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Detection of murine norovirus by reverse transcription loop-mediated isothermal amplification

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A B S T R A C T

Murine norovirus (MNV) has considerable genetical and biological diversity and is recognized worldwide as the most common contaminant in laboratory mouse colonies. This study developed a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method with the potential to detect a broad range of MNV. RT-LAMP, using a set of five primers containing mixed bases, obtained results under isothermal conditions at 62 °C for 90 min. Sensitivity of RT-LAMP was 50-fold less than that of two-step TaqMan real-time reverse transcription-polymerase chain reaction (TaqMan RT-PCR). Diagnostic performance of RT-LAMP on RNA extracted from mouse fecal specimens was compared with TaqMan RT-PCR and nested RT-PCR. MNV was detected in 54 of 120 mouse fecal specimens by RT-LAMP, and RT-LAMP had an estimated sensitivity and specificity of 96.4% and 100% compared with TaqMan RT-PCR, and 94.7% and 100% compared with nested RT-PCR. RT-LAMP, which does not require expensive instruments, might be useful for the screening of mice actively or persistently infected with MNV.

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1. Introduction

Murine norovirus (MNV), a member of the genus *Norovirus* in the family *Caliciviridae*, is a non-enveloped virus with positive sense single-stranded genomic RNA. The virus MNV-1 was the first strain discovered in severely immunocompromised RAG2/STAT1$^{-/-}$ mice (Karst et al., 2003). To date, many strains of MNV have been isolated from laboratory mouse colonies (Hsu et al., 2006; Müller et al., 2007; Thackray et al., 2007; Barron et al., 2011; Smith et al., 2012). In North America, 22.1% of 12,639 mouse sera were positive for anti-MNV-1 antibodies (Hsu et al., 2005). The high prevalence of MNV in laboratory mice was also confirmed by serological survey in Europe and Japan (Pritchett-Corning et al., 2009; Kitagawa et al., 2010). Currently, MNV is recognized as the most common contaminant in laboratory mice, and thought to be prevalent in mouse colonies worldwide. Biological diversity among MNV strains has also been reported. Clone CW3 of MNV-1 was rapidly cleared in wild-type C57BL/6 mice, while C57BL/6 mice were infected persistently with MNV-CR1, MNV-CR3, MNV-CR6, MNV-CR7, and MNV-WU11 (Thackray et al., 2007).

For modern biomedical research, laboratory mice are required to be free from infectious agents that may cause research interference. However, the effects of MNV infection on biomedical research are still unclear. MNV-CR6 infection had no significant effect on the immune response to vaccinia virus and influenza A virus infection (Hensley et al., 2009). MNV-4 infection did not affect bacteria-induced inflammatory bowel disease (IBD) in Smad3$^{-/-}$ mice, although the infection accelerated IBD progression in Mdr1a$^{-/-}$ mice (Lencioni et al., 2011). MNV-1 inhibited CD8 T cell responses to immunodominant murine cytomegalovirus (MCMV) epitopes in both BALB/c and C57BL/6 mice. However, MNV-1 did not affect MCMV titers in either mouse strain (Doom et al., 2009). Atg16L1^{HM}

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mice infected with MNV-CR6 exhibited multiple hallmarks of human Crohn's disease after dextran sodium sulfate (DSS) administration as opposed to mice infected with MNV-1.CW3 (Cadwell et al., 2010). Since MNV infection may interfere in experiments and the data analysis, the use of MNV-free laboratory mice is appropriate for biomedical research.

To detect MNV with sensitivity and specificity, reverse transcription-polymerase chain reaction (RT-PCR)-based assays have been developed. The end-point detection methods including nested RT-PCR are the most common in molecular diagnostic tests (Hsu et al., 2006; Ward et al., 2006; Compton, 2008; Kitajima et al., 2009; Ohsugi et al., 2013; Tajima et al., 2013). However, they are time-consuming, laborious and inconvenient. Real-time RT-PCR is a simple, rapid and sensitive technique compared to end-point RT-PCR (Baert et al., 2008; Belliot et al., 2008; Kitajima et al., 2010). In spite of that, the requirement for an expensive and sophisticated instrument and the higher running costs prevent its use becoming popular.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP), a nucleic acid amplification technique, was reported for the first time in 2000 (Notomi et al., 2000). Molecular detection with RT-LAMP replacing RT-PCR has been reported for the rapid diagnosis of various RNA viruses such as coronaviruses (Hong et al., 2004; Hanaki et al., 2013), human norovirus (Fukuda et al., 2006), and influenza viruses (Jayawardena et al., 2007; Kubo et al., 2010; Dinh et al., 2011). RT-LAMP is considered a simple, rapid, and cost-effective method compared to end-point RT-PCR and real-time RT-PCR.

