/plasmas and 169 COVID-19 patients' sera/plasmas, confirmed by rRT-PCR, were analyzed by the b-ELISA assay. In addition, a total of 27 animal sera (5 horses, 13 rats, 2 cats and 7 dogs) were employed in order to evaluate the b-ELISA in other animal species.

Results: Out of the 169 patient samples, 129 were positive for IgG anti-SARS-CoV-2 and 40 were negative when they were tested by ELISA COVIDAR® IgG. When a cut-off value of 5.0 SDs was established, 124 out of the 129 COVID-19 positive samples were also positive by our developed b-ELISA (sensitivity: 96.12%). Moreover, the test was able to evaluate the humoral immune response in animal models and also detected as positive a naturally infected cat and two dogs with symptoms, whose owners had suffered the COVID-19 disease.

Conclusion: The obtained results demonstrate that the method developed herein is versatile, as it is able to detect antibodies against SARS-CoV-2 in different animal species without the need to perform and optimize a new assay for each species.

1. Introduction

Since the first cases reported in December 2019 of coronavirus disease 2019 (COVID-19) caused by a novel virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) there have been over 508 million confirmed cases and over 6 million deaths reported worldwide (as of 26th April 2022) (WHO: https://covid19.who.int/) (World Health Organization, 2020; Gorbalenya et al., 2020). The ongoing global pandemic has been underway for more than 2 years causing serious health and economic impacts around the world.

The virus causes a disease spectrum ranging from asymptomatic to severe acute respiratory distress syndrome (ARDS), and death. The signs

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and symptoms of COVID-19 disease are different from patient to patient. Most of them, of which the elderly and immunocompromised are most at risk, complain of flu-like symptoms, including dry cough, fever, fatigue and headache. The most common complications include pneumonia, acute respiratory distress syndrome, septic shock, and cardiovascular manifestations (Xu et al., 2020; Nanshan et al., 2020). There are also asymptomatic patients as demonstrated in several reports (Zhiliang et al., 2020; Yanrong et al., 2020; Mizumoto et al., 2020; Xingfei et al., 2020).

SARS-CoV-2 is a single-stranded positive sense RNA virus encapsulated within a membrane envelope. The most characteristic feature is the spike (S) glycoprotein on its surface that mediates entry into host cells by interacting with angiotensin converting enzyme 2 (ACE2) (Ke et al., 2020). That is why S protein immediately became the main target in the development of antibody tests kits, as well as the focus on therapeutic vaccines (Salvatori et al., 2020; Smith et al., 2021).

Diagnostic tests are needed for monitoring and prognosis of the different stages of the disease. Since the beginning of the pandemic by SARS-CoV-2, various assay kits and tests have been developed for the diagnosis of COVID-19. Currently, commercially available COVID-19 detection kits can be divided into three categories: i) molecular assays of SARS-CoV-2 RNA based on real-time reverse transcription polymerase chain reaction (rRT-PCR) techniques, isothermal amplification and genome sequencing; ii) serological assays that detect antibodies produced by individuals exposed to SARS-CoV-2 based on lateral flow immunoassay (LFIA), chemiluminescence immunoassay (CLIA) or enzyme-linked immunosorbent assay (ELISA); and iii) LFIA-based antigen detection kits (Ruhan et al., 2020). These three categories are complementary. Whereas PCR and antigen-based assays are used to diagnose infection, serological tests are used to assess antibody responsiveness to the virus or vaccination (Krishnan et al., 2020; Pokhrel et al., 2020; Hu et al., 2021; James et al., 2021; Peroni et al., 2021; Wang et al., 2021).

rRT-PCR tests detecting viral RNA, considered the gold standard for the diagnosis of COVID-19, indicate current viral infection, and are an essential part of contact tracing and testing. However, these have some limitations, such as false negative results due to inappropriate sample collection, sample type and sampling technique, together with a short positive time of SARS-CoV-2 RNA in most infected individuals (Azzi et al., 2020; Coupeau et al., 2020; Lee et al., 2020; Lu et al., 2020; Petrillo et al., 2020). Conversely, serological assays are gaining importance because they can be utilized for a long duration of time after infection. Antibodies can typically be detected within 2–3 weeks of infection and provide an indirect measurement of immune responsiveness (Krishnan et al., 2020; Sethuraman et al., 2020; Xu et al., 2020).

