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Production of equine sera as a potential immunotherapy against COVID-19

Mariana V. Cepeda[1], Juan C. Jiménez[2], Flor H. Pujol[3], Héctor R. Rangel[3], Carlos Bello[1], José Cubillan[3], María L. Serrano[4], Tony Chacón[1], Antonietta Saba[1], Miguel A. López[1] and Alexis Rodríguez-Acosta[1,5]

[1]Biotecfar C.A, Facultad de Farmacia, Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela.
[2]Instituto de Inmunología "Dr. Nicolás Bianco", Facultad de Medicina, Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela.
[3]Laboratorio de Virología Molecular, Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas, Miranda, República Bolivariana de Venezuela.
[4]Unidad de Química Medicinal, Facultad de Farmacia, Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela.
[5]Laboratorio de Inmunoquímica y Ultraestructura, Instituto Anatómico "Dr. José Izquierdo", Facultad de Medicina, Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela.

Key words: Equine antiserum; anti-RBD; immunotherapy; pandemic; SARS-CoV-2; equine coronavirus.

Abstract. Emerging viruses such as the COVID-19-inducing virus, SARS-CoV-2, represent a threat to human health, unless effective vaccines, drugs or alternative treatments, such as passive immunization, become accessible. Animal-derived immunoglobulins, such as equine immunoglobulins might be useful as immunoprophylaxis or immunotherapy against this viral disease. Therapeutic antibodies (Abs) for SARS-CoV-2 were obtained from hyperimmune equine plasma using the Spike protein receptor binding domain (RBD) as an immunogen. The presence of anti-RBD antibodies was evaluated by ELISA and the titres of neutralizing antibodies were determined in viral cell culture. Immunized horses generated high-titre of anti-RBD antibodies with antiviral neutralizing activity on Vero-E6 cells of 1/1,000. To minimize potential adverse effects, the immunoglobulins were digested with pepsin, and purified to obtain the F(ab')2 fragments with the protocol standardized by Biotecfar C.A ® for the production of snake antivenom. Pre-immune serum displayed an unexpected anti-RBD reactivity by ELISA (titre up to 1/900) and Western Blot, but no

Corresponding author: Alexis Rodríguez-Acosta, Facultad de Farmacia, Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela. Tel: +58 491 7243; E-mail: rodriguezacosta1946@yahoo.es

neutralizing activity. Modelling of the RBD of equine coronavirus showed that some of the known epitopes of SARS-CoV-2 RBD were structurally conserved in the equine coronavirus protein. This might suggest that some of the reactivity observed in the pre-immune serum to the SARS-CoV-2 RBD might be due to a previous exposure to equine coronavirus.

Producción de un suero equino como inmunoterapia potencial contra la COVID-19

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Palabras clave: Antisuero equino; anti-RBD; inmunoterapia; pandemia; SARS-CoV-2; coronavirus equino.

Resumen. Los virus emergentes, como el virus causante de la COVID-19, el SARS-CoV-2, representan una amenaza para la salud de la humanidad, mientras no estén disponibles vacunas, medicamentos o tratamientos alternativos eficaces, como la inmunización pasiva. Las inmunoglobulinas de producción animal, como las de los equinos, pueden ser útiles como inmunoprofilaxis o inmunoterapia contra esta enfermedad viral. Se produjeron anticuerpos terapéuticos (Abs) contra el SARS-CoV-2 a partir de plasma equino hiperinmune inmunizado con el dominio de unión al receptor (RBD) de la proteína de la espiga viral. La presencia de anticuerpos contra RBD se evaluó mediante ELISA y de anticuerpos neutralizantes por inhibición del crecimiento del virus en cultivos celulares. Los caballos inmunizados generaron títulos elevados de anticuerpos anti-RBD con actividad neutralizante antiviral en células Vero-E6 de 1/1.000. El suero preinmune mostró una reactividad anti-RBD por ELISA (título hasta 1/900) y Western Blot pero sin actividad neutralizante. Con el propósito de disminuir posibles efectos adversos, se realizó la digestión proteica con pepsina de las inmunoglobulinas y posterior purificación para obtener los fragmentos F(ab')2 empleando el protocolo utilizado por Biotecfar C.A® para la producción de los antivenenos de serpientes. El modelado del RBD del coronavirus equino mostró que algunos de los epítopos conocidos del RBD del SARS-CoV-2 se conservaban estructuralmente en la proteína del coronavirus equino. Esto podría sugerir que parte de la reactividad del suero preinmune al RBD del SARS-CoV-2 podría deberse a una exposición previa al coronavirus equino en el animal.

