Effects of Storage Temperature and Media/Buffer for SARS-CoV-2 Nucleic Acid Detection

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ABSTRACT

Objectives: *The increase in the number of patients with coronavirus disease 2019 (COVID-19) has delayed real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR), requiring proper shipping and storage conditions, especially in hot weather. This study aims to assess how some conditions, such as storage period, temperature, media or buffer, and sample types, affect the results of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RT-qPCR.*

Methods: *SARS-CoV-2–positive specimens were collected from Boramae Medical Center for 2 months (from May to June 2020) and stored in different media or buffers at different temperatures.*

Results: *As a result of examining confirmed patient samples, RT-qPCR results were not significantly affected by 2°C to 8°C storage until after 7 days. When stored at 20°C to 22°C or above 35°C, the results were affected negatively even after 1 day. Higher storage temperatures resulted in a lower probability of detecting viral nucleic acids because of degradation. Samples stored in pH-controlled media or buffer were more stable than those stored in nonbuffer states.*

Conclusions: *These results emphasize the importance of storage temperature and media or buffer and performing RT-qPCR for SARS-CoV-2 nucleic acid detection as soon as possible after sample collection.*

Key Points

- When stored at 2°C to 8°C, samples positive for severe acute respiratory syndrome coronavirus 2 in universal transport medium (UTM) or phosphate-buffered saline (PBS) showed no significant cycle threshold change by real-time reverse transcription–quantitative polymerase chain reaction for 7 days.
- The higher the storage temperature, the faster the nucleic acids degrade, and viral RNA could not be amplified in the molecular test even 1 day after storage.
- The virus and nucleic acid were more stable in the pH-controlled UTM or PBS than in the non–pH-controlled buffer.

Coronavirus disease 2019 (COVID-19), which was first reported in Wuhan, China, in late 2019, is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1-3] On March 11, 2020, the World Health Organization declared this disease a pandemic.[4] As of July 18, 2020, the global cumulative confirmed cases were approximately 13.8 million, with approximately 600,000 deaths (the United States recorded a cumulative 3.5 million confirmed cases and 130,000 deaths; Korea recorded a cumulative 13,000 confirmed cases and 300 deaths).[5] By July 18, the number of daily confirmed cases was approximately 260,000 and increasing each day.[5] However, if we could consider all unofficial numbers that could not be included because of a lack of adequate testing and treatment, the actual cumulative number of confirmed cases would be much higher.

The diagnostic workup of current active SARS-CoV-2 infection is based on molecular tests.[6-8] Respiratory specimens are collected from suspected

patients, nucleic acids are extracted, and real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) is used for diagnosis. RT-qPCR, which is approved for emergency use, identifies 2 or more SARS-CoV-2 genes, the target genes are amplified, and the samples are interpreted as positive.[6-8] RT-qPCR helps not only to diagnose patients but also to determine the direction of treatment and to predict disease progression. The cycle threshold (C_t) value of the test can also be used to infer the severity and infectivity of the patients.[9-11] Another important aspect of RT-qPCR is that it is included in the criteria to release COVID-19 patients from isolation.[6-8] To confirm the clearance of the virus and to allow discharge from isolation in most countries, the patient has to be clinically recovered and/or have 2 negative RT-PCR results on sequential samples collected at least 24 hours apart.[6-8]

General laboratory medicine guidance regarding shipping and storage recommends storing samples in a refrigerator (2°C-8°C) immediately after collection.[12] Consequently, the US Centers for Disease Control and Prevention, the European Center for Disease Prevention and Control, and the Korea Centers for Disease Control and Prevention also recommend storage of SARS-CoV-2 specimens at 2°C to 8°C for up to 72 hours after collection.[6-8] If a delay in testing or shipping is expected, specimens should be stored at −70°C or below.[6-8] However, given exponentially increasing cases—in addition to insufficient medical staff, collection kits, refrigeration and freezing facilities, and test equipment—the results could be delayed. Inevitably, most samples have been collected at designated walk-thru and drive-thru screening centers (far from medical institutions),[13,14] and it could be difficult to follow this guidance because of a lack of storage facilities. As countries located in the northern hemisphere entered summer and many countries, including the United States, were updating the maximum number of daily confirmed cases, the recommended shipping and storage temperature should be reconsidered. In this study, we aimed to determine how temperature and storage media influence the results of SARS-CoV-2 RT-qPCR.

