A cell-based ELISA as surrogate of virus neutralization assay for RBD SARS-CoV-2 specific antibodies

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ABSTRACT

SARS-CoV-2, the cause of the COVID-19 pandemic, has provoked a global crisis and death of millions of people. Several serological assays to determine the quality of the immune response against SARS-CoV-2 and the efficacy of vaccines have been developed, among them the gold standard conventional virus neutralization assays. However, these tests are time consuming, require biosafety level 3 (BSL3), and are low throughput and expensive. This has motivated the development of alternative methods, including molecular inhibition assays. Herein, we present a safe cell-based ELISA-virus neutralization test (cbE-VNT) as a surrogate for the conventional viral neutralization assays that detects the inhibition of SARS-CoV-2 RBD binding to ACE2-bearing cells independently of species. Our test shows a very good correlation with the conventional and molecular neutralization assays and achieves 100% specificity and 95% sensitivity. cbE-VNT is cost-effective, fast and enables a large-scale serological evaluation that can be performed in a BSL2 laboratory, allowing its use in pre-clinical and clinical investigations.

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1. Introduction

COVID-19 is a disease caused by the novel coronavirus SARS-CoV-2 and has become a serious health issue for all mankind. To date (December 2021), more than 5 million all over the world have deceased as a consequence of the infection [1].

SARS-CoV-2 is a single strand RNA virus composed by four structural proteins: the spike, envelope, membrane and nucleocapsid [2]. The spike is displayed as homotrimer in the surface of the viral particle and is responsible for the binding to the receptor in human host cells, through its receptor-binding domain (RBD). This receptor is the angiotensin converting enzyme 2 (ACE2), although other proteins like TMPRSS2, furin-like proteases and cathepsins are involved in priming of spike and further fusion of viral and cellular membrane [3,4].

COVID-19 has mobilized huge efforts from the medical and scientific community for the discovery of efficacious therapies, although the best resources are now concentrated in vaccination to stimulate the immunity against the virus [5]. Vaccines pursue to elicit a robust antibody response against the virus as many reports highlight the relevance of neutralizing antibodies (NAbs) in the clearance of this pathogen, without ruling out the role of cellular response in orchestrating an effective defense [6,7]. The characterization of the humoral immune response against SARS-CoV-2 constitutes a critical parameter to determine protection against the virus [8]. Therefore, it is necessary to understand the quality, quantity, and duration of the response of antibodies during

the different stages of COVID-19, in the period of convalescence and vaccination.

In this sense, it is very important the evaluation of the neutralizing potential of SARS-CoV-2 specific antibodies through different types of serological tests [9,10]. The tests can be used either to detect binding (lateral flow immunoassay (LFIA), ELISA and chemiluminescent immunoassay (CIA)) [10–12] or neutralizing antibodies (conventional virus neutralization tests (cVNT), pseudovirus neutralization tests (pVNT) and molecular inhibition assays (mVNT)) [13–22]. Within the neutralization tests, cVNT is considered the reference standard method to determine the functional ability of antibodies to prevent infection by SARS-CoV-2 [23,24]. These assays require the use of BSL3 facilities, a highly trained personnel and are laborious and expensive [25,26]. Alternative techniques like the pVNT have been proposed to overcome the practical disadvantages of the former, but still need the use of viruses [20]. In addition, both tests are time-consuming (2 to 4 days long) which, in turn, require aseptic techniques [17–21]. These limitations have encouraged the development of mVNT that have demonstrated to act as potential surrogates of neutralization [14–16,22]. Such technology relies on the use of purified recombinant RBD/spike and ACE2 variants and combine the rapidness and high throughput of conventional ELISAs. However, this type of assay albeit showing good correlation with cVNT is an artificial representation of virus/host interaction, as it does not involve the actual pathogen and cell receptor. Indeed, one drawback of mVNT is associated to the potential conformational changes of the coated proteins [27–31]. This gap demands a fine standardization depending on format of each pair RBD-ACE2 to achieve a reliable mimics of virus infection scenario and, in turn, to assess a more realistic neutralizing potential of RBD specific antibodies.

Here, we propose an alternative way to measure neutralizing SARS-CoV-2 antibodies using a cell-based ELISA-virus neutralization test (cbE-VNT) that can be easily performed in a short time (5 h) in BSL2 laboratory. This platform could be considered an intermediate approach between the mVNT and the cVNT as it takes advantage of using the ACE2 in a more natural context maintaining the use of purified RBD variants. This assay has proven to be useful in detecting NAbs from convalescents and immunized individuals (human/non-human) with high specificity and sensitivity. Although it was originally designed as a neutralization test, this cell-based ELISA could also be conveniently modified to study the ACE2 binding profile of RBD variants in a high throughput way (i.e., RBD mutants from a yeast or phage display library), with the gain of using closer to reality presentation of the receptor.

2. Materials and methods

2.1. Cells, viruses and recombinant proteins

Vero (ATCC® CCL81™) and Vero-E6 (ATCC® CRL1586™) (both African green monkey kidney epithelial cells) cells were cultured in Minimum Essential Medium Eagle (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), and sodium bicarbonate. All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

For SARS-CoV-2 virus isolation nasopharyngeal exudate from an asymptomatic Cuban patient diagnosed as positive for COVID-19 was inoculated in Vero-E6 (ATCC® CRL1586™), following the methodology published by Noa et al. The presence of virus was confirmed by visualization of the cytopathic effect from electron microscopy and the virus replication by real-time PCR techniques from culture supernatant viral RNA (rRT-PCR) and domestic ELISA for quantification of SARS-CoV-2 protein N [32].

RBD, ACE2 and irrelevant Fc-fusion proteins were generated at the departments of Chimeric Proteins and Protein Engineering of the Center of Molecular Immunology (Cuba), upon the coupling of either an Fc region of a human IgG1or a murine IgG2a, to RBD of SARS-CoV-2 S protein (residues 328–533), human ACE2 (residues 18–740), or PDL1. The irrelevant protein 6X-His tag-PDL2 was also generated at the department of Protein Engineering of the Center of Molecular Immunology (Cuba).

2.2. Human and animal sera used in this study

The sera from COVID-19 convalescents used in this study were donated by the Hematology and Immunology Institute (IHI; Cuba) and Finlay Vaccine Institute (FVI; Cuba) (Clinical Trial Number: RPCEC00000366) [33]. Sera from immunized convalescent patients with COVID-19 come from the phase 1 study of the FINLAY-FR-01A vaccine (Clinical Trial Number: RPCEC00000349) [34]. Samples from healthy donors were donated by FVI, corresponding with pre-vaccination sera from clinical trials of COVID-19 vaccine candidates (Clinical Trial Number: RPCEC00000332; RPCEC00000338; RPCEC00000340) [35–37]. The convalescent and healthy donors were collected with written informed consent and approved by the Ethics Committee of IHI and by the National Toxicology Center, respectively. Mouse and hamster anti-SARS-CoV-2 RBD sera were provided by FVI and come from preclinical in vivo studies with the RBD-TT conjugate/Al(OH)$_3$ [38].