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INTRODUCTION

SARS-CoV-2, the new coronavirus responsible for the disease COVID-19, belongs to the family *Coronaviridae*, genus *Betacoronavirus*, subgenera *Sarbecovirus*. The virus genome encodes for four structural proteins and several non-structural proteins. Among the structural proteins, the Spike protein (S) contains two domains: S1 that includes the receptor-binding domain (RBD), which interacts with the human receptor angio-

tensin-converting enzyme 2 (hACE2), and S2 that includes the fusion domain, which promotes the fusion of the viral envelope with the cellular membrane, to promote viral entry, through an early or late endosomal pathway (1, 2) SARS-CoV-2 RBD has been documented to enclose immune epitopes proficient of stimulating antibodies able to neutralise viral invasion and impede viral entry by competing with hACE2 binding (3) or targeting the receptor-binding domain (RBD) (4). Several authors have found that SARS-CoV-2 RBD immunogen stimulated upper titres of neutralizing antibodies (5) and displaying a strong antibody response in the immunized mammals and non-human primates (6).-

Even if vaccines are becoming available to prevent COVID-19 (7), the pandemic is still highly active, and vaccination of all the population will not be feasible in a short term (8). Thus, therapeutic tools will still be needed for preventing the severe evolution of the disease.

Equine-derived polyclonal antibodies against several proteins have shown good efficacy against infectious diseases caused by highly pathogenic agents, such as Ebola (9), West Nile virus (10), H_5N_1 influenza virus (11), respiratory syncytial syndrome virus (12) and Middle East respiratory coronavirus syndrome (13).

Production of equine anti-SARS-CoV-2 antibodies have been reported in China (14-19) India (20) and Argentina (21).

The aim of this study was the production of F(ab')2 anti-RBD equine antibodies as a potential passive immunization medication against COVID-19.

MATERIALS AND METHODS

Virus antigen, plasma pools, animals and reagents

Protein RBD antigen, expressed in HEK293 line was purchased from Acro Biosystem, USA. Specific hyper-immune horse plasma (SHHP) was obtained of experimental animals from Biotecfar. C.A®. Mice (*Mus musculus*) NIH strain (18-20 g) of both sexes, purchased from the Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela, were used for toxicity assays. Bovine serum albumin (BSA), Tris-base and Tween-20 were from Sigma-Aldrich (USA). Pepsin (from porcine gastric mucosa) 0.7 FIP-U/mg) was from Merck (Germany). Goat anti-horse F(ab')2 IgG conjugated with horseradish peroxidase (HRP) was from LSBio (USA). All other chemicals for buffers and solutions were from Sigma-Aldrich (USA), unless otherwise stated.

Experimental horses

Three male horses (*Equus ferus caballus*) were selected between the ages of 3 and 5 years old from the Purebred Racing Race. These specimens were examined previous to starting the trial: general physical evaluation, equine infectious anaemia test, cellular and biochemical haematology and a complete health plan was implemented in accordance with national regulations. The specimens were evaluated weekly over and done with a complete physical examination and cellular and biochemical haematology (22). The horses were housed in individual stalls or boxes, feed with Bermuda grass hay, commercial concentrate and *ad libitum* water, respecting all national and international animal welfare conditions.

Ethical approval

This research has been approved by the Bioethics Committee of Hospital Universitario de Caracas, in Ordinary Meeting via online N° 06 dated December 22, 2020, following the norms obtained from the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Trained staff organised all the experimental methods relating to the use of live animals.

Selection of immunogen

In order to guarantee a safe handling of the material with which the horses were immunised, it was decided to work with the SARS-CoV-2 S protein RBD (N354D, D364Y), which was expressed from human 293 cells (HEK293) with a molecular mass of 27.0 kDa purchased from (Acro Biosystem, USA). The protein migrates as 33-35 kDa under reducing condition (SDS-PAGE) due to glycosylation (data not shown).

Horse immunization

The selected horses received a variable amount (100 to 600 μg/mL) of the RBD antigen in 1 mL of PBS: first injection of 100 μg of antigen in Complete Freund adjuvant at week 0; injection of 200 μg of antigen in Incomplete Freund adjuvant at week 1; injection of 300 μg of antigen in PBS buffer at week 2; injection of 600 μg of antigen in PBS buffer at week 3; injection of 600 μg of antigen in PBS buffer at week 5. The antigen injection was subdermal performed in the lateral region of the neck. The sampling was carried out after elapsed times and disinfection and asepsis of the jugular vein to recover the plasma was performed.

Preliminary sample collection (negative control)

The previous sample extraction was carried out from normal horses after disinfection and asepsis of the jugular vein to recover the blood.

Preparation of equine IgG immunoglobulins and F (ab')^2 fragments

Plasma samples were taken every 7 days (from day 14), storing them at 4°C prior to processing and analysis. Each sample was placed in a thermal bath at 56°C for one hour, and at that time were centrifuged at 2000 rpm for 15 min and then pass through a 0.45 μm filter. After the initial immunization, horse's plasmas were collected on the days indicated in the previous table, to be stored at 4°C for further processing and analysis.

Obtaining purified immunoglobulins

A pool of horse anti-RBD sera from the three horses was diluted with equivalent volumes of saline solution, and 1/2 volume of a saturated ammonium sulphate solution was included at that time.

The mixture was stired together softly at room temperature for 30 min and afterward centrifuged at 5000 rpm for 20 min. The precipitates were running in saline solution, previously, 1/3 volume of ammonium sulphate was slowly added. Subsequently, incubation of the sample was kept at room temperature for 30 min. Then, the solution was centrifuged at 5000 rpm for 20 min. The precipitates were dissolved in saline and dialysed overnight at 4 °C to remove the ammonium sulphate.

