Human orthopneumovirus, which is also known as the respiratory syncytial virus (RSV), is a primary causative agent of respiratory tract infections in children worldwide. Preterm children or those with underlying cardiopulmonary disorders are at a particularly high risk of developing severe and lethal RSV respiratory tract infections (1-3). Elderly and immunocompromised individuals are also susceptible to infections causing severe respiratory disease, highlighting the importance of preventing nosocomial and in-house RSV infections in hospitals and care centers for elderly people (4,5). The World Health Organization (WHO) has started the discussion for the global surveillance system for RSV, based on the global influenza surveillance and response system initiated in 2015. The US Centers for Disease Control and Prevention (CDC) has developed a genetic detection method based on real-time reverse transcription polymerase chain reaction (RT-PCR), which is used in global RSV surveillance. In Japan, immunoassay-based rapid antigen detection kits are widely used for the detection of RSV. In this study, an ultra-rapid real-time RT-PCR method for the rapid detection of RSV was developed using the PCR1100 device based on the US CDC assay in order to detect RSV in comparable time to rapid test kits. The ultra-rapid real-time RT-PCR could detect RSV viral RNA in less than 20 min while maintaining sensitivity and specificity comparable to conventional real-time RT-PCR using large installed instruments. Furthermore, combining ultra-rapid real-time RT-PCR with the M1 Sample Prep kit reduced the total working time for the detection of RSV from clinical specimen to less than 25 min, suggesting this method could be used for point-of-care RSV testing.

Human orthopneumovirus, which is also known as the human respiratory syncytial virus (RSV), is a leading cause of respiratory tract infections in children worldwide. The World Health Organization has taken steps toward establishing a global surveillance system for RSV, based on the global influenza surveillance and response system initiated in 2015. The US Centers for Disease Control and Prevention (CDC) has developed a genetic detection method based on real-time reverse transcription polymerase chain reaction (RT-PCR), which is used in global RSV surveillance. In Japan, immunoassay-based rapid antigen detection kits are widely used for the detection of RSV. In this study, an ultra-rapid real-time RT-PCR method for the rapid detection of RSV was developed using the PCR1100 device based on the US CDC assay in order to detect RSV in comparable time to rapid test kits. The ultra-rapid real-time RT-PCR could detect RSV viral RNA in less than 20 min while maintaining sensitivity and specificity comparable to conventional real-time RT-PCR using large installed instruments. Furthermore, combining ultra-rapid real-time RT-PCR with the M1 Sample Prep kit reduced the total working time for the detection of RSV from clinical specimen to less than 25 min, suggesting this method could be used for point-of-care RSV testing.
reverse (250 nM), 5′-TCTTTTTCTAGGACATTGTAYTGAAACAG-3′; probe (50 nM), 5′-FAM-CTGTGTATGTGGAGCCTTCGTGAAGCT-BHQ-3′ (8). The conventional real-time RT-PCR was performed using the AgPath-ID One-Step RT-PCR reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the reactions were run on a LightCycler 96 system (Roche, Basel, Switzerland), using the following reaction conditions: 50°C for 600 sec; 95°C for 600 sec; and 40 cycles of 95°C for 15 sec and 58°C for 60 sec. To avoid false-positive results, Cp values less than 35 were considered positive signals. For RSV subgrouping, the method described by Kuypers et al. (10) was used, with the minor modification of using 900 nM of each primer and 200 nM of the probe. The RSV in each sample was classified into the A or B subgroup, based on the group with the lowest Cp value. For ultra-rapid real-time RT-PCR, the cycling conditions and reaction components were as previously described (9) except for the primer concentrations; the RNase P gene was still used as the internal control. The KAPA3G Plant PCR kit (KAPA Biosystems, Wilmington, MA, USA) and FastGene Scriptase II (Nippon Genetics Co., Ltd. Tokyo, Japan) were used. The primer and probe concentrations were as follows: RSV forward, 688.9 nM; RSV probe, 688.9 nM; RSV probe P forward, 266.7 nM; RNase P reverse, 533.3 nM; RNase P probe, 400 nM.

The sensitivity of the assays was validated using RNA extracted from typical laboratory viral strains (Table 1). Using conventional real-time RT-PCR, 7.3–15.8 copies of viral RNA could be detected. By contrast, 0.9–15.8 copies of viral RNA could be detected using ultra-rapid real-time RT-PCR, suggesting that the reaction time could be reduced maintaining the sensitivity of the assay. On some occasions, the US CDC assay provided false-positive results when conventional real-time RT-PCR was used; therefore, the cut-off value was set to Cp < 35 to eliminate such false-positive findings (Table 1). Conversely, ultra-rapid real-time RT-PCR as run using the PCR1100 device provided no false-positive findings (Table 1) and no cross-reactivity to other respiratory viruses, including clinical isolates, was observed (Table 2).