Materials and Methods

Nucleic Acid Extraction and RT-qPCR

Nucleic acids from samples were extracted using the EMAG system (bioMérieux), and RT-qPCR was performed using the Standard M nCoV Real-Time Detection Kit (SD Biosensor). The analysis was conducted using the CFX96 Real-Time Detection System (Bio-Rad). The PCR included 40-cycle amplification, following the manufacturer's instructions, and was interpreted as positive when both *E* and *RdRp* were amplified in fewer than 36 cycles ($C_t \leq 36$). In this study, for simplicity, the analysis was based on *RdRp* alone.

Respiratory Samples in Universal Transport Medium vs Phosphate-Buffered Saline

A total of 132 SARS-CoV-2–positive respiratory specimens were collected from Boramae Medical Center for 2 months (from May to June 2020). When the 132 respiratory samples (76 were a combination of nasopharyngeal swabs [NPS] and oropharyngeal swabs [OPS], whereas the rest were sputum) were separated according to the C_t values of *RdRp*:, "$C_t \leq 10$ cycle" for 12 of 132 (9.1%), "$10 < C_t \leq 20$ cycle" for 46 of 132 (34.8%), "$20 < C_t \leq 30$ cycle" for 58 of 132 (43.9%), and "$30 < C_t \leq 36$ cycle" for 16 of 132 (12.1%).

Of the 132 samples, we selected 80 and divided them into 2 groups of 40; half were NPS or OPS contained in universal transport medium (UTM; Noble Bioscience), and the other half were sputum in phosphate-buffered saline (PBS). The distribution of *RdRp* C_t values in both groups was structured similarly to that of the total 132 positive samples. Distribution for the UTM group was as follows: "$C_t \leq 10$ cycle" for 3 of 40 (7.5%), "$10 < C_t \leq 20$ cycle" for 15 of 40 (37.5%), "$20 < C_t \leq 30$ cycle" for 17 of 40 (42.5%), and "$30 < C_t \leq 36$ cycle" for 5 of 40 (12.5%). Distribution for the PBS group was as follows: "$C_t \leq 10$ cycle" for 4 of 40 (10.0%), "$10 < C_t \leq 20$ cycle" for 13 of 40 (32.5%), "$20 < C_t \leq 30$ cycle" for 18 of 40 (45.0), and "$30 < C_t \leq 36$ cycle" for 5 of 40 (12.5%).

For the 80 selected samples, the first molecular test date was set as the reference date (D0), and the remaining sample after the first test was divided into 0.6-mL aliquots and placed in a refrigerator (2°C-8°C), at room temperature (20°C-22°C), and in an incubator at 35°C to 40°C. Next, sequential molecular tests were conducted 1 day (D1), 2 days (D2), and 1 week (D7) later with the stored samples to confirm serial C_t values according to storage temperature and storage length.

Statistical Analysis

The results of the 2 groups were compared using a Student *t* test. One-way ANOVA with post hoc Tukey analysis was used when comparing 3 groups. All statistical analyses were carried out using SPSS, version 26.0 (IBM Corp), and $P < .05$ was considered statistically significant.