Preparation of F(ab′)2 Fragments

For the preparation of F(ab')2, the protocol standardised by Biotecfar C.A® was used, Pepsin was activated in order to eliminate the Fc fragment from total IgG. Briefly, the pH of the horse anti-RBD immunoglobulins was upheld to 3.3 with 1 mol/L HCl. The total IgG was diluted in a 1: 2 ratios and then the pepsin was added at a concentration of 1.25 g/L. The enzymatic digestion reaction was carried out at 30°C for 30 min. Then was stopped to adjust the pH and carry out the first precipitation of contaminating proteins. It was filtered and the pH of the filtrate was adjusted for a second precipitation with ammonium sulphate, to recover the hyper-immune proteins. This preparation was diafiltered with distilled water, before evaluation, and the protein was stored at 4°C until use.

Analysis of antibodies against SARS CoV-2 by SDS-PAGE

The $F(ab')_2$ purity (5μg) was analysed by non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% SDS-PAGE). After running, the SDS-PAGE were stained with Coomassie brilliant blue. Gel images after decolouration were cap-

tured with a ChemiDoc MP imaging system (Bio-Rad).

Determination of equine antibody titres by Enzyme Linked Immunosorbent Assay (ELISA)

RBD was diluted to 1 μg/mL in PBS pH 7.4, and added to 96-well polystyrene plates (100 μL/well). The microtitre plates were incubated at 4°C overnight. The plates were then washed three times with PBS-Tween 20 (0.05%) and blocked with 5% skimmed milk for 1 h at 37°C. Subsequently, the wells were washed three times and the samples (serum or diluted antibodies, 100 μL volume) were added to each well; as negative controls PBS or nonspecific antibodies were used. The plates were placed at 37°C for one hour and at that point incubated with horseradish peroxidase (HRP), and conjugated to anti-horse IgG antibodies at room temperature for another hour. After washing, 3,3',5, 5'-tetramethyl-benzidine was added. The plates were placed in the dark room for approximately 15 minutes with the chromogens and the reaction was stopped by adding 50 μL well of 2M H_2SO_4. Finally, the absorbance was measured at 450 nm with a microplate reader (BioTek, USA), and values twice times greater than controls were considered positive.

Immunoblotting assays

The equine IgG immunoglobulins and F $(ab')^2$ fragments reactivity versus the SARS-CoV-2 RBD protein were estimated using western immunoblotting (23).

With this purpose, the gel was incubated for 10 min in transfer solution (50 mM Tris-HCl at pH 8.0, containing 380 mM glycine, 0.1% SDS and 20% methanol). Afterward, the gel was located in a transfer chamber, permitting the proteins to pass from the polyacrylamide matrix to a nitrocellulose membrane. This process was carried out at 180 milliamps (mA) for 2 h.

Next, the transfer, the nitrocellulose membrane was blocked for 2 h at room temperature with a 0.2 M PBS solution at pH 7.0, with 5% (w/v) skimmed milk and 0.1% (w/v) Tween 20. Following, three washes were carried out for 5 min, each with a solution of 0.05% (w/v) Tween 20 and 0.2 M PBS at pH 7.0. The membrane was incubated over at room temperature for another 90 min, with equine IgG immunoglobulins and F $(ab')^2$ fragments diluted to 1:10 in blocking solution. Next, after the incubation interval, the membrane was washed three times. Straightaway after the washes, the secondary antibody anti-equine IgG (coupled to horseradish peroxidase) diluted 1:10 in blocking solution was added. Then, the membrane was incubated at room temperature for another 90 min and washed, as specified. The electrophoretic bands recognised by equine IgG immunoglobulins and F $(ab')^2$ fragments were visualised and the image was analysed.

Anti-SARS-CoV-2 serum toxicity test

The toxicity assay of anti COVID-19 serum was carried out by intraperitoneal injection of 0.5 mL of the serum in NIH mice. The clinical toxicological manifestations were recorded during a period of 7 days. None of the mice showed signs of clinical toxicity after seven days of anti-SARS-CoV-2 serum injection. All animals were considered healthy with a normal behaviour.

Cell line

Vero-E6 cells was maintained in RPMI-1640 medium (Gibco, NY, USA) supplemented with 8% foetal bovine serum (FBS, Gibco, NY, USA) were cultured in an incubator at 37 °C with 5% CO_2.

Neutralization assay

Vero-E6 cells were seeded in 96-well plates with 2×10^4 cells/well overnight in 8% FBS-RPMI medium supplemented with 1600 U/mL of penicillin, 800 μg/mL of streptomycin and 10 μg/mL of amphotericin B. Horse sera were diluted in culture medium and incubated with 10 TCID50 (10 times the tissue culture infective dose, which produce cytopathic effect in half of the plates)

of SARS-CoV-2 at 37°C for 1 h. The mixture was then added to the cells and incubated at 37 °C for 1 h. The cells were washed with PBS and incubated in the culture medium. The cytopathic effect (CPE) was examined with an inverted microscope after three days post-infection. The neutralization titre was defined as the dilution of sample where 50% of the wells exhibited no CPE (24).

