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Development of fluorescent reverse transcription loop-mediated isothermal amplification (RT-LAMP) using quenching probes for the detection of the Middle East respiratory syndrome coronavirus

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ABSTRACT
Clinical detection of Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) in patients is achieved using genetic diagnostic methods, such as real-time RT-PCR assay. Previously, we developed a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for the detection of MERS-CoV [Virol J. 2014. 11:139]. Generally, amplification of RT-LAMP is monitored by the turbidity induced by precipitation of magnesium pyrophosphate with newly synthesized DNA. However, this mechanism cannot completely exclude the possibility of unexpected reactions. Therefore, in this study, fluorescent RT-LAMP assays using quenching probes (QProbes) were developed specifically to monitor only primer-derived signals. Two primers sets (targeting nucleocapsid and ORF1a sequences) were constructed to confirm MERS cases by RT-LAMP assay only. Our data indicate that both primer sets were capable of detecting MERS-CoV RNA to the same level as existing genetic diagnostic methods, and that both were highly specific with no cross-reactivity observed with other respiratory viruses. These primer sets were highly efficient in amplifying target sequences derived from different MERS-CoV strains, including camel MERS-CoV. In addition, the detection efficacy of QProbe RT-LAMP was comparable to that of real-time RT-PCR assay using clinical specimens from patients in Saudi Arabia. Altogether, these results indicate that QProbe RT-LAMP assays described here can be used as powerful diagnostic tools for rapid detection and surveillance of MERS-CoV infections.

1. 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 (Assiri et al., 2013; Azhar et al., 2014). As of 15 March 2018, there have been 2144 confirmed cases, with 750 deaths, reported from 27 countries [The World Health Organization (WHO), Global Alert and Response (GAR), Coronavirus infections, updated on 15 March 2018, http://www.who.int/csr/don/15-march-2018-mers-oman/en/].

Abbreviations: ADV, adenovirus; ATCC, American Type Culture Collection; BIP, backward inner primer; CoV, coronavirus; FFU, focus forming unit; FIP, forward inner primer; HBoV, human bocavirus; HCoV, human coronavirus; MERS, Middle East respiratory syndrome; MPV, metapneumovirus; N, nucleocapsid; ORF, open reading frame; PBS, phosphate-buffered saline; PIV, parainfluenza virus; PFU, plaque forming unit; QProbe or QP, quenching probe; RSV, respiratory syncytial virus; RT-LAMP, reverse transcription-loop-mediated isothermal amplification; TCID50, 50% tissue culture infectious dose; upE, upstream E

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According to the case definition of the WHO, at least two distinct genomic targets are required for a positive diagnosis [WHO, GAR, Revised interim case definition for reporting to WHO – Middle East respiratory syndrome coronavirus (MERS-CoV), updated on 3 July 2013, http://www.who.int/csr/disease/coronavirus_infections/case_definition/en/index.html]. Therefore, many genetic diagnostic methods have been developed for the stable and reliable diagnosis of MERS-CoV infections. Currently, the main diagnostic method of MERS-CoV is real-time RT-PCR assays, and the primer/probe sets [upE and open reading frame (ORF) 1a] developed by Corman et al. are widely used as standard assays (Corman et al., 2012a,b).

The loop-mediated isothermal amplification (LAMP) method amplifies specific nucleotide sequences using a set of four or six unique primers (Nagamine et al., 2002; Notomi et al., 2000). This method is relatively quick and user-friendly; amplification signals can be readily detected within an hour, and it only requires a single incubation temperature. As such, various LAMP assays have been developed for the detection of a wide range of pathogens, such as bacteria (Adhikari et al., 2009; Geojith et al., 2011; Ueda and Kuwabara, 2009), parasites (Arimatsu et al., 2012; Wang et al., 2010), and viruses (Hong et al., 2004; Imai et al., 2006; Mahony et al., 2013; Shirato et al., 2007; Ushio et al., 2005) including MERS-CoV (Bhadrav et al., 2015; Lee et al., 2016; Shirato et al., 2014).

