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RT-qPCR investigation of *post-mortem* tissues during COVID-19

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Abstract
In 2020, there were numerous cases in Kazakhstan with clinical symptoms of COVID-19 but negative PCR results in nasopharyngeal and oropharyngeal swabs. The diagnosis was confirmed clinically and by CT scans (computed tomography). The problem with such negative PCR results for SARS-CoV-2 infection confirmation still exists and indicates the need to confirm the diagnosis in the bronchoalveolar lavage in such cases. There is also a lack of information about confirmation of SARS-CoV-2 infection in deceased patients. In this study, various tissue materials, including lungs, bronchi, and trachea, were examined from eight patients who died, presumably from SARS-CoV-2 infection, between 2020 and 2022. Naso/oropharyngeal swabs taken from these patients in hospitals tested PCR negative for SARS-CoV-2. This study presents a modified RNA isolation method based on a comparison of the most used methods for RNA isolation in laboratories: QIAamp Viral RNA Mini Kit and TRIzol-based method. This modified nucleic acid extraction protocol can be used to confirm SARS-CoV-2 infection by RT-qPCR in the tissues of deceased patients in disputed cases. RT-qPCR with RNA of SARS-CoV-2 re-extracted with such method from *post-mortem* tissues that were stored at –80 °C for more than 32 months still demonstrated high-yielding positive results.

Keywords: COVID-19; *Post-mortem* tissues; RNA extraction; RT-qPCR; SARS-CoV-2

Highlights:
- SARS-CoV-2 RNA was confirmed in *post-mortem* tissues from patients who tested PCR negative.
- A new RNA extraction TRIzol-based protocol is developed.
- SARS-CoV-2 RNA was detected in re-extracted tissues after long storage at –80 °C.

Introduction

During the pandemic, which lasted more than three years, starting on March 11, 2020 (WHO, 2023), Central Reference Laboratory (CRL) in Almaty was the first in Kazakhstan to conduct SARS-CoV-2 testing and served as an advisory center for various aspects of SARS-CoV-2 research. Two hospitals sought the CRL's expertise for confirming *post-mortem* diagnoses of eight patients. According to the information provided by the hospitals, all patients were pregnant woman who had died of pneumonia of unknown origin and had negative *pre-mortem* PCR analyses for SARS-CoV-2 infection. This problem of negative PCR tests with suspected COVID-19 was acute at that time. By the end of the pandemic, there was a total of 1,498,668 confirmed cases and 19,071 deaths (Johns Hopkins Coronavirus Resource Center, 2023) in Kazakhstan. Of these, only 13,843 deaths were confirmed by PCR analysis of nasopharyngeal (NP) or oropharyngeal (OP) swabs (KZ Health Ministry, 2023). Accurate statistics on deaths from COVID-19 have been complicated by the fact that initially cases of pneumonia with symptoms of COVID-19 infection but with negative PCR tests were registered as pneumonia of unknown etiology separately from laboratory-confirmed cases of COVID-19 (Gaipov et al.,

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2021). After consulting with the WHO, the country has decided to attribute these cases to COVID-19 since August 1, 2020. By August 2020, mortality from pneumonia of unknown etiology had a 2.3-fold increase compared to 2019 (Agency for Strategic planning and reforms of the Republic of Kazakhstan, Bureau of National statistics, 2020). An additional diagnostic of bronchoalveolar lavage samples for confirmation was performed only if bronchoscopy was required for other reasons not related to the COVID-19 diagnosis protocol (Clinical protocol for the diagnosis and treatment of coronavirus infection COVID-19 in adults in Kazakhstan, 2022).

Several reports showed cases of pneumonia caused by COVID-19 with NP- or OP- negative PCR results in Kazakhstan during the pandemic (Filchakova et al., 2022; Nguyen et al., 2020; Yegorov et al., 2021; Zhussupov et al., 2021). This could be due to the accuracy of PCR testing. RT-qPCR remains the "gold standard" for clinical diagnostic detection of SARS-CoV-2 (Hong et al., 2020), in addition to laboratory, clinical-epidemiological, and radiologic findings. However, although RT-PCR specificity is very high, sensitivity varies from 33% to 80% (Wiersinga et al., 2020), depending on several factors, such as time from exposure, accuracy in PCR testing, chosen targets in PCR reaction, and adequacy of the sample collection – including insufficient amount of virus RNA in the nasopharyngeal swab from the upper tract in the late stages of the disease (Teymouri et al., 2021). Consequently, 20%–67% of nasal swabs may be false negative (Wiersinga et al., 2020). Nevertheless, such cases are relatively understudied and hold considerable interest. Laboratory confirmation of COVID-19 diagnosis in patients exposed to COVID-19 but who tested PCR negative is crucial, not only for the treatment protocol and the assessment of the epidemiological situation, but also for *post-mortem* diagnosis in cadavers due to legal aspects. Moreover, due to the impossibility of immediate analysis or carrying out autopsies on all corpses, in some cases confirmatory analyses must be performed on exhumed bodies or in long stored tissue samples to assess the presence of SARS-CoV-2 RNA.

