

# Toward to establish selection criteria for *rapid* serological tests for COVID-19

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## Abstract

COVID-19, the new pandemic is associated to SARS-CoV-2 virus infection. Social distancing and the testing have been the principal measures that have shown to be effective for the reduction of critical cases. Although the gold standard for diagnosis of COVID-19 is the RT-PCR, rapid serological tests could also be used for prevalence studies, and for epidemiological monitoring. In order to characterize the humoral immune response, we analyzed eight immuno-chromatographic test and one ELISA test, as a verification or secondary validation analysis used positive and negative control serum samples. Sera from negative and positive individuals [asymptomatic or symptomatic individuals, outpatient or inpatient (intensive care unit)] were analyzed, and the following results were found: of all these rapid tests, only 4 exhibit clear banding patterns for IgG and two of these also showed results for IgM (only in a few symptomatic patients). Instead, with an ELISA test a preferential recognition was observed for symptomatic patients who were critically ill, whereas in asymptomatic individuals it did not show more than 25% of positivity. Understanding and validating molecular and serological tests are an essential component for the design of public health measures to response to the pandemic.

## Hacia la construcción de criterios para la selección de pruebas serológicas rápidas para COVID-19

### Resumen

COVID-19 la nueva pandemia está asociada con la infección por el virus SARS-CoV-2. La distancia social y el análisis masivo de muestras son las principales medidas que se han mostrado efectivas para la reducción de los casos críticos. Aunque el estándar de oro para el diagnóstico de COVID-19 es la RT-PCR, las pruebas serológicas rápidas podrían también ser usadas en estudios de prevalencia, así como en la vigilancia epidemiológica. Con el fin de caracterizar la respuesta inmune humoral, analizamos ocho pruebas inmunocromatográficas y una prueba de ELISA, en un proceso de verificación de desempeño o validación secundaria, usando sueros control positivos y negativos. Los sueros de pacientes positivos y negativos [individuos asintomáticos o sintomáticos, pacientes ambulatorios u hospitalizados (en cuidados intensivos)] fueron analizados, encontrando los siguientes resultados: de todas las pruebas rápidas examinadas solamente 4 mostraron un claro patrón de bandas para IgG, de éstas, dos de ellas también mostraron resultados para IgM (solamente en pocos pacientes sintomáticos). En cambio, con la prueba de ELISA un reconocimiento preferencial fue observado en pacientes sintomáticos con enfermedad crítica, mientras que los individuos asintomáticos no mostraron más de un 25 % de positividad. Comprender y validar las pruebas serológicas son componentes esenciales en el diseño de las medidas de políticas públicas como respuesta a esta pandemia.

## Introduction

The new pandemic associated to SARS-CoV-2 virus, causes a disease known as COVID-19<sup>1</sup>. Besides of documented rapid spreading, the virus is associated with a case fatality risk of at least 1.4%, specially related to older or other comorbidities such as cardiac involvement, a critical situation that is worsened given the lack of a specific treatment or vaccine<sup>1</sup>. Spreading of this new coronavirus in humans could be measured if positive (symptomatic and asymptomatic) cases could be diagnosed, identified and isolated, in order to define individual treatments when necessary, as well as the application of various collective health measures<sup>3</sup>.

Given the specificity of antigen-antibody reactions, serological tests based on this immunochemistry are commonly used for diagnostic or prognostic purposes for various infectious diseases. Serological tests do not usually meet strict diagnostic standards, but if used in combination with molecular tests, they can be very useful in surveillance strategies. When well-characterized antibodies are available, the detection of antigens in biological samples meets the criteria to be a diagnostic test. If what is being searched for in a serological test are the antibodies that the infected individual has generated as a response to the pathogen, this cannot be characterized as a diagnostic test, and all that can be affirmed is that the individual has been or is currently is infected, regardless of

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whether that individual is symptomatic or not. In the case of COVID-19, it is safe to assume that an infected asymptomatic individual is highly infectious. Because of this, it is important to complement a serological test with a follow-up molecular test, in order to confirm whether the individual is currently infected<sup>8</sup>. Therefore, combined use of molecular and serological tests can help guide public policies regarding epidemiologic surveillance, particularly once the disease has spread widely within a population<sup>7-9</sup>. However, the performance of some of these tests has been questioned because of their low sensitivity, increasing the percentage of false negatives (asymptomatic but infectious individuals).

With the understanding that SARS-CoV-2 infection may be asymptomatic or mild in about half of the cases, the immune response is expected to play a critical role in natural disease control, as has been shown for other newly-onset viral respiratory diseases impacting public health, like SARS and MERS<sup>10, 11</sup>. The number of infected individuals that have recovered is also expected to be significant, so that they manage to limit the spread, in what is known as the generation of collective or herd immunity.

