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Nucleoprotein-based ELISA for detection of SARS-COV-2 IgG antibodies: Could an old assay be suitable for serodiagnosis of the new coronavirus?

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A R T I C L E I N F O

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A B S T R A C T

Objectives: We evaluated the performance of a nucleoprotein-based enzyme-linked immunosorbent assay (ELISA) for detection of IgG antibodies to SARS-CoV-2.

Methods: The ELISA was based on serum IgG reactivity to a 46-kDa protein derived from the recombinant SARS-CoV2 nucleoprotein. Assay sensitivity was assessed using serum samples from 134 COVID-19 confirmed cases obtained > 15 days after symptom onset. Specificity was determined by testing sera from 94 healthy controls. Cross-reactivity was evaluated with sera from 96 individuals with previous dengue or zika virus-confirmed infections, with 44 sera from individuals with confirmed infections to other respiratory viruses or with bacterial and fungal infections that cause pneumonia and with 40 sera negative for SARS-CoV-2 nucleoprotein by commercial ELISA kits.

Results: The majority of subjects were male and ≥ 60 years old. Assay sensitivity was 90.3 % (95 % confidence interval 84.1 %–94.2 %) and specificity was 97.9 % (92.6 %–99.4 %). There was no cross-reactivity with sera from individuals diagnosed with dengue, zika virus, influenza virus, rhinovirus, adenovirus, respiratory syncytial virus, seasonal coronavirus, *Mycobacterium tuberculosis*, *Staphylococcus* (*S. aureus* and coagulase-negative), *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and the fungus *Aspergillus fumigatus*. The level of concordance of our test with results from commercial ELISA kits was 100 %.

Conclusion: The nucleoprotein-based ELISA was specific for detection of IgG anti-nucleoprotein antibodies to SARS-CoV-2. It utilizes a frequently employed low expense assay protocol and is easier to perform than other currently available commercial SARS-CoV2 antibody detection tests.

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1. Introduction

In late 2019, the Severe Acute Respiratory Syndrome – Coronavirus-2 Virus (SARS-CoV-2), the cause of Coronavirus Infectious Disease-2019 (COVID-19), emerged in China and subsequently rapidly spread worldwide within a few months (Andersen et al., 2020). The ability to accurately determine who is infected with this virus is a critical step in achieving control of this pandemic. Diagnostic tests for COVID-19 fall into two main categories: molecular tests that detect viral RNA, and serological assays that detect anti-SARS-CoV-2 specific immunoglobulins. Analysis of nasopharyngeal and oropharyngeal secretions for viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) is the gold standard for diagnosis of acute COVID-19 (Andersen et al., 2020). Serological assays, while more limited than RT-PCR in detecting SARS-CoV-2 infection at its initial stage, is easier to perform and much less expensive. Determination of IgG-positivity complements molecular testing by confirming exposure and identifying the magnitude of the immune response in infected individuals (Böger et al., 2020; Theel et al., 2020). Serological testing for SARS-CoV-2 IgG antibodies has been recommended in the following situations: screening of recovered COVID-19 patients for convalescent plasma donation, for population seroprevalence studies, and for monitoring the immune response of candidate COVID-19 vaccines (Theel et al., 2020).

The aim of the present study was to evaluate the performance of a classic protocol-based ELISA to detect SARS-CoV-2 IgG anti-nucleoprotein antibodies. Sera from individuals admitted to the hospital for suspected COVID-19 infection were assayed. The nucleoprotein was chosen as the target of the assay since it is an immunodominant antigen and consistently employed to detect antibodies against other coronaviruses in both humans and animals (Guo et al., 2020a; Abdelwahab et al., 2015; Leung et al., 2004).

2. Methods

2.1. Design

This was a cross-sectional diagnostic accuracy study in which a SARS-CoV-2 serodiagnosis protocol was evaluated.

2.2. Setting and study subjects

Sera from 134 Covid-positive symptomatic individuals were evaluated. The majority (85.8 %) were hospitalized (intensive care unit = 105; ward = 10) either at the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (USP)* (a public hospital) or at *Hospital Sírio Libanês* (a private hospital) in São Paulo, Brazil, between March and May 2020. The remainder were seen as outpatients. All individuals presented with acute respiratory symptoms and were positive for SARS-CoV-2 by RT-PCR in respiratory secretions. Age, sex, case severity, and date of the onset of symptoms were retrieved from medical records and from the laboratory information system of the two hospitals. Their blood samples were collected > 15 days (20.9 %) or ≥ 20 days (79.1 %) from the onset of symptoms. The historical control group was composed of 94 clinically healthy blood donors whose serum samples were sent to our laboratory from 2007 to 2017, prior to the emergence of SARS-CoV-2.

