Identification of Immunohistochemical Reagents for In Situ Protein Expression Analysis of Coronavirus-associated Changes in Human Tissues

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Abstract: We studied the suitability of commercially available monoclonal antibodies (mAbs) for the immunohistochemical (IHC) detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) in standard archival specimens. Antibodies were screened on HEK293 cells transfected with viral nucleoprotein, S1 subunit and S2 subunit of spike protein and on untransfected cells, as well as a panel of normal tissue. Lung tissue with presence of SARS-CoV2 confirmed by in situ hybridization (ISH) was also used. A total of 7 mAbs were tested: (1) mAb 001 (Sino Biological, 40143-R001), (2) mAb 007 (Sino Biological, 40150-R007), (3) mAb 019 (Sino Biological, 40143-R019), (4) mAb 1A9 (GeneTex, GTX632604), (5) mAb ABM19C9 (Abeomics, 10-10007), (6) FIPV3-70 (Santa Cruz, SC-65653), and (7) mAb 6F10 (BioVision, A2060). Only 2 mAbs, clone 001 to the nucleoprotein and clone 1A9 to the S2 subunit spike protein displayed specific immunoreactivity. Both clones showed strong staining in the acute phase of COVID-19 pneumonia, mostly in areas of acute diffuse alveolar damage, but were not completely congruent. Viral protein was also found in kidney tubules, endothelia of multiple organs and a nasal swab of a patient with persistent SARS-CoV2 infection. The other tested reagents were either poorly reactive or demonstrated nonspecific staining in tissues and lesions not infected by SARS-CoV2. Our study demonstrates that rigid specificity testing is mandatory for the evaluation of mAbs to SARS-CoV2 and that clones 001 to nucleoprotein and 1A9 to S2 subunit spike protein are useful for the in situ detection of SARS-CoV2.

Key Words: SARS-CoV2/COVID-19, immunohistochemistry, specific monoclonal antibodies

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The sudden on-set and worldwide dissemination of infections by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) has led to a pandemic challenge of global health and a severe health care crisis. SARS-CoV2 is a member of the family of coronaviruses. The latter are RNA viruses which can infect humans as well as animals. Six coronaviruses infecting humans have been previously identified, 4 of which cause mild respiratory symptoms.[1] Two strains, however, SARS-CoV and MERS, which can cause severe and potentially fatal lung disease, have led to minor epidemic spread mostly in Asia and the Mediterranean in 2003 and 2012, respectively.[2–5] COVID-19, the infectious disease of SARS-CoV2, is characterized by severe pulmonary disease but may also involve other organs, many of which are affected by thrombi.[6–9]

Accurate characterization of pathomorphologic changes is mandatory for the understanding of virus-associated changes and immunohistochemical (IHC) detection of SARS-CoV2 viral proteins is essential for the appropriate interpretation of histologic findings. In most recent publications, little emphasis has been placed on characterizing the available IHC reagents used for this purpose. Consequently, in the present study we have developed an approach to test antibodies to detect SARS-CoV2 for their suitability in IHC assays applied to formalin-fixed paraffin-embedded tissues. While most tested monoclonal antibodies (mAbs) proved to be unsuitable, we have identified 2 commercially available mAbs to the viral nucleoprotein and to the spike protein S2 subunit, respectively, which rendered consistent and strong immunostaining for the IHC analysis of virus-associated changes.

MATERIAL AND METHODS

Cell Line Transfectants

HEK293 cells transfected with various SARS-CoV2 proteins were obtained commercially (RayBiotech, Peachtree Corner, GA). HEK293 cells transfected with the following viral proteins were employed: nucleoprotein, S1 subunit spike protein, S2 subunit spike protein, untransfected. After harvesting, the cells were washed twice

in PBS and fixed in 4% buffered formaldehyde solution, and pelleted in a gel matrix (Histogel, Richard Allan Scientific, San Diego, CA) and embedded in paraffin.

In Situ Hybridization (ISH)

A chromogenic ISH method was employed to detect SARS-CoV2 RNA in tissue specimens. Assays were performed on a Leica-Bond-RX automated stainer platform (Leica Biosystems, Buffalo Grove, IL). A probe to SARS-CoV2 (RNAscope 2.5 LS Probe-V-nCoV2019-S; #848568; Advanced Cell Diagnostics/ACD, Newark, CA) was obtained commercially. Probe detection was performed with the chromogenic detection kit (RNAscope 2.5 LS Assay on Leica BOND RX-BROWN; Leica). Positive and negative controls were performed according to the manufacturer's recommendations.

