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Establishment of a RT-lamp method for the rabbit hemorrhagic disease virus detection

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ABSTRACT

To develop a new molecular biological method for the quick detection of rabbit hemorrhagic disease virus (RHDV), a set of 7 primers were designed according to the conserved sequence of the capsid proteinVP60gene of RHDV published in GenBank, and the reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay was established through preparation of target gene fragments, optimization of reaction conditions, sensitivity and specificity tests. Results showed the RT-LAMP method for the RHDV detection had a ladder-like pattern of amplication bands from about 213 bp incubation at 64°C for 45min by using agarose gel electrophoresis,and with good sensitivity and specificity,the detection limit could reach about 5 copies of cloned viral genomic fragments, which was more sensitve than that of traditional RT-PCR, and no amplifications for gene fragment of European brown hare syndrome virus, P*asteurella multocida*, E.*coli* and S*almonella* from rabbits detection by this RT-LAMP approach.

KEYWORDS

Rabbit hemorrhagic disease virus;
RT-LAMP;
VP60 gene;
Detection;
RT-PCR.

INSTRUCTION

Rabbit hemorrhagic disease (RHD), an important OIE Listed disease, is a highly contagious and rapidly fatal viral disease of both domestic and wild rabbits(Oryctolagus cuniculus)[1]. It was caused by rabbit hemorrhagic disease virus (RHDV), which is a non-enveloped RNA virus with a diameter of about 36-40 nm, and the genome consists of a positive-sense single-stranded RNA molecule of 7437nt in size. The disease is characterized with the typical pathological lesions including necrotic hepatitis, hemorrhaging and edema of lungs and trachea, short incubation period (24-48 h after infection),and high mortality rate of 60-100%[2]. Since RHD was first described in China in 1984, it has been reported in many countries successively throughout the world[3], and has resulted in the deaths of nearly a quarter billion free-living and domestic rabbits and generated signify- cant losses to rabbit farming industries and trade. RHD is still endemic in some areas sometimes, studies on the rapidly diagnosis and prevention of RHD are still important and should be paid attention[4,5].Although there were many common RHD detection methods were reported such as virus isolation, ELISA[6] and RT-PCR[5], but these techniques might delay disease diagnosis for the limits of sensitiv-

ity, or requirements of laboratory facilities, etc.

As a novel nucleic acid amplification method, which was reported recently[7], the RT-LAMP assay has been used for the detection of many pathogens because of advantages of rapidity, sensitivity, and non-requirements of any expensive special equipment such as a thermal cycler. In this paper, based on the sequence analysis and alignment of the capsid protein(VP60) gene of RHDV published in GenBank, 2 primers were designed for the preparation of target gene fragments by RT-PCR and a set of 5 primers were designed for the RT-LAMP, and a new molecular biological method for RHDV detection was described and assessed.

EXPERIMENTAL

Reagents and sample

TIANprep Mini Plasmid Kit(DP103)and 2×Taq PCR Master Mix were provided by TIANgen Biotech(Beijing) Co.,Ltd., Betaine (Z0041-1) was provided by Shanghai Kayon Biotechnology Co.,Ltd., DNA Maker DL 2000(D501A), Primerscript RT reagent Kit (BK1901), TaKaRa rTaqTM (DR001 AM), *Taq I* (D1051A) and RNAiso Plus were provided by TaKaRa Biotechnology (Dalian) Co.,Ltd., Bst polymerase (M0275) was provided by Beijing New England Biolabs Co.,Ltd., RHDV-Sch01, gene fragments of European brown hare syndrome virus (pGM-T-EBHSV), *Pasteurella multoc -ida*, E.*coli* and *Salmonella* from rabbits were provided by the animal quarantine Lab of Sichuan Agricultural University; RHDV positive samples which collected from the experimentally infected rabbits with the RHDV-Sch01were stored at -70 °C.

Primer preparation.

