INTRODUCTION

Middle East respiratory syndrome (MERS) is an emerging respiratory disease caused by the MERS coronavirus (MERS-CoV). MERS has been endemic mainly in Saudi Arabia since 2012 (1,2). As of August 26, 2019, a total of 2,458 confirmed cases, with 849 deaths, have been reported from 27 countries (World Health Organization [WHO], https://www.who.int/emergencies/mers-cov/en/). According to the case definition of the WHO, amplification of at least two different genomic targets is required for positive diagnosis (WHO, http://www.who.int/csr/disease/coronavirus_infections/case_definition/en/index.html). Therefore, the likelihood of positive diagnosis of the MERS-CoV increases as the number of sensitive genetic diagnostic methods used increases. Various genetic assays have been developed, such as real-time reverse transcription polymerase chain reaction (RT-PCR) (3,4), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (5,6), and reverse transcription recombinase polymerase amplification (RT-RPA) (7). Of these, the real-time RT-PCR assays developed by Corman et al. are widely used (these are the WHO standard), wherein a primer/probe set targeting the upE region of the MERS-CoV genome is used for the first screening test and a set targeting the open reading frame (ORF) 1a region is used for the confirmation test (3,4). In the Corman assay, positive results in both tests are required for a positive diagnosis of MERS-CoV.

Real-time RT-PCR assays are commonly used to detect the genomes of pathogens; this wide prevalence is one of their advantages. However, conventional laboratory real-time RT-PCR assays require large laboratory instruments, and amplification takes approximately 2 h. These disadvantages limit rapid diagnosis. Here, an ultra-rapid real-time RT-PCR test was established comprising a multiplex assay for upE and ORF1a running on a mobile PCR1100 device. As few as five copies of the MERS-CoV RNA can be detected within 20 min using the standard WHO assays in the mobile PCR device, with the sensitivity and specificity being similar to those of a conventional real-time PCR instrument such as the LightCycler, thereby enabling timely intervention to control MERS-CoV infection.
case of the conventional real-time RT-PCR.

Recently, an ultra-rapid mobile PCR device, the PicoGene PCR1100 (Nippon Sheet Glass, Tokyo, Japan), has been developed. This device enables ultra-rapid real-time PCR by moving the reaction mix through air pressure between areas of two different temperatures in the device through shallow grooves in the chips (i.e., there is no requirement to increase or decrease temperature), thereby facilitating rapid execution of a (maximally) three-color multiple real-time RT-PCR assay. Here, we developed an ultra-rapid real-time RT-PCR system featuring the multiplex Corman assay on the PCR1100 for rapid detection of the MERS-CoV.

MATERIALS AND METHODS

Viruses: The MERS-CoV EMC strain was kindly provided by Dr. Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. Human orthopneumoviruses [respiratory syncytial virus (RSV), Long, A2, B WW/14617/85 (B1 wild type), and 18537] were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human metapneumovirus (HMPV; Sendai-H/2404/2003) was obtained from the Virus Research Center, Sendai Medical Center, Japan. Human coronavirus (HCoV)-229E isolates ATCC VR-740, Sendai-H/1121/04, and Niigata/01/08 (12) were used. HCoV-NL63 (Amsterdam I) was supplied by Dr. Lia van der Hoek, University of Amsterdam, The Netherlands. HCoV-OC43 ATCC VR-1558 was used. SARS coronavirus (the Frankfurt strain) was supplied by Dr. J. Ziebuhr, University of Würzburg, Germany. Human respiriviruses [parainfluenza viruses (PIV) 1 (strain C35) and 3 (strain C243)] were obtained from ATCC. Adenoviruses (ADVs) (serotype 3, strain G.B.; serotype 4, strain RI-67; and serotype 7, strain Gomen) were also obtained from ATCC. The viruses were propagated and titrated using HEp-2, HeLa, RD, Vero, VeroE6, LLC-MK2, or Vero/TMPRSS2 cells (13); otherwise, copy numbers were calculated using real-time RT-PCR (14). Influenza viruses [Flu; A/California/7/2009 (H1N1pdm), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008] were propagated and titrated using MDCK cells. Clinical isolates of HCoV-OC43 (Tokyo/SGH-36/2014, LC315646; Tokyo/SGH-61/2014, LC315647; Tokyo/SGH-06/2015, LC315648), HKU1 (Tokyo/SGH-15/2014, LC315650; Tokyo/SGH-18/2016, LC315651), and NL63 (Tokyo/SGH-15/2017, LC488390; Tokyo/SGH-18/2018, LC488389; Tokyo/SGH-24/2018, LC488388) were isolated and propagated using human bronchial tracheal epithelial cells (Lifeline Cell Technology, Frederick, MD, USA), cultured, and allowed to differentiate at an air-liquid interface, as previously described (15). Copy numbers were calculated using virus-specific real-time RT-PCR (16).

