Clinical performance and potential of a SARS-CoV-2 detection kit without RNA purification steps

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Abstract

Objectives: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is rapidly spreading globally. Early diagnosis plays an essential role in controlling the infection. Therefore, early and accurate SARS-CoV-2 detection assays along with easy operation are required. The aim of this study was to compare the clinical performance of the Ampdirect™ 2019-nCoV Detection Kit (SHIMADZU assay), which does not require RNA purification steps, with that of the preexisting SARS-CoV-2 detection assays, which use a purified RNA template.

Methods: A total of 71 samples (65 nasopharyngeal specimens and 6 sputum specimens) were collected from 32 individuals, including patients infected with SARS-CoV-2 and those with suspected infection. The sensitivity and kappa (κ) coefficient were assessed between the SARS-CoV-2 detection assays using the reference standard, which was defined as a true positive result by any one of the four SARS-CoV-2 detection assays.

Results: The overall sensitivity and κ coefficient of the SHIMADZU assay were 86.0% (95% confidence interval [CI]: 77.9–94.2) and 0.83 (95% CI: 0.69–0.96), respectively. In particular, among the 18 samples collected within 10 days from symptom onset, the sensitivity and κ coefficient of the SHIMADZU assay were 100% and 1.0, respectively.

Conclusions: Although a relatively small number of samples was evaluated, the SHIMADZU assay showed good analytical performance and as such would be highly useful for the detection of SARS-CoV-2. The test can be performed easily and quickly and has the potential for future applications in situations where a highly sensitive diagnosis is required.

Keywords: assay; COVID-19; detection; real-time RT-PCR; SARS-CoV-2.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection was first detected in a patient in December 2019 in Wuhan, Hubei, China [1]. SARS-CoV-2 causes the coronavirus disease 2019 (COVID-19), which is mainly presented with fever and respiratory symptoms [2]. COVID-19 has spread widely in many countries, including Japan. As of April 4, 2021, the estimated number of people infected worldwide was 130.5 million, and the death toll was approximately 2,842,325 [3], stressing the need to prevent its spread. SARS-CoV-2 is highly contagious [4], and it has been reported that infected people, both symptomatic and asymptomatic, have a large amount of virus in the body, which contributes to the spread of infection [5, 6]. Therefore, early detection of infected patients is critical for appropriate infection control highlighting the need for sensitive and accurate SARS-CoV-2 detection assays. Currently, reverse transcription-polymerase chain reaction (RT-PCR) is the gold standard for detecting SARS-CoV-2. However, the RNA extraction step, which requires specialized techniques, takes approximately 3 h from sample collection to reporting.

In April 2020, Shimadzu Corporation released the Ampdirect™ 2019-nCoV Detection Kit, which enabled the...
Materials and methods

Sample collection

We collected 71 samples (65 nasopharyngeal specimens and 6 sputum specimens), including samples from the same patients collected at different times, from 14 patients confirmed or 18 patients suspected with COVID-19 at the Sapporo Medical University Hospital in February and March 2020. The sample size was relatively small, with 39 overlapping samples. The sputum specimens were collected in sterile Petri dishes or spitz tubes, and the nasopharyngeal specimens were collected using a kit containing a nasopharyngeal nylon-flocked swab and a tube containing universal transport medium (UTM) viral transport medium (Copan Diagnostics, Murrieta, CA, USA). The samples were kept for up to 48 h at 2–8 °C until RNA extraction and purification.

