RT-PCR diagnosis of COVID-19 from exhaled breath condensate: a clinical study

Makoto Sawano\textsuperscript{1,\ast}, Kyousuke Takeshita\textsuperscript{1}, Hideaki Ohno\textsuperscript{3} and Hideaki Oka\textsuperscript{4}

\textsuperscript{1} Center for Advanced Emergency Medicine and Critical Care, Saitama Medical Center, Saitama, Japan
\textsuperscript{2} Department of Clinical Laboratory Medicine, Saitama Medical Center, Saitama, Japan
\textsuperscript{3} Department of Infectious Diseases and Infection Control, Saitama Medical Center, Saitama, Japan
\textsuperscript{4} Department of General Medicine, Saitama Medical Center, Saitama, Japan

\ast Author to whom any correspondence should be addressed.

E-mail: sawano@me.com

Keywords: COVID-19, exhaled breath condensate, RT-PCR

Abstract

Current diagnostic testing for coronavirus disease 2019 (COVID-19) is based on detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in nasopharyngeal swab samples by reverse transcription polymerase chain reaction (RT-PCR). However, this test is associated with increased risks of viral dissemination and environmental contamination and shows relatively low sensitivity, attributable to technical deficiencies in the sampling method. Given that COVID-19 is transmitted via exhaled aerosols and droplets, and that exhaled breath condensate (EBC) is an established modality for sampling exhaled aerosols, detection of SARS-CoV-2 in EBC offers a promising diagnostic approach. However, current knowledge on the detection and load of the virus in EBC collected from COVID-19 patients remains limited and inconsistent. The objective of the study was to quantify the viral load in EBC collected from COVID-19 patients and to validate the feasibility of SARS-CoV-2 detection from EBC as a diagnostic test for the infection. EBC samples were collected from 48 COVID-19 patients using a collection device, and viral loads were quantified by RT-PCR targeting the E gene. Changes in detection rates and viral loads relative to patient characteristics and days since disease onset were statistically evaluated. Need for mechanical ventilation was significantly associated with higher viral load ($p < 0.05$). Need for oxygen administration or mechanical ventilation, less than 3 d since onset, and presence of cough or fever were significantly associated with higher detection rates ($p < 0.05$). Among spontaneously breathing patients, viral load in EBC attenuated exponentially over time. The detection rate was 86\% at 2 d since onset and deteriorated thereafter. In mechanically ventilated patients, detection rate and viral load were high regardless of days since onset. These results support the feasibility of using RT-PCR to detect SARS-CoV-2 from EBC for COVID-19 patients within 2 d of symptom onset.

1. Introduction

The current standard for diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection as the cause of coronavirus disease 2019 (COVID-19) is based on detection of viral ribonucleic acids (RNAs) in nasopharyngeal swab samples by reverse transcription polymerase chain reaction (RT-PCR) \cite{1}. However, sampling via nasopharyngeal swab frequently provokes sneezing or coughing, which increases the risks of viral dissemination and environmental contamination. Presently, rules limiting sampling conducted by medical personnel under strict safety measures are hindering the prevalence of PCR testing in Japan. Furthermore, a recent systematic review indicated that up to 54\% of patients with a final diagnosis of COVID-19 may test negative on the initial RT-PCR via nasopharyngeal swab samples \cite{2}. This high false-negative rate, despite the ultra-sensitivity of the PCR technique, is mainly attributable to technical deficiencies in sampling methods.
Currently, the only established modality for sampling exhaled aerosols (droplets and droplet nuclei with aerodynamic diameter ≤5 μm) is via collection of exhaled breath condensate (EBC). Given that COVID-19 is transmitted via exhaled aerosol and droplets [3], and that coronaviruses (not SARS-CoV-2) has been detected simultaneously among different specimens (nasal swab, throat swab, droplet and aerosol) [4], detection of SARS-CoV-2 RNA from EBC represents a promising approach [5].

Sampling of EBC is simple and non-invasive, requiring study participants to breathe into an EBC collection device such as the R-tube® (Respiratory Research, Austin, TX, USA) or placement of the device (R-tubeVent®) in the expiratory limb of a ventilator circuit for 5 min to collect approximately 1 ml of EBC. The advantages of EBC sampling over nasopharyngeal swab sampling include much lower risks of viral dissemination or environmental contamination (no sneezing or coughing), the lack of a need for training in special sampling techniques (participants can sample on themselves after a short introduction and sample stability).

By the end of 2020, four articles reporting the detection of SARS-CoV-2 RNA by RT-PCR in EBC collected from COVID-19 patients were indexed in MEDLINE [6–9]. However, detection rates varied widely between those studies (22.2%–93.3%), with only one study reporting quantification of the viral (RNA) load from two EBC samples [7]. As such, current knowledge on the detection and quantification of viral load in EBC collected from COVID-19 patients remains limited and inconsistent. The objective of this study was thus to clarify changes in RNA load in EBC over time following disease onset and to validate the feasibility of SARS-CoV-2 RNA detection from EBC (EBC-PCR test) for diagnosing COVID-19.

2. Methods

2.1. Study design, ethical considerations and participant selection criteria

The study was designed as a single-centered prospective observational study enrolling COVID-19 patients hospitalized in Saitama Medical Center, Saitama Medical University, Japan.

