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Absence of Severe Acute Respiratory Syndrome-Coronavirus-2 RNA in Human Corneal Tissues

Tarek Bayyoud, MD, Angelika Iftner,† Thomas Iftner, PhD,† Karl Ulrich Bartz-Schmidt, MD,* Jens Martin Rohrbach, MD,* Marius Ueffing, PhD,*‡ Michael Schindler, PhD,† and Sebastian Thaler, MD‡*

Purpose: To examine corneal tissue for severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2) positivity regarding implications for tissue procurement, processing, corneal transplantation, and ocular surgery on healthy patients. We performed quantitative reverse transcription-polymerase chain reaction qRT-PCR-testing for SARS-CoV-2 RNA on corneal stroma and endothelium, bulbar conjunctiva, conjunctival fluid swabs, anterior chamber fluid, and corneal epithelium of coronavirus disease 2019 (COVID-19) postmortem donors.

Methods: Included in this study were 10 bulbi of 5 COVID-19 patients who died because of respiratory insufficiency. Informed consent and institutional review board approval was obtained before this study (241/2020BO2). SARS-CoV-2 was detected by using a pharyngeal swab and bronchoalveolar lavage. Tissue procurement and tissue preparation were performed with personal protective equipment (PPE) and the necessary protective measures. qRT-PCR-testing was performed for each of the abovementioned tissues and intraocular fluids.

Results: The qRT-PCRs yielded no viral RNA in the following ocular tissues and intraocular fluid: corneal stroma and endothelium, bulbar-limbal conjunctiva, conjunctival fluid swabs, anterior chamber fluid, and corneal epithelium.

Conclusions: In this study, no SARS-CoV-2-RNA was detected in conjunctiva, anterior chamber fluid, and corneal tissues (endothelium, stroma, and epithelium) of COVID-19 donors. This implicates that the risk for SARS-CoV-2 infection using corneal or conjunctival tissue is very low. However, further studies on a higher number of COVID-19 patients are necessary to confirm these results. This might be of high importance for donor tissue procurement, processing, and corneal transplantation.

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From the *Department of Ophthalmology, University Hospital Tübingen, Tübingen, Germany; †Institute for Medical Virology, University Hospital Tübingen, Tübingen, Germany; and ‡Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany.

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Correspondence: Tarek Bayyoud, MD, Department of Ophthalmology, University Hospital Tübingen, Elfriede-Aulhorn-Str. 7, 72076 Tübingen, Germany (e-mail: tarek.bayyoud@med.uni-tuebingen.de).

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The ongoing severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2) pandemic is a global health threat that causes immanent hardships in medical practice involving logistics, patient management, surgery, and handling of infectious materials.[1–3] One of the first healthcare professionals to raise the alarm was the Chinese ophthalmologist Li Wenliang, who died from coronavirus disease 2019 (COVID-19) at the young age of 33, after being infected by one of his patients.[4] In general, ophthalmologists are at great risk because of close patient contact while performing a significant number of examinations and interventions. Despite the question, whether SARS-CoV-2 has the potential to be transmitted using ocular fluids, it is of special interest for the cornea and eye banking specialists to know whether corneal tissues are potentially infectious and possibly mediate the transmission of SARS-CoV-2 from corneal donors to recipients. Currently, there is a lot of discussion among corneal specialists regarding acute adjustments and changes to standard procedures in tissue procurement, processing, and transplantation (personal communication, tissue transplant section of the German Ophthalmological Society). A recent study confirmed that SARS-CoV-2 can invade the conjunctival epithelium and cause a full-blown picture of viral conjunctivitis.[5] The objective of this study was to evaluate corneal involvement in COVID-19 postmortem donors in the following tissues: corneal stroma and endothelium, bulbar-limbal conjunctiva, conjunctival fluid swabs, anterior chamber fluid, and corneal epithelium. Findings may have implications for corneal transplantation and in particular corneal tissue procurement and processing. Our secondary objective was to describe precautions taken and personal protective equipment (PPE) used during these tissue procurements.[6]

MATERIALS AND METHODS

Informed Consent and Approval of Independent Institutional Review Board

Informed consent, adherence to the tenets of the Declaration of Helsinki, and approval of an independent

ethics committee (institutional review board) was obtained before commencement of the study (241/2020BO2).

