Journal of Virological Methods 187 (2013) 274–277

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Short communication

Development of a reverse-transcription loop-mediated isothermal amplification method for detection of rabbit hemorrhagic disease virus

Dongwei Yuan[a,b], Dongchun Guo[a], Jiasen Liu[a], Changde Si[a], Qian Jiang[a], Huan Lin[a], Tiankuo Yang[a], Liandong Qu[a,*]

[a] Experimental Animal Research Center, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, Harbin, China
[b] College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, China

Article history:
Received 24 April 2012
Received in revised form 14 October 2012
Accepted 13 November 2012
Available online 23 November 2012

Keywords:
Rabbit hemorrhagic disease virus
Reverse transcription
Loop-mediated isothermal amplification
Sensitivity
Reliability

A B S T R A C T

Rabbit hemorrhagic disease virus (RHDV) causes haemagglutination and severe liver damage, with a high mortality rate. To develop a rapid and sensitive method for the surveillance of RHDV, a one-step reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay was established using a set of four primers specific for the VP60 gene segment of RHDV. The established assay was performed at 64 °C for 40 min under isothermal conditions, and the results were visualized directly by electrophoresis or as fluorescent signals under ultraviolet light. The detection limit of the RT-LAMP assay was 10 copies of viral RNA per reaction, which was comparable to quantitative real-time RT-PCR, and 100-fold more sensitive than standard RT-PCR. Furthermore, seven viral RNAs of field isolates in China could be detected successfully using this assay. Overall, the newly established RT-LAMP assay indicates the potential usefulness of the technique as a simple, rapid and sensitive procedure, and can visually detect RHDV infection without the need for any specialized equipment.

Rabbit hemorrhagic disease (RHD) is a highly contagious disease in rabbits of the species *Oryctolagus cuniculus* that is caused by the rabbit hemorrhagic disease virus (RHDV). The disease is characterized by disseminated intravascular coagulation (DIC) in combination with severe liver damage, which causes high morbidity and mortality in both wild and domestic adult rabbits (Ferreira et al., 2004). RHDV is a positive-sense, single-stranded RNA virus, a member of the genus *Lagovirus* of the family *Caliciviridae* (Parra and Prieto, 1990). The first outbreak of this disease was noticed in 1984 in China, and since then it has dispersed rapidly and widely across the rest of Asia and Europe, causing epidemics within a few years (Abrantes et al., 2012). The rapid detection of RHDV would provide important information to improve the control of the circulation of RHDV. Conventional virological methods for the detection of RHDV are the haemagglutination test, electron microscopy, enzyme linked immunosorbent assay (ELISA), immunostaining and Western blot (OIE, 2004). Recently, the detection of viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR has been developed to complement the primary diagnostic techniques for RHDV infection, and these methods have been applied widely because of their high sensitivity and specificity (Gall et al., 2007). However, these assays are time-consuming and laborious, and they require well-equipped laboratory facilities and submission of clinical specimens, which results in a delay in the diagnosis of RHDV. Therefore, a more convenient, rapid, sensitive, cost-effective and efficient assay is needed for detection of the virus.

Loop-mediated isothermal amplification (LAMP) is a novel method that can be used rapidly to amplify a specific nucleic acid with high specificity under isothermal conditions, with the use of four to six specifically designed primers (Notomi et al., 2000). It has emerged as a powerful gene amplification tool owing to its simplicity, speed, specificity and cost-effectiveness. So far, the LAMP method has been applied to the rapid detection of various microbes and pathogens, including protozoa, bacteria, and viruses, in the environment, food, and clinical samples (Dukes et al., 2006; Haridas et al., 2010; Huang et al., 2010; Kuan et al., 2010; Parida et al., 2007; Saitou et al., 2010). In addition, RNA also can be used directly as the starting material by utilizing reverse transcription coupled with LAMP in one step (Chen et al., 2008; Peyrefitte et al., 2008), which makes the technique ideal for the detection of RNA viruses such as RHDV. However, the application of the LAMP assay to the detection of RHDV has never been reported. In the present study,

* Corresponding author at: Experimental Animal Research Center, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, Harbin, Heilongjiang Province 150001, China. Tel.: +86 0451 51997176; fax: +86 0451 51997176.
E-mail address: qld@hvri.ac.cn (L. Qu).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.11.020

Table 1
Source of 26 isolates of RHDV including the reference RHDV isolate in the database used in this study.

