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Standardization of Viral Neutralization Assay Used in To the Determination of Neutralizing Antibodies in the Evaluation Studies of the Cuban Vaccines against COVID-19

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ABSTRACT

The epidemiological situation created by SARS-CoV-2 imposed the challenge of developing vaccines and demonstrating their efficacy through a test capable of detecting functional neutralizing antibodies. It was proposed to standardize of viral neutralization assay used in the determination of neutralizing antibodies in the evaluation studies of the Cuban vaccines against COVID-19. Serial doubling dilutions of the serum samples were used and mixed with equal volumes of strain D614G at different infective doses and concentrations of Vero E6. The neutralization titer was determined by the colorimetric method and the cytopathic effect. 24 hours were defined as virus harvest time, the fourth day as the optimal time for reading the assay, $2x10^4$ cells/well as cell concentration, and 100 $TCID_{50}$ as optimal infective dose. The analytical specificity was 100%, as well as the specificity and diagnostic sensitivity. There was a high correlation between the micro neutralization assay and the UMELISA anti RBD system and a strong association with the molecular neutralization test. There were no differences between the cytopathic effect readings between the two reading methods. It was shown that it is a sensitive, specific and precise method, which guarantees the feasibility of its use in the evaluation of vaccine candidates against COVID-19.

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Abbreviations

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus Type 2
COVID-19: Coronavirus Disease 2019
RBD: Receptor Binding Domain
Ab: Antibodies
Nab: Neutralizing Antibodies
rtRT-PCR: Real-Time PCR
CPE: Cytopathic effect
$TCID_{50}$: Tissue Culture Infective Dose Medium
VNT: Viral neutralization titer
SD: Standard Deviation
CV: Coefficient of Variation
OD: Optical Density

Introduction

Since its emergence in late 2019, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), the etiologic agent of coronavirus disease 2019 (COVID-19), has caused more than 768 million cases and more of 6.9 million deaths worldwide [1]. SARS-CoV-2 belongs to the genus Betacoronavirus of the *Coronaviridae* family. It is an enveloped virus, with a linear, single-stranded, positive-sense RNA genome, and encoding four structural proteins: spike (S), envelope, membrane, and nucleocapsid proteins [2-4]. The S protein binds to the host cell mediating entry of the virus. It is composed of the S1 subunits, which promotes adhesion, and S2, responsible for membrane fusion. The receptor binding domain (RBD) is the fraction of the S1 subunit that binds to host cell angiotensin-converting enzyme 2 (ACE2). SARS-CoV-2 uses this enzyme and the TMPRSS2 serine protease for penetration. In this way, the S protein is considered a target in the inhibition of cellular access of the virus [5-7]. Neutralizing antibodies (NAb) against SARS-CoV-2 are mainly stimulated by the RBD. These antibodies are produced by B lymphocytes weeks after infection or immunization and can protect cells from virus entry and confer protective immunity. NAbs can inhibit infection in the process of viral attachment and entry into host cells by interfering with the binding or interaction of the SARS-CoV-2 RBD and the host cell surface receptor, hACE2 [8-13]. Since the beginning of the SARS-CoV-2 pandemic, researchers have a specific interest in determining and quantifying NAbs, as it allows them to explore

the relationship between the level of NAbs and the severity of the disease; predict the possibility of reinfection in patients with COVID-19; determine protection and its durability in naturally infected individuals, evaluate the efficacy of immunotherapy and the immunogenicity of vaccine candidates [14,15].

The main serological tests developed for the detection of antibodies (Ab) are based on enzyme-linked immunosorbent assays (ELISA) that which have a high performance, great determination capacity and do not require the use of a containment laboratory for their performance. These tests, however, cannot discriminate between NAb and non-neutralizing. It is known that only a small subset of specific Abs against the virus is capable of neutralizing its infectivity and, therefore, protecting against future infections and diseases [16,17]. The neutralization assay is the most specific and sensitive serological test capable of evaluating and detecting functional NAbs, considered the gold standard. It consists of mixing a defined quantity of virus with serial dilutions of the serum to be evaluated. After incubation, to allow possible neutralizing activity, the virus/serum mixture is inoculated into a culture of susceptible cells and incubated at a suitable temperature for a determined time. It is then examined for virus-induced cytopathic effect (CPE) production or some other indicator of viral growth. The degree of infection is compared in the absence of serum and in the presence of e different dilutions of the serum. The dilution which 50% of the infection is inhibited, is called the 50 inhibitory concentration and the dilution of the serum that achieves, it is the neutralization titer. Several formats have been developed based on this principle, including the micro neutralization assay [14,18-21].