Extraction of nucleic acids from viral stocks: RNA was extracted using TRIzol LS, the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) from viral stocks, and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used for extractions from specimens, according to the manufacturers’ instructions. Viral DNA was extracted using the SimplePrep Reagent for DNA (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer’s instructions. Nasopharyngeal swab (NPS), nasal swab (NS), sputum, and bronchoalveolar lavage (BAL) specimens were obtained from Discovery Life Sciences (Los Osos, CA, USA). These were used in spike tests with the approval of the Research and Ethical Committee for the Use of Human Subjects of the National Institute of Infectious Diseases, Japan (approval #1003). For spike tests, infectious MERS-CoV was mixed with clinical specimens at various concentrations together with an RNase inhibitor and subjected to RNA extraction. A positive control RNA of ribonuclease (RNase) P, transcribed from T7 promoter-monoribinated PCR template, was also used in the validation tests.

Real-time RT-PCR: Corman assays (targeting the upE and ORF1a regions) were used to detect the MERS-CoV (3,4), using the following primers and probes: upE-forward, 5′-GCAACGGCGAGTTAGTT-3′; upE-reverse, 5′-GCTCTTACACGGGGACCCATA-3′; upE-probe, 5′-CTCTTCATATAATCGCCCCGAGCTCG-3′; ORF1a-forward, 5′-CCACTACTCCCCATTTGTCAGC-3′; ORF1a-reverse, 5′-CAGTATGTTAGTGCGCATATAAGCA-3′; and ORF1a-probe, 5′-TTGCAAATGGTTCGCCCCACT-3′. To detect single targets, both probes were labeled with fluorescein amide (FAM) and carboxytetramethylrhodamine ( TAMRA). Single-target detection was performed using the AgPath-ID One-Step RT-PCR reagents (Thermo Fisher Scientific) on a LightCycler 480 (Roche, Basel, Switzerland) or LightCycler 96 (Roche) system, following the manufacturer’s instructions. The amplification conditions of Corman et al. were utilized (3,4). For multiplex detection, at least one probe was labeled with FAM and ORF1a probe with Cy5, and Black Hole Quencher (BHQ) was used to quench both reactions. The RNase P gene served as the internal control, using the following primer/probe set described previously (17): forward, 5′-AGTATTGGACCTGTGGGACG-3′; reverse 5′-GAGCCGGCCTGCTCCACAAGT-3′; and probe HEX-5′-TTCGTGACCTGAAAGGGCTCGGC-3′-BHQ.

An ultra-rapid real-time RT-PCR assay employed the components of the KAPA3G Plant PCR Kit (KAPA Biosystems, Wilmington, MA, USA). FastGene Scriptase II (NIPPON Genetics, Tokyo, Japan) or SMART MMLV Reverse Transcriptase (TaKaRa Bio) served as the reverse transcriptase (RT). The components of the reaction mixture and the primer/probe mixtures are shown in Table 1. The real-time RT-PCR conditions were set for the PCR 1100: 55°C for 180 s; 95°C for 15 s; and 50 cycles of 95°C for 5 s, and 60°C for 7 s. The longest reaction time displayed on the device screen was 19 min, 17 s. For the displayed reaction time, the PCR 1100 device estimated the longest required time as 2 min, 15 s for initial stabilization of block temperatures. The time depends on the environment of the test performed. If the block temperatures are stabilized within a shorter duration than 2 min, 15 s, the total reaction time also becomes shorter. The assay sensitivity was calculated using the Reed-Muench method.
RESULTS

