Histopathology and SARS-CoV-2 Cellular Localization in Eye Tissues of COVID-19 Autopsies


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Ophthalmic manifestations and tissue tropism of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been reported in association with coronavirus disease 2019 (COVID-19), but the pathology and cellular localization of SARS-CoV-2 are not well characterized. The objective of this study was to evaluate macroscopic and microscopic changes and investigate cellular localization of SARS-CoV-2 across ocular tissues at autopsy. Ocular tissues were obtained from 25 patients with COVID-19 at autopsy. SARS-CoV-2 nucleocapsid gene RNA was previously quantified by droplet digital PCR from one eye. Herein, contralateral eyes from 21 patients were fixed in formalin and subject to histopathologic examination. Sections of the droplet digital PCR—positive eyes from four other patients were evaluated by in situ hybridization to determine the cellular localization of SARS-CoV-2 spike gene RNA. Histopathologic abnormalities, including cytokoid bodies, vascular changes, and retinal edema, with minimal or no inflammation in ocular tissues were observed in all 21 cases evaluated. In situ hybridization localized SARS-CoV-2 RNA to neuronal cells of the retinal inner and outer layers, ganglion cells, corneal epithelia, scleral fibroblasts, and oligodendrocytes of the optic nerve. In conclusion, a range of common histopathologic alterations were identified within ocular tissue, and SARS-CoV-2 RNA was localized to multiple cell types. Further studies will be required to determine whether the alterations observed were caused by SARS-CoV-2 infection, the host immune response, and/or preexisting comorbidities. (Am J Pathol 2023, 1–8; https://doi.org/10.1016/j.ajpath.2023.02.016)

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects tissues throughout the human body, including ocular tissues and fluids.¹ Specific to ocular tissues and fluids, SARS-CoV-2 RNA and/or protein has been detected in conjunctival swabs, cornea, choroid/sclera, lens, retina, optic nerve, and aqueous and vitreous humor.¹⁻⁷

SARS-CoV-2 infection, persistence, and replication, including ocular tissues from 38 coronavirus disease 2019 (COVID-19) autopsy cases were systematically investigated

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in a previous study.\(^1\) SARS-CoV-2 nucleocapsid (N) RNA was detected by droplet digital PCR (ddPCR) in ocular fluids and/or tissue in 23 of 38 cases (61%). Specifically, N RNA was detected in the cornea [8 of 19 (42%)], choroid/sclera [16 of 32 (50%)], lens [10 of 19 (53%)], retina [6 of 17 (35%)], optic nerve [17 of 29 (59%)], vitreous humor [5 of 35 (14%)], and aqueous humor [8 of 32 (25%)]. The copies of N RNA were lower overall in ocular tissues from individuals with longer disease duration and persisted for months in several tissue types. ddPCR-positive tissues and ocular fluids were also assayed for subgenomic RNA, a marker of recent viral replication. Subgenomic RNA was detected in ocular tissues of 7 of 22 cases tested (32%), including in the choroid/sclera of a patient who died 99 days into illness.\(^1\)

Despite these reported findings, information on the cellular localization of SARS-CoV-2 within ocular tissues and their associated pathologic findings is limited.\(^2,6,8,9\) Viral particle localization in specific ocular cells can provide pertinent information on the possible ocular effects of SARS-CoV-2 infection. The ocular tissues of the COVID-19 autopsy cohort described above were further examined for the purposes of evaluating macroscopic and microscopic histopathology and investigating the cellular localization of SARS-CoV-2 by performing in situ hybridization (ISH) of SARS-CoV-2 spike gene RNA.

**Materials and Methods**

**Postmortem Human Eye Procurement and Processing**

Postmortem human eyes (E1 to E25) from 25 patients with confirmed SARS-CoV-2 infection were obtained at autopsy performed at the NIH Clinical Center following consent of the legal next of kin as previously described.\(^1\) The study adhered to the tenets of the Declaration of Helsinki. The cases had a range of illness durations before death and were categorized as early (\(n = 11\)), mid (\(n = 7\)), or late (\(n = 7\)) by illness day (D) at the time of death being D14 or earlier, D15 to D30, or D31 or later, respectively (Supplemental Tables S1 and S2). Previously, one eye from E1 to E25 was freshly dissected, and tissues were preserved in RNA later for subsequent quantification of SARS-CoV-2 RNA by ddPCR.\(^1\) For the study described here, the contralateral eye from E1 to E21 was fixed in 10% neutral buffered formalin for a minimum of 72 hours with the globe intact, opened for macroscopic examination, and then embedded in paraffin for histopathologic evaluation. For cellular localization of SARS-CoV-2 RNA, one eye from E22 to E25 was dissected to isolate cornea, retina, choroid/sclera, and optic nerve. Each tissue type was divided into two pieces—one for ISH and one for SARS-CoV-2 ddPCR. The piece prepared for ISH was fixed for 24 hours in neutral buffered formalin because prolonged formalin fixation interfered with RNA preservation. This fixed piece was then transferred to 70% ethanol for a minimum of 2 days before impregnation with paraffin. The piece prepared for ddPCR was placed in RNA later media for nucleic acid preservation and subsequent SARS-CoV-2 RNA quantification. SARS-CoV-2 RNA quantification was used to guide selection of tissues for analysis by ISH.

