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Monoclonal and Polyclonal Antibodies as Biological Reagents for SARS-CoV-2 Diagnosis Through Nucleocapsid Protein Detection

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Abstract

SARS-CoV-2 is an enveloped, positive-strand RNA virus that contains four structural proteins: spike, envelope, membrane, and nucleocapsid (N-protein). The N-protein participates in virus RNA packaging and particle release, is conserved within SARS-CoV-2 isolates, is highly immunogenic, and is abundantly expressed during SARS-CoV-2 infection. For these reasons, the N-protein could be used as a marker for detecting SARS-CoV-2 in early infection when antibodies against SARS-CoV-2 have not been produced yet. This paper describes the production and characterization of mouse monoclonal antibodies (mAb) and rabbit polyclonal antibodies (pAb) specific for the M20P19 peptide (N-protein linear epitope) for detection purposes. For this study, B-cell hybridomas were generated from mice independently immunized with two different M20P19 peptide-carrier protein conjugates: (1) meningococcal protein P64K; and (2) the keyhole limpet hemocyanin (KLH). Rabbits were also independently immunized with these two immunogens. Study results demonstrated that the M20P19 peptide was very immunogenic in mice and rabbits, and both mAb and pAb specifically recognized the non-conjugated M20P19 peptide, conjugated M20P19 peptide, and N-protein with high affinity and specificity, which could allow SARS-CoV-2 detection by different analytical techniques. This study corroborated that specific and high affinity constant mAb and pAb against the M20P19 peptide can be used as biological reagents for specific and rapid SARS-CoV-2 detection, mainly in tissue samples.

1.0 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new type of coronavirus first reported in Wuhan City, Hubei Province, China.[1] This virus has a round, elliptical, and often pleomorphic form with a diameter of approximately 60–140 nm. The virus genome is a positive-strand RNA containing 29,891 nucleotides that encode 9860 amino acids.[2] SARS-CoV-2 is composed of four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). The S protein subdomain S1 contains the receptor-binding domain (RBD) that can specifically bind angiotensin-converting enzyme-2 (ACE2), and the S2 subdomain drives viral and host membrane fusion. The RBD presents 40% of amino acid identity with other coronaviruses and a relatively high homology among SARS-CoV-2 isolates.[2] For this reason, the S protein is considered to be the main target of SARS-CoV-2 prophylactic vaccines and antiviral compounds developed to inhibit membrane fusion.[3,4]

The World Health Organization (WHO) and US Centers for Disease Control and Prevention (CDC) recommend, as the gold-standard diagnostic technique, detection of SARS-CoV-2 RNA by means of real-time reverse transcription-polymerase chain reaction (RT-PCR) technique. Samples are obtained from the upper respiratory tract (*e.g.,* nasal and oropharyngeal swabbing) and the lower respiratory tract (*e.g.,* expectorated sputum, endotracheal aspirate, bronchoalveolar lavage).[4,5]

When RT-PCR produces a positive result, there is no doubt that the patient is infected with SARS-CoV-2. This detection method is robust and sensitive (500–5000 copies of viral RNA/mL). However, a negative test result could mean several things: the person is not currently infected, virus was not present at the sampling spot, the sample was of poor quality or not acquired properly, or lastly, it was too early/late in the infection to detect replicating virus.[6,7] On the other hand, reliable serologic tests specific for SARS-CoV-2 structural proteins could play a valuable role in detecting the virus in

a significantly larger segment of the population.[8]

The SARS-CoV-2 N-protein has a very high homology with SARS and MERS coronavirus N-proteins. It is particularly immunogenic and abundantly expressed in infected cells. Therefore, as many ongoing investigations have demonstrated, SARS-CoV-2 N-protein is considered to be one of the most appropriate biological reagents for serological diagnosis, primarily in the early stages of infection when SARS-CoV-2-specific antibodies have not been produced yet.[9]

This paper describes the generation and characterization of mouse monoclonal antibodies (mAb) and rabbit polyclonal antibodies (pAb) specific for the M20P19 peptide, a linear epitope: PKKDKKKKADETQALPQRQKK located at the C-terminal domain (CTD) of SARS-CoV-2 N-protein to be used in virus diagnosis, research (quantification of N-protein production), and tissue sample pathology analysis.

2.0 Materials and Methods

2.1 SARS-CoV-2 N-Protein Sequence

The SARS-CoV-2 full-length N-protein amino acid sequence was obtained from the US National Library of Medicine GenBank (MN908947).

2.2 Biological Reagents

M20P19 peptide and overlapping peptides were produced by the Synthetic Peptide Unit of the Center for Genetic Engineering and Biotechnology (CIGB) of Havana, Cuba. The P64K carrier protein was produced in *Escherichia coli* (*E. coli)* at the CIGB of Havana[10,11] while the KLH carrier protein was supplied by Sigma-Aldrich. The recombinant SARS-CoV-2 N-protein was produced by the CIGB of Sancti Spíritus, Cuba, and the CB.SCoV-2-PN.14 mAb horseradish peroxidase (HRP) was produced by the Monoclonal Antibody Department of the CIGB of Havana. Goat pAb conjugates were supplied by the CIGB of Sancti Spíritus.

2.3 Immunization of Animals

Ten 10-week-old female BALB/c mice were primed once a week for six weeks via subcutaneous immunization. Specifically, five of the mice received 50 µg of M20P19 peptide/P64K conjugate each week and five others were immunized at the same frequency with 50 µg of M20P19 peptide/KLH conjugate. Beforehand, the conjugates were emulsified with Complete Freund's Adjuvant (Sigma-Aldrich) for the first dose and Incomplete Freund's Adjuvant (Sigma-Aldrich) for subsequent doses. Three days before splenectomy, one of the M20P19 peptide/P64K-immunized mice was sensitized intraperitoneally with 50 µg of the same conjugate. For this process, Incomplete Freund's Adjuvant was used to emulsify the conjugates.

Simultaneously, five F1 rabbits were also primed (1000 µg each) and then sensitized (500 µg each) using the same conjugates, adjuvants, and protocols. All animal experiments were performed following the CIGB Animal Care and Use Committee recommendations (in accordance with international guidelines for the ethical treatment of animals).