SARS-Co-2 viral RNA can persist in cadaveric tissues for a very long time – in *post-mortem* respiratory swabs it can be detected for over 35 days (Beltempo et al., 2021; D'Errico et al., 2020). To confirm the diagnosis in cadaver tissues, the quality of the isolated RNA and the virus preservation in various human tissues under different conditions become critical. Many countries have developed their own *post-mortem* protocols for confirmation of SARS-CoV-2 infection (Musso et al., 2021). Several methods of SARS-CoV-2 RNA extraction from tissues have been reported (Amirouche et al., 2021; Bruce et al., 2020; Gokulan et al., 2021; Sablone et al., 2021). Our goal was to establish a quick and efficient protocol for investigation of *post-mortem* tissues and to verify the virus preservation in cadaveric tissue samples stored for a long period at –80 °C.

Materials and methods

Samples receipt

In 2020, 2021, and 2022, various *post-mortem* tissues were obtained from eight patients at the official request of two hospitals in Kazakhstan. All patients were pregnant woman. Unique codes were assigned to the samples, resulting in 38 samples labeled as T1 to T38. The autopsies were conducted at the hospitals by pathologists in compliance with national and international safety protocols (COVID-19 Autopsy Project, 2020). The hospitals obtained all necessary ethical permissions. The study was reviewed and approved by the Ethics Committee of the National Center for Biotechnology (Approval No. 4 issued on September 8, 2020). Samples were transported at –20 °C to the laboratory within a timeframe of one to three days. The tertiary packaging was opened in a class A2 biosafety cabinet. Subsequent processing of the samples was carried out in a flexible film isolator (FFI, Public Health England). The tissues were: lungs (tissue sections and tissue washouts), bronchi (tissue sections and tissue washouts), trachea (tissue sections and tissue washouts), as well as rectal swabs and nasopharyngeal swabs. All samples were either dry or in physiological liquids. A brief overview of the samples obtained is provided in Table 1, for more detailed information see Suppl. Table 1, 2.

Sample processing

Tissue samples were processed as follows: washouts, a small 2–3 mm tissue section moist with tissue fluids, and 5–6 mm large dry pieces were placed in 2 ml tubes containing 700 µl of Viral Transport Medium (Thermo Fisher Scientific), along with four 5 mm glass beads (Fisherbrand) for homogenization. Homogenization was carried out using an MM-400 Mixer Mill (Retsch) for 2 minutes at 30 Hz frequency.

Following homogenization, the samples were briefly centrifuged, and 100 µl of the supernatant was transferred into new 1.5 ml tubes for RNA isolation. To increase the yield of total RNA, 20 µl of 20 mg/ml proteinase K (Qiagen) was added prior to extraction to each sample, followed by incubation at 56 °C in TDB-120 thermostat (Biosan). For comparison, an alternative extraction method was employed in these samples which were not treated with proteinase K prior to extraction and were directly incubated immediately after the homogenization step. Samples were completely solubilized during the incubation period of six hours, with stirring every 2 hours on a vortex.

Isolation of RNA

In 2020 total RNA was extracted from the tissue samples using two methods: a QiaAmp Viral RNA mini kit (Qiagen) according to the manufacturer's protocols, and TRIzol™ LS Reagent (Thermo Fisher Scientific) according to a modified protocol. Both protocols used proteinase K pre-treatment. The TRIzol protocol was as follows: 400 µl of TRIzol was added to 100 µl of each sample, homogenized and incubated at room temperature (RT) for 5 minutes. The mixture was then briefly spun and 100 µl of chloroform was added. After thorough vortexing for 1 minute, the mixture was incubated at RT for an additional 3 minutes. The mixture was then centrifuged at 12,000 g for 15 minutes at 4 °C, and the supernatant was carefully transferred to new tubes. Subsequently, 250 µl of isopropanol was added, vortexed thoroughly and incubated for 25 minutes at RT. The mixture was then centrifuged at 20,000 g for 30 minutes at 4 °C. The supernatant was discarded without disturbing the pellet, and 1 ml of 75% ethanol was added. Vortexing was performed vigorously, followed by centrifugation at 20,000 g for 5 minutes at 4 °C. The supernatant was discarded again, and the pellet was air-dried for 15 minutes. The RNA was eluted in 20 µl of TE-buffer (Invitrogen, Thermo Fisher Scientific) and stored at –80 °C before RT-qPCR.