2.3. SARS-CoV-2 RBD and S protein direct binding

Vero and Vero-E6 cells were seeded at different densities in 96-well cell culture plates (COSTAR®, Corning Incorporated). After 24 or 48 h of seeding, the cells were fixed by adding 100 μl of 4% paraformaldehyde (PFA: Sigma-Aldrich #MKCJ2721) followed by incubation at room temperature (RT) for 20 min and quenching for 5 min at RT with 50 μl of 0.3% H_2O_2, in PBS. The cells were blocked with 200 μl/well of assay buffer (3% of bovine serum albumin (BSA: Sigma #114 K0580) in PBS) during 1 h, at RT. Next, 50 μl of human Fc-RBD (RBD-hFc) or mouse Fc-RBD (RBD-mFc) fusion proteins, or SARS-CoV-2 Spike S1 (Sino-Biological-Inc #40591-V08H3) at different concentrations were added and incubated for 2 h at RT. RBD-mFc binding was detected with 100 μl of anti-mouse IgG antibody conjugated to peroxidase (1:5,000; Jackson #115–035-003) for 1 h at RT. Alternatively, RBD-hFc binding was revealed with 100 μl of anti-human IgG biotin antibody (1:5,000, 30 min, RT; Jackson #109-065-098), followed by the addition of 100 μl of streptavidin conjugated peroxidase (1:20,000, 30 min, RT; Sigma #S5512). The binding of Spike was detected with 100 μl of mouse produced anti-RBD S1 CBSS-RBD antibody (10 μg/mL) (CIGB #202) for 1 h at RT. After, it was added 100 μl of anti-mouse IgG biotin antibody (1:5,000, 1 h, RT; Jackson #115-066-071) followed by the addition of 100 μl of streptavidin conjugated peroxidase (1:25,000, 30 min, RT; Sigma #S5512). Finally, the 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Sigma #T0440) was added and plates were light-protection incubated for 15 min at RT. The reaction was stopped using 10 M H_2SO_4. The optical density (OD) at 450 nm was measured using a microwell reader (BioTek). All incubations were followed by three washing steps with PBS. Recombinant proteins and antibody conjugates were diluted in assay buffer

2.4. Cell based ELISA-virus neutralization test (cbE-VNT)

The general methodology described in Section 2.3 was followed. Vero cells were seeded at a density of 40,000 cell/wells in 96-well cell culture plates (COSTAR®, Corning Incorporated). After 48 h, the cells were fixed, quenched and blocked. Serial dilutions of sera

were pre-incubated with RBD-Fc at a final concentration of 20 ng/mL, for 1 h at 37 °C. RBD-mFc was used for human samples and RBD-hFc was used for animal samples. Mixtures were added to the plates (50 μl/well) and incubated for 2 h at RT. The detection of RBD-mFc and RBD-hFc binding to ACE2 of Vero cells was performed as described in Section 2.3. All incubations were followed by three washing steps with PBS. RBD-Fc fusion proteins, sera and antibody conjugates were diluted in assay buffer.

Inhibition mediated by both human or non-human samples was calculated and expressed as percentage according to the next formula: Inhibition (%) = $[1-(OD_{450nm}$ sample/OD_{450nm} maximal recognition)] × 100. Maximal recognition corresponds to RBD-mFc or RBD-hFc (20 ng/mL). For determination of ID50 (half-maximum inhibitory serum dilution) in the cbE-VNT, dilutions were log transformed and data was adjusted to a log(inhibitor) vs normalized response with variable slope non-linear regression.

For the work with frozen cells, Vero-coated microplates were fixed, quenched and stored at −80 °C with PBS-BSA 3%. Seven days later, plates were thawed for 40 min at 37 °C and used as described above.

2.5. Molecular virus neutralization test (mVNT)

Microtiter plates (Maxisorp, Thermo Scientific) were coated with 250 ng/well of ACE2-hFc or ACE2-mFc in carbonate-bicarbonate buffer, 0.1 M (pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 200 μl/well of 2% of skim milk in PBS-Tween 0,05% (PBST) during 1 h, at 37 °C. Serial dilutions of sera were pre-incubated with RBD-Fc (final concentration: 20 ng/mL), for 1 h at 37 °C. RBD-mFc was used for human samples and RBD-hFc was used for animal samples. Mixtures were added to the plates (50 μl/well) and incubated for 2 h at 37 °C. The binding of RBD-mFc was detected by addition of alkaline phosphatase (AP) conjugated anti-mouse IgG antibody (1:1,000; Sigma #A9316) for 1 h at 37 °C. The binding of RBD-hFc was detected through incubation with AP conjugated anti-human IgG antibody (1:1,800; Sigma #A3188). Finally, p-nitrophenylphosphate (Sigma #N9389) diluted at 1 mg/mL in diethanolamine buffer (pH 9.8) was added, and plates were incubated at RT for 30 min, protected from light. The OD at 405 nm was measured in a microwell system reader (Bio-Tek). In all steps other than blockade, samples and reagents were added using a final volume of 50 μl/well. Three washing steps with PBST followed each incubation.

Inhibition was calculated and expressed as percentage according to the next formula: Inhibition (%) = $[1-(OD_{405nm}$ sample/OD_{405nm} maximal recognition)] × 100. Herein, maximal recognition corresponds to wells incubated only with RBD-mFc or RBD-hFc (20 ng/mL). For determination of ID50 in the mVNT, dilutions were log transformed and data was adjusted to a log(inhibitor) vs normalized response with variable slope non-linear regression.

2.6. Conventional virus neutralization test (cVNT)

The cVNT assay was performed following the recommendation of Manenti et al. with few modifications [17]. Animal or human serum samples were heat-inactivated for 30 min at 56 °C. Two-fold serial dilutions, starting from 1:10 to 1:2,560 were then mixed with an equal volume of viral solution containing 100 TCID50 of SARS-CoV-2 (Strain 2025, Cuban Collection at NLCD). After incubating for 1 h at 37 °C each serum-virus mixture was added in duplicate to a cell plate containing a semiconfluent Vero-E6 monolayer (10^4 cell/well). After 4 days of incubation, the colorimetric reaction was carried out according to the protocol described by Manenti et al. The highest serum dilution showing an OD representing the 50% of average OD value from control cell wells, was considered as the neutralization titer and is represented as

neutralizing titer 50 (NT50). Control cell wells involve Vero-E6 monolayer with mixture of virus-serum.

2.7. Statistical and graphical analysis

GraphPad Prism program (version 7 for Windows, California USA) was used for graphical representation and statistical analysis of the results, as well as ID50 determination. Normality and variance homogeneity of the samples were analyzed using Shapiro-Wilk and Bartlett tests, respectively. Statistical differences were further determined with two-way ANOVA followed by Tukey's post-hoc. Correlations between cVNT, mVNT and cbE-VNT were analyzed with Pearson or Spearman correlation coefficients.

3. Results

3.1. RBD reactivity standardization on cell-based ELISA

Taking into account previous experiences with molecular inhibition assays, we planned to establish a platform supported by a cell-based ELISA relying on RBD-cell interaction, for further neutralization studies. Then, for the establishment of the technique it was first studied certain variables such as cell lineage, cell number and growth kinetics.