2.4 Titration of Mouse and Rabbit Sera

Nunc PolySorp plates (Thermo Fisher Scientific) were independently covered with M20P19 peptide/KLH and M20P19 peptide/P64K conjugates. Meanwhile, Nunc MaxiSorp plates (Thermo Fisher Scientific) were only covered with nonconjugated M20P19 peptide. The enzyme-linked immunosorbent assays (ELISA) were done following standard protocol.[12] Wells were considered positive when their absorbances were higher than average absorbance plus three times standard deviation of preimmune serum absorbances.

2.5 Generation of Hybridomas

Hybridomas were generated following protocols described by Köhler and Milstein[13] with some modifications. All clones were screened to determine the presence of antibodies specific to the M20P19 peptide using the indirect antibody capture ELISA described in section 2.4. Then the subcloning process was performed by the limit dilution method.[14] Two hybridomas, CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436, specific for the M20P19 peptide (from the M20P19 peptide/P64K conjugate immunization scheme described in section 2.3) were finally selected for the biological reagent characterization.

2.6 Cell Counting and Viability Measurement

Cell counting and viability were measured by the trypan blue exclusion method using a hemocytometer as a counting chamber.[15]

2.7 Purification of mAb and pAb

Both mAb were purified from hybridoma cell culture supernatants by Protein A MabSelect™ SuRe™ matrix (Cytiva) affinity chromatography using 1.5 M glycine/3 M NaCl (pH 8.9) as adsorption buffer, and 100 mM citric acid (pH 6.0, 5.0, 3.0) as individual elution buffers. Columns were operated at 40 cm/h linear flow rate. After elution, the sample buffer was exchanged with 20 mM Tris/150 mM NaCl (pH 7.6) by size-exclusion chromatography using a XK26 column loaded with Zetadex-25 (emp Biotech) operated at 20 cm/h. The pAb were purified from rabbit sera following the same procedure. In this case, the elution buffer was 100 mM citric acid (pH 3.0). After each chromatographic

cycle, the matrix was cleaned with 200 mM NaOH at a flow rate of 130 cm/h and washed with purified water to allow matrix equilibration and reuse.

2.8 Quantification of Proteins

Total protein quantification was performed following the procedure described by Lowry *et al*.[16]

2.9 Measurement of mAb and pAb Purity by SDS-PAGE and Western Blot

An aliquot each of purified mAb and pAb preparations with approximately 20 µg of proteins were subjected to a 12.5% SDS-PAGE.[17] The western blot analysis was done following protocol described by Towbin *et al*.[18]

2.10 Isotyping of mAb

CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb isotypes were determined using commercial kits (OriGene).

2.11 Detection of SARS-CoV-2 N-Protein by Dot-Blot

A Hybond-C nitrocellulose membrane (Cytiva) was activated in methanol for 20 min and placed in a dot-blot apparatus (Trans-Blot® SD, BioRad) where 100 µL of each sample were applied. The membrane material was blocked with 5% non-fat powder milk (Oxoid Ltd.) dissolved in 150 mM PBS (pH 7.6) for 1 h at 37 °C. Subsequently, the mAb (CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436) and pAb (generated with M20P19 peptide/KLH and M20P19 peptide/P64K conjugates) were applied to detect SARS-CoV-2 N-protein with a goat anti-mouse and anti-rabbit IgG labelled with HRP (Sigma-Aldrich). Pieces of membrane were incubated again for 1 h with the respective HRP conjugates, and color development was visualized by using 150 mM PBS (pH 7.6), 10 mg/mL of 1,2'-diaminobenzidine (Anacrom Diagnósticos) and hydrogen peroxide (Sigma-Aldrich) at 30% in 12 mL volume. Reactions were stopped by adding water to the membrane.

2.12 Mapping of Epitope Recognized by the CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb

MaxiSorp well plates were coated with 10 µg/mL of each mAb. ELISAs were conducted following the same protocol described in section 2.4. M20P19 overlapping peptides used to map the specificity of mAb were:

- J20P114a (PKKDK)
- J20P115 (PKKDKKKKAD)
- J20P116 (PKKDKKKKADETQAL)
- J20P117 (QRQKK)
- J20P118 (TQALPQRQKK)
- J20P119 (KKADETQALPQRQKK)

2.13 Detection of SARS-CoV-2 N-Protein by Western Blot

This procedure was also done to demonstrate the recognition capacity of CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb and pAb by the SARS-CoV-2 N-protein. SDS-PAGE and western blot procedures were done as described in section 2.9; and in this case, the Vip3Aa protein was used as non-related protein.[19]

2.14 Estimation of mAb Affinity Constant

The CB.SCoV-2-M20P19.24 mAb affinity constant (K_{aff}) was determined by following the method described by Beatty *et al*.[20] using MaxiSorp plates coated with M20P19 peptide and later incubated with the antibody preparation.

2.15 Quantification of SARS-CoV-2 N-protein by ELISA

PolySorp plates were coated with 10 µg/mL of CB.SCoV-2-M20P19.24 mAb or pAb applied in 100 µL of coating buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$, and 500 mM NaCl [pH 8.5]) per well and incubated at 37 °C for 1 h. N-protein preparations (serial dilutions from 1000–31 ng/mL) were added to plates and incubated for 1 h at 37 °C. Meanwhile, Vip3Aa protein and 150 mM PBS/0.05% Tween-20 (pH 7.6) were used as the negative control and blank of immunoassays, respectively. After washing, plates were loaded with the anti-N-protein CB.SCoV-2-PN.14 mAb or a goat anti-pAb preparation (both with HRP) and incubated for 1 h at 37 °C. The process described in section 2.12 was followed for the remaining quantification steps.

