SARS-CoV-2 RNA screening in routine pathology specimens

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Summary

Virus detection methods are important to cope with the SARS-CoV-2 pandemics. Apart from the lung, SARS-CoV-2 was detected in multiple organs in severe cases. Less is known on organ tropism in patients developing mild or no symptoms, and some of such patients might be missed in symptom-indicated swab testing. Here, we tested and validated several approaches and selected the most reliable RT-PCR protocol for the detection of SARS-CoV-2 RNA in patients’ routine diagnostic formalin-fixed and paraffin-embedded (FFPE) specimens available in pathology, to assess (i) organ tropism in samples from COVID-19-positive patients, (ii) unrecognized cases in selected tissues from negative or not-tested patients during a pandemic peak, and (iii) retrospectively, pre-pandemic lung samples. We identified SARS-CoV-2 RNA in seven samples from confirmed COVID-19 patients, in two gastric biopsies, one small bowel and one colon resection, one lung biopsy, one pleural resection and one pleural effusion specimen, while all other specimens were negative. In the pandemic peak cohort, we identified one previously unrecognized COVID-19 case in tonsillectomy samples. All pre-pandemic lung samples were negative. In conclusion, SARS-CoV-2 RNA detection in FFPE pathology specimens can potentially improve surveillance of COVID-19, allow retrospective studies, and advance our understanding of SARS-CoV-2 organ tropism and effects.

Introduction

Identification and isolation of infected individuals with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is an effective preventive measure to limit the 2019 coronavirus disease (COVID-19) pandemic. Previous studies suggested that 40–45% of patients infected with SARS-CoV-2 develop only mild symptoms or even remain asymptomatic (Oran and Topol, 2020). Some patients may initially present with very mild respiratory or atypical, e.g., gastrointestinal, symptoms (Wang et al., 2020). This could limit the effectiveness in identifying infected individuals if the examination is indicated only by the presence of symptoms. Apart from the nasal and respiratory tract and the lung (Hou et al., 2020), several organs have been described as positive for viral RNA, especially the salivary gland, heart, liver, central nervous system, kidneys, lymph nodes, spleen and colon (Azzi et al., 2020; Lamers et al., 2020; Puell et al., 2020; Sekulic et al., 2020).

Pathologists analyse a wide variety of samples from virtually all tissues, most of which are formalin-fixed and paraffin-embedded (FFPE). The latter is an efficient
method for long-term preservation of proteins and nucleic acids but also inactivates infectious agents, including SARS-CoV-2. Therefore, the analysis of FFPE specimens does not demand the high biosafety precautions required for swabs or unfixed specimens. A smaller proportion of specimens analysed in pathology, particularly for perioperative diagnostics, biobanking and some cytologic analyses, are processed unfixed and are thus potentially infectious (Guerrini-Rocco et al., 2020). To date, the majority of SARS-CoV-2 RNA analyses have been performed on FFPE autopsy specimens or cell pellets (Guerrini-Rocco et al., 2020; Lean et al., 2020; Liu et al., 2020; Puelles et al., 2020). For the detection of SARS-CoV-2 in non-autopsy tissues, mainly case reports on a small number of individual patients and very few studies with more than 20 cases focusing on single organs have been published so far (Escher et al., 2020; Smithgall et al., 2020). In this single-centre study, we established a SARS-CoV-2 RNA detection protocol for FFPE material to (i) assess the feasibility of detecting viral RNA in samples from clinically diagnosed COVID-19 patients, (ii) evaluate the potential use of FFPE samples to screen for previously unrecognized infected patients, and (iii) consider the use of archival material, e.g. to screen for potential cases before identification of the first local index patients, as recently proposed (Deslandes et al., 2020). To search for previously unrecognized infected patients, given the previous data on the traceability of SARS-CoV-2 RNA in various tissues and organs, we focused on samples from oropharyngeal and sinonasal mucosa, salivary glands, lung, colon and kidney (Borczuk et al., 2020; Guerrini-Rocco et al., 2020; Puelles et al., 2020; Remmelink et al., 2020; Sekulic et al., 2020).

One of the first and most affected SARS-CoV-2 hotspots in Germany was in the catchment area of our centre in Aachen, with 22% asymptomatic patients (Streeck et al., 2020). Therefore, our Institute of Pathology was well positioned to address the above research questions using FFPE material from our diagnostic archive.

Results

Evaluation of two RNA isolation systems and RT-PCR SARS-CoV-2 RNA detection systems for FFPE specimen screening

Since tissue processing of FFPE specimens may influence RNA quality and quantity, we tested two different isolation systems for RNA isolation, i.e. automated magnetic particle purification and manual spin-column purification. The yield of total RNA was comparable for both methods (Fig. S1a). The RNA quality showed a strongly degraded RNA after isolation with both methods (Fig. S1b and c). However, viral SARS-CoV-2 RNA could be detected in all assessed positive samples (viral copy numbers of 8 to 12 000 µl⁻¹) independently of the RNA isolation method used (Fig. S1d and e). For further sample processing, we used automated magnetic particle purification.

