

ESTABLISHMENT AND APPLICATION OF REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION FOR DETECTION OF RABBIT HEMORRHAGIC DISEASE VIRUS

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ABSTRACT

A Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method to detect *Rabbit hemorrhagic disease virus* (RHDV) was established. Total RNA was extracted from the fresh liver of 53 rabbits died of RHDV collected from different region in Jiangsu and Shandong province during spring in 2007. A pair of primers was designed according to the sequences of RHDV published in GenBank, which could be amplified a 269 bp segment. The result of sequencing showed that the homology was 95.8-98.5% compared with the reference sequence published in GenBank. The least amount of RHDV that could be detected was 3.32 ng/ μ l, which also showed its high sensitivity and, what's more, the sensitivity was 4×10^4 times higher than that of hem agglutination (HA). The detection of RHDV by RT-PCR was established successfully. Compared to HA, RT-PCR could detect RHDV in all the viscera except for in feces, and HA only in liver, while kidney, spleen, blood and lung were positive. Samples kept at -20°C for 12 months could be detected by this RT-PCR. All above results indicate that this RT-PCR method has strong specificity, high sensitivity and good repetition, and can be used in RHDV clinical diagnosis, epidemiology study and quarantine of rabbit products such as meat.

Key words: Rabbit hemorrhagic disease virus (RHDV), RT-PCR, Viscera distribution.

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) was a non cultivable calici virus that infected rabbits with hepatocellular necrosis and disseminated intravascular coagulation and caused epidemics of acute death (Xu and Chen, 1989; Gunn and Nowotny, 1996). Since 1984, when it was first found at Wuxi city Jiangsu province, this virus had caused great economic loss in China and other countries (Liu *et al.*, 1984; Gregg *et al.*, 1991). Rabbit hemorrhagic disease virus (RHDV) was identified as a calicivirus (Mitro and Krauss, 1993), a positive-sense, single-stranded RNA virus that is antigenically related to the calicivirus *European brown hare syndrome virus* in hares. The complete genome of the virus has been elucidated for the German isolate and shown to comprise a single-stranded, positive-sense RNA genome of 7,437 nucleotides (nt). The genome of RHDV contains two open reading frames (ORFs). The long ORF of 2,344 codons (ORF1), which codes for a polyprotein containing nonstructural proteins as well as the virion coat protein, is at the C terminus. There was 98.2-99.0% homology of the long ORF gene in the relevant sequences published in GenBank (Yan *et al.*, 2001). The short ORF of 118 codons (ORF2), which codes for a minor structural protein (VP10), is at the N terminus.

There are some methods in common use to detect RHDV at present, such as HA (Liu *et al.*, 1984; Gong *et al.*, 2003), hem agglutination inhibition (HI) (Gong *et al.*, 2003; Shi, 1988), enzyme linked immunosorbent assay (ELISA) (Liu *et al.*, 2006; Qin *et al.*, 2007), Electron Microscope (Capucci *et al.*, 1998), RT-PCR (Hu *et al.*, 2006; Zhou *et al.*, 2006) which can provide significant clinical diagnosis. HA and HI are easy to operate, but they are not sensitive and cannot detect minim virus. ELISA is sensitive, but its specificity is often not assured because the antigen is not purified completely. Electron microscope is fast and intuitionist, but its outlay is too expensive. RT-PCR, as a

modern molecular biological method, can be used in detection of large numbers of samples and investigation of epidemiology. In this study, a series of experiments were carried out, such as sensitive experiment, specificity experiment, in order to establish stable RT-PCR method to detect RHDV, and the RHDV distribution in different organ.

MATERIALS AND METHODS

Clinical samples

Fresh organ specimens were used in this study. Fresh-frozen liver samples collected from 53 RHD rabbits from different region in Jiangsu and Shandong province were used to establish the RT-PCR method. The original diagnosis was based on histopathology and confirmed by the HA and electron microscopy. All of the samples were stored at -20°C.