2.16 Detection of SARS-CoV-2 by an Indirect Immunohistochemical Assay with pAb

A novel molecular detection process by Liu *et al*. (2020)[21] was followed for this study. Tissues fixed in 10% formalin/150 mM PBS (pH 7.0) for 24 h were processed and embedded in paraffin blocks for histology analysis. Embedded sections 4 µ thick were placed on pre-treated TOMO® slides (Matsunami) and deparaffinized in xylene, then rinsed with degraded alcohol (100–90–80–70–30%) and distilled water. Endogenous peroxidases were blocked with a saturating amount of H_2O_2, and tissue antigens were recovered with neutral citrate buffer at 9°C. Later, sections were covered with an optimal diluted primary antibody preparation (pAb specific for M20P19 peptide) and incubated overnight at 4°C. Subsequently, pre-diluted HRP-labeled anti-pAb and anti-mouse IgG (secondary antibody) preparations were applied at room temperature for 45 min. Finally, the immunoreaction was revealed with 1,2'-diaminobenzidine for about 3 min. The substrate-chromogen solution was rinsed off the slides, which were then lightly counterstained with Mayer's hematoxylin, dehydrated, cleared with Xyless II (ValTech Diagnostics), and coverslipped.

3.0 Discussion

Since the COVID-19 pandemic began, many countries have encountered difficulties covering all SARS-CoV-2 testing with the RT-PCR technique. Therefore, the development of reliable, sensitive, rapid, cost-effective, and widely applied diagnostic tests based on SARS-CoV-2 structural protein detection are very much in demand. This technology is particularly relevant in the early stages of infection when the human body has not begun producing specific antibodies to fight SARS-CoV-2.

For instance, N-protein has been detected in SARS-CoV-2 patient sera as early as day 1 post-infection. In a comparative study undertaken to detect N-protein, SARS-CoV-2-specific IgG and RNA identified during the early stages of infection demonstrated a higher efficiency for N-protein detection.[22–24] However, the selection of commercially available SARS-CoV-2-validated diagnostic kits based on antigen detection is limited.[25, 26] One of the new technologies available for COVID-19 detection is the lateral flow immunoassay (also known as a rapid diagnostic test [RDT]), which meets minimum performance requirements for specificity (97%). It is simpler and faster to perform than the nucleic acid amplification tests. This technique relies on SARS-CoV-2 viral protein detection using nasal and other respiratory secretion samples, providing results in < 30 min.[27] Such products could play a significant role in guiding patient management and public health decisions.

To help address the demands created by this novel virus, our paper describes the development of biological reagents specific for N-protein. Uses can include SARS-CoV-2 detection, research (N-protein production quantification), and tissue sample pathological analysis.

Structurally, the SARS-CoV-2 N-protein possesses more than 400 amino acids distributed in three highly conserved domains.[11] As with most proteins, it also contains multiple linear epitopes[28], which are usually proper targets to raise a humoral immune response to select the valuable antibodies for diagnosis. That is why the SARS-CoV-2 N-protein linear epitope (M20P19 peptide: PKKDKKKKADETQALPQRQKK) was chosen to produce mAb and pAb for this study. This epitope corresponds to amino acids 368–388 of SARS-CoV-2 N-protein, has shown the strongest immunogenicity capacity, and the N-terminal region has a high degree of homology with N-protein of SARS and MERS coronaviruses. Therefore, antibodies specific for this region could also be used as reagents to detect SARS and MERS viruses.

4.0 Results

4.1 Animal Immunization and Serum Titration

Two groups of mice and rabbits were immunized with M20P19 peptide conjugated with two different carrier proteins (P64K and KLH), respectively. **Figure 1** illustrates mouse titers after the fourth immunogen injection. Titers of mice immunized with M20P19 peptide/P64K conjugate were measured by covering ELISA plates with M20P19 peptide/KLH conjugate and with M20P19 peptide alone in

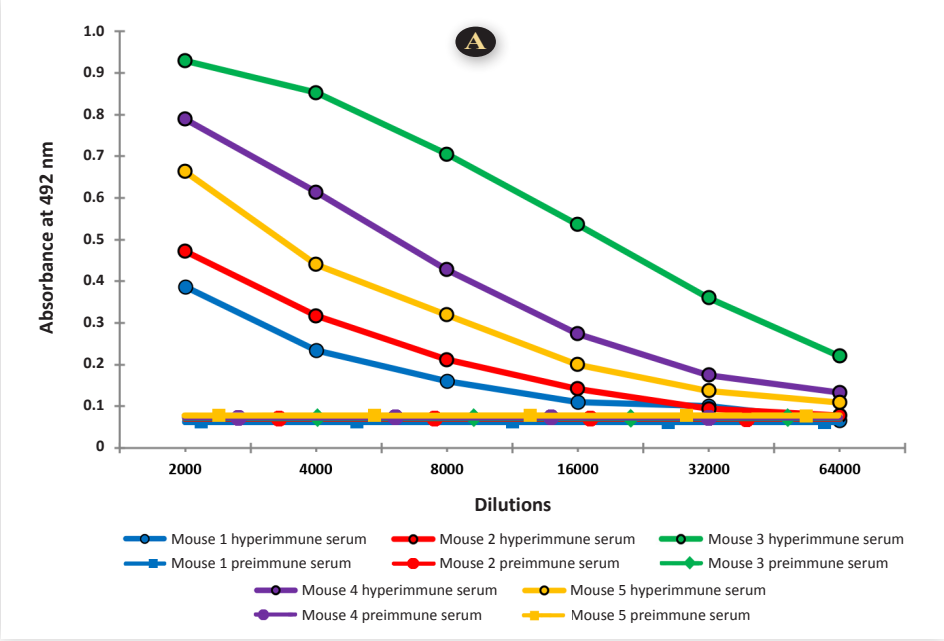


FIGURE 1A. Serum titration of mice after the fourth immunization with the M20P19 peptide/P64K conjugate.