RT-LAMP requires a primer set including a pair of outer primers (F3 and B3) and a pair of inner primers (FIP [F1c-F2] and BIP [B1c-B2]) that recognize six distinct regions on a target sequence (Notomi et al., 2000). Additionally, one or two loop primers (LF and/or LB) are also designed to accelerate the LAMP reaction (Nagamine et al., 2002). The intricate primer set can be designed automatically using online primer design software programs such as PrimerExplore (Fujitsu Limited). However, the programs have difficulty in designing a set of broadly reactive LAMP primers for an RNA virus consisting of many variants. To solve this problem, multiple sets of LAMP primers have been used to detect RNA viruses (Fukuda et al., 2006; Dinh et al., 2011; Zhang et al., 2011). In this study, a set of LAMP primers with the potential to detect MNV isolates that have been reported worldwide were designed manually, and the broad reactivity and sensitivity of RT-LAMP were compared with those of TaqMan RT-PCR. Finally, the diagnostic performance of RT-LAMP was examined using RNA extracted from mouse fecal specimens.

2. Materials and methods

2.1. Viruses and RAW264 cell line

MNV-1.CW1, obtained from American Type Culture Collection (ATCC number: PTA-5935), and MNV-S7-PP3, isolated from a laboratory mouse in The University of Tokyo, Bunkyo, Tokyo, Japan (Kitagawa et al., 2010) were used in this study. The RAW264 cell line (RCB0535), provided by the RIKEN BioResource Center (RIKEN BRC, Tsukuba, Ibaraki, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, was maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Osaka, Japan) supplemented with 10% (v/v) fetal calf serum (FBS; Thermo Scientific, Yokohama, Kanagawa, Japan), 2 mM L-glutamine and 50 μg/ml gentamicin at 37 °C in humidified 5% CO_2. MNV-1.CW1 and MNV-S7-PP3 were propagated by infecting subconfluent monolayers of RAW264 cells in DMEM, respectively. After 4-days infection, the cells were frozen and thawed twice, followed by centrifugation at 8000 × *g* for 5 min. The supernatants were stored at −80 °C until use.

The infectious titer was determined by an endpoint titration assay using RAW264 cells cultured in 48-well culture plates. A 200-μl aliquot of RAW264 cells (1×10^6 cells/ml) prepared in DMEM was added to each well. Then 50 μl of 10-fold serially diluted virus stock in DMEM was placed into five wells per dilution. After 1 h incubation at 37 °C in humidified 5% CO_2, 250 μl DMEM containing 10% FBS was added to each well and cultivated for a further 4 days. The 50% tissue culture infectious dose ($TCID_{50}$) values were calculated using the Reed and Muench method (1938).

2.2. Sample processing and RNA extraction

The mice used in this study were deposited in the RIKEN BRC, a central core facility for mouse resources in Japan, by 19 laboratories or institutes in Japan and one laboratory in USA. Mouse fecal specimens were collected from 120 cages in reproduction rooms for rederivation. The collection of fecal specimens was approved by the Animal Experimentation Committee of the RIKEN Tsukuba Institute (Approval no. JITSU13-002) and was conducted in accordance with the RIKEN guidelines for animal research. One or a few fecal pellets were suspended in 0.5–0.7 ml PBS (40–320 mg/ml) using toothpicks. Solutions were clarified by centrifugation at 5000 × *g* for 5 min, and the supernatants were stored at −20 °C until RNA extraction. Viral RNA was extracted from the MNV culture supernatant or fecal supernatant using the High Pure Viral RNA Kit (Roche Applied Science, Minato, Tokyo, Japan) according to the manufacturer's instructions. Viral RNA was eluted with a 50-μl elution buffer attached to the kit and stored at −80 °C until use.

2.3. MNV sequence alignment and primer design

MNV-specific primers for RT-LAMP were designed on the well-conserved open reading frame 1 (ORF1)–ORF2 junction region. For this purpose, 72 strains of complete or nearly complete MNV genome sequences (GenBank: AB435514, AB435515, AB601769, AY228235, DQ223041–DQ223043, DQ285629, DQ911368, EF014462, EF531290, EF531291, EF650480, EF650481, EU004654–EU004683, EU482057, EU482058, EU854589, FJ446719, FJ446720, GQ180108, HQ317203, JF320644–JF320653, JN975491–JN975498, JQ658375, JQ237823, JX048594) and 35 strains of partial MNV genome sequences including the ORF1–ORF2 junction region (GenBank: AB469416, AB684730–AB684736, EF531292, HM044221, JN975522–JN975546) in the NCBI GenBank database were aligned using Genetyx-Mac v16 (Genetyx Corp., Shibuya, Tokyo, Japan). A set of primers was designed manually on the highly conserved regions of each gene, based on criteria reported previously (Hanaki et al., 2013): F1c and B1c have no mismatch within the last five nucleotides at both the 5′ and 3′ ends of the primer, and others have no mismatch within the last five nucleotides at the 3′ end of the primer. Primers were synthesized by Life Technologies (Chuo, Tokyo, Japan).