Antibodies and Immunohistochemistry

Commercially available mAbs to SARS-CoV2 antigens were selected based on their claimed specificity for defined viral antigens (nucleocapsid, spike protein, S1/S2 subunit). Initial reagent choice was based on alleged suitability for IHC. However, since several of the first tested mAbs did not reveal any or unspecific immunostaining, subsequent antibodies were obtained irrespective of their alleged suitability for IHC. Because of the challenges with specificity and consistency with polyclonal antibodies, we focused on monoclonal reagents. The reagents and their properties are listed in Table 1. It is important to consider that SARS-CoV and SARS-CoV2 are closely related viruses with highly similar genomic and protein sequences hampering the generation of diagnostic antibodies specific for one of the viruses but not the other.[10–13] On the basis of this similarity, several anti-SARS-CoV2 reagents in the form of peptide sequences of the original SARS-CoV were used as immunogens for the present antibodies while manufacturers tested for reactivity to both SARS-CoV viruses (Table 1). Immunohistochemical staining was performed on a Leica Bond-III (Leica) platform. A polymeric secondary kit (Refine, Leica) was used for the detection of the primary. All antibodies were tested at various concentrations ranging from 0.1 to 10 μg/mL. Different antigen retrieval steps were employed comprising of heating in low pH (ER1, Leica) or high pH (ER2, Leica) retrieval buffer solution or enzymatic digestion (Enzyme 1, Leica). Our standard Bond-III protocol consists of a 30 minutes primary incubation and a 30 minutes heat-based antigen retrieval step or 10 minutes enzyme digestion at the Bond-III default temperatures.

Tissues

In order to rule out potential cross reactivities, all antibodies were tested on a panel of 10 normal tissues in a carrier-based multi-tissue block consisting of spleen as a carrier and cores of 5 mm of the following tissues: placenta, lung, kidney, liver, tonsil, testis, colon, pancreas, and skin.[14] Thereafter, tissues including lung, heart, and kidney from 3 autopsies of patients who had died from COVID-19 pneumonia were assayed for primary antibodies, which had passed the initial screening. All 3 cases had shown a strong hybridization signal in areas of COVID-19-associated pneumonia or acute diffuse alveolar damage with the ACD probe for SARS-CoV2. Formalin-fixed smears of 3 RT-PCR assayed nasal swabs from 3 SARS-CoV2 positive and 3 negative patients were also available for evaluation. The smears were directly applied to immunohistochemical slides, air dried, and then fixed for 10 minutes in 10% neutral buffered formalin and again air dried. Immunohistochemical staining was performed within three days of sampling and similar to paraffin sections except the omission of the deparaffinization step.

RESULTS

In the initial phase of the study, all antibodies were tested on pellets of HEK293 cells, untransfected or transfected with one of the following SARS-CoV2 proteins: nucleoprotein, S1 subunit spike protein, or S2 subunit of the spike protein (Fig. 1). This allowed for an accurate assessment of staining specificity and sensitivity comparing the actual immunostaining of cell pellets with the target antigen for each antibody as published in respective data sheets issued by manufacturers and without relying solely on lung tissue with yet to be determined pathologic changes. All antibodies were also tested on ten normal tissues as well as an autopsy lung with ISH-confirmed presence of SARS-CoV2 (Fig. 2). Antibodies which showed specific staining in the initial step, were used for further testing. Of the 7 tested mAbs, only 2 demonstrated suitability for IHC.