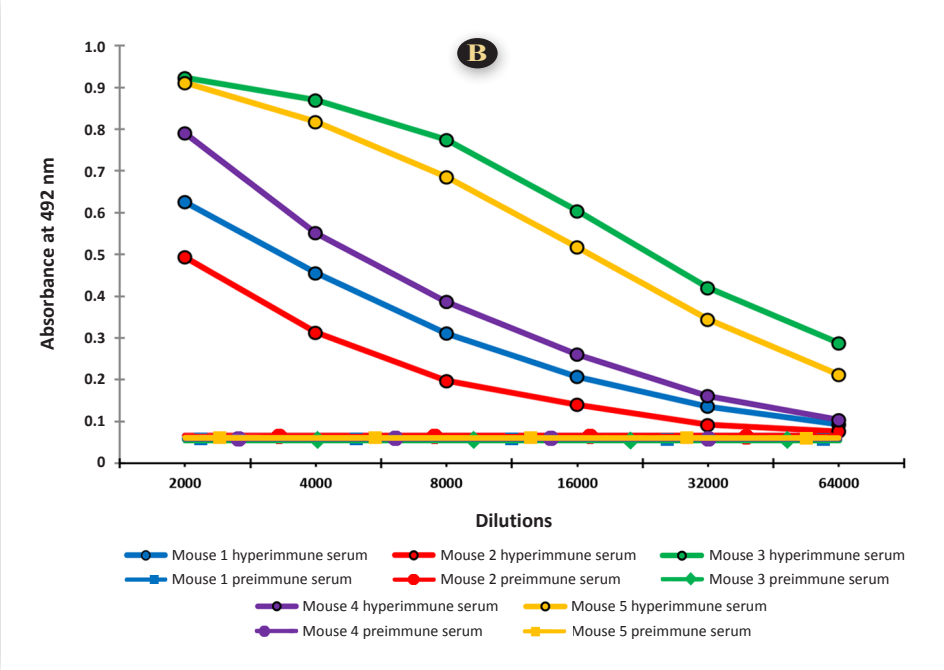


FIGURE 1B. Serum titration of mice after the fourth immunization with the M20P19 peptide/KLH conjugate.

MaxiSorp ELISA plates. The opposite analysis was also done to measure titers of mice immunized with M20P19 peptide/KLH conjugate. As it can be noted, titers of mice immunized with M20P19 peptide/P64K conjugate (**Figure 1A**) was as follows: one mouse (1:16,000), two mice (1:32,000), and two mice (1:64,000), whereas titers estimated in the sera of mice immunized with M20P19 peptide/KLH conjugate were: one mouse (1:16,000), one mouse (1:32,000), and three mice (1:64,000) (**Figure 1B**). These results indicated that the M20P19 peptide was able to induce a relatively strong immune response, in the context of these two different carrier proteins and immunization protocols in all immunized animals.

On the other hand, **Figure 2** shows titration of rabbit sera

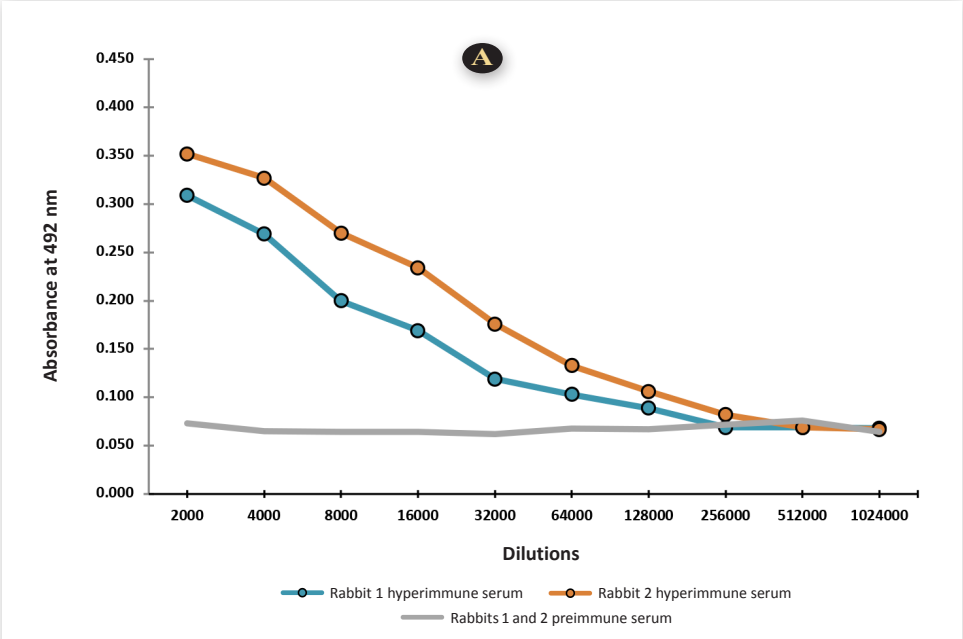


FIGURE 2A. Serum titration of rabbits after the third immunization with the M20P19 peptide/P64Kconjugate.

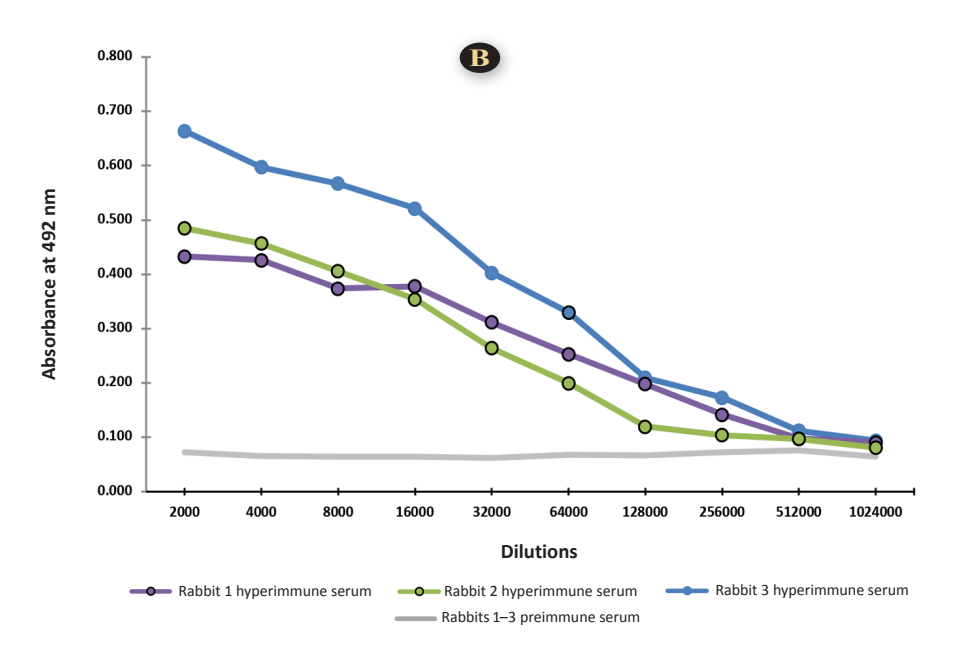


FIGURE 2B. Serum titration of rabbits after the third immunization with M20P19 peptide/KLH conjugate.

against the M20P19 peptide. Since the titers for all rabbits was ≥1:128,000, a very strong humoral immune response was raised against the M20P19 peptide in just three immunization doses, confirming that the M20P19 peptide was much more immunogenic in rabbits than in mice. Detailed results revealed titers of two rabbits (1:128,000) immunized with the M20P19 peptide/P64K conjugate (**Figure 2A**), and one rabbit (1:128,000), one rabbit (1:256,000), and one rabbit (1:512,000) immunized with M20P19 peptide/KLH conjugate (**Figure 2B**), demonstrating that KLH promoted a higher humoral response in rabbits than P64K.