Antibodies tests require some knowledge of those proteins to which the immune system responds, triggering the production of antibodies that flag or neutralize the virus. In the case of SARS-CoV-2, the N protein and the receptor-binding domain (RBD) of the S1 subunit of the S glycoprotein are the most commonly used antigens. These methods typically detect IgM, IgG or total antibody, with IgG being the most common immunoglobulin measured in SARS-CoV-2-serological assays (Espejo et al., 2020).

The repertoire of epitopes offered by the ectodomain (amino acids 15 to ~1210) of the Spike protein (1 to 1273) is much higher than that obtained only with RBD (amino acids 319 to ~540). It is also known that there is a proportion of neutralizing antibodies that are directed against areas of Spike protein outside the RBD, such as the N-terminal domain (Alenquer et al., 2021; Graham et al., 2021; Weisblum et al., 2020).

Furthermore, considering the continued need for the development of new and effective treatments against COVID-19 infection, it is important to have serological tests available for the detection of SARS-CoV-2-specific antibodies in non-human species.

This would be particularly useful in the following cases:

- Evaluation of the humoral immune response in those animal models in which new vaccines are being studied.
- Monitoring of the production process of hyperimmune equine sera used for the treatment of COVID-19.
- Identification of the natural reservoirs and intermediate hosts of the SARS-CoV-2 virus, which seek to prevent the establishment of new zoonotic reservoirs.

In this sense, the aim of the present work was to develop a novel bridge enzyme-linked immunosorbent assay (b-ELISA) for SARS-CoV-2 antibodies detection in human and other species, employing recombinant Spike protein (S protein) as a unique antigen, which is produced at high scale in insect larvae.

2. Materials and methods

2.1. Human sera collection

Serum/plasma samples were obtained from individuals who fasted for approximately 8 h. Collected samples were stored at −20 °C until tested. The study comprised the following cohorts of participants:

2.1.1. Healthy control individuals

Control serum/plasma ($n = 82$) was obtained from samples collected from healthy individuals before the outbreak of SARS-CoV-2. The sample collection was approved by the Ethics Committee of José de San Martín Clinical Hospital, University of Buenos Aires (UBA), Buenos Aires, Argentina. All subjects were informed about the purpose of the study, and a signed consent for study participation was obtained.

2.1.2. COVID-19 patients

Serum/plasma samples were collected from a total of 169 COVID-19 cases confirmed to be infected with SARS-CoV-2 by real-time RT-PCR (rRT-PCR) on samples from the respiratory tract. These samples were provided by the Biobank of Infectious Diseases (BBEI) of the Institute for Biomedical Research on Retroviruses and AIDS (INBIRS). Out of the 169 samples, 129 and 40 samples were positive and negative, respectively, for SARS-CoV-2 IgG, as determined using the SARS-CoV-2 COVIDAR® IgG ELISA assay (Laboratorio Lemos SRL, Argentina). Sample collection and protocols were approved by the Ethics Committee of BBEI-INBIRS and the Ethics Committee in Clinical Research of the School of Pharmacy and Biochemistry, UBA. All subjects were informed about the purpose of the study, and they signed consent for study participation.

2.1.3. Horse polyclonal plasma against S protein or RBD

Equine polyclonal anti-S antibodies were obtained through immunization of one mixed-breed 4 to 10 years-old, 300 to 450 kg horse, as follows. The horse was initially primed subcutaneously with 100 μg S protein in a 30% (*v*/v) complete Freund adjuvant (CFA) (F5881, Sigma-Aldrich, St. Louis, MO, USA) emulsion in saline, boosted two weeks later with 100 μg recombinant RBD (rRBD) in 30% (v/v) incomplete Freund adjuvant (IFA) (F5506-Sigma-Aldrich) emulsion in saline, then boosted 2 times at weekly intervals with 100 μg S protein in a 20% (v/v) dilution in saline of a stock $Al(OH)_3$ suspension and finally two weekly spaced booster doses, first with 400 μg S protein and the second with 200 μg S protein in a 20% (v/v) dilution of a stock $Al(OH)_3$ suspension in saline. One week after the final booster dose horse's blood was extracted in two sequential days, its plasma obtained by citrate addition, separated and stored refrigerated until use.

On the other hand, four hyperimmune equine plasma samples from horses hyperimmunized against rRBD protein during an anti-SARS-CoV-2 immunotherapeutics production were kindly gifted by INPB Institute.