3.1.1. Cell lines

The cell lines Vero and Vero-E6 have been extensively used in many studies of SARS-CoV-2 virus, due to the high homology of green monkey ACE2 with that of humans (97.1% of similarity and 94.8% of identity) [39,40] and the high expression of this receptor in their membranes [41,42]. Vero cells are used for virus amplification, while Vero-E6 are employed mainly in cVNT [3,14,17], which make them good candidates to be used in a cell-based ELISA as source of ACE2 receptor.

First, a comparison between both SARS-CoV-2 permissive cell lines was performed, in order to select the one best recognized by a RBD-Fc fusion protein. Both cell lines were seeded at 10,000 cells/well in 96-well plates and 24 h after they were fixed and incubated with different concentrations of RBD-hFc or RBD-mFc. The binding of RBD-hFc was detected with an anti-human biotin antibody followed by a streptavidin-peroxidase conjugate. In the case of RBD-mFc, the binding was revealed by the use of a peroxidase-coupled anti-mouse antibody. For both settings, a final washing step preceded the addition of TMB peroxidase substrate.

Both RBD-hFc and RBD-mFc recognized cell membrane ACE2 in a concentration-dependent manner (Fig. 1A and B), exhibiting higher reactivity in Vero cell line. The specificity of the interaction is supported by the non-recognition of the irrelevant proteins used (PDL1-Fc fusion proteins). Additionally, the signal-to-noise, (S/N), referred to as OD_{450nm} of the highest reactivity sample/OD_{450nm} of the conjugate control (blank), was higher in Vero cells-coated plates (Fig. 1C), which suggests a higher sensitivity of the assay using this cell line. Taking into account this result, we chose Vero cells to continue the standardization of the technique.

3.1.2. Cell number and growth kinetics

To augment the sensitivity of the assay, different cell densities and growth times after cell seeding were evaluated. In general, the increase in cell number rendered higher OD_{450nm} signals. As shown in Fig. 2, the highest OD_{450nm} values obtained upon the interaction between RBD proteins and ACE2 bearing cells were observed with 40,000 cells/well grown after 48 h. Thus, we selected this cell density to continue with the study.
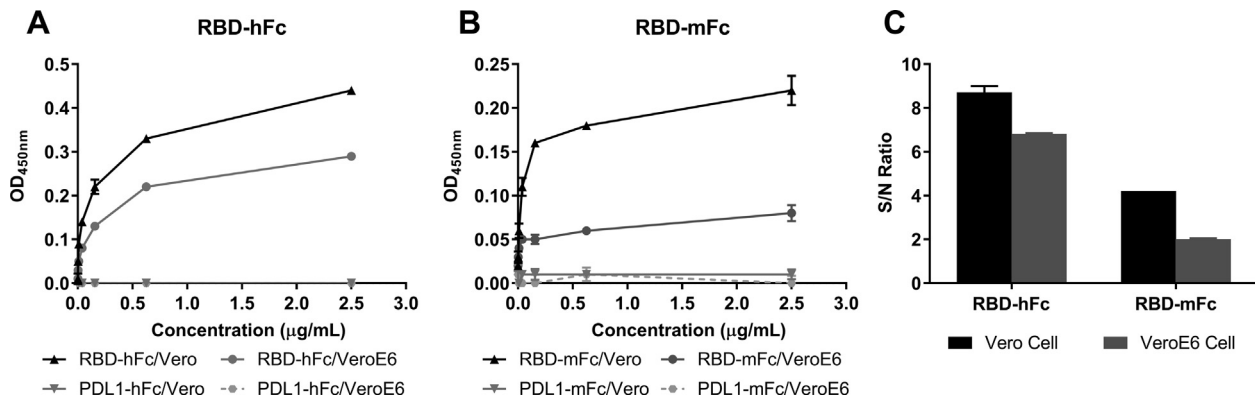
Fig. 1. Cell line selection for RBD binding to ACE2 in a cell-based ELISA. Binding of RBD-hFc **(A)** and RBD-mFc **(B)** to ACE2 of Vero and Vero-E6 cells. PDL1-hFc and PDL1-mFc were used as irrelevant fusion proteins. Graphics represent the mean OD450nm for technical duplicates of two independent experiments ± SD. **(C) Signal-to-noise** (S/N) ratio of RBD-hFc and RBD-mFc in Vero and Vero-E6 cells is represented.
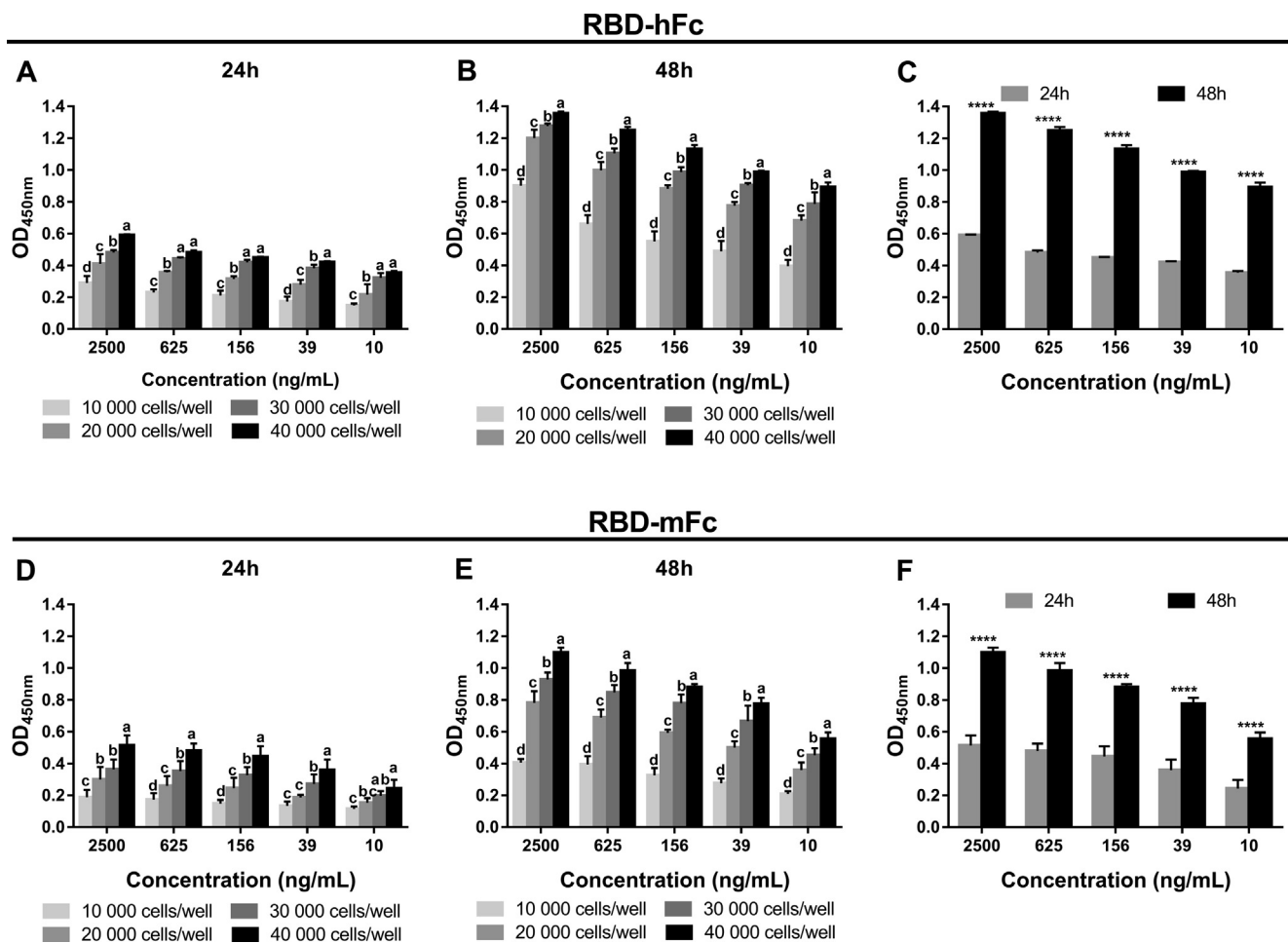