Using a SARS-CoV-2 RNA standard with a defined viral copy number (twofold dilution), we validated two different one-step RT-PCR methods (TaqMan and RealStar) in single- and multiplex approaches with different primer and probe sets for SARS-CoV-2 RNA detection (Fig. 1A), and evaluated RNA detection in FFPE samples in this context. Using the TaqMan method, we analysed the RT-PCR efficiencies of the SARS-CoV-2 E gene, the RdRp gene and the N gene as previously suggested (Corman et al., 2020), in a singleplex approach for selecting two assays to establish a dual-target assay. We found that only the E gene assay was within the range of efficient RT-PCR, and the RdRp and N gene assay were out of range (> 110%). The RdRp-gene assay had been suggested previously as a confirmatory test (Corman et al., 2020). Therefore, we additionally analysed the RT-PCR efficiency of the E and RdRp gene in a multiplex approach as a potential method to save resources, but we found that the efficiency was impaired in the multiplex approach (Fig. 1B). The RealStar method is a simple single-tube assay that detects the SARS-CoV-2 S gene and the B-βCoV E gene in a multiplex approach as a dual-target assay. We measured amplification and, using linear regression, found that RT-PCR efficiency for the SARS-CoV-2 S gene and the B-β-CoV E gene were within the range of efficient RT-PCR (90–110%) (Taylor et al., 2010). We have compared the TaqMan singleplex and multiplex method of the E gene and RdRp gene by the Bland–Altman plot, showing the bias for both is close to zero, indicating no systematically different results. In general, we observed higher Ct values (1–3.5 C\textsubscript{T} values) for detection of the RdRp gene in the SARS-CoV-2 RNA standard dilution compared to detection of the E gene at the same viral copy number. Based on the linearity of the SARS-CoV-2 RNA standard dilution series, we additionally determined the limit of detection (LOD) within a 95% confidence interval as viral copy number µl⁻¹. Comparing the TaqMan singleplex to multiplex approach, we found that the LOD for both the E and the RdRp genes decreased from the singleplex to the multiplex approach (E gene: 7 to 14 viral copy numbers µl⁻¹, RdRp gene 14 to 27 viral copy numbers µl⁻¹). For the TaqMan singleplex approach of the N gene, we found that the LOD was even lower (55 viral copy numbers µl⁻¹) than the LOD of the E and RdRp genes. The LOD of the RealStar multiplex approach was in the same range as the LOD of the singleplex approach of the E and RdRp genes (SARS-CoV-2 S gene: 14 viral copy numbers µl⁻¹, B-βCoV E gene: 7 viral copy numbers µl⁻¹, Table 1). To evaluate RT-PCR methods in
FFPE samples, we used diluted RNA (1:50) isolated from the trachea and lung samples of clinically confirmed COVID-19 autopsy cases. Because of the good RT-PCR efficiency results, we compared the TaqMan singleplex approach and RealStar multiplex approach for the E gene assay, and found that in the same samples, SARS-CoV-2 RNA detection was positive with the TaqMan method but negative with the RealStar method (Table 2). Therefore, we established a workflow for SARS-CoV-2 RNA detection using the TaqMan RT-PCR singleplex approach with the E gene assay for screening RNA isolated from FFPE tissue samples from patients. For confirmatory testing (dual-target assay), we established the RdRp gene assay (Fig. S2).

**Detection of SARS-CoV-2 RNA in the COVID-19 patients cohort**

In the COVID-19-positive patient cohort, the relatively small number of tissue samples (n = 47 samples from 34 patients, Table S1) was not surprising because invasive procedures are kept to a minimum in COVID-19 patients. The 47 samples included seven specimens in which SARS-CoV-2 RNA was detectable (two gastric biopsy specimens, one small bowel resection specimen and one colon resection specimen, one pleural resection specimen, one lung specimen and one FFPE specimen from a cytospin preparation of a pleural effusion (Fig. 2A and D; patient 23, Table 4)). Using fluorescence *in situ* hybridization (FISH), intracytoplasmic SARS-CoV-2 RNA was detectable in cells morphologically identified as macrophages in the pleural effusion sample (Fig. 2B′), supported by hematoxylin-eosin staining (Fig. 2C′). This sample, as well as autopsy lung samples from COVID-19 patients (Fig. 2B″, C″, D), allowed us to confirm the specificity of the RT-PCR method with an independent FISH approach using a different target RNA sequence.

In patients with mild disease progression (i.e. not requiring mechanical ventilation, Table 3) and earlier COVID-19 stage (i.e. < 14 days after first symptoms/
Table 1. Detection of SARS-CoV-2 in RNA standard dilution series by the various RT-PCR methods.