A reverse transcription (RT)-LAMP assay for the detection of MERS-CoV was developed by our group recently (Shirato et al., 2014), which employs a primer set targeting the viral nucleocapsid (N) sequence, comparable to standard real-time RT-PCR assays. In the LAMP assay, positive signals are indicated by turbidity that results from magnesium pyrophosphate precipitation following LAMP reaction. However, in this mechanism, the possibility of unexpected signals derived from primer dimer and/or non-primer reactions cannot be excluded (Nijiri, 2012). There is also a possibility of detecting turbidity if the host-derived DNA makes LAMP product non-specifically. Thus, if unexpected signals are detected, it is very difficult to identify the origin of the signal. The validity of MERS-CoV detection by previous RT-LAMP assay has been confirmed (Shirato et al., 2014), but the mechanism of turbidity detection can be improved.

Fluorescence dye (calcine, etc.) or DNA intercalator can be added for fluorescence monitoring (http://looppamp.eken.co.jp/c/tech/detect_index.html), which may help to improve turbidity detection. The addition of DNA intercalator was used in Zika virus detection (Kuroski et al., 2017). However, the detection principle of these methods is the same as turbidity detection. Using fluorescence labeled primer can solve the problem of non-primer-derived signals. Recently, Fowler et al. (2016) reported RT-LAMP assays for detection of vesicular stomatitis, foot and mouth diseases, and swine vesicular disease viruses using fluorescence labeled forward inner primers (FIPs) or backward inner primers (BIPs). However, this study used 5’ end-labeled primers. Therefore, if the fluorescent primer causes non-specific extension at the 3’ end, unexpected signals will be detected. To avoid non-specific signals, melting curve analysis of the LAMP amplicon is useful to confirm amplification of the targeted sequence (Fowler et al., 2016; Kuroski et al., 2017). However, melting curve analysis requires incubation of at a higher temperature than that LAMP, and requires additional time after amplification, which negates the main advantage of LAMP.

In this study, to address these problems, a quenching probe 3G (QProbe) was used for monitoring RT-LAMP. In QProbe, the fluorescence dye is labeled at the 3’ end of the primer. Therefore, the extension of the primer sequence is blocked by dye even if the primer anneals non-specifically at its 3’ end. Use of QProbe can detect primer-derived signals only, and thus can avoid detecting non-specific amplification caused by fluorescent primer. In addition, to validate a positive MERS-CoV diagnosis, an additional primer set (targeting the ORF1a region) for use in QProbe RT-LAMP assays was developed to enable to confirm MERS cases only by QProbe RT-LAMP.

2. Materials and methods

2.1. Viruses

MERS-CoV EMC strain was kindly provided by Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, the Netherlands. MERS-CoV was propagated and titrated using Vero cells. Human respiratory syncytial viruses (RSV; Long, A2, B WV/14617/85 and 18537) were obtained from the American Type Culture Collection (ATCC). 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 and Sendai-H/1121/04 (Shirato et al., 2012) were used. HCoV-NL63 was supplied by Dr. Lia van der Hoek, University of Amsterdam, the Netherlands. HCoV-OC43 isolate ATCC VR-1558 was used. SARS coronavirus (Francfurt strain) was supplied by Dr. J. Ziebauer, University of Würzburg, Germany. Human parainfluenza viruses (PIV) 1 (strain C53) 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 obtained from ATCC. Viruses were propagated and titrated using HEp-2, HeLa, RD, Vero cells, or LLC-MK2 cells (Shigirane et al., 2008). 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) and HKU1 (Tokyo/SGH-15/2014, LC315050: Tokyo/SGH-18/2016, LC315051) were isolated and propagated using human bronchial tracheal epithelial cells (Lifeline Cell Technology, Frederick, MD, USA) that were cultured and differentiated at the air–liquid interface.

2.2. Construction of primers for QProbe RT-LAMP

For amplification of the N sequences, the primer set reported previously was utilized (Shirato et al., 2014). The primer set for the amplification of the ORF1a region was constructed using the online LAMP primer design software (PrimerExplorer V4; http://primerexplorer.jp/e/) based on the sequence of the MERS-CoV EMC strain (GenBank JX869059.2). The nucleotide sequence and concentration of primers used in each reaction are listed in Table 1. For the detection of the RT-LAMP reaction by fluorescent signals, the QProbe was used (Nippon Steel & Sumikin Eco-Tech Corp., Tsukuba, Japan) (Tani et al., 2009). For primer sets targeting N and ORF1a, QProbes were constructed based on LB primers, and several nucleotides were added to LB primers (Table 1). The final reaction mixture contained 1 pmol of QProbe-LBs and the six general MERS-CoV primers.