The test is read-out using the classical method of detection by microscopy or by some colorimetric method such as neutral red analysis. This is a test of cell viability, based on the ability of living cells to incorporate and combine neutral red within lysosomes, and is generally used in adherent cells. The absorption of neutral red by the cell can decrease as a result of irreversible changes in the cell membrane. The amount of dye incorporated into the cell is measured by spectrometry, and is directly proportional to the number of cells with intact membranes [22]. The neutralization assay is a fundamental test in studies of virology, immunology, vaccine evaluation and epidemiology. However, it has limitations due to the high infectivity and pathogenicity of SARS-CoV-2, which needs to be handled in security level 3 facilities. The objective of this work is standardized of viral neutralization assay used in the determination of neutralizing antibodies in the evaluation studies of the Cuban vaccines against COVID-19.

Materials and Methods

All operations involving the cultivation of SARS-CoV-2 were performed at the Level 3 Biological Safety Facility (NSB3) of the Civil Defense Scientific Research Center (CICDC) (Mayabeque, Cuba).

Samples

Panel separate of 1: 30 serum samples from COVID-19 convalescent.

Panel 2: Made up of 40 serum samples, negative for antibodies against SARS-CoV-2 (collected in January 2019), with the presence of Ab and/or antigens to other viral agents: T-cell lymphotropic virus (n=5), Hepatitis C virus (HCV) (n=10), Hepatitis B virus (HBV) (n=10), Human Immunodeficiency Virus type-1 (HIV-1) (n=10) and Virus of Human Immunodeficiency type-2 (HIV-2) (n=5).

Panel 3: Composed of 40 samples from patients with respiratory manifestations, initially suspected of COVID-19, who were negative by real-time PCR (rtRT-PCR) for SARS-CoV-2.

Panel 4: Made up of 79 serum samples from healthy individuals, negative for HIV, HBV, HCV markers and Ab against SARS-CoV-2, obtained in January 2019.

Panel 5: Made up of 394 samples from immunized individuals with different levels of Ab against SARS-CoV-2.
Serum samples were inactivated at 56°C for 30 minutes (min) before being assayed.

Cell Substrate

VERO E6 cells [Vero 76, clone E6, Vero] (ATCC ® CRL-1586™) monkey kidney epithelial cell line (Cercopithecus aethiops), were cultured with Minimum Essential Medium (MEM) (Gibco, UK), supplemented with 10% fetal bovine serum (FBS) (Capricorn, Germany), 25 mM L-glutamine (Sigma, EU), 2 µg/mL sodium bicarbonate (MercK, Germany) (M10) and incubated at 37° C in a humid atmosphere with 5% CO_2. Cell banks were prepared to limit and control the number of passages.

Viral Strain

The strain D614G lineage B1 of SARS-CoV-2 CUT 2010-2025 (GISAID Code: EPI-ISL-7495115) was used, isolated from the nasopharyngeal exudate of an asymptomatic Cuban patient, diagnosed with COVID-19 by the rtRT-PCR assay, according to Noa et al (2020) [24].

Procedure

Modified Micro Neutralization Test

The assay was standardized according to the method developed by Manenti et al. Briefly, serial doubling dilutions (from 1/5) of the heat-inactivated serum samples were mixed with equal volumes of 100 mean tissue culture infective dose ($TCID_{50}$) of the CUT2010-2025 strain, the mixture was incubated for 60 min at 37°C, 100 µL of the serum-virus mix was added to semi-confluent Vero E6 monolayers ($2x10^4$ Vero E6 cells/well) in 96-well microtiter plates. The cells were incubated during four days in a humid atmosphere with 5% CO_2 [22]. In each assay, a cell control with Vero E6 cells, 100 $TCID_{50}$ viral control, positive control serum and negative control serum were incorporated.