Tissues extracted in 2021–2022 were processed using the modified TRIzol-based protocol with proteinase K pre-treatment. The same protocol was applied to all tissue samples that had been preserved at –80 °C for the second extraction to check the influence of storage on the RNA quality. These preserved samples were thawed all at once and kept at 4 °C until completely thawed. The duration of tissue storage ranged from 2 to 32 months (Table 1). The quality of RNA was measured

Table 1. Brief information on patient's info, tissue type, data of death, and time of storage before second RNA extraction

Patient #	Sample #	Tissue	Date of death	Interval of storage at –80 °C before second RNA extraction	Patient age	Diagnosis at admission
I	T1–T4	Lung, bronchi	July, 2020	2 years and 8 months (32 months)	29	Acute bilateral viral pneumonia in lower lobe. Type 2 respiratory failure. COVID-19? (not identified). Moderate anemia. Gestational edema-proteinuria-hypertension (GEPH). Pregnancy 33 weeks. Myopic astigmatism.
II	T5–T6	Lung, trachea	July, 2020	2 years and 8 months (32 months)	36	Bilateral viral pneumonia. Type 3 respiratory failure. COVID-19? (not identified). "Transverse Lie". Multiparous. Class III obesity. Moderate anemia. Bad Obstetric History (BOH). Pregnancy 30–31 weeks.
III	T7–T11	Lung, bronchi, trachea	Aug, 2020	2 years and 7 months (31 months)	37	Bilateral pneumonia. Type 1–2 respiratory failure. Intrauterine insemination (IUI). Uterine scare after second caesarean section. Infertility anamnesis. Abnormal placentation. Bad Obstetric History (BOH). Symptoms at admission: Cough or difficulty in breathing, shortness of breath, dry cough, fever up to 38 °C, fourth day of illness. Pregnancy 26 weeks. Negative PCR test for COVID-19.
IV	T12–T16	Lung, bronchi, trachea	Aug, 2020	2 years and 7 months (31 months)	34	Community-acquired bilateral pneumonia (CAP) in lower lobe. Type 3 respiratory failure. Pregnancy 26 weeks. COVID-19? (not identified).
V	T17–T21	Lung, bronchi, trachea	Aug, 2020	2 years and 7 months (31 months)	32	Bilateral viral pneumonia. Type 3 respiratory failure. Mild anemia. Pregnancy 31–32 weeks. COVID-19? (not identified).
VI	T22–T24	Lung, trachea	July, 2021	1 year and 8 months (20 months)	41	Symptoms at admission: Nausea and Vomiting 2–3 times per days. General condition is satisfactory.
VII	T25–T31	Lung, bronchi, trachea, rectal swab	Aug, 2021	1 year and 8 months (20 months)	30	Not available
VIII	T32–T38	Lung, bronchi, trachea, rectal swab, *post-mortem* naso-pharyngeal swab	Dec, 2022	2 months	32	Not available

spectrometrically on Nanodrop 2000C (Thermo Scientific), Table 3.

Real time RT-PCR

To confirm COVID 19 diagnosis in samples extracted in 2020, real-time RT-PCR was performed following the Charité/Berlin protocol (Corman et al., 2020) for SARS-CoV-2 detection. For this purpose, the "Sarbeco" primers targeting the E gene were used. Additionally, the "HKU" primers targeting the N gene were used for results confirmation according to the School of Public Health protocol (Chu et al., 2020). The SuperScript III Platinum™ One-Step qRT-PCR Kit (Thermo Fisher) was utilized for the RT-qPCR. Primers, probes, and positive controls were synthetized at the National Center for Biotechnology (Astana, Kazakhstan). Samples with a cycle threshold (Ct) of less than 40 were considered positive. Each reaction was performed in triplicate and an average threshold cycle (Ct) was determined.