2.3. Extraction of nucleic acids from virus stocks

RNA was extracted from viral stocks using TRizol LS, TRizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or MagnaPure Compact Nucleic Acid Isolation kit (Roche, Basel, Switzerland), according to the manufacturer’s instructions. Viral DNA was extracted using the SimplePrep Reagent for DNA (Takara-Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Total RNA and genomic DNA were quantified using standard methods of measuring the OD value. For sensitivity assays, to isolate RNA from virion only, Vero cells were infected with MERS-CoV, and incubated for 4 days. Cell supernatants were then collected and centrifuged at 1500 × g for 30 min at 4°C, and the supernatants were treated with RNaseA (Nippongene, Tokyo Japan) at a concentration of 10 μg/ml for 30 min at 37°C to exclude non-viral RNA as previously reported (Shirato et al., 2014). The MERS-CoV RNA copy number was calculated based upon the standard curve generated by real-time RT-PCR assay using the upE primer set (Corman et al., 2012a) and a positive control RNA template. Total RNAs were diluted in
To evaluate the sensitivities of each primer set for detection of various target sequences, point mutations were introduced into the N and ORF1a sequences on plasmid by site-direct mutagenesis. The control RNA transcripts with the incorporated mutations were generated as described above.

To synthesize the control RNA for amplification of ORF1a region, the EMC strain sequence (1000-2000) was subcloned into pGEM-T Easy vector (Promega). The resultant RNA transcripts were quantified based on the OD value, and the copy number was calculated. The RNA was diluted in ribonuclease-free water containing 10μg/mL of yeast RNA. RT-PCR was performed using upE and ORF1a primer sets (Corman et al., 2012a,b) were performed using a QuantiTect Probe RT-PCR kit (Qiagen) and a LightCycler 480 or LightCycler96 Instrument (Roche) as per the manufacturers' instruction. The amplification conditions as previously reported were utilized (Corman et al., 2012a,b).

### 2.5. Real-time RT-PCR

Real-time RT-PCR assays using upE and ORF1a primer sets (Corman et al., 2012a,b) were performed using a QuantiTect Probe RT-PCR kit (Qiagen) and a LightCycler 480 or LightCycler96 Instrument (Roche) as per the manufacturers' instruction. The amplification conditions as previously reported were utilized (Corman et al., 2012a,b).

### 2.6. Processing of clinical specimens

All experiments using human clinical specimens were approved by the Research and Ethical Committee for the Use of Human Subjects of the National Institute of Infectious Diseases, Japan (Approval #746); the Ethical Committee of Showa General Hospital (Approval #REC-094); and the Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Kingdom of Saudi Arabia (Approval #121-16). Clinical specimens diagnosed to be positive for other respiratory pathogens were used for the evaluation of non-specific reaction in MERS-CoV-negative specimens. From January 2014 to February 2016, 19 nasopharyngeal aspirates, secretions, or swabs were collected from patients presenting with influenza-like illnesses at the outpatient pediatrics clinic of Showa General Hospital. Parents or legal guardians of all children/minor participants provided written informed consent. Specimens were sent to the Special Infectious Agents Unit, King Abdulaziz University, Kingdom of Saudi Arabia, using lyophilized reaction mixtures in 12 stripe tubes and an ESEQuant TS2 tube scanner (Qiagen). The specimens were tested for MERS-CoV using the QProbe RT-LAMP assay, the protocol described above.

The QProbe RT-LAMP assay using MERS-CoV positive specimens was performed in the Special Infectious Agents Unit, King Abdulaziz University, Jeddah, Saudi Arabia, using lyophilized reaction mixtures in 12 stripe tubes and an ESEQuant TS2 tube scanner (Qiagen). Specimens used for validation were archived specimens collected from MERS cases since 2014. These were stored at −80°C until testing. Total RNA (5μL) extracted from MERS-CoV-positive specimens that were pre-tested by real-time RT-PCR was mixed with 20μL of RT-PCR-grade water (Thermo Fisher Scientific), and was subsequently added to each well in
Fig. 1. a) Schematic representation of quenching probe (QProbe). QProbe is labeled with fluorescent dye at the cytosine residue at the 3’ end. When the QProbe hybridizes with the target, fluorescence is quenched by the guanine residue present in the target sequence. b) Images of detecting fluorescence quenching. Fluorescence RT-LAMP (N and ORF1a) was performed with serially diluted MERS-CoV viral RNA using the LightCycler480 instrument. The wavelength used for signal detection is the same as FAM. Negative signal is represented by an upper line. Positive signal is represented by a reverse S-shaped curve. NC, negative control.
QP: Quenching probe.

the tube strip, and then used for MERS-CoV detection. Quenching signals were detected using the ESEQuant TS2 tube scanner at 63 °C for 30 min.

