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Short communications

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Summary

At the end of 2019, pneumonia caused by novel coronavirus 2019 (nCoV) emerged in Wuhan city, China. Many airline travelers moved between Wuhan and Japan at that time, suggesting that Japan is at high risk of invasion by the virus. Diagnostic systems for 2019-nCoV were developed with urgency. Two nested RT–PCR assays and two real-time RT–PCR assays were adapted to local Japanese conditions. As of 8 February 2020, the assays developed have successfully detected 25 positive cases of infection in Japan.

At the end of 2019, pneumonia caused by novel coronavirus 2019 (nCoV) emerged in Wuhan city, China. Because many airline travelers moved between Wuhan and Japan at that time (1), Japan was at high risk of invasion by the virus. Therefore, we urgently established diagnostic systems for 2019-nCoV as the national diagnostic laboratory in Japan.

We first developed a conventional reverse transcription (RT)–PCR assay for 2019-CoV. Because neither the viral RNA nor test controls were available, many primer sets were constructed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and their performances were evaluated with real test specimens. In the diagnostic tests for the first confirmed case of 2019-nCoV infection in Japan, nine specific primers and pan-coronaviral primers were used and primer set 1 (open reading frame 1 [ORF1] set) produced a specific band. The 2019-nCoV sequence was decoded from the amplicon (2). Primers for the spike (S) protein (S set) also detected the 2019-nCoV sequence in different specimens from the same patient (3). Therefore, these two primer sets were considered suitable for the diagnosis of 2019-nCoV infection. To increase the sensitivity and specificity of the assays, primers for a second PCR were constructed, and special primers were also prepared to sequence the PCR products (Table 1). Total RNA was extracted from the specimen with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden,

Germany), according to the manufacturer's instructions. First-strand cDNA was synthesized with SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) using random and oligo-dT primers. The PCR was performed with Quick Taq® HS DyeMix (Toyobo, Osaka, Japan), with the primers at final concentrations of 800 nM. The PCR thermal cycling conditions were: 94 °C for 1 min; 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 68 °C for 1 min. The second (nested) PCR was performed with 1 μL of the first PCR product under the same conditions. The PCR products were purified with AMpure XP (Beckman Coulter, Brea, CA, USA), and directly sequenced with the sequencing primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific). As expected, these primers detected the 2019-nCoV-specific amplicons with no nonspecific reactions when the second confirmed case in Japan was diagnostically tested (4). Using test controls is important in the quality control of diagnostic tests. We constructed positive controls using PCR primers and the MERS-CoV sequences, enabling to check the correct combination of primer usage (Fig. 1a). The amplicon sizes of the controls in the second PCR were smaller than those obtained with PCR using viral RNA (Fig. 2a). This size difference allowed us to distinguish real positive bands from unexpected contamination of control RNAs.

After the nested RT–PCR assays for 2019-nCOV were established, a real-time RT–

PCR assay system was required to process large numbers of specimens. Corman et al. reported three real-time RT–PCR assays (based on the RNA-dependent RNA polymerase [RdRp] gene, envelope [E] gene, and nucleocapsid [N] gene) for detecting beta coronaviruses, including 2019-nCoV (5). Considering the distribution of diagnostic methods to the local health research institutes in Japan, the validation test was performed with the QuantiTect Probe RT–PCR Kit (QuantiTect, Qiagen). Because all local institutes are equipped this reagent, they could establish the test as soon as possible. Among the three primer sets, only the N gene set performed well against the synthesized control RNA template in the QuantiTect assay (data not shown). Therefore, the N gene set was used in our diagnostic test system as N set no. 1. Another candidates were constructed based on the 2019-nCoV sequence (MN908947.1) with the Primer 3 software (ver. 4.0, http://bioinfo.ut.ee/primer3-0.4.0/). We checked their specificity for 2019-nCoV by comparison with six other human coronavirus sequences. Then, they were validated using synthesized each specific control RNA. The primer set that targeted the N protein, but differed from the N set no. 1, had passed the quality check test and used as N set no. 2 (N2). Details of the sets are shown in Table 1. Both sets showed sufficient sensitivity (~5–50 copies for the control RNA) and no cross-reactivity with other respiratory viruses. The pathogens tested were those used previously (6). The real-time RT–PCR was performed