| Method | TaqMan singleplex | TaqMan singleplex | TaqMan singleplex | TaqMan multiplex | RealStar multiplex |
|--------|-------------------|-------------------|-------------------|------------------|-------------------|
|        | E gene Ct         | SARS-CoV-2        | RdRp gene Ct      | SARS-CoV-2       | N gene Ct         | SARS-CoV-2        | E gene Ct         | SARS-CoV-2       | RdRp gene Ct      | SARS-CoV-2       | B-ßCoV |
| Copy number | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n |
| 14000   | 26.03 | 0.075 | 5 | 29.22 | 0.090 | 5 | 30.69 | 0.194 | 5 | 25.24 | 0.563 | 5 | 29.17 | 0.033 | 5 | 25.67 | 0.067 | 5 | 26.64 | 0.044 | 5 |
| 7000    | 27.09 | 0.042 | 5 | 29.95 | 0.088 | 5 | 31.66 | 0.199 | 5 | 26.69 | 0.043 | 5 | 29.89 | 0.099 | 5 | 26.65 | 0.064 | 5 | 27.59 | 0.053 | 5 |
| 3500    | 27.98 | 0.102 | 5 | 30.98 | 0.077 | 5 | 32.45 | 0.206 | 5 | 27.57 | 0.046 | 5 | 30.43 | 0.086 | 5 | 27.54 | 0.027 | 5 | 28.53 | 0.062 | 5 |
| 1750    | 29.06 | 0.084 | 5 | 31.44 | 0.149 | 5 | 33.10 | 0.083 | 5 | 28.60 | 0.069 | 5 | 31.00 | 0.043 | 5 | 28.49 | 0.050 | 5 | 29.57 | 0.073 | 5 |
| 875     | 30.05 | 0.080 | 5 | 32.08 | 0.141 | 5 | 34.35 | 0.265 | 5 | 29.51 | 0.080 | 5 | 31.61 | 0.151 | 5 | 29.48 | 0.044 | 5 | 30.55 | 0.103 | 5 |
| 438     | 31.00 | 0.138 | 5 | 33.13 | 0.128 | 5 | 35.63 | 0.307 | 5 | 30.58 | 0.081 | 5 | 32.50 | 0.170 | 5 | 30.52 | 0.055 | 5 | 31.58 | 0.074 | 5 |
| 219     | 31.99 | 0.137 | 5 | 34.14 | 0.088 | 5 | 36.76 | 0.224 | 5 | 31.72 | 0.136 | 5 | 33.35 | 0.173 | 5 | 31.49 | 0.104 | 5 | 32.52 | 0.070 | 5 |
| 109     | 33.00 | 0.057 | 5 | 35.06 | 0.128 | 5 | 37.44 | 0.577 | 5 | 32.62 | 0.084 | 5 | 34.27 | 0.274 | 5 | 32.38 | 0.051 | 5 | 33.55 | 0.170 | 5 |
| 55      | 33.95 | 0.129 | 5 | 36.07 | 0.556 | 5 | 38.55 | 0.826 | 5 | 33.89 | 0.149 | 5 | 35.21 | 0.621 | 5 | 33.49 | 0.078 | 5 | 34.62 | 0.167 | 5 |
| 27      | 35.01 | 0.284 | 5 | 37.34 | 1.219 | 5 | 39.14 | 0.508 | 4 | 35.40 | 0.806 | 5 | 37.61 | 0.522 | 5 | 34.47 | 0.287 | 5 | 35.36 | 0.197 | 5 |
| 14      | 36.44 | 0.385 | 5 | 37.99 | 0.805 | 5 | 38.78 | 0.316 | 2 | 36.44 | 0.293 | 5 | 37.69 | 0.630 | 4 | 35.21 | 0.347 | 5 | 36.37 | 0.349 | 5 |
| 7       | 37.54 | 0.589 | 5 | 38.35 | 0.092 | 4 | 37.76 | 0.393 | 2 | 38.09 | – | 1 | 37.88 | – | 1 | 36.95 | 0.608 | 4 | 37.99 | 1.064 | 5 |
| 3       | 38.04 | 0.647 | 4 | 39.48 | 1.615 | 3 | ND | – | 0 | ND | – | 0 | ND | – | 0 | 37.55 | 1.036 | 4 | 38.81 | 0.715 | 4 |

*Ct* values for SARS-CoV-2 RNA detection using TaqMan singleplex (E, RdRp, and N gene), TaqMan multiplex (E, RdRp gene) and RealStar multiplex (S gene, E gene B-ßCoV) assays in two-fold SARS-CoV-2 RNA standard dilutions with defined copy numbers. Only TaqMan singleplex E and RdRp gene and RealStar multiplex assays have high LODs with 7–14 viral copy numbers μl⁻¹ (n = number of detectable values in five replicate measurements). ND, not detectable.
SARS-CoV-2 detection in the pandemic peak cohort

SARS-CoV-2 RNA was detected in two samples from the pandemic peak period in a previously unrecognized COVID-19 patient (Fig. 2A). The positive samples were bilateral tonsillectomy specimens from a female patient in her twenties with clinical symptoms of tonsillitis and peritonsillar abscess formation (139–169 viral copies $\mu l^{-1}$, Fig. 2B, C” and D). She remained without symptoms typical for COVID-19. The remaining $N = 221$ specimens from the head and neck, colon, lung and kidney during the pandemic peak were negative (Tables S2 and S3).

SARS-CoV-2 detection in the pre-pandemic cohort showed no positive cases

None of the $N = 93$ samples in the pre-pandemic cohort were positive for SARS-CoV-2 RNA (Fig. 2A, Table S4).

Discussion

This study established and validated SARS-CoV-2 RNA detection methods in non-autopsy FFPE tissues, showing its utility as a prospective and retrospective screening method and a tool to investigate organ tropism and effects of SARS-CoV-2. The RNA extraction is a bottleneck during PCR for SARS-CoV-2, and the availability of extraction kits may be limited during a pandemic. Therefore, we tested two different RNA extraction methods on clinically confirmed SARS-CoV-2-positive autopsy FFPE tissues. Our data suggested that the performance is not dependent on the RNA isolation method, and the results are consistent even in highly degraded RNA. For RT-PCR, we used commercially available kits that enable broad applicability, and validated the analytical efficiency and limit of detection, as suggested previously (Forootan et al., 2017; Vogels et al., 2020). We established a dual-target assay using the E gene assay (TaqMan) as first-line screening and the RdRp gene assay (TaqMan) as a confirmatory test; both assays in a singleplex approach.