All study protocols were approved by the ethics committee of the hospital (reference no. 2384). Protocols were explained to potential study participants and written consent for inclusion in the study was obtained on the day of admission, or the day after. This procedure was conducted inside a special quarantine ward for treating COVID-19 patients by a researcher wearing full personal protective equipment (PPE). After being photographed through the glass between the ‘red zone’ and ‘green zone’, these signed consent forms were disposed of as infectious medical waste in the ward. From the perspective of infection prevention, it is important not to remove documents that have been touched (signed) by an infected individual in the ‘red zone’.

In cases where the potential participant was on a ventilator or otherwise unable to confirm consent to participate in the study, the researcher contacted the legal proxy by phone to explain the protocol and to obtain consent. Importantly, contact was made by telephone due to the high likelihood that the proxy, usually a family member, might be equally infected by COVID-19. In this protocol, the consents were obtained from the proxies of mechanically ventilated patients 3–7 d after admission.

Between 6 July and 1 December 2020, a total of 84 patients were admitted to the hospital with a diagnosis of COVID-19 after providing a positive RT-PCR assay from nasopharyngeal swab samples. Of those 84 patients, 50 patients gave written consent to participate in the study (legal representatives for three patients on ventilators provided verbal consent) and were enrolled in the study.

2.2. EBC collection and safety measures

EBC collection was conducted using an R-tube® or R-tubeVent® device (Respiratory Research). The R-tubes were placed inside an aluminum sleeve surrounded by dry ice within an 8 cm diameter co-axial container [10, 11]. Alternatively, five sleeves were stored in a cooler box with dry ice and exchanged every minute. The whole device was disposed of as infectious medical waste after EBC collection, except for the sleeve, which was disinfected by submerging in 75 vol% ethanol before reuse.

Following the provision of informed consent, a researcher explained the usage of the R-tube to the study participant, while wearing full PPE inside the individual room of the patient in the ‘red zone’ of the COVID-19 ward. The subject was asked to hold the R-tube mouthpiece in the mouth and breathe freely for 5–7 min to collect 0.5–1.0 ml of EBC. After collection of sufficient EBC, the mouthpiece was removed, and both ends of the R-tube were sealed with caps.

2.2.1. EBC collection from mechanically ventilated patients

Of the 48 patients enrolled in the study, three were under totally controlled mechanical ventilation receiving continuous administration of sedatives and muscle relaxants at the time of EBC collection. None of the patients underwent mechanical ventilation before admission. Table 1 shows ventilator parameters, minute ventilation volume, and period of mechanical ventilation, at the time of EBC collection for the three patients. Settings of the ventilator parameters had been changed as needed according to arterial blood gas results. These three patients were placed on a ventilator on the day of or the day after admission, but difficulties in obtaining the consent according to the protocol caused significant delay of EBC collection.
Table 1. Ventilator parameters, minute ventilation volume and period of mechanical ventilation at the time of EBC collection for the three mechanically ventilated patients.

| Mechanically ventilated patient | #1               | #2               | #3               |
|---------------------------------|------------------|------------------|------------------|
| Ventilator parameters           | Mode: BIPAP      | Mode: BIPAP      | Mode: BIPAP      |
|                                 | FiO2: 0.8        | FiO2: 0.7        | FiO2: 0.6        |
| P(high): 25cmH₂O                | P(high): 20cmH₂O | P(high): 20cmH₂O |
| P(low): 12cmH₂O                 | P(low): 10cmH₂O  | P(low): 10cmH₂O  |
| T(high): 1.2 s                  | T(high): 1.2 s   | T(high): 1.2 s   |
| T(low): 1.8 s                   | T(low): 3.0 s    | T(low): 3.0 s    |
| Minute ventilation volume       | 8.4–9.2 l min⁻¹ | 6.2–7.2 l min⁻¹ | 7.1–8.2 l min⁻¹ |
| Period of mechanical ventilation| 2 d              | 7 d              | 6 d              |

BIPAP: Biphasic positive airway pressure. FiO2: Fraction of oxygen in inspiratory gas. P(high): Airway pressure during ‘high pressure phase’. P(low): Airway pressure during ‘low pressure phase’. T(high): Duration of ‘high pressure phase’. T(low): Duration of ‘low pressure phase’. IV: Intravenous administration.

![Diagram](A)

**Figure 1.** (A) (Top) shows a schematic diagram of the ventilator circuit generally used for COVID-19 patients. (B) (Bottom) shows the diagram of the ventilator circuit modified for EBC collection. AHD: active humidification device. HEPA filter: high efficiency particulate air filter. VENT: ventilator.

Figure 1(A) shows a schematic diagram of a ventilator circuit commonly used for COVID-19 patients. An active humidifier (AHD) was installed at the inspiratory limb to control the humidity and temperature of the inlet gas, and a high-efficiency particulate air (HEPA) filter was installed at the gas exhaust port of the ventilator to prevent contamination of the environment. Figure 1(B) (bottom) shows the diagram of the ventilator circuit modified for EBC collection. The researcher installed the R-tubeVent at the...
patient side of the expiratory limb for 5–7 min to collect 0.5–1.0 ml of EBC. Since exhaled gas from the patient mixes with inhaled gas at the expiratory limb during the expiratory phase, the AHD was turned off during EBC collection to minimize dilution of EBC by vapors in the inhaled gas. Attachment and removal of the R-tubeVent was performed within 10 s of halting the ventilator to minimize contamination of the environment.