RNA extraction and molecular assays

The UTM containing nasopharyngeal specimens were vortexed for 5 s and were then used for RNA extraction and purification. For the sputum specimens, calcium-magnesium-free phosphate-buffered saline was added at a volume of 1–3 times that of the sputum, suspended with a vortex mixer, and the suspension (approximately 1 mL) was transferred to a 1.5 mL sterile microcentrifuge tube. The suspension was centrifuged at 20,000 × g at 4 °C for 30 min, and RNA was extracted from the supernatant of the suspension. The RNA extraction step of the Ampdirect™ 2019-nCoV Detection Kit (Shimadzu Corporation, Kyoto, Japan; SHIMADZU assay) was performed by adding a treatment solution to the sample. A mixture of the sample (5 µL) and the treatment solution (5 µL) was pretreated at 95 °C for 5 min and then added to the PCR solution. For the preexisting SARS-CoV-2 detection assays, the RNA extraction step was performed using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. Briefly, 140 µL of each sample was mixed with 560 µL of buffer AVL/carrier RNA and kept at room temperature for 10 min. After the addition of 560 µL of 99.5% ethanol, the sample was washed in two steps using buffer AW1 followed by buffer AW2. RNA was eluted in a volume of 60 µL of buffer AVE. The extracted RNA was stored at −80 °C until further use. LightMix Modular EAV RNA Extraction Control (TIB Molbiol, Berlin, Germany) was added as an internal control for nucleic acid extraction and amplification processes.

Table 1 and Supplementary Methods present an overview of the four SARS-CoV-2 detection assays used in this study. All samples were measured in duplicate, and the cycle threshold (Ct) values were averaged. The preexisting SARS-CoV-2 detection assays were performed using the LightMix Modular SARS, Wuhan CoV E-gene, LightMix Modular SARS, and Wuhan CoV N-gene kits (TIB Molbiol, Berlin, Germany; Roche assay) and the Loopamp SARS-CoV-2 detection kit (EIKEN CHEMICAL, Tokyo, Japan; Eiken assay) according to each manufacturer’s instruction. The N2 assay of the Japanese National Institute of Infectious Diseases (NIID) assay was based on the Pathogen Detection Manual 2019-nCoV Ver.2.6 [7].

Sequence analysis

Five samples (sample no. 65, 68, 69, 70, and 71) were positive in only one of the four SARS-CoV-2 detection assays sequenced at Eurofins.

Table 1: Summary of the SARS-CoV-2 detection assays evaluated in this study.

| Assay          | Principle                          | Target gene | Extraction required | Volume of template RNA/total reaction volume, µL | Positive criteria | Instrument Run timea | Thermal cycling condition | Ct value < 40                                                                 |
|---------------|------------------------------------|-------------|---------------------|-----------------------------------------------|------------------|----------------------|-------------------------|--------------------------------------------------------------------------------|
| SHIMADZU assay | Real-time reverse transcription-polymerase chain reaction | N gene      | No                  | 10/25                                         | Ct value < 40    | CFX-96 Deep Well >90 min | 10 min at 42 °C, 1 min at 95 °C, 45 cycles of 5 s at 95 °C, and 15 s at 60 °C | 30 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, and 60 s at 60 °C |
| NIID assay    | Real-time reverse transcription-polymerase chain reaction | N gene      | Yes                 | 5/20                                          | Ct value < 40    | LightCycler 480 System >200 min | 5 min at 55 °C, 5 min at 95 °C, 45 cycles of 5 s at 95 °C and 15 s at 60 °C, and 15 s at 72 °C | 5 min at 55 °C |
| Roche assay   | Real-time reverse transcription-polymerase chain reaction | E, N-gene   | Yes                 | 5/20                                          | Ct value < 39    | LightCycler 480 System >150 min | 35 min at 62.5 °C          | A rise of the amplification curve during reaction time |
| Eiken assay   | Reverse-transcription loop-mediated isothermal amplification | N, RdRP-gene| Yes                 | Yes                                           |                  | LoopampEXIA >95 min         | 35 min at 62.5 °C          | 35 min at 62.5 °C |

aTime required for sample preparation and PCR detection. NIID, National Institute of Infectious Diseases; RdRP, RNA-dependent RNA polymerase; Ct, cycle threshold.
Genomics (Ebersberg, Germany). Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific).