To evaluate possible cross-reactivity of the SARS-CoV-2 ELISA with IgG antibodies to other viral infections, such as dengue and zika virus that are still circulating in Brazilian urban areas following recent epidemic episodes, 96 serum samples from patients with RT-PCR-confirmed dengue (n = 76) or zika infections (n = 20) collected between 2010 and 2019 were tested. In addition, serum samples from individuals who produced vaccine-induced antibodies against the three influenza viruses (n = 20), or who had severe acute respiratory syndrome due to rhinovirus (n = 1), adenovirus (n = 1), respiratory syncytial virus (n = 1) and to four seasonal coronaviruses - HCoV 229E (n = 1), NL63 (n = 1), HKU (n = 1) and OC43 (n = 1) - were also tested. Cross-reactivity with pneumonia-related bacteria such as *Mycobacterium tuberculosis* (n = 10), *Staphylococcus aureus* (n = 2), *Staphylococcus* coagulase negative (n = 1), *Streptococcus pneumoniae* (n = 1), *Klebsiella pneumoniae* (n = 1) or the fungal pathogen *Aspergillus fumigatus* (n = 2) were also evaluated. Additionally, we evaluated the concordance of our ELISA test results with results obtained by a reference panel of 40 sera that were uniformly negative when assayed in different commercial SARS-CoV2 kits.

All control samples had been stored at −80 °C and previously unthawed aliquots were tested.

2.3. IgG anti-nucleoprotein detection

The protocol was performed using a qualitative nucleoprotein-based ELISA to detect SARS-CoV-2 IgG antibodies. PolySorp NUNC 96 wells microplates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with a recombinant SARS-CoV2 nucleoprotein antigen generated in an *E.coli* expression vector with purity > 90 % as indicated by the manufacturer (GenScript Inc., NJ, USA, 46 KDa protein). The optimal concentration of the antigen and serum dilutions were chosen after block titration assays. The plates were coated with the recombinant SARS-CoV-2 nucleoprotein at a concentration of 1 μg/mL diluted in 0.05 M carbonate-bicarbonate buffer and incubated in a humid chamber at 4 °C overnight. After each incubation step, the plates were rinsed with phosphate-buffered saline containing 0.05 % Tween 20 (PBS-T). The microtiter wells were blocked with PBS-T supplemented with 5 % skim milk by a 2 h preincubation at room temperature. The serum samples, diluted 1:200 in PBS-T supplemented with 2 % skim milk, were added to the wells and incubated for 1 h at 37 °C. Following the rinsing steps, the wells were incubated with horseradish peroxidase-conjugated anti-human IgG (Sigma Aldrich, San Louis, MO, USA) diluted 1:20,000 in the same buffer solution. The chromogenic solution TMB and substrate (Siemens Healthcare, Marburg, Germany) were used for detection. The optical density (OD) in individual plate wells was measured at 450/650 nm in an automatic ELISA reader.

The cut-off value for a positive reaction was operationally defined as the mean OD plus three standard deviations as determined by testing serum samples from 94 healthy blood donors obtained pre-pandemic.

Standard negative and positive controls as well as a threshold control (with OD equal to the cut off value) were analyzed in each ELISA run. The results were expressed as Reactivity Index (RI) by dividing the OD of each sample by the OD of the threshold control. The results were considered positive if the RI value was ≥ 1.

2.4. Statistical analysis

Categorical variables were summarized as percentages, and continuous variables as median and interquartile ranges. Sensitivity and specificity were calculated following standard guidelines; 95 % confidence intervals (95 % CI) were calculated by the exact binomial method. To evaluate the statistical hypothesis, we used the Chi-square test or the Fisher exact test when appropriate (n ≤ 5) for categorical variables, or the Wilcoxon rank sum for continuous variables. All analyses were performed using the R software for windows statistical computing, version 3.6.3.

3. Results

Demographic

Demographic (gender and age) and clinical data (level of care required and time from symptom onset to serology collection) of the study subjects, divided by the presence or absence of IgG anti-nucleoprotein antibodies is shown in Table 1. There were no differences between seropositive and seronegative cases in these parameters

Table 1
Nucleoprotein-based ELISA results for detection of SARS-CoV-2 IgG according to clinical and demographic variables.

	IgG Seropositive n = 121 n (%)	IgG Seronegative n = 13 n (%)
Sex		
Female	44 (89.8)	5 (10.2)
Male	77 (90.6)	8 (9.4)
Age (years)		
<40	14 (93.3)	1 (6.7)
40−59	11 (84.6)	2 (15.4)
60−79	42 (93.3)	3 (6.7)
≥80	54 (88.5)	7 (11.5)
Level of care required		
ITU	105 (89.0)	13 (11.0)
Ward	10 (100)	0 (0.0)
Outpatient	6 (100)	0 (0.0)
Time from symptom onset to serology collection - days median (IQR*)	27 (20−35)	22(18−30)

*IQR, Interquartile range.

by univariate analysis. In both groups, the majority of subjects were male, > 60 years of age and were admitted to an intensive care unit. The mean time from onset of symptoms to blood collection was between 22–27 days.