However, the quality of both molecular and serological tests is a sensitive concern for governments. If these tests do not show high levels of sensitivity and specificity, false negative results (undetected infectious individuals) would be responsible for dissemination hotspots, which could not be properly isolated and monitored<sup>9</sup>. The concern is understandable: due to the urgency of testing, some health agencies have relaxed the rules for health registration mechanisms, which has in turn led to the use of evidence with questionable sensitivity and specificity. With molecular testing these parameters are relatively clear; with serological tests they are not.

Commercial immunochromatographic tests to detect anti-IgG and anti-IgM antibodies against SARS-CoV-2 virus have the following characteristics:

- i) They show widely varying ranges of sensitivity and specificity, parameters which are closely correlated to the time of infection<sup>6, 12, 13</sup>. However, few commercial tests indicate in their inserts the variables related to the kinetics of the infection, an aspect relevant to their interpretation and clinical correlation.
- ii) The manufacturers show validation of their tests with variable numbers of serum samples, and generally do not report the results of these validations in the product insert. When it is reported, the ranges vary from 20 to 1,300 samples<sup>6</sup>. This leads to a lack of reliability with some of these tests, especially those with limited sizes in the validation population.
- iii) Very few manufacturers report positivity correlation rates with a molecular test. In those cases where it is reported, the values range from 37.5% to 95% for IgG, and 81.25% to 97.1% for IgM. Given that the possibility of finding IgG antibodies increases after the 10th day post-infection, a high positivity rate will allow for the detection of individuals in earlier stages of the disease.

Although the COVID-19 pandemic has accelerated the production of molecular and serological tests for diagnostic and epidemiological purposes, these products must be accompanied by the proper health records of their countries of origin and that of other countries that may decide to endorse them. These are usually processes that review documentation only, and do not entail an analytical verification of the claims set forth in the documentation. After the controversies regarding the use of some immunochromatographic tests of Chinese origin, the Chinese Ministry of Commerce, the General Consumer Administration of China and the Chinese National Medical Products Administration (NMPA) announced on March 31 the elimination of the "Fast Track" route for the registration of such products<sup>14</sup>. After this date, Chinese authorities decided to require additional evidence from manufacturers, so these products do not currently have export permits. Apparently, only a few companies were able to meet the more stringent conditions now required to obtain a sanitary registration in China.

For the case of Colombia, an adequate selection of the serological tests to be used for the detection of anti-SARS-CoV-2 IgM/IgG has to take into account the provisions referred to in Decree 3770 of 2004 of the country's Ministry of Health and Social Protection. This means reviewing in detail the sensitivity and specificity ranges of the tests, ensuring they are in accordance with the time points of infection with SARS-CoV-2. Only those that report positivity correlation rates with molecular tests and report the number of samples with which the developers carried out the validation of the test, should be considered. Furthermore, as part of the support and quality requirements, it is desirable that the test should have a current sanitary registration in the country of origin, and an endorsement or certification from other regulatory agencies such as the FDA or the European Union, or that they are listed by regulatory agencies such as the World Health Organization. Considering the shortage of diagnostic products, the Colombian Ministry of Health and the consensus of experts have decided to introduce serological tests as part of the response in the context of care and follow-up<sup>15</sup>.

A serological test that is useful for seroconversion and seroprevalence studies, either for management of either individual cases or for epidemiological surveillance, should identify the generation of a humoral immune response (neutralizing antibodies) in all symptomatic or asymptomatic infected individuals.

## Materials and Methods

### Study Design

Demo samples of "fast" serological tests were received from companies or persons interested in marketing them, in varying number and conditions.

Verification of the packaging, as well as test presentations, were performed for each test prior to its use and summarized in Table 1. A random internal number was assigned to each test. The number of samples run for each test was in accordance to the available number of demo tests delivered by each supplier.

The ELISA NovaTec<sup>®</sup> test for IgM, IgG and IgA was given by Quimiolab. The sera were tested according to the manufacturer's instructions. For the calculation of the arbitrary units of immunoglobulins (AUI), the average optical densities (at 450 nm) for the cut-off control were first established. AUIs of each serum sample was calculated as follows: OD of the serum sample x the dilution factor/cut-off control.

### Serum Samples

The serum samples correspond to individuals positive for SARS-CoV-2, as diagnosed by RT-PCR as follows: **A**: asymptomatic; **SD**: symptomatic patients hospitalized at home with clinical follow-up of public health system in Bogotá; **SH**: inpatients (general ward or intensive care Unit) of Clínica Colsanitas in Bogotá. Negative patients (**N**): who were symptomatic from other disease (Non-COVID-19) and RT-PCR negative for SARS-CoV-2.