### Table 2. SARS-CoV-2 RNA detection in FFPE tissues.

| Tissue     | Dilution | TaqMan Singleplex | RealStar Multiplex |
|------------|----------|-------------------|-------------------|
|            | pos./neg.| Ct-SARS-CoV-2 E gene | Viral copy number $\mu l^{-1}$ | pos./neg. | Ct-SARS-CoV-2 S gene | Ct-B-9CoV E gene |
| Trachea    | 1:50     | Positive 34.50     | 43                 | Negative   | ND               | ND               |
| Lung       | 1:50     | Positive 34.88     | 33                 | Negative   | ND               | ND               |
| Lung       | 1:50     | Positive 34.45     | 4429               | Negative   | ND               | ND               |

Isolated and diluted (1:50) RNAs from FFPE tissue (trachea, lung) from clinically confirmed COVID-19 autopsy cases were positive at 33–44 viral copy numbers $\mu l^{-1}$ in the TaqMan singleplex (E gene) approach, but negative for SARS-CoV-2 in the RealStar multiplex approach.

test, SARS-CoV-2 RNA was found in two gastric biopsies (patients 5 and 6, Table 3), but was not detectable in soft tissues, normal and neoplastic oral mucosa, lymph nodes, salivary gland, ovarian and peritoneal lavage fluid and placenta (Table 3). In patients with mild disease in later COVID-19 stages (> 14 days after first symptoms/test), SARS-CoV-2 RNA was detectable in a pleural resection specimen from a patient 48 days after the first positive test (patient 13, Table 3), while other samples, including pleural effusion from the same patient (patient 13, Table 3), pulmonary tissues, lymph node, liver and colon, were negative.

In patients with severe disease (i.e. requiring mechanical ventilation, Table 4) in early COVID-19 (< 14 days after first symptoms/test), samples from pleural effusion (patient 23, Table 4) and small bowel (patient 24, Table 4) were positive for SARS-CoV-2 RNA, while soft tissue was negative. In later COVID-19 stages (> 14 days after first symptoms/test) in severe disease courses, one large bowel sample was positive (patient 32, Table 4), while other samples from small and large bowel, thymus neoplasia, bone marrow and one pleural effusion were negative for SARS-CoV-2 RNA. Morphologically, no characteristic features of viral infection or sequelae of viral infection were evident in extrapulmonary tissues.

Lung samples from three patients with onset of COVID-19 symptoms long before surgery, i.e. 50–81 days, showed pulmonary morphologies consistent with the severity of clinical COVID-19 disease progression, e.g. focal fibrosis in disease course (patient 14, Table 3, Fig. 3A' and A'') and diffuse fibrosis with nearly complete obliteration of the alveolar spaces in severe disease courses (patient 29, Table 4, Fig. 3B' and B''). In one patient, a pulmonary biopsy 76 days after the first symptoms was still positive for SARS-CoV-2 RNA (patient 15, Table 3). Morphologically, the biopsy showed pulmonary fibrosis. Confounding factors such as previous lung disease, radiation to the lungs or a history of tobacco abuse were not known to exist in these patients.
are in line with a previous study (Corman et al., 2020). A dual-target assay is included in most commercially available molecular diagnostic kits for SARS-CoV-2 RNA detection to increase sensitivity and specificity, but single-target assays are also available (Afzal, 2020). Our results show typical analytical RT-PCR efficiencies for the target E gene assay, and the detection limit of the RdRp gene was higher than that of the E gene. When
Table 3. Patient characteristics of COVID-19-Positive Patients with mild disease course.

| Patient No. | Sex  | Age range (d) | Duration of ventilation (d)/hospitalization (d) | First symptoms (d +/- sampling) | SARS-CoV-2 RNA from FFPE µl | Localization | Indication for surgery/sampling | Specimen received as | Histologic result |
|-------------|------|---------------|-----------------------------------------------|--------------------------------|----------------------------|--------------|---------------------------------|-------------------|-----------------|
| Mild disease course without mechanical ventilation |      |               |                                               |                                |                            |              |                                 |                   |                 |
| 1<sup>a</sup> | M    | 76–80         | 0/1                                          | Unknown                        | neg                         | Appendix     | Appendicitis                     | Formalin-fixed resection | Appendix with acute serositis |
| 2<sup>b</sup> | F    | 81–85         | 0/27                                         | Asymptomatic                   | neg                         | Right kidney | Neoplasia                        | Formalin-fixed resection | Urothelial cancer |
| 3<sup>b</sup> | M    | 61–65         | 0/18                                         | 3; upper airways               | neg                         | Left lateral oral floor | Neoplasia             | PERIOPERATIVE fresh-frozen section | Tumour-free oral mucosa |
| 4<sup>b</sup> | F    | 31–35         | 0/1                                          | Asymptomatic                   | neg                         | Anterior oral floor | Neoplasia             | Formalin-fixed resection | SCC of oral mucosa |
| 5<sup>a</sup> | M    | 71–75         | 0/9                                          | Unknown                        | pos                         | Gastric antrum | Gastritis                         | Formalin-fixed biopsy | Chronic antrum gastritis, no intestinal metaplasia, no helicobacter pylori |
| 6<sup>a</sup> | M    | 51–55         | 0/18                                         | Asymptomatic                   | pos                         | Gastric antrum | Gastritis                         | Formalin-fixed biopsy | Chronic antrum gastritis, intestinal metaplasia, no helicobacter pylori |

