Highly sensitive detection of SARS-CoV-2 RNA by multiplex rRT-PCR for molecular diagnosis of COVID-19 by clinical laboratories

Takayuki Ishige[a,*], Shota Murata[a], Toshibumi Taniguchi[b], Akiko Miyabe[a], Kouichi Kitamura[a], Kenji Kawasaki[a], Motoi Nishimura[a], Hidetoshi Igari[b], Kazuyuki Matsushita[a]

[a] *Division of Laboratory Medicine, Chiba University Hospital, 1-8-1 Inohana, Chuo-ward, Chiba-city, Chiba 266-8677, Japan*
[b] *Department of Infectious Diseases, Chiba University Hospital, 1-8-1 Inohana, Chuo-ward, Chiba-city, Chiba 266-8677, Japan*

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ABSTRACT

Background: The detection of SARS-CoV-2 RNA by real-time reverse transcription–polymerase chain reaction (rRT-PCR) is used to confirm the clinical diagnosis of COVID-19 by molecular diagnostic laboratories. We developed a multiplex rRT-PCR methodology for the detection of SARS-CoV-2 RNA.
Methods: Three genes were used for multiplex rRT-PCR: the *Sarbecovirus* specific E gene, the SARS-CoV-2 specific N gene, and the human *ABL1* gene as an internal control.
Results: Good correlation of C_q values was observed between the simplex and multiplex rRT-PCR methodologies. Low copies (< 25 copies/reaction) of SARS-CoV-2 RNA were detected by the novel multiplex rRT-PCR method.
Conclusion: The proposed multiplex rRT-PCR methodology will enable highly sensitive detection of SARS-CoV-2 RNA, reducing reagent use and cost, and time required by clinical laboratory technicians.

1. Introduction

The global pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was declared by the World Health Organization (WHO) in March 2020 [1]. Currently, clinical diagnosis of COVID-19 is confirmed by detecting SARS-CoV-2 RNA by molecular diagnostic laboratories using the real-time reverse transcription–polymerase chain reaction (rRT-PCR) [2–5].

We analyzed two regions of the N genes used to detect SARS-CoV-2 RNA by simplex one-step rRT-PCR using the previously described primer/probe sets N_Sarbeco and NIID_2019-nCOV_N (NIID-N) [3]. However, this method requires improvement as a molecular diagnostic test used by clinical laboratories. The WHO recommended the detection of at least two different targets on the COVID-19 virus genome [6]. For example, Corman et al. recommended the E gene assay as the first-line screening tool, followed by confirmatory testing with the RdRp gene assay [2]. However, our experiments and previous reports show that the N_Sarbeco set is less sensitive than the NIID-N set [3]. In cases where NIID-N is weakly positive and N_Sarbeco is negative, we have found it difficult to judge whether results are true positives or false positives, indicating the need for another assay with sensitivity equal to the NIID-N set to detect two regions of the COVID-19 genome with high

sensitivity. In addition, an internal control assay is required for evaluating the qualities of the specimen, nucleic acid extraction step, and RT-PCR amplification [6].

Here, we developed a multiplex rRT-PCR methodology for the simultaneous detection of two regions of the SARS-CoV-2 genome (E and N genes), and of the human *ABL1* gene. The qualities of the clinical specimen and of the RT-PCR assay can be evaluated by using the human *ABL1* gene as an internal control (IC) [7].

2. Materials and methods

2.1. Primers and probes

Three genes were designed: a *Sarbecovirus*-specific E gene (E_Sarbeco, target), a SARS-CoV-2-specific N gene (NIID-N, target), and a human *ABL1* gene (IC). The primers and probes were referred from previous research [2,3,7] and the sequences are described in Table 1. Primers and probes were purchased from Integrated DNA Technologies (Coralville, IA, USA). The 4 × primer/probes mixes were prepared at the following concentrations: E_Sarbeco and NIID-N, 0.8 μM of primer and 0.4 μM of probe; *ABL1* gene, 0.4 μM of primer and 0.2 μM of probe.

* Corresponding author.
  E-mail address: ishige-t@chiba-u.jp (T. Ishige).

Table 1

Primers and probes used in multiplex rRT-PCR.

Name	Sequence ($5' > 3'$)
NIID-N	
NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC
NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC
NIID_2019-nCOV_N_P2	FAM-ATGTCGCGC/ZEN/ATTGGCATGGA-IBFQ
E_Sarbeco	
E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R	ATATTGCAGCAGTACGCACACA
E_Sarbeco_P1	HEX-ACACTAGCC/ZEN/ATCCTTACTGCGCTTCG-IBFQ
Human *ABL1* gene	
ABL1_ENF1003	TGGAGATAACACTCTAAGCATAACTAAAGGT
ABL1_ENR1063	GATGTAGTTGCTTGGGACCCA
ABL1_ ENPr1043	Cy5-CCATTTTTG/TAO/GTTTGGGCTTCACACCATT-IBRQ

2.2. Control samples

We used a synthetic RNA control with the N gene (CTRL-N) with a previously described sequence [3], gifted by the National Institute of Infectious Disease (NIID; Tokyo, Japan). We prepared another control sample (CTRL-C) using SARS-CoV-2 test positive total nucleic acid solution extracted from a clinical specimen and total RNA solution extracted from the K562 cell line.

