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Diagnostic performance of two serological assays for the detection of SARS-CoV-2 specific antibodies: surveillance after vaccination

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ABSTRACT
Massive vaccination programs are being carried out to limit the SARS-CoV-2 pandemic that started in December 2019. Serological tests are of major importance as an indicator of circulation of the virus and to assess how vaccine-induced immunity progresses.

An Enzyme-Linked Immunosorbent Assay (ELISA) and a Lateral Flow Assay (LFA) have been developed based on the SARS-CoV-2 recombinant Receptor Binding Domain (RBD) and the combination of Spike and Nucleoprotein, respectively. The validation with 1272 serum samples by comparison with INgezim COVID 19 DR showed good diagnostic performance (sensitivity: 93.2%-97.2%; specificity: 98.3%-99.3%) for detection of previous contact with SARS-CoV-2. Moreover, according to our results, these assays can help in the serosurveillance during and after vaccination, by detecting the humoral immune response as soon as 15 days post-vaccination and identifying low-respondents. Hence, these tests could play a key role in the progression to a COVID-19 free world, helping to adjust future vaccination protocols.

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1. Introduction

Since December 2019, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has infected more than 260 million people and produced around 5.2 million deaths around the world [1]. As a complementary tool to direct detection of the virus by molecular detection techniques, plenty serological tests have been described and commercialized, some detecting nucleocapsid, spike specific antibodies or both [2–7].

As of January 2022, the European Medicines Agency (EMA) had recommended 5 vaccines for authorization in the EU, all of them containing the gene sequence that codifies for the Spike protein (S) [8]. Furthermore, the majority of the vaccines that are currently in clinical trials elicit antibodies that target the S protein, including the receptor binding domain (RBD) [9].

S is a transmembrane glycoprotein that forms homotrimers protruding from the viral surface. This protein is formed by two functional subunits: S1 subunit, that binds to the host cell receptor through the RBD, and S2 subunit that allows the fusion of the viral and cellular membranes [10]. Entrance of SARS-CoV-2 in the cells is carried out through the binding of S to hACE2 (human Angiotensin Converting Enzyme 2), with comparable affinity to S of SARS-CoV, that may contribute to its efficient spread among humans [11, 12].

Although several epitopes within the S protein have been described to elicit neutralizing antibodies [13, 14], most of those produced upon natural infection target the RBD, blocking the binding of
the virus to hACE2 [15]. The Nucleoprotein does not elicit neutralizing antibodies, however, good correlation between antibody response to this protein and neutralizing antibody titer has been described [16].

Enzyme-Linked Immunosorbent Assay (ELISA) is a well-known lab technology, that allows the simultaneous analysis of high numbers of samples. In contrast, lateral flow assays (LFA) are one of the most widely used techniques for point-of-care testing and diagnosis due to its characteristics (user-friendly, low cost, rapid results, long-term stability over a wide range of climates) [17].

Since vaccines are mainly based on the S protein, it is important to develop tests to complement serological status determination. For that purpose, here, the comparison of an ELISA that detects RBD-specific antibodies, and a dual rapid test (LFA) for the differential detection of S and N-specific antibodies, is described. A collection of serum samples from naturally infected, vaccinated non-infected, vaccinated previously infected, and non-infected nor vaccinated people, has been analyzed to validate the two assays and determine their performance.

2. Materials and methods

2.1. Serum samples

A total of 1272 human serum samples were evaluated by the two assays described in this article. Serum samples were collected from selected volunteers (SARS-CoV-2 naturally infected, vaccinated non-infected, vaccinated previously infected, and non-infected nor vaccinated) from different hospitals and laboratories (Table 1). The sera from the Amsterdam UMC were collected through the Amsterdam Cohort Studies on HIV infection and AIDS. Samples were characterized as positive or negative to previous infection by the commercial CE-certiﬁed ELISA Ingezim COVID 19 DR (N-ELISA) [2] for the statistical evaluations. Data relative to PCR were considered when available. The seroneutralization assay (SNT) previously described [18] was used as reference to determine the presence of neutralizing antibodies. Reference sera from the World Health Organization [11] were used for determination of the Limit of detection (LoD) for the different assays [19].

2.2. Production of recombinant proteins

Recombinant expression of Nucleoprotein was carried out as described [2].