Based on the sequence analysis of RHDV VP60 gene published in GenBank(accession number: NC 001543), 2 primers(P1/P2)for RT-PCR and 5 primers for RT-LAMP(listed in table 1)were carefully designed using DNAStar and Primer Explorer V4 software (http://primerexplorer.jp/e/),respectively. All these primers were prepared by TaKaRa Biotechnology (Dalian) Co.,Ltd.

TABLE 1 : Primers used in this study

Primer name	Sequence (5'–3')	Products Size(bp)	Reference Sequence
RT- PCR			NC_001543
P1	GGCAATGACAACAGGTGGAA（754-773）	749	
P2	CTGAGCGAAAGCCCAATTGT（1502-1483）		
RT- LAMP			
RF3：	GGTTTTCCACGTGCAACAG （827-809）	213	
RB3：	AATGACATGTCAGGGAAGCC（1022-1003）		
RFIP：	GATGGTCAATGTCGGCAAACCGCTGGAACCTGAACGGCAG		
RBIP：	TGGGAACAACTCCACCAACGTGCAACCTGGGAGATAGGGT		
RBLP：	TTTGGTACGCTAATGCTGGGT		

Preparation of target gene fragments

- **Isolation of RHDV RNA and RT reaction:**

RHDV RNA were extracted from the positive samples of experimentally infected rabbits with RHDV-Sch01 using the total RNA isolation reagent kit(RNAiso Plus reagent,Cat.No.BK1501, TaKaRa Biotechnology (Dalian) Co.,Ltd.,China) and stored at -70 ºC. A total reaction volume of 10 μL, containing 2 μL of 5×Primerscript buffer, 0.5μL of Primerscript RT Enzyme Mix, 0.5μL of Oligo dT Primer(50 μmol/L), 0.5μLof Random 6 mers(100 μmol/L), 2.5μL of total RNA extracted above, 4.0μL of RNase–free ddH_2O, was used for reverse transcription reaction following the manufac -turer's instructions of Primerscript RT reagent Kit(BK1901), and carried out at 37ºCfor15 min, and 85ºC for 5s, then the RT products (cDNA)were collected for further study.

- **PCR reaction**

PCR was carried out in a total volume of 50 μL, containing 3 μL of RT products (cDNA), 1.0 μL of

each primer(P1 and P2, 10 μmol/L), 25 μL of 2×Taq PCR Master Mix, and 20 μL of RNase–free ddH_2O, and performed at the following reaction conditions, denaturation at 95 ºC for 5 min followed by 30 cycles at 94 ºCfor40 s, a gradient of annealing degree from55ºCfor 45s, and 72 ºCfor 60 s, and terminated by an extension at 72 ºC for 8 min.5μL of PCR products were analyzed in 1.0% agarose in TAE buffer gel containing 0.5 mg/mL greenview using electrophoresed and photographed under a UV imaging system(Bio-Rad).

- **Construction of recombinant plasmids**:

The PCR products were separated and purified according to the instructions of TIANgel Midi Purification Kit(DP209), directly cloned into pMD-19T Vector to construct recombinant plasmid, and trans- formed into the DH5α competent cells, then the recombinant plasmids were identified using agarose electrophoresis, PCR and sequencing[8].

Basic RT-LAMP reaction.

The RT-LAMP reaction mixture (25 μL) contained 2.5μL of 10×ThermoPol buffer, 1μL (8 units) of BstDNA polymerase, 3μL of MgCl2(25mM),3μL of betaine (8 mM),6μL of dNTP(2.5 mM),1μL of each primers (RF3 (5μM),RB3(5μM), RFIP(50μM), RBIP(50 μM),and RBLP(25μM)), 1μL of template(the recombinant plasmids) and 3.5 μL ddH_2O. The reaction mixture was incubated at 65 °C for 1 h then heated at 80 ºC for 2 min in a laboratory water bath (DSY21-8, China), and the amplified products were analyzed in 15g/L agarose in TAE buffer gel. The results could also be visualized directly with the naked eye according to the white precipitate of magnesium pyrophosphate generated in the reaction or the green color changed under 254 nm UV light by the addition of diluted SuperSYBR (61201-50, TIANDZ,Chian). To confirm the specificity of amplification products, 20μL of the reaction mixture was digested with *Taq I* at 65 °C for 2 h.