3.1. Assay performance

Assay performance of the nucleoprotein-based ELISA to detect SARS-CoV-2 IgG antibodies is presented in Fig. 1. There were no cross-reactions when serum samples from individuals positive for antibodies to dengue, zika, other respiratory viruses (rhinovirus, adenovirus, respiratory syncytial virus) and four seasonal coronaviruses (HCoV 229E, NL63, HKU and OC43) or pneumonia-related bacteria and a fungus were tested. Based on the 134 RT-PCR-confirmed COVID-19 positive cases, the ELISA sensitivity was 90.3 % (95 % CI 84.1 %–94.2 %). Considering only the samples provided at least 20 days (98/106) after the onset of symptoms, the sensitivity was 92.5 % (95 % CI 85.8 %–96.1 %). The ELISA specificity based on 94 serum samples from healthy pre-pandemic blood donors was of 97.9 % (95 % CI 92.6 %–99.4 %). The level of concordance of our test with results obtained with a panel of negative sera tested in different SARS-CoV-2 serological commercial ELISA kits was 100 %.

4. Discussion

In this study, we confirmed the validity of a nucleoprotein-based ELISA for detection of SARS-CoV-2 IgG antibodies in sera obtained >15 days from symptom onset. This time period was chosen since a prior study demonstrated that the median time for IgG anti-SARS-CoV-2 appearance was 14 days after the onset of symptoms (Guo et al., 2020b).

The specificity of our ELISA was 97.9 %, which is comparable to other assays with a similar format (Böger et al., 2020; Deeks et al., 2020; Lisboa Bastos et al., 2020; Caini et al., 2020). There were no false positives when testing 44 sera from individuals with vaccine-induced antibodies to influenza viruses, or who had severe acute respiratory syndrome or pneumonia due to other causes. In addition, we did not observe any cross-reactivity with sera from individuals with a history of infection with common cold coronaviruses such as HCoV-229E, -NL63, -OC43 and -HKU1. This paralleled findings obtained with other widely used SARS-CoV-2 serological assays (Deeks et al., 2020; Brochot et al., 2020). Different seasonal coronaviruses have been detected by RT-PCR with a frequency varying from 1.5 to 11 % among patients presenting with respiratory infection in different regions in Brazil (Bezerra et al., 2011; Trombetta et al., 2016; HLDS et al., 2019; Matsuno et al., 2019; Góes et al., 2020) One study has reported a 10 % cross-reactivity with SARS-CoV-2 among sera from healthy blood donors with a history of seasonal coronaviruses This was attributed to antibody binding to conserved epitopes in the nucleocapsid or spike proteins (Ng et al., 2021). While a potential low level cross-reaction between IgG antibodies to the seasonal coronavirus and to the pandemic SARS-CoV-2 virus is possible and needs to be considered, this is of minor concern in the epidemiological context of the Covid pandemic (Deeks et al., 2020; Ng et al., 2021; Yue et al., 2021).

In countries like Brazil, where dengue fever and other arboviruses are endemic problems, the possibility of cross-reactions with SARS-CoV-2 could be a challenge (Nath et al., 2020; Yan et al., 2020; Lustig et al., 2020; Lorenz et al., 2020). Although these arboviruses do not cause respiratory disease, they can be difficult to distinguish from COVID-19 as both can elicit similar extra-pulmonary manifestations such as fever, cutaneous rash and conjunctivitis. We tested 96 serum samples from patients with RT-PCR-confirmed dengue (n = 76) or zika infections (n = 20), collected between 2010 and 2019 and did not observe any cross-reactivity.

The limitations of our study must be considered. Most patients in the present study (85.8 %) were hospitalized and elderly, suggesting that their infections were severe. Previous studies have suggested that the magnitude of the antibody response to SARS-CoV-2 was related to clinical severity (Hachim et al., 2020). We cannot comment, therefore,