Fig. 2. Determination of optimal cell density and growth time of Vero cells for RBD reactivity cell-based ELISA. Binding of RBD-hFc (upper panel: **A-C**) or RBD-mFc (lower panel: **D-F**) to ACE2 of Vero cells. **(A-F)** Bars represent means of OD_{450nm} duplicate samples of three independent experiments ± SD. In **(C)** and **(F)** is represented the result corresponding to plates coated with 40,000 cells/well. Different letters indicate significant differences and "ns" means not significant, *$p < 0.01$, **$p < 0.001$, ****$p < 0.0001$ by two-way ANOVA with Tukey's post-hoc.

3.1.3. Evaluation of ACE2 binding of SARS-CoV-2 Spike S1

Next, reactivity with SARS-CoV-2 Spike Glycoprotein-S1 was determined following the optimal setting previously established with RBD-Fc proteins, but using another system for ACE2 binding detection. This alternative relies on a monoclonal antibody specific for RBD and further anti-mouse biotin/streptavidin-peroxidase.

When comparing different RBD formats, S1 reactivity was much lower than that of the RBD-Fc fusion proteins previously evaluated (Fig. S1A) and the S/N ratios of the S1 was lower (S/N < 5) than RBD-Fc (8 < S/N < 12) (Fig. S1B) at the same concentrations. This result prompted us to implement ACE2-binding neutralization with RBD-Fc proteins.
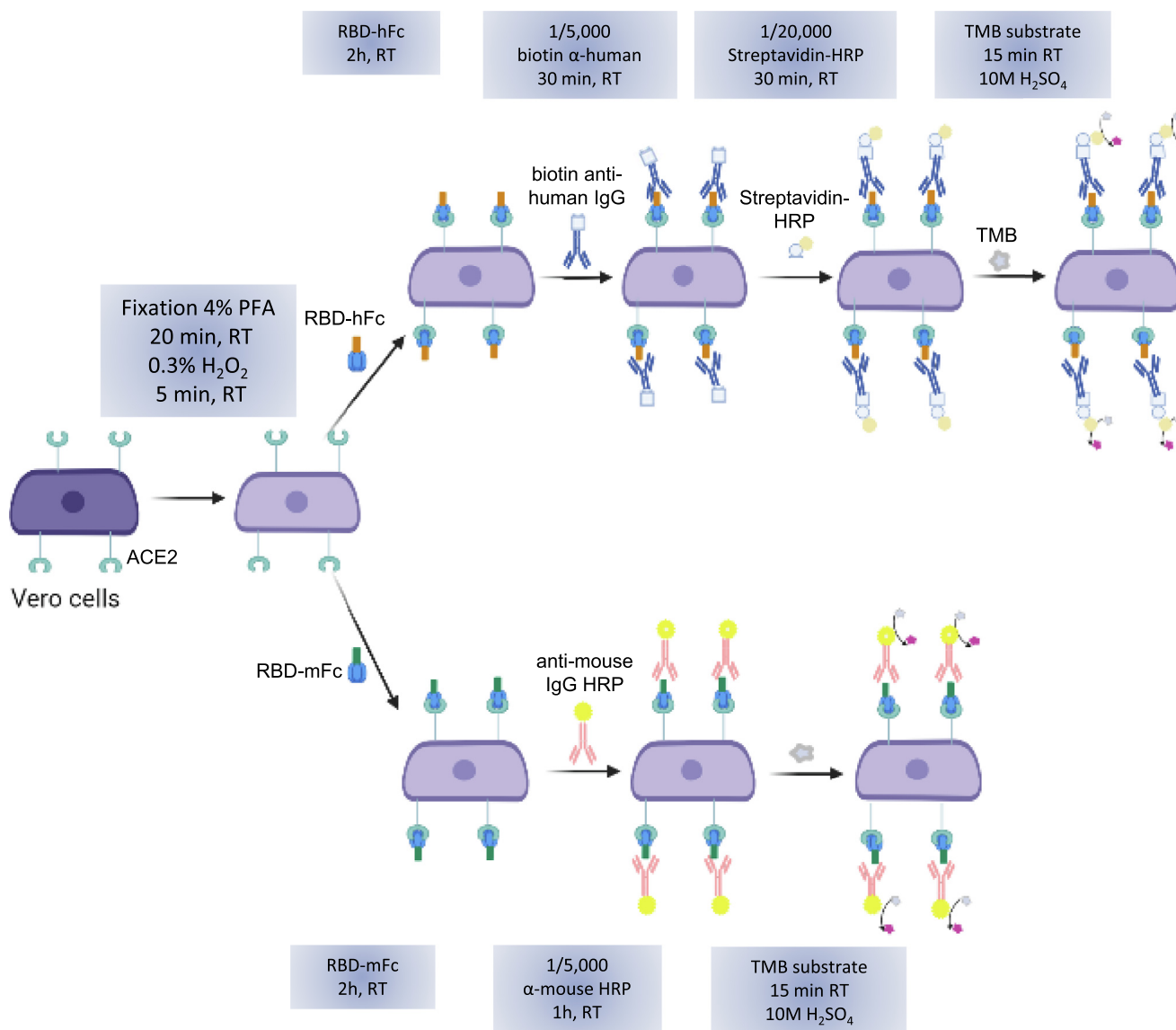
Fig. 3. Cell-based ELISA to measure direct binding of RBD-Fc to ACE2 on fixed Vero cells. The illustrations were created using BioRender. HRP: horseradish peroxidase

For subsequent experiments, 40,000 Vero cells/well incubated for 48 h and fixed with 4% PFA, were used. The final protocol of the RBD-Fc reactivity in cell-based ELISA is shown in Fig. 3.