2.2.2. Transportation of EBC samples to PCR laboratory
The R-tube itself was sealed in a plastic bag after disinfection of the outer wall, which was achieved by spraying with 75 vol% ethanol, conducted inside the ‘red zone’. The outer surface of the bag containing the R-tube was disinfected by spraying 75 vol% ethanol and this was in turn placed into an additional plastic bag held by another researcher waiting in the ‘yellow zone’. The bag (triple-sealed EBC sample) was dropped into a cooler box containing dry ice in the ‘green zone’ and immediately transported to a PCR laboratory certified as Biosafety Level 2+. Alternatively, the bag was stored at ultra-low temperature (–89 °C) in a deep freezer [11].

Before leaving the ‘red zone’, the researcher removed the PPE and disposed of them as infectious medical waste. The researcher then entered the ‘yellow zone’, and disinfected their hands and forearms with a quick drying hand sanitizer containing 75 vol% ethanol. Finally, the researcher entered the ‘green zone’ and washed their hands with soap and running water for at least 30 s.

After completing the amplification and quantification of viral RNA in the PCR laboratory, the specimens were sealed in the specimen container, outside of which was sterilized with 75 vol% ethanol. The container and R-tube were double-sealed in a plastic bag and immediately disposed of as infectious medical waste inside the PCR laboratory.

2.3. Detection and quantification of SARS-CoV-2 RNA in EBC
In the PCR laboratory, 400 µl of collected EBC was transferred from the R-tube to a specimen container inside an isolated cabinet (Biosphere Class 2 bench). After disinfection of the outside wall with 75% vol% ethanol, the container was taken out of the cabinet and introduced into a nucleic acid purifier (MagNA Pure Compact®, 480; Roche Molecular Systems, Pleasanton, CA, USA) to obtain 50 µl of purified nucleic acid solution. A quantity of 10 µl of the solution underwent amplification through RT-PCR for detection and quantification of SARS-CoV-2 RNA using a real-time RT-PCR device (LightCycler® 480; Roche Molecular Systems) and a PCR assay kit (LightMix® Modular SARS-CoV, COVID-19 E-gene; Roche Diagnostics, Indianapolis, IN, USA). The LightCycler is capable of simultaneously running RT-PCR processes on 64 samples in isolated closed circuits. After each measurement, circuits were thoroughly decontaminated to eliminate any impact on the next measurement. These procedures for detecting SARS-CoV-2 RNA were the same as those performed on the nasopharyngeal swabs, except that the maximum PCR cycle was extended from 40 to 55 to increase assay sensitivity. Detection of viral RNA by RT-PCR assay was determined as a significant rise in the PCR curve from baseline, i.e. an increase in the accumulation of PCR products. The LightMix targets the E gene of SARS-CoV-2 RNA for the RT-PCR assay.

Quantification of the viral RNA load from EBC was achieved by employing the comparative threshold cycle (Ct) method [12]. Ct is defined as the PCR cycle at which the accumulated amount of PCR product achieves an arbitrary threshold. This method allows precise determination of the viral RNA load in a sample, relative to that of a positive control with known viral RNA load, by comparing the Ct of the sample and the positive control included in the PCR assay kit.

Prior to this study, a series of SARS-CoV-2 RNA solution with concentration of 2.0 × 10^5, 2.0 × 10^4 and 2.0 × 10^3 copies ml⁻¹ were prepared by stepwise dilution of a standard solution with concentration of 2.0 × 10^5 copies ml⁻¹. A calibration curve for the RT-PCR assay was obtained by measurement of the series in triplicate. From the calibration curve, the efficiency of the RT-PCR assay was estimated as 97% (1.94-fold per PCR cycle) and the viral RNA load of the positive control was determined as 1.7 × 10^3 copies ml⁻¹ (1360 copies per reaction).

Another series of the viral RNA solution with high (1.7 × 10^5 copies ml⁻¹), medium (1.7 × 10^4 copies ml⁻¹), and low (1.7 × 10^3 copies ml⁻¹) concentration were prepared by stepwise dilution of the positive control. A quantity of 100 µl of each solution were mixed with 300 µl of saline (the saline mixture) or EBC collected from a healthy adult volunteer, who had negative COVID-19 nasopharyngeal swab PCR test and had not received COVID-19 vaccine (the EBC mixture). All mixtures underwent the nucleic acid purification and quantification by the RT-PCR assay in triplicate to estimate the recovery. The recovery of the saline mixtures with high, medium, and low concentration were 23.5 ± 2.0%, 22.8 ± 2.0%, and 21.3 ± 9.4% (mean ± standard deviation), respectively. While the recovery of the EBC mixtures were 21.7 ± 0.9%, 19.1 ± 3.5%, and 18.3 ± 4.0%, respectively. Student’s t test revealed no significant difference between the recovery of the saline and the EBC mixture (p = 0.40, 0.20, and 0.65 for high, medium, and low concentrations). Based on these findings, the recovery of SARS-COV-3 RNA by the nucleic acid purifier was approximated as 20%.
2.4. Characteristics of patients at the time of EBC collection

