Journal of Virological Methods 187 (2013) 222–227

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Reverse transcription-loop-mediated isothermal amplification for the detection of rodent coronaviruses

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Article history:
Received 6 June 2012
Received in revised form 11 October 2012
Accepted 22 October 2012
Available online 30 October 2012

Keywords:
Loop-mediated isothermal amplification
Mouse hepatitis virus
Rat coronavirus
Rodent coronavirus
RT-LAMP

A B S T R A C T

Mouse hepatitis virus (MHV) is one of the most prevalent viruses detected in laboratory mouse colonies. Enterotropic strains predominate in natural infections, and molecular techniques for the detection of MHV shedding in feces are powerful enough to diagnose active infections. A reverse transcription-loop-mediated isothermal amplification (RT-LAMP) technique was developed for the detection of rodent coronaviruses within 90 min. The specificity of this technique was confirmed by its ability to detect all 17 different strains of MHV and 6 strains of rat coronaviruses as well as its failure to detect human, bovine, and porcine coronaviruses nonspecifically. The sensitivity of RT-LAMP was 3.2-fold higher than that of reverse transcription-polymerase chain reaction (RT-PCR) and 31.6-fold lower than that of nested RT-PCR. An evaluation of the diagnostic performance of RT-LAMP performed in duplicate using mouse fecal specimens showed that the sensitivity and specificity with respect to nested RT-PCR were 85.7% and 100%, respectively. RT-LAMP assays would be suitable for monitoring active MHV infection in mouse colonies.

1. Introduction

Mouse hepatitis virus (MHV), a species in the genus *Betacoronavirus*, family *Coronaviridae*, is one of the most prevalent viruses in mouse colonies throughout the world. MHV, which has a 31-kb single-strand positive RNA genome, changes through mutation and genetic recombination and can be classified serologically into many strains (Lai, 1996; Makino et al., 1986). The strains are divided into two groups, enterotropic or polytropic, according to patterns of tissue tropism, tendency to disseminate, and virulence (Homberger et al., 1998). Enterotropic strains, such as D, DVIM, and NuU, are less virulent than polytropic strains, such as 1, 2, 3, JHM, and A59; adult immunocompetent mice infected with enterotropic strains show no clinical symptoms (Homberger, 1997). However, enterotropic strains are considered to be more contagious than polytropic strains. MHV infection can seriously affect the quality of biomedical research (Boorman et al., 1982; Cook-Mills et al., 1992; Torrecilhas et al., 1999). MHV can also cause a chronic wasting disease that eventually results in death in immunocompromised mice such as athymic nude mice. MHV is transmitted by contact with apparently healthy carrier mice through respiratory aerosols, feces, direct contact, and fomites. Early detection of carrier mice is therefore very important for eliminating this highly contagious infection from mouse colonies. For this purpose, methods of directly detecting viral RNA using reverse transcription-polymerase chain reaction (RT-PCR) have been developed (Besselsen et al., 2002; Casebolt et al., 1997; Homberger et al., 1991; Matthaei et al., 1998; Smith et al., 2002; Wang et al., 1999; Yamada et al., 1998). However, RT-PCR is a time-consuming and labor-intensive detection method and is highly prone to cross contamination between samples.

An isothermal gene amplification technique called loop-mediated isothermal amplification (LAMP) has been developed using a specially designed primer set and a DNA polymerase with strand displacement activity (Notomi et al., 2000). The simple addition of a reverse transcriptase to the reaction enables the technique to amplify RNA molecules (RT-LAMP). The principles of the LAMP reaction have been well documented by the developers (Notomi et al., 2000; Tomita et al., 2008). RT-LAMP has several advantages over RT-PCR. RT-LAMP can be carried out at a constant temperature in a single tube, and the reaction can be completed typically within 1 h (Kubo et al., 2010; Lan et al., 2009; Thai et al., 2004). The result can be judged by the naked eye without opening the reaction tube, using a metal indicator, hydroxy naphthol blue (Goto et al., 2009). The theoretical specificity of RT-LAMP is superior to that of RT-PCR because of the use of 4 oligonucleotide primers that recognize a total of 6 distinct sequences. In other words, it is more