3. Results

3.1. Sensitivity of the QProbe RT-LAMP assay

The detection principle of QProbe is shown as in the schematic diagram of Fig. 1; fluorescence from the fluorophore bound to the cytosine residue at the 3′ end of the QProbe is quenched by the guanine residue present in the target sequence during hybridization (Fig. 1a). The positive signal is shown as quenching of fluorescence, which generates a reverse sigmoid curve (Fig. 1b). In contrast, negative signals due to the lack of fluorescence quenching generate a straight line (Fig. 1b). The detection limit of the QProbe RT-LAMP assay was determined using serially diluted MERS-CoV RNA templates and was evaluated in comparison to those of real-time RT-PCR (upE and ORF1a) and RT-LAMP (turidity) assays (Table 2). Although target regions of QProbe RT-LAMP assays were different from real-time RT-PCR assays, the validation was performed using copy number-determined viral RNA, and each amplification was performed using the same samples. As reported previously, both real-time RT-PCR and RT-LAMP assays are capable of detecting MERS-CoV RNA at a copy level as low as 20 (Corman et al., 2012a, b; Shirato et al., 2014). As shown in Table 2, QProbe RT-LAMP assays, which targeted N and ORF1a sequences, were able to detect MERS-CoV RNA at a similar level, comparable to real-time RT-PCR and RT-LAMP. These data indicate that the sensitivity of QProbe RT-LAMP assays is similar to that of existing genetic diagnostic methods.

3.2. Specificity of the QProbe RT-LAMP assays

Next, the specificity of QProbe RT-LAMP was determined using various respiratory virus isolates (Table 3). For both N and ORF1a primer sets, no cross reaction was detected with other respiratory pathogens included in this study. Similarly, no cross-reactivity was observed in the QProbe RT-LAMP assay where clinical specimens positive for other respiratory pathogens (determined by real-time RT-PCR) were utilized (Table 4). These data demonstrate that QProbe RT-LAMP possessed a high specificity for the diagnosis of MERS-CoV.

To evaluate the accuracy of QProbe RT-LAMP in detecting MERS-CoV from human specimens, QProbe RT-LAMP assays were performed using seven total RNAs extracted from clinical specimens that were initially confirmed to be MERS-CoV-positive by real-time RT-PCR (upE) (Table 5). Two MERS-CoV negative specimens were used as negative controls. Taking into consideration the recent MERS case occurrence rate, it was difficult to obtain fresh specimens; therefore, stored specimens were used for validation. Specimens deemed to be positive had quantification cycle values of 20.2–30.9 for the upE set. Using the N and ORF1a primer sets, QProbe RT-LAMP confirmed a positive diagnosis for all seven positive samples and a negative diagnosis for the other two. In short, the QProbe RT-LAMP assays developed in this study were capable of detecting MERS-CoV from human clinical specimens.

3.3. Validations for mismatched sequences

The primer sets utilized in this study were constructed based on the conserved region of the N protein and ORF1a from the MERS-CoV EMC strain. However, significant genetic variations are present in these viral genomic regions as demonstrated by the large amount of sequences registered in GenBank. As shown in Table 1, 300 and 278/9 variations of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the FIP primers had a high mismatch rate in the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4). A comparison of the identities of nucleotide sequences for N and ORF1a have been registered in the database, with the majority of them showing a complete match to our primers (see Supplemental figure). In particular, the F2 region; the identities were 81.7% for N set and 86.0% for ORF1a set (Table 4).
Developed, which include RT-LAMP (Bhadra et al., 2015; Shirato et al., 2012a,b). However, PCR amplification involves a relatively long run-time and may be unsuitable for field-based studies. Recently, real-time RT-PCR assays have been proven to be highly sensitive and specific; therefore, they are easy to use, and they do not require large equipment for processing. RT-LAMP assays are more suitable for field-based studies. As demonstrated using clinical specimens (Table 5), this assay could be run in a portable device (e.g., ESEQuant TS2) and be completed in 30 min or less for accurate diagnosis of MERS-CoV. Real-time RT-PCR is the most commonly used technique for the detection and confirmation of MERS-CoV infection. According to the case definition outlined by the WHO, positive amplification of at least two different virus-specific genomic targets is required for case confirmation. Two real-time RT-PCR assays were developed by Corman et al., using primer sets targeting upE and ORF1a region. These assays have been proven to be highly sensitive and specific; therefore, they are used as the standard diagnostic method for MERS-CoV (Corman et al., 2014) and reverse transcription isothermal recombinase polymerase amplification (RT-RPA) (Wahed et al., 2013). In this study, the QProbe RT-LAMP targeted different positions in the MERS-CoV genome [ORF1a (nt 1572–1753) and N] from Corman’s assays [ORF1a (nt 18265–18314) and upE]. Two positives in the QProbe RT-LAMP or real-time PCR assays are enough, to confirm the presence of MERS-CoV. However, this means if the specimen is positive in two of four sets, it can be considered positive for MERS-CoV; if one of the real-time RT-PCR assay is negative, one positive QProbe RT-LAMP is sufficient for case confirmation, and vice versa. Thus, these techniques have improved the sensitivity and diagnostic outcomes of MERS-CoV by increasing the number of viral genomic targets available for amplification.