Phylogenetic analysis

Coronavirus RBD were aligned using DNAman version 5.2.2 (Lynnon Biosoft, Canada) and BLOSUM matrix. Phylogenetic tree constructed with Poisson correction and 100 bootstrap replicas. RBD alignment was also analysed for protein conservation using PRALINE sequence alignment (https://www.ibi.vu.nl/programs).

Protein modelling

The crystal structure of the RBD of SARS-CoV-2 bound to neutralizing antibody CR3022 (PDB code 6W41) was retrieved and selected for the comparative analyses. Homology structural model of the RBD of Equine coronavirus, was generated using the crystal structures of the RBD domain of the human coronavirus OC43(PDB code 6nzk) and the beta-coronavirus HKU1 (PDB code 5kwb) as templates. The model was obtained with the Phyre2 modelling server using the intensive modelling mode (25). The quality of the models was established via ProSA (26) and PROCHECK programs (27). The Deep-View/Swiss-PdbViewer 4.01 and Biovia Discovery Studio Visualizer 17.2.0 were used to superposition and visualisation of protein structures.

B-cell epitope prediction

EIDB (Immune-Epitope-Database and Analysis-Resource) were used to predict linear and discontinuous B-cell epitopes using Bepipred and ElliPro with default parameter settings (28). Biovia Discovery Studio Visualizer 17.2.0 software was used to examine the positions of discontinuous epitopes on the 3D structures (29).

Protein-protein docking

The crystal structure of the RBD of SARS-CoV-2 bound to neutralizing antibody CR3022 (PDB code 6W41) was downloaded from Protein Data Bank. In addition, the homology model for the RBD of Equine coronavirus was assayed. The viral spike RBD and the structural model of the ECoV RBD were evaluated against the CR3022 antibody through molecular docking. Then, the obtained binding patterns and affinity estimations were analysed and compared. This process was performed through two steps; first, ProABC-2 (30) was used to identify the paratope residues in CR3022 and the information was utilized to drive the modelling of antibody–antigen complexes using HADDOCK2.4 web server (31) and the best model was further refined with HADDOCK Refinement interface. Then, the resulting docking data were processed and analysed by using the tools of PRODIGY software (32). Finally, results were evaluated considering binding energies and main interacting residues in each complex.

RESULTS

Horse immunization

Three male horses were immunized with the Spike RBD viral recombinant protein. No major adverse effects or chronic clinical alterations were observed for any of the horses. The evaluation of immunized horse's sera binding to the SARS-CoV-2 RBD as assessed by ELISA, further validated the qualities of RBD antigens tested in this study and is shown in Fig. 1. Antibody titers were developed since the first week of immunization, increasing with the boosts of weeks 1, 2, 3 and 5, reaching a titer of 1/24,300 maintained after 50 days. Some antibody reactivity was observed for the pre-immune equine serum at 1/100 (not shown in the Fig.1: OD 1.1 in pre-immune serum vs 3.4 in the immunised horse), 1/300 and even 1/900 (Fig. 1). This reactivity was not observed when the ELISA plates were not coated with the RBD antigen (OD 0.1 in the pre-immune serum).

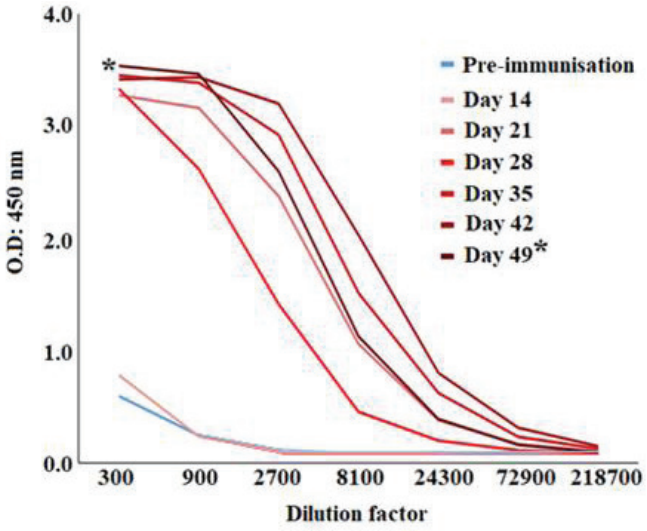

Fig. 1. Evaluation of horse sera binding to the SARS-CoV-2 RBD as measured by ELISA. One (1.0) μg/mL of RBD protein was coated and 7-fold serially diluted serum was supplemented after blocking. Experiments were carried out in triplicate and the error bars denote ± SE, n = 2. Statistical significance was defined as * $P < 0.05$. O.D: optical density.

Anti-SARS-CoV-2 serum toxicity test

None of the mice showed signs of clinical toxicity after seven days of anti-SARS-CoV-2 serum injection. All animals were considered healthy with a normal behaviour.