Several factors deemed the remaining 5 antibodies unsuitable for IHC after the initial screening. Although antibody ABM19C9, generated to the C-terminal region of the spike protein showed immunopositivity in the pellet of spike protein S2 subunit transfected HEK293 cells, there was also weak staining in the nucleoprotein and S1 subunit transfected cells. Furthermore, no consistent or strong immunostaining was present in lung tissue with COVID-19 pneumonia. According to its specifications, clone FIPV3-70 was raised against the nucleoprotein of coronavirus. However, it gave no immunoreactivity in all cell pellets and was also negative in SARS-CoV2-positive lung tissue (Figs. 1, 2). Clone 019 was generated to the nucleoprotein but displayed immunoreactivity in all HEK293 pellets including the untransfected cells (Fig. 1). There was strong immunopositivity in the SARS-CoV2-positive lung tissue and staining in lung was compatible with the detection of viral antigens. However, all tested normal tissues also stained intensely positive (Fig. 2). Consequently, clone 019 was not pursued. Clone 6F10 displayed immunopositivity in only a subset of the HEK293 cells transfected with the nucleoprotein, which is its proposed target antigen. Clone 6F10 remained negative in the SARS-CoV2-positive lung tissues, except for focal unspecific staining in smooth muscle of bronchi and blood vessels and was hence eliminated from evaluation due to lack of sensitivity. Similarly, clone 007 generated to the S1 subunit of the spike protein stained only in

TABLE 1. Monoclonal Antibody Clones, Manufacturers, Order Information, Detected Antigen/Immunogen as Well as Application as Provided by Manufacturer

Clone	Designation	Species	Manufacturer	Order#	Reagent provided Total Volume (Concentration)	Immunogen (as per Manufacturer)	Specificity (as per Manufacturer)	Applications	Immunohistochemical Staining, Cell Pellets	Immunohistochemical Staining, tissue
001	SARS-CoV nucleoprotein/NP antibody	Rabbit	Sino Biological	40143-R001	100 µl (500 µg/mL)	SARS-CoV nucleoprotein	SARS-CoV nucleoprotein/NP, has cross-reactivity in ELISA and WB with SARS-CoV2 (2019-nCoV) nucleoprotein/NP protein	WB, ELISA, IHC-P, FCM, ICC/IF, IF, IP	Strong immunostaining of nucleoprotein pellet	Staining of COVID-19-positive tissue
007	SARS-CoV2 (2019-nCoV) spike antibody	Rabbit	Sino Biological	40150-R007	100 µl (500 µg/mL)	Not stated	SARS-CoV-2 (2019-nCoV) spike protein (S1 subunit), SARS-CoV2 (2019-nCoV) spike protein (RBD), has cross-reactivity in ELISA with SARS coronavirus spike protein (S1 subunit), SARS coronavirus spike RBD	ELISA, FCM, ICC/IF, IHC-P, IP	Subset of cells of S1-RBD cell pellet immunopositive	Negative in COVID-19-positive tissue
019	SARS-CoV2 nucleoprotein/NP antibody	Rabbit	Sino Biological	40143-R019	100 µl (1 mg/mL)	Purified, recombinant SARS-CoV-2 nucleoprotein	SARS-CoV2 (2019-nCoV) nucleoprotein/NP protein	WB, ELISA, FCM, ICC/IF, IHC-P, IP	Immunostaining of all cell pellets	Ubiquitous staining in normal tissues
1A9	SARS-CoV/SARS-CoV2 (COVID-19) spike antibody	Mouse	GeneTex	GTX632604	100 µl (1 mg/mL)	SARS coronavirus, SARS coronavirus 2; S2 subunit	SARS-CoV spike and SARS-CoV2 spike proteins (S2 subunit), no cross-reactivity with MERS-CoV spike protein	WB, ICC/IF, FACS; ELISA, IP	Immunostaining of all S2 spike protein cell pellets	Staining of COVID-19-positive tissues
ABM19C9	Coronavirus (COVID-19) spike antibody	Mouse	Abeomics	10-10007	200 µl (500 µg/mL)	Partial length recombinant coronavirus spike protein, C-term region		ELISA, WB	Immunopositive subpopulation of S2-pellet cells; weak staining in S1 and nucleoprotein pellets	Negative in COVID-19-positive tissue
FIPV3-70	Coronavirus antibody	Mouse	Santa Cruz	SC-65653	1 mL (100 µg/mL)	Coronavirus	Binds to the nucleoprotein of SARS-CoV2 (COVID-19)	WB, IF, IHC	Negative	Negative
6F10	Anti-SARS-CoV2 NP antibody	Mouse	BioVision	A2060	50 µl (2.6 mg/mL)	Synthetic peptide targeting amino acids 300-400 of SARS-CoV2 nucleoprotein		WB, ELISA, sandwich ELISA	Subset of cells of nucleoprotein pellet	Negative (smooth muscle staining)

ELISA indicates enzyme-linked immunosorbent assay; FCM, flow cytometry; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; P, paraffin; RBD, RNA binding domain; WB, western blot.