2.1.4. Rat polyclonal sera against S protein

Serum from 13 rats (pre-immune and immunized with S protein) were selected from the samples collected from the Service provided by

the Facultad de Farmacia y Bioquímica, UBA, for the determination of antibodies against SARS-CoV-2.

2.1.5. Samples of pets that cohabited with COVID-19 patients

Serum sample from 2 cats (one of them RT-PCR+ for SARS-CoV-2) and 2 dogs with symptoms were kindly provided by Nadia Fuentealba from Laboratorio de Virología, Facultad de Ciencias Veterinarias (FCV), Universidad Nacional de La Plata (UNLP), La Plata, Buenos Aires, Argentina. All the animals were in close contact with their COVID-19-positive owners. Each owner gave their written consent to allow the collection of samples from their pets, and all the protocols were approved by the Animal Care and Use Institutional Committee (CICUAL) from the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina, under the protocol code 105–4- 20 P. Lastly, sera from 5 dogs collected before the COVID-19 pandemic were tested for control purposes.

2.2. Expression of S protein in insect larvae and purification by cromatography

S protein was expressed as a fusion protein with a histidine tag in insect larvae as previously described by our group (Smith et al., 2021). Briefly, batches of 500 fifth-instar *Rachiplusia nu* larvae (AgIdea, Pergamino, Argentina) were injected with 50 μL of the recombinant baculovirus stock (diluted to 1×10^7 PFU/ml) which was obtained using the Bac to Bac® baculovirus expression system (ThermoFisher Scientific, USA) in our laboratory. Larvae were selected by fluorescence under UV light, harvested at day 4 post-infection and frozen immediately at −80 °C until they were processed for analysis. The recombinant S protein was obtained with high purity in one step using Immobilized Metal-Ion Affinity Chromatography (IMAC) as reported previously by Smith et al. (2021).

2.3. Biotinylation of S protein

The purified S protein was subjected to buffer exchange to phosphate buffered saline (PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4) using a PD-10 desalting column (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions. The desalted protein (250 μg) was then incubated for 2 h at 0 °C with a 800-fold molar excess of sulfo-NHS-biotin (Pierce Biotechnology, Rockford, IL, USA). Free biotin was removed on a new PD-10 desalting column.

Biotinylated S protein was stored at −80 °C with the addition of glycerol to a final concentration of 10% and 100 mM of L-Arginine.

2.4. SARS-CoV-2 antibodies detection by bridge enzyme-linked immunosorbent assay (b-ELISA)

2.4.1. Reagents

The coating buffer was PBS, the blocking buffer was 3% skim milk in PBS, the washing buffer was PBS containing 0.05% Tween 20 (PBS-T). Reagent dilutions were prepared in 3% skim milk, in PBS-T (PBS-MT). Streptavidin-Horseradish Peroxidase was purchased from Jackson ImmunoResearch Laboratories, Inc. The 3,3′,5,5′-tetramethyl-benzidine/ H_2O_2; Single Component TMB Peroxidase EIA Substrate Kit, (BioRad, Hercules, CA, USA) was employed as the chromogenic substrate. Except when otherwise indicated, incubations were performed at RT, washing steps were performed with PBS-T and 50 μl per well were added in each incubation step.

2.4.2. b-ELISA protocol

The protocol employed was based on that previously described with minor modifications (Villalba et al., 2007). Briefly, polystyrene microplates (Maxisorp, NUNC, Roskilde, Denmark) were coated over night at 4 °C with 2 μg/ml of purified S protein per well, washed three times with PBS, blocked for 1 h with 200 μl of blocking buffer, and washed six times with PBS-T. Serum/plasma samples were added in duplicate to the coated microplates and incubated for 20 min. Plates were then washed with PBS-T six times and 50 ng of S protein-biotin per well were added. After another 20 min of incubation, plates were washed with PBS-T six times and bound S protein-biotin was detected by the addition of Streptavidin-Horseradish Peroxidase diluted 1/300. After 20 min of incubation at 37 °C, microplates were washed with PBS-T five times plus one final washing step with 200 μl of PBS; the chromogenic substrate was added, and plates were incubated for 15 min in the dark. The colour reaction was stopped with 4 N H_2SO_4. The oxidized substrate was measured at 450 nm with an ELISA plate reader MultiskanFC (Thermo Scientific Labsystems, USA). The schematic description of this protocol is shown in Fig. 1. The blank control was made by replacing serum/plasma samples with PBS-MT. The positive control of the assay was the hyperimmune equine serum anti-S protein. Results were calculated as specific absorbance (A = the mean of each sample minus the mean of the blank control) and expressed as Standard Deviation score (SDs).