According to the incubation period reported by Lauer *et al*<sup>16</sup> the likely date of infection was calculated based on the reported onset of symptoms in patients positive for COVID-19 (5.5 days). Thus, the serums of patients positive for COVID-19 were collected between 12- and 35-days post-infection.

### Results

In this preliminary serological test of the verification of characteristics of the different tests analyzed, we report a negative match rate of 100% for all the assays tested: No anti-IgG or anti-IgM was detected in patients with RT-PCR for SARS-CoV-2 negative, according to immunochromatographic tests. 3 out of 8 (37.5%) negative patients for SARS-CoV-2 showed positive results by ELISA-measured IgA, which showed a low specificity of this technique.

A 100% positive match rate level for IgG was found for tests identified as 3, 5, and 9. The lowest match rate corresponds to 70% regarding the molecular test. For positive match rates for IgM, a 100% rate was found for tests identified as 3 and 6. The lowest match rate for IgM corresponds to 30% (number test 8). The result of rapid test identified with number 1 was associated with a recognition of 50% of the sera from COVID-19 patients. Although the ability to discriminate two immuno-

globulins is announced in the test insert, the cassette only allows showing a single recognition band for both IgM and IgG, preventing the characterization of the evolution of the infection. Test number 4 showed IgG recognition in 9 of the 10 positive control sera analyzed, 90% for IgG and 70% for IgM, while test 10 showed a recognition by sera positive control for IgG and IgM of 77% for each immunoglobulin.

A comparative analysis of the recognition of positive control serums according to the evolution time of the infection, for assays that had the highest number of tests (marks number 4, 6, 8 and 10, with 20 tests each) is showed in Table 3.

In line with our analysis of the different tests, figure 1 shows the sera recognition bands of patients identified as 18, 19 and 20, taken on days 12 to 17, 18 to 23 and 24 to 35 after infection, respectively.

With the use of ELISA, positives could be more objectively discriminated from negatives (weak recognition bands generated by immunochromatography), and although the result is not rapid, the reliability and sensitivity are improved with respect to immunochromatography. The results of the preliminary immunoglobulin analysis are showed in Table 4. For IgM analyzed by ELISA, 25 % (2 out of 8 individuals) of recognition was found by groups of asymptomatic and symptomatic individuals with mild symptoms (hospitalized at home). 75 % of hospitalized symptomatic individuals had detectable levels of IgM. The two patients hospitalized for COVID-19 who were negative for IgM had between 12 and 17 days after symptoms appeared. Patients negative for SARS-CoV-2 were not recognized by the IgM ELISA test. At the IgG level, 62.5 % of COVID-19 patients followed at home were positive for this immunoglobulin, while 8/8 (100 %) of hospitalized patients were positive. When analyzing the level of antibodies represented in the AUI for each of the symptomatic COVID-19 groups (SD and SH, Table 4), at least twice as much as AUIs are observed in the hospitalized versus home-monitored group. ELISA for IgA shows a nonspecific recognition, represented by a 37.5 % positivity in the group of negatives for SARS-CoV-2 (hospitalized by Non-COVID-19 cause). 25 % and 87.5 % of home-monitored and hospitalized symptomatic individuals, respectively, showed positivity by IgA ELISA tests.

**Table 1.** Serological anti-IgG and anti-IgM lateral flow assays, with a cassette presentation. The assigned number corresponds to the internal identification code of the test. The number of tests used corresponds to the number of serum samples used, as well as to the available demo tests. The observations record the presentation of each of the tests. N/A: Demo test was sent without information.

Assigned Number	Name	Number of Samples Used	Observations
1	Green Spring <sup>®</sup>	20	A single cassette with both anti-immunoglobulins (IgG and IgM) overlaid in line
3	Livzon <sup>®</sup>	10	Two cassettes, one with anti-IgG and another with anti-IgM
4	Medicscann <sup>®</sup>	20	A single cassette with both anti-immunoglobulins (IgG and IgM) in line
5	Sure Biotes VR products <sup>®</sup>	10	A single cassette with both anti-immunoglobulins (IgG and IgM) in line
6	Cellex <sup>®</sup>	20	A single cassette with both anti-immunoglobulins (IgG and IgM) in line
8	Liming Bio <sup>®</sup>	20	A single cassette with separated anti-IgG and anti-IgM
9	Innovita <sup>®</sup>	18	Two cassettes, one with anti-IgG and another with anti-IgM
10	Aria <sup>®</sup>	20	Two cassettes, one with anti-IgG and another with anti-IgM

**Table 2.** Characteristics of each patient whose sample was used in this performance check of “rapid” immunochromatographic and ELISA serological tests. \* Both sera samples provided from same patient took with a day of difference. <sup>a</sup> Negative volunteer (N); <sup>b</sup> Hospitalized symptomatic patients (SH); <sup>c</sup> Asymptomatic volunteers (A); <sup>d</sup> Symptomatic patients in house hospitalization (SD). ND: No Data; NA: Does Not Apply.