2.3. Sample preparation and viral RNA extraction

Thirty sputum samples from patients who underwent routine clinical SARS-CoV-2 tests (simplex RT-PCR with NIID-N) were prepared using a DTT solution containing 20 mM DTT in Dulbecco's phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA). The sputum samples were mixed well with the DTT solution at a volume ratio of 2:1. After incubation for 10 min at room temperature, the samples were centrifuged at 1500*g* for 5 min. Nucleic acids were extracted from 200 μL of supernatant and viral RNA was extracted using a Maxwell RSC Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) using a Maxwell RSC Instrument (Promega). Total nucleic acid was eluted in 50 μL of nuclease-free water.

2.4. Multiplex rRT-PCR

A Cobas z 480 instrument (Roche Diagnostics, Mannheim, Germany) was used for rRT-PCR. All reagents were brought to room temperature prior to preparing the master mix because preparing the mix on ice can result in occasional non-specific signal increases. Each 10-μL reaction mixture contained 2.5 μL of 4 × TaqPath 1-Step Multiplex Master Mix (No ROX; Thermo Fisher Scientific, Waltham, MA, USA), 2.5 μL of 4 × primer/probes mix, and 2.5 μL of extracted total nucleic acid. The final concentrations of primers and probes were 0.2 μM (target primers), 0.1 μM (target probes), 0.1 μM (IC primer), and 0.05 μM (IC probe). The cycling program was as follows: uracil DNA glycosylase incubation at 37 °C for 2 min; reverse transcription at 55 °C for 10 min; polymerase activation at 95 °C for 2 min; and 45 cycles of PCR at 95 °C for 3 sec and 60 °C for 30 sec (signal acquisition). The filter combinations were 465–510 (FAM), 540–580 (HEX), and 610–670 (Cy5). The melt factor, quant factor, and max integration times were 1, 10, and 2 sec, respectively. No color compensation experiment was performed because Cobas z 480 instruments contain calibrated filters. The C_q values were determined by the manual Fit Points method.

3. Results

3.1. One-step RT-PCR reagents and probe chemistries

Simplex rRT-PCR with NIID-N was performed using a synthetic RNA control with 2500 copies/reaction as the rRT-PCR template. Each experiment was performed in duplicate. First, we compared the three one-step RT-PCR reagents TaqPath 1-Step Multiplex Master Mix, QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany), and LightCycler Multiplex RNA Virus Master (Roche); the average C_q values and standard deviations (SD) were 24.5 (0.11), 26.9 (0.064), and 26.8 (0.11), respectively. Second, we compared two probe chemistries: FAM/TAMRA (a single-quenching probe) and FAM/ZEN/IBFQ (a double-quenching probe). The average difference between the maximum and minimum fluorescence intensities and SD were FAM/TAMRA 9.1 (0.21) and FAM/ZEN/IBFQ 10.1 (0.30). The signal-to-noise ratio was 11% higher using FAM/ZEN/IBFQ and thus TaqPath 1-Step Multiplex Master Mix and the double-quenching probes were selected because of their low C_q value and improved signal-to-noise ratio, respectively.

3.2. Limit-of-detection (LOD) of simplex one-step RT-PCR with NIID-N

The LOD was analyzed using serially diluted synthetic control RNA samples (2500, 250, 25, 5 and 2.5 copies/reaction). The C_q values and detection rates are shown in Table 2. The slope, intercept, and R^2 were − 3.23, 35.6, and 0.997, respectively. The calculated PCR efficiency [10ˆ(−1/slope)] was 2.04. The detection rate was 100% up to 25 copies/reaction. The calculated LOD with 95% detection probability was 21 copies/reaction.

3.3. Multiplexing

We compared the three primer/probe sets NIID-N, N_Sarbeco and E_Sarbeco. The E_Sarbeco set was selected because its sensitivity was well characterized previously [2,4,8]. The C_q values of the same SARS-CoV-2 positive samples were 26.2 (NIID-N), 29.9 (N_Sarbeco), and 25.5 (E_Sarbeco). The NIID-N and E_Sarbeco sets were selected for multiplexing because of their sensitivities. In addition, the human *ABL1* gene was included as an IC in the novel multiplex rRT-PCR assay to evaluate the qualities of the clinical specimens (e.g., sputum and nasopharyngeal swabs), nucleic acid extraction, and RT-PCR.