No.	Isolate	Year	Country	GenBank accession no.
1	NJ/China	1985	China	AY269825.1
2	TP*	2002	China	AF453761.1
3	XJ1/China*	2002	China	GU339228.1
4	XJ2/China*	2002	China	GU339229.1
5	CD/China*	2004	China	AY523410.1
6	Whn/China01/2005	2005	China	DQ069280.1
7	Whn/China02/2005	2005	China	DQ069281.1
8	Whn/China03/2005	2005	China	DQ069282.1
9	WHNRH	2005	China	DQ280493.1
10	HYD*	2005	China	JF412629.1
11	JX/CHA/97*	2005	China	DQ205345.1
12	SH/China*	2006	China	FJ794180.1
13	YL	2006	China	DQ530363.1
14	TC/China/2007	2007	China	JN165233.1
15	WF/China	2007	China	FJ794180.1
16	NJ-2009	2009	China	HM623309.1
17	FP/China/2009	2009	China	JN165235.1
18	BJ/China/2009	2009	China	JN165236.1
19	09-SD	2010	China	GU564448.1
20	XA/China/2010	2010	China	JN165234.1
21	99–05	1999	France	AJ302016.1
22	Triptis	1999	Germany	Y15442.1
23	Lowa2000	2000	USA	AF258618.2
24	00-REU	2002	France	AJ303106.2
25	03–24	2003	France	AJ969628.1
26	CUB5-04	2005	Cuba	DQ841708.1

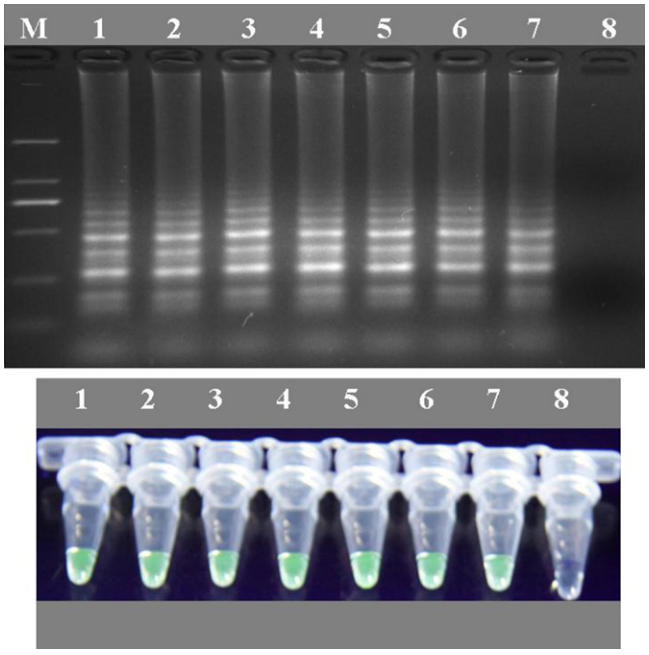

Fig. 1. Reliability assay of the RT-LAMP. Seven RHDV isolate strains in China were subjected to RT-LAMP using the primers shown in Table 1, and the RT-LAMP products were examined by both 2% agarose gel electrophoresis (upper panel) and visually inspection for fluorescence signal (lower panel). M: DL2000 DNA Marker; Lane 1, RHDV-TP isolate strain; Lane 2, RHDV-HYD isolate strain; Lane 3, RHDV-SH isolate strain; Lane 4, RHDV-CD isolate strain; Lane 5, RHDV-XJ (c1) isolate strain; Lane 6, RHDV-XJ (c2) isolate strain; Lane 7, RHDV-JX isolate strain; Lane 8, negative control.

a simple reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) assay was described for the rapid detection of RHDV using primers specific for the VP60 gene segment of RHDV. The established RT-LAMP assay is a simple, rapid, and sensitive test that could usefully be applied for the diagnosis of RHDV infection.