Note: SARS-CoV-2 in pathology samples.
Clinically confirmed COVID-19 patients with mild COVID-19 infection without mechanical ventilation. Time of surgery/sampling was set as zero, times are given as days +/- before/after surgery/sampling. DAD, diffuse alveolar damage; FFPE, formalin-fixed paraffin-embedded; MET, metastasis; RCC, renal cell carcinoma; SCC, squamous cell carcinoma.

a. Deceased.
b. Dismissed.
| Patient No | Sex | Age range | First symptoms (d +/- sampling) | Duration of ventilation (d) / hospitalization (d) | SARS-CoV-2 RNA from FFPE | Viral copy number/µl | Localization | Indication for surgery/sampling | Specimen received as | Histologic result |
|------------|-----|-----------|---------------------------------|-----------------------------------------------|--------------------------|---------------------|-------------|---------------------------------|------------------|------------------|
| 17         | M   | 66–70     | Unknown                         | Unknown                                       | neg                      | .                   | Soft tissue | Necrosis                        | Formalin-fixed resection | Decubitus ulcer   |
| 18         | M   | 61–65     | Unknown                         | Unknown                                       | neg                      | .                   | Pleural cavity | Pleural effusion                | Formalin-fixed biopsy | Macrophages; mesothelia |
| 19         | M   | 56–60     | Unknown                         | Unknown                                       | neg                      | .                   | Left distal main bronchus and carina, upper/lower left lung lobe | Bronchitis, mesothelia | Bronchitis with squamous metaplasia |
| 20         | M   | 56–60     | 3/18                            | Unknown                                       | neg                      | .                   | Pleural cavity | Pleural empyema | Formalin-fixed resection | Pleuritic |
| 21         | M   | 46–50     | 30/30                           | Unknown                                       | neg                      | .                   | Intrabronchial space, endotracheal tube suction | Endobronchial biopsy | Necrotic material, fibrin |
| 22         | F   | 66–70     | 12/33                           | 1; upper airways                              | neg                      | .                   | Cubital joint | Exclusion of osteomyelitis | Formalin-fixed resection | Synovitis |
| 23         | F   | 56–60     | 8/8                             | –7; lower airways                             | pos                      | 141                 | Pleural cavity | Pleural effusion | Formalin-fixed biopsy | Macronphages; mesothelia |
| 24         | F   | 51–55     | 14/14                           | –14; upper airways                            | pos                      | 2                   | Small bowel | Ischemia | Formalin-fixed resection | Ischemic enteritis |
| 25         | M   | 56–60     | 18/18                           | –21; upper airways                            | -22                     | -11; upper airways | Mediastinum | Neoplasia | Perioperative fresh-frozen section | Thymic neoplasia |
| 26         | M   | 56–60     | 27/27                           | –19; lower airways                             | -22                     | -11; lower airways | Mediastinum | Neoplasia | Formalin-fixed resection | Ischemic colitis |
| 27         | M   | 71–75     | 12/21                           | –20; upper airways                            | -19                     | –19; upper airways | Colon ascendens | Ischemia | Formalin-fixed resection | Ischemic colitis |
| 28         | F   | 66–70     | 26/26                           | –25; lower airways                            | -22                     | -11; lower airways | Colon ascendens | Bleeding | Formalin-fixed resection | Ischemic colitis |
| 29         | M   | 56–60     | 36/50                           | –44; upper & lower airways                    | -47                     | upper & lower airways | Colon ascendens | Lung nodules | Formalin-fixed resection | Haemorrhagic lung infection |
Table 4. (Continued)

| Patient No | Sex | Age range | Duration of ventilation (d) / hospitalization (d) | ECMO (yes/ no) | First symptoms (d +/- sampling: localization) | Positive test for SARS-CoV-2 (d +/- sampling: localization) | SARS-CoV-2 RNA from FFPE | Viral copy number/µl | Localization | Indication for surgery/ sampling | Specimen received as | Histologic result |
|------------|-----|-----------|-----------------------------------------------|----------------|---------------------------------|---------------------------------|----------------|----------------|-------------|----------------------------|-------------------|-----------------|
| 30<sup>b</sup> | M   | 26–30     | 61/75                                        | Yes            | Unknown                          | –53; lower airways –36; upper airways | neg              | Bone marrow iliac crest | Hemophagocytic lymphohistiocytosis | Formalin-fixed biopsy | Tillinear maturation, normocellular, abundant macrophages |
| 31<sup>b</sup> | M   | 56–60     | 71/97                                        | Yes            | –60                             | –55; upper airways –35; lower airways | neg              | Colon sigmoidum | Diverticulitis | Formalin-fixed resection | Colon sigmoideum | Diverticulosis    |
| 32<sup>a</sup> | F   | 66–70     | 66                                           | No             | –60                             | –57; upper airways 0; upper airways | neg              | Coecum          | Peritonitis   | Formalin-fixed resection | Coecum       | Collitis         |
| 33<sup>a</sup> | F   | 66–70     | 76/76                                        | No             | Unknown                          | –59; lower airways –46; sputum | neg              | Colon          | Ulcus        | Formalin-fixed resection | Colon         | Hyperplastic colon mucosa |
| 34<sup>a</sup> | M   | 46–50     | 82/83                                        | Yes            | –82                             | –81; upper airways –46; lower airways | neg              | Middle right lung lobe | Lung nodules | Formalin-fixed resection | Middle right lung lobe | Lung infection |