with the QuantiTect Probe RT–PCR Kit and LightCycler 480 (or 96) (Roche, Basel, Switzerland). The thermal cycling conditions were: 50 °C for 30 min; 95 °C for 15 min; and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The N2 set was also used in the diagnostic test for the second confirmed case of 2019-CoV infection in Japan, with a positive result, consistent with the nested RT–PCR (4). Both sets were used to confirm the third and fourth positive cases of infection in Japan, in parallel with nested RT–PCR, demonstrating the equivalent sensitivity and specificity of the real-time RT–PCR and nested RT–PCR assays. Thereafter, the national diagnostic test in our laboratory was mainly performed with the real-time RT–PCR assay. Like the positive controls for nested RT–PCR, those for real-time RT–PCR contained marker sequences (*Bam*HI site and a check probe sequence) (Fig. 1b). When laboratory contamination is possible, for example when a positive signal is detected in the negative control, the addition of a VIC-labeled check probe (VIC-AGCTAGCGCATTGGATCTCG-MGB) to the reaction mixture allowed us to detect the contamination.

Finally, the nested RT–PCR and real-time RT–PCR assays for 2019-nCoV were distributed with positive controls to 84 prefectural and municipal public health institutes and 13 quarantine depots. As of 8 February 2020, 25 cases of 2019-CoV infection have been confirmed in Japan (7). The real-time RT–PCR assay has shown satisfactory

performance. We knew that the reverse primer of the N2 set has a one-nucleotide mismatch (C29277G, Table 1) with the sequence in the current database (MN908947.3), because it was constructed based on first reported sequence (MN908947.1). Validation of the version 3 sequence of the N2 reverse primer (N2 ver.3) was performed with serially diluted viral RNA and the control RNA (Fig. 2b). The N2 and N2 ver.3 sets showed almost the same quantitation cycle (Cq) values for both the viral RNA and control RNA, indicating that this mismatch did not affect the amplification and that both the ver.1 and ver.3 primers are available for the detection of 2019-nCoV.

Conflict of interest

The authors declare that they have no conflicting interests.

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Table 1. Primer sets for nested RT–PCR and real-time RT–PCR.

Nested RT-PCR				
Application	Name*	Direction	Sequence (5' to 3')	Expected size (bp)
ORF1a set				
1st PCR	NIID_WH-1_F501	Sense	TTCGGATGCTCGAACTGCACC	413
1st PCR	NIID_WH-1_R913	Antisense	CTTTACCAGCACGTGCTAGAAGG	
2nd PCR	NIID_WH-1_F509	Sense	CTCGAACTGCACCTCATGG	346
2nd PCR	NIID_WH-1_R854	Antisense	CAGAAGTTGTTATCGACATAGC	
Sequencing	NIID_WH-1_Seq_F519	Sense	ACCTCATGGTCATGTTATGG	
Sequencing	NIID_WH-1_Seq_R840	Antisense	GACATAGCGAGTGTATGCC	
S protein set				
1st PCR	WuhanCoV-spk1-f	Sense	TTGGCAAAATTCAAGACTCACTTT	547
1st PCR	WuhanCoV-spk2-r	Antisense	TGTGGTTCATAAAAATTCCTTTGTG	
2nd PCR	NIID_WH-1_F24381	Sense	TCAAGACTCACTTTCTTCCAC	493
2nd PCR	NIID_WH-1_R24873	Antisense	ATTTGAAACAAAGACACCTTCAC	
Sequencing	NIID_WH-1_Seq_F24383	Sense	AAGACTCACTTTCTTCCACAG	
Sequencing	NIID_WH-1_Seq_R24865	Antisense	CAAAGACACCTTCACGAGG	