SDs = (A-Ac)/SDc, in which Ac is the mean specific absorbance from pre-pandemic control samples (approximately 20 normal control sera in each assay), and SDc is the corresponding standard deviation between measurements for those control samples. The cut-off value of the assay was set at SDs = 5.0.

2.5. Statistical analysis

The selection of optimal cut-off values was based on curves constructed by plotting the calculated specificity and sensitivity vs. the corresponding cut-off values. The performance of the b-ELISA was analyzed by determining the area under the curve (AUC) of receiver operating characteristic (ROC) curves. Statistical significance was assessed by parametric tests: Student's *t*-test for unpaired samples with Welch correction; or non-parametric tests: Mann-Whitney *U* test for unpaired data, when applicable. Spearman coefficient of correlation (r_s) was calculated to evaluate inter-assay correlation. The degree of agreement between COVIDAR® IgG ELISA and the developed b-ELISA was evaluated by calculating the kappa statistic. A kappa value of 0.01–0.20 was indicative of slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, almost perfect or perfect agreement (Cohen, 1968; Koch et al., 1977; Landis and Koch, 1977a, 1977b).

All calculations were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). A p value <0.05 was considered statistically significant.

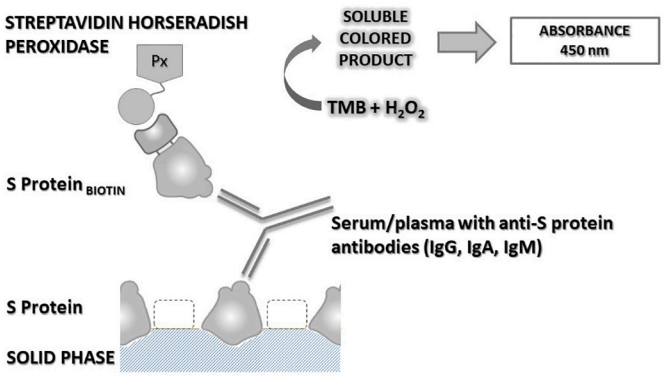

Fig. 1. Schematic representation of b-ELISA protocol for detection of anti-S protein antibodies.

3. Results

3.1. Expression of recombinant S protein in insect larvae, purification and biotinylation

As previously described, recombinant S protein was efficiently expressed in *Rachiplusia nu* insect larvae after 4 days of infection. The larvae were harvested under a UV lamp using fluorescence as an indicator of infection. The clarified homogenate from the larvae was obtained and the purification was done by IMAC. The yield of recombinant trimeric S protein was 15 μg/g of larvae with high purity. The gp64 signal peptide was effective to target S protein to the secretory pathway and its localization to hemolymph. This strategy facilitates its recovery from the larvae extract as a high-quality trimeric version as previously described by Smith et al. (2021). The one-step purification allowed to obtain recombinant trimeric S protein with a sufficient degree of purity for its subsequent labelling with biotin and to use it as an antigen in the immunoassay coating.

The recovery of recombinant S protein was analyzed by reducing SDS-PAGE showing one band with the expected molecular weight (≈ 150 kDa) for the full-length engineered protein (monomer) in the fractions obtained before and after purification. This demonstrated that it was correctly glycosylated and did not suffer protease degradation. The identity of S protein was confirmed by WB analysis using a specific anti-Histidine monoclonal antibody (Smith et al., 2021).

3.2. SARS-CoV-2 antibodies detection by bridge enzyme-linked immunosorbent assay (b-ELISA)

The recombinant trimeric S protein expressed in insect larvae *R. nu* was used for the development of a b-ELISA aimed to detect anti-S specific antibodies of different isotypes and from different species, not only in human samples. For this purpose, we employed 169 rRT-PCR positive human samples also tested by COVIDAR® IgG (ELISA for SARS-CoV-2 IgG antibodies, which was approved for the commercialization in Argentina by the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica -ANMAT-). This assay is a heterogeneous, non-competitive immunoenzymatic assay, based on the indirect method for the detection of specific IgG antibodies against the SARS-CoV-2 virus present in human serum or plasma. In the b-ELISA design described herein the S protein was used as the immobilized antigen coating the microplate wells and was also biotinylated in order to reveal the specific interaction of anti-S antibodies in the reaction (Fig. 1). This design gives the assay high specificity and versatility as it can be used for the study of antibodies present in different animal species, not just human samples.