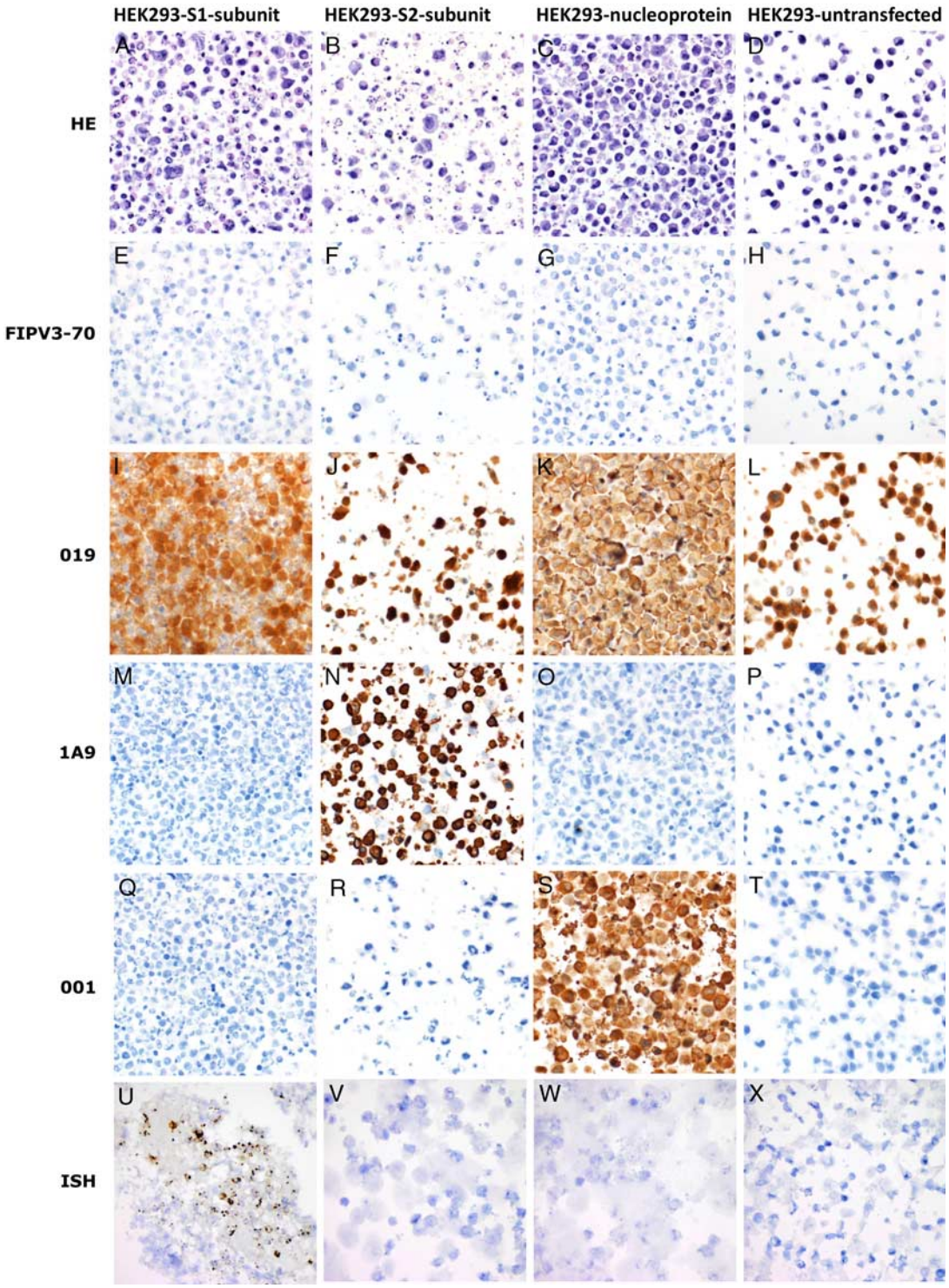

FIGURE 1. Pellets of HEK293 cells transfected with SARS-CoV2: S1 and S2 subunit of spike protein, nucleoprotein and untransfected cells. A–D, Hematoxylin eosin stain/H&E. E–H, mAb FIPV3-20 to nucleoprotein, no immunolabeling of any pellet. I–L, mAb 019, intense immunostaining of all cell pellets. M–P, mAb 1A9, exclusive immunoreactivity of HEK293 cells transfected with spike protein S2 subunit. Q–T, mAb 001, homogeneous staining of HEK293 cells expressing nucleoprotein. U–X. In situ hybridization with probe to S1 subunit positive in corresponding HEK293 cells.