2.4. Phylogenic analysis

Seventy-two complete or nearly complete MNV genome sequences between F3 and B3 primers (308 nt) were examined for phylogenetic analysis. The phylogenetic tree was constructed by the MAFFT version 7 online program (http://mafft.cbrc.jp/alignment/software/), based on the neighbor-joining method, with the evolutionary distances computed by the Jukes–Cantor model (Katoh and Standley, 2013). The statistical

support for each node was evaluated by bootstrap analysis with 1000 replicates.

2.5. Artificial DNA standards

Ten pUC19 plasmids containing a chemically synthesized 400-bp DNA fragment of the ORF1–ORF2 junction region of MNV were manufactured by Fasmac (Atsugi, Kanagawa, Japan). The strains and the sequence positions used in the constructions were as follows: 1.CW1 (GenBank: DQ285629, nt 4801–5200), 4 (GenBank: DQ223043, nt 4801–5200), Apo960 (GenBank: JN975492, nt 4776–5175), Berlin/04/06 (GenBank: DQ911368, nt 4801–5200), KHU-1 (GenBank: JX048594, nt 4801–5200), MT30-2 (GenBank: AB601769, nt 4801–5200), NIH-2747 (GenBank: JF320647, nt 4801–5200), S7-PP3 (GenBank: AB435515, nt 4801–5200), TW2007 (GenBank: EU482058, nt 4527–4926), and WU11 (GenBank: EU004663, nt 4804–5203). Ten-fold serial dilutions ranging from 1.0×10^7 to 1 copy per 2-μl of each plasmid were produced using molecular grade water.

2.6. LAMP and RT-LAMP

LAMP was performed in a 0.2-ml microtube with a 20-μl reaction mixture containing 2 μl plasmid standard, 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM LB, 1.4 mM each of dNTPs, 6.4 U *Bst* DNA polymerase, Large Fragment (New England BioLabs, Sumida, Tokyo, Japan), and 120 μM hydroxy naphthol blue trisodium salt (CAS No. 63451-35-4; Sigma–Aldrich Japan, Shinagawa, Tokyo, Japan) in LAMP buffer (20 mM Tris–HCl [pH 8.8], 8 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween-20, and 0.8 M betaine). The reaction mixture for RT-LAMP was identical to those described above for LAMP except that 5 μl RNA extract was used as template instead of 2 μl plasmid standard and 0.45 U cloned AMV Reverse Transcriptase (Life Technologies) was included in the reaction mixture. For the negative controls in RT-LAMP, the RNA extract was substituted with RNA extract from 1.3×10^7 $TCID_{50}$/ml of feline calicivirus (FCV) or molecular grade water. The samples were placed in a thermocycler (Applied Biosystems 2720; Life Technologies) and incubated at 62 °C for 90 min. Successful gene amplification was indicated by a color change of the reaction solution from purple to sky blue under ambient light (Goto et al., 2009).

2.7. TaqMan PCR and TaqMan RT-PCR

Two-step TaqMan RT-PCR was performed with modifications (Kitajima et al., 2010). To synthesize cDNA, a High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used. A 10-μl reaction mixture containing 5 μl RNA extract, 4 mM dNTPs, 1× RT Random Primers, 25 U of MultiScribe Reverse Transcriptase, and 10 U of RNase Inhibitor in 1× RT buffer was incubated at 25 °C for 10 min, at 37 °C for 120 min, and then at 85 °C for 5 min. TaqMan PCR master mixture was prepared in a 20 μl-reaction volume: 10 μl of 2× Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan), 2 μl cDNA or plasmid standard, 2 μl of 4 μM each of the primers (forward, 5′-CCGCAGGAACGCTCAGCAG-3′; reverse, 5′-GGYTGAATGGGGACGGCCTG-3′), 0.5 μl of 12 μM TaqMan MGB probe (5′-FAM-ATGAGTGATGGCGCA-MGB/NFQ-3′, Life Technologies) and 5.5 μl of molecular grade water. PCR amplification was performed using a LightCycler Nano (Roche Applied Science) under the following conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of amplification with denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s. The results were analyzed by software in LightCycler Nano. Quantification of viral cDNA in the reaction was calculated using a standard curve constructed by amplifying known amounts of MNV-S7-PP3 plasmid.