3.2. Establishment of cbE-VNT to detect inhibition of RBD binding to ACE2 expressing cells

Considering previously established conditions, a cell-based inhibition assay (cbE-VNT) was designed (Fig. 4), aiming to evaluate the ability of SARS-CoV-2 RBD specific Abs elicited by natural infection or vaccine, to block the binding to ACE2 receptor on cell membrane. For this test, RBD-Fc fusion proteins were used at a final concentration of 20 ng/mL (non-saturating concentration), that guarantees S/N ratio of 5 for RBD-mFc and 6 for RBD-hFc, allowing suitable discrimination between the signal and the background (Fig. S2A and B). In this method, RBD-mFc or RBD-hFc are pre-incubated with human or non-human hyperimmune sera, respectively, and the binding is measured as described in Materials and Methods.

3.2.1. cbE-VNT with human and non-human hyperimmune sera

As proof of concept, it was first demonstrated the capability of this assay to detect NAbs in the sera of COVID-19 convalescents. By using this approach, a cohort of 110 sera from PCR-confirmed COVID-19 cases and 114 healthy donors, was studied. A cutoff at 15% inhibition was chosen from mVNT of more than 400 healthy human sera. The study of RBD binding inhibition at 1/100 serum dilution for the whole cohort resulted in 100% specificity and 95% of sensitivity of the technique (Fig. 5). Additionally, sera of 29 recovered individuals were evaluated at different dilutions and showed to be able to inhibit the binding of RBD-mFc to ACE2 expressing cells in a dose-dependent manner (Fig. 6A). Moreover, inhibition profile of cbE-VNT and the mVNT previously established in our lab with the same RBD-Fc proteins were very similar (Fig. S4A).

After proving the capability of the cbE-VNT to detect naturally induced SARS-CoV-2-RBD specific NAbs, we decided to study the neutralizing potential of sera from mice, hamsters (Fig. 6C-D) and humans (Fig. 6B) previously vaccinated with SARS-CoV-2 RBD-based immunization schemes. Similarly to what occurred with convalescents sera, for all samples inhibition decreased with
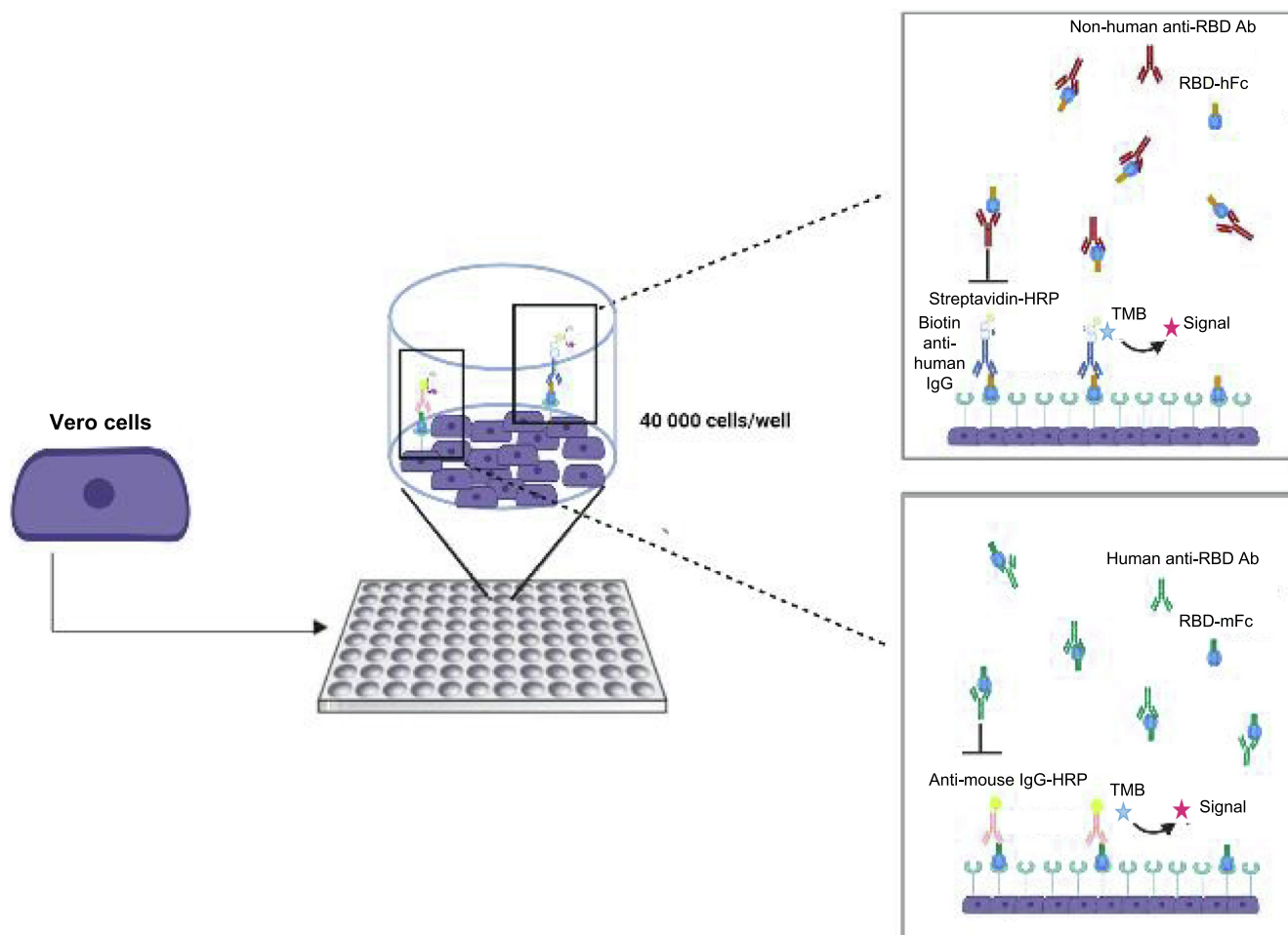
Fig. 4. Detection of SARS-CoV-2 NAbs by a cell based ELISA-virus neutralization test (cbE-VNT). The illustrations were created using BioRender. HRP: horseradish peroxidase
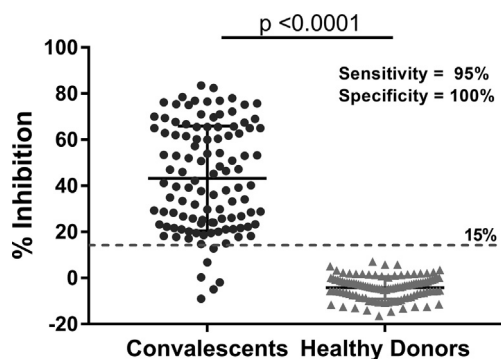

Fig. 5. Specificity and sensitivity of cbE-VNT. Sera (final dilution 1/100) from COVID-19 convalescents (n = 110), or healthy controls (n = 114) were evaluated. Inhibition percentages are shown, as mean ± SD of duplicate values. The horizontal lines indicate the median values. The dotted lines represent the cutoff at 15% inhibition. The P value was calculated from unpaired two-tailed Mann Whitney test.

sera dilution, indicating its dose-dependence and suggesting its specificity (Fig. 6B-D). Furthermore, this result points out the technique is species-independent. In agreement with recovered individual samples, the inhibition curves coming from cbE-VNT were similar to those of the mVNT (Fig. S4B-D). In all cases, specificity

was supported by non-inhibition with pre-immune and healthy donor sera (Fig. 6B-D).