The PCR1100 is a very small real-time PCR device powered by an alternating current supply or a portable battery (Fig. 1A). For PCR reactions, specific chips with hollow grooves are required (Fig. 1B). Reagents are applied after peeling the front cover seal (Fig. 1B, i), which is subsequently resealed. The chip is set in place after peeling the back cover seal (Fig. 1B, ii). The device comprises three heated panels (Fig. 1C). Panel 1 is used for the RT reaction. PCR proceeds through back-and-forth movements between panels 2 and 3. The PCR mixture moves through the grooves by air pressure; the air is first passed through air filters on the chip (Fig. 1B, iii). The PCR is performed under closed conditions to prevent environmental contamination. Fluorescence is

Table 1. Components of the PCR1100 RT-PCR mixture

| Component                | Concentration | Volume (μL) | Final concentration |
|--------------------------|---------------|-------------|---------------------|
| 2× buffer (Plant 3G kit) | 0.4 mM dNTPs and 1.5 mM MgCl₂ | 10.0 | dNTPs, 0.2 mM |
| MgCl₂ (Plant 3G kit)     | 25 mM         | 1.35       | MgCl₂, 3.1875 mM   |
| Primer/probe mix         | See table on the right side | 0.8 | |
| Reverse transcriptase    | 200 U/μL      | 1.15       | 23 U/μL            |
| DNA polymerase (Plant 3G kit) | 2.5 U/μL | 1.5 | 0.375 U/μL |
| Dithiothreitol (DTT; RT kit) | 100 mM | 0.2 | 1 mM |
| RNA                      | 5.0           |            | 5.0                |
| Total                    | 20            |            | 20                 |

Table 2. Components of primer/probe mix

| Component                  | Volume of 100 mM stock (μL) | Final concentration (nM) |
|----------------------------|-----------------------------|--------------------------|
| upE forward                | 0.085                       | 425                      |
| upE reverse                | 0.145                       | 725                      |
| upE probe                  | 0.08                        | 400                      |
| ORF1a forward              | 0.085                       | 425                      |
| ORF1a reverse              | 0.145                       | 725                      |
| ORF1a probe                | 0.08                        | 400                      |
| RNase P forward            | 0.04                        | 200                      |
| RNase P reverse            | 0.08                        | 400                      |
| RNase P probe              | 0.06                        | 300                      |
| Total                      | 0.8                         |                          |

Fig. 1. (Color online) Photographs of the PicoGene PCR1100 (panels, A-D). A) A photograph of the front. The device is approximately 20 cm in length. The PCR results are shown on the central screen. B) A photograph of a PCR1100 chip; a shallow groove is apparent. The reagent insertion slot is covered by the front seal (i). The vent filters (iii) are covered by the back seal (ii). C) The three heating panels. Panel 1 is used for RT. PCR proceeds via back-and-forth movements between panels 2 and 3. The PCR mixture is moved through the grooves by air pressure. D) The monitor screen image after amplification. The amplifications are plotted in three colors. Blue shows FAM, green shows VIC, and red shows Cy5 fluorescence. Currently, Japanese is the only display language of the PCR1100.
monitored as the mixture moves between panels 2 and 3 (Fig. 1C). The amplifications can be observed in real time on the monitor screen and the cycle thresholds are calculated automatically (Fig. 1D).