Limit of viral detection

The artificially synthesized RNA containing the N gene target from Shimadzu Corporation was diluted with Tris-EDTA buffer. The limit of detection (LOD) test was performed using the SHIMADZU assay. The diluted artificially synthesized RNA (100, 50, 25, 12.5, 6.3, 3.1, 1.6, and 0.8 copies/reaction) was added before the PCR step, and eight replicates were measured using the CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The detection rate for each serial dilution series was then calculated. Probit analysis was used to estimate LOD.

Definitions and analyses

Currently, there is no gold standard for comparative studies of SARS-CoV-2 infection. In this study, to avoid false positives, a true positive result for SARS-CoV-2 was defined as a positive result obtained using any one of the four SARS-CoV-2 detection assays, whereas a true negative result was defined as negative results obtained using all the SARS-CoV-2 detection assays. The sensitivity, specificity, and kappa (κ) coefficient, which indicated agreement among the assays, were assessed among the SARS-CoV-2 detection assays using the defined positive result as a reference. The number of days from symptom onset to sample collection was stratified and analyzed as less than 10 days and more than 10 days. We also analyzed the time course of the results of SARS-CoV-2 detection assays in six samples that were followed up for four or more days. Furthermore, we classified 70 samples measured using all four SARS-CoV-2 detection assays into four groups according to the number of positive results obtained using the detection assays (1–4). The Ct and threshold time (Tt) values of each detection assay were statistically analyzed.

Statistical analyses

The sensitivity of the assays was compared using McNemar’s test. Using probit analysis, the LOD was determined as the lowest detectable dilution (copies/reaction) at which the synthetic RNA was positive with a 95% probability of detection. Ct and Tt values were compared using the Mann–Whitney U test. Statistical significance was set at p<0.05. All statistical analyses were performed using IBM SPSS Statistics version 24 (IBM Corp., Chicago, IL, USA) and JMP Pro15 statistical software (SAS Institute, Cary, NC, USA).

Ethical approval

All experiments involving human subjects complied with all relevant national regulations and institutional policies and were in accordance with the tenets of the Helsinki Declaration (as revised in 2013). This study was approved by the authors’ institutional review boards (https://web.sapmed.ac.jp/byoin/rinshokenkyu/; number 312–241). Sapporo Medical University Hospital approved this study and waived the requirement for informed consent based on the retrospective design of the study.

Results

Clinical overview

The median age of the 32 patients with confirmed and suspected SARS-CoV-2 infection was 62 years (range, 22–82 years); 20 (62.5%) patients were male. A total of 71 samples (65 nasopharyngeal specimens and 6 sputum specimens) were collected and tested. The samples for which the date of symptom onset was obtained from the electronic medical records were collected on median day 4 (IQR: 8–18).

Clinical performance of four SARS-CoV-2 detection assays

Out of the total, 44 (62%) samples were true positive, and 27 (38%) were true negative, as per our definition (Supplementary Table 1). A total of 37 (86%) samples were positive in the SHIMADZU assay, which was the highest, followed by 36 (82%) in the Roche assay, 32 (73%) in the NiID assay, and 21 (48%) in the Eiken assay. Eight samples (Sample No. 64–71, Supplementary Table 1) were positive in only one SARS-CoV-2 detection assay. Of these, five, two, and one samples were positive in the SHIMADZU, Roche, and NiID assays, respectively. Sequence analysis was performed on five samples (samples No. 65, 68, 69, 70, and 71). The results showed that the sequence in all five samples was in perfect agreement with that of SARS-CoV-2.