Colorimetric Method with Neutral Red

At the end of the incubation period of the micro neutralization assay, the supernatant of the 96-well plates was removed, 100 µL of 0.02 % neutral red solution in Phosphate Buffered Saline pH 7.2 (PBS) was added to each well and the plate was incubated for one hour at room temperature (25+ 2°C) in the dark. At the end of the incubation time, the neutral red solution was removed and the cells were washed twice with a 0.05% solution of Tween 20 (Chem Lab NV, Belgium) in PBS. 100 µL of lysis buffer (96% ethanol [Labochem® international; Germany]/ultrapure water/glacial acetic acid [Merck, Germany] in the volume ratio 50:49:1) was added per well and the plates were incubated for 15 min at room temperature (20-25ºC) in the dark. The reading was carried out at a wavelength of 540 nm in a PR 621 Plate Reader (Immunoassay Center, Cuba) [22].

Interpretation of Results

Viral neutralization titer (VNT) by CPE: VNT was considered the highest serum dilution in which CPE was not visualized. VNT by colorimetric method: The highest dilution of the serum evaluated with an OD value greater than the cut-off value was considered as

VNT. The cut-off value was calculated as the average of the cell control optical density values divided by two [22].

Standardization of The Micro Neutralization Assay Procedures
Propagation and Titration of The Virus in Vero E6 Cells
The SARS-CoV-2 virus was inoculated into three 75cm2 flasks (Nunc, Denmark) containing Vero E6 cells, seeded 10^6 cells/mL in M10 medium and incubated for 18 to 24 hours (h) at 37°C in humid atmosphere with 5% CO_2. Subsequently, the supernatant was removed and the semi confluent cell monolayer was washed twice with PBS. This solution was removed and virus was added at a multiplicity of infection (MOI) of 0.07. It was incubated for one hour at 37°C in a humid atmosphere with 5% CO_2. Once the incubation time had concluded, 10 mL of MEM medium supplemented with 4% FBS and 80 µg/mL of gentamicin (Sigma, USA) (M4) was added and incubated at 37ºC in a humid atmosphere with 5% CO_2. A cultured of uninoculated Vero E6 cells was used to rule out cell death, if any, from CPE. Each culture flask was subjected to a cycle of freezing (-85°C) and thawing (37°C) at 24, 48, and 72 h. Subsequently, it was harvested and centrifuged at 500 x g. The supernatant was filtered through a 0.45 µm cellulose acetate (CA) membrane (Nalgene, Mexico) and 500 µL aliquots were made and frozen at -85°C until titration was carried out. This experiment was repeated three times. The corresponding harvest time with the maximum viral production and a CPE of 80 to 95% was selected.

The virus was titrated by the endpoint method, in 96-well plates (Nunc, Denmark). To serial dilutions in base 4 of the virus, in medium supplemented with 2% FBS and 40 µg/mL of gentamicin (Sigma, USA) (M2), Vero E6 cells in suspension were added, at a concentration of 10^4 cells/well. Eight replicates of each dilution were made in three independent experiments. The plates were incubated at 37°C in a humid atmosphere with 5% CO_2 and were observed daily under an inverted light microscope (MoticTMAE 2000, China) to visualize the presence or absence of CPE. TCID50 was evaluated on the fourth and seventh day and was calculated by the Reed & Muench method [23-25].

Evaluation of Cell Concentration for Micro Neutralization Assay
Cell suspensions were prepared with 10^4, $2x10^4$ and $2.5x10^4$ cells/well. Each cell suspension was diluted in M10 medium and dispensed 100 µL/well into 96-well plates. Vero E6 cells were incubated at 37°C in a humid atmosphere with 5% CO_2. Between 18 and 24 h after sowing, the medium was changed and MEM supplemented with 2% SFB and 80 µg/mL of gentamicin (M2) was added, incubated under the same conditions during four days. Cell viability was determined by the trypan blue exclusion method (Merck, Germany). As criteria for acceptance of cell concentration, the confluence of the cell monolayer > 85% and cell viability > 95% were taken.

To study the repeatability of the cultures between the wells and the intermediate precision between days, three plates were seeded on three different days, with the optimal cell concentration, under the previously mentioned conditions. After this period, the cultures were stained with the 0.02% neutral red dye (Sigma, EU) and the reading was carried out.