First, we evaluated sensitivity (Table 2). The upE and ORF1a primer/probe sets detected MERS-CoV RNA at the level of several copies after a singleplex reaction using a general real-time RT-PCR instrument (the LightCycler), as previously described (3,4). Using the ultra-rapid real-time RT-PCR, multiplex assays detecting upE, ORF1a, and RNase P simultaneously detected the internal control and MERS-CoV RNA, and the sensitivity for viral RNA was approximately five copies. We next evaluated specificity (Table 3). As Corman et al. reported, the upE and ORF1a primer/probe sets did not cross-react with any other respiratory pathogens in the singleplex reactions (3,4). After the

| Copies/reaction | 5,000 | 500 | 50 | 5 | 0.5 | 0.05 |
|-----------------|-------|-----|----|---|-----|------|
| **LightCycler (singleplex)** |       |     |    |   |     |      |
| upE             | 6/6²  | 6/6 | 6/6 | 3/6 | 6/6 | 0/6  |
| ORF1a           | 6/6   | 6/6 | 6/6 | 5/6 | 0/6 | 0/6  |
| **PCR1100 (multiplex)** |       |     |    |   |     |      |
| upE             | 3/3   | 3/3 | 3/3 | 5/6 | 0/6 | 0/3  |
| ORF1a           | 3/3   | 3/3 | 3/3 | 3/6 | 1/6 | 0/3  |
| RNase P¹        | 3/3   | 3/3 | 3/3 | 6/6 | 6/6 | 3/3  |

¹: Each sample contained 45,000 copies of control RNA (encoding RNase P).
²: Positive/number.

| Virus Strain | Amount / reaction | PCR1100 | upE | ORF1a | RNase P¹ |
|--------------|------------------|---------|-----|-------|----------|
| MERS-CoV     |                  |         |     |       |          |
| EMC          | 1×10⁵ copies     | +       | +   | +     |          |
| SARS-CoV     |                  | -       | -   |       |          |
| Frankfurt    | 1×10⁴ TCID₅₀     |         |     |       |          |
| HCoV-229E    | VR-740           | 2.5×10⁴ PFU | -   |       | +        |
| Sendai-H/1121/04 | 5×10⁴ PFU |         | -   |       | +        |
| Niigata/01/08 | 2×10⁴ PFU       |         | -   |       | +        |
| HCoV-NL63    | Amsterdam I      | 1×10⁵ FFU | -   |       | +        |
| Tokyo/SGH-15/2017 | 6.2×10⁴ copies |         | -   |       | +        |
| Tokyo/SGH-18/2018 | 3.3×10⁴ copies |         | -   |       | +        |
| Tokyo/SGH-24/2018 | 5.1×10⁴ copies |         | -   |       | +        |
| HCoV-OC43    | VR-1558          | 2.5×10⁴ TCID₅₀ | -   |       | +        |
| Tokyo/SGH-36/2014 | 2×10⁴ copies |         | -   |       | +        |
| Tokyo/SGH-61/2014 | 1×10⁴ copies |         | -   |       | +        |
| Tokyo/SGH-06/2015 | 1×10⁴ copies |         | -   |       | +        |
| HCoV-HKU1    | Tokyo/SGH-15/2014 | 5×10⁴ copies | -   |       | +        |
| Tokyo/SGH-18/2016 | 5×10⁴ copies |         | -   |       | +        |
| Other respiratory viruses |          |         |     |       |          |
| RSV, Long    | 5×10⁴ copies     | -       | -   |       | +        |
| RSV, A2      | 1×10⁶ copies     | -       | -   |       | +        |
| RSV, CH/18537| 5×10⁴ copies     | -       | -   |       | +        |
| RSV, B1      | 1×10⁶ copies     | -       | -   |       | +        |
| Human metapneumovirus | Sendai-H/3404/2003 | 1.2×10⁶ PFU | -   |       | +        |
| Human respirovirus 1 | PIV1, C-35 | 1.2×10⁷ PFU | -   |       | +        |
| Human respirovirus 3 | PIV3, C-243 | 1×10⁷ PFU | -   |       | +        |
| Human adenovirus 3 | G.B. | 2×10⁷ TCID₅₀ | -   |       | +        |
| Human adenovirus 4 | RI-67 | 2×10⁷ TCID₅₀ | -   |       | +        |
| Human adenovirus 7 | Gomen | 2×10⁷ TCID₅₀ | -   |       | +        |
| Influenza viruses |          |         |     |       |          |
| H1N1pdm      | A/California/7/2009 | 4×10⁷ TCID₅₀ | -   |       | +        |
| H3N2         | A/Victoria/210/2009 | 1.25×10⁷ TCID₅₀ | -   |       | +        |
| B            | B/Brisbane/60/2008 | 1.25×10⁷ TCID₅₀ | -   |       | +        |