We analyzed the overall sensitivity, specificity, and κ coefficient using 70 samples measured using all four assays because the SHIMADZU assay could not test one sample owing to sample shortage (Table 2). The overall sensitivity and κ coefficient of the SHIMADZU assay N1 + N2 primer set were 86.0% (95% confidence interval [CI]: 77.9–94.2) and 0.83 (95% CI: 0.69–0.96), respectively, which were the highest among the four SARS-CoV-2 detection assays. Among 18 samples (28%) collected within less than 10 days from the onset of symptoms, high clinical performance was observed in all SARS-CoV-2 detection assays, in particular the SHIMADZU assay N1 + N2 primer set, and all agreed with the defined positive sample (Table 3). Although the clinical performance of all SARS-CoV-2 detection assays decreased in 46 samples (72%) collected after 10 days or more from symptom onset,
Table 2: Overall diagnostic performance of four SARS-CoV-2 detection assays in 70 samples.

| Primer       | Sensitivity (95% CI), % | Specificity (95% CI), % | Kappa (95% CI) |
|--------------|-------------------------|-------------------------|--------------|
| SHIMADZU assay N1 | 83.7 (75.1–92.4) | 100b | 0.80 (0.66–0.94) |
| N2           | 41.9 (30.3–53.4)       | 100b | 0.36 (0.21–0.51) |
| N1 + N2      | 86.0 (77.9–94.2)       | 100b | 0.82 (0.68–0.96) |
| NIIID assay N2 | 72.1 (61.6–82.6)      | 100b | 0.67 (0.50–0.83) |
| Roche assay E | 81.4 (72.2–90.5)       | 100b | 0.77 (0.63–0.91) |
| N             | 34.9 (23.7–46.0)       | 100b | 0.29 (0.15–0.43) |
| E + N        | 81.4 (72.2–90.5)       | 100b | 0.77 (0.63–0.91) |
| Eiken assay N + RdRp | 46.5 (34.8–58.2) | 100b | 0.40 (0.24–0.56) |

4 Calculated against the defined reference standard. 95% CI could not be calculated. *p<0.05, compared with the defined reference standard. CI, confidence interval; NIIID, National Institute of Infectious Diseases; RdRp, RNA-dependent RNA polymerase; Overall diagnostic performance of four SARS-CoV-2 detection assays was evaluated with 70 samples because the SHIMADZU assay could not test one sample due to sample shortage.

Table 3: Overall diagnostic performance of four SARS-CoV-2 detection assays in 18 samples with less than 10 days between onset and testing.

| Primer       | Sensitivity (95% CI), % | Specificity (95% CI), % | Kappa (95% CI) |
|--------------|-------------------------|-------------------------|--------------|
| SHIMADZU assay N1 | 100b | 100b | 1.00b |
| N2           | 81.8 (64.0–99.6)       | 100b | 0.78 (0.50–1.00) |
| N1 + N2      | 100b | 100b | 1.00b |
| NIIID assay N2 | 90.9 (77.6–100.0)      | 100b | 0.89 (0.67–1.00) |
| Roche assay E | 90.9 (77.6–100.0)       | 100b | 0.89 (0.67–1.00) |
| N             | 81.8 (64.0–99.6)       | 100b | 0.78 (0.50–1.00) |
| E + N        | 90.9 (77.6–100.0)       | 100b | 0.89 (0.67–1.00) |
| Eiken assay N + RdRp | 81.8 (64.0–99.6) | 100b | 0.78 (0.50–1.00) |

4 Calculated against the defined reference standard. 95% CI could not be calculated. CI, confidence interval; NIIID, National Institute of Infectious Diseases; RdRp, RNA-dependent RNA polymerase.

The SHIMADZU assay N1 + N2 primer set had a sensitivity of 81.3% (95% CI: 70.0–92.5) and a κ coefficient of 0.73 (0.53–0.92), which was the highest among all assays (Supplementary Table 2).

Detection limit of the SHIMADZU assay

The detection limit test indicated that the SHIMADZU assay could detect up to 1.0 copies/reaction of N1 and up to 4.2 copies/reaction of N2 (Table 4).

Time course of the results of SARS-CoV-2 detection assays

Figure 1, Supplementary Table 3, and Supplementary Figure 1 show the time course of Ct and Tt values of each SARS-CoV-2 detection assay for six cases. The Ct and Tt values were low in the early stages of symptom onset and increased over time. The SHIMADZU, NIIID, and Roche assays could detect SARS-CoV-2 in all samples up to day 11 (Patient No. 19, 24, and 25); SARS-CoV-2 could not be detected in some samples after day 12. The Eiken assay could detect the virus up to day 8; however, it was not detected in some samples after day 9.