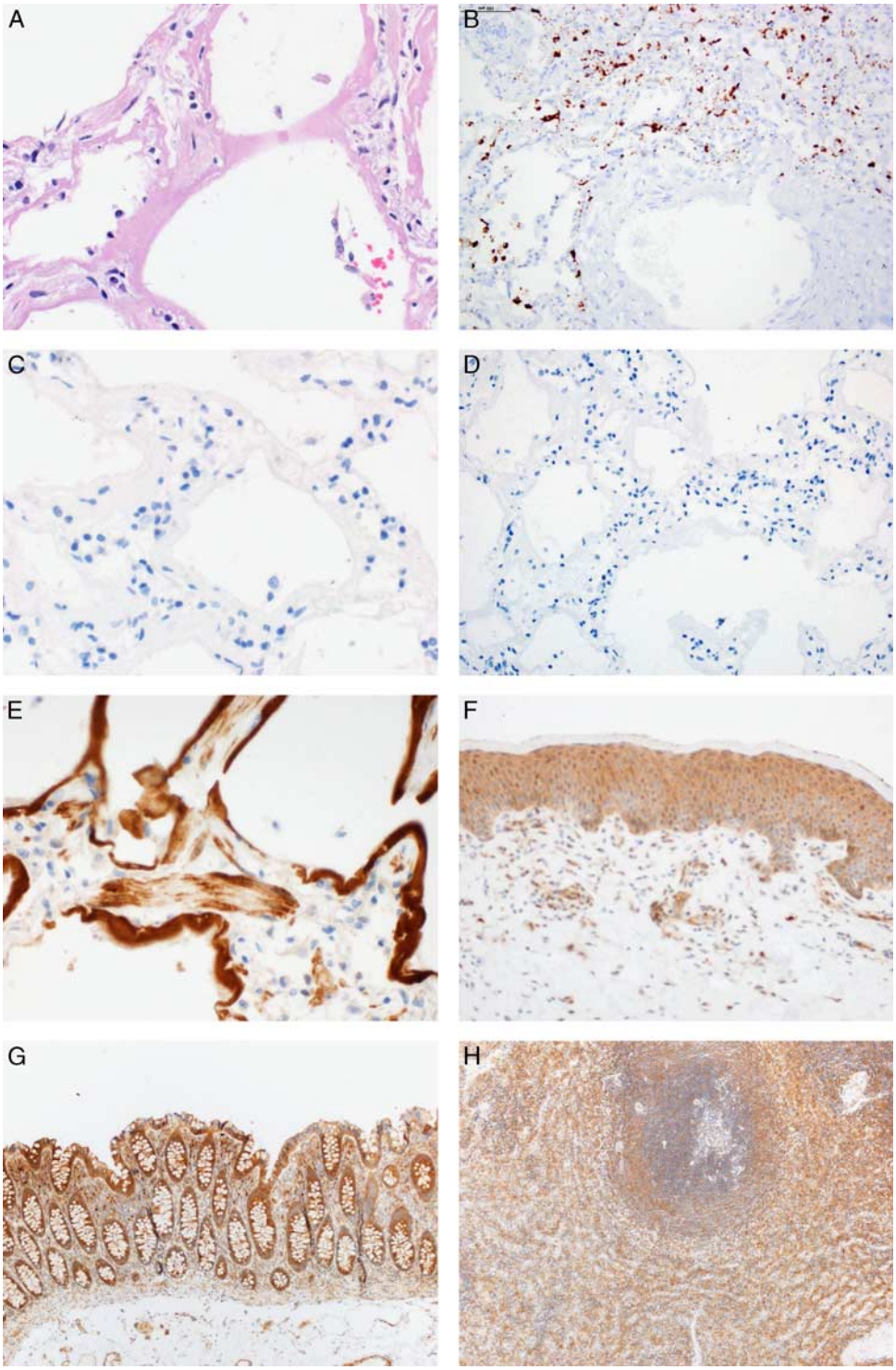

FIGURE 2. H&E stain (A), in situ hybridization (B) and immunohistochemical staining (C–H) of SARS-CoV2 positive lung (A–E) and SARS-CoV2 negative tissues (F–H); ISH probe to S1 spike protein (B) demonstrating SARS-CoV2 viral RNA in lung tissue(B); SARS-CoV2-positive lung tissues negative for mAb FIPV3-20 (C) and mAb 007 (D); mAb 019, intense immunolabelling of hyaline membranes in lung (E), extensive nonspecific immunolabelling of skin (F), colon (G), and spleen (H). full color online