Subsequently, the ultra-rapid real-time RT-PCR method was validated using clinical specimens. Nasal and nasopharyngeal (NP) swabs from patients with respiratory infections, including those of RSV, were obtained from Discovery Life Sciences (Los Osos, CA, USA) and used after approval from the ethics committee of our institute (approval no. 1001). As shown in Table 3, both conventional real-time RT-PCR and ultra-rapid real-time RT-PCR could detect RSV RNA in specimens from patients diagnosed with an RSV infection. Moreover, no cross-reactivity was observed in specimens from patients diagnosed with other respiratory viruses. These findings suggest that the performance of the ultra-rapid real-time RT-PCR is sufficient for use in practical use.

RNA extraction is most commonly conducted using a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) or TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA, USA). The conventional real-time RT-PCR was performed using the AgPath-ID One-Step RT-PCR reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the reactions were run on a LightCycler 96 system (Roche, Basel, Switzerland), using the following reaction conditions: 50°C for 600 sec; 95°C for 600 sec; and 40 cycles of 95°C for 15 sec and 58°C for 60 sec. To avoid false-positive results, Cp values less than 35 were considered positive signals. For RSV subgrouping, the method described by Kuypers et al. (10) was used, with the minor modification of using 900 nM of each primer and 200 nM of the probe. The RSV in each sample was classified into the A or B subgroup, based on the group with the lowest Cp value. For ultra-rapid real-time RT-PCR, the cycling conditions and reaction components were as previously described (9) except for the primer concentrations; the RNase P gene was still used as the internal control. The KAPA3G Plant PCR kit (KAPA Biosystems, Wilmington, MA, USA) and FastGene Scriptase II (Nippon Genetics Co., Ltd. Tokyo, Japan) were used. The primer and probe concentrations were as follows: RSV forward, 688.9 nM; RSV probe, 688.9 nM; RSV probe P forward, 266.7 nM; RNase P reverse, 533.3 nM; RNase P probe, 400 nM.

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Table 1. The sensitivities of the ultra-rapid real-time RT-PCR for RSV

| Target | Positive/number |
|--------|----------------|
| **Long** |                |
| LightCycler RSV | 3/3            |
| PCR1100 RSV | 4/4            |
| RNSpase P | 4/4            |
| **A2** |                |
| LightCycler RSV | 3/3            |
| PCR1100 RSV | 4/4            |
| RNSpase P | 4/4            |
| **CH18537** |            |
| LightCycler RSV | 3/3            |
| PCR1100 RSV | 4/4            |
| RNSpase P | 4/4            |
| **B1** |                |
| LightCycler RSV | 3/3            |
| PCR1100 RSV | 4/4            |
| RNSpase P | 4/4            |
| **NC** |                |
| LightCycler RSV | 0/16           |
| PCR1100 RSV | 0/12           |
| **Sensitivity (copies)** | |
| 500 | 50 | 5 | 0.5 |

1: Each sample contained 500 copies of control RNA (encoding RNase P). 2: Negative control (NC) is water contains 10 μg/ml of Yeast RNA as carrier.
Scientific). Therefore, even if the reaction time is reduced to 20 min, RNA extraction remains a time-consuming process. To overcome this, the M1 Sample Prep Cartridge kit for RNA (Biomeme, Philadelphia, PA, USA) was used. This kit enables the extraction of RNA from specimens within 5 min only by pumping action of filter-attached syringe. To obtain RNA from clinical specimens, a sample volume of 400 μL was sufficient for rapid RNA extraction, and the sample was assayed immediately using ultra-rapid real-time RT-PCR. Twenty specimens (11 subgroup-B, 7 subgroup-A, and 2 non-infected specimens) were tested; using this method, we obtained 18 positive and 2 negative results. Thus, combining ultra-rapid real-time RT-PCR with the M1 Sample Prep kit reduced the total working time to less than 25 min without compromising the performance