3.4. Verification of multiplex rRT-PCR using SARS-CoV-2-positive samples

We compared the C_q values of simplex and multiplex rRT-PCR using six SARS-CoV-2 RNA-positive samples (Fig. 1) and observed good correlation between the targets. However, the C_q values of N_Sarbeco showed an average delay of 6.4 cycles (a minimum of 5.5 to a maximum of 7.5). Similar results for C_q values were obtained using simplex NIID-N, multiplex NIID-N and multiplex E_Sarbeco. These results indicate that the sensitivity of NIID-N was not decreased by multiplexing, and E_Sarbeco and NIID-N had equal sensitivity. We also tested another nucleic acid extraction kit (High Pure Viral RNA Kit, Roche). Although the C_q values were slightly higher than those obtained using a Maxwell RSC Viral Total Nucleic Acid Purification Kit (minimum − 0.8, average

Table 2

Sensitivity of simplex rRT-PCR with NIID-N.

Copies/reaction	C_q: Average (SD)	Detected/tested (%)
2500	24.5 (0.13)	6/6 (100%)
250	27.8 (0.25)	12/12 (100%)
25	31.2 (0.63)	12/12 (100%)
5	33.6 (1.41)	13/18 (72%)
2.5	34.0 (1.09)	11/18 (61%)

Slope, − 3.23; intercept, 35.6; R^2, 0.997; efficiency, 2.04.
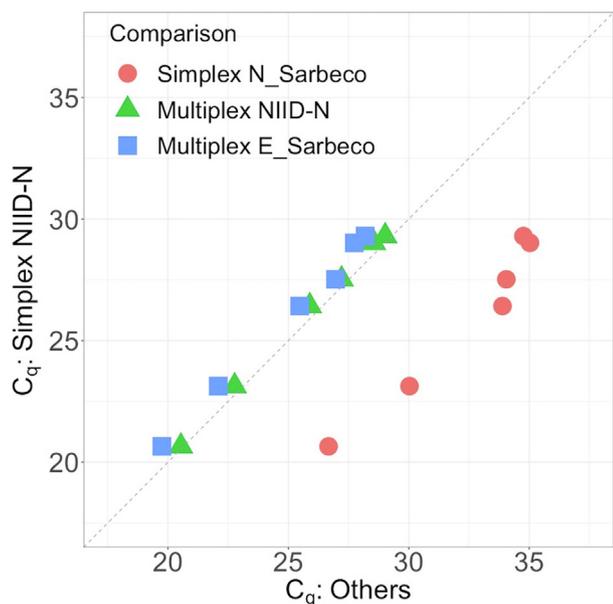
Fig. 1. Comparison of C_q values. Six samples were analyzed by simplex rRT-PCR (NIID-N and N_Sarbeco) and multiplex rRT-PCR (NIID-N and E_Sarbeco). Simplex rRT-PCR with NIID-N was used as a reference (y axis). The dashed line indicates the y = x line.

2.0, maximum 3.2), both NIID_N and E_Sarbeco were detected from all six samples.

3.5. Highly sensitive detection of the SARS-CoV-2 E and N genes

The SARS-CoV-2 test results obtained using the novel multiplex rRT-PCR are shown in Fig. 2. Twenty-four clinical specimens were analyzed. Four positive and twenty negative results were confirmed by simplex rRT-PCR. The four positive samples were NIID-N-positive and the N_Sarbeco-negative results were confirmed by simplex rRT-PCR. Although these samples had low copies (< 25 copies/reaction as determined by NIID-N), a concordant result was obtained using the novel multiplex rRT-PCR method. In addition, in one of the four samples, the novel multiplex rRT-PCR method could simultaneously detect two regions of SARS-CoV-2 RNA (N and E genes) and the human *ABL1* gene. Four target positive samples (NIID-N/E_Sarbeco: positive/positive, N = 1; positive/negative, N = 3) were obtained from the same patient. The SARS-CoV-2 RNA of these samples calculated by NIID-N were 15, 16 (also determined by E_Sarbeco, 19 copies/reaction), 5, and 2 copies/reaction. Twenty samples provided negative results for SARS-CoV-2. The C_q values for the *ABL1* gene in these samples were less than 35 cycles (minimum 24.6, average 28.6, maximum 32.3), and the assay quality was validated.

4. Discussion

We developed a multiplex rRT-PCR methodology for the molecular diagnosis of COVID-19. The sensitivity of RT-PCR is affected by various factors, such as the nucleic acid extraction method, the one-step RT-PCR reagent, and the primer/probe sets. Optimization experiments are required prior to using this methodology in clinical settings.