Real-time RT-PCR			
Name	Sequence (5’ to 3’)	Position**	Concentration
Nucleocapsid protein set no. 1			
N_Sarbeco_F1	CACATTGGCACCCGCAATC	28706-28724	600nM
N_Sarbeco_R1	GAGGAACGAGAAGAGGCTTG	28833-28814	800nM
N_Sarbeco_P1	FAM-ACTTCCTCAAGGAACAACATTGCCA-BHQ***	28753-28777	200nM
Nucleocapsid protein set no. 2 (N2)			
NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	29125-29144	500nM
NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC	29282-29263	700nM
NIID_2019-nCOV_N_R2ver3	TGGCACCTGTGTAGGTCAAC		
NIID_2019-nCOV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ***	29222-29241	200nM

*: Positions in primer name refer to sequence MN908947.1.

**: Positions refer sequence MN908947.3.

***: TAMRA quencher also shows equal reactivity.

Fig. 1

a)

ORF1a set

GGG**TTCGGATGCTCGAACTGCACC**TCATGGCAACAATGCACAGGCTCT
ATCCAAATTAGCTAGCGAGCTATCTAATACTTTTGGTGCTATTTCCGCCT
CTATTGGAGACATCATACAACGTCTTGATGTTCTCGAACAGGACGCCCA
AATAGACAGACTTATTAATGGCCGTTTGACAACACTAAATGCTTTTGTT
GCACAGCAGCTTGTTCGTTCCGAATCAGCTGCTCTTTCCGCTCAATTGG
CTAAAGCTATGTCGATAACAACTTCTG**CCTTCTAGCACGTGCTGGTAAA**
GTTTATTGACACTAAGTTTTTTTTTTTTTTT

NIID_WH-1_F501 NIID_WH-1_F509
MERS-CoV(JX869059.2, 24536-24755)
NIID_WH-1_R854 **NIID_WH-1_R913**

S protein set

GGG**TTGGCAAAATTCAAGACTCACTTT**CTTCCACCAACAATGCACAGG
CTCTATCCAAATTAGCTAGCGAGCTATCTAATACTTTTGGTGCTATTTCCG
CCTCTATTGGAGACATCATACAACGTCTTGATGTTCTCGAACAGGACGC
CCAAATAGACAGACTTATTAATGGCCGTTTGACAACACTAAATGCTTTT
GTTGCACAGCAGCTTGTTCGTTCCGAATCAGCTGCTCTTTCCGCTCAAT
TGGCTAAAGATAAAGTCAATGAGTGTGTCAAGGCACAAGTGAAGGTGT
CTTTGTTTCAAAT**CACAAAGGAATTTTTATGAACCACA**
TTTATTGACACTAAGTTTTTTTTTTTTTTT

WuhanCoV-spk1-f NIID_WH-1_F24381
MERS-CoV(JX869059.2, 24536-24785)
NIID_WH-1_R24873 **WuhanCoV-spk2-r**

T is replaced by U.

Fig. 1

b)

N set no.1

GGGACTTCCCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACT
GAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCT
GCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGC
CAAAAGGATCCAGCTAGCGCATTGGATCTCGCGGCAGTCAAGCCTCTT
CTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGC
AGCAGTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGAT
GCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAGAGCA
AAATGTCTGGTAAAGGC
358 base

N set no.2 (N2)

GGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCA
GGAACTAATCAGACGGATCCAGCTAGCGCATTGGATCTCGCAAATTG
CACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATT
GGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGCTG
CCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTT
TGCTGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGC
CTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACC
GCAGAGACAG
337 base