These results are not coincident with those reported by T. Li *et al.*[29] who demonstrated a relatively weak-to-middle humoral response in mice after immunizing with a M20P19 peptide/KLH conjugate (1:5000). However, differences in these results could have been because their titer evaluation conditions were different from those applied in this work (*e.g.*, covering plate with 1 µg/mL), and researchers did not report detailed information on immunization schemes used. Differences in titers against M20P19 peptide were also observed in a paper by M. Li *et al.*[28] where rabbit immunizations produced a maximum titer of 1:100,000.

Coincidently, immunizations with the conjugates used did not induce a strong humoral response against the M20P19 peptide in the first three injections. As expected, average titers against respective carrier proteins were almost three times higher than titers against the M20P19 peptide, corroborating the idea that conjugating the peptide to P64K or KLH did not modify immunogenicity of these carrier proteins in these two animal models. Also, corroborating these two carrier proteins would be useful carrier moieties for repeated immunizations against this peptide. Lastly, titers of mouse and rabbit sera estimated covering ELISA plates with M20P19 peptide alone did not show differences with those estimated covering ELISA plates with respective conjugates. Thus, both covering reagents can be used indistinctly to estimate the immunized animal titers.

4.2 Generation of Hybridomas

The generation of hybridomas is a complex, five-step procedure: (1) antigen production; (2) animal immunization to generate specific and high-affinity mAb; (3) B-cell isolation; (4) fusion with myeloma cells; and (5) multiplication of specific hybridomas for mAb purification. While it usually yields multiple specific hybridomas, sometimes the amounts of selected/specific hybridomas do not correlate with the high titers measured in immunized animals. For instance, our study results evidenced 97.4% of fusion efficiency, but only 1.2% of the wells (3, 24, 118, 222, 349, 435, 436, 530, and 582) contained antibody-producing cells against the M20P129 peptide. After subcloning nine positive clones, only two producers of CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb were finally chosen for further characterization.

4.3 Purification of mAb and pAb

The protein A chromatography step is an extremely important clearance (obtaining >90% purity) and recovery/capture (>70%) step. Plus, the same purification protocol could be used to purify different antibody molecules, since

only small changes in pH values of elution buffer must be produced to elute antibodies of different classes and subclasses. After the protein A chromatography step, about 85% mAb recovery was estimated for both mAb (CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436) and 70% recovery for both pAb preparations (M20P19 peptide/P64K or KLH conjugates). Differences in recovery values can be explained by non-purified material characteristics (cell culture supernatants and sera) applied to columns. The huge majority of both mAb molecules eluted in pH 6.0 fractions, which corresponds with the pH needed to elute low affinity antibodies by protein A. As measured by SDS-PAGE, antibody molecular purity was >98% in all elution fractions, which is too high for application of these biological reagents. Subsequent western blot analysis also corroborated a typical IgG identity pattern (under reducing conditions) and very high purity for both kinds of purified antibody preparations (data not shown).

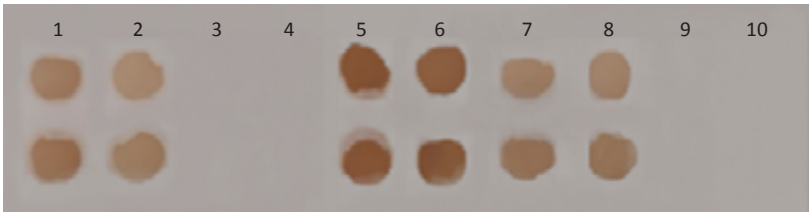


FIGURE 3. Results of SARS-CoV-2 N-protein detection by dot-blot. The N-protein was transferred onto a nitrocellulose membrane and plotted with mAb and pAb generated with M20P19 peptide conjugates containing either P64K or KLH carrier proteins:

Lane	Protein	Antibody Solution (1 µg/mL)	Detection
1	N-protein	CB.SCoV-2-M20P19.24 mAb	Goat anti-mouse IgG Ab
2		CB.SCoV-2-M20P19.436 mAb	
3	P64K	CB.SCoV-2-M20P19.24 mAb	
4		CB.SCoV-2-M20P19.436 mAb	
5	N-protein	CB.SCoV-2-M20P19/P64K pAb conjugate	Goat anti-rabbit IgG Ab
6		CB.SCoV-2-M20P19/KLH pAb conjugate	
7	P64K	CB.SCoV-2-M20P19/P64K pAb conjugate	
8	KLH	CB.SCoV-2-M20P19/KLH pAb conjugate	
9	P64K	CB.SCoV-2-M20P19/KLH pAb conjugate	
10	KLH	CB.SCoV-2-M20P19/P64K pAb conjugate	

4.4 Detection of SARS-CoV-2 N-Protein by Dot-Blot

Molecular characterization was performed to determine potential biological reagents for SARS-CoV-2 detection. Dot-blot is similar to other blotting techniques, except that it does not provide information regarding size of hybridized DNA, RNA, or proteins. Nonetheless, it is especially well-suited for probing a given antigen with different antibodies or for semi-quantitation of a given biological molecule in a mixture by serial dilutions. Study results demonstrated high specificity in both antibody preparations, since neither mAb recognized the P64K carrier protein. The pAb preparations, however, demonstrated to be specific for M20P19 peptide, but also for respective carrier proteins (**Figure 3**).

TABLE 1. Epitope mapping results for CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb performed using small linear peptides spanning the full length of M20P19 peptide.