single HEK293 cells transfected with the S1 subunit spike protein. No immunostaining could be achieved in the SARS-CoV2-positive lung tissue.

Clones 1A9 and 001 both showed strong and homogeneous staining in the HEK293 cells transfected with their matching immunogens, S2 spike protein and

nucleoprotein, respectively (Fig. 1). Both antibodies remained negative in normal tissue but displayed strong staining in samples of COVID-19 pneumonia. Consequently, staining of clones 001 and 1A9 had passed the initial screening for sensitivity and specificity for the detection of SARS-CoV2. Clone 001 worked best at a dilution of 1:5K (0.1 μg/mL), while 1A9 displayed best immunoreactivity at 1:1K (1 μg/mL). High pH buffer (ER2, Leica, 30') worked best for both reagents.

Both clones strongly labelled hyaline membranes of acute diffuse alveolar damage in autopsy tissues from patients with previously confirmed COVID-19 pneumonia. They demonstrated strong immunoreactivity in alveolar macrophages as well as the alveolar lining, predominantly of type II pneumocytes with reactive hyperplasia (Fig. 3). Interestingly, the extent of staining was more prevalent with mAb 001 to the nucleoprotein than with mAb 1A9 to the S2 subunit spike protein. The signal of mAb 001 was more intense and widespread in hyaline membranes and pneumocytes (Fig. 3) as compared with clone 1A9. This expression pattern was congruent with the presence of viral RNA as detected by ISH (not shown). The staining difference between mAb 001 and 1A9 is also exemplified in the mucinous content admixed with cell detritus present in some bronchi (Fig. 3). Clone 001 also showed focal staining in the endothelial cells of septal blood vessel (Fig. 3). The presence and the amount of viral protein depended on the stage of inflammation and decreased parallel to organizing changes. The exact pulmonary pathology including the presence of viral proteins as highlighted by mAbs 001 and 1A9 are exceeding the scope of the present study and are outlined in detail in a separate manuscript.[15]

To analyze reactivity of both clones outside the lung, we also tested various other autopsy tissues from patients with COVID-19. In one kidney, focal Immunopositive