Based on sequence data for the RHDV VP60 gene available in GenBank, the sequences of 44 pathogenic RHDV strains and 35 non-pathogenic rabbit calicivirus (RCV) strains were retrieved and subjected to phylogenetic analyses using the Molecular Evolutionary Genetics Analysis (MEGA) Version 5.0 program. Table 1 lists the information of 26 isolated strains of RHDV for primer design, including the reference virus RHDV-TP strain, based on which a conserved sequence of the RHDV-VP60 gene (nt 850–1041) was selected as the target for RT-LAMP. A primer set for RT-LAMP assay was designed using the Primer Explorer V 4.0 software program for LAMP primer design (http://primerexplorer.jp). Details of the primers are listed in Table 2.

The reliability of the newly established RT-LAMP assay was tested in seven RHDV field isolates from China (RHDV-TP isolate strain; RHDV-HYD isolate strain; RHDV-SH isolate strain; RHDV-CD isolate strain; RHDV-XJ1 isolate strain; RHDV-XJ2 isolate strain; RHDV-JX isolate strain). Total viral RNA was extracted from virus stocks using the Axyprep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, California, USA) according to the manufacturer's instructions. The RT-LAMP was performed in a total volume of 25 μL and incubated in a water bath at 64 °C for 40 min. The reaction mixture contained 2.5 μL of 10× Thermopol Buffer (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), 12 U Bst DNA polymerase (New England Biolabs, Beijing, China), 10 U M-MLV RTase (TaKaRa, Dalian, China), 10 μM each of F3 and B3 primers, 50 μM each of FIP and BIP primers, 5 mmol $MgSO_4$, 10 μM betaine (SIGMA, Shenyang, China), 0.4 mM deoxynucleoside triphosphate (dNTP, TaKaRa) and viral RNA template. The RT-LAMP product was detected by DNA electrophoresis or SYBR Green I staining, according to a previously described protocol (Huang et al., 2010; Mori and Notomi, 2009). A positive amplification was indicated by a ladder-like pattern on the gel or fluorescence signals under ultraviolet (UV) light. As shown in Fig. 1, all the RHDV isolates were amplified successfully by the RT-LAMP assay and showed positive results. To determine the detection limit of the RT-LAMP assay for RHDV, reference viral stock RNA was extracted and quantified by spectrophotometry, and a panel of RNAs with concentrations ranging from 10^0–10^8 copies per assay was prepared. The lowest copy number of RNA detectable under the conditions described above was defined as the detection limit. The amplification results were compared with those of RT-PCR and real-time RT-PCR performed in parallel using the same template. The standard RT-PCR and quantitative real-time RT-PCR reaction conditions were described previously (Gall et al., 2007). As shown in Fig. 2A, the detection limit of standard RT-PCR was 10^3 copies viral RNA per reaction. However, the detection limit of the RT-LAMP assay was approximately 100-fold lower at 10 copies viral RNA per reaction (Fig. 2C), while the sensitivity was comparable to that of quantitative real-time RT-PCR (Fig. 2B). Positive reactions were also observed by fluorescence using the naked eye (Fig. 2D). RT-LAMP is more sensitive than standard RT-PCR and more convenient than quantitative real-time RT-PCR, this will enable RT-LAMP to be applied as an accurate molecular diagnostic tool in practicality tests.

This study established and used a one-step RT-LAMP method for the detection of RHDV for the first time in China. This newly established RT-LAMP assay offers several advantages compared to conventional diagnostic techniques. First, the high sensitivity of the assay; the detection limit of the RT-LAMP assay was 10 copies viral RNA per reaction, which is comparable to quantitative real-time RT-PCR and 100-fold more sensitive than standard RT-PCR. Secondly, the RT-LAMP assay is a cost-effective, simple and rapid method; the amplification reaction was accomplished in an isothermal water bath for 40 min, with no specialized PCR equipment. Although high quality purified RNA is still required, this characteristic means that RT-LAMP represents a comparatively simple and rapid molecular detection tool for laboratory testing, where budgets may be limited and state-of-the-art equipment rare. Thirdly, the method