Recombinant expression of Spike trimeric and RBD proteins was performed by transfection of suspension cultures of Human embryonic kidney 293-F (HEK293) cells. The codon-optimized DNA sequence encoding the RBD residues 331-524 (GeneBank accession number NC_045512) was ordered to Integrated DNA Technologies, ampliﬁed by PCR, and inserted into the plasmid pCMV6-AC-FC-S (Origene), generating the plasmid pCMV6-RBDmFc. A second plasmid, pCMV6-RBDHis, was generated by modifying the described RBD sequence by PCR adding a 6-histidine sequence at the 3’ region followed by a stop codon. The transfections were performed in a proportion of 1 μg plasmid/ml cell culture, yielding recombinant proteins expressed as a C-terminal fusion with a mouse Fc (RBDmFc) or with a 6His tag (RBDHis). The proteins were collected from the supernatant of HEK293 cell suspension cultures four days post-transfection, and subsequently puriﬁed by afﬁnity chromatography to protein G or Ni2+ for RBDmFc and RBDHis, respectively. Both proteins were analyzed by SDS-PAGE expecting theoretical molecular weights of 49.2 kDa (RBDmFc) and 22.8 kDa (RBDHis). To use the puriﬁed RBD protein as a detection tool in the Double Recognition ELISA (DR-ELISA), RBDmFc was labeled with Horseradish Peroxidase (HRP) [20].

S protein was expressed fused to a 6-histidine tag as previously described [21]. The transfection procedure was the same as for RBD proteins except that the culture was collected six days post transfection.

Table 1

| Collection | Origin | N | Comments |
|------------|--------|---|----------|
| Negative (445) | 1 | 192 | Analyzed at the HGUGM by trained staff. Complementary analysis by Abbott SARS-CoV-2 IgG. |
| 2 | 61 | | |
| 3 | 100 | | |
| 4 | 8 | | |
| 5 | 84 | | |
| Positive (368) | 1 | 241 | Analyzed at the HGUGM by trained staff. Complementary analysis by Abbott SARS-CoV-2 IgG. |
| 2 | 41 | | |
| 3 | 50 | | |
| 4 | 2 | | |
| 5 | 34 | | |
| Vaccinated (293) | 1 | 77 | Pfizer-BioNTech (53), Moderna (2), Oxford-AstraZeneeca (7) |
| 4 | 62 | Pfizer-BioNTech (87), Moderna (13), Oxford-AstraZeneeca (54) |
| 5 | 154 | | |
| Interferences (19) | 3 | 19 | β-hCG (7), bilirubin (5), hemoglobin (4), lipids (3). |
| Cross-reactivity (82) | 3 | 30 | Positive to antibodies speciﬁc against VHH (1), Adenovirus (2), Zoster Herpes virus (4), Rubella virus (4), Cytomegalovirus (4), Epstein-Barr (3), HCV (1), HBV (1), rheumatoid factor (3), and anti-nuclear antibodies (7). |
| 1 | 12 | Positive to antibodies speciﬁc against HCV, viremic (6) and non-viremic (6). |
| 6 | 40 | Positive to antibodies speciﬁc against another human Coronavirus: OC43 (14), NL63 (12), 229E (12), OC43/HKU1 (1) and OC43/229E (1). |
| SNT (60) | 7 | 60 | Classified as positive (35) and negative (25) by the seroneutralization assay. |
| Reference sera | WHO | 5 | International reference panel 20/268 |

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2.3. Double recognition ELISA for detection of antibodies anti-RBD (InGezim COVID RBD-DR; RBD-ELISA)

A DR-ELISA was developed as previously described [2, 22] with some modifications. The above-described proteins were tested: RBDHIs rendered better specificity results and RBDMFc was more suitable for conjugation. Briefly, 1 ng/μL of the RB protein (RBDHIs) was used to coat 96-well plates and was incubated overnight at 4°C in 50 mM carbonate buffer, pH 9.6. After washing the wells with PBS, 0.05% Tween 20 (PBST) using a manual washer, a blocking step was performed with Stabilzyme SELECT Stabilizer (SurModics, Inc.) for 1 h at room temperature (RT). The plate was incubated with serum samples diluted 1:5 in PBST with 2.5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at RT. Duplicates of positive (pool of human SARS-CoV-2 positive sera) and negative (dilution buffer) controls were included in each plate. The wells were washed as described above and incubated with the HRP-conjugated RBDMFc protein for 30 min at RT. Finally, after a washing step as above mentioned, the plate was incubated for 15 min with the substrate (TMB-MAX, Neogen Corporation), and the reaction was stopped by the addition of 0.5 M sulfuric acid. The absorbance was measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices, LLC). Results were presented as S/P, defined as: (sample OD – negative control OD)/(positive control OD – negative control OD) × 10.