2.8. Nested RT-PCR

Two-step nested RT-PCR developed by Kitajima et al. (2009) was performed with modifications. The RT reaction was carried out using a High Capacity cDNA Reverse Transcription Kit as described above. Fifty-μl of the first PCR mixture consisted of 5 μl cDNA, 0.4 μM each of the outer primers (forward, 5′-GCCATGCATGGTGAAAAG-3′; reverse, 5′-CATGCARACCAGGCGCATAG-3′), 0.2 mM each of dNTPs, and 2.5 U Ex Taq (Takara Bio) in 1× Ex Taq buffer attached to the enzyme. The samples were placed in a thermocycler (Mastercycler ep gradient S; Eppendorf, Chiyoda, Tokyo, Japan) and subjected to the following thermocycling conditions in the given order: activation of DNA polymerase at 94 °C for 3 min; 40 cycles of: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s; and a final elongation step at 72 °C for 7 min. The second PCR was performed with a 50-μl reaction mixture consisting of 2 μl of the first PCR product, 0.4 μM each of the inner primers (forward, 5′-ACARTGGATGCTGAGACC-3′; reverse, 5′-CAACCACCTTGCCAGCAG-3′), 0.2 mM each of dNTPs and 2.5 U Ex Taq in 1× Ex Taq buffer. The samples were subjected to thermocycling for the first PCR. Amplification products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

3. Results

3.1. Design of RT-LAMP primers

To design RT-LAMP primers, the whole genome sequence of MNV-1.CW1 was aligned with other complete or nearly complete genome sequences of 71 MNV strains and the partial genome sequences of other 35 MNV strains. First, the RT-LAMP primer sets were designed on the relatively conserved ORF1–ORF2 junction using PrimerExplore v4 (http://primerexplorer.jp/e/). However, there were one or more mismatches between a set of primers and the aligned genes, and some of the mismatches were located in the 3′-terminal region (data not shown). Then a set of primers comprising two outer (F3 and B3), two inner (FIP and BIP) and one loop (LB) primers that recognized seven distinct regions was designed manually. The location of the primer set is shown in Fig. 1, and the primer sequences are listed in Table 1.

3.2. Broad reactivity and sensitivity of LAMP compared with TaqMan PCR

MNV plasmids were used to evaluate the broad reactivity and sensitivity of LAMP. To construct the plasmids, 10 strains were chosen from different branches on the neighbor-joining phylogenetic tree of 72 MNV nucleotide sequences between primers F3 and B3 (Fig. 2). The evaluations of LAMP were carried out in triplicate using 10-fold serial dilutions of respective MNV plasmids, and compared with those of TaqMan PCR (Table 2). LAMP had the same detecting ability among the 10 MNV strains, and the reliable detection limit was 1000 copies. By contrast, the TaqMan PCR showed a different detection ability among the MNV strains, and the detection limit ranged from 10 to 1000 copies. It was estimated that the detection sensitivity of LAMP was 1–100-fold less than that of TaqMan PCR for the detection of the plasmids in this study.

3.3. Sensitivity of RT-LAMP compared with TaqMan RT-PCR

The detection sensitivity of RT-LAMP was analyzed in triplicate using 10-fold serial dilutions of MNV-1.CW1 RNA and MNV-S7-PP3 RNA, and compared with that of TaqMan RT-PCR (Table 3). MNV-1.CW1 and MNV-S7-PP3 are located on different