Eighty two human control sera/plasmas and 169 COVID-19 patients' sera/plasmas, cases confirmed by rRT-PCR were analyzed by the b-ELISA assay described herein. Samples collected from 169 patients comprised 129 and 40 samples that tested positive and negative, respectively, for SARS-CoV-2 IgG as determined by ELISA COVIDAR® IgG. To calculate the coefficient variation, a positive serum from a COVID-19 patient was employed. The intra-assay coefficient variation was 5.57% ($n = 3$) and the inter-assay coefficient variation was 10.6% (n = 3). The test performance was optimized in terms of sensitivity and specificity by evaluating the effect of different cut-off values (in SDs) on receiver operating characteristic curves (ROC) (Fig. 2A). As shown in Fig. 2B, the area under the ROC curve (AUC) was 0.9905, indicating that the method had high accuracy to distinguish between samples from the two groups under study (Carter et al., 2016).

When a cut-off value of 5.0 SDs was established, 124 out of 129 COVID-19 samples that tested positive by COVIDAR® IgG ELISA Test, were also positive by our developed ELISA (sensitivity: 96.12%) (Fig. 3).

On the other hand, there were 2 samples that tested positive for SARS-CoV-2 antibodies by b-ELISA, but negative for COVID-19 IgG by the indirect ELISA method. The concordance between the two immunoassays was 96%, with a kappa statistic of 0.888, demonstrating high agreement between the two methods. The specificity, calculated as 100% minus the percentage of true negative samples (normal human sera, $n = 82$) detected as positive, was 100.0%. The median SDs range of true negative samples was −0.17 (range: −2.07 to 3.45) and the median SDs range of COVID-19 patients´ samples was 39.25 (range − 0.31 a 121.6) for COVIDAR® IgG positive and − 0.03 (range − 1.63 a 33.06) for COVIDAR® IgG negative.

3.3. Comparison of SARS-CoV-2 antibodies detected by indirect ELISA and b-ELISA using recombinant S protein produced in insect-larvae

Ninety-five control sera/plasmas and 99 patient's sera/plasmas were tested in parallel by b-ELISA and the Indirect ELISA previously published (Smith et al., 2021). Even though the methods demonstrated a correlation coefficient of 0.52 and a regression slope of 4.22 ± 0.29 (95% confidence interval), qualitative result concordance between the two

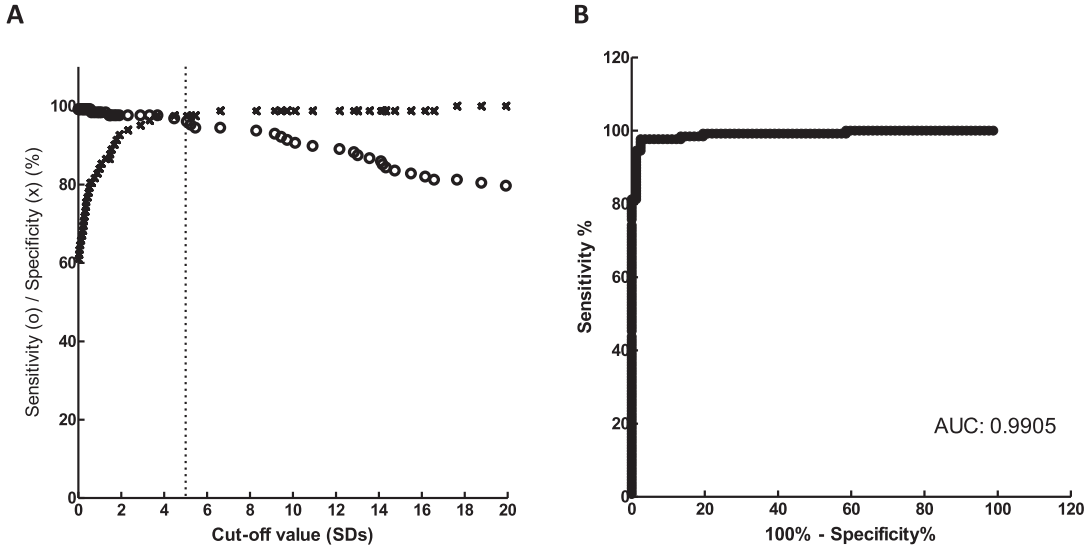

Fig. 2. Analysis of the performance of b-ELISA resulting from the study of 82 sera/plasmas from normal control individuals and 129 sera from patients with COVID-19 IgG+ by COVIDAR® IgG ELISA test. (A) Sensitivity curve (o) and specificity (x) as function of the possible cut-off values. The vertical dashed line indicates the cut-off value with the optimized sensitivity and specificity parameters (cut-off = 5.0). (B) ROC curve analysis of b-ELISA, AUC is included.