Determination of the Infective Dose of SARS-CoV-2 For the Micro Neutralization Assay
To determine the infective dose of SARS-CoV-2 to be used in the micro neutralization assay, three doses of virus were compared: 25, 100, and 200 $TCID_{50}$ in 50 µL. Double serial dilutions of samples (Panel 1) were mixed with an equivalent volume of the viral suspension; the dilutions were made with the M2 medium. The serum-virus mixture was incubated for one hour at 37°C in a humid atmosphere with 5% CO_2, added to a 96-well plate with a monolayer of Vero E6 cells seeded $2x10^4$ cells/well. The plates were incubated for four days under the same conditions. Visualization of the CPE was performed under an inverted light microscope and colorimetric with neutral red. A cell control with Vero E6 cells and a viral control of each dose of virus were included in each plate.

Evaluation of the Micro Neutralization Assayneutralization Assay Performance Evaluation
For the evaluation of the performance of the standardized neutralization assay, what was regulated by regulation No. 41 related to the validation of analytical methods, of the Center for State Control of Medicines, Medical Equipment and Devices (CECMED), regulatory agency of the Republic of Cuba, was followed [26]. Analytical specificity: To evaluate this parameter, the samples of Panel 2 and 3 were used, undiluted and in dilutions 1:5, 1:10. Diagnostic specificity: To calculate this parameter, the samples from Panel 4 were used. Diagnostic sensitivity: Panel 1 was used to evaluate this parameter.

Correlation of Micro Neutralization Assay with the Umelisa SARS-CoV-2 Anti Rbd and The Molecular Neutralization Test
The samples belonging to Panel 5 were studied to determine the correlation between the neutralization assays and the Umelisa SARS-CoV-2 anti RBD (Immunoassay Center, Cuba), which is a quantitative assay that allows determining the levels of IgG to the RBD protein of the virus in serum or plasma samples and the molecular neutralization assay that relies on antibody-mediated blockade of the RBD: hACE2 interaction. The latter uses recombinant RBD-mouse-Fc (RBD-Fcm) and the host cell receptor (hACE-Fc) as coating antigen. Human antibodies against RBD can block RBD-Fcm interaction with hACE2-Fc. RBD-Fcm that was not inhibited can bind to hACE2-Fc and is recognized by an alkaline phosphatase-conjugated anti-murine monoclonal antibody. The ratio of inhibition of the RBD: hACE2 interaction at a serum dilution of 1/100 and the maximum mean molecular virus neutralization titer (mVNT) are calculated; mVNT is the serum dilution that inhibits 50% of the RBD: hACE2 interaction [27].

Comparison Read Out Methods
In order to compare read out methods the neutralization test (visualization of the CPE and colorimetric with neutral red), the samples of Panel 1 were used, which were evaluated by two operators at two different moments.

Statistical Analysis
Statistical analysis was performed using the Graphpad Prism version 8.0.1 for Windows system (Graphpad Software Inc, San Diego, California, USA), measures of central tendency and dispersion (mean, standard deviation [SD], maximum and minimum values were estimated). Friedman's non-parametric test and Dunn's multiple comparison test were applied to compare the viral titers obtained in the experiment on the days of virus harvest, the geometric means of the NAb titers of the experiments to determine the infective dose of SARS -CoV-2 for the micro neutralization assay and the methods for reading the assay. The data resulting from the experiment on the days of reading the microtiter assay were analyzed with the Student's t test. The value of $p < 0.05$ was considered statistically significant. To demonstrate the precision of cell cultures for the micro neutralization assay, the mean, standard deviation (SD), and coefficient of variation

(CV) of the optical density (OD) were calculated. To determine the correlation of the neutralization assays with the Umelisa SARS-CoV-2 anti RBD and with molecular neutralization, Spearman's correlation coefficient was used.

Results and Discussion

The spread of SARS-CoV-2 and the impact of COVID-19 gave rise to an emergency response from the global scientific community. This response focused on the development of multiple pandemic control tools. With unprecedented speed, vaccines were developed and, with emergency use approval, have been supplied in different parts of the world [25]. Cuba is part of the small group of nations that have managed to develop specific vaccine projects against SARS-CoV-2 with three vaccines and two vaccine candidates. Therefore, it became necessary to have an assay that would allow evaluating the immunogenicity and level of protection of the immunogens [28].