2.4. Double recognition LFA for simultaneous detection of SARS-CoV-2 N and S-specific antibodies (InGezim COVID 19 N/S DUAL CROM)

This double LFA comprises two parallel strips, one for the detection of N-specific antibodies (N-LFA) and another one for S-specific antibodies (S-LFA). The single assay, for the detection of N-specific antibodies, has been already described [2]. Thus, the assay for the detection of S-specific antibodies is reported.

2.4.1. Capture reagents

Recombinant S protein diluted at 1 mg/mL in 20 mM Tris-HCl buffer, pH 7.5 containing sucrose and sodium azide was used as the test line capture reagent. As control line capture reagent, a monoclonal antibody against the control protein was employed. These reagents were dispensed in two parallel lines on a nitrocellulose membrane and the resulting membranes were dried for 5 min at 45°C, sealed and stored at room temperature under dry conditions.

2.4.2. Detector reagents

The S protein was covalently conjugated to red latex beads whereas blue latex particles were covalently conjugated with the control protein. Prior to protein conjugation, bare latex beads were washed and activated with EDC and NHS. Then, S protein was coupled to a surface concentration of 1 mg/mL and, after blocking the non-reactive functional groups, particles were diluted to a concentration of 1% in Tris-HCl 10 mM pH 8.2.

In order to prepare the conjugate solution, the S-latex and control-latex particles were diluted at a concentration of 0.2% and 0.15% respectively, and the mixture was dispersed onto the conjugate pad, dried for 30 min at 45°C and stored at room temperature under dry conditions.

2.4.3. Assembling of chromatographic strips

The nitrocellulose membrane, conjugate pad, sample pad and wicking pad were pasted on a plastic card and protected with a cover tape. The master card was then cut into strips of 4.2 mm width, and both N and S-specific Ab strips were assembled into cassettes.

2.4.4. Test procedure

The double test was designed to be used with serum or blood samples. Serum samples were fresh, refrigerated at 2 to 8°C or frozen at –20°C. Blood samples were fresh or refrigerated up to 4 days at 2–8°C and collected with anticoagulant (EDTA, heparin or citrate).

To perform the test, twenty microliters of blood or ten microliters of serum were applied to the round window of each cassette and followed by addition of 3 drops of the shared running buffer (Tris-HCl pH 7.5, NaCl, casein and NaN3). Results were interpreted after 10 minutes.

2.5. Statistical analysis

MedCalc 10 software was used for statistical analysis. Receiver Operating Characteristics (ROC) curves analyses were performed to establish the optimal cut-off value for each assay, as well as sensitivity, specificity, and likelihood ratio (LR). Regarding samples from vaccinated subjects, results were presented in box-whisker plots according to time postvaccination.

3. Results

3.1. Recombinant proteins

The three proteins expressed (RBDMFc, RBDHIs and S [21]) showed the expected apparent molecular weights considering their potential N-glycosylation pattern (3, 2, and 6 potential N-glycosylation sites, respectively). The mean expressions yields were between 10 and 20 mg/L. The purity degree was >99%, according to Coomassie staining of SDS-PAGE gels (Fig. 1).

3.2. Diagnostic performance

First, the potential diagnostic application of the newly developed assays was evaluated with samples collected from naturally infected and non-infected volunteers. Analyzing signal intensity (S/P for ELISA and test-line intensity for LFA), best cut off values were established according to a ROC curve analysis (Fig. 2).

As shown in Fig. 2 and detailed in Table 2, the new assays exhibited good diagnostic parameters and agreement with the commercial ELISA used as reference in the present study. Moreover, all the assays exhibited a high positive LR (+LR), >10, and a low negative LR (−LR), indicating that the performance of the assays is optimal for diagnosing past infection. Additionally, the tests’ LoD was assessed with WHO reference sera (Table 2).

Regarding RBD-ELISA (Fig. 2A), an optimal cut off was established at 2.5 S/P, obtaining a sensitivity of 95.1% (117/123) and a specificity of 99.0% (310/313). All the samples that gave a negative result with the N-ELISA, but positive with the RBD-ELISA, also gave a positive result by the commercial test INCPG-402, ALLTEST, able to detect antibodies to the S protein, and thus considered as positive samples.
A good agreement was obtained between both ELISAs (Cohen’s kappa coefficient 0.94, Table 2).