Clinically confirmed COVID-19 patients with severe COVID-19 infection on mechanical ventilation. Time of surgery/sampling was set as zero, times are given as days +/- before/after surgery/sampling. DAD, diffuse alveolar damage; ECMO, extracorporeal membrane oxygenation; FFPE, formalin-fixed paraffin-embedded.

<sup>a</sup> Deceased.

<sup>b</sup> Dismissed.
comparing the Ct values of the E and RdRp gene assays of the SARS-CoV-2 RNA standard dilution with each other, we found that the Ct values at the same viral copy number for the RdRp gene were generally higher than the Ct values of the E gene, indicating lower virus detection rates by the RdRp gene assay. A possible reason for this observation could be the detection of genomic and subgenomic RNA, since the RdRp gene is present only in genomic RNA (ORF1b) and the E gene is present in the genomic and also in subgenomic RNA (Alexandersen et al., 2020; Kim et al., 2020). Therefore, we used the linear regression of the standard SARS-CoV-2 RNA for the E gene to determine the viral load in samples. Previous studies attributed the lower virus detection to mismatch of the RdRp reverse primer and described the assay as not reliable for samples with < 1000 copy numbers μl⁻¹ (Corman et al., 2020). In contrast, we found LOD at a viral copy number of 14 μl⁻¹ using the RdRp gene assay. However, in the clinically confirmed COVID-19 patient cohort, we found seven positive samples using the detection of the E gene, two with a higher viral load (> 140 viral copies μl⁻¹) and five with a comparatively low viral load (< 7 viral copies μl⁻¹), whereas viral RNA from samples with low viral load was undetectable using the RdRp gene assay. This suggests that detection of the RdRp gene in RNA isolated from FFPE samples is less efficient compared to the SARS-CoV-2 RNA standard, but the RdRp gene can be detected up to at least 140 viral copies μl⁻¹. Taken together, our established SARS-CoV-2 RNA detection method provides a reliable screening method for RNA isolated from FFPE specimens.

The virus detection method can be used to analyse viral spread in different organs and disease mechanisms by correlating pathological and molecular findings with virus presence in specimens from previously confirmed COVID-19 patients. Previous studies in autopsies from deceased COVID-19 patients suggested that a low to very low viral load can be detected in extrapulmonary organs (Best Rocha et al., 2020; Menter et al., 2020; Polak et al., 2020; Puelles et al., 2020; Sekulic et al., 2020; Wichmann et al., 2020). However, these are all severe and fatal cases, and it remained unclear whether such viral spread is also found in less severe and non-fatal cases. For this, analyses of pathology specimens might likely be the only available approach. In four out of 16 patients (25%) with a mild disease course not requiring mechanical ventilation, we detected SARS-CoV-2 RNA in two gastric biopsies at a very early stage of infection (one day before and one day after the first positive swab, respectively) and in later stages in pleural and lung tissues (48–73 days after the first positive swab). Interestingly, we found the highest viral copy number of all positive samples in a gastric biopsy sample from an asymptomatic patient. To our knowledge, detection of viral RNA in gastric tissues has previously not been reported. This is likely because mostly post-mortem tissues were analysed previously, which are usually subject to strong autolytic changes in the stomach. We detected SARS-CoV-2 RNA in three out of 18 patients (17%) with a severe disease course requiring mechanical ventilation. We identified a SARS-CoV-2 RNA-positive FFPE sample from a pleural effusion cytospin preparation of a severely ill COVID-19 patient in the early phase of infection (< 14 days after first symptoms, patient 23). Because the sample contained predominantly macrophages, this suggests the presence of the virus in these cells. We confirmed and visualized this finding with direct detection of viral RNA by FISH in these cells, in agreement with previous reports showing SARS-CoV-2 RNA in pleural effusion fluid (Lescure et al., 2020; Mei et al., 2020). Finally, we found one positive small bowel sample in an earlier stage (14 days after first symptoms) and one colonic resection specimen, interestingly in a late stage 57 days after the first positive test, confirming previous reports of positive RNA detection in the colon and wastewaters (Lammers et al., 2020; Lodder and de Roda Husman, 2020; Remmelink et al., 2020).

Of the 15 PCR-negative samples from COVID-19 patients with severe symptoms, one patient was in an asymptomatic early phase of the disease (one day before the first symptoms), and eleven were in a later phase of the disease, i.e., > 14 days after the first symptoms. In the later phase of the disease, the virus was likely already eliminated from the organism by the immune system, and therefore was not detectable in other colon or lung samples. The negative SARS-CoV-2 RT-PCR of the lung samples, some of which showed progressive circumscribed or organized diffuse alveolar damage, suggests ongoing tissue damage in the absence of the virus and thereby might help to differentiate direct effects from sequela of COVID-19. The data also confirm the concept of fibrotic pulmonary late effects of COVID-19 (Polak et al., 2020).