Optimization of reaction conditions

In the optimum conditions assay, the RT-LAMP reaction mixture as described above were used, and the reaction conditions were determined to be as follows: a gradient of incubation degree from60°C to65°C for 30 min,45 min and 1 h, respectively, and terminated at 80°C for 2 min. In all, 5 μL of RT-LAMP products were analyzed using 15g/L agarose gel electrophoresis.

Specificity of RHDV RT-LAMP.

The specificity of the RT-LAMP was assessed by comparing RHDV with EBHSV (recombinant plasmids constructed pGM-T- EBHSV),Pasteurella multocida, E.coli and Salmonella from rabbits. The DNA extraction of *Pasteurella multocida*, E.*coli* and *Salmonella* from rabbits by the common method according to the reported literature[8].Then amplifications of the RHDV, pGM-T-EBHSV(constructed), *Pas- teurella multocida*, E.*coli* and *Salmonella* from rabbits were performed using the optimum reaction system and conditions for RT-LAMP determined above in this study.

Sensitivity of RHDV RT- LAMP.

To assess the sensitivity of RHDV RT-LAMP, the recombinant plasmids containing the RT-PCR product of RHDV (constructed above) were estimated by ND-1000 ultraviolet spectrophotometer (Nano Drop Co.,Ltd,USA) and amplified in a serial of 10-fold dilution by using the optimum reaction system and conditions for RT-LAMP determined above, and compared with the RHDV RT-PCR detection method established in reported literature[9].

Primary application of RHDV RT- LAMP

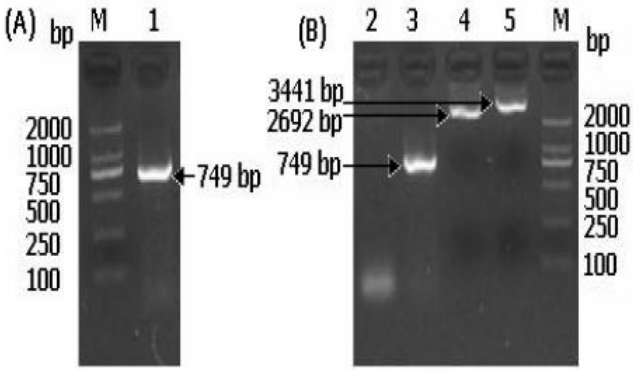
To evaluate the reliability of RHDV RT-LAMP, 5 RHDV experimentally infected samples and 30 clinical samples collected from markets were detected by using the RT-LAMP with RT-PCR established in reported literature in parallel.

RESULTS

Construction of recombinant plasmids

In order to develop the RT-LAMP for RHDV detection, a 749 bp length target DNA fragment of VP60 gene of RHDV was synthesized using RT-PCR method(see Figure 1(A)), and directly cloned into pMD-19T Vector to construct recombinant plasmid named pMD-19T-RHDV, then the recom- binant plasmids were identified using agarose electrophoresis, PCR and sequencing as described in this study(see Figure

1(B)), The results showed the target DNA fragment of VP60 gene of RHDV (as shown in Figure 1) were successfully synthesized, which had 100% homology with the reference sequence published in GenBank, and pMD-19T- RHDV was extracted and stored at -20 °C for the template used in this study.



M: DNA marker DL2000; 1: RT-PCR products of RHDV-Sch01 RNA; 2: RT-PCR products of pMD -19T vector; 3: RT-PCR products of pMD-19T- RHDV; 4: pMD-19T vector;5: pMD-19T-RHDV