Determination of equine antibody titres by Enzyme Linked Immunosorbent Assay (ELISA)

The evaluation of immunised horse's sera binding to the SARS-CoV-2 RBD as measured by ELISA binding to RBD, further validated the qualities of RBD antigens tested in this study is showed in Fig. 1.

Purification and characterisation of the F(ab')2 preparation

The purity of the F(ab')2 anti-RBD S protein fragments was evaluated by 10% SDS-PAGE under reduced conditions. Three conspicuous bands between ~ 90 and 150 kDa bands were observed (data not shown).

Specificity of anti SARS-CoV-2 to RBD S protein via Western blot

Western Blot analysis of the antibody reactivity showed the specific recognition of the RBD antigen by the immune serum, together with another band of higher molecular weight, also recognised by other immune equine sera, probably a contaminant of the antigen preparation. Some recognition of the RBD band was observed in the pre-immune serum, together with the other upper band (Fig. 2, band 5).

Determination of the neutralizing activity of the F(ab')2 preparations

Two F(ab')2 preparations (1 and 2, at the middle and the end of immunization, respectively) were evaluated for the presence of total and neutralizing antibodies against SARS-CoV-2 RBD (Fig. 3). As expected, the titre of both anti-RBD antibodies and neutralizing antibodies increased with time and immunization boosts. The titre of neutralizing antibodies was approximatively 1/550 for the first F(ab')2 preparation and 1/2,300 for the second one. Pre-immune and F(ab')2 preparations against other antigen (anti-venom against snake or scorpion) did not produce any viral neutralization, even at 1/30 dilution (data not shown).

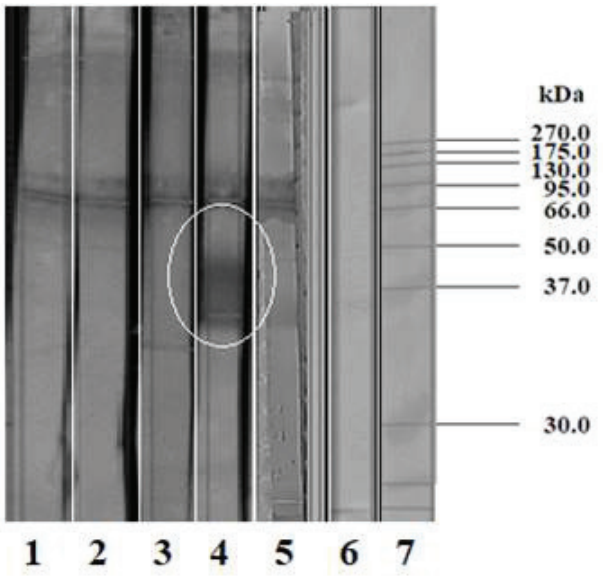

Fig. 2. Western blotting. (1 and 2) Polyvalent anti-ophidic sera. (3) Anti-scorpion serum. (4) anti-RBD S protein [oval]. (5) Normal equine serum. 6) Membrane stained with red Ponceau. (7) Molecular mass markers. RBD band is shown with a circle.

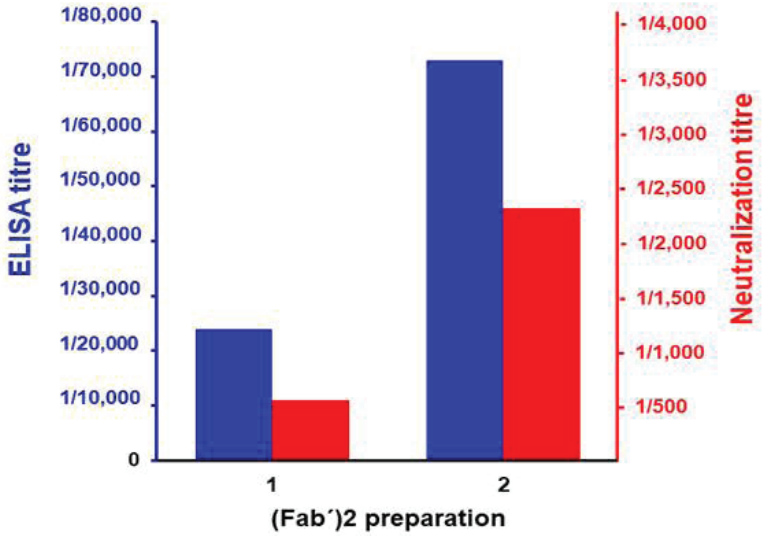

Fig. 3: Anti-RBD and neutralizing titres in F(ab')2 preparations produced at different immunization times. F(ab')2 preparation 1 was obtained after 3 immunizations and preparation 2 after the complete scheme. The neutralization titre is the highest dilution of serum that prevents infection of 50% of replicate inoculations. Neutralization tests were run in triplicate for Preparation 1 and duplicate for Preparation 2, with 3 replicas each time.