**Histopathology**

Each eye was cut horizontally along the pupillary-optic nerve head (P-O) axis. For other lesions outside the P-O section, a segment through the lesion was also obtained for histologic analysis. After macroscopic examination of the open eye under a stereo dissecting microscope, all P-O sections and segments were then processed for routine paraffin embedding and sectioning. Consecutive sections of 4-μm thickness were sliced from each paraffin block and stained with hematoxylin and eosin or periodic acid–Schiff reagent.

**Immunohistochemistry**

CD61, a marker of platelet aggregation and thrombosis, and cytomegalovirus (CMV) expressions were evaluated on formalin-fixed, paraffin-embedded (FFPE) slides using immunohistochemistry. Briefly, the presence of CD61 was detected by immunohistochemistry performed on FFPE sections with an anti-CD61 mouse monoclonal antibody (clone 2f2; Roche Diagnostics, Mannheim, Germany) performed on a Roche Discovery Ultra instrument with 3,3′-diaminobenzidine detection per clinical laboratory protocol. The presence of CMV was also detected by a similar method with an anti-CMV antibody (clone CCH2+DDG9; Agilent Dako, Santa Clara, CA).

**SARS-CoV-2 Spike RNA ISH**

ISH was performed using manual RNAscope 2.5 HD Reagent Kit-BROWN (catalog number 322350; Advanced Cell Diagnostics, Newark, CA) according to the user manual (322360-USM) and as previously described.\(^1\) Briefly, FFPE slides were deparaffinized with xylene followed by 100% ethanol and then treated with hydrogen peroxide at room temperature for 10 minutes to block endogenous peroxidase activity. After antigen retrieval at 99°C for 15 minutes, the slides were incubated with protease at 40°C for 20 minutes. For each ocular tissue, RNAscope Probe-V-nCoV2019-S (catalog number 848561; Advanced Cell Diagnostics), RNAscope Positive Control Probe-Hs-PPB (catalog number 313901; Advanced Cell Diagnostics), and RNAscope Negative Control Probe-DapB (catalog number 310043; Advanced Cell Diagnostics) were applied to three sequential slides and incubated at 40°C for 2 hours. After rinsing, the ISH signal was amplified using six amplifiers and incubated with a dianaminobenzidine substrate for 10 minutes at room temperature. Slides were then
counterstained with 50% hematoxylin, air-dried, and mounted.

**Results**

**Autopsy Cohort Overview**

Postmortem ocular specimens were collected from 25 cases (E1 to E25) with confirmed COVID-19 between April 2020 and December 2021. E24 was the only case that received a COVID-19 vaccine. Eight of the 25 cases (32%) were female, and the mean age at the time of death was 58 years (range, 6 to 91 years). Twenty-four of the 25 (96%) cases had at least one comorbidity, and 15 (60%) cases had three or more comorbidities, with hypertension [15 (60%)] and obesity [10 (40%)] being the most common. The median interval from symptom onset to death was 18 days (range, 4 to 204 days), and the median interval between death and autopsy was 23 hours (range, 10 to 106 hours). Systemic steroids and systemic anticoagulation were commonly administered to treat COVID-19. Case-by-case demographic and clinical data can be found in Supplemental Table S1.

