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Commentary

Avidity assay to test functionality of anti-SARS-Cov-2 antibodies

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1. Commentary

The ongoing status of the Coronavirus pandemic in Brazil, as notoriously reported (<https://covid.saude.gov.br/>), highlights the public health efforts still necessary to comply with the World Health Organization (WHO) directives in order to control and eradicate the coronavirus disease 2019 (COVID-19) and prevent coronavirus spread around the world.

Brazil is the fifth largest country in the world in terms of territory (8,514,876 km²), only, behind Russia, Canada, China and the United States. By way of comparison, Brazil is slightly smaller than the whole Europe, which has about 10.5 million km². Besides Brazilian geographic space, also the natural environment of our country is large and diverse, presenting the most distinct characteristics. Hence, Coronavirus pandemic must also be considered from this perspective, since the challenge to control the disease is unique in a Country with those characteristics.

Until immunity will be successfully induced in large population throughout the world, SARS-CoV-2 infections are likely to become endemic seasonally, causing, therefore the COVID-19 in millions of people worldwide, even in the future. Large-scale testing to identify infected individuals, isolation of infected cases, and tracing and quarantine of the contacts are crucial steps to control the disease. Thus, reliable, inexpensive, and preferentially, point-of-care (POC) diagnostic tests are really welcome [1], but can be a challenge. While the main direct diagnostic method used worldwide, the RT-PCR, depends on expensive equipment and skilled personnel to be performed, the POC diagnostic available to detect antigen or anti-SARS-CoV-2 antibody, such as lateral flow tests, lacks sensibility.

Indirect diagnostic, based on antibody detection, also depends on the development of humoral immune response to the infection, what can take some days or even weeks to be detectable [2], reducing the sensibility of the diagnostic tests in the early stages of infection. In order to achieve high sensibility, specificity and POC diagnostic tools, new labelled or label-free platforms of detection

of the virus or anti-virus antibodies have been developed, including near-infrared-fluorescence amplification by nanostructured plasmonic gold substrates [3]; silicon photonics biosensors or nanoplasmonic ones, such as surface plasmonic resonance (SPR) or localized SPR (LSPR), with promising results [4]. On the other hand, enzyme linked immunosorbent assay (ELISA) is an enzyme-labeled, well established platform to perform diagnostic. The indirect ELISA to detect anti-SARS-CoV-2 antibody also has the limitation of lack of sensibility during the first days of infection, however, a modified ELISA to measure antibody avidity can be a valuable tool to measure how antibodies affect the natural course of viral infections, what is really important to study immunity [5,6]. Moreover, since antibodies persist for a relatively long period of time, indirect diagnostic tools are valuable to surveillance and epidemiological studies [7]. Although IgM and IgG can be detected as early as two days after symptoms appearance the sensitivity of the serological tests increases up to 14 days in PCR positive patients, showing a heterogeneous response in the population [8], with IgG been detected in up to 100% of individuals by day 14 post infection [2,4].

The avidity (“functional affinity”) of an antibody is measured thru the overall strength of the interactions between the antibody and the antigen. The avidity of virus specific IgG antibody is usually low during primary viral infection and increases with time, as described [9–11]. At the moment, we can use the avidity test for the evaluation of the functionality of antibodies produced in order to cope with bacteria, parasites or even with other virus [12–15]. Additional methods to access the presence of receptor-binding antibodies into the patient serum, and consequently, to access antibody functionality have been developed. Walker et al. [16] used two different platforms with this objective, an ELISA test that can measure competition and blocking of the angiotensin-converting enzyme 2 (ACE2) receptor to the SARS-CoV-2 spike protein with anti-spike antibodies and a SPR based method, that can quantify both antibody binding to the SARS-CoV-2 spike protein and blocking to the (ACE2) receptor in a single experiment.

The production of antibodies in response to infection or vaccination is an important process to prevent infectious diseases. The reaction of the antibody to the antigen is a non-covalent interaction [17]. It has been shown that affinity of antibodies increases with time, through a process named affinity maturation, which is

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a consequence of B-cells somatic hypermutation. This reaction occurs in the germinal centers and requires the support provided by follicular dendritic cells and T-helper cells, thus generating antibodies that bind faster and with more strength to the antigen [18]. Antibody avidity can be measured by different methods [14], and the most common is a modified ELISA [17]. Although the COVID pandemic is recent and long term immunity studies had no time to be performed, an increase in anti-Sars-Cov-2 IgG avidity over time is expected, due to its similarity with SARS [3,19], for which significant increase in number of samples presenting high avidity antibodies from 11 to 269 days after infection was observed [19]. Moreover, for other infections, antibody avidity has been used to differentiate recent from past or primary from secondary infections, being also a potential tool for assessing vaccination efficacy, immunity and convalescent plasma-based antibody quality [3,19,20].

Up to now, as we know, in order to perform neutralization assays it is necessary to deal with infectious Coronavirus, making the use level 3 security labs mandatory. In order to simplify and verify antibodies functionality during different phases of infection [21] or vaccine evaluation [22,23] it would be interesting to use level 2 labs, instead. Thus, we propose to use the avidity assay, by means of a modified ELISA using a recombinant antigen and a chaotropic agent, such as thiocyanate, to measure the strength of the antigen-antibody reaction. If a test like this will have a good correspondence to neutralizing assay it can have a huge applicability, due to the lack of the necessity of working at level 3 labs.