Analysis of the possible cross-reactivity of antibodies against equine coronavirus RBD and SARS-CoV-2 RBD

Since the equine pre-immune serum exhibited some degree of reactivity, both by ELISA and Western Blot, the presence of epitopes shared between the RBD of the Equine Coronavirus (ECoV) and SARS-CoV-2 (33) was evaluated. ECoV and SARS-CoV-2 RBD shares only 25% protein homology (Fig.

4A). Three domains known as main epitope regions in the SARS-CoV-2 RBD did not exhibit neither more conservation. Only one of them (epitope region 2) shared 30 % of sequence identity among them (Fig. 5B). However, structural modelling of the ECoV RBD showed that epitope domains 1,2 and 3 share structural homology (Fig. 4B).

ECoV and SARS-CoV-2 RBDs were also analysed in order to evaluate not only identical amino acids but also conservative substitutions, particularly in the structurally conserved regions (Fig. 5).

Additionally, by docking studies, the binding free energy of a monoclonal antibody against SARS-CoV-2 RBD (CR3022), to the EqCoV RBD was -10.5 Kcal/mol, similar to the one obtained for SARS-CoV-2 RBD (-14.9 Kcal/mol). CR3022 interacts with EqCoV RBD and recognizes a discontinuous epitope that includes several residues on the same region (epitope 2) than with SARS-CoV-2 (Fig. 6). Although the SARSCoV2 and ECoV RBD epitopes do not completely overlap and different binding modes were determined, the interaction energy suggests that CR3022 might show some degree of ECoV recognition. Interestingly, the epitope of CR3022 does not overlap with the ACE2-binding site of the RBD SARS-CoV-2 (34).

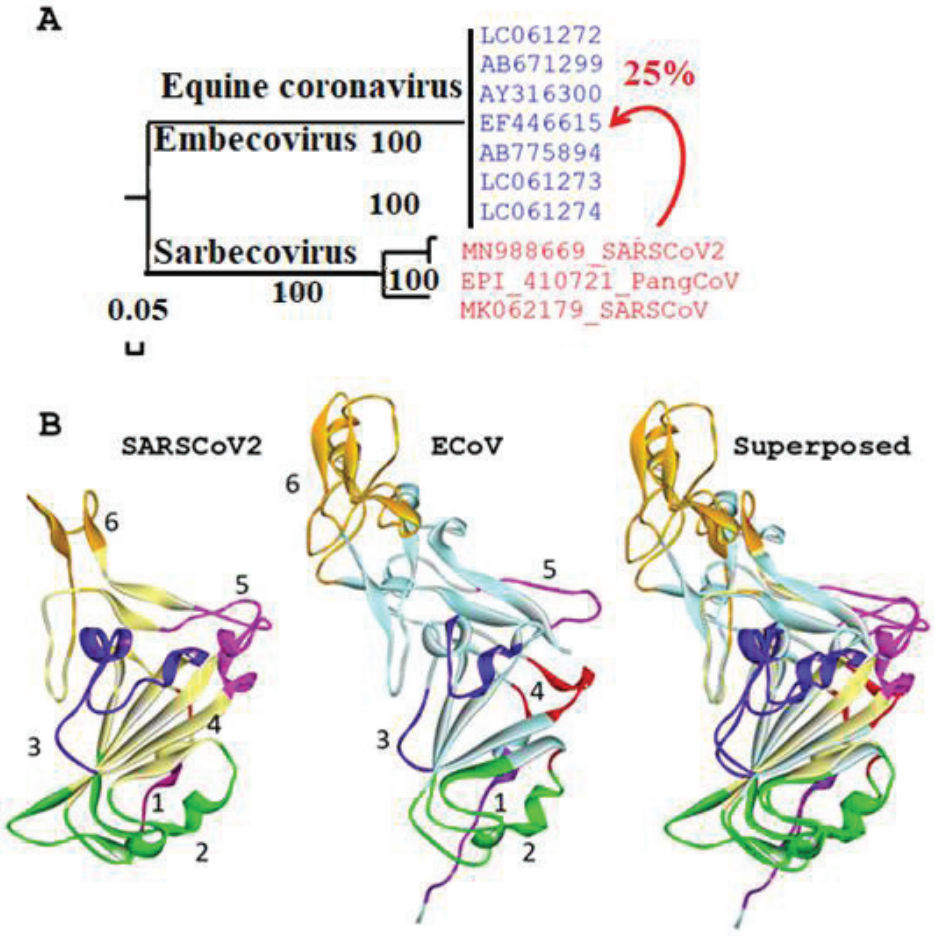

Fig. 4. Protein and structural homology between ECov and SARS-CoV-2 RBD. (A) Phylogenetic analysis of RBD proteins from different coronavirus. Percent identity between ECoV and SARS-CoV-2 RBDs is shown. The sequences are entitled with their accession or GISAID number. **(B) Structural modelling of EqCoV RBD and comparison with SARS-CoV-2 structure.** Epitope domains are shown in colours.