During the development of vaccine candidates, it is important to demonstrate that the virus capable of producing an infection can be efficiently neutralized and evidenced by using an in cur sic letter system. In the case of vaccines against COVID-19, methods have been used to demonstrate the ability of NAbs to block the interaction of the virus spike protein and ACE2 of the host cell [29].

Standardization of the micro neutralization assay propagation and titration of the virus in cell culture

The virus harvest time was defined as 24 hours, which corresponds to the day of maximum viral production. The CPE in the cell monolayer at 24 h was 80% and an increase of 95 and 100% was observed at 48 and 72 h, respectively. Figure 1 shows the viral titers of the three experiments in which different virus harvest times were evaluated. At 24 h, the highest viral titer was obtained with mean values of $10^{6.691}$ $TCID_{50}$/mL ± 0.14 SD; while the viral titer at 48 and 72 h after infection was $10^{6,483}TCID_{50}$/mL ± 0.06 SD and $10^{6,328}$ $TCID_{50}$/mL ± 0.02 SD, respectively. According to the Friedman test, there were significant differences ($p<0.05$) between the viral titers at 24 and 72h, and there were no differences between the viral titers at 24 and 48h, nor between 48 and 72h.

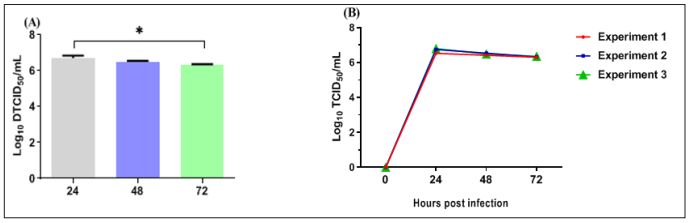

Figure 1: Viral Titer at Three Harvest Moments of The Virus

A- Viral titers at 24, 48 and 72 h post infection (n=3). A significant increase in the viral titer was achieved after 24 h. According to the Friedman test ($p < 0.05$), there were statistically significant differences between the viral titers obtained at 24 and 72 h (*), the error bars indicate the standard deviation between the three independent means B–Curve of the Replication kinetics of three independent experiments.

The comparison of the infectivity of the virus on the fourth and seventh day is shown in Figure 2. The viral titers on the fourth and seventh day of virus culture ($10^{6.417}$ ± 0.115 SD and $10^{5.972}$ ± 0.413 SD $TCID_{50}$/mL, respectively) did not show significant differences, according to the Student t test. The fourth day was selected as the optimal time to read the test since the highest viral

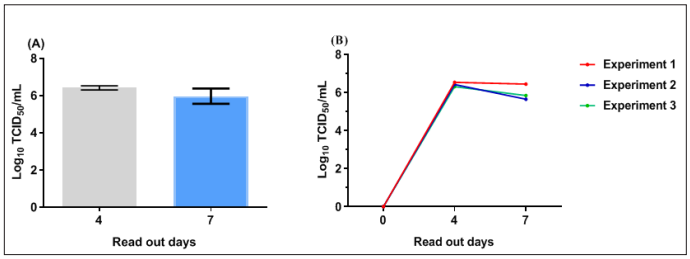

Figure 2: Viral Titer A Two Moments of Reading the Viral Microtiter Assay, Fourth and Seventh Day Post Infection

A - Viral titers on the 4th and 7th days of reading, in triplicate (n=3). A higher viral titer was reached on the 4th day of reading without significant differences with the 7th day, according to Student's t-test, the error bars indicate the standard deviation between the three independent means. B-Viral titer of three independent experiments at two reading times of the micro neutralization assay.

Cell Concentration for Micro Neutralization Assay

The concentration $2x10^4$ Vero E6 cells/well met the established acceptance criteria for the neutralization assay (85% cell monolayer confluence and 95% viability); while with 10^4 cells/well the confluence was <85% and for $2.5x10^4$ cells/well, the viability was less than 95%. The mean values of the colorimetric reading in the three assays with $2x10^4$ cells/well (0.9728 ±0.1126 SD, 1.021 ± 0.08183 SD and 1.028 ± 0.08402), showed a CV of 11.58%; 8.02% and 8.17% respectively.