Peptides	Amino Acid Sequences	CB.SCoV-2-M20P19.24 mAb (Absorbance at 492 nm)	CB.SCoV-2-M20P19.436 mAb (Absorbance at 492 nm)
M20P19	PKKDKKKKADETQALPQRQKK	1.133	1.113
J20P114a	PKKDK	0.061	0.055
J20P115	PKKDKKKKAD	0.065	0.356
J20P116	PKKDKKKKADETQAL	1.198	0.367
J20P117	QRQKK	0.059	0.050
J20P118	TQALPQRQKK	0.074	0.054
J20P119	KKADETQALPQRQKK	1.221	0.051

In this study, binding of CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb was assessed using an ELISA, covering well plates with six small peptides of whole M20P19 peptide sequence. As shown in **Table 1**, the CB.SCoV-2-M20P19.24 mAb recognized peptides M20P19 (absorbance=1.133), J20P116 (absorbance at 4927 nm=1.198) and J20P119 (absorbance=1.221). These peptides share amino acid sequence ETQAL (Glu-Thr-Gln-Ala-Leu), which is close to the middle of the M20P19 peptide sequence. Interestingly, omitting amino acid E (Glu) in the J20P119 peptide sequence avoided the CB.SCoV-2-M20P19.24 mAb total recognition by J20P119 peptide, dropping down absorbance from 1.221 to 0.074. Conversely, the CB.SCoV-2-M20P19.436 mAb recognized a different molecular determinant. The specific sequence recognized by this mAb was KKKAD (Lys-Lys-Lys-Ala-Asp), which is represented

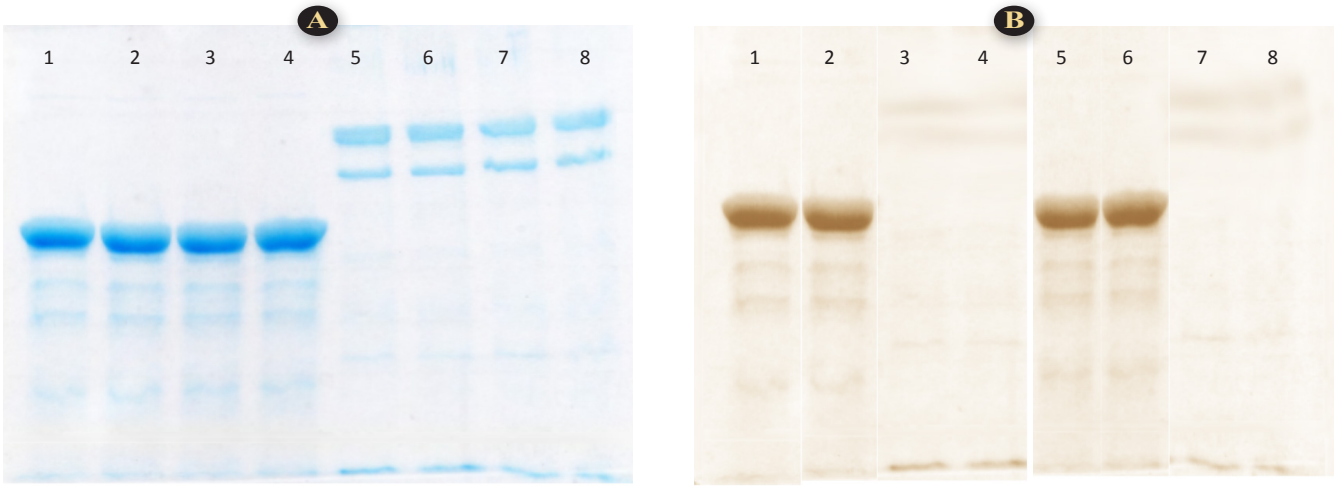
in peptides M20P19 (absorbance = 1.113), J20P115 (absorbance = 0.356), and J20P116 (absorbance = 0.367). This sequence is located in the middle position within the M20P129 peptide and before the epitope recognized by the CB.SCoV-2-M20P19.24 mAb. However, absorbance measured against J20P155 and J20P116 peptides with the CB.SCoV-2-M20P19.436 mAb was low, which can be explained by a modification of molecular determinant conformation after interaction with the plate surface, or by a lesser interaction of these small peptides with ELISA-plate surface. This is something that cannot be corroborated without a positive control in the assay. Similar to the CB.SCoV-2-M20P19.24 mAb, eliminating one amino acid (Lys) provoked complete loss of molecular determinant recognition with the J20P199 peptide (Lys-Lys-Ala-Asp, absorbance = 0.050).

In order to demonstrate the potential for these mAb and pAb to be used for the identification of SARS-CoV-2 N-protein, a western blot technique was also done using electrophoretically separated SARS-CoV-2 N-protein and Vip3Aa as non-related protein. These two proteins have important differences in size (SARS-CoV-2 N-protein, 50 kDa; Vip3Aa inactive form, 85 kDa; and Vip3Aa active form, 66 kDa). Results showed visualization of SARS-CoV-2 N-protein bands, corroborating recognition of this inner linear epitope and potential applicability of these mAb and pAb preparations for SARS-CoV-2 N-protein detection by western blot (**Figure 4**), since linear epitopes are not destroyed by electrophoresis conditions.

The K_{aff} of an antibody to its antigen is another key parameter to define mAb performance as biological reagents in immunoassays. Beatty *et al.*[20] reported a method of analysis of sigmoid serial dilution curves to measure the K_{aff} of antibodies in solution. The application of this method here revealed an K_{aff} of $8.13 \pm 1.80 \times 10^{-12}$ M for the CB.SCoV-2-M20P19.24 mAb (**Figure 5**). This K_{aff} value does not differ from those measured in several mAb isolated after relatively long immunization schemes, and it should be enough to capture antigens, even in very low concentrations.