Concerning the dual LFA, best results were obtained when comparing tests specific for the detection of antibodies to the same protein of the virus (N), obtaining 10 false negative samples among the 359 positive samples included (97.2% sensitivity). Only 3 false positives were detected among the 430 negative samples evaluated with the N-LFA, showing a specificity of 99.3% (Fig. 2C), one of them obtained from a person with a positive PCR result more than 60 days prior to the blood extraction point. Optimal agreement was obtained between the 2 assays (Cohen’s kappa coefficient 0.967, Table 2). For the detection of S-specific antibodies with the S-LFA (Fig. 2B), a sensitivity of 93.2% was obtained, detecting 8 false negative samples among the 118 positive samples included in the assay. Within those samples, six also showed a negative result with the RBD-ELISA. In addition, the specificity was established at 98.3%, detecting 4 false positive samples among the 237 negative samples evaluated. Three of them were the same samples described as false positive for the RBD-ELISA. According to the Cohen’s kappa coefficient determination, established as 0.923 (Table 2), the S-LFA showed a good agreement with the N-ELISA. Moreover, the two assays for the specific detection of antibodies to the S protein of the SARS-CoV-2 (RBD-ELISA and S-LFA) exhibited an almost perfect agreement between them, with a Cohen’s kappa coefficient of 0.979.

3.3. Cross-reactivity with other pathogens and interferences

In order to determine potential cross-reactivity in the reported assays, samples containing antibodies specific to other viruses were analyzed (VIH, Adenovirus, Zoster Herpes, Rubeola, Citomegalovirus, Epstein-Barr, Hep C, Hep B, Influenza A and B). No cross-reactivity was observed with these viruses, except for one sample, positive for Epstein-Barr antibodies that was positive for S-specific antibodies when analyzed by S-LFA, but negative for the other assays.

Furthermore, samples containing antibodies to other human Coronavirus (hCoV) were analyzed by S-LFA (n = 40 [23]) and RBD-ELISA (n = 10), being all of them negative by both assays, indicating no cross-reaction with any other hCoV.

Several analytes that could cause interferences in the assays were also studied [24]. Samples with an increased content of Beta human chorionic gonadotropin (β-hCG), bilirubin, hemoglobin or lipids were analyzed, showing that samples with concentrations up to 1.9 ng/dl β-hCG, 48 mg/dl hemoglobin, 800 mg/dl triglycerides and 7 mg/dl bilirubin respectively, do not affect the test result.

Table 2
Diagnostic performance of the developed assays compared to Ingezim COVID 19 DR.

| Assay | Sensitivity % (95% CI) | Specificity % (95% CI) | +LR Value (95% CI) | -LR Value (95% CI) | Cohen’s kappa coefficient | LoD, (BAU/ml) |
|-------|------------------------|------------------------|-------------------|-------------------|--------------------------|-----------------|
| Ingezim® COVID RBD-DR | 95.1 (89.7-98.2) | 99.0 (97.2-99.8) | 99.2 (95.2-103.4) | 0.049 (0.01-0.2) | 0.949 | 33 |
| Ingezim® COVID 19 N/S DUAL CROM (N) | 97.2 (94.9-98.7) | 99.3 (98.0-99.8) | 139.34 (136.7-142.0) | 0.028 (0.008-0.1) | 0.967 | 25 |
| Ingezim® COVID 19 N/S DUAL CROM (S) | 93.2 (87.1-97.0) | 98.3 (95.7-99.5) | 15.60 (14.7-16.5) | 0.072 (0.03-0.2) | 0.923 | 23 |

* LoD calculated with WHO reference serum 20/144 and expressed as BAU/ml to the corresponding target proteins of each assay (RBD, S or N).
3.4. Vaccine-induced immunity

A cohort of 293 serum samples from vaccinated individuals was analyzed by the two developed serological assays (ELISA and LFA) for the specific detection of antibodies to the S protein of SARS-CoV-2. Individuals had been inoculated with vaccines from Pfizer/BioNTech, Moderna, or Oxford/AstraZeneca. Samples were obtained at different times during vaccination and were categorized as: less than 15 days post-first dose; more than 15 days post-first dose but before the second one; less than 15 days post-second dose; more than 15 days post-second dose. In the case of vaccination with AstraZeneca, only samples after first dose were evaluated.

