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Short Communication

RNase in the saliva can affect the detection of severe acute respiratory syndrome coronavirus 2 by real-time one-step polymerase chain reaction using saliva samples

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded RNA virus that causes coronavirus disease 2019, which spread worldwide immediately after the first patient infected with this virus was discovered in Wuhan, China, in December 2019. Currently, polymerase chain reaction (PCR) specimens for the detection of SARS-CoV-2 include saliva, nasopharyngeal swabs, and lower respiratory tract-derived materials such as sputum. Initially, nasopharyngeal swab specimens were applied mainly to the PCR detection of SARS-CoV-2. There was a risk of infection to healthcare workers due to coughing or sneezing by the subjects at the time of sample collection. In contrast, saliva specimens have a low risk of droplet infection and are easy to collect, and their application to PCR testing has been promoted. In this study, we have determined the detection limit of SARS-CoV-2 in saliva samples and examined the effects of storage temperature and storage time of saliva samples on the PCR detection results. As a result, 5×10^3 copies of SARS-CoV-2 could be detected in 1 mL phosphate-buffered saline, whereas 5×10^4 copies of SARS-CoV-2 were needed in 1 mL saliva to detect the virus by real-time one-step PCR. Interestingly, SARS-CoV-2 (5×10^3 copies/mL) could be detected in saliva supplemented with an RNase inhibitor. Concerning the saliva samples supplemented with an RNase inhibitor, the optimal temperature for sample storage was −20 °C, and PCR detection was maintained within 48 h without problems under these conditions. These finding suggest that RNase in the saliva can affect the detection of SARS-CoV-2 by PCR using saliva samples.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded RNA virus classified into β-coronaviruses, together with severe acute respiratory syndrome coronavirus in 2002 and Middle East respiratory syndrome coronavirus in 2012 [1]. Outbreaks of pneumonia caused by SARS-CoV-2 were reported in Wuhan, Hubei Province, China, in December 2019, and coronavirus disease 2019 (COVID-19) quickly spread across the world. In March 2020, COVID-19 was declared a pandemic by the World Health Organization. In Japan, the first case of pneumonia with a history of stay in Wuhan was confirmed in January 2020. In response to the rapid spread of COVID-19, a state of emergency was declared by the government in April 2020, and the number of domestic cases fell once but then began to rise again, with the cumulative number of cases exceeding 120,000 in November 2020.

SARS-CoV-2 infects human cells through the binding of a spike-like glycoprotein in the envelope and its receptor, angiotensin-converting enzyme 2 (ACE2) [2]. Epithelial cells of the upper and lower airway mucosa [3] and vascular endothelial cells [4] have been reported as the main cells that express ACE2 *in vivo*. The clinical manifestations of COVID-19 range from relatively mild symptoms such as fatigue, cough, and fever to severe pneumonia with rapid respiratory distress syndrome, which varies from case to case. It has also been reported that immunothrombosis caused by a viral infection of vascular endothelial cells has been found in severe cases of COVID-19 [5,6].

Currently, saliva, nasopharyngeal swabs, and lower respiratory tract-

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derived specimens (sputum or tracheal aspirate) were subjected to polymerase chain reaction (PCR) testing for SARS-CoV-2. Saliva was approved for use as a specimen in June 2020. Before that time, nasopharyngeal swabs were the most commonly used specimens, and the risk of infection to healthcare workers due to the subject's coughing or sneezing during the sample collection was considered an issue. In contrast, saliva specimens have been applied to PCR testing because the risk of droplet infection is low and the collection is easy.

In this study, we employed real-time one-step PCR to determine the detection limit of SARS-CoV-2 in saliva samples. Although it is desirable to test specimens immediately after collection, they may need to be stored in cases where the specimens overflow and the transportation from remote locations to the investigation center is required. Therefore, the effects of storage temperature and storage time of saliva specimens on the PCR results were also examined.

2. Methods and materials

2.1. SARS-CoV-2 RNA

AcroMetrix™ Coronavirus 2019 (COVID-19) RNA Control (Thermo Fisher Scientific, Rockford, IL, USA), which contain the N, S, E, and Orf1ab regions of the SARS-CoV-2 genome, was used.

2.2. Saliva samples

Saliva samples from two healthy volunteers were used. Saliva was collected directly into a container from the mouth. First, a series of experiments were carried out using saliva derived from a volunteer (A; 23 years old, male). Next, some missing data due to the incomplete study design were compensated by additional experiments using saliva from another volunteer (B; 23 years old, male). The volunteers refrained from eating and drinking for 2 h before collecting saliva.