Sars-Cov-2 Infective Dose for the Micro Neutralization Assay

Samples from Panel 1 neutralized all three infective doses of the virus. Statistical analysis showed significant differences between NAb titers when using 25, 100 and 200 $TCID_{50}$ of virus. According to the results, 100 $TCID_{50}$ were selected. Figure 3 shows the NAb titers obtained with the three doses of virus.

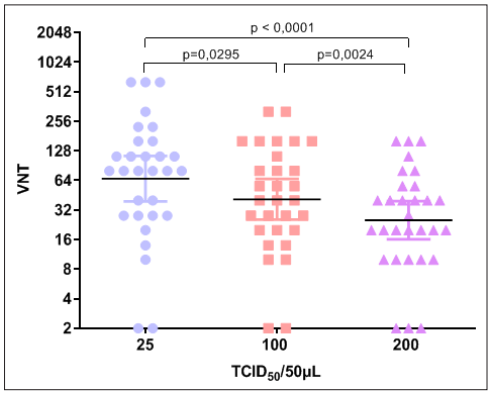

Figure 3: Virus Neutralization Titers (VNT) of Serum Samples From COVID-19 Convalescent Against Three Different Mean Infective doses of SARS-CoV-2

According to the Friedman test ($p < 0.05$) there were statistically significant differences between the geometric means of the VNT obtained: between 25 and 100 $TCID_{50}$ (p=0.0295), between 100 and 200 $TCID_{50}$ (p=0.0024) and between 25 and 200 $TCID_{50}$ ($p < 0.0001$). The error bars indicate a 95% confidence interval between the means of the VNTs.

Evaluation of the micro neutralization assay Neutralization assay performance evaluation

The neutralization assay did not show non-specific reactions with samples from patients with the presence of antibodies against other

viral infections, nor with serum samples from people with non-COVID19 respiratory diseases; while the 79 sera from individuals with no history of COVID19, belonging to Panel 4 did not show VNT against SARS-CoV-2, with an analytical and diagnostic specificity of 100%, respectively. The assay detected NAb in the 30 samples from COVID-19 convalescent belonging to Panel 1, with a diagnostic sensitivity of 100%.

Correlation of the Neutralization Assay with the Umelisa SARS-CoV-2 Anti Rbd and the Molecular Neutralization Test

The correlation between the micro neutralization assay and the Umelisa SARS-CoV-2 anti RBD was significant ($p<0.0001$), with a correlation coefficient of 0.8430 (CI: 0.8108 to 0.870), as shown in Figure 4A. Similarly, the correlation between the micro neutralization assay and the molecular neutralization assay demonstrated a strong association ($p<0.0001$), with a value of $r=0.8598$, (CI: 0.8294 to 0.8836) as shown in Figure 4B.

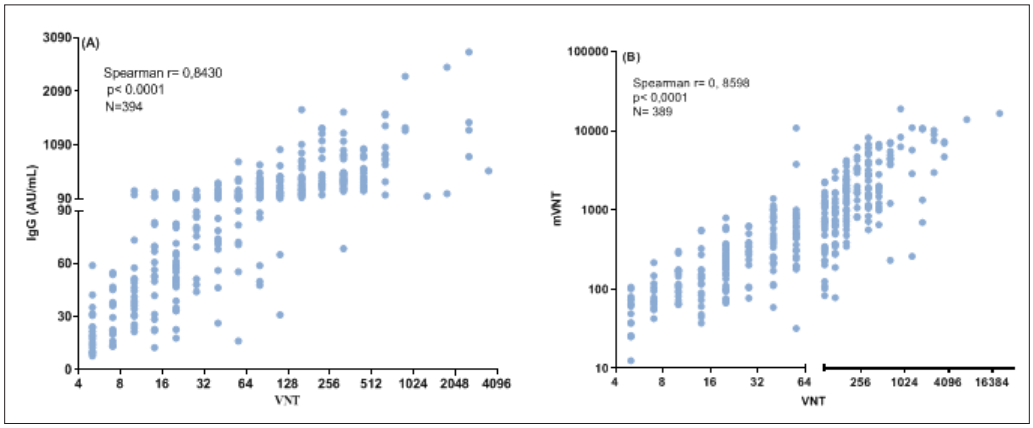

Figure 4: Analytical Correlation Between Serological Assays to Detect Antibodies Against SARS-CoV-2

A- Evaluation of the sera from Panel 5 with the micro neutralization assay and the Umelisa SARS-CoV-2 anti RBD. B-Evaluation of the sera from panel 5 with the micro neutralization assay and the molecular neutralization test.