The results of RT-LAMP can be detected at the endpoint by checking magnesium pyrophosphate precipitation or fluorescent signal generated by DNA intercalators under ultraviolet light (Mori et al., 2001). Because they are easy to use, and they do not require large equipment for processing, RT-LAMP assays are more suitable for field-based studies. However, in turbidity monitoring, the salt accumulation accompanied by the LAMP reaction can be induced by primer dimers and/or non-primer reactions (Njiru, 2012). It is possible to detect unexpected increase in turbidity derived from non-primer signal, such as fragments of host DNA. Therefore, in this study, we developed a fluorescent RT-LAMP method with the addition of QProbes, in which only fluorescence quenching-derived from the probes was measured as a positive signal (Kurata et al., 2001). As such, QProbes provide additional specificity for detection as they bind to unique nucleotides that are only present in the target sequence and amplicon by LAMP primers. This means that a positive signal in the QProbe RT-LAMP assay is dependent on the primer reaction only. In addition, labeling the 3′ end with fluorescence dye abrogates non-specific signals derived from primers because extension of the QProbe is physically blocked by the dye. Thus, QProbe allows for highly specific detection under isothermal conditions and in a short time without melting curve analysis. Furthermore, materials used in QProbe RT-LAMP assays can be prepared as lyophilized form and packaged into diagnostic kits, increasing product integrity during shipment and handling. As demonstrated using clinical specimens (Table 5), this assay could be run in a portable device (e.g., ESEQquant TS2) and be completed in 30 min or less for accurate diagnosis of viruses.

### Table 4
**QProbe RT-LAMP assays using clinical specimens positive for other respiratory viruses.**

| Specimen Type | Detected viruses | Primer set |
|--------------|------------------|------------|
| Number | Name | Cq | Name | Cq | Name | Cq |
| 1 | Nasal secretion | HCoV-HKU1 | 23.7 | ADV2 | 33.1 | Rhino | 31.6 |
| 2 | Nasal aspiration | HCoV-OC43 | 18.3 | RSV A | 34.3 | ADV4 | 24.8 |
| 3 | Nasal secretion | HBoV | 25.1 | Rhino | 26.9 |
| 4 | Nasal aspiration | PIV3 | 28.9 | Rhino | 19.7 |
| 5 | Nasal aspiration | RSV B | 21.0 | HBoV | 31.9 |
| 6 | Nasal aspiration | ADV2 | 28.3 | Rhino | 19.7 |
| 7 | Nasal aspiration | PIV4 | 32.0 |
| 8 | Nasal aspiration | RSV B | 19.5 |
| 9 | Nasal aspiration | HBoV | 25.0 |
| 10 | Nasal aspiration | ADV2 | 27.2 |
| 11 | Nasal swab | FluA, H3 | 19.1 |
| 12 | Nasal secretion | FluA, H1pdm | 22.2 |
| 13 | Nasal swab | FluA, H1pdm | 18.6 |
| 14 | Nasal aspiration | FluA, H3 | 20.6 |
| 15 | Nasal aspiration | FluA | 21.0 |
| 16 | Nasal aspiration | HBoV | 27.2 |
| 17 | Nasal swab | FluA, H1pdm | 22.2 |
| 18 | Nasal swab | FluA, H1pdm | 18.6 |
| 19 | Nasal aspiration | FluA | 20.6 |
| 20 | Nasal aspiration | FluA | 21.0 |
| 21 | Nasal aspiration | FluA | 27.2 |
| 22 | Nasal aspiration | FluA | 19.1 |
| 23 | Nasal aspiration | FluA | 22.2 |
| 24 | Nasal aspiration | FluA | 18.6 |
| 25 | Nasal aspiration | FluA | 20.6 |
| 26 | Nasal aspiration | FluA | 21.0 |
| 27 | Nasal aspiration | FluA | 27.2 |
| 28 | Nasal swab | FluA, H1pdm | 22.2 |
| 29 | Nasal swab | FluA, H1pdm | 18.6 |
| 30 | Nasal swab | FluA | 20.6 |
| 31 | Nasal swab | FluA | 21.0 |
| 32 | Nasal swab | FluA | 27.2 |