To confirm SARS-CoV-2 infection in samples extracted in 2021–2022, as well as in samples after thawing, the real-time RT PCR was performed using the multiplex "Sarbeco/SARS-CoV-2 Screen" kit (National Center for Biotechnology, Kazakhstan). The 2020 samples have also been cross-checked with this kit. It detects two viral target genes: S and *N* genes of SARS CoV 2 and one reference gene *RPS23S* (ribosomal protein S23) as a control of RNA extraction. Samples with a Ct of less than 40 for at least one of the viral genes and the RPS23S gene were considered positive for SARS-CoV-2 RNA. Amplification was conducted in 20 µl of reaction volume, including 5 µl RNA sample, 10 µl 2× Buffer Nova, 0.5 µl qPCR Enzyme Mix, and 4.5 µl of nuclease-free water. The PCR amplification program was as follows: reverse transcription at 37 °C for 20 min, activation at 95 °C for 7 min, and 40 cycles of 95 °C for 10 s, 56 °C for 30 s, 72 °C for 10 s. Real time RT-PCR was conducted using Quant Studio 5 thermal cycler (Applied Biosystems).

Sequencing

One PCR-positive sample was sequenced on ABI 3500 XL Genetic Analyzer (Applied Biosystems). The Applied Biosystems BigDye terminator cycle sequencing 3.1v kit (Thermo Fisher Scientific) was utilized according to the manufacturer's instructions. Primers for the complete surface glycoprotein (*S*) gene are given in Table 2. BLAST analysis of the sequence obtained was performed using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2. Primers for the complete surface glycoprotein (S) gene sequencing

Primer name	Direction	Nucleotide sequence	Position in genome*
FwS	F	(5')gagagagatatttcaactgaaatctatc	*S*: 1393–1420
RvS	R	(5')accaacaccattagtgggttgg	*S*: 1491–1512
07-seqRT	R	(5')ctcttgcttggttttgatggatc	*S*: 2422–2444
31-revPCR	F	(5')tcagcacctcatggtgtagt	*S*: 3163–3182
06-seqRT	R	(5')aaggatcataaactgtgttgttgac	*S*: 3397–3421
Sfull-Sal_F	F	(5')tagtcgaccaatgtttgtttttcttgttttattgccact	*S*: 1–29
Sfull-Kpn_R	R	(5')aaggtaccgtttatgtgtaatgtaatttgactcctttga	*S*: 3795–3824

* Positions in virus genome are given for the first fully sequenced genetic variant (NCBI Reference Sequence: NC_045512).

Statistical analysis

To calculate the RNA expression of target genes, mean Ct values of the target gene N and reference *RPS23* gene obtained using "Sarbeco/SARS-CoV-2 Screen" kit were calculated. The Student *t*-test was used to determine the significance of the difference between the means of two sets of data. Normalization of expression based on *RPS23S* gene expression was performed. Mean normalized Ct values (ΔCt) were calculated as reported (Muller et al., 2002), and mean normalized expression (MNE) was calculated in Microsoft Excel-based software application QGene (https://www.qgene.org).

Results

Comparison of two RNA extraction methods via Nanodrop

We have established a protocol for *post-mortem* tissues investigation during the pandemic based on the comparison of two extraction methods for the samples obtained in 2020. Results of the comparison of the two methods of RNA isolation, using QIAamp columns and the TRIzol reagent, showed a statistically significant difference (see Table 3), except for T9 and T11 tissues. The OD absorbance ratio for A260/A280 ranged from 1.55 ± 0.21 to 2.43 ± 0.07 for RNA samples obtained using QIAamp and proteinase K (except T6) and from 1.72 ± 0.04 to 2.10 ± 0.01 for RNA samples obtained using TRIzol and proteinase K, in both cases making it sufficient for downstream applications.

Comparison of two RNA extraction methods via RT-qPCR

The RNAs from the 2020 *post-mortem* tissue samples (21 tissues) extracted with two methods described above were used for RT-qPCR with Sarbeco/HKU primers (see Suppl. Table 2). Tissues of patient III showed a "negative" result for SARS-CoV-2 presence. For the tissues extracted with TRIzol isolation method, gene *E* of SARS-CoV-2 was detected in eight of 21 tissues, and gene *N* – in 12 of 21. For the QIAamp extraction, gene *E* was found in four of 21 tissues, and gene *N* – in 11 of 21 tissues.

RNA isolation in 2020 was carried out without proteinase K due to its absence, resulting in low efficiency of RNA extraction and higher Ct values in RT-qPCR (data not shown). The preliminary addition of proteinase K significantly improved the identification of SARS-CoV-2 in *post-mortem* tissues and leads to an increase in RNA quality, nearly doubling the quantity of positive results.