3.2.2. cbE-VNT with pre-coated plates

In order to improve the precision inter-assay of cbE-VNT and to have ready-to-use plates we evaluated a pre-coating strategy of the cells into the wells, by using sera from immunized COVID-19 convalescents. Fresh cell-coated plates were used as controls.

As shown in Fig. S3 A and B, the percentage of inhibition at 1/100 dilution and ID50 were very similar to those obtained with fresh cell-coated plates. This result indicates the possibility of low temperature preserving of plates and minimizing the inter-day variability of the method.

3.2.3. Correlations between cell-based, molecular and conventional VNT

To compare the results rendered by this cell-based inhibition ELISA with other established virus neutralization tests, a comparative study was made based on correlations. The inhibition titers (ID50) of sera from different animal species in cbE-VNT and mVNT exhibited a very good positive correlation (Fig. 7C-D), as well as the percentages of RBD-ACE2 binding inhibition of different dilutions obtained by both techniques (Fig. S5C-D). In addition, there was an excellent positive correlation between percentages of inhibition and ID50 values obtained by both methods with human sera (Fig. 7-A-B and S5A-B). The results indicate the similarity in the outcome from both techniques, cbE-VNT and mVNT.
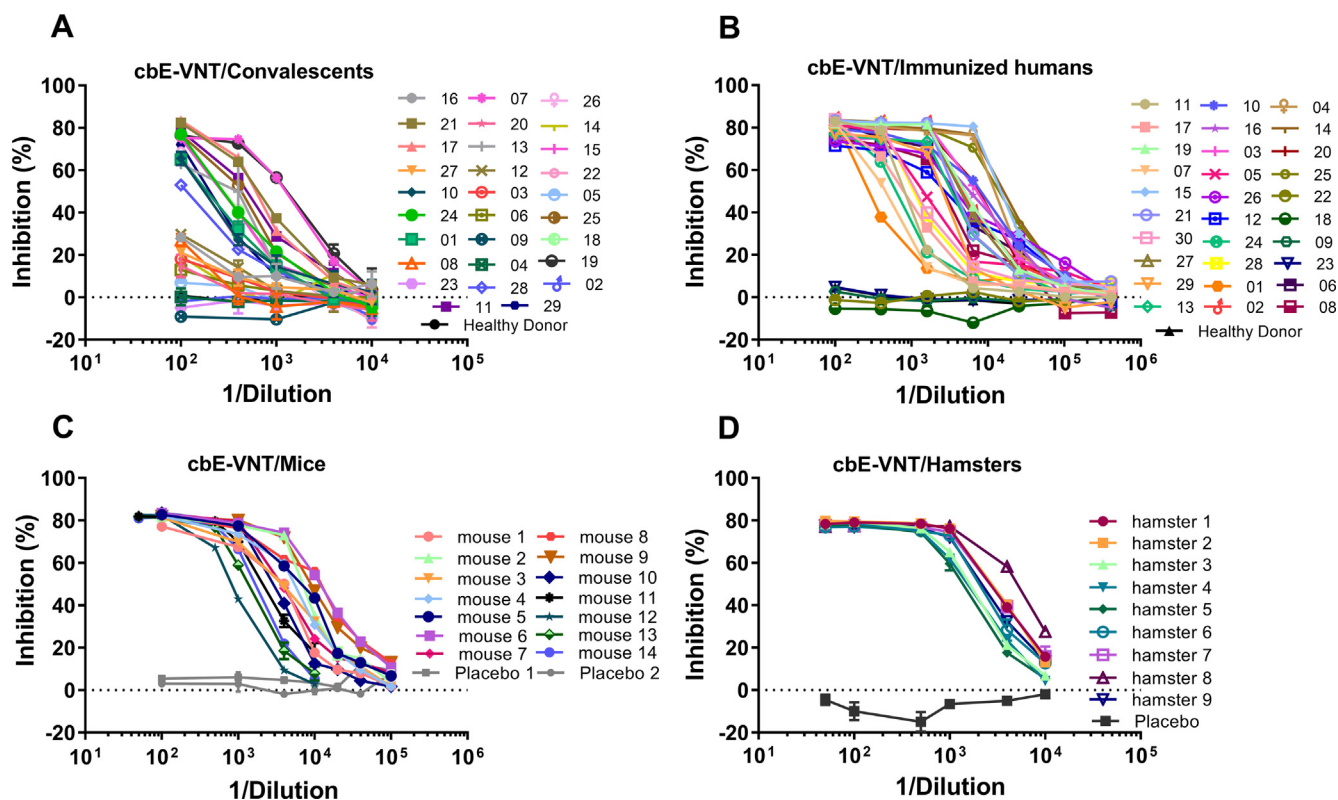
Fig. 6. Inhibition of RBD binding to ACE2 receptor by sera from immunized individuals, as measured by cbE-VNT. Sera from COVID-19 convalescents (n = 29) **(A)**, recovered people immunized with FINLAY-FR1A dimeric RBD recombinant vaccine (n = 30) **(B)** or mice (n = 14) **(C)** and hamsters (n = 9) **(D)** immunized with RBD-TT/AlOH$_3$ were evaluated. Placebo and healthy donors sera were used as negative controls. Inhibition percentages are shown as mean ± SD of duplicates values **(A, C-D)** or of triplicates values **(B)**.

Similar analysis was performed with neutralization titers resulting from cVNT (NT50) and cbE-VNT. In this case, both titers exhibited good correlation in mice (r = 0.84, 95% confidence interval (CI) 0.54–0.95, p < 0.001; Fig. 8A) and human sera (r = 0.93, 95% CI 0.85–0.97, p < 0.0001; Fig. 8B). Indeed, cbE-VNT demonstrated a better correlation with the cVNT than mVNT, when compared Pearson coefficients and R^2 (Fig. 8C-D). Moreover, similar high correlation with cVNT (r = 0.92, 95% CI 0.82–0.96, p < 0.0001) was observed for cbE-VNT with frozen plates, supporting their use (Fig. S3C). In summary, these results argue in favor of cbE-VNT as a surrogate for cVNT.

4. Discussion

Many immunization approaches against SARS-CoV-2 are currently used all over the world to control the COVID-19 pandemic. The vaccines already registered or in ongoing clinical trials seek for the induction of a protective immune response mainly based on high titers of NAbs. Furthermore, the study of the levels and functionality of the SARS-CoV-2 specific antibodies has been used to evaluate the humoral response elicited by different vaccines and to identify the best convalescent plasma donors for hyperimmune plasma therapy [43].

