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Performance of Recombinant Nucleocapsid Protein-Based Constructs for Serological Diagnosis of SARS-CoV-2 Infection

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HIGHLIGHTS

- Recombinant constructs of SARS-CoV-2 N-protein for serosurveillance purpose.
- ELISA using three N-based constructs detect IgG across COVID-19 disease categories.
- The C-terminus N-protein portion, rCoV-7, showed the better diagnostic performance.
- Useful for epidemiology of SARS-CoV-2 in populations vaccinated with S-protein based vaccines.

Abstract: Since the inception of COVID-19 pandemic, there has been a challenging race for the development of precise diagnostic tests. Specific SARS-CoV-2 serological assays are the main tools used to estimate the rate of past infections or herd immunity in epidemiological studies, in addition to being helpful in guiding public health management policies. In this study, an in-house ELISA based on the construct of SARS-CoV-2 nucleocapsid (N) proteins, named rCoV2, rCoV4, and rCoV7, showed diagnostic performance for the detection of IgG antibodies. Sensitivity was evaluated in serum samples from patients with mild to moderate or severe COVID-19 infections, which were collected at different time points, while specificity was evaluated using pre-pandemic sera. In samples from mild to moderate cases obtained ≥16 days after the onset of symptoms, the sensitivities for rCoV2, rCoV4, and rCoV7 were 66.7%, 75%, and 77.8%, respectively. For samples from severe cases, the sensitivity was above 80% for all constructs. All proteins showed high specificity (94–98%). Overall, rCoV7 (C-terminus N-protein portion) showed better diagnostic performance, with 62.3% sensitivity in moderate and severe cases and 96.6% specificity. The SARS-CoV-2 ELISA using N-protein-based constructs could be a promisor tool for investigate the epidemiology of COVID-19 and monitor population-level serosurveillance.

Keywords: COVID-19; serodiagnosis; Nucleocapsid protein; ELISA; recombinant protein.

INTRODUCTION

The COVID-19 pandemic, an emergent respiratory disease caused by SARS-CoV-2 virus [1,2], has infected more than 753 million people, resulting in approximately 6.8 million deaths worldwide [3]. After more three years since the start of the COVID-19 pandemic, SARS-CoV-2 continues to spread globally. The advancement of the pandemic demands a worldwide race for the development of better laboratory diagnostic tests applicable in different medical and epidemiological settings. Molecular tests are the more accurate choice for diagnosing current infections [4]. Although antibody testing diagnosis is not currently used to assess active COVID-19 infection or immunity after COVID-19 global vaccination [4], it still plays a significant role in monitoring serosurveillance, helping to develop population-based seroprevalence studies in many countries [5–9]. Additionally, serological assays are still useful for distinguishing infection-induced from vaccine-induced responses in people who were immunized with schedules based only on spike antigen.

The humoral immune response induced by a SARS-CoV-2 infection includes the production of antibodies of several immunoglobulin isotypes that target SARS-CoV-2 proteins, most notably structural spike (S) and nucleocapsid (N) proteins [10]. The N protein is located within the viral particle and is essential for viral replication and packaging viral RNA into new virions [11]. Data of SARS-CoV and other coronaviruses have shown that this protein is highly immunogenic and abundantly expressed during acute and chronic infections [12–14]. The SARS-CoV-2 N-protein contains five distinct RNA-binding domains (RBD): a disordered N-terminal domain (NTD) (residues 1–50), an RNA-binding domain (RBD) (residues 51–174), a predicted disordered central linker (LKR) (residues 175–246), a dimerization domain (residues 247–365), and a predicted disordered C-terminal domain (residues 366–419) [15].

Few studies have focused on the SARS-CoV-2 N-protein as a diagnostic target [9,16–20]. Furthermore, IgG titers against the SARS-CoV-2 N protein can be correlated with the severity of COVID-19 cases in non-vaccinated populations [21]. Although entire proteins or larger molecules have a higher potential to show better sensitivity results because they present more epitopes, we hypothesized that the use of smaller molecules with selected immunodominant regions will help increase specificity during diagnosis. In order to select the main immunogenic regions of the SARS-CoV-2 N-protein for diagnostic purposes, we developed three recombinant constructs of the antigen: the full-length (rCoV2), the C-terminal region of the N-protein (rCoV7), and a chimeric containing the E1 and E2 immunodominant epitopes repeat in tandem (rCoV4).

Thus, the aim of this study was to evaluate the diagnostic performance of COVID-19 IgG assays using three different constructs of the SARS-CoV-2 N-protein and assess their correlation with the time of disease onset.