Confirmation of viral SARS-CoV-2 RNA presence with "Sarbeco/SARS-CoV-2 Screen kit"

To confirm the results obtained with Sarbeco/HKU primers for patients I-V received in 2020 and an additional three patients received in 2021–2022 (Table 1), we used a registered commercial "Sarbeco/SARS-CoV-2 Screen kit". All tissues were extracted with TRIzol reagent with proteinase K pre-treatment according to developed protocol. All samples showed positive results for the reference gene, indicating the presence of human RNA in the samples (Fig. 1).

The analysis revealed the presence of SARS-CoV-2 RNA in at least one type of the examined *post-mortem* tissues in six out of eight patients. Patients III (tissues T7–T11) and VIII (T32–T38) showed negative results for the presence of SARS CoV 2. All examined types of tissues of these patients showed no detectable results for any of the target virus genes (Fig. 1). The results for patient III are consistent with those obtained with Sarbeco/HKU primers.

The SARS-CoV-2 RNA was detected in different tissues; most frequently it was detected in the lungs, followed by bronchial and tracheal tissues. All RNA isolated from large, dried pieces of tissue showed negative PCR results (T4, T8, T14, T17 tissues). Thus, RT-qPCR analysis showed that SARS-CoV-2 RNA is hardly detectable in large dry organ pieces, which were transported without fluid. Washouts from small pieces of lung and tracheal tissues showed low Ct values (T1, T30 tissues).

Tissue storage study

The expression of gene *N* analyzed using the "Sarbeco/SARS-CoV-2 Screen kit". The expression of *RPS23S*, internal control human gene, was detected in each tissue (Suppl. Table 2). Ct results were obtained for two types of RNA samples: freshly extracted RNA and RNA extracted from samples stored at –80 °C for 2–32 months (Fig. 2). The results obtained for RNA extracted for 38 samples immediately after receiving were comparable to those obtained for RNA extracted from the same tissues that were stored at –80 °C for 2–32 months (depending on data of reception), except for tissues T21 and T23 where no viral RNA was detected after re-extraction. For sample T17, positive result after re-extraction was detected at Ct = 39.9 which is close to the kit's detection limit. Tissues T4, T14, T31 were negative both times. At the same time, it is not possible to compare the values of normalized expressions in this experiment, since the extraction was conducted only once, and it is impossible to calculate a significant statistical difference. The RT-qPCR were made in triplicate (see Suppl. Table 2).

Table 3. RNA concentration for the fresh extracted samples received in 2020

Patient #	Sample #	Tissue type	Material type	RNA extraction				Student's
				with QIAamp		with TRIzol		*t*-distribution
				Nanodrop data, RNA concentration (ng/µl) and OD ratio				*p*-value
					OD 260/280	ng/µl	OD 260/280	*p*
I	T1	lung (left)	washout	89.3 ± 0.2	1.87 ± 0.03	322.4 ± 0.8	2.10 ± 0.01	0.05
	T2	bronchi (left)	washout	82.0 ± 0.8	1.74 ± 0.04	122.0 ± 0.2	1.94 ± 0.03	0.05
	T3	lung (left)	tissue cut	19.8 ± 0.1	1.40 ± 0.14	25.1 ± 0.7	1.97 ± 0.03	0.003609
	T4	bronchi (bifurcation)	tissue cut (dry piece)	9.7 ± 0.3	1.79 ± 0.07	39.7 ± 0.4	1.87 ± 0.05	0.000001
II	T5	lung (left)	tissue cut	40.5 ± 0.4	1.94 ± 0.03	70.9 ± 0.4	1.99 ± 0.01	0.000014
	T6*	trachea	tissue cut	92.8 ± 0.2	3.05 ± 0.17	94.5 ± 0.2	1.84 ± 0.02	0.009228
III	T7	trachea	tissue cut	2.4 ± 0.1	1.61 ± 0.08	55.0 ± 0.3	1.73 ± 0.08	0.000000
	T8	bronchi (right)	tissue cut (dry piece)	11.5 ± 0.2	1.67 ± 0.06	52.0 ± 0.1	1.74 ± 0.09	0.000000
	T9	lung (left)	tissue cut	40.6 ± 0.2	2.43 ± 0.07	41.7 ± 0.4	1.76 ± 0.05	**0.090894**
	T10	bronchi (left)	tissue cut	23.9 ± 0.7	1.64 ± 0.01	53.0 ± 0.7	1.81 ± 0.04	0.000086
	T11	lung (right)	tissue cut	28.6 ± 0.4	3.13 ± 0.05	29.3 ± 0.5	1.87 ± 0.04	**0.354224**
IV	T12	trachea	tissue cut	40.4 ± 0.2	1.90 ± 0.06	115.8 ± 0.4	1.78 ± 0.11	0.000000
	T13	lung (right)	tissue cut	60.4 ± 0.1	1.81 ± 0.11	117.0 ± 0.4	1.84 ± 0.12	0.000001
	T14	lung	tissue cut (dry piece)	20.8 ± 0.1	1.93 ± 0.07	33.6 ± 0.1	1.77 ± 0.12	0.000003
	T15	lung	washout	161.7 ± 0.3	1.62 ± 0.15	179.2 ± 0.2	2.10 ± 0.02	0.000019
	T16	bronchi	tissue cut	35.2 ± 0.8	1.60 ± 0.15	96.7 ± 0.6	1.78 ± 0.14	0.000009
V	T17	trachea	tissue cut (dry piece)	27.0 ± 0.1	1.55 ± 0.21	48.0 ± 0.8	1.86 ± 0.08	0.000124
	T18	lung (right)	tissue cut	32.1 ± 0.1	1.58 ± 0.23	59.8 ± 0.4	1.82 ± 0.05	0.000007
	T19	lung (left)	tissue cut	39.0 ± 0.5	1.80 ± 0.06	113.8 ± 0.3	1.72 ± 0.04	0.000001
	T20	bronchi (left)	tissue cut	41.3 ± 0.4	1.97 ± 0.01	46.9 ± 0.3	1.85 ± 0.05	0.001526
	T21	lung (left)	tissue cut (dry piece)	10.5 ± 0.3	1.45 ± 0.16	42.4 ± 0.4	1.84 ± 0.07	0.000008