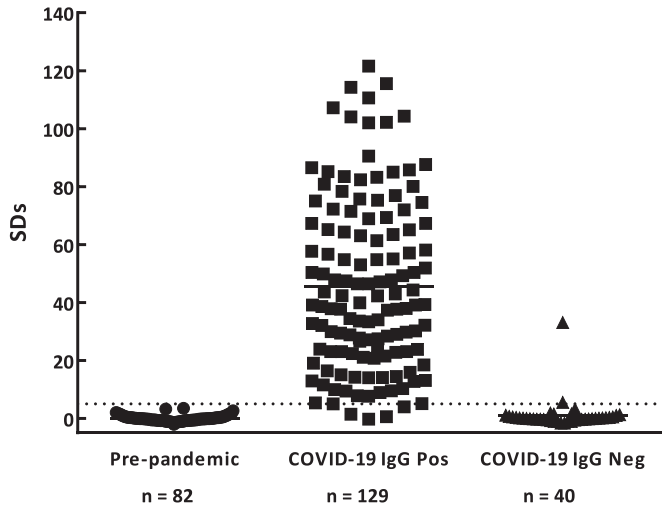

Fig. 3. SARS-CoV-2 antibody test results obtained by b-ELISA from pre-pandemic samples and samples obtained from seropositive (COVID-19 IgG Pos) and seronegative (COVID-19 IgG Neg) individuals as determined by indirect ELISA (COVIDAR® IgG). Results are expressed as Standard Deviation score (SDs). The cut-off value (SDs > 5.0) is indicated by a dotted line and median SDs for each group is indicated by a solid line (*$p < 0.0001$).

assays was 96%, with Cohen's kappa statistic of 0.94 (*i.e.* high qualitative agreement). Although there are no significant differences in terms of sensitivity and specificity obtained with both ELISA designs, the dynamic range is much wider for b-ELISA than for Indirect-ELISA (Fig. 4 and Table 1). Furthermore, another advantage of b-ELISA is that it requires less operational assay time (2–3 h *vs.* 5 h for Indirect ELISA).

3.4. b-ELISA application in animal models

A total of 27 animal sera (5 horses, 13 rats, 2 cats and 7 dogs) were employed in order to evaluate the b-ELISA in other animal species different from human (Fig. 5). The humoral immune response was studied in horses ($n = 4$) immunized with S protein or RBD to obtain hyperimmune sera for the treatment of COVID-19 patients. On the other hand, the b-ELISA was applied in the follow-up of the immune response in rats ($n = 13$) immunized with S protein. Likewise, the test was able to

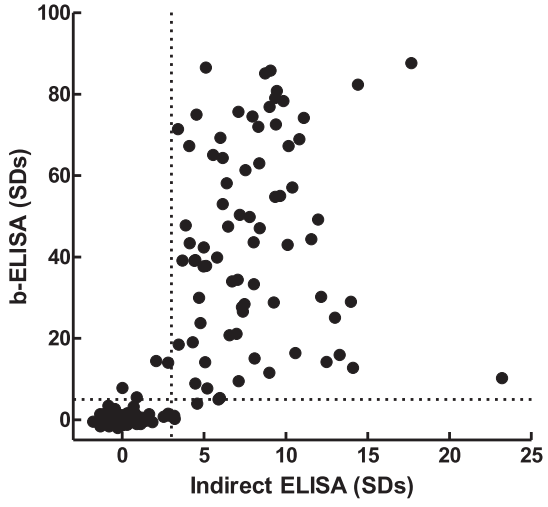

Fig. 4. Correlation between b-ELISA and Indirect ELISA results. The regression slope was 4.22 ± 0.29 and the correlation coefficient (r^2) was 0.52. Dotted lines indicate the cut-off value for each assay (b-ELISA SDs > 5.0; indirect ELISA SDs > 3.0).

detect as positive a naturally infected cat and two dogs with symptoms, whose owners had suffered the COVID-19 disease.