MATERIAL AND METHODS

Samples and patients

This study was performed at the Federal University of Pelotas (UFPel) and was approved by the local ethics committee (approval number: 4.043.628). Sixty-nine symptomatic patients who tested positive for SARS-CoV-2 by real-time PCR (RT-PCR) of nasopharyngeal swab samples between March and May 2020 were selected for this study. Patients were then either admitted to the *Hospital Escola da UFPel* (HE UFPel) intensive care unit (n=10), the *Hospital de Clínicas de Porto Alegre* (HCPA) infectious disease ward (n=20), or *Fundação de Medicina Tropical Dr. Heitor Vieira Dourado* (FMT-HVD, AM) (n=39). Clinical severity during hospitalization was retrieved from patient records. The participants were stratified into three groups. The first group consisted of patients from FMT-HVD who had mild to moderate symptoms, and their blood samples were collected > 1 or ≥ 16 days from the onset of COVID-19 symptoms. These first group consisted of 14 male and 25 female patients with a median age of 45.7 years (23 – 76 years). The other two participant groups were patients from HCPA and HE UFPel, considered to have severe COVID-19 disease if hospitalization at the intensive care unit was required. The clinical classification followed criteria to patient management determined by Ministry of Health of Brazil [22], which is based on WHO technical guidance [23]. These patients were hospitalized, and their blood samples were collected at 0 and 7 days (n=20; HCPA) or between 0 and 7 days (n=10; HE UFPel) after a positive RT-PCR assay result. HCPA group consisted of 6 male and 4 female patients with a median age of 61.9 years (43 – 77 years). HE UFPel group consisted of 14 male and 6 female patients with a median age of 63.7 years (44 – 81 years). Written informed consent was obtained from all the participants enrolled in this study.

The specificity of the assay was determined by analyzing 60 patient samples that were positive for antibodies against HIV (n=49), HIV + *Toxocara* (n=8), and hepatitis B and D (n=3) obtained before the occurrence of SARS-CoV-2 (2004 – 2019). Serum samples were acquired from the Universidade Federal do Rio Grande (FURG). All samples used in the study were stored at -20 °C until use.

Antigen production

The full length of the N-protein of SARS-CoV-2 (1–419 aa) (rCoV2), the C-terminal region of the N-protein (160–406 aa) (rCoV7), and a chimeric containing the E1 (160–181 aa) and E2 (370–406 aa) immunodominant epitope repeats in tandem (E1-E2-E1-E2-E1-E2-E1-E2) from the N-protein (rCoV4) were used as detection antigens. For rCoV4 construct, a rigid linker ($EAAAK_{3\times}$) was added to connect each epitope repeatedly, in tandem. The selected amino acid sequences were used to design three codon-optimized synthetic genes for *E. coli* heterologous expression, which were synthesized and cloned into the pET28a vector by Epoch Life Science (USA). All N-protein constructs were expressed in *E. coli* Star (DE3) cells and characterized using an anti-His-HRP antibody. Recombinant proteins were purified by affinity chromatography using HisTrap™ HP 1 mL columns with pre-charged Ni Sepharose™ using the AKTA START™ automated liquid chromatography system (GE Healthcare, USA). Fractions containing the purified proteins were extensively dialyzed in PBS and stored at -20 °C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Brazil) as per the manufacturer's instructions.

In-house ELISA using N-protein-based constructs

For in-house indirect ELISA, the ideal concentrations of each assay reagent and ideal conditions for each step were previously standardized with pools of positive (in RT-PCR) and negative (pre-pandemic) serum samples described in 2.1 section. ELISA was performed under optimal conditions as follows: Nunc-Immuno MicroWell MaxiSorp™ plates were coated with rCoV4 (50 ng/well) or rCoV2 and rCoV7 (100 ng/well) diluted in carbonate-bicarbonate coating buffer (0.05 M; pH 9.6) for 18 h at 4 °C. The coating solution was removed and the plates were blocked with 5% (w/v) non-fat milk solution in PBS-T (PBS, 0.05% Tween 20). Serum samples were diluted in PBS-T with 2.5% skimmed milk powder and added at 1:100 (rCoV4) or 1:200 (rCoV2 and rCoV7) dilutions (100 µL/well). Anti-human IgG (Thermo Scientific, USA) 1:4000 was used as the secondary antibody. Reactions were developed by adding o-phenylenediamine dihydrochloride (Sigma-Aldrich, USA) diluted in citrate-phosphate buffer (0.2 M; pH 4.0) with 0.02% (v/v) H_2O_2 and incubating for 15 min at room temperature. The reactions were stopped by adding 3% H_2SO_4 (v/v). The optical density (OD) was measured at 492 nm (Biochrom EZ Read 400 microplate reader), and the mean values were obtained

from serum samples assayed in duplicate. Plates were washed three to five times with 300 µL of PBS-T/well between each step, and the incubation steps were carried out for 1 h at 37 °C.

