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BRIEF REPORT

Detection of SARS-CoV-2 by RNAscope® *in situ* hybridization and immunohistochemistry techniques

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Abstract

In situ hybridization (ISH) and immunohistochemistry (IHC) are essential tools to characterize SARS-CoV-2 infection and tropism in naturally and experimentally infected animals and also for diagnostic purposes. Here, we describe three RNAscope®-based ISH assays targeting the ORF1ab, spike, and nucleocapsid genes and IHC assays targeting the spike and nucleocapsid proteins of SARS-CoV-2.

Abbreviations

COVID-19	coronavirus disease 2019
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
ISH	*In situ* hybridization
IHC	Immunohistochemistry
BSL2	Biosafety level 2
MOI	Multiplicity of infection
FFPE	Formalin-fixed paraffin-embedded
S	Spike
N	Nucleocapsid
ORF1ab	Open reading frame 1ab

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The world is currently experiencing the devastating effects of the coronavirus disease 2019 (COVID-19) pandemic caused by the newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 has severely challenged the health care systems in most countries around the world, with increased demand for rapid diagnosis and treatment of seriously ill patients. It has been demonstrated that SARS-CoV-2 can infect domestic (i.e., cats and ferrets) and wild animals (e.g., tigers, lions, and mink), causing increasing concerns amongst animal owners [1].

In situ hybridization (ISH) and immunohistochemistry (IHC) techniques allow visualization of viral nucleic acid and protein antigens, respectively, within tissues and cells. These methods offer a semi-quantitative identification of target nucleic acids and proteins, respectively, while conserving topological information of expression within cells and tissues, with respect to specific cellular/tissue structures. This critical information is, in fact, lost with other detection methods, such as western blotting, qPCR/RT-qPCR, or single-cell RNAseq, for which cells and tissues must be dissociated. ISH and IHC are well established and widely used in research and routine laboratory diagnostics [2, 3]. Since the 1970s, RNA ISH has been a valuable tool for investigating molecular mechanisms of cellular and molecular pathology. Currently, multiple approaches exist to carry out RNA ISH [4–9], and among them, the RNAscope® technology excels for robustness, specificity, and sensitivity [6, 7, 10–13]. This technique takes advantage of a variation of the branched DNA or "tree" amplification method. In contrast, IHC performance depends heavily on the existence of a specific antibody with high affinity for its antigen with minimum background staining and good performance in formalin-fixed tissues.

The development of suitable preclinical animal models is paramount for studying COVID-19 pathogenesis and evaluating the efficacy of vaccines and therapeutics (i.e., antivirals). For this purpose, the development of SARS-CoV-2-specific ISH and IHC are both critical for the assessment of viral distribution, cell tropism, and cytopathology within tissues, complementing classical histopathology, various molecular tools, and serological assays. Also, validation of these tools can be of significant utility for postmortem diagnosis of SARS-CoV-2 in animals (and humans) within the context of veterinary diagnostic laboratories using formalin-fixed tissues, which render the virus inactive and safer to test under BSL2 conditions. Thus, the objective of this study was to develop RNAscope® ISH and IHC methods for the detection of SARS-CoV-2-specific antigen and RNA in infected cells that can be utilized for both research (e.g., studies involving experimentally and naturally infected animals) and diagnostic purposes.

For this study, confluent Vero cells (CCL-81™, ATCC, Manassas, VA, USA) were infected with the WA1 strain of SARS-CoV-2 (USA-WA1/2020 strain; BEI Resources, ATCC, Manassas, VA, USA) at a multiplicity of infection (MOI) of 1. Twenty-four hours postinfection, mock-infected and SARS-CoV-2-infected monolayers were fixed in 10% formalin, and cell pellets were embedded in paraffin. Here, we briefly describe the SARS-CoV-2-specific ISH and IHC procedures. The list of reagents, including catalog numbers, as well as detailed protocols for these assay, can be obtained by contacting the authors.