Code	Age	Gender	Admission Date	Date of Onset of Covid-19 Symptoms	Symptoms	Previous Contact to Covid-19	Travel within Last Month	Place of Attention	Date of Molecular Testing	Date of Serologic Testing
1 N <sup>a</sup>	15	Female	07/04/2020	NA	Non Covid-19	NO	NO	Inpatient	08/04/2020	08/04/2020
2 N	26	Female	08/04/2020	NA	Non Covid-19	NO	NO	Emergency Room	08/04/2020	08/04/2020
3 N	46	Male	07/04/2020	NA	Non Covid-19	NO	NO	Inpatient	07/04/2020	07/04/2020
4 N	30	Male	05/04/2020	NA	Non Covid-19	NO	NO	Emergency Room	08/04/2020	08/04/2020
5 N	30	Female	06/04/2020	NA	Non Covid-19	NO	NO	Inpatient	08/04/2020	08/04/2020
6 N	22	Male	04/04/2020	NA	Non Covid-19	NO	NO	Inpatient	08/04/2020	08/04/2020
7 N	66	Male	26/02/2020	NA	Non Covid-19	NO	NO	Inpatient	08/04/2020	08/04/2020
8 N	40	Male	08/04/2020	NA	No Covid-19	NO	NO	Outpatient	08/04/2020	08/04/2020
9 N	36	Female	07/04/2020	NA	Non Covid-19	NO	NO	Inpatient	08/04/2020	08/04/2020
10 N	39	Male	08/04/2020	NA	Non Covid-19	NO	NO	Emergency Room	08/04/2020	08/04/2020
16 <sup>a</sup> SH <sup>b</sup>	73	Male	27/03/2020	12/03/2020	Respiratory syndromes, cough, occasional green expectoration, dyspnea	NO	NO	ICU	27/03/2020	06/04/2020
17 <sup>a</sup> SH	73	Male	27/03/2020	12/03/2020	Respiratory syndrome, cough, occasional green expectoration, dyspnea	NO	NO	ICU	27/03/2020	05/04/2020
18 SH	60	Female	28/03/2020	24/03/2020	Asthenia, adynamia, choking sensation, dry cough without secretions, 39 °C fever, nausea, emesis, liquid stools without mucus or blood	YES	NO	Inpatient	28/04/2020	05/04/2020
19 SH	66	Female	29/03/2020	22/03/2020	Cough with secretions, 39 °C fever, general malaise, asthenia, adynamia, Hypoxemia (oxygen saturation 85)	NO	NO	ICU	29/03/2020	06/04/2020
20 SH	83	Female	29/03/2020	28/03/2020	Epigastralgia, dry cough, mucus- and blood-free diarrhea	NO	YES	Inpatient	29/03/2020	04/04/2020
21 SH	47	Female	07/04/2020	08/03/2020	Dry cough, diarrhea, one fever episode of 39 °C, productive cough (4 days previous), dyspnea,odynophagia and headache, burning sensation in both eyes	YES	NO	Inpatient	29/03/2020	07/04/2020
22 SH	70	Male	31/03/2020	21/03/2020	Flu-like symptoms, diarrhea, fever, desaturated, respiratory difficulty, altered state of consciousness, septic shock of pulmonary origin, severe pneumonia	NO	NO	ICU	31/03/2020	08/04/2020
23 SH	66	Male	31/03/2020	21/03/2020	Unquantified fever, asthenia, adynamia, loss of appetite, weight loss, diarrhea	NO	YES	Inpatient	30/03/2020	02/04/2020
24 SH	72	Male	23/03/2020	19/03/2020	Dyspnea, dry cough, fever	NO	NO	ICU	23/03/2020	02/04/2020
25 SH	28	Male	25/03/2020	19/03/2020	Progressive dyspnea, dry cough, chills, functional class deterioration, multilobar pneumonia, previous Covid-19 diagnosis	NO	NO	ICU	19/03/2020	08/04/2020
26 SH	33	Male	29/03/2020	23/03/2020	Cough, 39°C fever, asthenia, adynamia, osteomyalgia, high-intensity headache, dyspnea, decreased urination	YES	NO	ICU	29/03/2020	08/04/2020
27 SH	59	Male	31/03/2020	23/03/2020	Respiratory symptoms, nasal congestion, dry cough, general discomfort, 39°C febrile peaks, dyspnea, difficulty breathing, 66% desaturation, polypnea	NO	YES	ICU	31/03/2020	08/04/2020