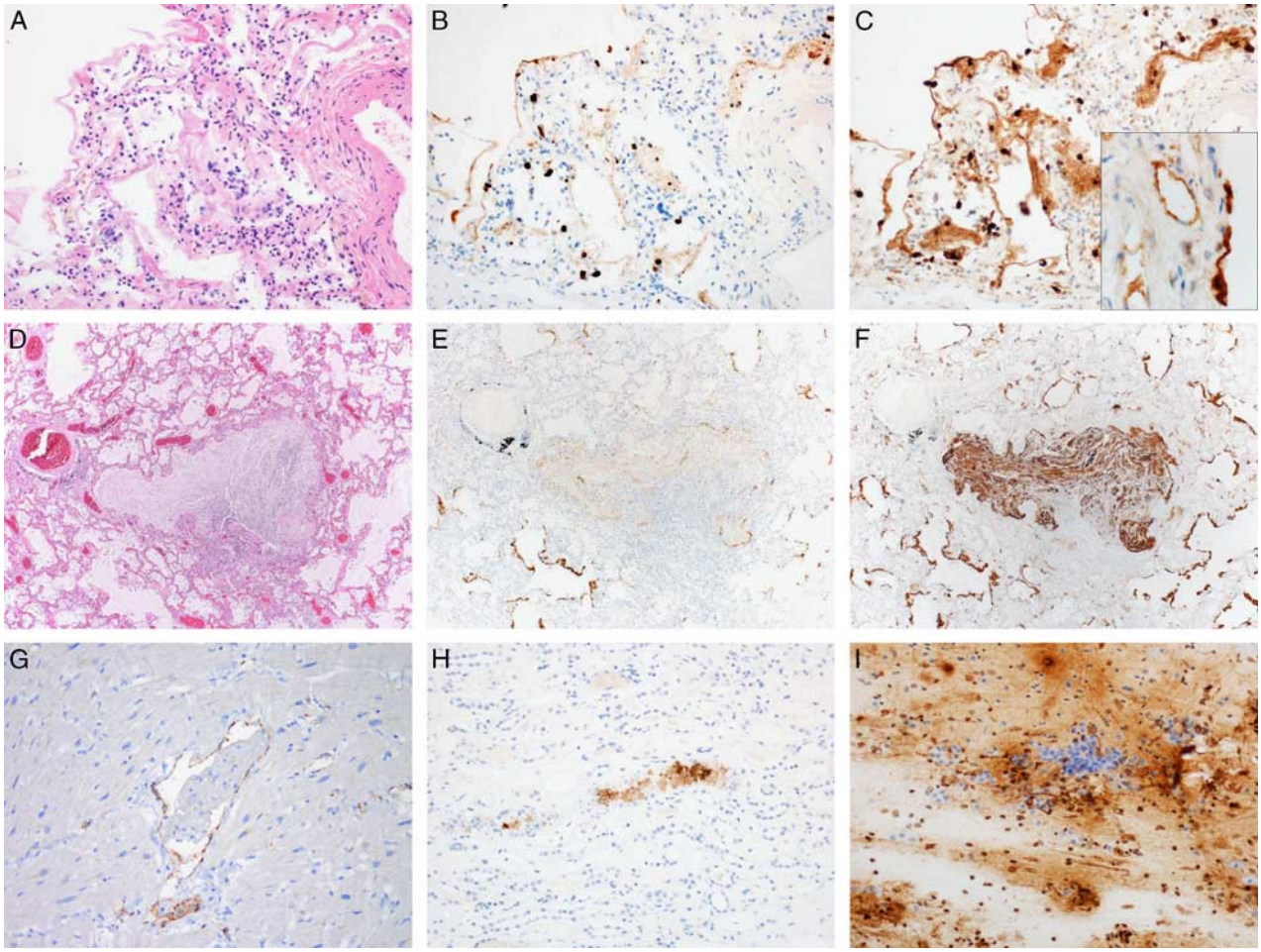

FIGURE 3. Analysis of autopsy tissue from patients who died from COVID-19: H&E stain (A, D) immunoreactivity with mAb 1A9 to S2 subunit spike protein (B, E) and mAb 001 to nucleoprotein (C, F–I); COVID-19 pneumonia in acute phase diffuse alveolar damage with serial sections stained with H&E (A), mAb 1A9 (B), and mAb 001 (C); extensive immunostaining of hyaline membranes and alveolar macrophages for both spike protein and nucleoprotein. mAb 001 staining in endothelia of focal septal vessels and pneumocytes (inset); mucus filled bronchus (D) H&E stain with weakly positive S2 spike protein content (E) and strong signal for nucleoprotein (F); heart muscle with mAb 001 immunopositive endothelia; mAb 001/nucleoprotein-positive content in tubule of SARS-CoV2-positive autopsy kidney (H) and smear of nasal swab stained with mAb 001 positive for SARS-CoV2 (I). full color online

proteinaceous precipitates were present in the lumen of single tubules with mAb 001 and to a lesser extent with mAb 1A9 (Fig. 3). In the myocardium, small vessels from a patient with prolonged COVID-19 infection and recurrent thrombotic events, strongly labelled in a linear manner along the endothelial lining with mAb 001, while mAb 1A9 remained negative (Fig. 3). Finally, we tested smears from the nasal cavity from 3 COVID-19 patients with mAb 001. There was strong immunostaining of the mucus and some of the epithelial cells in 1 patient. Swabs from all three SARS-CoV2 negative patients remained all negative by IHC. Clones 1A9 and 001 were also tested in >100 normal tissues, tumors, and inflammatory lung specimens from SARS-CoV2 negative patients. No staining was observed in non–COVID-19-related lesions.

DISCUSSION

The fast and widespread dissemination of COVID-19 earlier this year, made it clear that proper assessment of virus-related pathomorphologic changes is needed. Consequently, the primary aim of this study was to identify reliable, commercially available monoclonal antibodies for the detection of viral antigens in standard archival pathological specimens.