In this sense, different serological tests have been developed to measure the neutralizing potential of the humoral immune response [13–22]. The cVNT is the "gold standard" method for the assessment of NAbs [23,24]. Indeed, recent studies have shown a correlation between NAbs levels and the protective efficacy for different vaccines [44]. However, cVNT requires the work with live virus and therefore, the use of BSL3 facilities, highly trained and

competent personnel and appropriate personal protective equipment [25,26]. On the other hand, platforms based on pseudotyped viral particles have been adapted to measure NAbs against SARS-CoV-2 and despite using BSL2 laboratories, they are not appropriate for large-scale production, even in developed countries. Moreover, both pVNT and cVNT require two to four days to obtain the results, which demands working in aseptic conditions [14,18–21].

Alternatively, immunoenzymatic assays have tried to mimic the virus binding to host cell through a protein–protein interactions involving purified recombinant RBD and ACE2. This platform is the base for inhibition tests (mVNT) to detect NAbs in an easy, safe and rapid manner [13–16,22]. Nevertheless, since the coating process in the experimental procedure involves the passive adsorption of proteins via hydrophobic interactions, conformational changes of the coated protein molecules may occur, resulting in the exposition of regions that may not be present in the native state [27–31]. This might lead to interactions of RBD with regions of ACE2 that do not naturally exist, and in turn, do not mimic exactly the interaction RBD-ACE2.

The present work proposes an alternative platform to cVNTs, pVNT or mVNTs for the determination of virus blocking antibodies. To date, cell-based ELISAs have been widely used in determination of SARS-CoV-2 infection [45], or evaluation of SARS-CoV-2-specific antibody response [46] but scarcely one research (to our knowledge) has reported its use in determination of NAbs against this virus. Thus, the cbE-VNT becomes a novel and attractive procedure that allows a large-scale serological evaluation that can be performed in a BSL2 laboratory in a short period of time (5 h to 6 h) with a non-limiting and unexpensive source of ACE2, as these are Vero cells.
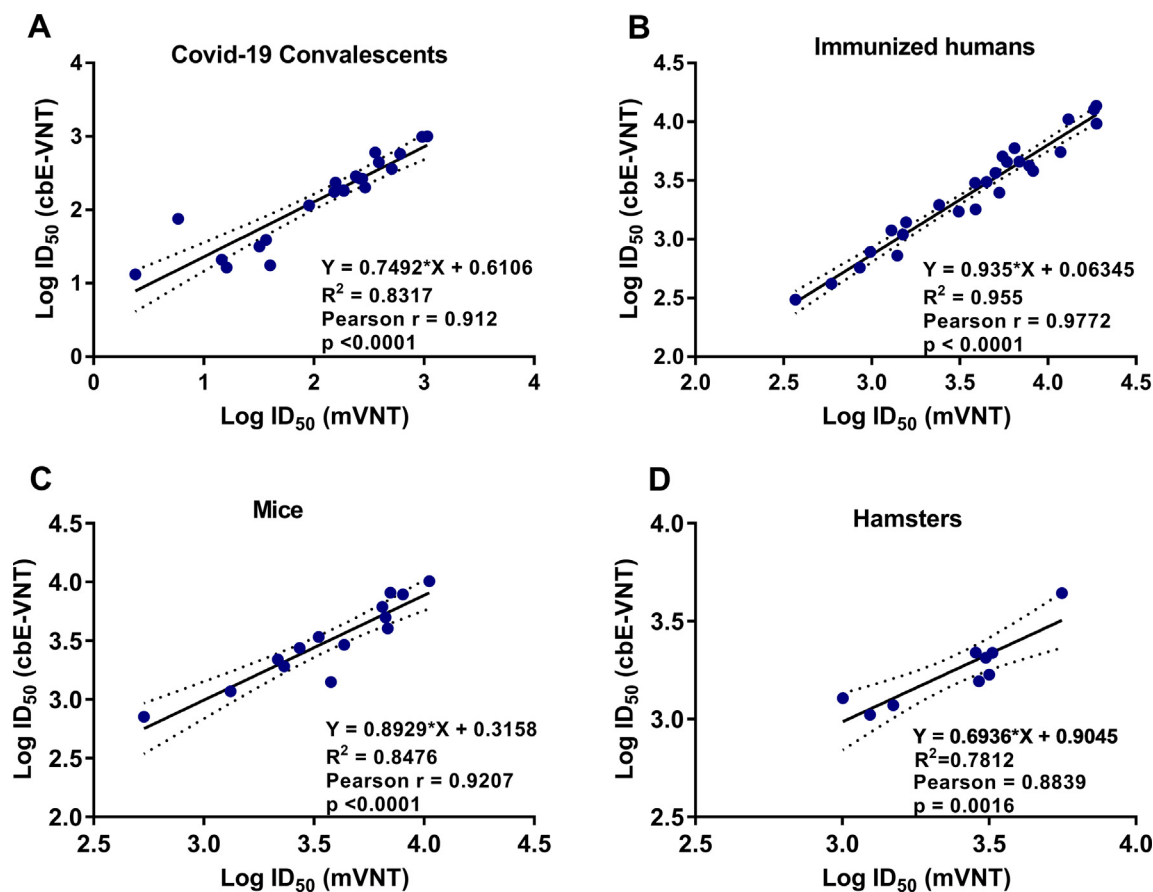
Fig. 7. Correlation between cbE-VNT and mVNT. The neutralization titers of hyperimmune sera of different species: COVID-19 convalescents (**A**), immunized humans (**B**) mice (**C**) and hamsters (**D**) were used for the analysis. Linear regression and correlations were performed in GraphPad Prism using Pearson's correlation coefficients. Statistical significance was calculated using the two-tailed test. The data presented are the log of the ID50 value for cbE-VNT and for mVNT. The dashed lines indicate the standard deviations of the linear regression plots.

Additionally, the use of Vero cells determines the presence of ACE2 receptor in a natural context which could be a more realistic representation of virus/host interaction, and in turn, measurement of a closer to actual neutralization capacity, if compared with a system based on protein–protein interactions. We don't rule out the use of other ACE2 bearing cells for future adaptations of this method, but the easy culture and fast growth rate of Vero cells make them excellent candidates for this technique. Indeed, these characteristics supported their selection over Vero-E6 cells. During the standardization of the method, the use of SARS-CoV-2 Spike was explored, but the reactivity of the whole viral protein rendered a low signal-to-noise ratio, non-suitable for inhibition assays like this one. Furthermore, it has been reported that if not all, most of NAbs target the RBD of SARS-CoV-2 Spike [47], which validates its utilization for any molecular neutralization study. Moreover, the selection of recombinant RBD over SARS-CoV-2 Spike in mVNT has been also documented by other groups [14].

One of the advantages of the cbE-VNT assay is the possibility to be easily adapted to detect NAbs of any species. Indeed, the present work proposes two versions of the assay for evaluation of samples from human and non-human species. This guarantees the application of the method in both clinical and preclinical studies, with animal models. Noteworthy, this highly sensitive (95%) method demonstrated to have a very high specificity (100%), comparable to that reported for mVNT [14,15], which could allow to provide reliable results due to the perfect discrimination of healthy people from those with some level of NAbs response.