Code	Age	Gender	Admission Date	Date of Onset of Covid-19 Symptoms	Symptoms	Previous Contact to Covid-19	Travel within Last Month	Place of Attention	Date of Molecular Testing	Date of Serologic Testing
28 SH	66	Female	24/03/2020	17/03/2020	Non-quantified fever, general discomfort, asthenia, adynamia, vomit, edema, dysuria, pollakiuria	NO	NO	ICU	26/03/2020	08/04/2020
29 SH	50	Male	26/03/2020	16/03/2020	Coughing without expectoration, dyspnea, 38 - 39°C febrile peaks, diarrhea without blood or mucus, no tolerance in oral pathways, general discomfort, dysphagia	NO	NO	Inpatient	26/03/2020	08/04/2020
30 SH	84	Female	03/03/2020	25/03/2020	Dry cough, white expectoration in the last 4 days, hyaline rhinorrhea, moderate dyspnea, somnolence, time disorientation	YES	NO	Inpatient	22/03/2020	08/04/2020
0004 A <sup>c</sup>	36	Female	NA	NA	NA	NO	NO	Outpatient	22/03/2020	22/03/2020
0012 A	39	Male	NA	NA	NA	NO	NO	Outpatient	22/03/2020	22/03/2020
0027 A	40	Male	NA	NA	NA	NO	NO	Outpatient	22/03/2020	22/03/2020
0062 A	22	Male	NA	NA	NA	NO	YES	Outpatient	22/03/2020	22/03/2020
0091 A	50	Female	NA	NA	NA	NO	YES	Outpatient	22/03/2020	22/03/2020
0101 A	29	Female	NA	NA	NA	NO	YES	Outpatient	22/03/2020	22/03/2020
0194 A	58	Male	NA	NA	NA	NO	YES	Outpatient	22/03/2020	22/03/2020
0240 A	78	Female	NA	NA	NA	YES	NO	Outpatient	24/03/2020	30/03/2020
8 SD <sup>d</sup>	35	Female	NA	29/03/2020	Respiratory syndromes	ND	ND	Home- care treatment	02/04/2020	19/04/2020
14 SD	ND	Male	NA	8/04/2020	Respiratory syndromes	ND	ND	Home- care treatment	08/04/2020	19/04/2020
16 SD	ND	Female	NA	09/04/2020	Respiratory syndromes	ND	ND	Home- care treatment	09/04/2020	19/04/2020
18 SD	ND	Female	NA	03/04/2020	Respiratory syndromes	ND	ND	Home- care treatment	10/04/2020	19/04/2020
19 SD	ND	Female	NA	06/04/2020	Respiratory syndromes	ND	ND	Home- care treatment	09/04/2020	19/04/2020
20 SD	ND	Female	NA	06/04/2020	Respiratory syndromes	ND	ND	Home- care treatment	13/04/2020	19/04/2020
28 SD	33	Female	NA	18/03/2020	Respiratory syndromes	ND	ND	Home- care treatment	25/03/2020	21/04/2020
32 SD	31	Female	NA	28/03/2020	Respiratory syndromes	ND	ND	Home- care treatment	31/03/2020	21/04/2020

**Table 3.** Immunochromatographic test reading. Results of the immunochromatographic assay with respect to the time of infection (5.5 days before appearance of the onset of COVID-19 symptoms.).

Sera / Test	Test 4		Test 6		Test 8		Test 10	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
<b>12 to 17 days after infection</b>								
18 SH	Neg	Pos D	Pos	Pos	Neg	Pos D	Pos	Pos
20 SH	Neg	Pos D	Pos	Pos	Neg	Neg	Pos	Neg
23 SH	Pos	Pos	Pos D	Pos D	Neg	Pos D	Pos D	Pos
<b>18 to 23 days after infection</b>								
19 SH	Pos D	Pos	Pos	Pos	Neg	Pos D	Pos D	Pos D
22 SH	Pos D	Pos	Pos	Pos	Pos	Pos	Pos	Pos
24 SH	Pos D	Pos	Pos	Pos	Neg	Pos	ND	ND
<b>24 to 35 days after infection</b>								
16 SH	Pos D	Pos	Pos D	Pos	Neg	Pos	Neg	Pos
21 SH	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
25 SH	Neg	Pos	Pos	Pos	Neg	Pos	Pos D	Pos D

Neg: Negative, no band appearance on the cassette. Pos: Positive, clear bands appear on the cassette. Pos D: Positive weak, corresponds to a faint band in the cassette membrane. ND: No Data: invalid test (no mark on the control band).