Characteristics of patients at the time of EBC collection were identified via questionnaires completed by each patient, observation by the researcher, and a review of the medical records. Completed questionnaires were disposed of after having been photographed through the glass between the ‘red zone’ and ‘green zone’. Collected characteristics included age, sex, days since disease onset, presence of radiologically evident pneumonia, need for oxygen administration or mechanical ventilation, and manifestation of symptoms known to be typical of COVID-19 (namely cough, fever, sore throat, headache, dysphagia, olfactory disturbance, and diarrhea). Disease onset was defined as the date on which the patient first recognized at least one of the symptoms. Radiological evidence of pneumonia was assessed by a radiologist not otherwise involved in the study, based on findings from chest x-ray or computed tomography. Fever was defined as a body surface temperature $\geq 37.5 \, ^\circ C$, or the need for antipyretics. The cutoff value was set according to the Infectious Disease Prevention Act in Japan. The act is based on the 95% confidence interval (CI) of body surface temperature for healthy adults being $>36.3 \, ^\circ C$ and $<37.5 \, ^\circ C$ (mean 36.9 $^\circ C$ and standard deviation 0.3 $^\circ C$).

Manifestation of the symptoms was identified in 45 patients, excluding three mechanically ventilated patients, who were receiving continuous administration of sedatives and muscle relaxant, and control of body temperature. The dosage and period of COVID-19 specific treatments, such as corticosteroids and antivirals, that the patient received prior to EBC collection were reviewed.

2.5. Statistical analysis

The statistical significance of differences in detection rates or viral RNA load in EBC ($\log_{10}$ copies ml$^{-1}$) between the two groups of patients was evaluated using the $\chi^2$ test with Yeats’ correction or Welch’s $t$ test, respectively. Time-course changes in load ($\log_{10}$ copies ml$^{-1}$) relative to days since onset were evaluated employing linear regression modeling. All statistical analyses were conducted using R version 3.5.1 software [13]. The significance level was set at 5% ($p < 0.05$).

3. Results

3.1. Patient characteristics

Fifty EBC samples from the 50 patients were collected on the day of or the day after admission. SARS-CoV-2 RNA was detected and quantified in 15 of 48 samples, but two samples were lost in storage because of a malfunction of the deep freezer.

The 48 patients from whom EBC were collected and analyzed comprised 31 male patients and 17 female patients with a median age of 53 years and an interquartile range (IQR) of 43.8–64.3 years. Median days from disease onset to EBC collection was 5 d (IQR, 3–7 d). At the time of EBC collection, 19 patients (40%) presented with radiologically evident pneumonia, 9 patients (19%) needed oxygen administration under spontaneous breathing and 3 patients (6%) needed mechanical ventilation. Among the 45 spontaneously breathing patients, 26 (54%) manifested cough, 19 (40%) fever, 13 (27%) sore throat, 8 (17%) headache, 12 (25%) dysphagia, 8 (17%) olfactory disturbance and none (0%) diarrhea.

3.2. Viral RNA load in EBC

Viral RNA loads were quantified for the 15 EBC samples from which viral RNA was detected by RT-PCR assay. The viral RNA load in the EBC samples showed bimodal distribution, those collected from 12 spontaneously breathing patients ranged from $1.1 \times 10^2$ to $2.8 \times 10^5$ copies ml$^{-1}$ (median $8.5 \times 10^2$ copies ml$^{-1}$), and those collected from three mechanically ventilated patients ranged from $3.5 \times 10^3$ to $4.0 \times 10^4$ copies ml$^{-1}$ (median $2.9 \times 10^3$ copies ml$^{-1}$). As such, a distribution analysis was conducted on the viral RNA load in 12 EBC samples collected from spontaneously breathing patients. Figure 2 shows histograms representing the distribution of both viral RNA load (copies ml$^{-1}$) (figure 2(A)) and both logarithmically transformed values (log$_{10}$ copies ml$^{-1}$) (figure 2(B)) in EBC collected from the spontaneously breathing patients. Although the Shapiro–Wilk test did not reject normality for either distribution at the 5% significance level ($p = 0.22$ and $p = 0.32$, respectively), histograms revealed that the distribution of logarithm-transformed values was more consistent with a normal distribution. Viral RNA loads were thus converted to logarithmic values (log$_{10}$ copies ml$^{-1}$) before quantitative analysis to allow application of parametric statistical methods.

Viral RNA load in the 15 EBC samples ranged from $2.0 \log_{10}$ copies ml$^{-1}$ to $4.5 \log_{10}$ copies ml$^{-1}$, with a mean of $3.1 \log_{10}$ copies ml$^{-1}$ and standard deviation of $0.70 \log_{10}$ copies ml$^{-1}$. Table 2 compares viral RNA load in EBC between patients with and without each of the characteristics. Among patient characteristics at the time of EBC collection, only the need for mechanical ventilation at the time of EBC collection was significantly associated with higher viral RNA load in EBC ($p < 0.05$).

Figure 3 shows logarithmically transformed values of viral RNA load in EBC (log$_{10}$ copies ml$^{-1}$) plotted against days from disease onset. Linear regression of plots from spontaneously breathing patients was conducted by assigning logarithmically transformed values of viral RNA load as the response variable ($Y$) and days since onset as the explanatory variable ($X$), to obtain a linear regression equation expressed as $Y = AX + B$. Estimates of coefficients $A$ (slope) and
Figure 2. (A) (Top) shows a histogram representing the distribution of SARS-CoV-2 virus (RNA) load (copies ml$^{-1}$) in EBC collected from spontaneously breathing COVID-19 patients. (B) (Bottom) shows a histogram representing distribution of logarithm (base = 10)-converted values of the load (log$_{10}$ copies ml$^{-1}$). The solid curve represents the normal distribution.