To test our hypothesis we modified an assay developed to test avidity of anti-*Neisseria lactamica* OMV antibodies [24]. In our study, the ELISA plate was adsorbed with receptor binding domain (RBD) from SARS-Cov-2 protein S. The detection of neutralizing antibodies was performed using the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Inc.), following the manufacturer instructions, with murine sera. Sera were obtained from Swiss mice previously immunized with two antigenic formulation composed by recombinant RBD and *N. meningitidis* cellular components (OMVs) used in comparison to RBD alone (control). The antigens were administered twice intramuscularly and twice intranasally.

Table 1 summarizes our findings. In general, as higher the avidity as higher the virus neutralization, except for control animals, immunized with the RBD domain alone, in which an avidity between 18 and 25% corresponded to very low virus neutralization (up to 4%). However, it worth mentioning that in all cases, low level of avidity (up to 25%) corresponded to virus neutralization smaller

than 50%. On the other hand, when virus neutralization was superior to 55% the avidity index were intermediate (38–45%).

We have found a good correspondence between both techniques (ELISA-avidity × neutralization), with an overall numeric correlation of 0.75. If we do not consider the control animals, the Pearson correlation is 0.97, corresponding to a very strong correlation.

In humans, it was shown that IgG avidity increases over the duration of the infection. This avidity remained elevated during the period of observation. The high levels of avidity were associated with older age, male sex and hospitalization. A strong positive correlation between anti-spike titre and avidity and between these two indicators and the occurrence of virus neutralization was also demonstrated. However, the avidity of anti-nucleocapsid IgG was not statistically correlated with neutralization [25].

With these results we demonstrated that commercial neutralization methodology cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Inc.) can be used with murine sera and that a good correspondence between ELISA-avidity assay and neutralization was observed. Thus, although additional tests performed with naturally infected human sera are worthy of investigation, we postulated that ELISA-avidity could be used as an alternative to the neutralization assay, or an additional methodology to test antibody functionality, what has been demonstrated to have correlation with disease progression [26], besides having application to help the establishment of parameter for plasma donor screening for COVID treatment, as previously reported [25].

2. Methodology

Five Swiss female mice per group were immunized with recombinant receptor binding domain (rRBD) from SARS-Cov-2 protein S alone (controls) or with two different preparations: rRBD plus aluminum hydroxide at 0.1 mM and cellular compounds (OMV) (10 µg/ml) of *N. meningitidis* strains B:8:P1.6 (Prep1) or C:2a.P1.5 (Prep2). The antigens were prepared 1 h before immunizations. Animals were immunized twice intramuscularly and twice intranasally. Immunizations were given after 30 days apart. Sera were collected from ocular plexus 15 or 45 days after each immunization. All procedures with animals were approved by our Institutional Animal Care and Use Committee (protocol number 03/2012 extended to 2022). The avidity index of IgG antibodies was performed based on the methodology described by Vermont et al. (2002). Plates were adsorbed with rRBD. The procedure followed the same steps of ELISA assay, with an additional step after

Table 1
IgG Avidity index (%) and neutralization (%) antibodies in the serum of Swiss mice immunized with rRBD (control) from SARS-Cov-2 S protein or with two different antigenic preparations containing rRBD. Animals were immunized twice intramuscularly and twice intranasally. Sera were collected 15 or 45 days after the first (A) or the second (B) intranasal immunization.

| (A) After the first dose i. n | | | | | | |
|--------------------------------|---------|-------|-------|---------|-------|-------|
| Days after immunization | 15 | | | 45 | | |
| | Control | Prep1 | Prep2 | Control | Prep1 | Prep2 |
| Avidity index (%) | 25 | 12 | 15 | 18 | 43 | 38 |
| Neutralization (%) | 3 | 25 | 30 | 4 | 67 | 55 |
| (B) After the second dose i. n | | | | | | |
| Days after immunization | 15 | | | 45 | | |
| | Control | Prep1 | Prep2 | Control | Prep1 | Prep2 |
| Avidity index (AI) | 22 | 13 | 18 | 25 | 43 | 45 |
| Neutralization (%) | 2 | 29 | 28 | 1 | 77 | 67 |

*Avidity index: The criterion for assessing antibody avidity is as follows: above 50% high avidity; between 30 and 49% intermediate avidity; below 29% low avidity. Neutralization: The average optical density (OD) of the negative control was used to calculate the inhibition %. Results of each individual samples were calculated using the formula: (1 – OD value of sample/OD value of negative control) × 100. rRBD plus aluminum hydroxide at 0.1mM (control); or cellular compounds (OMV) (10 µg/ml) of *N. meningitidis* strains B:8:P1.6 (Prep1) or C:2a.P1.5 (Prep2).

serum incubation as previously described [27]. The criterion for assessing antibody avidity is as follows: above 50% high avidity; between 30 and 49% intermediate avidity; below 29% low avidity [27]. The detection of neutralizing antibodies was performed using the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Inc.), following the manufacturer instructions. Briefly, mice sera samples, positive and negative controls were diluted at 1:10 in sample dilution buffer, while HRP-RBD conjugated was diluted at 1:1000 in RBD dilution buffer. The samples and HRP-RBD solution were mixed at 1:1 vol and incubated at 37 °C for 30 min. Then, the mixtures were transferred for 96 well plates coated with ACE-2 and incubated at 37 °C for 15 min. Plates were washed four times with washer buffer. The substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added and plates were incubated for 15 min, at room temperature, in the dark. The reaction was stopped by adding the stop solution and read at 450 nm in a microplate reader (Molecular Devices). The Inhibition rate, in percentual, was calculated using Excel (Microsoft Office), using the following equation: Inhibition = $(1 - \text{OD value of sample} / \text{OD value of negative control}) \times 100$. In our studies, the antigenic preparation used showed antibodies with neutralizing capacity and a good correlation with avidity, however more studies are needed [28].

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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