¹: Each sample contained 50,000 copies of control RNA (encoding RNase P).
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Spike tests: As MERS-CoV-positive clinical specimens are not available in Japan, spike tests were performed using mixtures of the MERS-CoV EMC isolates and clinical specimens. NPS and NS specimens were used to mimic the tests with upper respiratory tract specimens, and sputum and the BAL specimens to mimic the tests with lower respiratory tract specimens. As shown in Table 4, the detection kinetics of the single- and multiplex assays are similar, along with their sensitivities. These results suggest that the ultra-rapid real-time RT-PCR assay is capable of detecting MERS-CoV with a sensitivity similar to that of the conventional real-time RT-PCR assay, including in specimens from the upper and lower respiratory tract.

DISCUSSION

We developed an ultra-rapid real-time RT-PCR assay (a multiplex Corman assay) to detect MERS-CoV using a mobile PCR device, the PicoGene PCR1100. The assay detected MERS-CoV RNA with a sensitivity and specificity equivalent to those of the conventional real-time PCR instruments, even in clinical specimens. The Corman assay for MERS-CoV detection is the standard WHO assay (https://www.who.int/csr/disease/coronavirus_infections/mers-laboratory-testing/en/).

We found that the standard WHO assay for MERS-CoV detection can be performed within 20 min, without compromising quality.

The greatest advantage afforded by the PCR1100 is a reduced total reaction time, which is mediated by moving the reaction mixture between two regions of different temperatures, i.e., no change in temperature is needed, thereby reducing the assay duration. The Corman assays used TaqMan probes (3,4). The KAPA3G DNA polymerase was used for performing the ultra-rapid real-time RT-PCR, as this was the only polymerase tested that could drive the TaqMan assay during ultra-rapid real-time RT-PCR on the PCR1100. In the TaqMan assay, probe removal through the 5′ to 3′ exonuclease activity of Taq DNA polymerase is essential (18), but it is a time-consuming process. We found that the KAPA3G DNA polymerase reduced the extension time, i.e., the probe-disassembly time, to 7 s. The denaturing step required 5 s because the upper temperature limit of the PCR1100 was 95°C. Thus, the time required for one TaqMan RT-PCR cycle was 12 s. To maintain the sensitivity, the number of PCR cycles was increased to 50, but the reduced PCR-cycle time enabled the assay to conclude within 20 min, including 180 s for the RT reaction.

In this study, RNA purified with an extraction kit was used as a template. The ultra-rapid real-time RT-PCR reduced the reaction time from approximately 2 h to less than 20 min; however, RNA extraction remained time-consuming for both the ultra-rapid and conventional real-time RT-PCR. Currently, RNA extraction for real-time RT-PCR can be performed with a commercial

Table 4. Results of spike tests

| Specimen derived from | Copies/reaction | 1,000 | 100 | 10 | 1 | Sensitivity (copies) |
|-----------------------|----------------|-------|-----|----|---|---------------------|
| NPS \(^1\)            | Single         | upE   | N.T. | 4/4 | 3/4 | 0/4 | 5.6 |
|                       |                | ORF1a | N.T. | 4/4 | 2/4 | 0/4 | 10.0 |
| Multiple              | upE            | N.T.  | 6/6 | 3/6 | 0/6 | 10.0 |
|                       | ORF1a          | N.T.  | 6/6 | 5/6 | 0/6 | 4.6  |
|                       | RNase P        | N.T.  | 6/6 | 6/6 | 6/6 |     |
| NS \(^2\)             | Single         | upE   | N.T. | 4/4 | 1/4 | 0/4 | 17.8 |
|                       |                | ORF1a | N.T. | 4/4 | 0/4 | 0/4 | 31.6 |
|                       | RNase P        | N.T.  | 6/6 | 6/6 | 6/6 |     |
| Sputum                | Single         | upE   | 4/4 | 2/4 | 0/4 | N.T. | 100 |
|                       |                | ORF1a | 4/4 | 1/4 | 0/4 | N.T. | 178 |
|                       | RNase P        | 6/6 | 6/6 | 6/6 | 6/6 |     |
| BAL \(^3\)            | Single         | upE   | N.T. | 4/4 | 0/4 | 0/4 | 31.6 |
|                       |                | ORF1a | N.T. | 4/4 | 1/4 | 0/4 | 17.8 |
|                       | RNase P        | N.T.  | 6/6 | 6/6 | 6/6 |     |