These samples were analyzed by the immunological assays described, as well as with N-ELISA. As observed in Fig. 3A, for vaccinated but noninfected individuals, antibody titer to the S protein increased after vaccination. Up to 15 days post-first dose, only 23.9% (17/71) individuals had a detectable antibody titer, reaching 97.3% after 15 days post-second dose, and only one negative sample was detected which was confirmed negative by another commercial test (INCPG-402, ALLTEST). As expected, antibodies against N protein remained undetectable in noninfected vaccines for all samples tested (Fig. 3B).

On the other hand, previously infected people (12 till 2 months after a positive PCR results) exhibited a very different immunological behavior after vaccination. As shown in Fig. 3C, after 15 days post first dose, all the people included in the study developed high anti-S antibody titers. Both assays showed a sensitivity in vaccinated and previously infected individuals of 100%. In some samples, N-specific antibodies were still detectable, whereas in others the titer had considerably decreased and was not detectable by neither ELISA nor LFA. As expected after vaccination, and in contrast to anti-S antibodies, anti-N antibodies decreased with time (Fig. 3D).

3.5. Seroneutralization

In order to study the ability of the RBD-ELISA and S-LFA to detect neutralizing antibodies, the surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike protein–protein interaction (cPass SARS-CoV-2 Neutralization Antibody Detection Kit, GeneScript, cPass) was used in parallel, focusing on the antibodies specific to S protein. To this aim, a selection of sera from the described collections classified as negative (noninfected according to the commercial N-ELISA) and positive (vaccinated) were analyzed by cPass, RBD-ELISA (n = 80, 54 positives, 26 negatives) and S-LFA (n = 54, 39 positives, 15 negatives). The results indicated that the sensitivity and specificity parameters were in the same range for both RBD-ELISA (98.3% and 96.6%, respectively) and S-LFA (94.8% and 100%). The same applied for the agreement (Cohen’s kappa coefficient) between cPass TM and the studied assays (0.915 for RBD-ELISA and 0.911 for S-LFA).

Furthermore, a direct comparison between RBD-ELISA and seroneutralization (SNT) was performed with samples extracted at different times post vaccination (58) and 2 samples from infected non-vaccinated. The results showed a sensitivity of 85.7% and a specificity of 92.0% (Cohen’s kappa coefficient 0.764). Only 7 discordant sera (5 false negative, 2 false positive) were found. These sera had SNT titers below 8 and corresponded to times before the second vaccination dose (3 samples), long post-infection time (5 months, 1 sample) or one month after a single-dose vaccine administration (3 samples).

4. Discussion

Antibody tests that detect the responses to SARS-CoV-2 RBD and trimeric Spike protein can help in tracking the effect of vaccination programs.

In this article, two serological assays for the surveillance of the antibody response to infection and vaccination have been described: an ELISA that detects RBD-specific antibodies and a Dual rapid test that detects S and N-specific antibodies. Due to the high prevalence the disease can have, the simultaneous detection of N and S-specific antibodies could be helpful for the determination of the immunological status pre- and post-vaccination.

Both tests have demonstrated high sensitivity and specificity, as well as +LR and −LR that make them optimal assays for the diagnosis of SARS-CoV-2 infection. Good agreement has been observed with all the assays compared to the commercial ELISA Ingezim COVID 19 DR used as reference, as well as between assays specific for the detection of antibodies to the same protein. When comparing tests for the detection of specific antibodies to the S or RBD proteins with the commercial ELISA (specific for the detection of N-antibodies), slightly lower values of specificity were observed. However, most of the samples classified as false positive also gave a positive result with a commercial assay with the ability to detect antibodies to S protein. If those samples were considered positive, the specificity would be...
100% for RBD-ELISA and 99.6% for S-LFA. This observation may indicate that some of the people tested, developed immunoglobulins against N protein but not against S protein (4.3% of infected-individuals’ samples) and vice versa (12.1% of infected-individuals samples) during SARS-CoV-2 infection and/or that the level of antibodies against N protein decreased with time, as some published data are pointing out [25, 26]. In the lateral flow assay, if a positive result to either N or S was considered positive, the sensitivity of the assay would increase, justifying the use of a dual test as the one described (INGezim COVID 19 N/S DUAL CROM).