We investigated the feasibility of using pathology specimens to screen for SARS-CoV-2. We did this because (i) even during the first pandemic peak in our region, SARS-CoV-2 testing was only performed when patients were symptomatic, whereas some infections are known to be mild or even asymptomatic, (ii) false-negative results, especially depending on the stage of the disease, are not uncommon, (iii) no additional patient intervention is required to perform this test, (iv) in case of a positive result, improved tracing of infection would be possible, (v) in case of a positive result, additional and more complex molecular analyses on these tissues would be possible, e.g. cell-specific virus detection using
FISH. Compared to FISH, RT-PCR has the advantage of higher throughput, easier interpretation and much easier quantification. Therefore, we would recommend a sequential approach, screening samples with RT-PCR for the presence of SARS-CoV-2 RNA first, followed by the methods allowing the localization of the positive signal to specific cells using FISH (von Stillfried and Boor, 2021). For this analysis, we focused on periods of local pandemic peaks and on tissues that were most frequently positive for SARS-CoV-2 RNA in previous studies. Interestingly, among the n = 223 samples analysed, we found one previously unrecognized positive patient who underwent bilateral tonsillectomy. In addition to identifying new COVID-19 patients, this might also help to improve occupational hazard assessment for staff involved in specimen acquisition, e.g. in surgery, and processing, e.g. in pathology. The analysis of SARS-CoV-2 RNA can provide information on potential exposure to such unrecognized infectious specimens, which is particularly important in perioperative diagnostics, biobanking or cytology, where fresh unfixed specimens are processed directly.

Finally, this method also allows retrospective studies to be performed on archived FFPE material. A previous study reported a retrospective positive SARS-CoV-2 RNA detection in a respiratory specimen from December 2019 in France, well before the onset of the pandemic in Europe (Deslandes et al., 2020). In our cohort focusing on the most affected lung tissues, we did not find a single positive case between December 2019 and February 2020.

Limitations

The study has several limitations. These include the single-centre design and the relatively small sample size, especially for samples from COVID-19 confirmed patients. Despite this, our screening could identify a previously unrecognized case of COVID-19 and also indicated no viral spread beyond gastrointestinal tract, lungs and pleural cavity in mild COVID-19 cases. We believe that future larger and multicentre studies could further address these limitations, potentially in a similar approach to COVID-19 autopsies, for which we recently established a national registry aimed at collecting data from all COVID-19 autopsies in Germany to enable large-scale analyses (www.DeRegCOVID.ukaachen.de) (von Stillfried et al., 2020).

In COVID-19-positive patients, we did not have information on the date of first symptoms or the first test in five of the 34 cases, limiting the interpretation of these data. Another limitation is the overall reduced number of specimens sent for histopathologic analysis during the lockdown. Starting in calendar week 13 in 2020, all hospitals were asked to postpone all elective procedures to keep ICUs free for COVID-19 patients and minimize the risk of transmission to patients within the hospital. This was reflected in a 12% reduction in pathology specimens received at our centre compared with the same time in previous years (data not shown), which has also been reported previously (Stathonikos et al., 2020). Besides, the surgical staff was reduced to minimize potential exposure to infection, and many patients were also reluctant to seek medical attention during this time (Dinmohamed et al., 2020). Even though our centre was located in one of the hardest-hit early hotspots in Germany, the number of positive cases was still very low compared with the pandemic activity in some other countries. Therefore, our results cannot be generalized to other centres, countries or pandemic situations. Another limitation includes the use of FFPE material itself. Formalin is known to result in RNA degradation, which might reduce the sensitivity. However, in positive cases, SARS-CoV-2 RNA was detectable even at higher dilutions, when RNA concentration was not detectable or the RNA was strongly degraded, e.g. in autopsy cases.

Finally, our assay approach was not quantitative in terms of viral RNA copy numbers. Accurate quantification is probably not well possible for these samples given the variability in the preanalytical phase and thus potential virus and RNA degradation, i.e. unclear and variable time between surgery/sample collection and formalin fixation and unclear and variable duration of formalin fixation itself. However, from a diagnostic point of view, a dichotomous positive/negative result seems to be sufficient for most applications.

Experimental procedures

Histology samples & cohort description

We routinely analysed diagnostic FFPE samples archived at the Institute of Pathology, University Hospital Aachen. The diagnostic process had been completed for all samples. The study was approved by the local ethics committee (EK 304/20, EK 119/20 and EK 092/20). Details of the total number of identified samples, selection and exclusion criteria, topography of the samples and final numbers of selected samples included into the study are shown in Fig. S3 and outlined in the Supplementary Methods in more detail.

RNA isolation from FFPE specimens and SARS-CoV-2 RNA detection

We extracted RNA from FFPE tissue using a Maxwell® 16 LEV RNA FFPE Purification Kit (Promega GmbH, Walldorf, Germany) on the Maxwell® 16 IVD instrument (Promega GmbH) or with the ReliaPrep™ FFPE Total
RNA Miniprep System (Promega GmbH) according to the manufacturer’s instructions. The workflow for sample preparation is shown in Fig. S2. We stored the RNA samples at −80°C until further processing.