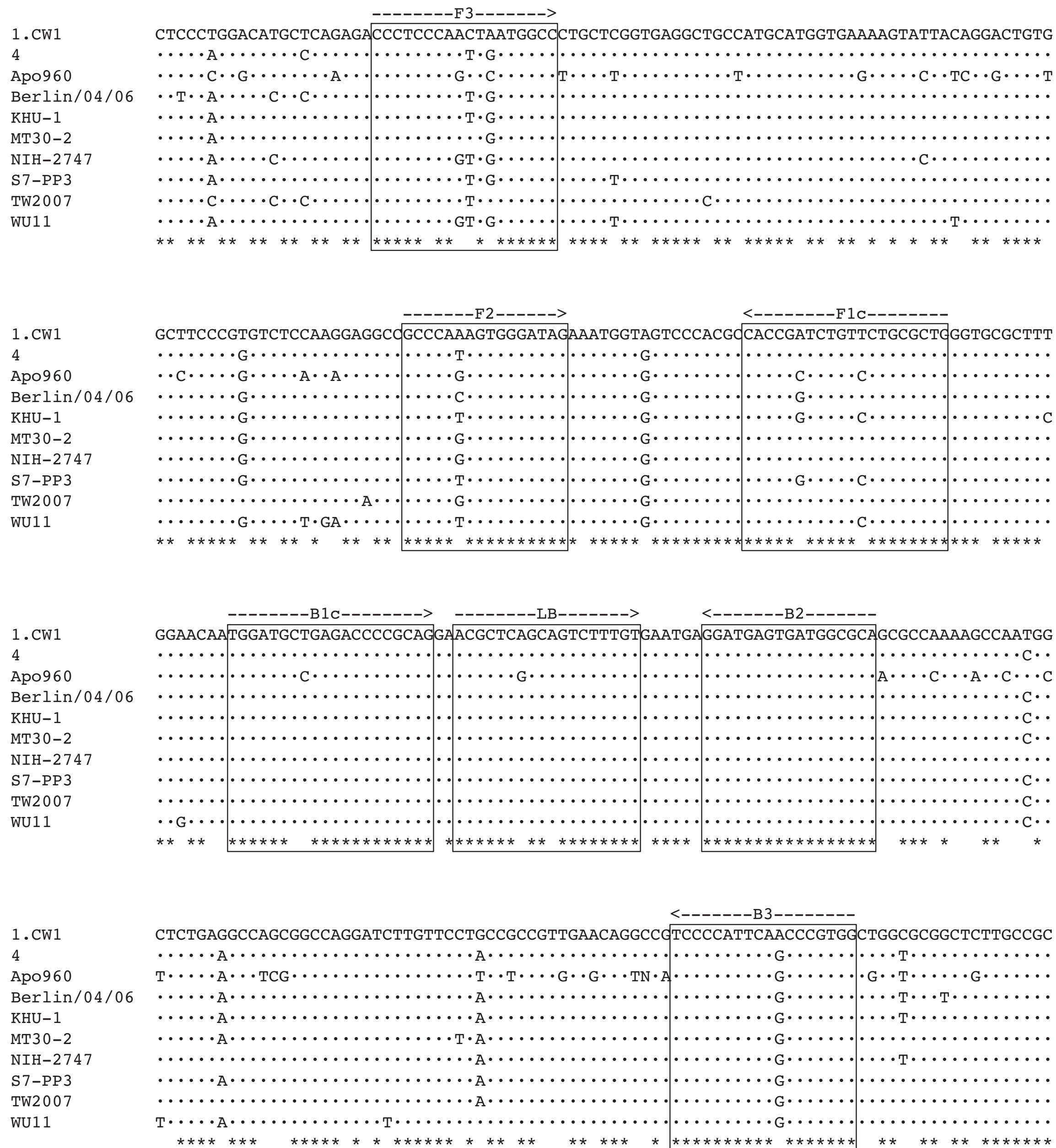

Fig. 1. Sequence alignment of MNV genes for LAMP primer design. Alignment of nucleotides 4833 to 5180 of MNV-1.CW1 (GenBank: DQ285629) with nine different strains. Arrows indicate the sequences from 5′ end to 3′ end used for the respective primers, and nucleotides that matched those of MNV-1.CW1 are represented as dots. Asterisks below the alignment show consensus nucleotide sequences among complete or nearly complete genome sequences of 72 MNV strains and the partial genome sequences of another 35 MNV strains. FIP and BIP primers contain two distinct sequences, F1c plus F2 and B1c plus B2, respectively.

branches of the phylogenetic tree (Fig. 2). Viral RNA was prepared from 1.2×10^6 $TCID_{50}$/ml of MNV-1.CW1 and MNV-S7-PP3 culture supernatants. The RT-LAMP including visual detection was completed in about 90 min, and the reliable detection limit was 10^4-fold diluted viral RNA, which was estimated as 2.4 $TCID_{50}$/reaction. In contrast, the TaqMan RT-PCR required about 3 h, and the reliable detection limit was 10^5-fold diluted viral RNA, equal to 4.8×10^{-2} $TCID_{50}$/reaction. Thus, the RT-LAMP was 50-fold less sensitive than

Table 1
Broadly reactive RT-LAMP primers for the detection of MNV strains.

Primer name	Genome position[a]	Length (bases)	Sequences[b] (5′ to 3′)
F3	4854–4871	18	CCCTCYCARYTVATGGCC
B3	5161–5144	18	CCACGGGYTGAATGGGGA
FIP (F1c-F2)	4996–4977, 4944–4959	41	CAGCGCAGRACAGANCGGTG-TTTTT-GCCCANAGTGGGATAG
BIP (B1c-B2)	5014–5033, 5076–5060	37	TGGATGNYGAGACCCCGCAG-TGCGCCATCACTCATCC
LB	5036–5053	18	ACGCTCRGCRGTCTTTGT

[a] Corresponding nucleotide position of MNV-1.CW1 (GenBank: DQ285629).
[b] Mixed bases in degenerate primers are as follows: R, A or G; V, A, C, or G;Y, C or T; N, any. Underlined portion indicates a 5 thymidine spacer.