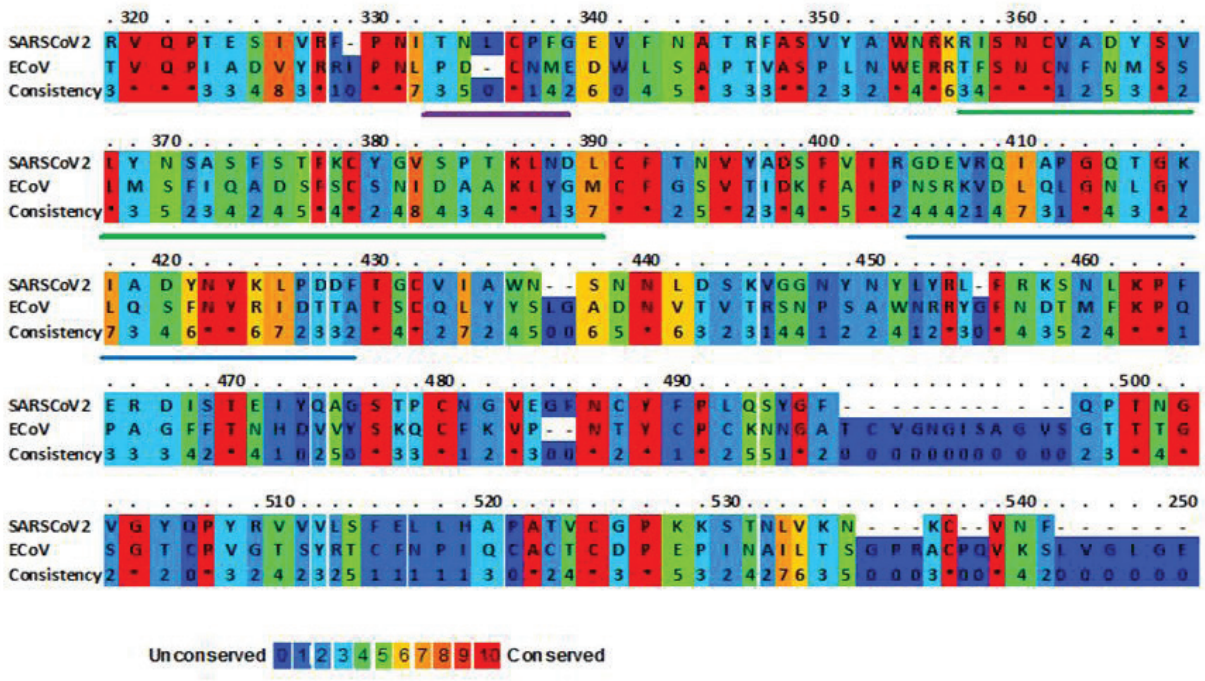

Fig. 5. Analysis of conservative substitutions between SARS-CoV.2 and ECoV RBD. All epitope domains are underline in colours.

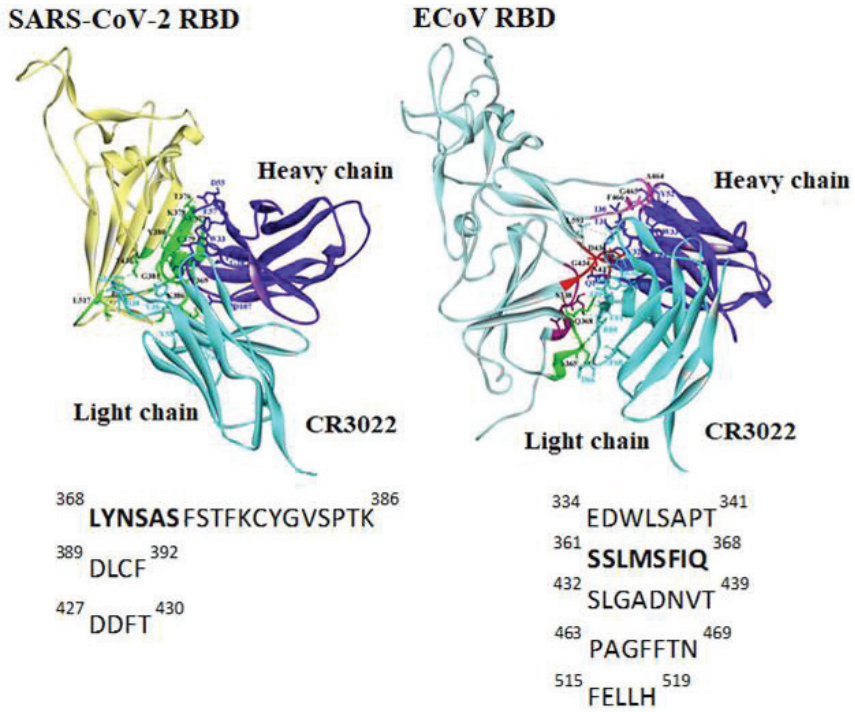

Fig. 6. A. Interaction of monoclonal antibody CR30221 with Coronavirus RBDs. Binding free energies calculated for the interaction of CR3022 with SARS-CoV-2 RBD or ECoV RBD are shown. **B. Aminoacids of the respective RBDs involved in the interaction with CR30221** are observed.