Data analysis

For in-house ELISA assays, receiver operating characteristic (ROC) analysis was performed and area under the curve (AUC) was determined to optimize the cut-off and confirm the accuracy of the tests. ROC analysis was performed using a sample panel of 69 positive and 60 pre-pandemic previously described at "2.1 Samples and patients" section. The curve was built using GraphPad PRISM software v.8 by calculating the sensitivity and specificity as a function of varying cut-off values as previously described [24]. Cutoff values were defined as the mean OD value (492 nm) of negative samples plus two values of their standard deviation (StDev). The analytical sensitivity and specificity data were processed using Microsoft Excel. The sensitivity and specificity of each serological assay were assessed separately in symptomatic and hospitalized subjects according to the time elapsed from the onset of symptoms and blood collection or by combination of both. Further statistical analysis and generation of figures was performed using GraphPad PRISM software v.8. Comparisons of antibody levels across patient groups were carried out using Kruskal-Wallis test (Dunn's multiple comparisons test). A P value < 0.05 was considered statistically significant.

RESULTS

Expression of recombinant N-proteins

All the gene constructs (Figure 1a) were successfully expressed in *E. coli* BL21 (DE3) Star bacteria; the products were soluble proteins whose expected molecular masses were approximately 48 (rCoV2), 39 (rCoV4) and 30 (rCoV7) kDa (Figure 1b). The recombinant proteins were detected using the anti-His antibody (Figure 1c).