Figure 2. (A) (Top) shows a histogram representing the distribution of SARS-CoV-2 virus (RNA) load (copies ml$^{-1}$) in EBC collected from spontaneously breathing COVID-19 patients. (B) (Bottom) shows a histogram representing distribution of logarithm (base = 10)-converted values of the load (log$_{10}$ copies ml$^{-1}$). The solid curve represents the normal distribution.

$B$ (intercept) in this equation were $-0.15$ and $3.36$, and the 95% CIs were $-0.29$ to $-0.01$ and 2.83–3.89, respectively (figure 3(A)). Significant negative estimation ($p < 0.05$) of coefficient $A$ (slope) indicated exponential attenuation of the viral RNA load (copies ml$^{-1}$) in EBC over time after onset. In contrast, no significant correlation was seen between the viral RNA load (log$_{10}$ copies ml$^{-1}$) and days in mechanically ventilated patients (figure 3(B)).

Among three mechanically ventilated patients, one received intravenous administration of Dexamethasone (6.6 mg d$^{-1}$ × 2 d) and Remdesivir (200 mg on day 1 and 100 mg on day 2), and other two received only Dexamethasone (6.6 mg d$^{-1}$ × 6 or 7 d), prior to EBC collection. In contrast, no spontaneously breathing patient received either prior to EBC collection. Table 3 shows the viral RNA load in EBC collected from the mechanically ventilated patients, days since disease onset, and the dosage and period of Dexamethasone and Remdesivir administered, at the time of EBC collection. No consistent correlation was found between the viral RNA load in EBC or the dosage and period of COVID-19 specific treatments.

3.3 Detection rate of viral RNA in EBC

Table 4 compares the detection rate of viral RNA in EBC between patients with and without each of the characteristics. Among characteristics at the time of EBC collection, the need for oxygen administration,
shows the detection 18 times higher than EBC from spontaneously breathing log for spontaneously breathing patients and 4.5 2.9 log 19 patients and revealed median loads of quantification of the viral RNA load were negligible. findings indicated that the matrix effect by EBC on EBC, regardless of the RNA concentration. These the solution mixed with saline and that mixed with difference in the recovery of the viral RNA between As described in the section 4.Discussion collection at 9–16 d after onset (figure 17–16). In spontaneously breathing patients, regardless of the relatively delayed col-

| Characteristics at the time of EBC collection | Patients with the characteristic (log_{10} copies ml^{-1}) | Patients without the characteristic (log_{10} copies ml^{-1}) | p-value |
|-----------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|---------|
| Age >52 years                                  | 3.2 ± 0.8                                                 | 2.8 ± 0.5                                                 | 0.16    |
| Male (vs female)                               | 3.0 ± 0.9                                                 | 3.2 ± 0.3                                                 | 0.64    |
| Radiologically evident pneumonia               | 3.0 ± 0.3                                                 | 2.7 ± 0.5                                                 | 0.10    |
| Need for O_2 administration                    | 3.4 ± 0.8                                                 | 2.8 ± 0.4                                                 | 0.12    |
| Need for mechanical ventilation                | 3.5 ± 0.9                                                 | 2.8 ± 0.4                                                 | <0.05†  |
| <3 d since onset                               | 4.2 ± 0.6                                                 | 2.9 ± 0.4                                                 | 0.26    |
| Cough*                                        | 2.9 ± 0.5                                                 | 2.9                                                     | —       |
| Fever*                                        | 2.9 ± 0.4                                                 | 2.9 ± 0.7                                                 | 0.98    |
| Sore throat*                                   | 2.7                                                     | 2.9 ± 0.5                                                 | —       |
| Nasal discharge*                               | 2.6                                                     | 2.9 ± 0.5                                                 | —       |
| Headache*                                     | 3.1                                                     | 2.9 ± 0.5                                                 | —       |
| Dyspnea*                                      | 3.2 ± 0.1                                                 | 2.8 ± 0.5                                                 | 0.06    |
| Dysgeusia*                                    | 2.9 ± 0.6                                                 | 2.9 ± 0.4                                                 | 0.96    |
| Impaired olfaction*                            | 2.5 ± 0.8                                                 | 3.0 ± 0.2                                                 | 0.43    |
| Diarrhea*                                     | 0.9                                                     | 2.9 ± 0.4                                                 | —       |

Values of viral RNA loads are expressed as mean ± standard deviation. The 'p-value' represents the probability that the hypothesis ‘the difference between values is equivalent to 0’ is true. † indicates that the probability is sufficiently small to dismiss the hypothesis (p < 0.05). * indicates that only patients breathing spontaneously are included.

4. Discussion

As described in the section 2, there was no significant difference in the recovery of the viral RNA between the solution mixed with saline and that mixed with EBC, regardless of the RNA concentration. These findings indicated that the matrix effect by EBC on quantification of the viral RNA load were negligible.