Relationship between the number of positive detection assays

Compared with the number of positive detection assays, the median Ct and Tt values were significantly lower in the group in which all assays were positive than in any other group (Figure 2, Supplementary Figure 2, and Supplementary Table 4). In the SHIMADZU assay N1, the NIIID assay N2, and the Roche assay E, the median Ct values were less than 35, whereas the number of positive assays tended to decrease when the Ct values were 35 or higher.
Discussion

We compared the clinical performance of the SHIMADZU assay with that of three preexisting molecular tests using 71 samples from 32 patients with confirmed and suspected COVID-19. The sensitivity and $\kappa$ coefficient of the SHIMADZU assay were higher than those of the NIID, Roche, and Eiken assays, and particularly remarkable in the samples collected within 10 days after symptom onset. It has the potential to be applied to situations in which a highly sensitive diagnosis is required.

The newly developed SHIMADZU assay enables performing real-time RT-PCR without RNA purification, thereby substantially shortening the measurement time. This technology, called Ampdirect Technology [8], is also used in preexisting kits, such as those used for norovirus detection. In addition, the SHIMADZU assay can reduce the risk of human error, cost, and human resources required for RNA extraction. The SHIMADZU assay had a sufficient “detection power” of 1,000 copies/mL for N1 and 4,200 copies/mL for N2 in the detection limit. Patients with COVID-19 are normally reported to have more than $10^4$ copies/mL of virus load [9]. Therefore, using artificially synthesized RNA, our study indicated that the SHIMADZU assay could adequately detect SARS-CoV-2 infection in patients with COVID-19. We presumed two explanations for the higher “detection power” in the SHIMADZU assay. First, real-time RT-PCR was performed directly on the samples. In preexisting real-time RT-PCR assays, the reaction may be inhibited by various PCR inhibitors derived from living organisms [10]. However, Ampdirect in the pretreatment reagent (sample treatment reagent) of the SHIMADZU assay suppresses these PCR inhibitors [8]. Thus, real-time RT-PCR can be performed directly on biological samples. Second, preexisting SARS-CoV-2 detection assays are affected by purification efficiency during the RNA purification process [11]. Thus, the SHIMADZU assay, which does not require an RNA purification step and is not affected by PCR inhibitors derived from the samples owing to insufficient purification or RNA purification efficiency, could be highly sensitive. The SHIMADZU assay has the potential to detect low virus copy numbers that are difficult to detect using conventional SARS-CoV-2 detection assays. In the future, it may apply to situations in which more sensitive tests are required, such as tests using various specimens (nasal vestibular, nasal discharge, urine, and stool) that have a low exposure risk for examiners or those with close contact with no symptoms who have recently come into contact with patients with COVID-19.

Matsumura et al. [12] compared the clinical performance of SARS-CoV-2 detection assays, including those of the NIID, Roche, and Eiken assays. The sensitivity and $\kappa$...
Table 4: Detection limit of the SHIMADZU assay for SARS-CoV-2 with serial dilutions of artificially synthesized RNA.

| Copies/reaction | 100 copies/reaction | 50 copies/reaction | 25 copies/reaction | 12.5 copies/reaction | 6.3 copies/reaction | 3.1 copies/reaction | 1.6 copies/reaction | 0.8 copies/reaction | Limit of detection (copies/reaction) |
|-----------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------------|
| N1 (positive rate) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 6/8 (75%) | 1.095% CI |
| N2 (positive rate) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 8/8 (100%) | 7/8 (87.5%) | 5/8 (62.5%) | 4/8 (50%) | 4.2 (4.0–4.4) |

95% CI could not be calculated. CI, confidence interval: The detection limit by probit analysis was determined as the lowest detectable dilution (copies/reaction) at which the synthetic RNA was positive with a 95% probability of detection.