**Macroscopic Retinal Lesions**

Pathologic findings (E1 to E21) are described in Supplemental Table S2 alongside previously reported SARS-CoV-2 RNA ddPCR results from contralateral ocular tissues of the same case. Six of the 21 eyes examined showed potential COVID-19–associated macroscopic lesions in the retina. Specifically, one eye (E18) had vascular sheathing similar to vasculitis in multiple branch retinal vessels (Figure 1A). Two eyes (E2 and E11) showed focal whitish cotton-wool spots in the posterior pole (Figure 1B). Two eyes had focal retinal hemorrhages in the posterior pole (E8 and E17) and midperipheral retina (E8) (Figure 1C). One eye (E19) showed several patches of subretinal whitish infiltrates (Figure 1D). Microscopic examination showed the whitish infiltrates being severe retinal edema and disorganization, accumulation of serous fluids in the retinal outer

![Figure 1](https://example.com/figure1.png)
plexiform layer, as well as debris and strands in the vitreous (Figure 1D, inset). In terms of lesions not associated with COVID-19, four eyes (E3, E7, E13, and E17) were pseudophakic with residual cortex material in the peripheral capsule; E3 also showed two old, large hypopigmented chorioretinal scars with focal hyperpigmentation in the peripheral retina. A large, whitish, fragile soft mass (myelin expulsion due to artifact) attached to the optic nerve head was noted in case E14. All the other 11 eyes (E1, E4-6, E9, E10, E12, E15, E16, E20, and E21) were grossly unremarkable.

Microscopic Retinal and Choroidal Lesions

Retinal lesions potentially associated with SARS-CoV-2 infection are summarized in Supplemental Table S2 and Figure 1, E–L. They include small neovascularization (Figure 1E), retinal sclerotic vessels (Figure 1F), retinal vascular occlusion (Figure 1G) with fibrin/thrombi in retinal vessels (Figure 1H), and vitreous hemorrhages. Eleven (E1, E4, E5, E7, E11, E13-15, E17, E18, and E21) of the 21 eyes (52.4%) contained cytoid bodies (the histopathologic constituent of clinical cotton-wool spots) in the nerve fiber layer of the neuroretina (Figure 1, F, H and I) and/or on the optic nerve head (Figure 1J). Focal loss of photoreceptor cells (Figure 1, E, I–L) was noted in seven eyes (33.3%) (E6, E8, E11, E16, E17, E18, and E19). Fourteen eyes (66.7%) (E1, E2, E4, E6-8, E12 to 15, and E18 to 21) showed retinal/choroidal vascular dilation, congestion, and tortuosity (Figure 1, F–H, J, and K). In addition, serous retinal edema in the inner and outer nuclear layers was noted in six eyes (28.6%, E3, E8, E12, E13, E17, and E19) (Figure 1, D and K), whereas optic nerve head edema was seen in five eyes (23.8%) (E2, E4, E9, E13, and E21) (Figure 1J). Fluid accumulation in the outer plexiform layer was also noted in two eyes (9.5%) (E8 and E11 in the macula) (Figure 1K).

Only one eye (E18) had viral inclusions, which stained negative for CMV, in a few retinal ganglion cells (Figure 1L, inset). Except for one eye (E3) showing minimal to mild lymphocyte infiltration on the optic nerve and one eye with minimal choroidal mononuclear inflammation (E16), no inflammatory cells were noted in the other ocular tissues or the other eyes. Importantly, cytoid bodies, retinal edema, or vascular congestions/hemorrhage were observed either alone or in combination in 20 of the 21 eyes (95.2%) and were associated with ddPCR positivity in the contralateral eye in 14 cases (Supplemental Table S2).

Positive CD61 staining of platelet thrombi (Figure 2), sometimes associated with fibrin and mononuclear cells, was observed in the retinal vascular wall in five eyes (E7, E13, E14, E17, and E18), all with positive ddPCR results in the contralateral eye and the presence of cytoid bodies in the nerve fiber layer of the retina. One eye (E18) also showed retinal vascular occlusion (Figure 1G) and neovascularization (Figure 1E). No CMV positivity was detected in any cases.

Cellular Localization of SARS-CoV-2 Spike Gene RNA by ISH

ISH for SARS-CoV-2 spike gene RNA was performed on ocular tissues from E22 to E25 (Figures 3 and 4). SARS-CoV-2 RNA was detected mostly in the outer nuclear layer (photoreceptor cells) of the retina in case E22 (Figure 3A). Some SARS-CoV-2 RNA was also detected in the inner nuclear layer and in the ganglion cells of the E22 retina. Examination of E23 demonstrated the presence of SARS-CoV-2 within retinal inner and outer nuclear cells and a few ganglion cells (Figure 3B). No signal was consistently observed using a standard negative control probe for the bacterial gene _DapB_ to demonstrate specificity of the assay. As examples, images of retinal tissues (from E22 and E23) treated with the negative control probe are shown in Figure 3, C and D, respectively. A focus of SARS-CoV-2 RNA was detected in the scleral fibroblasts of E24 (Figure 4A). Additionally, analysis of E25 demonstrated the presence of SARS-CoV-2 RNA within oligodendrocytes of the optic nerve (Figure 4B) as well as the retinal cells (Figure 4C) and corneal epithelium (Figure 4D).