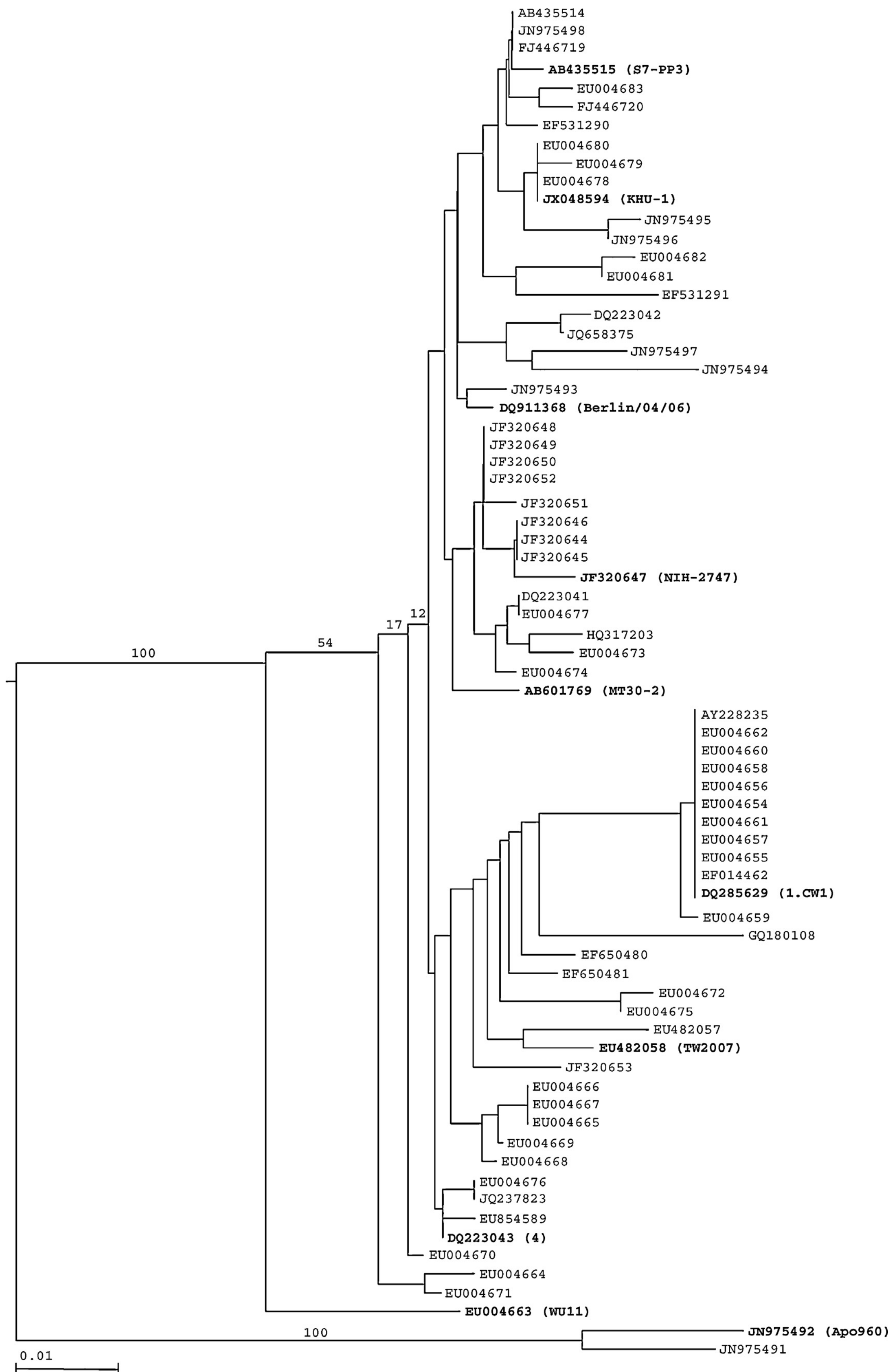

Fig. 2. Phylogenetic relationships among MNV strains. Phylogenetic analysis of 72 MNV strains isolated from laboratory and wild mice. The tree was constructed using the neighbor-joining method based on the 308-nucleotide sequences between F3 and B3 primers using MAFFT version 7. The bootstrap values are labeled at the major nodes as a percentage of 1000 iterations. Strains used for construction of MNV plasmids are in bold, and the names are shown in parenthesis after the GenBank accession numbers, respectively. The scale bar represents 0.01 nucleotide substitutions per position.

Table 2
Detection limit of LAMP vs. TaqMan PCR using 10-fold serially diluted plasmid standards.