Forward **Probe** **Reverse** **PC check (VIC)** **BamH I**

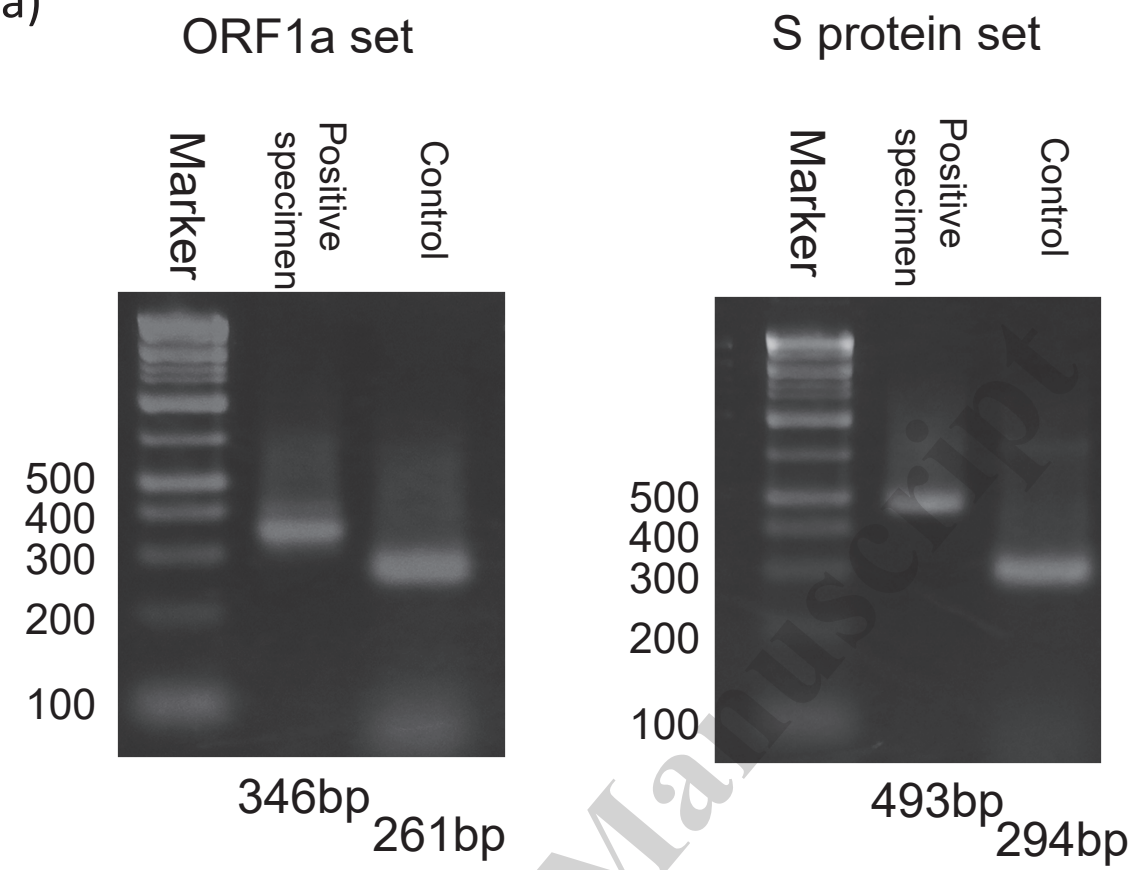
T is replaced by U.

Figure 1. Positive control sequences for a) nested RT–PCR, and b) real-time RT–PCR.

Primer sequences are shown in different colors.

Fig. 2

a)



b)

	Viral RNA				Control RNA		
Copies	N	N2	N2 ver.3	Copies	N	N2	N2 ver.3
5000	34.5	31.9	31.7	5000	29.7	28.4	28.4
500	37.7	34.8	34.3	500	32.9	31.4	31.3
50	39.3	37.1	35.2	50	35.6	34.5	34.3
5	>40	39.5	39.3	5	36.5	36.6	35.7

Figure 2. Images of amplification. a) Electrophoresis after second PCR. Second PCR amplicons were visualized with 4% agarose gel electrophoresis and ethidium bromide staining. b) Validation assay using the N2 ver.3 reverse primer. Number shows the average Cq value of three independent tests.