Stability of Low Viral Load Samples

Additional experiments were conducted to determine how susceptible the viral nucleic acids were to hot weather, depending on the type of sample and media or buffer. Various samples containing a small amount of virus (*RdRp* C_t values ranged from >30 to ≤36) were selected; these were obtained from patients with confirmed COVID-19 admitted to Boramae Medical Center for 2 months (from May to June 2020). Finally, we chose 5 respiratory (NPS or OPS) samples in UTM, 5 respiratory (sputum) samples in PBS, 5 respiratory (sputum) samples in distilled water (DW), 5 stool samples in PBS, and 5 serum samples without media or buffer. Unfortunately, in the case of urine from patients with COVID-19, all samples were negative during the study; therefore, virus-positive urine samples could not be chosen. The selected samples were incubated at 35°C to 40°C, and then RT-qPCR analysis was performed at D1 and D2.

Quantitative Test

The EDX SARS-CoV-2 standard reference materials (Exact Diagnostics) were synthetic RNA transcripts containing genes of SARS-CoV-2, namely, *E*, *N*, *Orf1ab*, *RdRp*, and *S*. We diluted the materials at a ratio of 1:10 to generate 100,000, 10,000, 1,000, and 100 copies/mL, and RT-qPCR was repeated 5 times at each concentration.

Ethics

This study was certified as exempt by the Boramae Medical Center institutional review board (07-2020-037).

Results

There was no significant difference in qualitative positivity between the 2 media or buffer conditions (ie, UTM and PBS) when they were stored at 3 different temperatures and compared for 7 days ($P = .921$ [D0], $P = .888$ [D7] at 2°C-8°C, $P = .836$ [D7] at 20°C-22°C, and $P = .807$ [D7] at 35°C-40°C). However, RT-qPCR C_t values differed depending on the temperature ❚Figure 1❚ and ❚Figure 2❚. When stored at 2°C to 8°C, all 80 samples showed no significant change for 7 days (Figure 1A). When stored at 20°C to 22°C, 1 sample in UTM (D0, $C_t = 34.17$ → D2 and D7, no fluorescence signal) and 2 samples in PBS had a negative change (D0, $C_t = 33.52$ → D1, D2, and D7, no fluorescence signal; D0, $C_t = 32.18$ → D7, no fluorescence signal). Moreover, the mean RT-qPCR C_t values of the D7 group were significantly different from those of the D0 group (Figure 1B). When stored at 35°C to 40°C, the mean of RT-qPCR C_t values gradually and prominently increased as the storage time increased Figure 2. In addition, 3 samples in UTM (D0, $C_t = 34.17$ → D1,
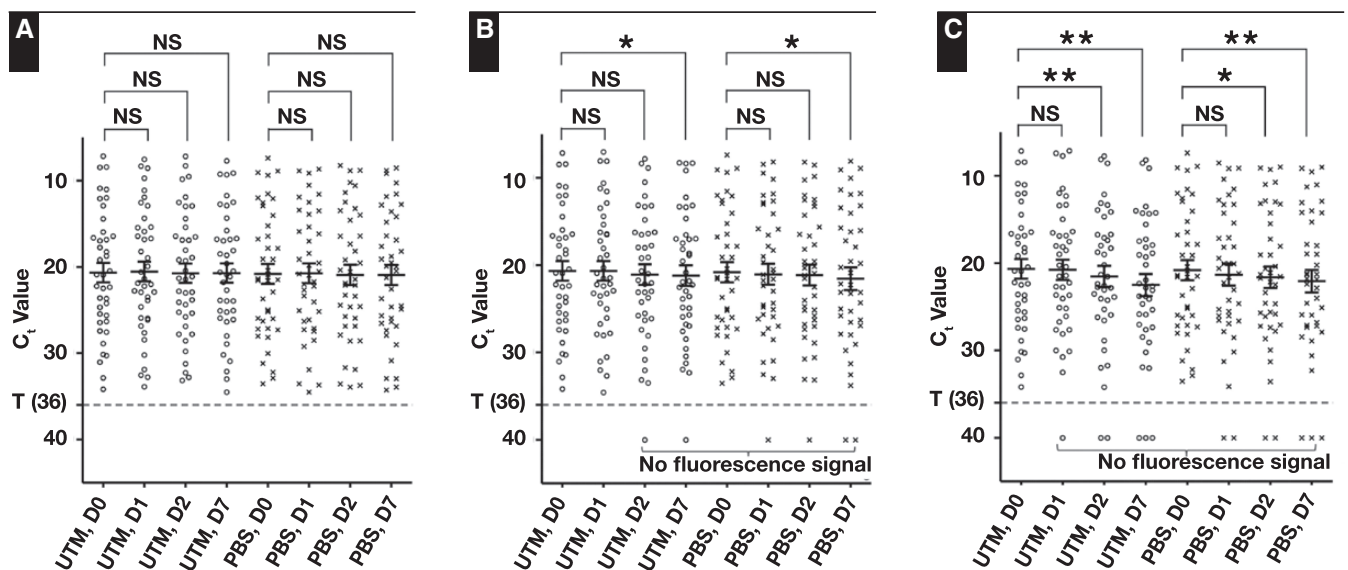