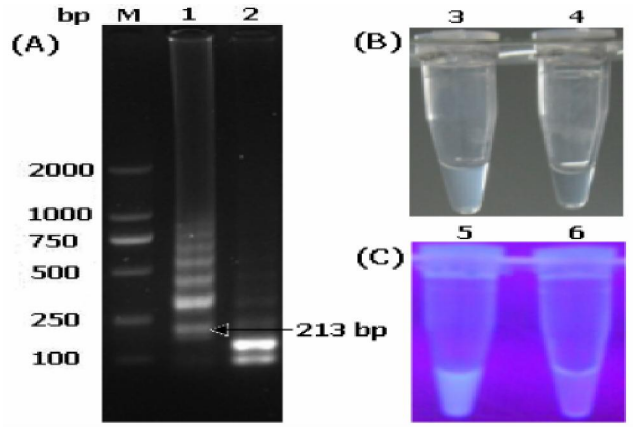
Figure 1 : Agarose gel electrophoresis results of target DNA fragment of VP60 gene of RHDV(A) RT- PCR result of VP60 gene of RHDV(B) Identi- fication results of recombinant plasmids

Basic RT-LAMP reaction

The RHDV basic RT-LAMP was carried out using pMD-19T-RHDV (constructed) as template and incubated at 65 °C for 1 h, then the amplification products were analyzed by using agarose gel electrophoresis, enzyme- digestion and visual inspection as described in the method section above. The results (as shown in Fig.2)showed the target template pMD-19T-RHDV was amplified at 65 °C with a ladder-like pattern bands from about 213 bp on the gel (Figure 2(A) lane1), which specificity were conformed by the two smaller digestion prodcuts from 100 bp to 213 bp with *Taq*'!(Figure 2(A) lane2). The positive reaction could also be visualised clearly by the white precipitate of mag- nesium pyrophosphate(Figure 2(B) tube3) and the green color produced after the addition of diluted Supper SYBR(Figure 2(C) tube5), while the ddH_2O or pMD-19T vector template control reactions had no amplifications and visual inspection changes (Figure 2 tube4 and 6).

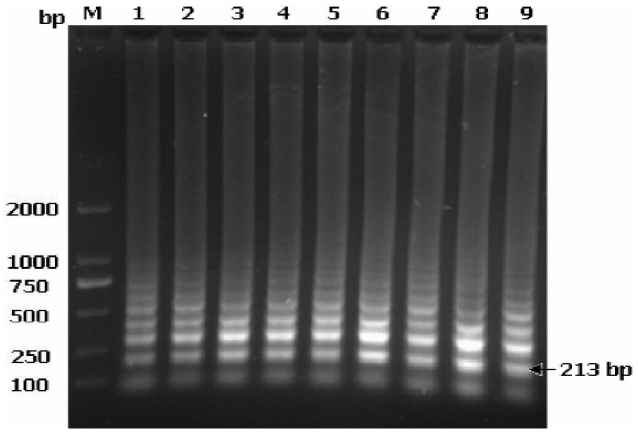
Optimization of reaction conditions

The RT-LAMP reaction conditions were optimized by different amplification temper-atures and reaction time. The results (as shown in Figure 3) showed that lane3, lane4, lane5, lane6, lane7, lane8 and lane9 had more clearer ladder-like reaction bands. Then the optimal temperature and reaction times for the RT-LAMP were selected at 64 °C for 45 min for the specificity and amplification efficiency, and the 25 μL reaction volume as described in the method section above was determined, and the optimum RT reaction volume components and conditions were the same as the instructions of Primerscript RT reagent Kit.



(A)Agarose gel electrophoresis; (B) Visual inspec- tion by turbidity; (C) visual inspection by adding Supper SYBR;M: DNA marker DL2000;1,3,5: RT-LAMP reaction products of pMD-19T-RHDV ; 2:The positive RT-LAMP products digested with *Taq*'!.4,6:Negative control (pMD-19T vector);

Figure 2 : Results of RHDV basic RT-LAMP



M: DNA marker DL2000; 1:60°C 1h; 2:61°C 1h; 3: 62°C 1h; 4: 63°C 1h; 5: 64°C 1h; 6: 65°C 1h; 7: 64°C 30min; 8: 64°C 45 min;9:64°C 1h