Tissue Procurement

Specific Guidelines, Assessment of the Environment for Tissue Procurement, and Personal Protective Equipment

Guidelines for the enucleation team (2 persons): To be checked before the enucleation of a COVID-19 postmortem donor:

1. Place of enucleation defined as an area and/or room needing permission to access (time spent at location has to be documented);
2. Place of enucleation is not allowed to be used by another person at time of tissue extraction;
3. Any kind of aerosol and/or turbulence has to be prevented;
4. The necessary equipment has to be discarded after usage and/or disinfected depending on the specific utensils used;
5. To preclude any kind of self-harm PPE has to be used appropriately. This includes the following:
   - Surgical hand disinfection;
   - Gowns (overalls and apron), double gloves (as indicator system), and hood;
   - Face mask (FFP-3 level: 0.6 μm/99% filtration),
   - Surgical instruments with tray;
   - Disposal of infectious wastes in a one-time lockable container and of sharp utensils in a suitable, second container.

Enucleation and Preparation Protocols

A routine tissue procurement protocol for corneal banking was used for the left globe of each donor. The respective right globe was kept naïve during the enucleation and preparation steps.

Enucleation

The enucleation was performed at the designated COVID-19 autopsy room of the Institute for Pathology and Neuropathology of the University Hospital of Tübingen . The average time of death to retrieval was 21 hours.

The following steps were performed:

- Final check of the set of instruments;
- Double check identity of postmortem donor and consent form;
- Confirm cause of death (COVID-19);
- Documentation of donor side (right/left), place, and time of enucleation;
- Proper usage of PPE including fitting test of FFP-3/N-95,
- Inspection of body bag and corpus;
- Preparation of transport media and vessel [left globe: sterile gauze, 10 mL NaCl, 10 mL gentamicinsulfate (5 mg/mL); right globe: sterile gauze, 10 mL NaCl; mark each vessel: COVID-19 donor tissue];
- Flushing of the superior and inferior fornix of the left globe (Betaisodona; 1:10 diluted in sterile NaCl equivalent to 1% of free iodine, flushing with sterile NaCl after 5 minutes), periocular wiping with Betaisodona; right globe is kept naïve;
- Appropriate usage of provided drape, vessel, and PPE;
- Perform enucleation with provided single-use surgical set (eyelid blocker, forceps, scissors, and hooks) to obtain an intact globe with conjunctivae (5–10 mm);
- Prosthesis selection, insertion, and closure of palpebral fissures;
- Transfer of each globe into specific transport vessel and a relockable container marked "COVID-19 donor tissue";
- Disposal of used PPE and potentially infectious materials.

Transport

- Transport by using a relockable, marked container ("COVID-19 donor tissue");
- Temperature is recorded and kept between 33.8°F and 50°F (+1 to +10°C) using cooling packs and box avoiding contact to ice (Libero T1, Elpro, Switzerland); direct preparation and further testing of donor tissue or storage at 42.8°F (6°C).

Preparation

The preparation was performed at a BSL2 laboratory (under a sterile workbench) of the Institute for Medical Virology of the University of Tübingen. The average time of death to preservation was 31 hours. The following steps were performed:

- Use of PPE including fitting test of FFP-3/N-95;
- Disinfection of the sterile workbench (Descosept-AF, desiccation of 15 minutes);
- Disinfection of globe (left) in diluted iodine solution [5 minutes in Betaisadona (7.5%, Braun, #3864154)/NaCl (250 mL, Fresenius Kabi, PZN-00809049) 1:20] and thorough rinse (in 50 mL NaCl); right globe is kept naive;
- Preparation of a corneoscleral donor tissues/fluids (surgical set: surgical forceps, 15-mm trephine, 30G-cannulas, Kolibri-forceps, Vannas-scissors, Westcott-scissors, hockey knife, and vacuum holder), and extraction of tissue samples for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) testing on SARS-CoV-2 RNA (4 samples per type of tissue/fluid: corneal stroma and endothelium, bulbar conjunctiva, conjunctival fluid swabs, anterior chamber fluid, and corneal epithelium);

RNA extraction and quantitative reverse transcription-polymerase chain reaction based on quality approved protocols with controls:

- Addition of 600 μL RLT (1 mL RLT, 10 μL ß-mercaptoethanol RNeasy Kit, QiaCube, QiaSymphony DSP Virus/Pathogen Kit, Qiagen, Hilden, Germany) and one 5mm-steel ball (Qiagen #69989) to each sample;
- Dissolution in ball mill (Fa. Retsch, 2 minutes, level 100);

- Purification in shredder pillar [Qiagen (#79656), ↺ centrifugation at 2 minutes at 14,000 rpm; addition of same volume of 70% EtOH with DEPC-H2O, nonvortex mix, 700 μL for RNeasy spin column, ↺ centrifugation at 15 seconds at 14,000 rpm, repeat with remaining RLT/EtOH mix];
- qRT-PCR using the RealStar SARS-CoV-2 RT-PCR Kit 1.0 (altona Diagnostics GmbH, Hamburg, Germany) and LightMix Modular SARS-CoV (COVID19) kit (TIB Molbiol Syntheselabor GmbH, Berlin, Germany).