4. Discussion

There is an important hurry to limit economic damage, to get people back to work, and to reopen borders, and those individuals whose immunity can be demonstrated should be able to return to work, with lower risk of severe illness. In this sense, several ELISA procedures for anti-SARS-CoV-2 antibodies detection have previously been reported (Deeks et al., 2020; Xiang et al., 2020; Machado et al., 2021; Meng et al., 2021; Safiabadi Tali et al., 2021). Most of these are based on surface-bound antigen and detection of total bound IgG with labelled xenogeneic anti-immunoglobulin antibody. Frequently, the use of such labelled anti-immunoglobulin antibody decreases the signal-to-background ratio. It was proposed that one way to reduce the background signal is to use the labelled specific antigen to detect bound antibody.

There is a lot hanging on the uniqueness of S protein from insect larvae. In terms of the specificity of serological tests in which it is used, the more unique it is, the lower the odds of cross reactivity with the immune response to other coronaviruses. As previously demonstrated by our group (Smith et al., 2021), the recombinant version of the S protein expressed in insect larvae *R. nu* was immunochemically suitable to be used as an immobilized antigen in an indirect ELISA design for the detection of anti-S specific IgG antibodies in human serum/plasma samples.

There are several different ELISA formats for the detection of SARS-CoV-2 antibodies, the most commonly used methods are indirect ELISA and double-antigen sandwich ELISA, also known as bridge-ELISA (Younes et al., 2020; Mohit et al., 2021; Safiabadi Tali et al., 2021). An important limitation in the performance of these assays is the antigen used to coat plates. For instance, when using SARS-CoV-2 nucleocapsid protein there is a high risk of interferences due to cross-reaction with other coronaviruses such as MERS-CoV and SARS-CoV-1 and with other types that are known to cause the common cold (HKU1, 229E, OC43, NL63) (Sun and Meng, 2004). Those methods may produce false positive results since nucleocapsid protein is the most conserved viral protein among human betacoronaviruses. Furthermore, when using RBD instead of the entire S protein, the repertoire of immunogenic epitopes used for antibody capture is more limited, which may confer sub-optimal test sensitivity. Our b-ELISA uses the whole S protein as antigen for the detection of antibodies against SARS-CoV-2 giving the assay high specificity and sensitivity. There are commercially available bridge ELISA assays - eg. Platelia SARS-CoV-2 Total Ab, BIORAD®; and WANTAI SARS-CoV-2 Ab ELISA, WANTAI®- that differ from the assay described herein mainly in the antigen used (Brochot et al., 2020). The former uses recombinant nucleocapsid protein of SARS-CoV-2 and the latter uses the receptor-binding domain of SARS-CoV-2 S protein for coating the plates. Both assays use antigen-conjugated with horseradish peroxidase as the second step for antibody detection. For our b-ELISA procedure, Spike protein conjugated to biotin may increase test sensitivity.

The b-ELISA protocol described in this work, based on anti-SARS-CoV-2 antibodies crosslinking of immobilized S protein and liquid-phase S protein–biotin, using the trimeric S protein expressed in insect larvae as the unique antigen, was highly specific (100%) and highly sensitive (96.12%), to detect anti-SARS-CoV-2 antibodies in human samples, when comparing it with the commercially accepted COVIDAR® IgG ELISA. As it was shown in Fig. 3, there is a discrepancy between the amount of COVID-19 positive patients detected by both assays. This can be justified because the latter method uses a mixture of S protein and RBD as coating antigens and it only detects IgG antibodies against SARS-CoV-2. On the contrary, the b-ELISA design described in this work uses, S protein expressed in an eukaryotic system – insect larvae - which is a low-cost alternative to cell culture based production and that also conserves its correct glycosylation pattern without

Table 1
Analytical parameters of indirect ELISA and b-ELISA from normal human controls and COVID-19 patients.