DISCUSSION

COVID-19 is one of the most important highly pathogenic viral disease at present. The COVID-19 pandemic has keep on for almost a year since the beginning of 2021, and it is expected that this severe viral pathology will pursue as an endemic disease for many years (33). The response to the international health crisis necessitates a fast answer from the medical organizations in the form of a prompt treatment, while vaccines become available for all individuals. Passive immunotherapy with equine antibodies may help to improve the medical treatment of the SARS-CoV-2.

In this study, we report the preparation of hyper-immune equine sera to demonstrate their protective efficacy against SARS-CoV-2 virus, using a virus neutralization assay. The production of equine antibodies was obtained by immunising horses with the recombinant RBD antigen. High titres of anti-RBD antibodies were obtained in horses after a five-boost immunization scheme. Western Blot analysis confirmed the specific recognition of these antibodies of the RBD protein. To minimise potential adverse effects, the immunoglobulins were digested with pepsin, and purified to obtain the F(ab')2 fragments. The F(ab')2 preparation unambiguously recognize the RBD antigen and exhibited anti-SARS-CoV-2 neutralizing activity. Moreover, the ratio of neutralizing to binding antibodies obtained in the present work and the seroconversion rate observed in horses after 49 days of immunization indicates the high quality of the antibodies generated by using the RBD spike protein as immunogen. Other studies have described slightly higher titres of antibodies against RBD than the one found in this study (15,21). These differences might be due to the immunization schemes used in each study. In our study, the RBD dose was lower than the one used in these studies, but with an additional booster dose. The titres of neutralizing antibodies found in the current report were comparable to the ones previously described (15,21). The hyper-immune equine sera was purified to obtain the F(ab')2 fragments with the protocol standardised by Biotecfar C.A ® for the production of snake and scorpion antivenom, and produces a satisfactory yield and adequate purity of preparations. The antivenoms generated using ammonium sulphate fractionation protocol have proven safe and effective in patients suffering snakebite and scorpion envenoming.

Pre-immune serum exhibited some degree of cross reactivity with the SARS-CoV-2 RBD by ELISA and Western Blot, but not in the neutralization test. In contrast to the F(ab')2 preparations against ophidic or scorpionic venoms, the pre-immune serum of the horses used in this study exhibit a light specific recognition of the SARS-CoV-2 RBD by Western Blot.

ECoV belongs to *Betacoronavirus* species, subgenus *Embecovirus* and was first isolated from a diarrheic foal in the USA in 1999 (35). Since then, several cases of ECoV infections have also been reported in adult horses from the USA, Europe and Japan. This coronavirus has been detected in faecal samples from horses with clinical signs such as anorexia, lethargy, fever and, less frequently, diarrhoea, colic and neurologic deficits. The morbidity rate varies from 10% to 83% during outbreaks, with low mortality (35, 36).

Seroprevalence of equine coronavirus in the U.S.A is relatively high: 9.3% (37). Even if SARS-CoV-2 and ECoV belong to different subgenera, the cross-reactivity observed in the pre-immune serum prompt us to evaluate if these two coronaviral RBDs might share some epitopes. Structural modelling of ECoV and SARS-CoV-2 RBD S showed that these two proteins shared the structural motifs of three of the main epitope domains recognised in the SARS-CoV-2 RBD. Moreover, docking analysis showed that one monoclonal antibody produced against SARS-CoV-2 RBD exhibited a similar binding energy to

the ECoV RBD. It is worth to mention that the epitope of CR3022 does not overlap with the ACE2-binding site of the RBD SARS-CoV-2 (34).

Poly-specific interaction of an antibody with two epitopes unrelated in terms of sequence homology has been described (37). In this study, no neutralizing activity was found associated to this pre-immune cross-reactivity. Nevertheless, (15) observed a partial neutralizing activity in pre-immune horse serum at low dilution. This observation is in agreement with the cross-reactivity found in this study.

Cross reactivity with other coronaviruses in immunoassays, detecting antibodies against SARS-CoV-2 in humans has also been suggested (38). It has been also proposed that previous exposure to other human coronaviruses might bring some protection for COVID-19 (39). Moreover, some cross-neutralization activity against SARS-CoV-2 has been described in some intravenous immunoglobulin preparations (40). Ladner *et al*. (41) proposed that the SARS-CoV-2 proteome reveals regions of conservation with endemic human coronaviruses (CoVs), but it keep unidentified to what degree of these may be cross-recognized by the antibody response. The SARS-CoV-2 response seems to be modulated by previous CoV exposures and which have the capacity to increase roughly neutralizing responses

To conclude, in the current work, we prepared horse anti-RBD S protein serum by immunizing equines with the SARS-CoV-2 RBD. In our program, the horses could be immunized numerous times, and satisfactory titres of antibodies are predictable to be achieved from hyper-immune horse's plasma for the SARS-CoV-2 therapeutic (42). Working with a scheme comprising increase immunogen injections, high-titre of horse antisera were achieved, and F(ab')2 fragments have been mass-produced using good manufacturing practices (GMP), in order to be used in human clinical studies.

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