For RNAscope® ISH, a total of three antisense probes targeting the nucleocapsid (N, nucleotide [nt] 28,274-29,533), spike (S, nt 21,563-25,384) and open reading frame 1ab (ORF1ab, nt 266-13,467) of SARS-CoV-2 WA1 strain (GenBank accession number MN985325.1) were designed and manufactured by a commercial company (Advanced Cell Diagnostics [ACD], Newark, CA; Table 1). Four micron sections of formalin-fixed paraffin-embedded mock-infected and SARS-CoV-2-infected Vero cells were mounted on positively charged Superfrost® Plus Slides (VWR, Radnor, PA). The RNAscope® ISH assay was performed using an RNAscope 2.5 HD Red Detection Kit (ACD) as described previously [10, 14, 15]. Briefly, deparaffinized sections were subjected to target retrieval for 15 min at 98-102 °C in 1X Target Retrieval Solution, dehydration in 100% ethanol for 10 min, and Protease Plus treatment for 20 min at 40 °C in a HybEZ™ oven (ACD). Slides were subsequently incubated with a ready-to-use probe mixture for 2 h at 40 °C in the HybEZ™ oven, and the signal was amplified using a specific set of amplifiers (AMP1-6) as recommended by the manufacturer). The signal was detected using a Fast Red solution (Red B: Red A in a 1:60 ratio) for 1-10 minutes at room temperature. Slides were counterstained with 50% Gill hematoxylin I (Sigma Aldrich, St Louis, MO) for 2 min, and bluing was performed using 0.02% ammonium hydroxide in water. Slides were finally mounted with Ecomount® (Biocare, Concord, CA). Probes specific for dihydrodipicolinate reductase B mRNA of *Bacillus subtilis* (DapB) and peptidylprolyl isomerase B (PPIB) were used as negative and positive controls to assess the assay specificity and RNA integrity, respectively. The antisense probes targeting the N, S, and ORF1ab genes generated equal and very strong intracytoplasmic and membranous signals in SARS-CoV-2-infected Vero cell pellets. In contrast, there was no staining in mock-infected Vero cells or Vero cells hybridized with the DapB probe (Fig. 1).

For IHC, 4 µm sections of formalin-fixed paraffin-embedded mock-infected and SARS-CoV-2-infected Vero cells were mounted on positively charged Superfrost® Plus slides and subjected to IHC using three different antibodies directed to the nucleocapsid (N) and one antibody directed to the spike (S) protein (Table 2). IHC was performed using the automated BOND-MAX and a BOND Polymer Refine Red Detection Kit (Leica Biosystems, Buffalo Grove, IL) as described previously [10]. Following automated deparaffinization, heat-induced epitope retrieval (HIER) was performed using a ready-to-use citrate-based solution (pH 6.0; Leica Biosystems) at 100 °C for 20 min. Sections were then incubated with each antibody (Table 2) for 30 min at room temperature, followed by a rabbit anti-mouse IgG (30 minutes) and/or a polymer-labeled goat anti-rabbit IgG coupled with alkaline phosphatase (30 minutes). Fast Red was used as the chromogen (15 minutes), and counterstaining was performed with hematoxylin. Slides were mounted using a permanent mounting medium (Micromount®, Leica Biosystems). Infected and mock-infected sections were incubated without the primary antibodies as controls. The antibodies specific for the N and S proteins showed equivalent cytoplasmic labeling only in SARS-CoV-2-infected Vero cells (Fig. 2). However, among the four antibodies used in this study, clone 6F10, specific for the N protein, showed the most intense staining of the infected cells.

Here, we describe the development of three antisense probes for the detection of three gene targets of SARS-CoV-2 (namely the ORF1ab, S, and N genes) using the

Table 1 Antisense probe targets for detection of SARS-CoV-2 strain WA1 (GenBank accession number MN985325.1) by RNAscope® ISH

Probe name	Target	Nucleotide position	Source
V-nCoV-N	Nucleocapsid (N)	28,274-29,533	Advanced Cell Diagnostics
V-nCoV2019-S	Spike (S)	21,563-25,384	Advanced Cell Diagnostics
V-nCoV-orf1ab-O1	ORF1ab	266-13,467	Advanced Cell Diagnostics