| Virus Type | Virus | Strain | Amount/reaction | RSV | RNaseP |
|------------|-------|--------|-----------------|-----|--------|
| Pneumoviruses | Human orthopneumovirus | RSV/A/NIID/2347/14 | 5 × 10^4 copies | + | + |
| | | RSV/A/NIID/2367/14 | 5 × 10^4 copies | + | + |
| | | RSV/A/NIID/2470/14 | 5 × 10^4 copies | + | + |
| | Human metapneumovirus (hMPV) | Sendai-H/3404/2003 | 1.2 × 10^9 PFU | - | + |
| | | IA10-2003 | 7 × 10^4 IU | - | + |
| | | Sendai/0256/2015 | 1.9 × 10^5 IU | - | + |
| | | Sendai/414/2013 | 2.7 × 10^5 FFU | - | + |
| | | Sendai/1052/2011 | 2.8 × 10^3 FFU | - | + |
| Other respiratory viruses | Human respirovirus 1 (Parainfluenzavirus 1, PIV1) | PIV1, C-35 | 1.2 × 10^2 PFU | - | + |
| | | PIV1, NIID/79081/1/2019 | 1.3 × 10^3 copies | - | + |
| | Human respirovirus 3 (Parainfluenzavirus 3, PIV3) | PIV3, C-243 | 2 × 10^4 PFU | - | + |
| | Human Rubulavirus 2 (Parainfluenzavirus 2, PIV2) | PIV2, NIID/56606/2/2019 | 7.3 × 10^2 TCID₅₀ | - | + |
| | Influenza virus (Flu) | H1N1pdm | A/California/7/2009 | 4 × 10^3 TCID₅₀ | - | + |
| | | H3N2 | A/Victoria/210/2009 | 1.25 × 10^5 TCID₅₀ | - | + |
| | | B | B/Brisbane/60/2008 | 1.25 × 10^3 TCID₅₀ | - | + |
| | Human adenovirus 3 (ADV3) | G.B. | 2 × 10^4 TCID₅₀ | - | + |
| | Human adenovirus 4 (ADV4) | RI-67 | 2 × 10^4 TCID₅₀ | - | + |
| | Human adenovirus 7 (ADV7) | Gomen | 2 × 10^4 TCID₅₀ | - | + |
| MERS-CoV | | EMC | 5 × 10^5 copies | - | + |
| Severe acute respiratory syndrome coronavirus (SARS-CoV) | SARS-CoV-2 | Japan/AU/004/2020 | 5 × 10^4 copies | - | + |
| | | Japan/TY/WK-501/2020 | 5 × 10^4 copies | - | + |
| | | Japan/TY/WK-521/2020 | 5 × 10^4 copies | - | + |
| Human coronavirus 229E (HCoV-229E) | VR-740 | 3.7 × 10^9 copies | - | + |
| | Human coronavirus NL63 (HCoV-NL63) | Sendai-H/1121/04 | 5 × 10^4 PFU | - | + |
| | Human coronavirus OC43 (HCoV-OC43) | Amsterdam I | 3.6 × 10^5 copies | - | + |
| | | Tokyo/Sgh/15/2017 | 3.1 × 10^4 copies | - | + |
| | Human coronavirus HKU1 (HCoV-HKU1) | Tokyo/Sgh/18/2016 | 5 × 10^3 copies | - | + |

*: Each sample contained 500 copies of control RNA (encoding RNase P).
of the assay. These findings suggest that this method could be used for point-of-care RSV testing instead of the currently used immunological test kits.

Currently, the cost of real-time RT-PCR assay is more expensive than immune-based rapid test kits, so it is required further efforts to cut it. However, the sensitivity of real-time RT-PCR assay is extraordinary higher than immune-based rapid test kits. For the correct diagnosis and surveillance, the transition to use real-time RT-PCR-based technique will be required in Japan.

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Conflict of interest None to declare.

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| Virus diagnosed | Swab source | Virus diagnosed | Subgroup/serotype | Positive/Test numbers |
|-----------------|-------------|----------------|-------------------|----------------------|
| RSV             | Nasal       | RSV            | A                 | 4/4                  |
|                 |             |                | B                 | 16/16                |
| RSV             | NP          | RSV            | A                 | 6/6                  |
|                 |             |                | B                 | 26/26                |
| ADV             | NP          | ADV            | C                 | 0/2                  |
| hMPV            | NP          | hMPV           | 0/2               |
| Flu             | NP          | Flu            | A                 | 0/2                  |
| PIV1            | NP          | PIV1           | 0/2               |
| PIV2            | NP          | PIV2           | 0/2               |
| PIV3            | NP          | PIV3           | 0/2               |