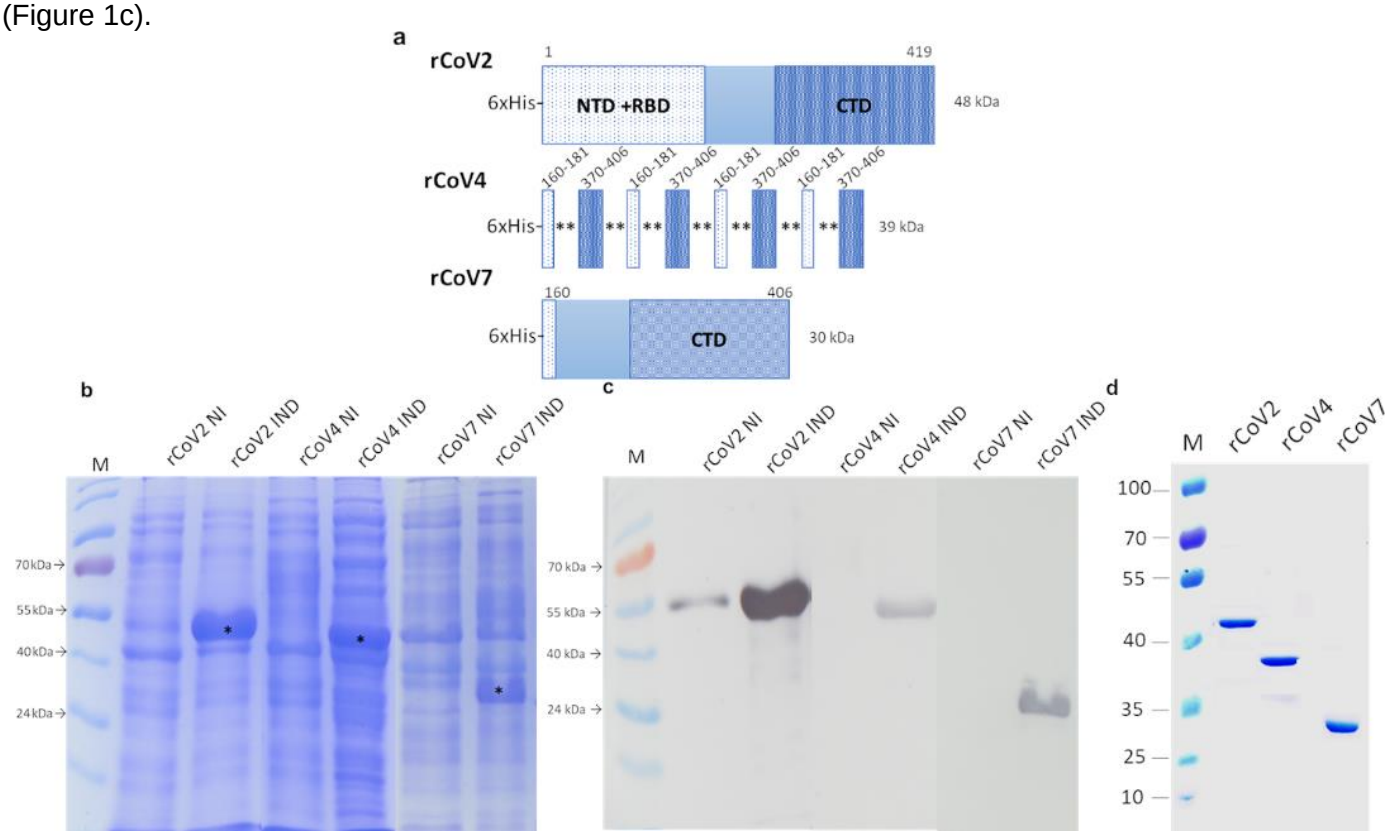

Figure 1. Construction, expression and characterization of the N-protein based constructs. (a) Schematic representation of the N-based constructs. (b) Expression of recombinant proteins (rCoVs) in *E. coli* Star strain analyzed by 12% SDS-PAGE and Coomassie staining. (c) Western blotting analysis of rCoVs, following transfer the nitrocellulose membranes were probed with an anti-His-HRP antibody. *target recombinant proteins; (d) Purification of rCoVs analyzed by 12% SDS-PAGE and Coomassie staining. M, PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Scientific), NTD, N-terminal domain; CTD, C-terminal domain; RBD, RNA-binding domain.

Diagnostic performance of IgG rCoVs in mild to moderate cases

All sera used were diagnosed as positive for COVID-19 via RT-PCR. For mild to moderate cases, the sensitivity of IgG ELISA for recombinant constructs was categorized by days since symptom onset (≤ 5 days, 6-10 days, 11-15 days and >16 days). The sensitivity results obtained for the three N-based constructs are summarized in Table 1. Overall, serum levels of IgG antibodies increased in parallel over time. The sensitivity for rCoVs was ≤50% during the two weeks after the onset of symptoms (days ≤5-15), with higher sensitivity levels observed at >16 days following the onset of symptoms for all antigens (>65%). Among the three N-based constructs, the highest sensitivity was for rCoV7 in all time intervals, ranging from 40.0% to 77.8%. When grouping the time points evaluated, overall sensitivity for rCoV7 was 53.8%, while the overall sensitivities for rCoV2 and rCoV4 were 38.5% and 30.8%, respectively (Table 1, Figure 2). The sensitivity for rCoV7 was statistically significantly different ($P < 0.05$) from that for the rCoV4 protein.

Table 1. Diagnostic sensitivity performance of SARS-Cov-2 N-based constructs.

IgG - mild to moderate symptoms[1]	***n***	**rCoV2**	**rCoV4**	**rCoV7**
Sensitivity [95%CI]				
≤ 5 Days	10	40.0% [30.9-49.8]	20.0% [13.3-28.9]	50.0% [40.3-59.6]
6-10 Days	10	20.0% [13.3-28.9]	10.0% [5.5-17.4]	40.0% [30.9-49.8]
11-15 Days	10	30.0% [21.9-39.6]	30.0% [21.9-39.6]	50.0% [40.3-59.6]
> 16 Days	9	66.7% [57.3-75.4]	75% [65.7-82.5]	77.8% [68.9-84.9]
Total	**39**	**38.5% [29.0-47.7]**	**30.8% [22.7-40.6]**	**53.8% [44.2-63.4]**
IgG - severe cases[2]	***n***	**rCoV2**	**rCoV4**	**rCoV7**
Sensitivity [95%CI]				
HCPA #0[3]	10	50.0% [40.3-59.6]	50.0% [40.3-59.6]	60.0% [50.2-69.0]
HCPA #7[4]	10	100.0% [96.3-100]	80.0% [71.1-80.6]	100.0% [96.3-100]
HE-UFPel #0-7	20	85.0% [76.7-90.6]	85.0% [76.7-90.6]	80.0% [71.1-86.6]
Total[5]	**40**	**73.3% [63.5-80.7]**	**73.3% [63.5-80.7]**	**73.3% [63.5-80.7]**
Overall performance[5]	**79**	**53.60% [44.2-63.4]**	**49.3% [58.6]**	**62.3% [52.2-70.9]**

[1]Days after the onset of symptoms
[2] Days after a positive RT-PCR result
[3]*Hospital de Clínicas de Porto Alegre* (HCPA) - blood samples collected at day 0
[4]*Hospital de Clínicas de Porto Alegre* (HCPA) - blood samples collected at day 7
[5]For HCPA samples were considered only the first blood sample collected after a positive RT-PCR (HCPA #0) 95% CI: 95% confidence interval