4.5 Applicability of Biological Reagents

Finally, proving applicability of mAb and pAb preparations generated through this work was studied in quantification of SARS-CoV-2 N-protein in human sera, mammalian cell culture samples, and immunohistochemical assays to detect SARS-CoV-2 in tissue samples of patients infected with SARS-CoV-2. We chose to include pAb in the immunohistochemical analysis because recognition



Lanes	Protein	Molecular Weight
1–4	N-protein	50 kDa
5–8	Vip3Aa (inactive)	85 kDa
5–8	Vip3Aa (active)	66 kDa

Lane	Protein	Antibody Solution (1 µg/mL)	Detection
1	N-protein	CB.SCoV-2-M20P19.24 mAb	HRP-goat anti-mouse IgG Ab
2		CB.SCoV-2-M20P19.436 mAb	
3		CB.SCoV-2-M20P19.24 mAb	
4		CB.SCoV-2-M20P19.436 mAb	
5	Vip3Aa	CB.SCoV-2-M20P19/P64K pAb	HRP-goat anti-rabbit IgG Ab
6		CB.SCoV-2-M20P19/KLH pAb	
7		CB.SCoV-2-M20P19/P64K pAb	
8		CB.SCoV-2-M20P19/KLH pAb	

FIGURE 4. Results of: **(A)** SDS-PAGE; and **(B)** western blot tests performed to detect the SARS-CoV-2 N-protein with CB.SCoV-2-M20P19.24 and CB.SCoV-2-M20P19.436 mAb and pAb.

of this kind of preparation is less dependent on molecular determinant sequence and conformation, and on the presence of blocking components in infected cells.

To explore the real diagnostic value of SARS-CoV-2 N-protein quantification in serum from the early stages of infection, a double antibody sandwich ELISA was standardized. The assay was found to be practical, simple to perform, and in all cases, provided a clear discrimination between related and non-related samples. The assay detection limit was 15 ng/mL; quantification limit, 30 ng/mL; and standard curve linear range, 30–1000 ng/mL; with a correlation coefficient R^2=0.9975 (**Figure 6**). The ELISA took four hours to complete, but the time could be reduced if sample incubation times are reduced, which may be a substantial benefit when large numbers of samples require testing. Therefore, this ELISA is a specific and rapid method for quantifying N-protein amounts found in mammalian cell culture samples used to express the N-protein for research and production applications (2–18 µg/mL HEK-293 supernatant and 28% of total protein *E. coli* fermentation process).

Nevertheless, this sensitivity level is not enough to quantify N-protein from serum samples, since concentration values reported have been 10–3000 pg/mL.[29] This phenomenon has also been seen in other antigen-based quantification systems for respiratory diseases such as influenza in which concentrations of influenza virus in respiratory samples from patients is comparable to that of COVID-19 patients.

On the contrary, the sensitivity of such tests might be expected to vary from 34–80%. Thereby, half or more of COVID-19 infected patients might be missed by such tests. These assumptions urgently require further studies to demonstrate whether these systems can achieve higher sensitivities. An alternative to this problem could be the addition of a concentration step prior to N-protein quantification directly from serum samples. This ELISA system was also modified using the pAb preparation generated with M20P19 peptide/KLH conjugate

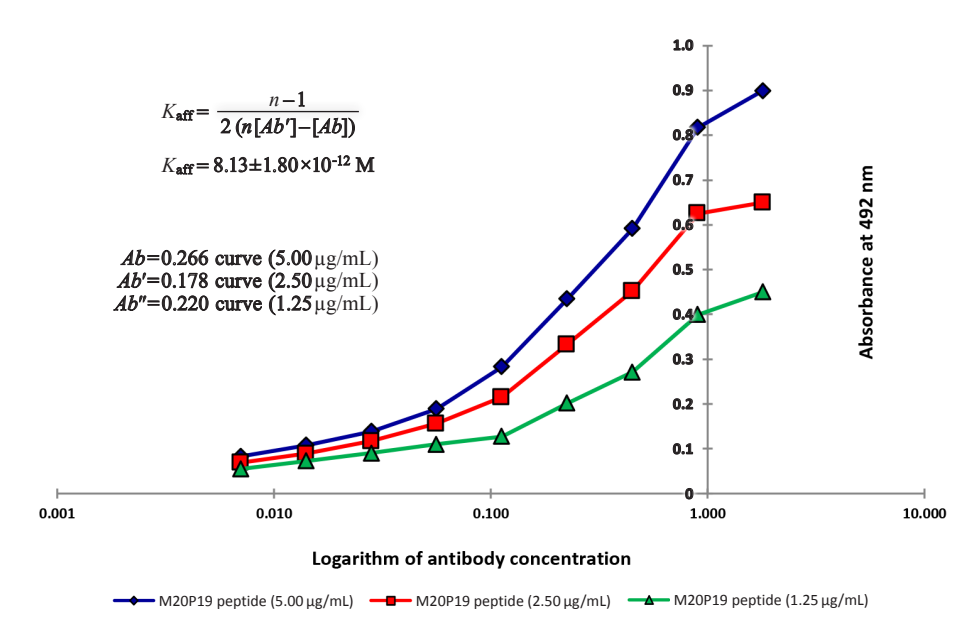


FIGURE 5. Experimental sigmoidal curves for anti-M20P19 peptide CB.SCoV-2-M20P19.24 mAb. Concentrations of the peptide in coating solutions were: 5.00, 2.25, and 1.25 µg/mL. *Ab*, *Ab′* and *Ab″* denote absorbance at 492 nm – 50 points of each curve.