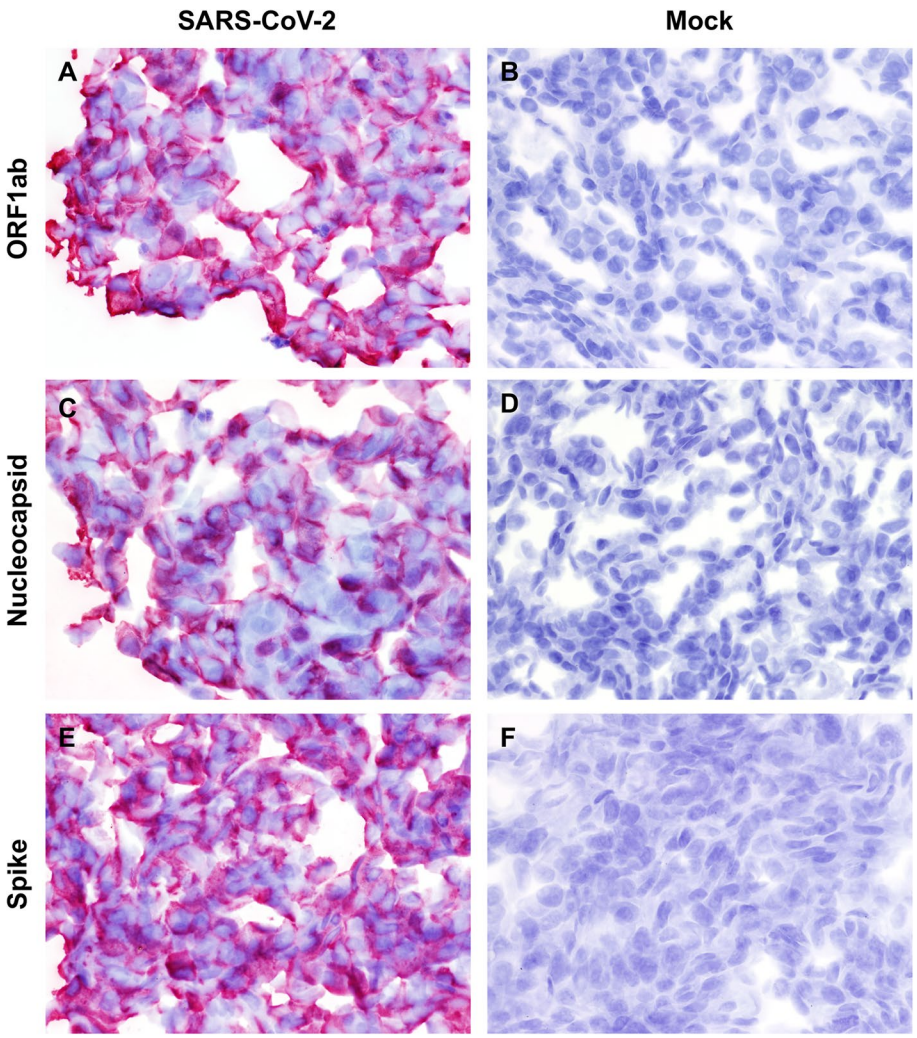

Fig. 1 Detection of SARS-CoV-2 RNA via RNAscope® ISH in SARS-CoV-2 WA1 strain-infected (A, C, E) and mock-infected (B, D, F) Vero cells. (A and B) ORF1ab, (C and D) nucleocapsid (N) and (E and F) spike (S)-specific RNAscope® ISH. Strong and diffuse intracytoplasmic labeling is evident in infected cells for all of the designed probes. No specific labeling is evident in mock-infected Vero cells incubated with SARS-CoV-2-specific probes. Fast Red, 400X

Table 2 Monoclonal and polyclonal antibodies used for immunocytochemical detection of SARS-CoV-2

Specificity	Clone	Species, isotype	Working concentration	Source
Nucleocapsid (N)	4B21	Mouse, IgG	0.25 µg/ml	Creative Diagnostics
Nucleocapsid (N)	6F10	Mouse, IgG	1 µg/ml	BioVision
Nucleocapsid (N)	NA*	Rabbit polyclonal	5 µg/ml	Thermo Fisher
Spike (S)	1A9	Mouse, IgG1	0.25 µg/ml	GeneTex

*NA, not applicable

highly sensitive and specific RNAscope®-based ISH assay. Concurrently, SARS-CoV-2-specific IHC assays were developed. Of the four commercial antibodies used for IHC, the monoclonal antibody clone 6F10 specific for the N protein provided the best staining of SARS-CoV-2-infected cells. The assays developed in this study are readily adaptable for the detection of SARS-CoV-2 in tissues from humans and animals, including those utilized as preclinical animal models of COVID-19 for studying the efficacy of vaccines and therapeutics. Furthermore, the development of IHC and ISH tools is of utmost significance for understanding the pathogenesis of SARS-CoV-2 by characterizing the viral tissue distribution/cellular tropism in animal models and humans. Most importantly, ISH and IHC will complement other quantitative molecular methods in the assessment of vaccine and therapeutic efficacy studies by effectively analyzing the dynamics of viral distribution and clearance within a tissue/cellular context. The tools developed and reported here can be easily multiplexed using automated systems and substantially contribute to understanding SARS-CoV-2 pathogenesis.