## Discussion

Lateral-flow immunodetection assays (immunochromatography for serology) have been employed in diagnostics since the 1980s<sup>17</sup>. The first available tests were designed for the detection of human chorionic gonadotropin, secreted in pregnancy. The quality of immunochromatographic tests and their value for detecting the corresponding analyte against which the detection antibodies have been developed, depend on several variables (ways to reach lower detection limits of Lateral Flow Immunoassays) that are increasingly better controlled (the design of the test, the material used in the recipient where sample containing the analyte is deposited, the material used for the interaction and movement of the reagents, the material in which the antigen-antibody reaction occurs, and the place where the reagents and biological material not coupled in the reaction are deposited)<sup>18,19</sup>. The sensitivity and specificity of immunochromatographic tests depend on these variables. Sensitivity therefore depends not only on the title or concentration of antigens, but on their ability to bind and remain in these supports and materials that allow immunodetection. If these variables are well controlled, the intensity in the coloration of the bands suggests a greater amount of analyte. In theory, a semi-quantitative titration could be made.

The sensitivity and specificity of each of serological tests must be carefully analyzed, as well as the correlation rates with molecular tests. Moreover, the number of samples with which the manufacturing companies carried out the respective validations is another important factor that must be considered. This study corresponds to a verification (not a validation) of the performance of eight different tests. Although an analytical method has been previously standardized, it is necessary to confirm whether it works properly, before proceeding to routine use. To this procedure by which the per-

formance of the method is evaluated to demonstrate that it meets the requirements for the intended use, which were established as a result of the validation (to identify IgG and IgM, specific for SARS proteins- CoV-2), is called secondary validation or verification. Unlike validation (which corresponds to the process necessary to demonstrate the performance characteristics set out when a method has been designed or developed, or when extensions or modifications have been made to a standardized method as a manufacturer or user), the verification process it is not always framed in analysis with statistical design. When it comes to qualitative procedures or subjective testing, positive and negative controls should be incorporated into verification processes, whenever possible. Validation is also necessary when seeking to demonstrate equivalence of the results obtained by two methods. Since higher antibody titers and seroconversions are detected in most individuals with severe or not severe symptomatic COVID-19 patients, but it could be negative in asymptomatic or pauci-symptomatic patients<sup>20-21</sup>. On the other hand, positive and negative control sera were confirmed by RT-PCR for SARS-CoV-2, and in this validation the RT-PCR result was considered the gold standard, although it has also been shown to be negative, especially in those with a longer period of time after the onset of symptoms<sup>6</sup>.

Although manufacturers do not reveal the characteristics of the anti-antibodies used, it is known that the system in which these are produced, and the variable affinity they present with the Fc fraction of immunoglobulins, in particular with IgM, induces greater complexity in their detection<sup>18</sup>. The diversity of hybridomas, of purification methods and of materials used as support, are factors that influence the sensitivity of each test, and are presumed to have been considered in the design of the technique, as well as in the reports that accompany the inserts in each kit.

Sera Test	Test 4	Test 6	Test 8	Test 10
18				
19				
16				

**Figure 1.** Recognition of representative sera of COVID-19 patients [with positive molecular (RT-PCR) diagnosis]. The characteristics at the intensity level of the bands could be correlated with the concentration or titers of anti SARS-CoV-2 antibodies, and therefore with the sensitivity of the test. Letter C in each cassette corresponds to control of procedure (other antibodies in sera). Letters G, M, or IgG or IgM, correspond to antibodies bind to the cassette membrane (anti-immunoglobulin IgG or anti-immunoglobulin IgM).

**Table 4.** IgM, IgG and IgA anti protein N SARS-CoV-2 production. AUI: Arbitrary Immunoglobulin Units. Results correspond to AUI calculated for each immunoglobulin. Shaded boxes represent results that are above the cutoff values. The cutoff value for IgM is > 3.8, for IgG > 6.0 and for IgA > 4.0.