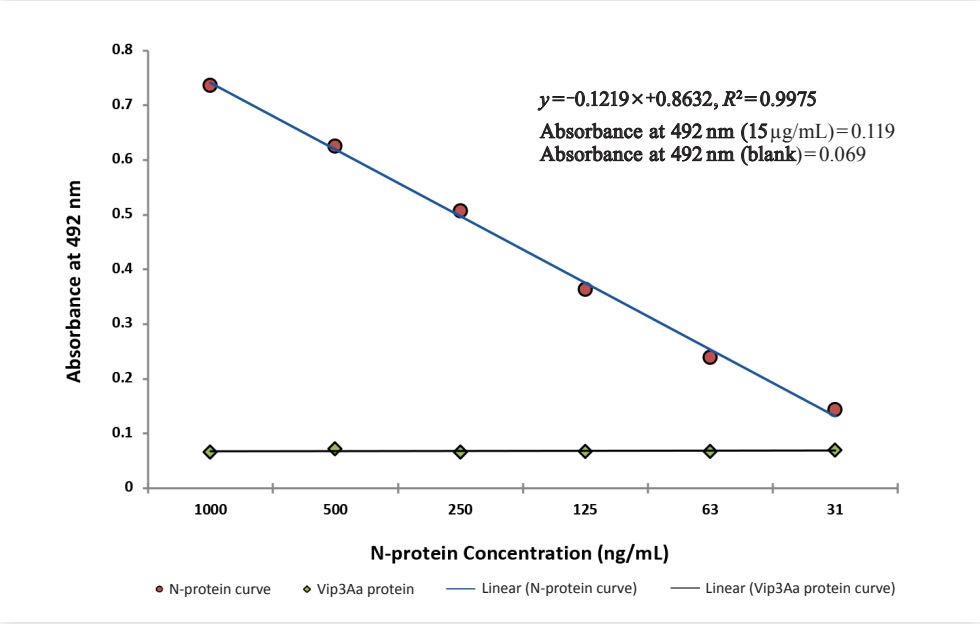


FIGURE 6. Standard curve of ELISA standardized to quantify SARS-CoV-2 N-protein. It was performed using the CB.SCoV-2-M20P19.24 mAb as the N-protein capture antibody and CB.SCoV-2-PN.14 mAb as the HRP-labelled antibody. The standard curve plotted absorbance values of six different standard curves, all using the same analysis techniques. *Bacillus thuringiensis* Vip3Aa protein was used as a non-related protein.

as the capture antibody. However, the quantification limit was unexpectedly lower (125 ng/mL of SARS-CoV-2 N-protein, and the linear range was from 125–2000 ng/mL, $R^2 = 0.9899$). Maybe the explanation for this unexpected issue lies in the fact that some kind of competition with the CB.SCoV-2-PN.14 mAb used as labelled antibody could be produced. Therefore, further experiments will be done to validate this SARS-CoV-2 N-protein quantification method based on other mAb combinations and signal amplification systems. Interestingly, findings observed by the N-protein purification process designers suggested a high aggregation of N-protein (approximately 300 kDa) out of the context of the virus or infected cells (*e.g.*, purification and storage buffers, and likely in serum too), which may have a negative influence on assay sensitivity. The authors speculate that the solution to this phenomenon could help increase detection and quantification limits of assays up to pg/mL level.

The final segment in this characterization study was to prove the capacity of the pAb in the detection of SARS-CoV-2 in tissue samples from infected patients. The histopathologic evaluation of biopsy and autopsy materials from COVID-19 patients is also of critical importance in understanding the pathophysiology and distribution of virus within different organs and tissues. With SARS-CoV-2 detection in infected tissue samples, the most remarkable changes are seen in the lungs.[30, 31] In this proof-of-concept study, lung tissue from patients with COVID-19 and a control sample were studied by immunohistochemical analysis. Included were three infected lung samples (removed at autopsy) and a control sample without pAb specific for the M20P19 peptide. Results revealed a similar pattern of reactivity in all tissue samples from patients with COVID-19 treated with pAb, since all autopsy lung samples showed focally positive cells for SARS-CoV-2 (**Figure 7**). Therefore, pAb generated with M20P19 peptide are suitable reagents for SARS-CoV-2 N-protein detection on lung tissue samples. Subsequent experiments have been carried out with CB.SCoV-2-M20P19.24 and CB.SCoV-2-PN.14 mAb. So far, similar recognition patterns have been observed with this CB.SCoV-2-M20P19.24 mAb in these kinds of samples (data not shown).

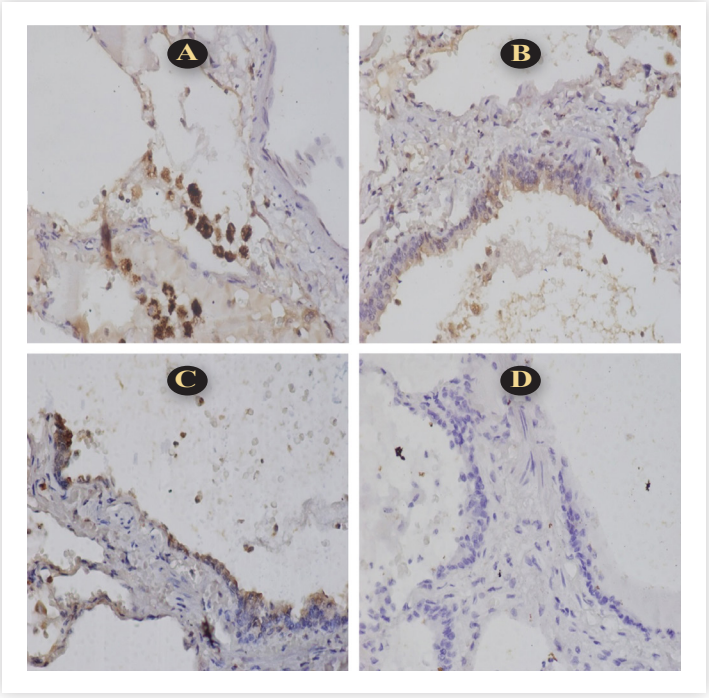


FIGURE 7. Lung samples processed by an immunohistochemical technique that identifies the presence of SARS CoV-2 N-protein using pAb specific for M20P19 peptide. The brown, globular-shaped cells in intracytoplasmic localization indicate specific reaction. **(A)** Type II pneumocytes; and **(B, C)** bronchioles with epithelial hyperplasia. The fourth sample **(D)** was the untreated negative control. (All photos are shown at ~40× magnification.)

5.0 Conclusions

Since the SARS-CoV-2 N-protein is abundantly expressed at early stages of SARS-CoV-2 infection, measurement of this protein in serum may have diagnostic value when specific antibodies have not been produced yet, shortening the serological diagnostic window period. Therefore, specific and high *K*aff CB.SCoV-2-M20P19.24 and CB.SCoV-2-PN.14-HRP mAb, and pAb specific for M20P19 peptide are biological reagents suitable for developing specific, reliable, and rapid detection systems for SARS-CoV-2 through N-protein. The ELISA standardized in this work is intended for N-protein quantification in mammalian cell culture samples, but not in serum samples of COVID-19 patients. The pAb generated with M20P19 peptide are suitable for SARS-CoV-2 N-protein detection on lung tissues samples.

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Conflicts of Interest

All authors were assessed for any conflicts of interest, and none were declared.

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