A mammal expression system using HEK293 transfected cells and a panel of normal tissues was deemed best for initial testing. This approach was considered superior to analyzing infected lung tissue with yet to be determined virally induced pathologic changes. Although all antibodies were eventually tested on SARS-CoV2-positive specimens, initial testing gave an accurate picture of reagents specificity. Specificity testing was supplemented by comparing antibody immunoreactivity pattern to the presence of viral RNA as detected by ISH. Vice versa, the ISH assay for SARS-CoV2 was also tested on the pellets of the transfected HEK293 cells. Only 2 of the 7 tested mAbs, clones 1A9 and 001, proved to be suitable for the IHC detection of viral proteins. Interestingly, most of the manufacturers' application recommendations for our tested clones were not congruent with the actual antibody properties as defined in our study. For example, clones 007 and FIPV3-70 were both recommended for the use in IHC. However, neither clone generated proper immunostaining. In contrast, manufacturer specification for clone 1A9 does not include IHC as potential application. Nevertheless, mAb 1A9 revealed excellent immunoreactivity in the present analysis.

Although experience with IHC detection of SARS-CoV2 is still limited, it has been addressed in a few recent studies. However, specificity analysis of the employed reagents has received little attention.[6,16–18] We are aware of only one study by Liu and colleagues employing extended specificity analysis of commercially available anti-SARS-CoV2 reagents.[19] Interestingly, SARS-CoV2 infected primate kidney cells were employed as test cell line as opposed to transfectants with defined viral proteins in our study. Moreover, specificity analysis comprising normal tissues or SARS-CoV2 negative pathologic specimens was not performed.[19] Nevertheless, only 2 of 6, 1 monoclonal and 1 polyclonal reagent were considered useful in their test setting.[19]

Importantly, our study demonstrates the need to include a wide variety of normal and SARS-CoV2 negative pathologic specimens, especially lung tissue to determine the reactivity spectrum of a reagent. For example, clone 019 appeared to stain well in COVID-19 pneumonia but immunostaining in various unrelated tissues including endothelia of peripheral vasculature disproved its specificity. A recent manuscript studying presence of viral proteins in CNS highlights the specificity issues of anti-SARS-CoV2 reagents since the authors observed staining in negative control tissues.[16] Another recent study analyzing the presence of SARS-CoV2 in peripheral vasculature, employed 2 polyclonal antibodies which appeared to have been tested solely in SARS-CoV2 positive controls and no negative control tissues are mentioned.[6] Given our findings, IHC results obtained with primary antibodies without preceding comprehensive specificity analysis should be considered with great caution.

Morphologically, our findings are partially congruent with previous studies in SARS-CoV1 and 2 localizing viral proteins in predominantly type II pneumocytes and alveolar macrophages.[12,17,18,20–23] However, we saw a strong focal endothelial component in some of the septal lung vessels. Interestingly, similar endothelial staining was also present in cardiac vessels of a patient with virus-related thrombi and restricted to mAb 001 to nucleoprotein, while mAb 1A9 remained negative. It should be emphasized that the testing of tissues was done in the context of evaluation of the tested primary reagents. The findings related to clinical disease course and histologic changes in the lung including IHC analysis employing mAbs 1A9 and 001 are detailed in a separate manuscript.[15] However, our preliminary data suggest that there is difference in the presence of nucleoprotein versus (S2 subunit) spike protein. To our knowledge, differential expression of viral proteins in SARS-CoV or SARS-CoV2 has not gained much attention in the past and awaits further elucidation.

We also demonstrate that mAb 001 can be successfully used to test smears of nasal swabs from SARS-CoV2 positive patient. Although a larger scale analysis is warranted, our findings indicate proof of virus via this detection method, especially since the sensitivity of molecular analysis of nasal swabs has been debated.

In conclusion, several of the commercially available monoclonal antibodies to SARS-CoV2 are not suitable for IHC in formalin-fixed paraffin-embedded tissues. However, we identified 2 clones, 001 to the nucleoprotein and mAb 1A9 to the S2 subunit spike protein which show excellent and most importantly specific immunoreactivity. Our preliminary analysis demonstrates viral protein in various organs and nucleoprotein appears to be more abundantly present than spike protein. Finally, we have demonstrated that the IHC detection of viral protein in nasal swabs is possible.

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