❚Figure 1❚ Distribution of the C_t value of SARS-CoV-2 *RdRp* respiratory samples in UTM or PBS. **A**, Storage at 2°C to 8°C for 7 days. **B**, Storage at 20°C to 22°C for 7 days. **C**, Storage at 35°C to 40°C for 7 days. The circles indicate C_t values of samples in UTM, and the x marks indicate C_t values of samples in PBS. Bars indicate the mean (SEM). Negative results (fluorescence signal not detected, not amplified by PCR) are included in the statistical calculations, calculated by substituting 40 (the maximum PCR cycle). *$P < .01$; **$P < .001$. T(36) indicates interpretation as positive, target cutoff $C_t ≤ 36$. C_t, cycle threshold; NS, not significantly different; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UTM, universal transport medium.
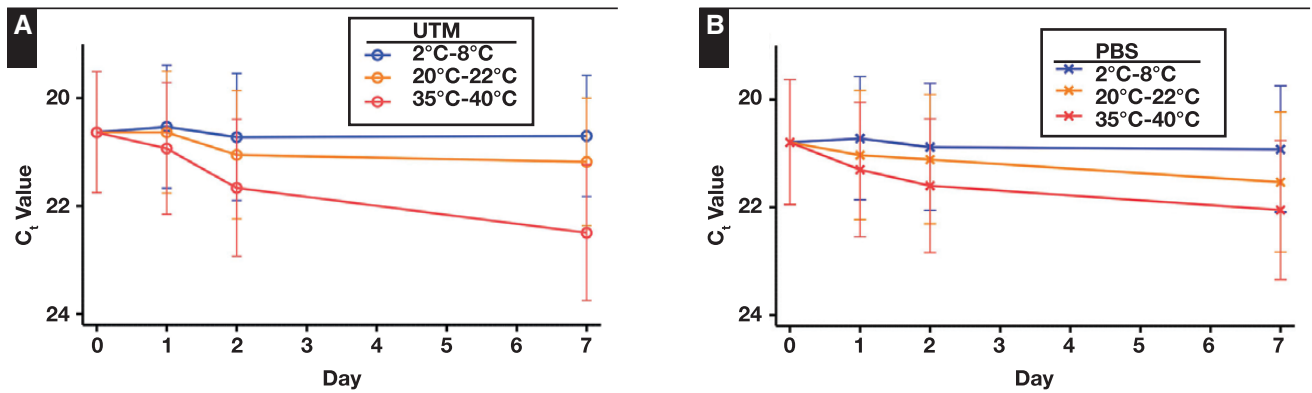
Figure 2 Seven-day follow-up of the C_t value of SARS-CoV-2 *RdRp* in UTM (n = 40) (**A**) and PBS (n = 40) (**B**). Bars indicate the mean (SEM). Negative results (fluorescence signal not detected, not amplified by PCR) are included in the statistical calculations, calculated by substituting 40 (the maximum PCR cycle). C_t, cycle threshold; PBS, phosphate-buffered saline; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UTM, universal transport medium.