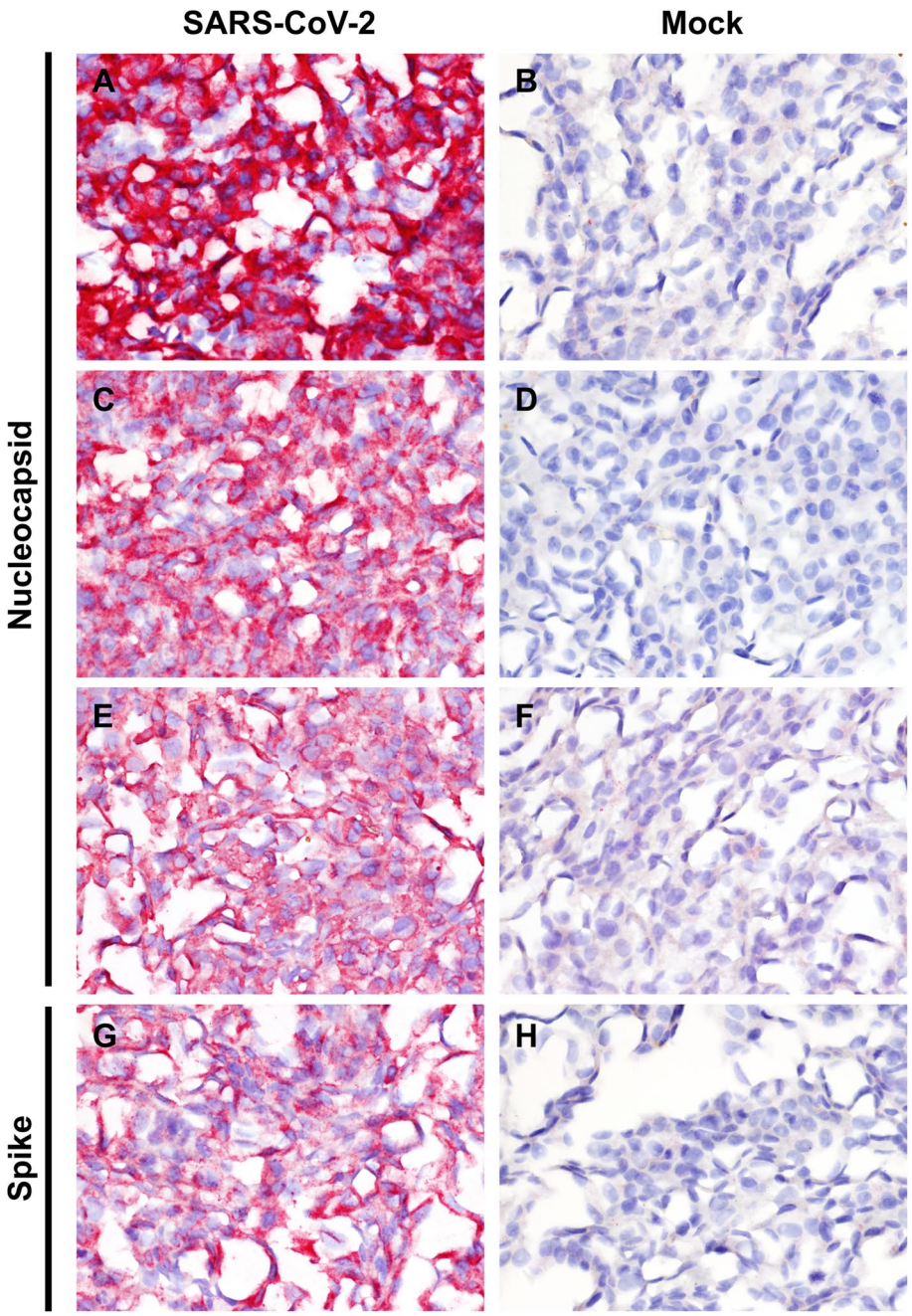

Fig. 2 Detection of SARS-CoV-2 antigen via IHC in SARS-CoV-2 WA1 strain-infected (A, C, E, G) and mock-infected (B, D, F, H) Vero cells. (A and B) anti-nucleocapsid (N) monoclonal antibody 6F10, (C and D) anti-nucleocapsid (N) monoclonal antibody 4B21, (E and F) anti-nucleocapsid (N) polyclonal antibody, and (G and H) anti-spike (S) monoclonal antibody 1A9. Strong and diffuse intracytoplasmic labeling is evident in infected cells with all antibodies. No specific staining is evident in mock-infected Vero cells. Fast Red, 400X

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Availability of data and material For further detail on protocols, please contact the authors directly.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Consent for publication All of the authors have agreed to the submission of this manuscript and to be responsible for its contents.

Ethics approval This article does not contain any studies with living animals performed by any of the authors.

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