Strains	Dilution of plasmid coding MNV ORF1–ORF2 junction region (copies)							
	10^7	10^6	10^5	10^4	10^3	100	10	1
1.CW1	3/NT	3/3	3/3	3/3	3/3	0/3	0/3	NT/0
4	3/NT	3/3	3/3	3/3	3/3	1/3	0/3	NT/0
Apo960	3/NT	3/3	3/3	3/3	3/3	1/0	0/0	NT/0
Berlin/04/06	3/NT	3/3	3/3	3/3	3/3	1/3	0/2	NT/0
KHU-1	3/NT	3/3	3/3	3/3	3/3	0/3	0/1	NT/0
MT30-2	3/NT	3/3	3/3	3/3	3/3	1/3	0/3	NT/0
NIH-2747	3/NT	3/3	3/3	3/3	3/3	2/3	0/3	NT/0
S7-PP3	3/NT	3/3	3/3	3/3	3/3	1/3	0/3	NT/0
TW2007	3/NT	3/3	3/3	3/3	3/3	0/3	0/3	NT/0
WU11	3/NT	3/3	3/3	3/3	3/3	0/3	0/3	NT/0

The results are shown as number of LAMP positives/number of TaqMan PCR positives ($N = 3$). NT, not tested.

Table 3
Detection limit of RT-LAMP vs. TaqMan RT-PCR using 10-fold serially diluted MNV RNA.

Strains	Dilution of MNV RNA						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1.CW1	3/3	3/3	3/3	3/3	1/3	0/1	0/0
S7-PP3	3/3	3/3	3/3	3/3	2/3	0/1	0/0

The results are shown as number of RT-LAMP positives/number of TaqMan RT-PCR positives ($N = 3$).

the TaqMan RT-PCR for the detection of the two strains of MNV RNA.

3.4. Detection of MNV in mouse fecal specimens

Diagnostic performance of RT-LAMP on RNA extracted from mouse fecal specimens was compared with TaqMan RT-PCR. Nested RT-PCR was also used to detect MNV RNA as the most sensitive reference method (Table 4). Of the 120 specimens, 54 specimens were confirmed positive by RT-LAMP. By contrast, 56 specimens were confirmed positive by TaqMan RT-PCR, and MNV RNA loads were in the range of 1.1×10^1 to 1.7×10^5 copies/reaction. The viral RNA loads in the two specimens, judged negative by RT-LAMP and positive by both TaqMan RT-PCR and nested RT-PCR, were less than 100 copies/reaction. None of the 64 negative specimens confirmed by TaqMan RT-PCR was positive by RT-LAMP. However, a negative specimen confirmed by both RT-LAMP and TaqMan RT-PCR was determined to be positive by nested RT-PCR.

4. Discussion

MNV is currently the most common contaminant worldwide in laboratory mouse colonies, and various effects of MNV infection on biomedical research have been reported. In addition, some strains can establish persistent infection even in immunocompetent mice, and infected mice shed the virus in feces over a long time period (Hsu et al., 2006; Thackray et al., 2007). Therefore, there is a necessity to eradicate MNV from mouse colonies in laboratory animal facilities. When a report of MNV elimination from mouse colonies or a health certificate is required to show that the colonies are free from MNV, RT-PCR-based assays are often used to monitor the infection (Kastenmayer et al., 2008; Ohsugi et al., 2013; Tajima et al., 2013). However, they are time-consuming, laborious, and expensive methods compared with serological assays. RT-LAMP does not require an expensive DNA polymerase kit, the expensive and sophisticated equipment required for real-time RT-PCR, and is not time-consuming and requiring of laborious electrophoretic analysis as for conventional RT-PCR. Thus, to overcome disadvantages of RT-PCR-based assays, we developed an RT-LAMP methodology with the potential to detect a broad range of MNV strains. Design of a specific and sensitive primer set is crucial for establishing a successful LAMP assay. However, no primer set designed automatically by PrimerExplore v4 allows the detection of all MNV strains in the GenBank database including Apo455 (GenBank: JN975491) and Apo960 (GenBank: JN975492) from wood mouse *Apodemus sylvaticus* (Smith et al., 2012). Thus, a set of primers that satisfied as much as possible, the recommended design features described in the PrimerExplore v4 guide, were configured manually in the highly conserved region of 308 bases between nucleotides 4854 and 5161 of MNV-1.CW1.