Current results indicate that: 1) the NIID-N set is slightly more sensitive than E_Sarbeco; 2) NIID-N and E_Sarbeco are a good combination for detecting two regions of the SARS-CoV-2 genome with high sensitivity; and 3) the human *ABL1* gene is useful as an IC for checking the qualities of the specimen, nucleic acid extraction step, and RT-PCR amplification. Therefore, we demonstrated that this highly sensitive multiplex rRT-PCR methodology can detect SARS-CoV-2.

The human *ABL1* gene is recommended as an IC gene for the molecular diagnosis and monitoring for minimal residual disease detection [7]. Many molecular diagnostic laboratories use this primer/probe set. We did not use the *ACTB* and *GAPDH* genes, which are also frequently used as ICs, because they are more abundantly expressed than the *ABL1* gene [9]. In multiplex PCR, the amplification of highly abundant targets may prevent the amplification of less abundant targets, possibly lowering the sensitivity of target detection.

The background of E_Sarbeco was slightly increased in later cycles. We judged these signals as negative results because: 1) reproducibility was poor (negative upon retesting with the same assay), 2) NIID-N did not show positive results, although it has equal sensitivity to E_Sarbeco, 3) the maximum signal intensities were significantly lower than the positive signals with low-copy samples, and 4) similar observations were reported previously [2,4]. Since these results may lead to false-positive results, care must be taken when judging weak positive signals.

The SARS-CoV-2 pandemic has resulted in a shortage of reagents related to the molecular diagnosis. The proposed multiplex rRT-PCR methodology will enable highly sensitive detection of SARS-CoV-2 RNA, reducing reagent use and cost, and time required by clinical laboratory technicians.

Declaration of Competing Interest

None declared.

Fig. 2. Results of SARS-CoV-2 detection using the novel multiplex rRT-PCR method. CTRL-C and CTRL-N included 400 copies/reaction of SARS-CoV-2 RNA and 25 copies/reaction of synthetic RNA containing the N region, respectively (red solid lines). The dotted (green) and dashed (blue) lines indicate negative and positive results for the clinical specimens, respectively. The horizontal lines indicate the threshold of noise band.

References

[1] World Health Organization (WHO), COVID-19. https://www.who.int/emergencies/diseases/novel-coronavirus-2019, 2020 (accessed 8 April 2020).

[2] V.M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D.K.W. Chu, T. Bleicker, S. Brünink, J. Schneider, M.L. Schmidt, D.G.J.C. Mulders, B.L. Haagmans, B. van der Veer, S. van den Brink, L. Wijsman, G. Goderski, J.L. Romette, J. Ellis, M. Zambon, M. Peiris, H. Goossens, C. Reusken, M.P.G. Koopmans, C. Drosten, Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR, Euro. Surveill. 25 (2020).

[3] K. Shirato, N. Nao, H. Katano, I. Takayama, S. Saito, F. Kato, H. Katoh, M. Sakata, Y. Nakatsu, Y. Mori, T. Kageyama, S. Matsuyama, M. Takeda, Development of Genetic Diagnostic Methods for Novel Coronavirus 2019 (nCoV-2019) in Japan, Jpn. J. Infect. Dis. (2020).

[4] R. Konrad, U. Eberle, A. Dangel, B. Treis, A. Berger, K. Bengs, V. Fingerle, B. Liebl, N. Ackermann, A. Sing, Rapid establishment of laboratory diagnostics for the novel coronavirus SARS-CoV-2 in Bavaria, Germany, February 2020, Euro. Surveill. 25 (2020).

[5] D.K.W. Chu, Y. Pan, S.M.S. Cheng, K.P.Y. Hui, P. Krishnan, Y. Liu, D.Y.M. Ng, C.K.C. Wan, P. Yang, Q. Wang, M. Peiris, L.L.M. Poon, Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia, Clin. Chem. 66 (2020) 549–555.

[6] World Health Organization (WHO), Coronavirus disease (COVID-19) technical guidance: Laboratory testing for 2019-nCoV in humans, Interim guidance. https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117, 2020 (accessed 8 April 2020).

[7] E. Beillard, N. Pallisgaard, V.H. van der Velden, W. Bi, R. Dee, E. van der Schoot, E. Delabesse, E. Macintyre, E. Gottardi, G. Saglio, F. Watzinger, T. Lion, J.J. van Dongen, P. Hokland, J. Gabert, Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program, Leukemia. 17 (2003) 2474–2486.

[8] S. Pfefferle, S. Reucher, D. Nörz, M. Lütgehetmann, Evaluation of a quantitative RT-PCR assay for the detection of the emerging coronavirus SARS-CoV-2 using a high throughput system, Euro. Surveill. 25 (2020).

[9] Y.L. Wang, J.W. Lee, E. Cesarman, D.K. Jin, B. Csernus, Molecular monitoring of chronic myelogenous leukemia: identification of the most suitable internal control gene for real-time quantification of BCR-ABL transcripts, J. Mol. Diagn. 8 (2006) 231–239.