2.3. Sample preparation

1) Using phosphate-buffered saline (PBS) as a solvent, samples were adjusted to achieve 5×10^4, 1×10^4, 5×10^3, 1×10^3, and 0 copies/mL of SARS-CoV-2 RNA.
2) Using healthy saliva as a solvent, samples were adjusted to achieve 5×10^4, 1×10^4, 5×10^3, 1×10^3, and 0 copies/mL of SARS-CoV-2 RNA.
3) Using healthy saliva with a 10th volume of an RNase inhibitor (40 U/μL; Promega, Madison, WI, USA) as a solvent, samples were adjusted to achieve 5×10^4, 1×10^4, 5×10^3, 1×10^3, and 0 copies/mL of SARS-CoV-2 RNA.

2.4. Real-time one-step PCR

SARS-CoV-2 detection kit (N2 set; Toyobo, Osaka, Japan) was used according to the manufacturer's instruction. Real-time one-step PCR was performed using Applied Biosystems® StepOnePlus™ (Thermo Fisher Scientific). The primers and probe used were 2019-nCoV_N2-Forward primer: TTACAAACATTGGCCCCGCAAA, 2019-nCoV_N2-Reverse primer: GCGCGACATTCCGAAGAA, and 2019-nCoV_N2-Probe: ACAATTTGCCCCCCCAGCGCTTCAG. PCR was run as follows: 45 cycles of 1 s at 95 °C for denaturation, 3 s at 50 °C for annealing, and 10 s at 55 °C for extension. All samples were considered duplicates, and the increase of the amplification curve of any one of the duplicates within 40 cycles was regarded as positive. The minimum concentration of a positive amplification curve was defined as the detection limit.

2.5. Examination of specimen storage temperature

Using healthy saliva with an RNase inhibitor as a solvent, the samples were adjusted to achieve 5×10^4 copies/mL of SARS-CoV-2 RNA. The samples were stored at 20 °C (room temperature), 4 °C (in a refrigerator), and -20 °C (in a freezer) for 4 h, and then real-time one-step PCR was performed as described above.

2.6. Consideration of specimen storage time

Using healthy saliva with an RNase inhibitor as a solvent, the samples were adjusted to achieve 5×10^4 copies/mL of SARS-CoV-2 RNA. The samples were stored at -20 °C (in a freezer) for 24 and 48 h, and then real-time one-step PCR was performed as described above.

2.7. Reproducibility of results

To determine the detection limit of SARS-CoV-2 RNA in mock specimens and its stability in different storage conditions, we carried out real-time one-step PCR six and three times, respectively. The results were reproduced without the examiners' variance.

3. Results

3.1. Detection limit

Real-time one-step PCR was performed on mock specimens prepared with PBS or healthy saliva as a solvent at a concentration of 5×10^4 to 1×10^3 copies/mL of SARS-CoV-2 RNA (Table 1). Representative PCR amplification curves are shown in Fig. 1. The detection limit of PBS as a solvent was 5×10^3 copies/mL, whereas the detection limit of healthy saliva as a solvent was 5×10^4 copies/mL. A similar study was performed using saliva with an RNase inhibitor as a solvent (Table 2). By adding an RNase inhibitor, the limit of detection of SARS-CoV-2 in the saliva was improved from 5×10^4 to 5×10^3 copies/mL.

3.2. Effect of specimen storage temperature

Using healthy saliva with an RNase inhibitor as a solvent, the samples adjusted to achieve 5×10^4 copies/mL of SARS-CoV-2 RNA were stored at 20 °C (room temperature), 4 °C (in a refrigerator), or -20 °C (in a freezer) for 4 h, and then real-time one-step PCR was performed (Table 3). The obtained PCR amplification curves are shown in Fig. 2. The storage of specimens at 20 °C (room temperature) or 4 °C (in a refrigerator) but not at −20 °C (in a freezer) for 4 h affected the PCR detection of SARS-CoV-2.

3.3. Effect of specimen storage time

Using healthy saliva with an RNase inhibitor as a solvent, the samples adjusted to achieve 5×10^4 copies/mL of SARS-CoV-2 RNA were stored at −20 °C (in a freezer) for 24 or 48 h, and then real-time one-step PCR was performed (Table 4). Under these conditions, the PCR detection of SARS-CoV-2 was maintained without problems for up to 48 h.

4. Discussion

Recent studies have demonstrated that saliva is a reliable tool to detect SARS-CoV-2 using real-time one-step PCR [7,8]. Detection of

Table 1
PCR amplification in mock specimens with PBS or healthy saliva as a solvent.

RNA (copy/mL)	PBS	Healthy saliva from volunteers A and B
5×10^4	+	+*
1×10^4	+	–
5×10^3	+	–
1×10^3	–	–

* One of the duplicates exhibited an increase of the amplification curve within 40 cycles.