\(^1\): Nasopharyngeal swab.
\(^2\): Nasal swab.
\(^3\): Bronchoalveolar lavage.
\(^4\): ORF is abbreviation of Open reading frame.
\(^5\): Not Tested.
\(^6\): Positive results/total.

ultra-rapid real-time RT-PCR, the multiplex assays also exhibited no cross-reactions with the genomes of any other respiratory viral pathogens. Thus, the sensitivity and specificity of the mobile device were equivalent to those of the LightCycler singleplex reaction, even when multiplex reactions were concluded within 20 min.
simple extraction buffer, such as CellAMP (TaKaRa-Bio) and RealTime ready Cell Lysis Kit (Roche). These buffers enable subsequent real-time RT-PCR analysis after a brief mixing procedure. Therefore, the total time for the diagnostic test for MERS-CoV can be shortened in combination with these types of simple extraction buffers, which is worth considering.

We have developed RT-LAMP assays for the rapid detection of MERS-CoV within 30 min (5,6). Based on the WHO case definition, detection of at least two distinct genomic targets is required for a positive diagnosis. Therefore, the target regions for the two RT-LAMP primer sets differ from those of the two Corman assays; a positive result in at least two of these four tests fulfills the WHO criteria. However, in Japan, the detection method for MERS-CoV is restricted to “PCR” based on a notification from the Ministry of Health, Labour and Welfare. Therefore, regardless of whether a MERS-CoV positive case fulfills the WHO criteria, if the detection method is not PCR, the case results cannot be considered positive in Japan. Importantly, containment measures for a patient with MERS-CoV-positive begin when the diagnosis test results fulfill the WHO criteria; however, the result of the Corman assay is required for an official announcement of a MERS case. Therefore, acceleration of the Corman assay has been validated in this study as much as possible. We believe that the findings of this study will be helpful for other countries in which Corman assays are the only method approved by the authorities.

As described above, a rapid diagnostic method is essential for the detection and isolation of superspreaders. The ultra-rapid real-time RT-PCR assay will enable timely intervention by accelerating administrative decisions. The viral titer is higher in the lower than in the upper respiratory tracts; thus, specimens from the lower tract are recommended for diagnosis of MERS-CoV (19–21). The ultra-rapid real-time RT-PCR assay detected MERS-CoV RNA not only in upper respiratory tract specimens but also in lower respiratory tract specimens. Furthermore, upon detection of MERS-CoV infection, the WHO strongly recommends repeated sampling of multiple bodily compartments (including the upper respiratory tract) for further PCR testing after PCR confirmation (WHO, https://www.who.int/csr/disease/coronavirus_infections/ mers-laboratory-testing/en/). It is important to determine the duration of viral shedding when implementing nosocomial infection control. Such testing is also essential to determine when a hospitalized patient with MERS can be discharged. Our ultra-rapid real-time RT-PCR assay will aid in performing numerous repeated tests by reducing the effort and valuable time of all staff involved in MERS control measures.

Acknowledgments We thank Ms. Miyuki Kawase for technical support. This work was supported by a Grant-in-Aid (Research Program on Emerging and Re-emerging Infectious Diseases, no. 19k0108030j0403, from the Japan Agency for Medical Research and Development (AMED); a Grant-in-Aid for Scientific Research (no. B:17H04642) from the Japan Society for the Promotion of Science; and a Grant-in-Aid from the Kawano Masanori Memorial Foundation for the Promotion of Pediatrics (no. 30-8).

Conflict of interests None to declare.

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