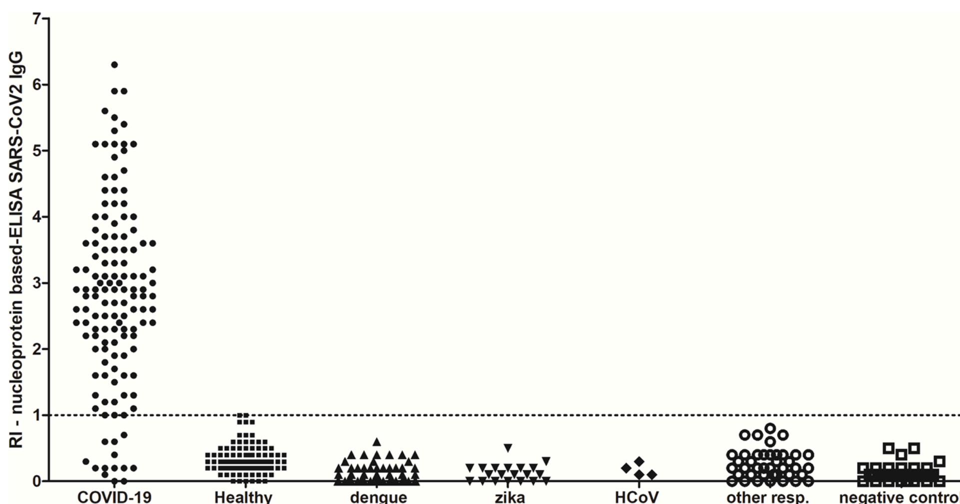

Fig. 1. Distribution of the Reactivity Index (RI) by using nucleoprotein based-ELISA for SARS-CoV-2 IgG antibody detection. Sera were from confirmed COVID-19 cases obtained > 15 after onset of symptoms (n = 134), from healthy blood donors obtained pre-pandemic (n = 94), from individuals with arboviruses infections (dengue, n = 76 and zika, n = 20), from individuals positive for HCoV 229E, NL63, HKU, and OC43 seasonal coronaviruses (n = 4), other respiratory infections (n = 40) and a panel of reference negative controls (n = 40). The RI was based on the relation between OD sample and OD threshold control. The RI ≥ 1.0 is defined as a positive result (dashed line).

on the sensitivity of our assay to detect IgG anti-nucleoprotein antibodies in mildly symptomatic or asymptomatic infected individuals. Similarly, limitations of our sera collection do not allow us to estimate the sensitivity of our ELISA to detect antibodies at < 15 days or >50 days after the onset of symptoms. It is important to emphasize that the duration and persistence of circulating antibodies against SARS-CoV-2 have not yet been established for most tests that are currently used (Deeks et al., 2020). Therefore, this limitation is not exclusive to our protocol. Our findings on sex are compatible with several studies reporting that the number of COVID-19 cases and their severity and lethality are higher in men compared to women (Jin et al., 2020; Spagnolo et al., 2020; Dudley and Lee, 2020). Unfortunately, we also were unable to obtain information on clinical outcome of the studied cases.

Despite these limitations, our ELISA has several advantages that should be highlighted. The sensitivity and specificity of our ELISA is high and equivalent to that of commercial kits or other *in-house* assays (Böger et al., 2020; Deeks et al., 2020; Lisboa Bastos et al., 2020; Caini et al., 2020). Our assay is technically easy to perform and can be implemented without the need for specialized equipment. This may be advantageous in resource-restricted areas where diagnostic capacity is limited. In addition, the cost of analysis by our ELISA is significantly lower when compared to that of commercial tests. An ELISA format also allows for the testing of large numbers of suspected cases in a short period of time. In Brazil, a country where the number of COVID-19 cases has been rapidly rising, this ELISA assay would be of value to clarify aspects related to the spread of the infection in the community and to establish optimal policies to fight the epidemic.

In conclusion, our nucleoprotein-based ELISA to detect IgG antibodies to SARS-CoV-2 is a specific and sensitive test to determine exposure to this virus two weeks from the onset of symptoms. It is a suitable and less expensive alternative to the serological tests that are currently available.

Ethical approval

The study was approved by the *Hospital das Clínicas* -University of São Paulo (HC-FMUSP) and *Hospital Sírio Libanês* ethnics committees (CAAE Registry No.:30419620.1.0000.0068 and 30701920200000068, respectively).

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CRediT authorship contribution statement

Tania Regina Tozetto-Mendoza: Conceptualization, Supervision, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Kelly Aparecida Kanunfre:** Supervision, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Lucy Santos Vilas-Boas:** Methodology, Supervision. **Evelyn Patricia Sanchez Espinoza:** Data curation. **Heuder Gustavo Oliveira Paião:** Methodology. **Mussya Cisotto Rocha:** Methodology. **Anderson Vicente de Paula:** Methodology. **Maura Salaroli de Oliveira:** Data curation. **Daniella Bosco Zampelli:** Data curation. **José Mauro Vieira:** Data curation. **Lewis Buss:** Formal analysis. **Silvia Figueiredo Costa:** Conceptualization, Data curation. **Ester Cerdeira Sabino:** Conceptualization, Funding acquisition. **Steven S. Witkin:** Data curation, Formal analysis, Writing - review & editing. **Thelma Suely Okay:** Formal analysis, Methodology, Writing - review & editing. **Maria Cassia Mendes-Correa:** Conceptualization, Funding acquisition, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

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