The present study quantified RNA loads for SARS-CoV-2 in EBC collected from COVID-19 patients and revealed median loads of 2.9 log_{10} copies ml^{-1} (8.5 × 10^2 copies ml^{-1}) for spontaneously breathing patients and 4.5 log_{10} copies ml^{-1} (2.9 × 10^3 copies ml^{-1}) for mechanically ventilated patients. Median viral RNA load in nasopharyngeal swab samples from COVID-19 patients has been reported as 4.9–5.1 log_{10} copies ml^{-1} [14–16], approximately 100–160 times higher than EBC from spontaneously breathing patients and 2–4 times higher than EBC from mechanically ventilated patients. A study that enrolled spontaneously breathing patients infected with a coronavirus (not SARS-CoV-2) reported a higher discrepancy (approximately 500–3000 fold) between the viral RNA load of nasopharyngeal swab and aerosol samples collected simultaneously [4]. However, it should be noted that the recovery of the viral RNA load through nucleic acid purification were not clarified in these reports and the comparison may be inaccurate. These discrepancies may be attributable to the dilution of respiratory tract fluid by vapor, which accounts for more than 99% of the contents in EBC [17, 18].

The present study also revealed attenuation of the viral RNA load in EBC over time from 2 d after onset of disease in spontaneously breathing patients (figure 3, top). The exponential decay curve for the viral RNA load in EBC resembles that reported in other samples (nasopharyngeal swabs, saliva, plasma, etc) collected over time from COVID-19 patients [19–21]. Those reports commonly indicated that peak viral burden in patients occurred no later than the date of onset. Therefore, assuming that peak viral RNA load in EBC also occurs no later than the date of onset appears reasonable.

Meanwhile, minimum viral RNA load detected in the study was 2.0 log_{10} copies ml^{-1} (1.7 copies per reaction) in EBC collected 7 d after onset from a spontaneously breathing patient. Considering that sensitivity of the RT-PCR assay is improved by extending the maximum PCR cycle, the detection limit of the study is consistent with the reported detection limit of 2.2 copies per reaction for the RT-PCR assay targeting the E gene of the virus [22].
Figure 3. (A) (Top) shows logarithm (base = 10)-converted values of SARS-CoV-2 virus (RNA) load (log_{10} copies ml^{-1}) in EBC collected from spontaneously breathing COVID-19 patients plotted against days from disease onset to EBC collection. Solid line represents predictions by linear regression analysis (Y = −0.15X + 2.42). Broken lines represent 95% CIs of the predictions. (B) (Bottom) shows plots of the load in EBC collected from mechanically ventilated patients.

Table 3. The viral RNA load in EBC collected from the three mechanically ventilated patients, days since disease onset, and the dosage and period of Dexamethasone and Remdesivir administration, at the time of EBC collection.

| Patient | #1          | #2          | #3          |
|---------|-------------|-------------|-------------|
| Viral RNA load in EBC | 4.5 log_{10} copies ml^{-1} | 3.5 log_{10} copies ml^{-1} | 4.5 log_{10} copies ml^{-1} |
| Days since disease onset | 9 d | 16 d | 15 d |
| Dosage & period of Dexamethasone IV | 6.6 mg day^{-1} × 2 d | 6.6 mg d^{-1} × 7 d | 6.6 mg d^{-1} × 6 d |
| Dosage & period of Remdesivir IV | Day1: 200 mg d^{-1} | — | — |
|                   | Day2: 100 mg d^{-1} | — | — |

IV: Intravenous administration.
The present study revealed that the detection rate of viral RNA in EBC was relatively high (86%) at 2 d after onset, then deteriorated and stayed relatively low (13–33%) at 3–7 d and was 0% at ≥8 d in spontaneously breathing patients. Given the attenuation of viral RNA load in EBC and the detection limit identified by the study, the deterioration and low detection rate in EBC collected ≥3 d after onset were attributable to low viral RNA load below the limit of detection in a majority of EBC samples. Furthermore, since peak viral RNA burden in patients was assumed to be present no later than the day of onset [19–21], the detection rate in EBC collected from the day of onset (day 0) or the day after (day 1) was estimated to be equal to or higher than that in EBC collected 2 d after onset.

In contrast, the detection rate was 100% for EBC collected from mechanically ventilated patients, regardless of the timing of EBC collection. This reflected the high viral RNA load in EBC (figure 3; bottom; figure 4: bottom). Several possible factors may have contributed to the high viral RNA load in EBC collected from mechanically ventilated patients. The first is the higher viral burden of most critically ill patients requiring respiratory support with mechanical ventilation. One study reported a higher detection rate for viral RNA in plasma collected from COVID-19 patients who required mechanical ventilation, compared to those did not [21]. This result indicated the high viral burden of patients in need of mechanical ventilation. The second was the high efficiency of EBC collection via a closed respiratory circuit. The third is contamination of the EBC samples by sputum or fluids adhering to the inner surface of the endotracheal tube or ventilator circuit during intubation or sustained mechanical ventilation. These sputum and fluids are likely to contain a large amount of accumulated viral RNA, and their contamination may have resulted in overestimation of the viral RNA load in EBC.

The present results were insufficient to determine whether one or more of these factors contributed to high viral RNA load in EBC collected from mechanically ventilated patients. This issue warrants further investigation.