Comparison Read Out Methods

As shown in Figure 5, the VNT obtained by the two reading methods, CPE and colorimetric, by the two operators did not show statistically significant differences with a value of $p=0.1748$.

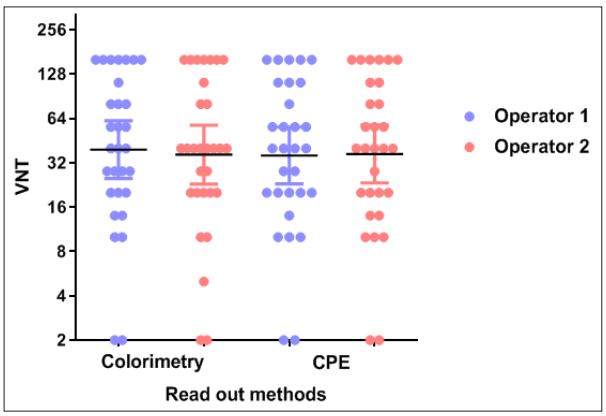

Figure 5: Virus Neutralization Titers (VNT) in Samples from Panel 1 of Sera from Convalescents to COVID-19 Determined by Two Operators by Two Reading Methods, Neutral Red Colorimetric and CPE Visualization

According to the Friedman test ($p < 0.05$) there were no statistically significant differences between the TNVs. The error bars indicate a 95% confidence interval between the means of the TNVs.

The viral neutralization assay is considered the gold standard, which uses human cells that express ACE2 receptors and interact with a mixture of infective SARS-CoV-2 virus and human or animal serum with or without the presence of NAb. However, these methodologies must be evaluated to demonstrate the performance of each parameter. As recommended by the United States Pharmacopeia (USP), the virus dose, the incubation time of the post-inoculation assay, and the detection method must be validated [14,17,30]. USP proposes inoculating susceptible cells with a relatively low multiplicity of infection (MOI < 0.1). In the study, for the production of the viral inoculum, the viral culture was expanded to a MOI of 0.07, as suggested by the USP [30]. With this dose of virus, maximum viral production was reached at 24 h. Manenti et al. (2020) obtained an increase in the viral titer between 48 and 52 hours of infection when using a MOI of 0.01. However, Aguilar et al. used an MOI of 0.1 to amplify the culture of the Hepatitis A virus (HAV) that they harvested on the seventh day [22-30].

The relationship between the day of reading the assay and the cell culture conditions has a crucial role in the calculation of the cut-off value and in the TNV of the serum. The study showed that the fourth day is the optimal time to read the assay, similar to Manenti et al. (2020). However, Salazar et al. carried out the reading at 72 h using a colorimetric method with crystal formaldehyde violet.

But Oguntuyo et al. in their study, performed the reading at 48 h when using a higher infectious dose [22,32,33].

The literature reports that the line cell excellence for isolation and replication of SARS-CoV-2 is Vero E6, due to its high expression of ACE2 receptor [21,22]. The results of previous investigations corroborate this fact, since this cell line was used for the isolation of the virus from the nasopharyngeal exudate of patients and for its adaptation in culture [24]. Cell culture infection may be inefficient if undergrown or overgrown cell monolayers are used. The results in relation with the selection of cell concentration coincide with that described by Amanat et al. who used $2x10^4$ cells/well in a 96-well plate for the microneutralization assay, however the author uses a viral dose of 600 $TCID_{50}$ in 60 µL and the infection process (virus-cell contact) is maintained for only 1 h (they eliminate the virus-cell mixture after one, add fresh medium and continue with the incubation), an element that also differs. Similarly, Dolscheid et al. in their study used the same cell concentration but with cells in suspension and incubated for 48 h [22,34]. On the other hand, Algaissi et al. describe a protocol for a micro neutralization assay to evaluate neutralizing antibodies against the Middle East Severe Respiratory Syndrome Coronavirus (MERSCoV) in which they use 10^4 cells/well and 120 $TCID_{50}$ in 60µL with a assay incubation time of 72 h [16].It was demonstrated that there is precision between the cell cultures of each plate and between different plates at different times, with a CV of less than 20%, acceptable values for tests in which cell cultures are used, according to the Cuban regulatory agency [26].