DNAse digest with an RNAse-Free DNase Set (#79254), purification with an RNeasy Mini Kit (Qiagen #74106):

- Dissolution of lyophilized DNAse in RNAse free water (550 μL);
- Addition of RW1 buffer (↺ centrifugation at 15 seconds at 14,000 rpm, wash column) and of DNAse (10 μL) to RDD buffer (70 μL), nonvortex mix, centrifugation;
- Addition of DNAse mix to center of the column (15 minutes RT);
- Add RW1 buffer (350 μL) to the column (↺ centrifugation at 15 seconds at 14,000 rpm), RPE (500 μL, ↺ centrifugation at 15 seconds at 14,000 rpm, wash column);
- Transfer column to the second collection tube (↺ centrifugation at 1 minute at 14,000 rpm) and new Eppendorf tube/microreaction vessel;
- Add RNAse free water (30–50 μL, ↺ centrifugation at 1 minute at 14,000 rpm);
- Second addition of RNAse free water (30–50 μL, ↺ centrifugation at 1 minute at 14,000 rpm) in case of >30 μg RNA.

Wuantitative reverse transcription-polymerase chain reaction:

- 10 μL of the RNA, positive, or negative control were used for qRT-PCR with the LightMix SarbecoV E-gene Kit (TIB MOLBIOL, 40–0776–96) in combination with the Roche LightCycler Multiplex RNA Virus Master (Roche, 07083173001). The positive control was supplied with the LightMix Kit and contained all diagnostic targets (E gene, N gene and RdRP) of SARS and SARS-CoV-2. As negative control, the water supplied with the Roche Master kit was used. The reaction mix was prepared as described in the manual.
- Data analysis was performed as described in LightCycler II operator's manual; in brief, color compensation was selected for multiplex assays and the "second derivative maximum method" was used. The results were shown in the 6-carboxyfluorescein channel.
- According to the producers' manual, the sensitivity is 5.2 copies per reaction. A hole genome, synthetic RNA control (Twist Bioscience, #MT007544.1) was also used in qRT-PCR; a consecutive dilution showed that down to 10 copies per reaction SARS-CoV-2 was detectable (linear correlation) (data not shown). The cutoff was defined as recommended in the LightMix Kit manual: C_P value for 10 copies (35.48 ± 0.2) plus 1 cycle and resulted in a C_P cutoff value of 36.48.

Postmortem pulmonary tissue samples from those who died of COVID-19 were tested for SARS-CoV-2 RNA using RT-PCR. All tested samples had positive SARS-CoV-2 results (unpublished data). Whether these samples were still infectious was not evaluated.

The interim guidance of the Centers for Disease Control and Prevention states concerning "Collection and Submission of Postmortem Specimens from Deceased Persons with Known or Suspected COVID-19": No data are currently available on the frequency of detection of SARS-CoV-2, the virus that causes COVID-19, by RT-PCR on postmortem swabs collected at different durations after death. If COVID-19 testing on postmortem swab specimens is being considered for a suspected COVID-19 case, SARS-CoV-2 RNA may still be detected up to 3 days postmortem and possibly longer based on available data from experiences with MERS-CoV and SARS-CoV; however, sensitivity may be reduced with a longer postmortem interval, and duration of illness may need to be considered in interpreting a negative result.[7] Per the United States Food and Drug Administration, respiratory viruses, in general, are not known to be transmitted by transplantation of human cell, tissue, or cellular- or tissue-based product, and there have been no reported cases of SARS-CoV, MERS-CoV, or any other coronavirus transmission via transplantation of ocular tissue.[8] European agencies advance a similar view as the US-centric agencies. This is outlined in the European CDC technical report "Infection prevention and control for COVID-19 in healthcare settings—first update, 12 March 2020." It references the "World Health Organization Interim Guidance for Collection and Submission of Postmortem Specimens from Deceased Persons Under Investigation (PUI) for COVID-19, 19 February 2020" (cited March 11, 2020; available from: https://www.cdc.gov/coronavirus/2019-ncov/hcp/guidance-postmortem-specimens.html).