Note – limitation: RNA concentration and quality data are not available for patients VI–VIII.

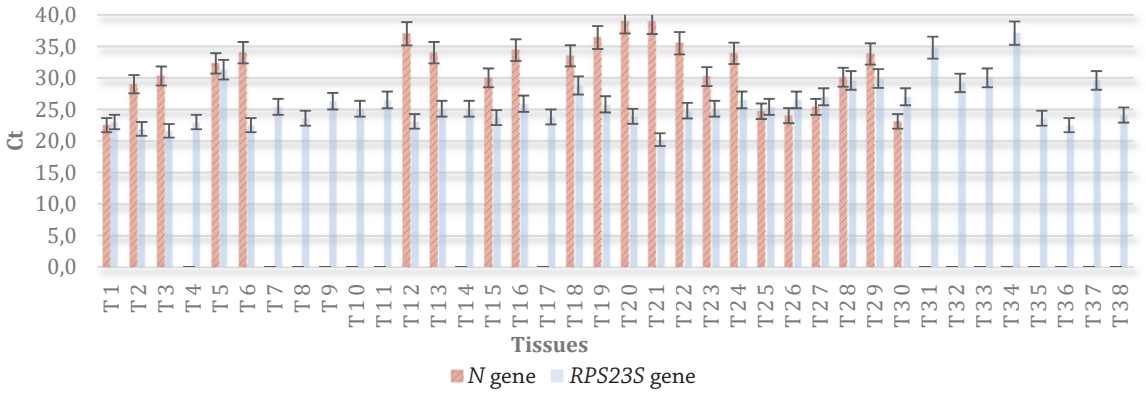

Fig. 1. RT-qPCR results for *N* and *RPS23S* genes expression using "Sarbeco/SARS-CoV-2 Screen kit" for all tissue samples T1–T38 for eight patients (TRIzol extraction).