	Indirect ELISA			b-ELISA		
	COVID-19 patients RT-PCR +		Normal Human Sera	COVID-19 patients RT-PCR +		Normal Human Sera
	COVIDAR® IgG+	COVIDAR® IgG-		COVIDAR® IgG+	COVIDAR® IgG-	
n	79	19	83	129	40	82
Mean (SDs)	7.83	−0.01	−0.09	45.69	1.026	−0.03
Median (SDs)	7.32	−0.39	−0.22	39.25	−0.031	−0.17
Range (SDs)	0.00 to 23.20	−1.33 to 3.17	−1.78 to 3.21	−0.31 to 121.6	−1.63 to 33.06	−2.07 to 3.45
Cut-off (SDs)	3.0			5.0		
Analytical Sensitivity*(%)	96.2			96.1		
Specificity† (%)	98.8			100		

* Percentage of patients COVIDAR® IgG positive that were also positive by indirect ELISA (Smith et al., 2021) or b-ELISA.
† 100 minus the percentage of false positives.

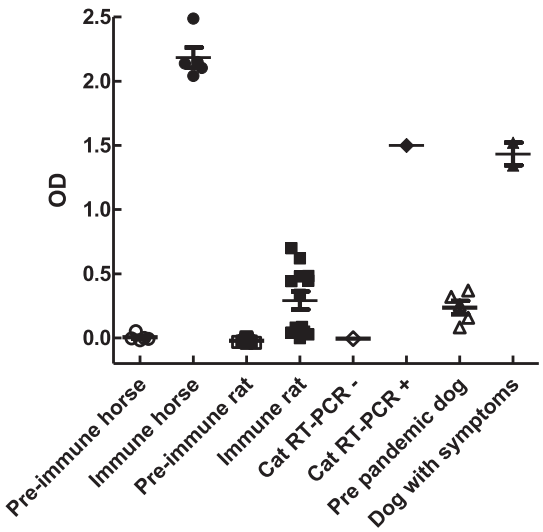

Fig. 5. Performance of the b-ELISA applied on animal sera or plasma (5 horses, 13 rats, 2 cats and 7 dogs). Serum samples from experimental immunization (horse and rat) or after RT-PCR-positive throat swab sample (cat) or with symptoms (dog) are indicated by black symbols. Negative control samples are shown by white symbols. Results are expressed as optical density (OD).

suffering protease degradation. Additionally, the method developed herein demonstrated its versatility, as it was able to jointly detect IgM, IgA and/or IgG antibodies anti-SARS-CoV-2 in different animal species (horses, rats, cats and dogs) without the need to perform and optimize a new assay to recognize each individual isotype of antibodies of each species. Wernike et al. (2020) published a multi-species ELISA for the detection of antibodies against SARS-CoV-2 in animals that employ different conjugates antibodies. However, when further species are to be tested, this method must be re-evaluated including the suitability of the conjugate for the particular species in question. Advantages of the b-ELISA test includes easy performance, is amenable to automation, and can be used to test for SARS-CoV-2 antibodies in human and non-human species.

5. Conclusion

In conclusion, the kit developed has high sensitivity, due to the use of the complete S protein (unlike the tests that use RBD), high specificity and low cross-reactivity (unlike the tests that use N protein), great versatility, since it detects total antibodies of different species (human samples and samples from different animal species can be included in the same plate) and lower cost than kits that use S protein from mammalian cells culture. Moreover, this kit simplifies the number of supplies since the S protein is used as the antigen immobilized in the solid phase as well as the molecule involved in the fluid phase detection of specific antibodies through the available paratope (S protein-biotin).

As it was mentioned before, the great versatility of the b-ELISA described herein allows its application in various situations such as: (i) monitoring the humoral immune response in individuals who have undergone the natural infection or in those who were vaccinated, (ii) evaluating the humoral immune response in animal models in which new vaccines are being studied, (iii) seeking the production process and neutralizing power of hyperimmune equine sera used for the treatment of COVID-19, and (iv) the identification of the natural reservoirs and intermediate hosts of the SARS-CoV-2 virus, which seek to prevent the establishment of new zoonotic reservoirs.

Declaration of Competing Interest

Silvina Sonia Bombicino, Aldana Trabucchi, Alexandra Marisa Targovnik, Federico Javier Wolman, Leonardo Gabriel Alonso, Matías Fingermann, Silvina Noemí Valdez, and María Victoria Miranda are career researchers of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET). Ignacio Smith and Gregorio Juan Mc callum are research fellows of CONICET. Adriana Victoria Sabljic is a research fellow of UBA. Juan Ignacio Marfía and Rubén Francisco Iacono belong to the career of Professional Support of CONICET. All the authors declare that there is no conflict of interests.

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