The present results support the feasibility of detecting SARS-CoV-2 RNA from EBC (EBC-PCR test) by RT-PCR assay for the diagnosis of COVID-19 within 2 d after disease onset in spontaneously breathing subjects (sensitivity ≥ 86%), and within 15 d after onset in mechanically ventilated patients (sensitivity 100%). This study also revealed increased sensitivity of EBC-PCR testing in subjects with a need for O2 administration or those with manifestations of cough or fever (table 2).

Several studies have reported transmission of the disease from presymptomatic patients, suggesting that peak viral burden may occur a day or two earlier than onset [20, 23, 24]. Given these findings, the feasibility of the EBC-PCR test may extend to screening presymptomatic patients, as potential spreaders of the disease. However, this issue warrants further investigation including sequential EBC collection from asymptomatic subjects who have been in close contact with known COVID-19 patients.

The major limitation of the present study involved the relatively low detection rate (31.2%) of viral RNA from EBC samples, and the small sample size (15 samples) of viral RNA load in EBC as a consequence. The small sample size precluded employment of multivariate analysis methods to clarify exact associations between viral RNA load in EBC and patient characteristics at the time of EBC collection.

### Table 4. Detection rates of viral RNA in EBC relative to patient characteristics at the time of EBC collection.

| Characteristics at the time of EBC collection | Patients with the characteristic | Patients without the characteristic | p-value |
|---------------------------------------------|---------------------------------|------------------------------------|---------|
| Age >52 years                                | 10/22 (45%)                    | 5/26 (19%)                         | 0.07    |
| Male (vs female)                             | 9/31 (29%)                     | 6/17 (35%)                         | 0.74    |
| Radiologically evident pneumonia            | 8/19 (42%)                     | 7/29 (24%)                         | 0.21    |
| Need for O2 administration                   | 7/9 (78%)                      | 8/39 (21%)                         | <0.01†  |
| Need for mechanical ventilation              | 3/3 (100%)                     | 12/45 (27%)                        | 0.04‡   |
| <3 d since onset*                            | 6/7 (24%)                      | 6/38 (37%)                         | <0.01‡  |
| Cough*                                       | 11/26 (42%)                    | 1/19 (5%)                          | <0.01†  |
| Fever*                                       | 9/19 (47%)                     | 3/26 (12%)                         | 0.01†   |
| Sore throat*                                 | 1/13 (8%)                      | 11/32 (34%)                        | 0.13    |
| Nasal discharge*                             | 1/2 (50%)                      | 11/43 (26%)                        | 0.47    |
| Headache*                                    | 1/8 (13%)                      | 11/37 (30%)                        | 0.42    |
| Dyspnea*                                     | 2/7 (29%)                      | 10/38 (26%)                        | 1.00    |
| Dysgeusia*                                   | 4/12 (33%)                     | 8/33 (24%)                         | 0.7     |
| Impaired olfaction*                          | 3/8 (38%)                      | 9/37 (24%)                         | 0.66    |
| Diarrhea*                                    | 0/8 (0%)                       | 12/37 (32%)                        | 0.09    |

Detection rates are expressed as positive samples/all samples (percentage). The ‘p-value’ represents the probability that the hypothesis ‘the difference between values is equivalent to 0’ is true.

† indicates that the probability is sufficiently small to dismiss the hypothesis (p < 0.05).

* indicates that only patients breathing spontaneously are included.
Figure 4. (A) (Top) shows detection rate of SARS-CoV-2 virus (RNA) in EBC collected from spontaneously breathing COVID-19 patients plotted against days from disease onset to EBC collection. Closed circles connected by solid line represent detection rates. Heights of the black and gray columns represent the number of EBC samples with positive and negative detection of viral RNA, respectively. (B) (Bottom) shows plots of the detection rate in EBC collected from mechanically ventilated patients.

A previous study reported that detection rates of viral RNA in EBC collected from patients with positive results from nasopharyngeal swab RT-PCR tests were 68.3% by RT-PCR assay targeting two genes (E and S genes) and 93.5% by an assay targeting all four genes (E, S, N and ORF1ab genes) [8]. That study enrolled spontaneously breathing hospitalized patients, and median time from disease onset to EBC collection was 13 d, longer than in the present study. Given the proportionality between detection rates
and number of targeted genes, the low overall detection rate in the present study could be attributed to the fact that the RT-PCR assay targeted a single gene (E gene).

In this context, future investigations by RT-PCR assay targeting four genes are expected to achieve higher detection rates and to extend the feasibility of EBC-PCR testing beyond 10 d after disease onset, or even later. Investigations are also expected to clarify the exact associations between viral RNA load in EBC and patient characteristics at the time of EBC collection by employing multivariate analysis. On the other hand, possible overestimation of the viral RNA load, which is derived from amplification of fragmented viral RNA, should be taken into consideration when interpreting RT-PCR assay results.

Another limitation of this study is that the study did not enroll spontaneously breathing patients, who received COVID-19-specific treatment (Dexamethasone, Remdesivir) prior to EBC collection. As a result, the study was unable to clarify the effect of the treatment on the viral RNA load in EBC and its attenuation over time. This limitation is attributable to the study protocol of collecting EBC as soon as possible after admission, and to the national guideline of recommending treatment only for patients with moderate to severe disease. This issue warrants further investigation including sequential EBC collection from increased number of the spontaneously breathing patients.