Histopathology

A histopathological macroscopic and microscopic examination using standard hematoxylin and eosin stains was performed on the extracted tissues.

Donor Characteristics and Clinical Aspects of COVID-19

The age of the donors ranged from 74 to 89 years (mean: 80 years; 1 woman; 4 men). Medical history included the following: arterial hypertension in all and diabetes mellitus in one patient. All donors were on antihypertensive drug regimens. One patient received in addition antihyperglycemic treatment. Three patients were on angiotensin-converting enzyme (ACE) inhibitor class of medications, 1 patient was on an angiotensin-receptor blocker, and another on the antihyperglycemic agent of the biguanide class (metformin). The mean time of hospitalization before demise was 15 days (±12.9 SD; range: 1–32 days) (Table 1).

All patients had initially unspecific symptoms that progressed to a full picture of COVID-19 with distinct dyspnea. Pharyngeal swabs and bronchoalveolar lavage fluid

TABLE 1. COVID-19 Patient Characteristics

				PMH		PDH	
Pat. ID	Age	Sex	Time of Hospitalization (d)	aHTN	DM	aHT	AGT
1	75	Male	9	Yes	No	Metoprolol	n/a
2	78	Male	8	Yes	Yes	HCT, bisoprolol, lercanidipine, candesartan	Metformin
3	87	Female	1	Yes	No	Torasemide, ramipril	n/a
4	89	Male	25	Yes	No	Ramipril, torasemide, thiazide	n/a
5	74	Male	32	Yes	No	Ramipril, bisoprolol	n/a

AGT, Antiglycemic treatment; aHT, Antihypertensive treatment; aHTN, arterial hypertension; DM, diabetes mellitus; HCT, hydrochlorothiazide; ID, identification number; PDH, past drug history; PMH, past medical history.

were tested positive for SARS-CoV-2-RNA by qRT-PCR. Coinfection by herpes simplex virus, cytomegalovirus, respiratory syncytial virus, parainfluenza, and influenza were excluded through qRT-PCR. The type of care included supportive, respiratory intubation, and machine-assisted support including extracorporeal membrane oxygenation and continuous venovenous hemofiltration (Table 2). Supportive care was administered to all patients, respiratory ventilation to 4 patients and machine-assisted support to 3 patients (1× extracorporeal membrane oxygenation and 2× hemofiltration). Organ system involvement was extensive in all cases and included the respiratory, gastrointestinal, and urogenital systems. The respiratory system was involved in all cases extending to acute respiratory distress syndrome in all and complicated by pleural effusion in 2, atrial fibrillation in 1, and myocardial infarction in 3 patients. A life-threatening organ dysfunction was diagnosed in all patients leading to the involvement of the gastrointestinal and genitourinary systems. Acute liver failure was seen in 2 patients and acute kidney failure in all patients. Multiorgan dysfunction syndrome was finally diagnosed in 3 patients. Laboratory parameters showed a leukocytosis combined with lymphopenia in 2 and a reduced hemoglobin concentration in 4 cases.

RESULTS

We report here the absence of SARS-CoV-2 RNA in corneal tissues obtained from COVID-19 postmortem donors using qRT-PCR. All tissue samples tested negative for SARS-CoV-2 viral RNA amplyfing the viral S and E genes (Table 3). All internal positive and negative controls were valid and included in each set of analyses. In addition, there was no difference noted in SARS-CoV-2 RNA detection between routine tissue procurement protocol for corneal banking used for the left globe of each donor and respective right globe which was kept naive during the enucleation and preparation steps.

The macroscopic and microscopic histopathological examinations performed confirmed in all globes normal extraocular and intraocular morphologies without histological signs of inflammation.

DISCUSSION

Recent studies suggest that clinical manifestations of ocular surface disease of COVID-19 are not common and are usually limited to the conjunctiva.[5,9–11] To our knowledge, our study may be the first suggesting the absence of SARS-CoV-2 RNA in conjunctiva and corneal tissue in COVID-19 cadaveric donors. Recently, a case of viral conjunctivitis of SARS-CoV-2 has been reported.[5] In addition, its RNA has been detected in tears and conjunctival secretions.[9] This suggests that the clinical spectrum of an ocular SARS-CoV-2 involvement might potentially be of greater extent.