Virus dose is an important variable in the neutralization assay. $TCID_{50}$ is used to quantify viral titers by determining the viral concentration at which 50% of infected cells present CPE [35]. In the assay, the dose of 100 $TCID_{50}$ in 50 µL was useful, since the antibodies present in the samples were capable of neutralizing the virus and it was selected as the optimal infective dose of SARS-CoV-2 for the micro neutralization assay, in accordance with the USP recommendations [30]. The geometric mean of the VNT obtained with the use of the lowest infective dose, were 1.6 and 2.6 times higher when compared with those achieved when using 100 and 200 $TCID_{50}$ in 50 µL, respectively. The VNTs of the trial in which 25 $TCID_{50}$ were used are more dispersed in relation to the trial that was carried out with 100 $TCID_{50}$. In addition, the geometric mean of the VNT obtained when using 100 $TCID_{50}$ is 1.6 times higher than with 200 $TCID_{50}$. Similar results were obtained by Manenti et al. (2020) when evaluating 83 serum samples with 25 and 100 $TCID_{50}$ of SARS-CoV-2 in 50 µL, where in some cases they reached VNT in a higher dilution with the use of the dose of 25 $TCID_{50}$/50 µL in relation 100 $TCID_{50}$/50 µL. Pisanic et al. (2023) used the same dose in their research (100 $TCID_{50}$) but in 100 µL [36]. However, Amanat et al. (2020) in their study used a much higher dose of virus as discussed above [21,22].

Umelisa SARS-CoV-2 anti RBD and the molecular neutralization test show a strong positive association with the viral microneutralization assay. The results of the study support that the total IgG titers against the spicule with the use of Umelisa SARS-CoV-2 anti RBD allow estimating the neutralizing capacity present in sera of people vaccinated or who have been infected with SARS-CoV-2. Similar results to those obtained in the investigation were reported by Lemos et al (2022) when demonstrating a positive and strong correlation between the viral micro neutralization assay and the Elecsys® Anti-SARS-CoV-2 System (Roche Diagnostics, Switzerland) [37]. Similarly, Perera et al. (2020), who developed an ELISA test, as well as Ojeda et al. (2021) using the COVIDAR® IgG ELISA (Laboratorios Lemos S.R.L, Argentina), found highly significant correlation values between NAb and anti-RBD IgG titers [19,38]. On the other hand, Pi et al. (2022) in their study demonstrated a better correlation between VNT and mVNT ($r = 0.91$, $P < 0.0001$) in human sera than those presented in the study [27].
The traditional method of reading the neutralization assay by visualizing the CPE with an inverted light microscope consists of monitoring the cell monolayer infected with SARS-CoV-2 to observe morphological changes. This method involves intense work and, above all, the experience of the observer, since it is difficult to estimate the relationship between the percentage of infected and uninfected cells [22].

Neutral red is a weak cationic dye that readily penetrates the cell membrane and accumulates intracellularly in lysosomes where it combines with the anionic part of the lysosomal matrix. The changes in the lysosomal membrane led to the fragility of lysosomes and gradually become irreversible. Such changes occurred by the action of xenobiotics result in a decrease in the absorption and combination of neutral red. Therefore, it is possible to distinguish viable, damaged or dead cells, principle on which the method is based [23].

In this study, it was shown that the colorimetric method turned out to be an effective method based on a quantifiable value of the optical density and that it makes it possible to increase the performance of the assay by allowing the processing of a larger number of samples, by reading the plates in an automated way.

Conclusion

The SARS-CoV-2 microneutralization method was standardized with adequate performance. The parameters evaluated demonstrated that it is a sensitive, specific and precise method, which guarantees the feasibility of its use to determine levels of neutralizing antibodies of individuals who were exposed to the infection and the evaluation of the sera of immunized individuals with the vaccine candidates against the COVID-19. The method allowed the evaluation of the serum samples from the preclinical and clinical trials of the vaccine candidates and Cuban vaccines against COVID-19.

Conflict of Interest

The authors declare that there is no conflict of interest.

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