5. Conclusion

RT-PCR assay of EBC collected from COVID-19 patients revealed exponential attenuation of the SARS-CoV-2 RNA load in EBC over time and deterioration of the detection rate ≥2 d after disease onset. These results support the feasibility of viral RNA detection in EBC as a diagnostic test for COVID-19 within 2 d after onset. Future investigation by RT-PCR assay targeting the four genes is expected to extend the feasibility of EBC-PCR testing up to 10 d after onset or even later.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

The authors would like to acknowledge biomedical laboratory scientists Chikako Matsuo and Yuki Watanabe (Saitama Medical Center) for their assistance in detection and quantification of SARS-CoV-2 RNA load in EBC, Joachim D Pleil for suggesting we submit the article on this study, Michael D Davis for his valuable technical advice on EBC collection, Anil Modak for constructive comments and advice on safety aspects, Nandor Macrzin for posing pertinent issues concerning COVID-19 related research, and finally many other editorial board members from the Journal of Breath Research for their encouraging comments.

ORCID iD

Makoto Sawano https://orcid.org/0000-0003-3433-6189

References

[1] Center of Disease Control and Prevention 2020 Interim Guidelines forCollecting, Handling, and Testing Clinical Specimens for COVID-19 (available at: www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html) (Accessed 15 January 2021)
[2] Arevalo-Rodriguez I et al 2020 False-negative results of initial RT-PCR assays for COVID-19: a systematic review PLoS One 15 e0242958
[3] Liu Y et al 2020 Aerodynamic analysis of SARS-CoV-2 in two Wuhan hospitals Nature 582 557–60
[4] Leung N H L et al 2020 Respiratory virus shedding in exhaled breath and efficacy of face masks Nat. Med. 26 676–80
[5] Khoubnasabjafari M, Jouyban-Gharamaleki V, Ghanbari R 2021 Use of exhaled breath condensate as a potential specimen for diagnosing COVID-19 Bioanalysis 12 1195–7
[6] Peng B et al 2021 Multi-route transmission potential of SARS-CoV-2 in healthcare facilities J. Hazard. Mater. 402 125771
[7] Zhou L et al 2020 Breath-, air- and surface-borne SARS-CoV-2 in hospitals J. Aerosol Sci. 15105693
[8] Ryan D J et al 2021 Use of exhaled breath condensate (EBC) in the diagnosis of SARS-CoV-2 (COVID-19) Thorax 76 86–8
[9] Sawano M, Takeshita K, Ohno H and Oka H 2020 A short perspective on a COVID-19 clinical study: ‘diagnosis of COVID-19 by RT-PCR using exhale breath condensate samples’ J. Breath Res. 14 042003
[10] Winters B R, Pleil J D, Angirah M M, Stiegel M A, Risby T H and Madden M C 2017 Standardization of the collection of exhaled breath condensate and exhaled breath aerosol using a feedback regulated sampling device J. Breath Res. 11 047107
[11] Davis M D, Winters B R, Madden M C, Pleil J D, Sessler C N, Wallace M A G, Ward-Caviness C K and Montpetit A J 2020 Exhaled breath condensate biomarkers in critically ill, mechanically ventilated patients J. Breath Res. 15 016011
[12] Livak K J and Schmittgen T D 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method Methods 25 402–8
[13] R Core Team 2018 R: a language and environment for statistical computing (Vienna: R Foundation for Statistical Computing) (available at: www.R-project.org/) (Accessed 15 January 2021)
[14] Pan Y, Zhang D, Yang P, Poon L L M and Wang Q 2020 Viral load of SARS-CoV-2 in clinical samples Lancet Infect. Dis. 20 411–2
[15] To KK-W et al 2020 Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study Lancet Infect. Dis. 20 563–74
[16] Kawasuki H et al 2020 Transmissibility of COVID-19 depends on the viral load around onset in adult and symptomatic patients PLoS One 15 e0234397
[17] Horváth I, Hunt J and Barnes P J 2005 On behalf of ATS/ERS task force on exhaled breath condensate. 2005 exhaled breath
condensate: methodological recommendations and unresolved questions Eur. Respir. J. 26 523–48
[18] Vogelberg C, Hirsch T, Rösen-Wolff A, Kerkmann M-L and Leupold W 2003 Pseudomonas aeruginosa and Burkholderia cepacia cannot be detected by PCR in the breath condensate of patients with cystic fibrosis Pediatr. Pulmonol. 36 348–52
[19] Zou L et al 2020 SARS-CoV-2 viral load in upper respiratory specimens of infected patients New Engl. J. Med. 382 1177–9
[20] He X et al 2020 Temporal dynamics in viral shedding and transmissibility of COVID-19 Nat. Med. 26 672–5
[21] Fajnzylber J et al 2020 Massachusetts consortium for pathogen readiness. 2020 SARS-CoV-2 viral load is associated with increased disease severity and mortality Nat. Commun. 11 5493
[22] Corman V M et al 2020 Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR Eurosurveillance 25 2000045
[23] Bai Y, Yao L, Wei T, Tian F, Jin D-Y, Chen L and Wang M 2020 Presumed asymptomatic carrier transmission of COVID-19 JAMA 323 1406–7
[24] Tong Z-D, Tang A, Li K-F, Li P, Wang H-L, Yi J-P, Zhang Y-L and Yan J-B 2020 Potential presymptomatic transmission of SARS-CoV-2, Zhejiang Province, China, 2020 Emerg. Infect. Dis. 26 1052–4