Figure 3 : The results of optimization of reaction conditions

Specificity and sensitivity of RHDV RT- LAMP

The specificity and sensitivity of RHDV RT-LAMP

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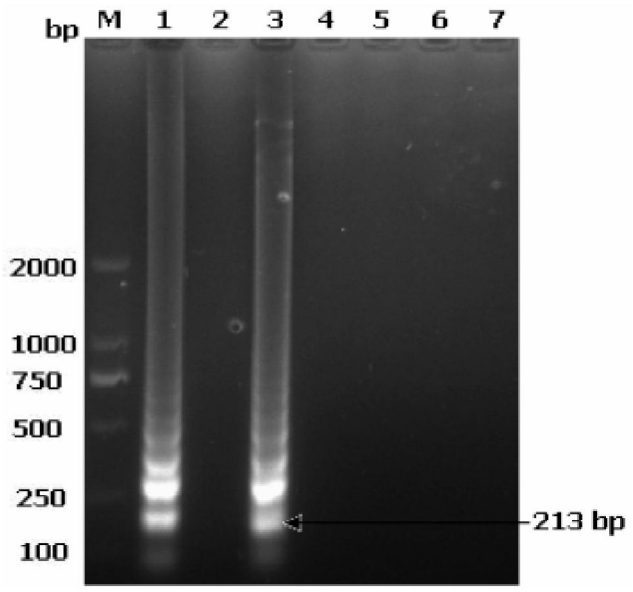
were assessed in the tests as described in the method section above. There was a clear ladder-like pattern of electrophoretic bands from about 213 bp observed only for the pMD-19T-RHDV and RHDV detection(see Figure 4, lane1 and lane3), and no specific amplification products were obtained for the detection of pGM-T-EBHSV, *Pasteurella multocida*, E.*coli* and *Salmonella* (see Figure 4).For the sensitivity, the expected size of amplification bands of 192 bp(see Figure 5(B) lane4, 50copies) or ladder-like pattern from about 213 bp (see Figure 5 (A)lane5,5 copies) could be observed when the pMD-19T-RHDV at different concentra- tions were tested(as shown in Figure 5), and this results indicated the sensitivity of detection limit by RT-LAMP could reach about 5 copies of cloned viral genomic fragments, which is higher than that of RT-PCR established.

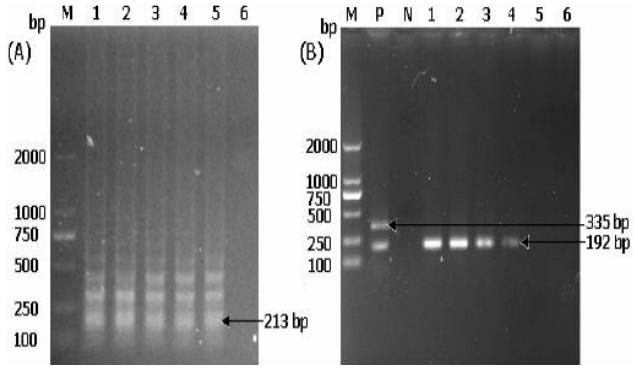


M: DNA marker DL2000; 1: pMD-19T-RHDV control; 2: ddH_2O 3: RHDV; 4: pGM-T-EBHSV ;5: E.*coli*; 6: P*asteurella multocida*; 7 : S*almonella*

Figure 4 : The specificity assay results of RHDV RT-LAMP on agarose gel electrophoresis

Primary application of RHDV RT- LAMP

The RHDV RT-LAMP was carried out by using 5 RHDV experimentally infected samples and 30 clinical samples with RT-PCR established in parallel. All of the 5 RHDV experimentally infected samples proved to be positive by the two methods, and there were not any amplification for the 30 clinical samples detection. The results indicated the good correlation between RHDV RT-LAMP and RT-PCR.



M: DNA marker DL2000;P:positive control of RT-PCR; N:negative control;1-6: the copies number of pMD-19T-RHDV was about 5×10^4, 5×10^3, 5×10^2; 50,5 and 0.5,respectively.