Discussion

This study illustrates the histopathologic characterization and molecular localization of SARS-CoV-2 within specific ocular tissues and cells in a cohort of 25 fatal COVID-19 cases. Abnormalities suggestive of indirect viral effect, including cytoid bodies, vascular changes, retinal edema, and minimal inflammation, were observed in all 21 eyes that underwent gross and histopathologic evaluation, irrespective of illness duration before death. Fourteen of 21 eyes (67%) were from cases whose contralateral eyes were positive for SARS-CoV-2 by ddPCR. To the best of our knowledge, this is the first report of localization of SARS-CoV-2 RNA in the retina demonstrated by ISH, including the inner and outer nuclear layers and ganglion cells. These findings are consistent with those of Casagrande et al who previously reported detection of SARS-CoV-2 RNA in retinal biopsies from three of 14 eyes by RT-PCR but differ...
from Bayyoud et al,\textsuperscript{10} who did not detect SARS-CoV-2 RNA in 10 cadaveric eyes from five deceased COVID-19 donors.

In this study, three of four eyes evaluated by ISH showed localization of SARS-CoV-2 to the retina. Importantly, these four eyes were dissected at autopsy and rapidly fixed to preserve RNA integrity, which contrasts with the standard approach for immersing the globe \textit{in toto}, risking over-fixation.\textsuperscript{11} Widespread SARS-CoV-2 dissemination in the cohort\textsuperscript{1} suggests hematogenous seeding of the eye with disruption of the blood-retinal barrier. However, localization of SARS-CoV-2 to oligodendrocytes of the optic nerve, focal neuronal loss in the optic nerve of three cases reported here, and the 59% optic nerve SARS-CoV-2 positivity rate that we previously reported\textsuperscript{1} raise the possibility of neuronal spread of SARS-CoV-2. There are conflicting reports of expression of angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) in human ocular tissues perhaps due to different methods for tissue preparation and protein detection.\textsuperscript{12} Nonetheless, some reports provide evidence of ACE2 and TMPRSS2 expression in multiple ocular tissues, including optic nerve; retinal

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\caption{Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike RNA by \textit{in situ} hybridization (ISH) in the retina. A–D: ISH for SARS-CoV-2 spike RNA (brown dots) primarily located in the outer nuclear layer of the retina (arrows, A) in case E22. Fewer positive signals are noted in the inner nuclear layer (A and B) and a retinal ganglion cell in case E23 (arrow, B). Panels A and B are with the SARS-CoV-2 spike RNA probe, and panels C and D are negative control probe for DapB. Scale bars = 20 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike RNA by \textit{in situ} hybridization (ISH) in the sclera, optic nerve, retina, and cornea. A–D: ISH for SARS-CoV-2 spike RNA in scleral fibroblasts in case E24 (A), optic nerve oligodendrocytes in case E25 (B), retinal outer and inner nuclear layers in case E25 (C), and corneal epithelium in case E25 (D). Scale bars = 20 μm.}
\end{figure}
neuronal, vascular, and perivascular cells; and visual processing centers of the brain, supporting the possibility of both neuronal and hematogenous infection routes.13,14

Given the expression of ACE2 and TMPRSS2 reported in cornea and conjunctival tissue,15 concern exists that the ocular surface can serve as a route for SARS-CoV-2 entry. Casagrande et al4 reported 55% SARS-CoV-2 RT-PCR positivity of corneal buttons in 11 deceased patients with COVID-19. Sawant et al7 reported 13% SARS-CoV-2 RT-PCR positivity of ocular specimens collected from 33 donors eliminated from corneal donation due to suspected or confirmed SARS-CoV-2 infection, with the highest rate of detection in vitreous and posterior corneal tissue. The study described here is the first to demonstrate localization of SARS-CoV-2 to the ocular surface using ISH, supporting the RT-PCR-based findings from previous studies (Figure 4, A and D).