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http://dx.doi.org/10.1016/j.jviromet.2012.10.008

difficult to design a set of LAMP primers than to design a pair of PCR primers for RNA viruses that mutate frequently (Arita et al., 2009; Dinh et al., 2011; Fukuda et al., 2006).This study aimed to develop a convenient and rapid molecular diagnostic technique for MHV infection. To accomplish this objective, a set of broadly reactive LAMP primers specific for rodent coronaviruses was designed. The sensitivity and specificity of this assay was compared with the existing RT-PCR and nested RT-PCR assays using isolated viruses. Finally, the diagnostic capability of the assay was examined using mouse fecal specimens.

2. Materials and methods

2.1. Viruses and cells

MHV strain 1 (MHV-1), MHV-2, MHV-3, MHV-JHM, MHV-A59, and MHV-S are prototype strains. MHV-D, MHV-DVIM, MHV-F-2D, MHV-IN-12, MHV-N, MHV-Nu, MHV-NuA, and MHV-NuU are low-virulence isolates (Hirano and Ono, 1990; Hirasawa et al., 1988). MHV-2-CC, MHV-JHM-CC, and MHV-A59-CC are low-virulence mutants of the respective strains 2, JHM, and A59 (Hirano et al., 1984). Viruses were propagated in DBT cells cultured in Eagle's minimum essential medium (EMEM), and virus titers were determined using a plaque assay as reported previously (Hirano et al., 1974). Rat coronavirus Parker strain (RCV-Parker) and 5 strains of sialodacryoadenitis virus (SDAV-681, SDAV-930, SDAV-K, SDAV-L-1, and SDAV-M) were grown in LBC cells cultured in EMEM (Hirano et al., 1985, 1986). Porcine hemagglutinating encephalomyelitis virus strain 67N (HEV-67N), HEV-NT9, and HEV-VW3 were grown in SK-K cells cultured in EMEM (Hirano et al., 1990). Porcine epidemic diarrhea virus strain 3 (PEDV-3) and porcine transmissible gastroenteritis virus strain TO (TGEV-TO) were grown in Vero and CPK cells, respectively, cultured in EMEM (Hofmann and Wyler, 1988; Honda et al., 1990). Two strains of bovine coronavirus (BCV-Kakegawa and BCV-Nebraska) were grown in BEK-1 cells cultured in EMEM containing 10% (v/v) tryptose phosphate broth (Inaba et al., 1976). Human coronavirus strain OC43 (HCV-OC43), which had been stored at −80 °C in a suckling mouse brain, was prepared as a 10% (w/v) mouse brain homogenate in EMEM. Each supernatant was collected and stored at −80 °C after removal of cell debris by centrifugation at 5000 × *g* for 5 min.

2.2. Fecal specimens

Sixty-nine fecal specimens from 45 cages (each housing 1–4 mice) were collected from 29 genetically engineered mouse strains deposited in the RIKEN BRC, a central core facility for mouse resources in Japan. Approximately 0.1 g of each fecal specimen (2–5 fecal pellets) was added directly to a bashing beads lysis tube (1 mm zirconia beads; Sarstedt, Chiyoda, Tokyo, Japan) and lysed with 0.6 mL of sterile water by bead beating in a vortex. The lysates were clarified by centrifugation at 5000 × *g* for 5 min. Each fecal supernatant was stored at −80 °C until RNA extraction.

2.3. Extraction and purification of viral RNA

Viral RNA was extracted from culture or fecal supernatant using the High Pure Viral RNA Kit (Roche Diagnostics, Minato, Tokyo, Japan) according to the manufacturer's instructions. Briefly, a 200 μL aliquot of the supernatant was dissolved in a lysis buffer containing poly-A and then bound to a glass fiber fleece. After wash and spin steps, viral RNA was eluted from the fleece using 50 μL of elution buffer and stored at −80 °C until use.