Figure 5 : The sensitivity assay results of RHDV RT-LAMP on agarose gel electrophoresis(A) The result obtained by RT-LAMP assay (B) The result according to RT-PCR assay by Wang et al.(2011)

DISCUSSION AND CONCLUSION

LAMP assay was regard as a powerful molecular tool for the DNA amplification and used widely in the many fields because of its advantages of rapidity, high sensitivity, high specificity and easy manipulation[10-12], but the requirements of the four based specially designed primers for the target amplification sequence may limit its application in the detectin for all the pathogens. Yuan et al (2013) had reported a one-step RT-LAMP method for the detection of RHDV using a set of 4 basic primers and detected 7 field RHDV isolates in China successfully[13]. In this paper, a new RT-LAMP method for RHDV detection was developed according to the VP60 gene segment using 4 basic special primers and 1 loop primer to improve the sensitivity,compared with the LAMP reported by Yuan et al. We all know European brown hare syndrome virus (EBHSV) and RHDV are both the two important members of genus Lagovirus in the family Caliciviridae, which had been con- formed by nucleotide and deduced amino acid sequence comparisons in the VP60 gene region, but many studies described recently about RHDV detection methods were few referred to EBHSV since it has not occurred in China until now, and this is the first report using a DNA fragment of 359 bp of EBHSV VP60 gene (synthesized) for the specificity evaluation of RHDV RT-LAMP in Chian.

In conclusion, the RT-LAMP assays described here

which performed at 64ºC for 45min in a laboratory water bath and visualized the results directly with the naked eye have good sensitivity and specificity, as few as 5 copies target gene could be detected, and the primary application of RHDV RT- LAMP showed the good correlation with RT-PCR established.Therefore this RT-LAMP method supply a new convenient and useful technology for the detection of RHDV in lab and field practice in China.

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REFERENCES

[1] G.LeGall, C.Arnauld, E.Boilletot, J.P.Morisse, D.Rasschaert; Journal of General Virology, **79**, 11 **(1998)**.

[2] B.Gromadzka, B.Szewczyk, G.Konopa, A.Fitzner, A.Kesy; Acta Biochim Pol., **53(2)**, 371 **(2006)**.

[3] F.Chen; Veterinary Lemology, 5th Edition. Bei jing, **(2008)**.

[4] M. T. McIntosh, S. C. Behan, F. M Mohamed, Z.Lu, K.E. Moran, T.G. Burrage, J.G Neilan, G.B. Ward, G. Botti, L.Capucci, S.A.Metw ally; Virology Journal, **4**, 96 **(2007)**.

[5] L.Yang, F.Wang, B.Hu, J.Xue, Y.Hu, B.Zhou, D.Wang, W.Xu; J Virol Methods., **151(1)**,24 **(2008)**.

[6] B.D.Cooke, A.J.Robinson, J.C.Merchant, A.Nardin, L.Capucci; Epidemiol Infect., **124(3)**, 563 **(2000)**.

[7] T.Notomi, H.Okayama, H.Masubuchi, T.Yonekawa, K.Watanabe, N.Amino, T.Hase; Nucl. Acids Res., **28(12)**, E63 **(2000)**.

[8] Z.Yang, G.Pang, Y.Wang, X.Yao, Y. Zhang, Z.Yang, K.Wang, X.Dai; Acta Agriculturae Boreali-occidentalis Sinica, **19**, 8 **(2010)**.

[9] Y.Wang, Z.Yang, X.Han, K.Wang, X.Yao, Y.Bai; Chinese Veterinary Science, **41(11)**, 1165 **(2011)**.

[10] S.Jayawardena, C.Y.Cheung, I.Barr, K.H.Chan, H.Chen, Y.Guan, J.S.Peiris, L.L.Poon; Emerg. Infect. Dis., **13**, 899 **(2007)**.

[11] X.Fang, W.Xiong, J.Li, Q.Chen; J Virol Methods, **151(1)**, 35 **(2008)**.

[12] S.Yin, Y.Shang, G.Zhou, H.Tian, Y.Liu, X.Cai, X.Liu;.J Biotechnol, **146**, 147 **(2010)**.

[13] D.Yuan, D.Guo, J.Liu, C.Si, Q.Jiang, H.Lin, T.Yang, L.Qu; J Virol Methods, **187**, 274 **(2013)**.