Non-CMV viral inclusion bodies, suggestive of active viral replication, were observed in only one eye (E18, with longer disease duration) within a few retinal ganglion cells (Figure 1L). However, the contralateral eye from this patient was SARS-CoV-2 positive in the aqueous not retina by ddPCR. It is not uncommon to present discrepancies between the two eyes in ocular diseases, including infection. CMV retinitis tends to be unilateral in presentation.16 In support of this finding, SARS-CoV-2 was observed in the retinal ganglion cell layer by ISH (Figure 3B). To the best of our knowledge, viral inclusions have not previously been reported in other histologic studies on postmortem ocular tissues following COVID-19 infection, although presumed SARS-CoV-2 viral particles have been reported in the retina of three patients with COVID-19 using transmission electron microscopy.7 The presence of viral inclusion bodies within the retina (ganglion cells) is classically associated with herpetic viral retinitis.17 Notably, in this case, CMV test results were negative, and no additional inflammatory infiltrate and retinal necrosis consistent with necrotizing retinitis were observed.

The lack of frank inflammatory cellular infiltration in the 21 eyes that underwent histologic evaluation is notable, given that 67% of contralateral eyes from these cases were ddPCR positive (Supplemental Table S2).1 The only exceptions were that E16 showed mild mononuclear choroidal inflammation and E3 showed minimal inflammation in the optic nerve, with the contralateral eye being SARS-CoV-2 ddPCR positive in both cases. The most common ocular histopathologic findings in the cohort were nerve fiber layer cytoid bodies; retinal vascular abnormalities, including dilation, congestion, tortuosity, and/or occlusion; and optic disc edema. These findings are consistent with microvascular insults in both early and late COVID-19 cases. However, all but one patient with cytoid bodies and retinal vascular dilation had concurrent cardiovascular risk factors, which might at least in part account for these findings. Clinical reports and autopsy studies indicate that multisystem microvascular injury and vasculopathy contribute to COVID-19 pathogenesis, including in the central nervous system, where acute hypoxic injury, microinfarcts, microhemorrhages, and hemorrhages have been observed.18,19 These observations and the lack of inflammatory cells in the eyes of patients with COVID-19 suggest that ocular microscopic changes are likely due to indirect rather than direct viral cytopathic effects.

Ocular microangiopathic changes, including the finding of platelet thrombi (Figure 2) in capillaries identified by CD61 expression in five cases, are supportive of thromboembolic events reported with SARS-CoV-2 infection.20 Specifically, one eye had CD61 positivity (E18) with histopathologic evidence of retinal vascular occlusion. CD61 has been implicated in having a central role in platelet activation and aggregation, and platelet microthrombi were described in early SARS-CoV-2 autopsy series.21 This could lead to microvascular ischemia, resulting in cytoid bodies in the retinal nerve fiber layers and optic nerve head. Reinhold et al20 reported in their cohort of 10 autopsy eyes congestion of the choriocapillaris and accumulation of multiple small thrombi within the choriocapillaris in eight eyes from four patients. Retinal microvascular changes have also been observed in studies of patients with COVID-19, including microhemorrhages, retinal vascular tortuosity, and cotton-wool spots, as well as quantitative alternations in the retinal vasculature.22 In addition to retinal microvascular changes, reports of retinal vein occlusion associated with COVID-19 have been described, including in younger patients without known risk factors. The hypercoagulable state associated with COVID-19 has been implicated in the pathogenesis of these retinal vein occlusions.23–27 Lastly, cases of optic neuritis have been reported following COVID-19 infection. In the study described herein, SARS-CoV-2 RNA was detected in the optic nerve oligodendrocytes in one eye (Figure 4B). Although the exact mechanisms remain unclear, antigenic mimicry and breakdown of the blood brain barrier have been postulated.28–30 Whether direct viral invasion can cause or potentiate these mechanisms is not known.

As referenced above, this study has limitations. Given that ocular history and clinical examinations from the donors are not available, it is not known whether any of these observed changes preceded SARS-CoV-2 infection. The diverse comorbidities of the donor cohort can contribute to the observed ocular pathology; consequently, it is difficult to draw clear conclusions about whether the pathology was solely and/or mainly caused by COVID-19 or not. In particular, many of the donors in the study cohort had cardiovascular comorbidities, which could contribute risk factors for more severe COVID-19 and ocular pathology. Despite these limitations, the study shows important new insights into SARS-CoV-2 ocular pathogenesis. Specifically, this is the first report to definitively localize SARS-CoV-2 to the retinal inner and outer nuclear cells, retinal ganglion cells, and ocular surface by ISH, validating previous studies that have exclusively used PCR-based methods. These observations highlight the need to better elucidate mechanisms of
SARS-CoV-2 infection and persistence in the eye, associated direct and indirect pathogenesis, and long-term ocular sequelae among COVID-19 survivors.

Acknowledgments


Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpam.2023.02.016.

References