We examined two different kits for RT-PCR analysis. We used the TaqMan™ Fast 1-Step Master Mix (Thermo Fisher Scientific GmbH, Dreieich, Germany) for the qualitative detection of the E gene, the RdRp gene (encoding the RNA-dependent RNA polymerase gene) and the N gene (nucleocapsid protein gene) by primer and probe sets labelled with fluorescent reporters and Quencher dyes. We used TaqMan® Exogenous Internal Positive Control reagents (Thermo Fisher Scientific GmbH, Dreieich, Germany) as internal PCR controls. We tested the singleplex approach of the TaqMan™ kit with each of the E, RdRp and N genes, the multiplex approach of the TaqMan™ kit with the E and RdRp genes and the multiplex approach of the RealStar® kit, in combination. To further evaluate the methods, we used diluted RNA isolated from FFPE tissue from clinically confirmed COVID-19-positive autopsies (lung and trachea).

**Evaluation of SARS-CoV-2 RNA detection by SARS-CoV-2 RNA standard**

We evaluated the two RT-PCR kits and the different primer and probe sets with the AmpliRun® SARS-CoV-2 RNA standard (bestbion dx GmbH, Cologne, Germany) provided with 14 000 viral RNA copies µl⁻¹. We prepared a twofold dilution series with 13 concentrations and measured each concentration as five replicates. We tested the singleplex approach of the TaqMan™ kit with each of the E, RdRp and N genes, the multiplex approach of the TaqMan™ kit with the E and RdRp genes and the multiplex approach of the RealStar® kit, in combination. To further evaluate the methods, we used diluted RNA isolated from FFPE tissue from clinically confirmed COVID-19-positive autopsies (lung and trachea).

**Fluorescence in situ Hybridization (FISH)**

We deparaffinized freshly cut 1-µm-thick paraffin sections from the RT-PCR-positive cytology sample and a
clinically confirmed COVID-19-positive autopsy lung sample, followed by dehydration with 100% ethanol. We performed FISH on the sections using the RNAscope® Multiplex Fluorescent Reagent Kit v2 assay (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer’s instructions.

Statistical and data analysis
Using a SARS-CoV-2 RNA standard, we created a twofold dilution series to determine a standard curve for each RT-PCR assay by linear regression of the measured $C_t$ values ($n = 5$) as a function of the logarithmic viral copy number with standard deviation (Fig. S4). We used the linear regression of the established SARS-CoV-2 RNA standard for the target E gene to determine the viral copy number $\mu l^{-1}$ in unknown samples (Fig. S4). We determined the limit of detection (LOD) of each RT-PCR assay based on the linearity of the twofold dilution series of the SARS-CoV-2 RNA standard by a 95% confidence interval (Forootan et al., 2017). Using the slope, we calculated the RT-PCR efficiency $E$ according to the equation $E = 100 \times (-1 + \frac{10}{\text{slope}})$ (Vogels et al., 2020).

Acknowledgments
The excellent technical help of Inge Losen and Cherelle Timm is gratefully acknowledged.

Author contributions
SVS, ED and PB designed and oversaw the study. SVS, SV and PB wrote the initial draft of the manuscript. SVS, RDB and SV assembled the cohort and collected material. SV carried out RNA extraction and RT-PCR experiments. SVS and RDB carried out histological analyses. PB had the conceptual idea, outlined and financed the study. All authors read and approved the final version of the article.

Conflict of interest
The authors declare that there is nothing to disclose.

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### Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Comparison of two RNA isolation systems (Promega vs. Zymo Research) using clinically confirmed COVID-19 autopsy tissues by total RNA yield (a), quality (b, c) and RT-PCR results in samples with (d) high (Patient No. 1–4) and (e) low viral load (Patient No. 5–8).

**Fig. S2.** Workflow for RNA isolation from FFPE specimens and SARS-CoV-2 RNA detection using RT-PCR. (a) RNA extraction. (b) RT-PCR amplification protocol used. (c) Interpretation of RT-PCR results.

**Fig. S3.** Flow chart of sample selection of patients’ cohorts. After identifying all eligible samples, we excluded cases without the availability of FFPE material in all cohorts. Next, we excluded cases from locations other than the predefined localizations in the peak pandemic cohort and the pre-pandemic cohort. Finally, we excluded FFPE samples with low tissue quantity or quality in all cohorts.

**Fig. S4.** Linear Regression and data analysis of the various RT-PCR methods. Determination of slope, y-intercept, R2 and RT-PCR efficiency by five replicates of 2-fold dilution series of the SARS-CoV-2 RNA standard of the approaches TaqMan singleplex (E, RdRp and N gene), TaqMan multiplex (E, RdRp gene) and RealStar multiplex (S gene and B-CoV E gene).

**Table S1.** Sample characteristics for COVID-19 Patients Cohort.

**Table S2.** Sample characteristics for the Pandemic Peak Cohort.

**Table S3.** Detailed topography of samples from the Pandemic Peak Cohort.

**Table S4.** Sample characteristics for Pre-pandemic Cohort.

**Table S5.** Primer and probe set overview used in TaqMan and RealStar RT-PCR method.

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