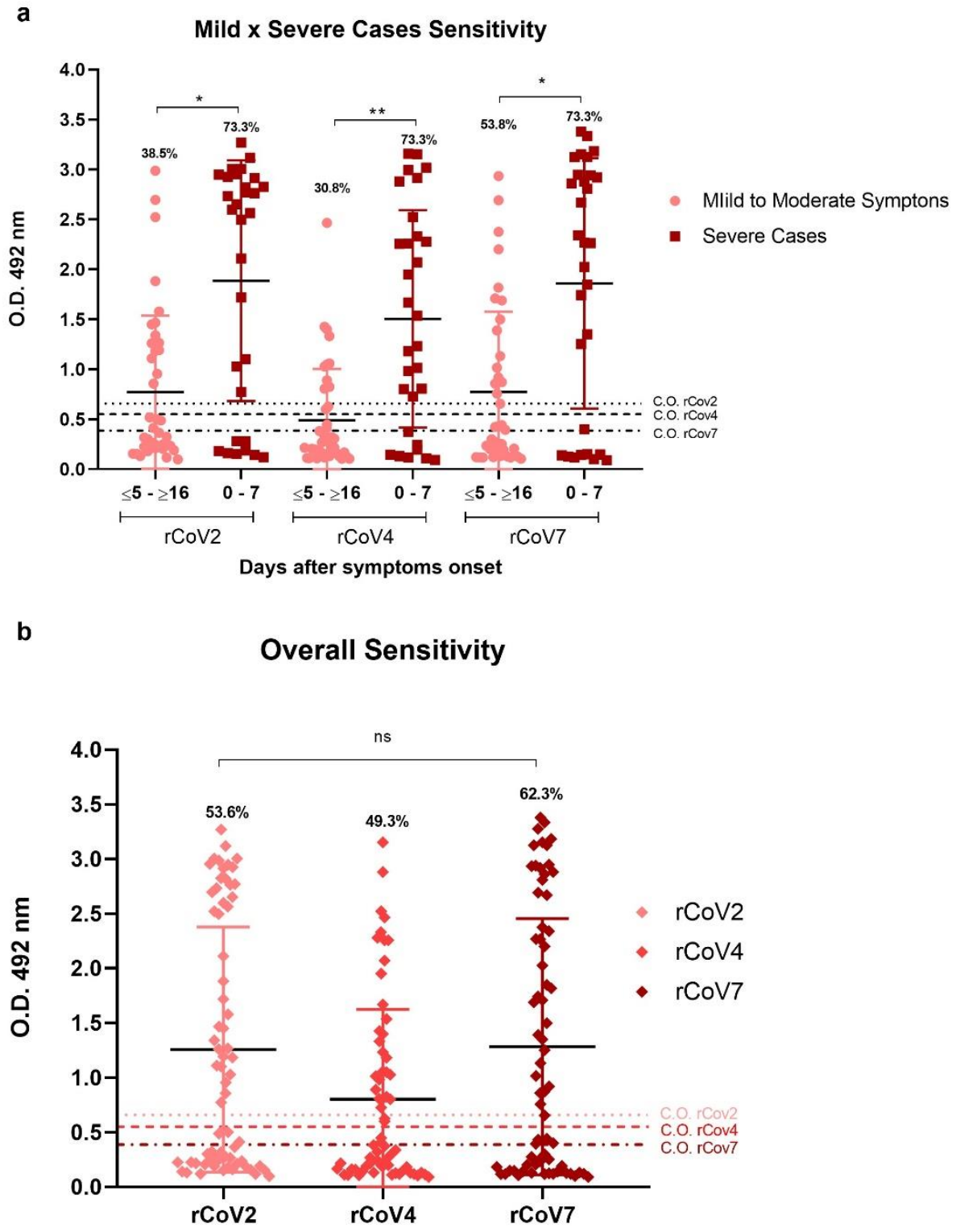

Figure 2. SARS-Cov-2 N-based constructs specific IgG responses in indirect ELISA. N-based constructs (rCoV2, rCoV4 and rCoV7) were used to detect IgG antibodies in sera from patients. (a) The sensitivity performance of the N-based constructs was evaluated with sera from SARS-CoV-2 mild to moderate cases (combining time points at 0 to 5 days, 6 to 10 days, 11 to 15 days, and more than 16 post onset of symptoms) compared to samples from severe cases collected at 0 (HCPA) or 0-7 days (HE), post a SARS-CoV-2 RT-PCR positive result. (b) The overall sensitivity performance was taken by combining data from acute and severe samples for each recombinant antigen and comparing the performance among them. Black dotted lines correspond to cut-off limits for each protein; OD: optical density; C.O.: Cut-off; * < 0.05 ** *P* < 0.01, ns: not significant.