Sera code	UAI	Sera code	UAI	Sera code	UAI	Sera code	UAI
<b>IgM</b>							
0004 A	1,17	1 N	2,57	8 SD	0,62	20 SH	2,06
0012 A	1,59	2 N	2,09	14 SD	1,40	21 SH	5,70
0027 A	1,73	3 N	1,27	16 SD	2,76	25 SH	14,36
0062 A	2,04	4 N	2,29	18 SD	1,51	26 SH	6,07
0091 A	2,00	5 N	2,59	19 SD	3,88	27 SH	11,29
0101 A	6,47	6 N	2,21	20 SD	3,21	28 SH	11,70
0194 A	4,46	7 N	2,88	28 SD	2,41	29 SH	16,87
0240 A	1,36	8 N	1,89	32 SD	4,11	30 SH	1,48
<b>IgG</b>							
0004 A	2,00	1 N	1,83	8 SD	1,14	20 SH	22,43
0012 A	2,34	2 N	1,74	14 SD	6,00	21 SH	14,25
0027 A	4,06	3 N	3,64	16 SD	10,18	25 SH	24,46
0062 A	3,50	4 N	4,59	18 SD	3,06	26 SH	14,98
0091 A	3,17	5 N	2,15	19 SD	4,55	27 SH	19,03
0101 A	4,82	6 N	4,00	20 SD	12,91	28 SH	22,17
0194 A	4,71	7 N	3,52	28 SD	10,12	29 SH	22,77
0240 A	2,92	8 N	4,47	32 SD	9,94	30 SH	8,96
<b>IgA</b>							
0004 A	1,92	1 N	2,65	8 SD	0,84	20 SH	Positive out of range
0012 A	1,99	2 N	3,18	14 SD	2,64	21 SH	2,24
0027 A	2,92	3 N	2,98	16 SD	2,86	25 SH	23,97
0062 A	2,12	4 N	4,95	18 SD	2,52	26 SH	18,24
0091 A	1,56	5 N	3,28	19 SD	1,67	27 SH	Positive out of range
0101 A	1,79	6 N	4,53	20 SD	7,12	28 SH	23,02
0194 A	2,79	7 N	3,73	28 SD	2,44	29 SH	25,65
0240 A	1,49	8 N	10,95	32 SD	5,91	30 SH	9,22

Considering the pattern of immunity against SARS-CoV-2, it has been proven that, as it is against other microbial agents, it is a predominantly humoral (IgG-based) response, and targeted against the N protein, which can be detected starting at day 4 post-infection, with an average seroconversion of 14 days in infected individuals<sup>13, 22</sup>. In another study in which antibodies were detected through ELISA, it was found that the average time point of seroconversion will occur between day 12 and 14, for IgM and IgG, respectively, and after this period increase rapidly from day 15, achieving an average seroconversion of 94.3% for IgM and 79.8% for IgG<sup>12</sup>. This study indicates that the combination of RT-PCR and antibody detection improves diagnosis, even in the early phase (first week). Although it is clarified that high antibody titers are not associated with the clinical evolution of the infected individuals. On the other hand, Jin et al. has described the positive rate and titer variance of IgG are higher than that of IgM in COVID-19<sup>23</sup>.

According to the speed and scale of the COVID-19 pandemic and the urgent public health needs of each country and

difficulties in obtaining supplies around the world, health authorities that have decided to use these serological tests have based their decisions on the review of the serological commercial and developers manufacturer's dossiers and inserts, focusing their attention on criteria such as the reported sensitivity and specificity of these serological tests. Considering all of the above, it is important to establish clear criteria that allow the definition of the best serological tests, based on the information they provide, which will then need to be verified and used for the purposes already described. This study showed that only for one of the serological tests referred to, the correlation rate with the molecular test corresponds to the information registered in the insert.

If a comparative validation test is considered, a larger number of samples will be required. Since this study was conducted with serum samples, it is recommended to perform a comparison with whole blood samples, with and without anticoagulant, although other authors (Li Z et al.) have evaluated this aspect and shown that differences in the type of sample used are not relevant<sup>13</sup>.

Inserts of the developers of the immunochromatographic assays indicate that the intensity of the color of the bands should not be associated with the anti-SARS-CoV-2 antibody titer. However, if a high affinity capture antibody is used, low reactivity plotted in weak intensity bands, if it could be correlated with the detected antibodies level. Despite the fact that in our study the number of positive control sera may be a limiting factor, as observed in Table 3, a trend towards the production of IgM and even detectable IgG can be postulated before 17 days post-infection (7 days from the report of symptoms) by some of the tests (6 and 10). Although they are not the same patients followed over time, the group of patients whose sera were collected between the 18th and 23rd post-infection, exhibited the most marked production of both immunoglobulins. A striking result suggests that after 24 days post-infection the antibody titers for both immunoglobulins (more marked for IgM) decline to patterns similar to those found before 17 days post-infection.