Although the target region by RT-LAMP is the highly conserved region within the MNV genome, it still contains polymorphisms that may affect its detection ability (Fig. 1). Thus, various strains of MNV were used to evaluate the broad reactivity and sensitivity of RT-LAMP. However, we could not collect MNV strains other than strains 1.CW1 and S7-PP3. Thus, 10 MNV plasmids were synthesized and used to evaluate the broad reactivity and sensitivity of LAMP. The MNV genome sequences for designing MNV plasmids were chosen based on both the phylogenetic relationship (Fig. 2) and the country of origin of strains: 1.CW1, 4, NIH-2747, and WU11 from United States; Apo960 from England; Berlin/04/06 from Germany; KHU-1 from Korea; MT30-2 and S7-PP3 from Japan; and TW2007 from Taiwan. LAMP using a manually designed primer set succeeded in the detection of plasmids containing one of the MNV strains listed in Fig. 1 with equal sensitivity (Table 2). MNV-1.CW1 RNA and MNV-S7-PP3 RNA were then used to evaluate the sensitivity of RT-LAMP. RT-LAMP using the designed primer set also succeeded in the detection of MNV-1.CW1 RNA and MNV-S7-PP3 RNA with equal sensitivity (Table 3). Thus, the primer set is expected to have the potential to detect MNV isolates worldwide with equal sensitivity. The specificity of RT-LAMP was confirmed by negative reactions in the presence of FCV RNA and RNA purified from feces of newly arriving MNV-free mouse strains (Charles River Laboratories Japan, Yokohama, Kanagawa, Japan) determined by Multiplexed Fluorometric ImmunoAssay® (data not shown). Moreover, the diagnostic performance of RT-LAMP exhibited 100% specificity compared with both TaqMan RT-PCR and nested RT-PCR in 120 clinical specimens (Table 3). The primer set is likely to be MNV genome-specific as indicated by in silico analyses using

Table 4
Comparison of detection of MNV from mouse fecal specimens by three methods.

Result	No. of samples with indicated result by		
	RT-LAMP	TaqMan RT-PCR	Nested RT-PCR
Positive	54	56	57
Negative	66	64	63
Total	120	120	120

Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and because the primer set did not amplify RNA purified from MNV-free mice (data not shown). The primer set is therefore expected not to amplify RNA purified from mouse feces other than those contained the MNV genome.

The detection limit of RT-LAMP was estimated to be 10-fold higher than that of LAMP by comparative sensitivity studies between LAMP and TaqMan PCR, and between RT-LAMP and TaqMan RT-PCR. One reason for the different detection limit might be that double stranded plasmid DNA could not be denatured sufficiently under the LAMP reaction condition as we previously reported (Goto et al., 2010).

For specific and efficient gene amplification, perfect base-pairing between the 3′ end of the primer and the target gene is essential (Dieffenbach et al., 1993). Thus, it is necessary to update the primer sequences to maintain the high sensitivity of gene amplification methods for detection of a broad range of MNV strains. This is an important point as previously developed RT-PCR-based methods have already lost their broad detection reactivity to known MNV strains. The RT-PCR test developed by Hsu et al. (2005) failed to amplify MNV-S7-PP3 RNA as a template (data not shown). BLAST searches (http://blast.ncbi.nlm.nih.gov/) showed that the forward primer has four mismatches including a mismatch at position −3 of the 3′ end to the RNA. Another RT-PCR test developed by Hsu et al. (2006) has a mismatch at position −2 of the 3′ end to the genomes of MNV-1.CW2 (GenBank: EU004654) and MNV-1.CW4 (GenBank: EU004655). The nested RT-PCR used in this study (Kitajima et al., 2009) has a mismatch between position −2 of the 3′ end of the outer forward primer and strain Lab12 genome (GenBank: JN975493). Another nested RT-PCR developed by Tajima et al. (2013) has a mismatch between position −2 of the 3′ end of the inner forward primer and MNV-CR11 genome (GenBank: EU004679). Regarding the TaqMan RT-PCR used in this study (Kitajima et al., 2010), the forward primer has a mismatch at position −5 of the 3′ end of Apo960 genome. The reverse primer has two mismatches at positions −14 and −17 of the 3′ end of Apo960 genome. Thus, primer mismatches in the TaqMan RT-PCR seem to have caused the reduced sensitivity in detecting strain Apo960 (Table 2).

Of 120 fecal specimens obtained from mouse cages deposited from 20 laboratory animal facilities or institutes, RT-LAMP detected MNV in 54 specimens. However, the reference methods TaqMan RT-PCR and nested RT-PCR detected MNV in 56 and 57 specimens, respectively. RT-LAMP exhibited 96.4% and 94.7% sensitivity compared with TaqMan RT-PCR and nested RT-PCR (Table 4). Thus, RT-LAMP is a cost-effective and practical alternative to RT-PCR-based assays and is suitable for monitoring active and persistent infection of MNV in most mouse facilities. However, updating the primer sequences is necessary to maintain sensitivity to detect a broad range of MNV isolates in the future, because this RT-LAMP method uses five primers recognizing seven distinct regions in the MNV gene.

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