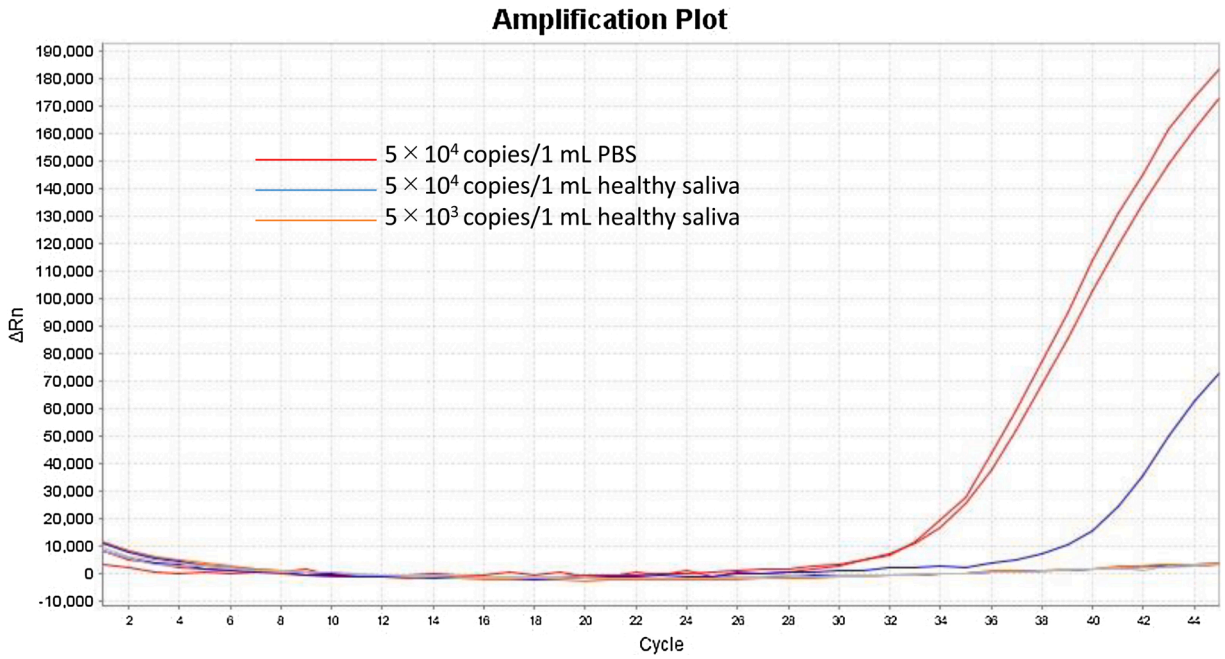

Fig. 1. Representative amplification curves of PCR detection of SARS-CoV-2 in mock samples with PBS or healthy saliva as a solvent.

Table 2
PCR amplification in mock specimens with healthy saliva from volunteers A and B as a solvent.

RNA (copy/mL)	Healthy saliva without RNase inhibitor	Healthy Saliva with RNase inhibitor
5×10^4	+*	+
1×10^4	–	+
5×10^3	–	+*
1×10^3	–	–

* One of the duplicates exhibited an increase of the amplification curve within 40 cycles.

Table 3
PCR amplification in mock saliva specimens from volunteer A with an RNase inhibitor.

Storage temperature	Storage time (0 h)	Storage time (4 h)
20℃	+	–
4℃	+	+*
−20℃	+	+

* Only one of the duplicates exhibited an increase of the amplification curve within 40 cycles.

SARS-CoV-2 using saliva samples collected in an acute phase was as accurate as using nasopharyngeal swab samples.

The detection limit of SARS-CoV-2 in real-time one-step PCR using mock specimens prepared in healthy saliva as a solvent was 5×10^4 copies/mL, whereas that of specimens prepared in PBS as a solvent was 5×10^3 copies/mL. This means that the sensitivity to detect SARS-CoV-2 in saliva samples is a 10th of that in PBS. In contrast, the detection limit of saliva specimens prepared with an RNase inhibitor was 5×10^3 copies/mL, which was comparable to the detection limit when PBS was used as a solvent. This suggests that SARS-CoV-2 RNA was degraded by the RNase present in the saliva.

The SARS-CoV-2 RNA used in this study was synthetic RNA, and we were unable to avoid the degradation by RNase in the saliva. It is not clear whether the RNase in the saliva affects the results when detecting SARS-CoV-2 by real-time one-step PCR using clinically available saliva specimens, as the RNA of SARS-CoV-2 is encapsulated in the envelope. Guest et al. reported that the saliva specimens were obtained at home by the subjects themselves and then sent to a laboratory, indicating that the samples contained sufficient RNA for SARS-CoV-2 detection [9]. This showed that the target RNA was stable in saliva specimens during transportation. Although it could be resistant to RNase rather than the synthetic RNA, it is necessary to examine whether there is a difference in detection sensitivity with or without an RNase inhibitor using saliva from individuals with SARS-CoV-2 infection.