Diagnostic performance of IgG rCoVs in severe cases

When analyzing samples from HCPA, higher sensitivity levels for the recombinant proteins were found in samples taken 7 days after a RT-PCR-positive result was observed, with rCoV2 and rCoV7 sensitivity reaching 100%. For HE-UFPel patients, samples were collected between 0 and 7 days after a positive RT-PCR result was obtained. Due to this variation of the time window, the sensitivity values were lower for all antigens (80-85%), with no statistically significant difference between them. For all the three antigens (rCoV2, rCoV4, and rCoV7), the overall sensitivity in samples from severe cases was 73.3% (Table 1), with no statistical difference between them. When grouping all time points from acute cases compared to all time points from severe cases, the capacity of all recombinant constructs to differentiate samples from severe cases differed significantly ($P < 0,05$) compared to that in the moderate course of disease (Figure 2a). On grouping all time points, acute samples and severe cases, no statistical difference was observed between sensitivities for the three constructs (Figure 2b). To calculate the overall sensitivity, in samples from severe patients, was considered only the first blood sample collected after a positive RT-PCR result obtained from HCPA patients.

Overall diagnostic performance of IgG rCoVs in acute and severe cases

When all positive samples were combined (mild to moderate and severe cases), the overall diagnostic performance of rCoV2, rCoV4, and rCoV7 antigens was 59%, 51.9%, and 65.8%, respectively (Table 1; Figure 2b). Although rCoV7 showed better sensitivity than the other antigens, no statistical difference was observed.

Overall, the panel of 60 pre-pandemic sera negative for SARS-CoV-2 showed low reactivity against the N-antigens used in the in-house ELISAs. Serum samples from three patients with HIV infection showed cross-reactivity with rCoV2, whereas only two and one HIV-positive serum samples were cross-reactive with rCoV4 and rCoV7, respectively. Assay specificities are shown in Table 2 and Figure 3, and the highest specificities were found using the rCoV4 antigen (98.3%), followed by rCoV7 (96.6%) and rCoV2 (94.9%). The same non-reactive serum samples were used to determine the cutoff values for the tests. The cutoff values of IgG against rCoV2 protein were calculated as mean + 2StDev (0.295 + 2[0.181] = 0.658), rCoV4 protein as mean + 2StDev (0.227 + 2[0.162] = 0.552), and rCoV7 protein as mean + 2StDev (0.217 + 2[0.085] = 0.387). Recombinant proteins revealed good diagnostic accuracy with an AUC of 0.7640 (95% confidence interval (CI):0.68-0.84) for rCoV2, 0.7416 (95% CI:0.66-0.83) for rCoV4, and 0.7630 (95% CI:0.68-0.85) for rCoV7.

Table 2. Overall diagnostic specificity performance of SARS-Cov-2 N-based constructs.

IgG - mild to moderate symptoms	**rCoV2**	**rCoV4**	**rCoV7**
Specificity* [95%CI]	93.3% [86.2-96.5]	98.3% [92.9-99.6]	96.7% [91.5-99.1]
Pre-epidemic sera (*n*)	60	60	60
True negative	93.3% (56/60)	98.3% (59/60)	96.7% (58/60)
False positive	6.6% (4/60)	1.7% (1/60)	3.3% (2/60)