Cq: quantification cycle value.

### Table 5
**QProbe RT-LAMP assays using clinical specimens positive for MERS-CoV viruses.**

| Specimen Type | Real-time RT-PCR | QProbe RT-LAMP |
|---------------|------------------|---------------|
| No. | upE | Cq | Primer set |
| 1 | + | 20.2 | + |
| 2 | + | 26.3 | + |
| 3 | + | 23.4 | + |
| 4 | + | 30.6 | + |
| 5 | + | 30.9 | + |
| 6 | + | 22.7 | + |
| 7 | + | 25.8 | + |
| 8 | + | > 40 | - |
| 9 | - | > 40 | - |

Cq: quantification cycle value.

B2 region (G29018T) slightly altered the amplification efficiency of RT-LAMP, leading to a five-fold decrease in detection sensitivity (Shirato et al., 2014). In contrast, the amplification efficiency in the QProbe RT-LAMP assays was not affected by mismatch in this region (Table 6). These findings indicate that the QProbe RT-LAMP assays could be used for the detection of all MERS-CoV isolates reported thus far, including for camels and humans.

### Discussion

Real-time RT-PCR is the most commonly used technique for the detection and confirmation of MERS-CoV infection. According to the case definition outlined by the WHO, positive amplification of at least two different virus-specific genomic targets is required for case confirmation. Two real-time RT-PCR assays were developed by Corman et al., using primer sets targeting upE and ORF1a region. These assays have been proven to be highly sensitive and specific; therefore, they are used as the standard diagnostic method for MERS-CoV (Corman et al., 2012a,b). However, PCR amplification involves a relatively long running process and may be unsuitable for field-based studies. Recently, other genetic diagnostic methods using different mechanisms have been developed, which include RT-LAMP (Bhadra et al., 2015; Shirato et al., 2014) and reverse transcription isothermal recombinase polymerase amplification (RT-RPA) (Wahed et al., 2013). In this study, the QProbe RT-LAMP targeted different positions in the MERS-CoV genome [ORF1a (nt 1572–1753) and N] from Corman’s assays [ORF1a (nt 18265–18314) and upE]. Two positives in the QProbe RT-LAMP or real-time PCR assays are enough, to confirm the presence of MERS-CoV. However, this means if the specimen is positive in two of four sets, it can be considered positive for MERS-CoV; if one of the real-time RT-PCR assay is negative, one positive QProbe RT-LAMP is sufficient for case confirmation, and vice versa. Thus, these techniques have improved the sensitivity and diagnostic outcomes of MERS-CoV by increasing the number of viral genomic targets available for amplification.

The results of RT-LAMP can be detected at the endpoint by checking magnesium pyrophosphate precipitation or fluorescent signal generated by DNA intercalators under ultraviolet light (Mori et al., 2001). Because they are easy to use, and they do not require large equipment for processing, RT-LAMP assays are more suitable for field-based studies. However, in turbidity monitoring, the salt accumulation accompanied by the LAMP reaction can be induced by primer dimers and/or non-primer reactions (Njiru, 2012). It is possible to detect unexpected increase in turbidity derived from non-primer signal, such as fragments of host DNA. Therefore, in this study, we developed a fluorescent RT-LAMP method with the addition of QProbes, in which only fluorescence quenching-derived from the probes was measured as a positive signal (Kurata et al., 2001). As such, QProbes provide additional specificity for detection as they bind to unique nucleotides that are only present in the target sequence and amplicon by LAMP primers. This means that a positive signal in the QProbe RT-LAMP assay is dependent on the primer reaction only. In addition, labeling the 3′ end with fluorescence dye abrogates non-specific signals derived from primers because extension of the QProbe is physically blocked by the dye. Thus, QProbe allows for highly specific detection under isothermal conditions and in a short time without melting curve analysis. Furthermore, materials used in QProbe RT-LAMP assays can be prepared as lyophilized form and packaged into diagnostic kits, increasing product integrity during shipment and handling. As demonstrated using clinical specimens (Table 5), this assay could be run in a portable device (e.g., ESEQquant TS2) and be completed in 30 min or less for accurate diagnosis of viruses.
Table 6
Sensitivity of QProbe RT-LAMP using sequence with mismatches to the N primer set.