Additionally, an increase in Ct and Tt values was observed over time after symptom onset. In Japan’s current discharge criteria, patients symptomatic for COVID-19 can be discharged 10 days after symptom onset with symptom improvement for at least 3 days [13]. Thus, negative PCR confirmation is not required. However, the sample for diagnosis is not always collected in the early stages of symptom onset. The sample may have a low viral load because patients asymptomatic for COVID-19 would have a lower viral load than those who are symptomatic for COVID-19 [14]. Therefore, it is desirable to use a more sensitive SARS-CoV-2 detection assay suitable for each facility.

The Ct and Tt values were significantly lower in the group in which all four assays were positive than in any other group. This finding indicated that the virus copy number would significantly affect the performance of the assays and cause discrepancies in their results. In particular, we observed that the real-time PCR assays, not only the SHIMADZU assay but also the NIH and Roche assays, showed high performance for the sample with a high virus copy number at the level of a Ct value less than 35. Although it is possible that the eight samples that tested positive using only one assay are false positives [15], five of these were sequence-analyzed and showed the same homology as that of SARS-CoV-2, which could indicate true positives. All the remaining three samples were positive only in the SHIMADZU assay. The N1 and N2 primer-probe sets of the SHIMADZU assay have the same nucleotide sequences as the primer-probe set recommended by the Centers for Disease Control and Prevention (CDC) [16]. In a previous report, the CDC primer-probe set demonstrated no cross-reactivity with other respiratory virus infections [17]. Therefore, false positives may be rare in the SHIMADZU assay; however, further research is needed to confirm this.

In the time course of patients with COVID-19, some patients (Patient No. 16, 19, and 21) retested positive after negative results. We assumed that the sample collection procedure may have affected the results. The Ct values of the samples retested positive were 35 or higher. Thus, it is necessary to carefully interpret the results when the copy number is low. Patient No. 18 remained positive according to all four SARS-CoV-2 detection assays, even though 14 days had passed since symptom onset. The patient had severe pneumonia and required oxygen administration on day 10 from symptom onset, mechanical ventilation support on day 12, and venenous extracorporeal membrane oxygenation on day 14. It has been reported that invasive viral pneumonia is associated with prolonged SARS-CoV-2 RNA shedding [18]. Patient No. 18 was also considered to continue to be positive by all four SARS-CoV-2 detection assays owing to persistent viral excretion; however, the virus activity was unknown.

This study has several limitations. First, a relatively small sample size was evaluated. Furthermore, the number of samples collected within 10 days from symptom onset was too small to evaluate clinical performance for diagnostic purposes. However, the SHIMADZU assay had high sensitivity and concordance even for samples with low viral copy numbers, and the performance of the test itself could be evaluated. Second, there may be variations in the sample collection method because different staff members collected the samples. Third, we used our definition of the positivity of the sample in this study, and not all samples were evaluated using sequence analysis. Therefore, we could not evaluate the specificity of the test for all the
Fourth, the SHIMADZU assay does not include the RNA purification step; the use of synthesized RNA results in a low LOD in comparison to that with the use of the real virus. Fifth, we were unable to add any direct PCR assays other than the SHIMADZU assay for comparison. For the SARS-CoV-2 detection assay, we used instruments and kits available in our hospital during the study period. Therefore, the comparison between the SHIMADZU assay and direct PCR assays should be examined in the future.

Conclusions

The SHIMADZU assay would be highly useful as a molecular assay for SARS-CoV-2 detection because of its simplicity, speed, and clinical performance compared to the preexisting assays. This would have the potential for future applications in situations where a highly sensitive diagnosis is required. However, caution must be exercised with false negatives, especially in cases of low virus copy numbers, even with molecular diagnostic tools, including the SHIMADZU assay. Considering infection opportunities, clinical symptoms, and the timing of sample collection, the results should be interpreted comprehensively.

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Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors’ institutional review board (https://web.sapmed.ac.jp/byoin/rinshokenkyu/)(number 312–241).

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