2.4. LAMP primer design

The whole-genome sequences of 8 MHV strains available in GenBank (MHV-1, accession number FJ647223; MHV-2, AF201929; MHV-3, FJ647224; MHV-JHM, NC_006852; MHV-A59, AY700211; MHV-MI, AB551247; MHV-Penn 97-1, AF208066; and MHV-S, GU593319) were aligned using Genetyx-Mac v16 (Genetyx Corp., Shibuya, Tokyo, Japan). The whole-genome sequence of RCV-Parker (GenBank ID: FJ938068) was also aligned with the MHV sequences. The highly conserved 5′-terminus sequence was selected as the target for primer design.

RT-LAMP needs a set of 4 primers comprising of 2 outer (F3 and B3) and 2 inner (FIP and BIP) primers (Fig. 1). The F3 primer is a sense sequence of the F3 region of the target gene and the B3 primer is an antisense sequence of the B3 region of the target gene. The FIP primer contains an antisense sequence of the F1c region at its 5′ end and a sense sequence of the F2 region at its 3′ end. BIP primer contains a sense sequence of the B1c region at its 5′ end and an antisense sequence of the B2 region at its 3′ end. Further, one or two loop primers (LB and/or LF) can be designed to accelerate the amplification reaction by binding to additional sites that are not accessed by internal primers (Nagamine et al., 2002). A set of 2 inner primers FIP (5′-CCGAGACCGTATTTGCCCATCT-CTCTGCCAGTGACGTGTC-3′) and BIP (5′-TGGGCCCCAGAATTTCCATGG-CCTCCTCTGACCTCTCAGG-3′), which recognize 4 distinct regions on the target sequence, and a loop primer LB (5′-CCGAACGCATCGGAGAAGTTG-3′) were designed with PrimerExplorer V4 online software (http://primerexplorer.jp/e/) developed by Fujitsu Systems East (Bunkyo, Tokyo, Japan). However, it was not possible to design the other loop primer (LF), located between the F2 and F1c regions, with the software. A set of 2 outer primers consisting of F3 (5′-CYTGGTCTTGTCATAGTGCT-3′) and B3 (5′-AAAATATCACGGATTATGGC-3′) were designed manually. Possible mismatches between the primers and all rodent coronavirus genes available in GenBank were also checked using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The locations of the primers are shown in Fig. 1. All primers were synthesized by Life Technologies Japan (Chuo, Tokyo, Japan).

2.5. RT-LAMP

RT-LAMP was performed in a 0.2 mL microtube with a 20 μL reaction mixture containing 4 μL of viral RNA, 1.6 μM of FIP and BIP, 0.8 μM of LB, 0.2 μM of F3 and B3, 1.4 mM of each dNTP, 0.45 U of Cloned AMV Reverse Transcriptase (Life Technologies Japan), 6.4 U of the large fragment of *Bst* DNA polymerase (New England BioLabs, Sumida, Tokyo, Japan), 20 U of RNasin Plus ribonuclease inhibitor (Promega KK, Cyuo, Tokyo, Japan), and 120 μM of hydroxy naphthol blue trisodium salt (CAS No. 63451-35-4; Dojindo Laboratories, Mashiki, Kumamoto, Japan) in 1 × 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]. Unless otherwise stated, the microtubes were incubated at 62 °C for 90 min in an Applied Biosystems 2720 thermal cycler (Life Technologies Japan). Successful gene amplification was judged by a color change of the reaction solution from purple to sky blue under ambient light (Goto et al., 2009).

2.6. RT-PCR

RT-PCR targeting a highly conserved viral membrane E1 glycoprotein gene was originally reported by Homberger et al. (1991). The primer sequences amplifying a 375-bp genome fragment were 5′-AATGGAACTTCTCGTTGGG-3′ and 5′-TAGTGGCTGTTAGTGTATGG-3′. The RT-PCR was carried out with PrimeScript One Step RT-PCR Kit Ver. 2 (Takara Bio., Otsu,