Considering all of the above, ELISA or chemoluminescence assays can be used to analyze processes such as seroconversion because higher sensitivity has been reported. However, not only technology is important: the proper selection of serum samples in the secondary validation phases is essential. Given that higher antibody titers as well as greater seroconversion rates are detected in individuals symptomatic for COVID-19, the positive control sera used in primary validation are collected from inpatients with severe or critical COVID-19 cases, which represents a limitation to evaluate sensitivity (including the asymptomatic individuals "innocent viral spreaders"<sup>24</sup>).

This trend could be problematic considering that the use of immunochromatographic serological tests has been suggested as a strategy to identify possible asymptomatic infectious carriers. In a preliminary study with sera from individuals positive for SARS-CoV-2 diagnosed by RT-PCR but asymptomatic, it was found that for two rapid serological tests, no more than 30% of the individuals were positive for any of the immunoglobulins. This may be because the viral load of patients with severe to critical symptomatic COVID-19, may be responsible for a detectable antibody response, while asymptomatic carriers have been associated with low viral load, which leads to low or null seroconversion<sup>20</sup>.

Taking into account the above, an optimal performance test should allow correlating the patient's immune status with their clinical evolution; also identify infectious asymptomatic patients who warrant isolation and follow-up. However, with the information obtained so far, the sensitivity of the tests for this epidemiological group would be lower than for the group of COVID-19 symptomatic patients. Both techniques appear to have limited use in asymptomatic carrier screening.

As a recommendation, decision makers are suggested to discuss in detail the following information considered in inserts or dossiers, and to choose tests with these characteristics:

i) Sensitivity (IgG, IgM or IgG/IgM) >80 % (including asy-

ymptomatic individuals positive for SARS-CoV-2, as diagnosed by RT-PCR);

- ii) Specificity (IgG, IgM or IgG/IgM) >80 %; (ii) Percentage of positive and negative correlation (match rate) with gold standard >80%;
- iii) Number of samples used in the validation process >100;
- (iv) Current sanitary registration in the test's country of origin;
- v) Inclusion in the list of the World Health Organization (March 30);
- vi) Technical Support from the company that markets the test, with a solid track record on the sale of reagents and/or laboratory instrumentation;
- vii) Availability and price.

For field studies it is desirable to choose tests whose cassettes include in the same line, with a clear separation, anti-IgG and anti-IgM results. For field studies, we do not recommend the use of tests with separate cassettes, or with independent lines for IgG and IgM, because it represents a double sample, and greater manipulation and exposure at the time of sampling. As in other in vitro analytical tests for diagnostic purposes, it would be desirable to have properly characterized control serums that allow for the verification of the performance of each test to be checked periodically in each laboratory.

It is possible that as the SARS-CoV-2 pandemic progresses, serological tests based on the detection of IgM / IgG antibodies will play a fundamental role in epidemiological surveillance. Besides, these tests can also play a role in the definition of seroprevalence, screening, identifying the recovered asymptomatic population that is able to return safely to the but the tests must be deployed appropriately.

With the data from this study we can suggest that the N protein used as an antigen in commercial serological tests (immunochromatography and ELISA), is a good immunogen for the follow-up of humoral response (IgA, IgM and IgG) in symptomatic COVID-19 patients (after two weeks of onset of symptoms). However, this protein is not identified by sera of asymptomatic patients. If the projected use of immunochemical tests involves the detection of infectious asymptomatic individuals in the framework of epidemiological surveillance (for particular isolations and follow-ups), such tests have a very limited usefulness. And although the quality of the tests and their performance in the field are associated with the sensitivity of these immunochemical techniques, in the case of COVID-19, the nature of the infection and the immunogenic characteristics of SARS-CoV-2 appear also to determine their value as a screening test. Recently, it has been described that the use of a recombinant Spike (S) protein from SARS-CoV-2 expressed in CHO cells in an ELISA test, allowed the early detection of antibodies in asymptomatic RT-PCR positive individuals (medical staff and close contacts of patients with COVID-19)<sup>24</sup>. This justifies the need to incorporate protein S as an antigen preferred in serological tests, in order to improve its scope and usefulness in epidemiological surveillance and cluster monitoring strategies (mainly asymptomatic individuals).



Lastly, determining seroconversion in healthcare workers, while at the same time monitoring them with a molecular test, is going to be of vital importance in the follow-up and care of this population. Understanding and validating serological and molecular tests is an essential component for the design of public health measures to response to the pandemic. Probably the integration in the strategies of molecular diagnosis and serological tests (with viral antigens as S protein alone or complementary to N protein) will contribute of important form not only in the containment of the epidemic but in its mitigation.

## Ethical disclosures

**Protection of human and animal subjects.** No experiments were performed in animal nor humans.

**Confidentiality of data.** Data were anonymized

**Competing interests.** None declared.

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**Conflict of interest.** The authors have no conflicts of interest to declare.

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