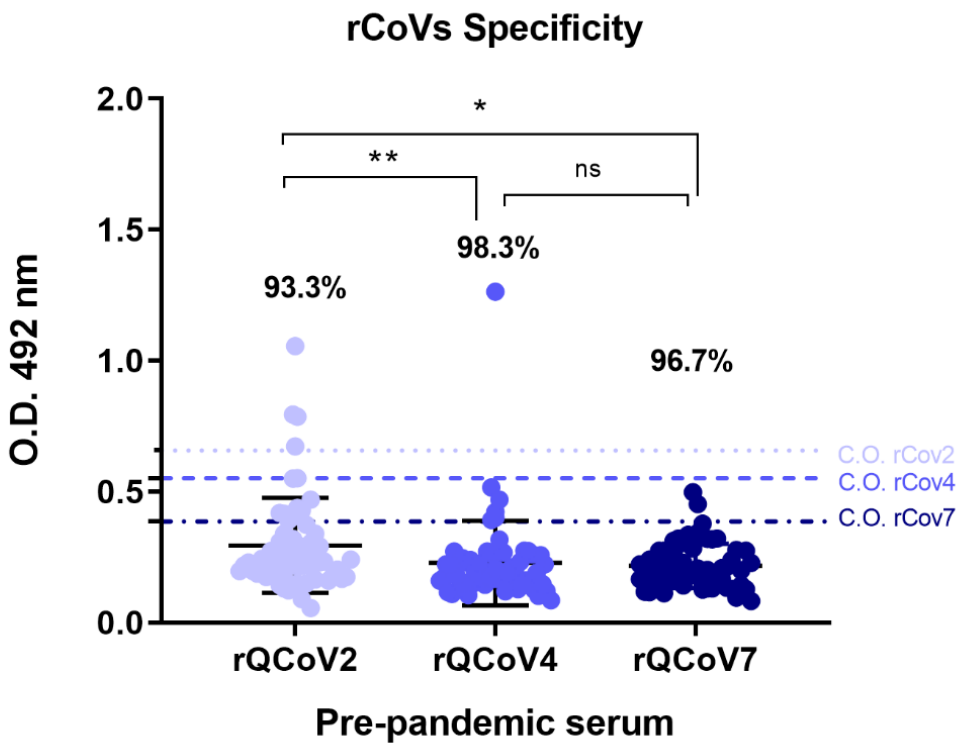

Figure 3. N-based constructs specificity IgG responses in indirect ELISA. Recombinant proteins (rCoV2, rCoV4 and rCoV7) were used to detect IgG antibodies in serum samples from non-infected individuals, collected before the COVID-19 pandemic. Dotted lines in shades of blue correspond to cut-off limits for tests. OD: optical density; C.O.: Cut-off; * $P < 0.05$; ** $P < 0.01$; ns: not significant.

DISCUSSION

In this study, we report the clinical performance of in-house ELISA tests using three different recombinant constructs (rCoV2, rCoV4, and rCoV7) for the detection of IgG antibodies against SARS-CoV-2 N-protein in serum samples of confirmed COVID-19 cases. The serum samples were divided into different groups according to the number of days after symptom onset and disease severity or blood sample collection after a RT-PCR positive result for severe patients. Our data showed that the three N-based constructs were able to detect specific anti-N IgG antibodies across all COVID-19 disease categories, with better performance in serum samples obtained from acute patients 16 days after symptom onset (sensitivity >65%), particularly for rCoV7 (77.8% sensitivity).

For patients with severe disease, sensitivity rates could reach >80% regardless of the antigens used. The three constructs showed an increase in antibody sensitivity rate with time and disease severity (Table 1; Figure 2), which is consistent with results found in previous antibody tests [17,25,26]. Seroconversion analysis in samples from patients infected with SARS-CoV-2 showed that anti-N antibody responses appear eight days after initial symptoms [25], and increase gradually within 1-3 weeks [27,28].

Despite the development and testing of many serological assays targeting the detection of anti-SARS-CoV-2 antigens during the early stages of infection, measurable antibody responses are more accurately detected between 7-14 days after illness onset [29–31]. In our study, lower sensitivity values (10-50%) were observed for all recombinant antigens in samples from mild to moderate cases collected less than two weeks after symptom onset, which may be due to low antibody titers in the early stages of the disease. This weakest performance is consistent with the seropositive rates from commercial tests using samples collected during the early stages of COVID-19 [26].

In the study performed by Rezaei and coauthors (2021) [32], commercial kits used to detect antibodies against N-protein in RT-PCR-positive patient samples collected < 7 and > 14 days after the onset of symptoms yielded a sensitivity of 60 and 63%, respectively. Interestingly, the same authors observed that the sensitivity towards the anti-S antibodies at < 7 days after the onset of symptoms was 30% reduced compared to that towards anti-N antibodies. When the overall data of sensitivity from mild to moderate cases were analyzed, the rCoV7 antigen showed a significant difference compared to rCoV4. It is important to highlight that in contrast to our study, the cohort of early samples (taken <7 days after symptom onset)

analyzed by Rezaei and coauthors (2021) [32] included samples from critical patients (47% of samples). Therefore, it is possible that these samples had higher antibody levels, which could have influenced the sensitivity rates at that time point. Other studies also show that antibodies responses to the N-protein of SARS-CoV-2 are more sensitive than to the S-protein [25,32], or not significantly different [17] for detecting early infection.

Patients with non-severe COVID-19 showed distinct kinetic profiles of humoral immune response compared to those with severe COVID-19, developing lower levels of viral-specific antibodies [10]. The study performed by Hashem and coauthors (2020) [33] showed that anti-N IgG antibodies were more likely to correlate with disease severity than anti-S1 IgG antibodies, especially when mild and severe cases were compared. The authors hypothesized that high levels of anti-N antibodies could indicate high virus replication in severely infected individuals compared to that in non-severely infected patients. Therefore, this protein could act as a useful prognostic marker for COVID-19. As expected, when serum samples from severe patients were evaluated, the sensitivity rates reached 100%, especially for rCoV7. In corroboration with these findings, previous data [34–36] demonstrated that the middle or C-terminal region of the SARS-CoV-2 N-protein is important for eliciting antibodies during immune response, which can help to explain the better performance of rCov7.

Some studies [1,37] have reported seropositivity rates above 90%; however, in most of these studies, analyses were performed with blood samples collected at 14 days or later after symptom onset, thereby excluding earlier samples. Since the sensitivity of most of the tests is mainly evaluated in hospitalized patients, it is unclear whether the tests are able to detect lower antibody levels likely seen in milder and asymptomatic COVID-19.

Other studies that used full N-protein also found lower antibody levels (<50%) in serum samples taken in the early phase (< 10 days), with significantly increased sensitivity (>80%) from the second week after symptom onset [38,39]. However, no specific stratification based on disease severity was described as performed in our study. A reliable diagnostic performance analysis should consider patient cohorts with mild and severe SARS-CoV-2 infections [40].