Like other neutralization assays (i.e., pVNT or mVNT) this ELISA is ultimately intended to be used as a surrogate for cVNT, the classical technique for evaluation of NAbs. On this regard, with human sera the correlation between cbE-VNT and cVNT (r = 0.93, P < 0.0001) was better than that between cVNT and mVNT developed in our laboratory (r = 0.91, P < 0.0001), pointing out the suitability of this method to screen a large number of samples for assessment of neutralization capacity. Even more, for human samples this correlation value (cbE-VNT vs cVNT) is higher than that between mVNT and cVNT from other groups, as reported by Byrnes et al. and Tan et al. (r = 0.74, P < 0.0001; r = 0.8591, P < 0.0001, respectively) [21,41]. It is noteworthy to highlight that the association between cbE-VNT and cVNT results seems to be better if compared with the correlation values between cVNT and pVNT informed in the literature (i.e., r = 0.7678, P < 0.0001 [21]; r = 0.89, P < 0.001 [20]; r = 0.8066, P = 0.0008 [19]). It is important to stress that the good correlation between cbE-VNT and cVNT observed in our study was not affected when using frozen precoated plates. The possibility of using these ready-to-use microplates enable a faster and more reproducible method for measuring NAbs. The optimization of the conditions and preservation times of the plates remain pending to perform.

Of note, the correlation between cbE-VNT and cVNT for mice was lower if compared with results with human sera. We must understand that although RBD-Fc is mimicking the ACE2 binding domain of the spike in the live virus, some RBD epitopes might not be equally exposed. Besides, some of these epitopes might be differentially recognized by other than humans immune repertoire
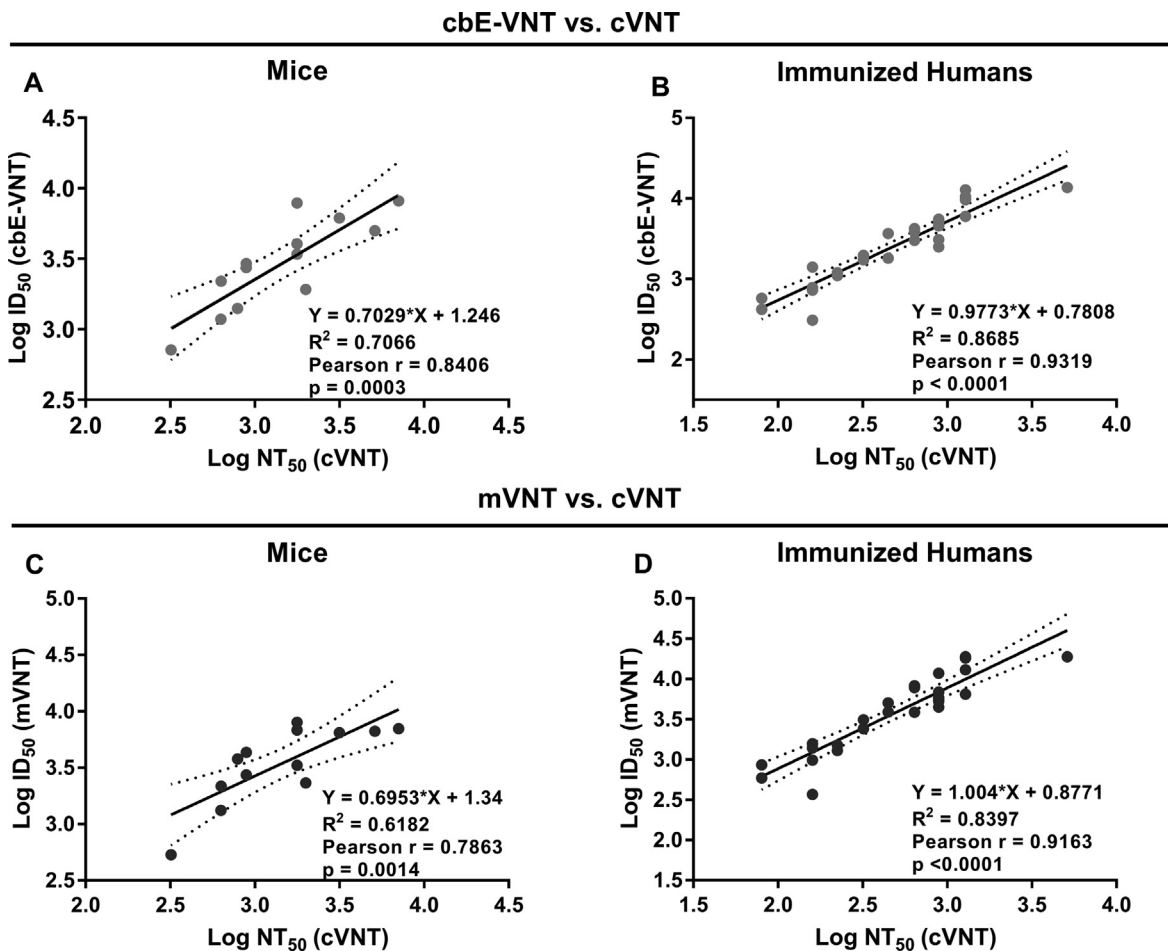
cbE-VNT vs. cVNT

A   Mice

$Y = 0.7029*X + 1.246$
$R^2 = 0.7066$
Pearson r = 0.8406
p = 0.0003

B   Immunized Humans

$Y = 0.9773*X + 0.7808$
$R^2 = 0.8685$
Pearson r = 0.9319
p < 0.0001

mVNT vs. cVNT

C   Mice

$Y = 0.6953*X + 1.34$
$R^2 = 0.6182$
Pearson r = 0.7863
p = 0.0014

D   Immunized Humans

$Y = 1.004*X + 0.8771$
$R^2 = 0.8397$
Pearson r = 0.9163
p <0.0001

Fig. 8. Correlation between cbE-VNT and cVNT (A-B) or mVNT and cVNT (C-D). The neutralization titers of RBD-immunized mice and humans sera were used for the analysis. Linear regression and correlations were performed in GraphPad Prism using Pearson's correlation coefficients. Statistical significance was calculated using the two-tailed test. The data presented are the log of the ID50 value for cbE-VNT or mVNT, and the log of NT50 of cVNT. The dashed lines indicate the standard deviations of the linear regression plots.

(in terms of dominancy) which, in consequence, could lead to distinct, and in this case, lower correlation values. Other influencing factor could be the smaller size of the sample for the study with rodents. Anyway, the correlation coefficient with cVNT titers in non-human samples is less relevant as the predictive value of this variable for protection is essentially meaningful in the clinical setting.

Summarizing, our results support the use of cbE-VNT to measure NAbs in an easy and safe manner and with the natural form of ACE2. The application of this method can be extended to monitor the durability of immune response and even to predict the potential impairment of neutralizing response against emergent SARS-CoV-2 mutants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Research ethics approval

The studies were approved by the Ethics Committee of Institute of Hematology and Immunology (IHI) and by the Ethics Committee of National Toxicology Center (CENATOX).

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ICMJE Criteria

All authors attest they meet the ICMJE criteria for authorship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.02.044.

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