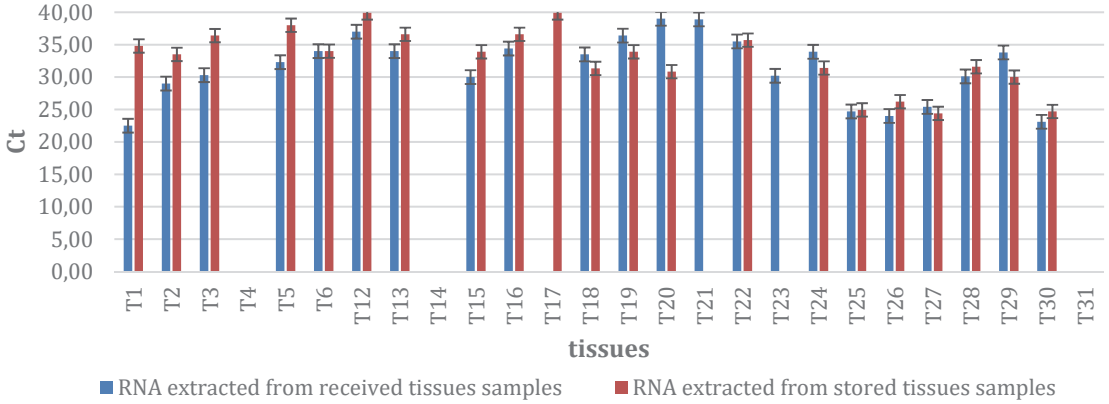

Fig. 2. Gene *N* expression with "Sarbeco/SARS-CoV-2 Screen kit" for positive patients for RNA extracted from received tissue samples and RNA extracted from the same tissue samples after storage from 2 to 32 months.

Confirmation by sequencing

To confirm RT-qPCR results by sequencing, one positive sample (T12, trachea tissue) was subjected to Sanger sequencing using primers for the complete surface glycoprotein (*S*) gene (Table 2); the sequence was uploaded to the NCBI database (OK354348.1). The result confirmed SARS-CoV-2 and showed 100% similarity to S gene isolated from human clinical sample (QRG21497.1, USA), and 98.65% similarity to S gene isolated from human nasopharyngeal swab (UAW54870.1, USA).

Discussion

RT-qPCR is a routinely used tool in the diagnosis of SARS-CoV-2 infection (D'Cruz et al., 2020; Hong et al., 2020). The ability to detect an RNA of a virus in *post-mortem* material directly depends on the quality of the material, type of material, RNA extraction method, and the specificity and sensitivity of RT-qPCR (Teymouri et al., 2021). However, the RNA quality extracted from various tissues is critical. Recently, progress has been made in extracting SARS-CoV-2 RNA from *post mortem* tissues (Musso et al., 2021; Sablone et al., 2021). However, some techniques are time consuming which sometimes played a significant role in diagnosis confirmation during the pandemic. Some extraction methods need a whole range of reagents, which might be difficult to obtain if supplies are limited, again during a pandemic. Therefore, optimization of RNA extraction for each tissue should be considered. The aim of this study was to determine the fastest and most efficient method for RNA isolation from pathological material, identifying the most suitable tissue for investigation of COVID-19 suspected cases, especially with pre-negative RT-qPCR results of NP and OP swabs.

Negative *pre-mortem* RT-qPCR results for COVID-19 in the deceased cases can be explained by various factors, including insufficient amount of virus RNA in NP in the late stages of the disease (Teymouri et al., 2021), contamination with RNAses, and technical issues of PCR testing (targets, sensitivity, cross-reactivity). Unfortunately, in this study, we did not have the information about PCR kits that were used to test the patients' swabs *pre-mortem*, and it was possible to obtain *post-mortem* nasopharyngeal swab samples only from the last patient. PCR-analysis of this sample showed negative results with both PCR methods. However, it was possible to detect SARS-CoV-2 RNA in *post-mortem* material obtained from six out of eight patients that were diagnosed with pneumonia of unknown origin with symptoms related to COVID-19. This can be partly explained by the phase of the disease and the localization of infection in certain organs depending on the course of the disease. There are findings in the literature suggesting that SARS-CoV-2 infection in the lungs of COVID-19 patients with respiratory failure can be detected during the acute phase and can be absent in the organizing phase in NP (Schaefer et al., 2020). Tissue samples from two patients, III and VIII, were negative for SARS-CoV-2 presence, despite the similar symptoms. Perhaps they had diseases similar to SARS-CoV-2 infection, seasonal coronaviruses, influenza, or other pneumonia, for which samples of negative patients were not tested in this study.