D2, and D7, no fluorescence signal; D0, C_t = 31.02 → D2 and D7, no fluorescence signal; D0, C_t = 32.85 → D7, no fluorescence signal) and 3 samples in PBS (D0, C_t = 32.18 → D1, D2, and D7, no florescence signal; D0, C_t = 32.87 → D1, D2, and D7, no florescence signal; D0, C_t = 33.52 → D7, no florescence signal) had a negative change. Furthermore, the mean RT-qPCR C_t values of the D2 and D7 groups were significantly different from those of D0 (Figure 1C).

Five samples with low SARS-CoV-2 viral loads were selected and incubated at 35°C to 40°C for 2 days: group 1, respiratory samples, NPS/OPS in UTM; group 2, respiratory samples, sputum in PBS; group 3, respiratory samples, sputum in DW; group 4, stool samples in PBS; and group 5, serum only, no buffer. The results are shown in **Figure 3**. Regardless of the sample type, the viral samples stored in UTM or PBS were occasionally detected even after 2 days; however, when stored in DW or incubated with serum alone, the majority of samples had a negative change. In addition, we obtained viral concentration–C_t value matching results by repeating the quantitative evaluation of the commercial reference substance 5 times. The mean C_t values of *E* and *RdRp* genes for each viral concentration were as follows: 100,000 copies/mL, 27.68 and 26.62 cycles; 10,000 copies/mL, 31.85 and 30.88 cycles; 1,000 copies/mL, 34.55 and 34.01 cycles; and 100 copies/mL, no fluorescence signal detection.

Discussion

As is known, viral nucleic acid detection does not necessarily mean that the host has infectivity.[15-17] Unfortunately, in this study, SARS-CoV-2 infectiveness was not assessed using viral culture; however, several

recent studies have reported that infectivity is extremely low 2 weeks or more after symptom onset and when the RT-qPCR C_t value is higher than 30.[9,10,16,20] Nevertheless, there could be 1- or 2-cycle variations for the C_t value due to differences in the sample-collection technique, the type of nucleic acid extraction, the PCR kit, or the patient's condition.[20,21] In this research, when samples with relatively high viral load (C_t ≤ 30 cycle) from patients with high infectiveness were stored in UTM and PBS, there was no effect on the qualitative judgment
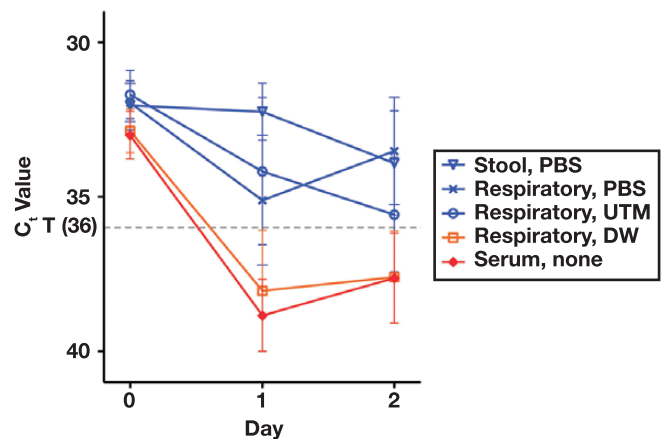

Figure 3 Follow-up of the C_t value of SARS-CoV-2 *RdRp* gene of samples with low viral load up to 2 days at 35°C to 40°C. Bars indicate mean (SEM). Negative results (fluorescence signal not detected, not amplified by PCR) are included in the statistical calculations, calculated by substituting 40 (the maximum PCR cycle). T (36) indicates positive interpretation, target cutoff C_t ≤ 36. C_t, cycle threshold; DW: distilled water; PBS, phosphate-buffered saline; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UTM, universal transport medium.