Recombinant full-length proteins or larger protein fragments from SARS-CoV-2 have been used as a more suitable strategy in most diagnostic tests available (reviewed in Deshpande and coauthors (2022) [41]) as they may present multiple epitopes for recognition. On the other hand, a more rational approach, that is the selection of immunogenic regions, would reduce non-specific cross-reactivity against homologous epitopes from other viruses, increasing specificity of the diagnostic test [42]. Although there was no significant difference between the diagnostic performances of rCoV7 (residues 160-406) and rCoV2 (residues 1-419), the rCoV7 protein showed the best sensitivity rate, with a few samples responding more robustly. Since rCoV2 represents the whole N-protein with a higher number of epitopes available, it is expected to perform better than other constructs. One hypothesis to explain this effect could be that immunodominant epitopes contained at the N-terminal portion of the whole recombinant N-protein (rCoV2) are not properly exposed for antibody recognition due to unfavorable conformational folding. Additionally, we analyzed the distribution of linear B-cell epitopes in the full-length SARS-CoV-2 N-protein using BepiPred 2.0 server [43] (data not shown). As expected, the C-terminal fragment, which comprises the rCoV-7 construct (residues 160-406), concentrates most of the N-protein epitopes (6/11), which may explain its higher immunogenicity. As mentioned above, previous data [34–36], which demonstrated higher immunogenicity of the middle or C-terminal region of the SARS-CoV-2 N-protein, are in accordance with the better results found in this study using rCov7.

The specificity for N-constructs was evaluated using pre-pandemic serum samples from people infected with other diseases, such as HIV and HCV. Low rates of antibody cross-reactivity in pre-pandemic individual samples were detected for N-based constructs (specificity >93% for all antigens). Despite the positive results, our study had some limitations. Cross-reactivity analysis might require more extensive exploration of SARS-CoV-1 and other human coronavirus (HCoV) respiratory viruses. N-proteins share little homology with the N-proteins of other common cold human coronaviruses (HCoVs) and may show cross-reactivity with antibodies from pre-pandemic blood samples [44,45]. Some evidence suggests the N-terminal domain (NTD) is the region with higher similarity to other HCoVs and may thus induce cross-reactivity [44,46]. More recently, Dobaño and coauthors (2021) [45] provided evidence that a small region of the C-terminal N-protein (residues 348-416) reacts specifically with SARS-CoV-2 antibodies. These data are in agreement with the specificity results obtained in our analyses, with rCoV2 (full N-protein) and rCoV7 (C-terminus region) showing lower specificity rates (93.3% and 96.7%, respectively) than rCoV4 (N-protein middle) (98.8%). Although rCoV4 is composed of tandem repeats of a short immunodominant fragment of the N-terminus (160-181 residues), it

is probable that specific SARS-CoV-2 residues in the other half of the construct (370-406 residues) play the main role in the better specificity performance obtained with rCoV4.

Regardless of some reports, the actual magnitude of the occurrence of cross-reactivity between SARS-CoV-2 N-protein and other common cold coronavirus proteins remains to be demonstrated. Several studies have shown that SARS-CoV-2 N-protein can be used specifically as an antigen to detect COVID-19 patients because the IgG antibodies against N-protein do not recognize N-proteins of seasonal coronaviruses [28,47,48]. The immunoassays based on the N protein from SARS-CoV-2 may contribute to finding diagnostic infected people and segregating them from the uninfected people [49]. The main vaccines used against COVID-19 are based on the S protein [50,51], so serological tests based on this target cannot be used to diagnose infection [52]. Some studies showed the N protein as an antigen in immunological tests to diagnose SARS-CoV infection after vaccination [52,53]. In this context, our ELISA using N-protein constructs described here may also represent a strategy to understand the dynamics of the immune response against SARS-CoV-2 in vaccinated and naturally infected people.

In conclusion, we developed and assessed the performance of an in-house ELISA for the detection of anti-SARS-CoV-2 N IgG antibodies. According to our findings, the N-based constructs showed good sensitivity, with rCoV7 showing a better performance (77.8%) for the detection of IgG antibodies in samples taken more than two weeks after symptom onset in mild-to-moderate COVID-19 cases. For severe cases, sensitivity rates were >85%, with that of rCoV7 reaching 100%. Specificity was >93% for the three antigens. The ELISA proposed here is useful for conducting seroepidemiological surveillance, especially when used to identify previous natural infections in vaccinated populations with schedules of formulations based on the S-protein or in non-vaccinated populations. Further studies are being carried out in order to investigate the diagnostic performance of the rCoVs to detect previous natural infections in vaccinated populations.

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