Related to the current Centers for Disease Control and Prevention interim guidance, we would like to point out that false negative testing may be due to timing of PCR testing, testing capability, postmortem interval, and length of hospitalization/duration of disease.[12] In addition, no test has been validated to date for testing in cadaveric donors. We note here that qRT-PCR was performed on COVID-19 cadaveric donor tissues and fluids. Thus, a false negative result might be more likely than during the acute phase of the disease. However, the number of eligible cases was limited

TABLE 2. COVID-19 Patients: Type of Care and Organ System Involvement

	Type of Care				Organ System Involvement				
Pat. ID	Supportive	Intubation	ECMO	Vv-Hemofiltration	ARDS	Sepsis	Liver Failure	Kidney Failure	MODS
1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
3	Yes	No	No	No	Yes	Yes	No	Yes	Yes
4	Yes	Yes	No	Yes	Yes	Yes	No	Yes	No
5	Yes	Yes	No	No	Yes	Yes	No	Yes	No

ARDS, acute respiratory distress syndrome; ECMO, extracorporeal membrane oxygenation; MODS, Multiple Organ Dysfunction Syndrome; Vv, venovenous.

TABLE 3. COVID-19 Postmortem Donor Tissues and SARS-CoV-2 qRT-PCR Results

Type of Ocular Tissue/Fluid	qRT-PCR for SARS-CoV-2 RNA on Right Eye	RNA Yields (Mean, ±SD in ng/μL)	qRT-PCR for SARS-CoV-2 RNA on Left eye*	RNA Yields (Mean, ±SD in ng/μL)
Conjunctival fluid swabs	vRNA undetectable	46.8 ± 33.9	vRNA undetectable	51.4 ± 29.3
Bulbar conjunctiva	vRNA undetectable	13.9 ± 7.8	vRNA undetectable	49.6 ± 81.7
Corneal epithelium	vRNA undetectable	52.4 ± 45.4	vRNA undetectable	16.2 ± 10.6
Corneal stroma and endothelium	vRNA undetectable	16.3 ± 19.7	vRNA undetectable	19.5 ± 14.0
Anterior chamber fluid	vRNA undetectable	6.5 ± 11.7	vRNA undetectable	7.1 ± 12.1

Total No. of COVID-19 postmortem donors: N = 5.
*Received routine procedure of tissue procurement for corneal banking.
qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction (S-/E-genes, positive/internal controls); vRNA, viral RNA.

because of informed consent of the next of kin or the patient himself before demise.

Therefore, future independent studies analyzing higher numbers of postmortem COVID-19 donors for SARS-CoV-2 RNA in ocular tissues are necessary and warranted. Furthermore, to clarify possible modes of transmission through conjunctiva or ocular tissues evidence of viral replication and cytopathology in living subjects suffering from COVID-19 should be analyzed in all phases of disease.

To our current knowledge, SARS-CoV-2 viral replication and its lytic activity restricts to epithelia. Therefore, corneal epithelial cells could potentially host the virus and when transplanted within corneal transplants may transmit the virus to the recipients of these transplants. This motivated us to analyze different types of corneal tissues and anatomically related fluids of COVID-19 tissue donors for the presence of SARS-CoV-2 RNA.

So far, little is known on the clinical spectrum of ocular disease caused by SARS-CoV-2 infection.[13,14] However, several modes of transmission of SARS-CoV-2 involving ocular tissue and tears are being discussed.[9–11] The ACE-2 receptor has been found to be a binding site of SARS-CoV-2.[15] Separately, the presence of ACE-2 receptor has been noted in ocular tissues.[15–18]

Owing to these uncertainties regarding a possible transmission, we have adapted our tissue procurement process for the collection of COVID-19 positive patients according to the current guidelines and described it in detail in the context of this study. Although the increased safety precautions mean an increased expenditure of time and material, we recommend taking them into account. No SARS-CoV-2 infection occurred in our collection team during this study.

In conclusion, this study shows the absence of SARS-CoV-2 RNA in postmortem COVID-19 donors in corneal tissues and anatomically related fluids. This implies that the risk for SARS-CoV-2 infection through corneal or conjunctival tissue may be very low and suggests that SARS-CoV-2 transmission through ocular tissue may be an unlikely event. Taking into account the limitations of this study, it suggests a low risk for viral transmission because of tissue procurement and processing of donor tissue for corneal transplantation surgery from individuals succumbed to SARS-CoV-2.

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