It has been recommended that the storage temperature of SARS-CoV-2 specimens should be set as low as possible [10], which is consistent with the results of this study. Although it was speculated that freezing and thawing of specimens could degrade RNA, no effect from a single freeze-thawing operation was observed in this study. A study using the H1N1 influenza virus, an RNA virus similar to SARS-CoV-2, has reported that repeated freeze-thaw cycles of up to seven times did not affect the virus detection results [11]. That report used nasopharyngeal swab fluid as a specimen. Future studies are needed to determine how many freeze-thaw cycles the saliva specimens can withstand when stored at −20 °C.

In the present study, the detection of SARS-CoV-2 was maintained within 48 h by freezing storage at −20 °C. The PCR test for SARS-CoV-2 must be rapid and it is considered that specimens stored for longer than 48 h are unlikely to be subjected to testing. Still, it is necessary to clarify the maximum length of time for which specimens can be stored at −20 °C in a future study.

In conclusion, when detecting SARS-CoV-2 by real-time one-step PCR using saliva specimens, we found that 5×10^3 copies/mL of SARS-CoV-2 were detected in saliva with an RNase inhibitor. It was also found that the specimens should be stored at −20 °C, and under these conditions, PCR detection was maintained for up to 48 h.

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Author contributions

Designed the study: YN, SM, and AI. Performed the experiments: YN, SK, KO, EI, KW, MY, YT, and SM. SK, KO, EI, and KW contributed equally

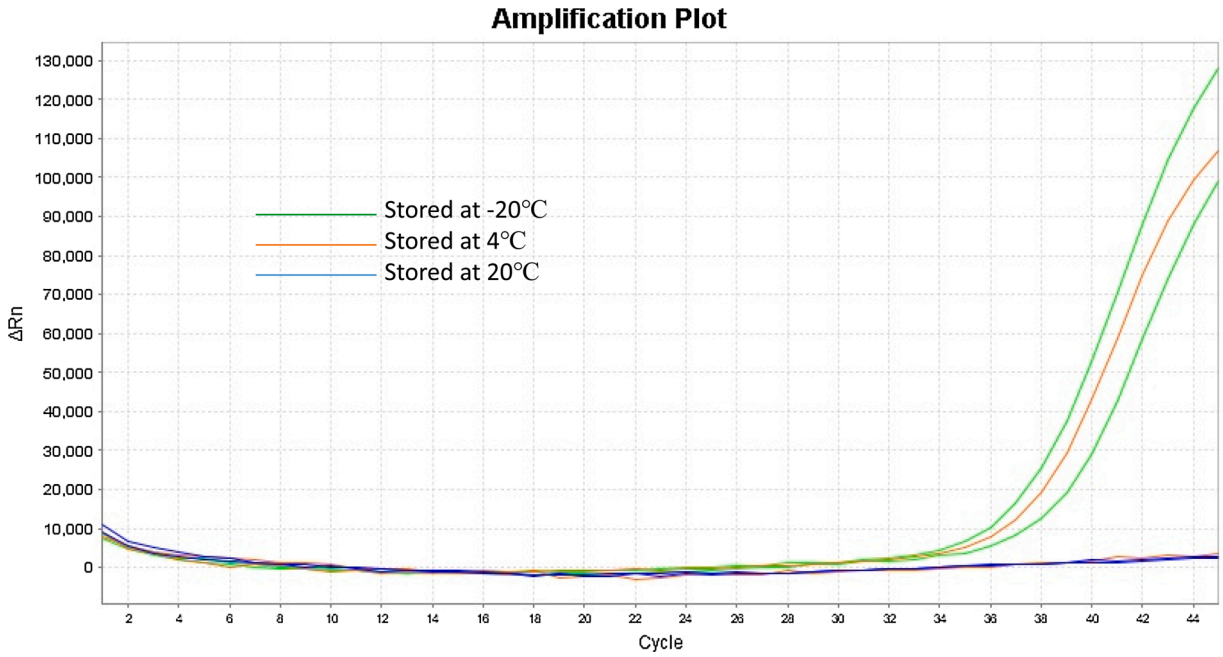

Fig. 2. Effect of storage temperature on PCR detection of SARS-CoV-2 in mock saliva samples with an RNase inhibitor.

Table 4
PCR amplification in mock saliva specimens from volunteer A with an RNase inhibitor.

Storage time (h)	Storage temperature (-20°C)
0	+
4	+
24	+
48	+

to this study. Analyzed the data: YN, MY, YT, RU, SM, UT, and AI. Wrote the manuscript: YN, SK, KO, EI, KW, and AI.

Ethics approval

The use of human materials was approved by the Ethics Committee of the Faculty of Health Sciences, Hokkaido University (approval number 20−20).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors report no declarations of interest.

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