The SARS-CoV-2 can be detected in different tissues (Poloni et al., 2022), particularly lung, bronchial, and tracheal tissues. Our study confirms these data, as the RNA of SARS-CoV-2 was most frequently detected in the lungs, followed by bronchial and tracheal tissues. Small pieces of tissues placed in viral transport media and dissolved with proteinase K before extraction showed increased possibility of SARS-CoV-2 detection. For confirmation of SARS-CoV-2 presence in tissues, we suggest using washouts from the lung and bronchial tissues, or small pieces of such tissues. It is also applicable to lethal cases suspicious for COVID-19, where swabs' analysis provided PCR-negative result, and lavage was not used for PCR *pre-mortem*. The literature highlights the need for early lower respiratory sampling, if possible, in patients with suspected COVID-19, especially cancer patients (Abid et al., 2021). During the 2003 outbreaks of the coronavirus SARS-CoV, as well as MERS-CoV in 2012, it was shown that both pandemic viruses tended to infect the lower respiratory tract and potentially cause acute respiratory syndrome (Ezhilan et al., 2021). For the MERS coronavirus, the WHO also recommended collecting lower respiratory tract samples whenever possible (WHO, 2018), based on results from tests on patients from different countries comparing viral load and genomic fraction yields among respiratory tract samples from different locations. Lower respiratory tract samples (tracheal aspirate and bronchoalveolar lavage) were shown to yield significantly higher viral loads and genomic fractions compared to upper respiratory tract samples, which is well documented in a review on pandemic coronaviruses (Al-Omari et al., 2019).

A significant role of proteinase K in the isolation of SARS-CoV-2 RNA from tissues was also confirmed. Proteinase K treatment is a valuable step in *post-mortem* PCR analysis that aids tissue digestion, removes PCR inhibitors, and increases nucleic acid yield (Qamar et al., 2017). It is widely used to improve the success rate and reliability of molecular analyses performed on various samples (Freppel et al., 2020; Mallmann et al., 2021). In our case, proteinase K was not used in the beginning of the pandemic due to its absence and inability to be purchased. However, later proteinase K significantly improved RNA isolation in both cases, using TRIzol reagent and QIAamp columns.

Post-mortem RNA degradation is considered a major concern in gene expression research as the global patterns of degradation in diverse *post-mortem* human tissues remain mostly unknown (Zhu et al., 2017). It has been shown that SARS-CoV-2 RNA can remain in cadaveric tissues for more than several weeks after death (Caniego-Casas et al., 2022). RNA samples are usually stored frozen at –20 °C or –80 °C or under liquid nitrogen. However, even at lower temperature some ribonucleases are still active (Ma et al., 2004). Some ribozymes can even be activated by freezing at –70 °C (Kazakov et al., 2006). We reviewed the storage of unextracted samples of *post-mortem* tissues under –80 °C conditions. A comparison of viral gene expression values for RNA extracted immediately after tissue receipt and for RNA re-extracted from the same tissue samples suggests that storage at –80 °C in viral transport media for more than two and up to 32 months preserves SARS-CoV-2 RNA and it can be detected by real-time RT-PCR. This may be critical in controversial COVID-19 cases when patient samples have been frozen after autopsy. The main limitation of this study was the absence of enough tissue material to repeat the experiment, to at least calculate a statistical difference between normalized expressions.

Conclusion

In six of eight patients who tested negative for SARS-CoV-2 by RT-qPCR testing of nasal and oropharyngeal swabs but had clinical symptoms of COVID-19, we confirmed the presence of

SARS-CoV-2 RNA in their tissues. The modified TRIzol-based RNA extraction protocol demonstrated its efficiency and a reproducibility in obtaining RNA from lung, bronchi, trachea, and cadaver swabs confirming via RT-qPCR the SARS-CoV-2 infection. Small cuts of lung and bronchial tissue samples and washouts are suggested to be used in *post-mortem* investigation.

Authors' contributions

ZB: Article concept, manuscript writing, PCR experiments; *EM:* Manuscript editing, sample receipt, biosafety control; *YP:* Data analysis, manuscript editing; *AN:* PCR experiments, experiment design; *AZ:* Primer design and sequencing; *DN:* Material receipt and storage, sequencing; *YO:* RNA isolation, manuscript editing; *SK:* RNA isolation, tissue treatment; *ZD:* RNA concentration measuring, tissue processing; *MK:* RNA concentration measuring, tissue processing; *AB:* standard operating procedures' writing; *AC:* PCR experiments design; *AM:* Visualization, PCR experiments; *NA:* Sample receipt, RNA isolation; *SA:* Coordination with hospitals, assistance with diagnoses; *GI:* Manuscript editing; *AD:* Agreements with hospitals; *SM:* General management, funding acquisition; *YS:* Agreements with hospitals, funding acquisition. All authors have read and agreed to the manuscript.

Ethics approval

The study was reviewed and approved by the Ethics Committee of the National Center for Biotechnology (approval #4 issued on September 8, 2020).

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Conflict of interest

The authors have no conflict of interest to declare.

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