Currently, there is a massive vaccination campaign with the aim to achieve herd immunity, which highlights the major importance to assess the durability of the immunity. This study showed that in vaccinated people previously infected, S-specific antibody levels measured by both tests were remarkably high, as early as 7 days after the first vaccine dose, independently of their N-specific antibody levels, as already described [4, 27]. In fact, 15 days after first dose, all the vaccinated and previously infected people, showed high antibody titer with all the tested vaccines. That result indicates that vaccination would act as a booster, as described by Wise [28], enabling vaccine supplies to be deployed effectively.

On the other hand, there is a gradual increase in S-antibody levels after first vaccine in non-infected people, but some of them remained negative even at early times after second dose. However, 15 days after completing vaccination schedule, all of them seroconverted except one, which was confirmed negative by an alternative assay. Although the current vaccines have demonstrated to be highly immunogenic, the possibility of low responders exists, and that is one of the reasons why serosurveillance is important. Furthermore, according to a small study in a nursing-home corresponding to 48 users and 32 employees, the immunological response was age-dependent, since 97% of the employees (average of 47 years old) showed a positive result 15 days after second vaccine dose, in contrast to only 65% of the elderly older than 85 years old (data not shown). Thus, the detection of hyporesponsiveness groups [29, 30] would be an important factor for the development of specific vaccination procedures for individual healthcare and for global control of the disease.

For the design of future vaccination protocols, it is important to determine not only the antibody titer, but also the neutralizing capacity of the circulating antibodies. In this work, the performance of RBD-ELISA showed good agreement with SNT and with a commercial ELISA that is a surrogate of neutralization (cPass). Taking this into consideration and that S-LFA and RBD-ELISA show excellent agreement (Cohen’s kappa coefficient 0.979), both tests can be useful for the prediction of neutralizing antibodies presence.

The emergence of SARS-CoV-2 mutations throughout the pandemic [31] highlighted the importance of determining if vaccines are efficacious against the new variants. Although further experiments are needed, preliminary results with the RBD-based ELISA using as antigen RBD corresponding to several variants (kindly provided by ICOSAGEN) and sera from vaccines (Pfizer/BioNtech or Moderna) has shown that the elicited antibodies also recognize Variants of Concern 20I/S:S:501Y.V1 (B.1.1.7, Alpha), 20H/S:S:501Y.V2 (B.1.351, Beta), 20J/S:S:501Y.V3 (P.1, Gamma) and 21A/S:478K (B.1.617.2, Delta; data not shown).

According to the data presented here, INGezim COVID 19 N/S DUAL CROM represents a valuable assay to detect SARS-CoV-2 infection due to its complementary information on N and S specific antibody response, increasing the overall sensitivity, and its ability to determine the extent of immunization after vaccination with the inherent advantages of rapid and point-of-care tests. Additionally, INGezim COVID RBD-DR provides the same information on vaccination response with the possibility of simultaneous determination of multiple samples.

Apart from the determination of seroprevalence through detection of previous infection, tracking the development of immunity will help policy makers to design vaccine administration programs and anti-COVID-19 measures.

**Author Contributions**

Alba Fresco-Taboada: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - Original Draft, Visualization, Marga García-Durán: Conceptualization, Formal analysis, Methodology, Validation, Investigation, Writing - Original Draft, Visualization, Cristina Aira: Investigation, Formal analysis, Writing - Original Draft, Visualization, Lissett López: Investigation, Writing - Review & Editing, Patricia Sastre: Resources, Writing – Review & Editing, Lia van der Hoek: Conceptualization, Resources, Writing - Review & Editing, Rogier W. Sanders: Methodology, Resources, Writing - Review & Editing, Barbara Holzer: Investigation, Writing - Review & Editing, Irene Zimpernik: Resources, Writing - Review & Editing, Eduardo López-Collazo: Resources, Writing – Review & Editing, Patricia Muñoz: Resources, Writing – Review & Editing, Paloma Rueda: Conceptualization, Project administration, Writing - Review & Editing, Carmen Vela: Conceptualization, Project administration, Writing - Review & Editing, Supervision.

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**Ethical statement**

Participation in the described studies was voluntary and without incentive in all the human serum samples collected. All Eurofins Ingenasa volunteers were informed about the study and gave written informed consent, obtaining as a result the evaluation on their serological status versus COVID-19. Moreover, samples provided by Public Organizations such as Hospitals or National Reference Centers were approved by the Hospital Ethics Committee, following the ethical principles established in the Declaration of Helsinki.

**Declaration of competing interests**

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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