| Position† | Accession No. | Sensitivity (copies) |
|-----------|---------------|---------------------|
| C28862T   | KM210278, KM210277, KM015348 | 15.8 |
| C28862T, T28880C | KJ782550 | 7.3 |
| C28865T, T29000C | KX108943 | 7.3 |
| C28865T, T28880C | KT368867, KT368866 | 7.3 |
| T28880C | KU710265, KU710264, KT777351, KT777350, KT61628, KT606055, KT368890, KT368887, KT368857, KT368856, KT368855, KT368854, KT368853, KT368852, KT368851, KT368850, KT368849, KT368848, KT368847, KT368846, KT368845, KT368844, KT368843, KT368832, KT368831, KT368830, KT368829, KT368828, KT368827, KP769415, KP223131, KM044034, KM044033, KM044032, KM027261, KM027260, KM027259, KM027258, KM027257, KM027256, KM027255, KJ692965, KJ712396, KJ713295, KJ650998 | 15.8 |
| T28880C, A28889G | KT368875 | 3.4 |
| T28928G | KT368834 | 3.4 |
| T28958C | KJ156905 | 15.8 |
| G28976A | KT368865, KT368864, KT368863, KT368862, KT368861, KT368860, KT368859, KT368858 | 3.4 |
| C28982T | KJ77102 | 15.8 |
| C28996T | KT121581, KT121580, KT121579, KT121578, KT121577, KT121576, KT121575, KT121574, KT121573, KT121572, KM027262, KJ813439, KP961221 | 15.8 |
| G29018T | KJ556336, KJ156944, KJ156883, KF958702, KF917527 | 15.8 |
| G29018A | KT368826 | 3.4 |
| C29021T | KT368873, JX869059 (QProbe) | 15.8 |
| C29021T | JX869059 (turbidity) | 7.3 |

Camel MERS-CoV sequences are indicated in bold.
† Based on EMC isolate (JX869059.2).

Table 7
Sensitivity of QProbe RT-LAMP using sequences with mismatches to the ORF1a primer set.

| Position† | Accession No. | Sensitivity (copies) |
|-----------|---------------|---------------------|
| C1604T    | KX108942, KX108941, KX108940, KX108939, KX108938, KX108937, KU242424, KU242423, KT751244, KT156561, KT156560, KP199393, KP199392, KP199391, KP199390, KP199298, KP199297, KP209313, KP209312, KP209311, KP209310, KP209309, KP209308, KP209307, KP209306, KJ650297, KJ650296, KJ650295, KJ650294, KJ361503, KJ361502, KJ361501, KJ361500, KJ361499, KJ156896, KJ156863, KF745068 | 7.3 |
| A1650C    | KX108947 | 1.6 |
| A1650G, C165ST | KX108944, KT368875, KT368874, KT368873, KT368872, KT368870, KT368869, KR012126, KR012125, KR012124, KR012123, KR012122, KJ713299, KJ713298, KJ713297, KJ713296, KJ713295 | 7.3 |
| C1685T    | KT861628, KT368824, KM027257, KJ556336, KJ156949, KJ156944, KJ156938, KJ156881, KF958702, KF917527 | 15.8 |
| C1696T    | KT368826 | 15.8 |
| T1718C    | KX108943 | 1.6 |
|          | JX869059 (QProbe) | 7.3 |

Camel MERS-CoV sequences are indicated in bold.
† Based on EMC isolate (JX869059.2).
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Competing interests
The authors declare that they have no competing interests.

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Appendix A. Supplementary data
Supplementary material related to this article can be found in the online version, at doi:10.1016/j.jviromet.2018.05.006.

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