(positive or negative) for up to 7 days at 2°C to 8°C, 20°C to 22°C, and even 35°C to 40°C. However, 115 kits had individual emergency use authorizations for molecular diagnostic tests for SARS-CoV-2 by the US Food and Drug Administration until July 18, 2020,[22] and the Standard M nCoV kit used in this study was very sensitive, with a limit of detection of 500 copies/mL. Even if other less sensitive kits were used, a negative change would be observed during 7-day storage at 2°C to 8°C.[23] Consequently, it must be considered that if other kits are used, the stability of the sample might not be maintained as well as in the current study.

This analysis, based on the quantitative reference tests at 20°C to 22°C, showed that a negative RT-qPCR result change from 1 day after storage was possible if the viral concentration was lower than 10,000 copies/mL (*RdRp* gene $C_t > 30.88$ cycles). At temperatures higher than 35°C, and even at high viral concentrations of 10,000 copies/mL or more (*RdRp* gene $C_t \leq 30.88$ cycles), there could be a negative change from 1 day after storage. However, when stored in UTM and PBS, it was possible to detect nucleic acids for a longer period than in DW and nonbuffer states. Because the media or buffer is pH controlled, UTM and PBS ensured nucleic acid stability even at high temperatures. These results are consistent with previous studies that reported the influence of pH and temperature on the infectivity and stability of several viral nucleotides.[24-30] Accordingly, we recommend that the samples should always be placed in media or buffer (eg, UTM or PBS), especially in the summer, to account for delayed shipping and refrigeration.

In this study, only *RdRp* PCR results were analyzed. The reason is that *RdRp* tends to be amplified approximately 1 cycle ahead of *E* on average among total positive samples, and when the viral load is lowered during treatment, the *RdRp* gene tends to reach negative conversion faster than the *E* gene. Therefore, the RT-qPCR C_t value of *RdRp* is more clearly separated into positive or negative than *E*; therefore, it is easier to analyze. If we used combined *E* and *RdRp* analysis (positive, "*E* and *RdRp* $C_t \leq 36$"; negative, "*E* and *RdRp* not amplified until maximum cycle number"; and inconclusive, "neither positive nor negative"), 1 negative sample at 20°C to 22°C, 3 negative samples at 35°C to 40°C, and 1 positive sample at 35°C to 40°C would be reclassified as inconclusive. However, the overall results of combined *E* and *RdRp* analysis did not significantly differ from those of only *RdRp* based on the following observations. First, when stored at 2°C to 8°C, all 80 samples were positive and showed no significant C_t change for 7 days. Second, samples with low viral load showed negative changes at more than 20°C to 22°C from 1 day after storage. Third, the higher the storage temperature, the faster the nucleic acid was degraded, and viral RNA was not amplified by RT-qPCR. Fourth, the virus and nucleic acids were more stable in the pH-controlled UTM or PBS.

This study has a few limitations. Normal saline, which is commonly used as a transport medium in general laboratories, was not evaluated as a buffer. In addition, because this study was retrospective, it was not possible to perform additional experiments by changing the buffer, and the sample volumes were very small because only archived samples were used. Based on the laboratory techniques and reagent conditions, C_t values of RT-qPCR showed a slight fluctuation. Therefore, samples near the limit of detection with a low viral load (with a high C_t value) might be changed as positive or negative, and it is necessary to test them several times.[20,21,31] However, the current study analyzed a limited number of samples that could be duplicated only at the point of the negative change.

Based on the findings of this study, we suggest that the detection of SARS-CoV-2 nucleic acid should be completed as soon as possible after sample collection. In addition, the storage of samples in pH-controlled media or buffer and at a low temperature, in case the molecular test is delayed, is recommended. Especially in hot weather, it is important to prepare adequate transport and refrigeration utilities. This study is expected to serve as a suitable reference for effective shipping and storage conditions to detect nucleic acids from this novel virus.

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