Establishment of RT-PCR assay

Viral RNA was extracted using the RNagents kit (Takara) following the manufacturer's instructions. Primers for RT-PCR were designed from known sequences based on the RHDV capsid protein VP60 with the help of the primer premier, version 5.0. The primer P1: 5'-CAGCCGTACTGAGCCAGATGTA- 3', The primer P2: 5'- AAGGACTAGTGTGGGAACAAGG- 3' was synthesized by Invitrogen. First-strand reverse transcription to produce cDNA was performed using Superscript II reverse transcriptase with the random oligo-dt primer for the reaction. PCR was used to amplify the cDNA, PCRs (25µl) typically consisted of template DNA (3-4µl of first-strand cDNA), 0.2 mM each dNTP, 2.0 to 2.5 mM MgCl₂, 0.5µM each primer, and 1 unit of Taq DNA Polymerase (Takara). Then the mixture was subjected to 30 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 50 s. Both positive and negative controls were included at every stage of these reactions. PCR product was cloned directly into pMD-19T easy vector (Takara) in order to sequence the fragment. After appropriate restriction enzyme digestion, the products were electrophoresed. The cloned fragment was sequenced by Takara Biotechnology Co., Ltd. to give approximately 269 bp of sequence for assembly and homology analysis.

Specificity experiment was carried out by comparing RHDV detection to Lapine rotavirus cell virus, Rabbit vesicular stomatitis virus, SPF rabbit liver and ddH₂O were taken as negative and blank control. Sensitivity experiment was carried out by diluting RHDV from RHD rabbit liver (HA=3) 2, 4, 8, 16(HA=0), 1×10^1 , 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 times serially, the highest dilution yielding a positive signal was considered as the detection limit of the assay.

RHDV Detection of samples

Virus distribution experiment was carried out using the established RT-PCR by detecting several viscera including liver, spleen, kidney, heart, thymus, lung, lymph, muscle, brain, spinal cord as well as feces. The results were compared to HA method. In addition, several RHDV livers that had been kept in -20°C for 2, 4, 12 months were also detected.

RESULTS AND DISCUSSIONS

Establishment of RT-PCR

After amplifying by RT-PCR, the products were electrophoresed. The result showed that a clear 269 bp fragment could be seen (Figure 1). The PCR product was cloned into pMD-19T easy vector (Takara). After appropriate restriction enzyme digestion, the result was showed (Figure 2) The sequencing result showed that the homology was 95.8-98.5% compared with the RHDV reference sequences published in GenBank. In specificity experiment, RHDV showed special fragment and Lapine rotavirus cell virus, rabbit vesicular stomatitis virus, SPF rabbit liver and ddH₂O showed negative (Figure 3), indicating its specificity. In sensitivity experiment, the segment could not be seen when the RHDV sample was diluted to 1.6×10^5 times (Figure 4). When the HA value of RHDV was zero, the RNA concentration of RHDV was $33.2 \mu\text{g}/\mu\text{l}$, the sample was diluted to 10^4 times, so the least concentration of RNA can be detected was $3.32 \text{ ng}/\mu\text{l}$.

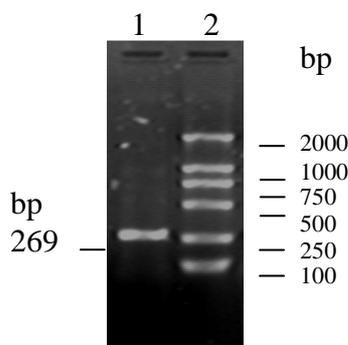


Figure 1: The amplified fragment of RHDV gene by RT-PCR 1 RT-PCR product; 2 DNA marker DL-2000

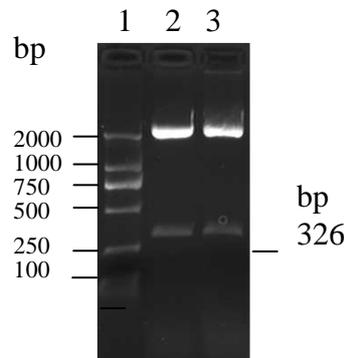


Figure 2: Enzyme digestion of recombinant plasmid RHDV gene/T-Vector 1, DNA marker DL2000; 2 and 3, RHDV Gene/T-Vector (*EcoRI* / *HindIII*)

RHDV Detection of samples

In virus distribution experiment, liver, heart, blood, spleen, lung and kidney were detected RHDV positive by HA method. All the samples of RHD rabbits could be detected RHDV positive by RT-PCR, expected for feces (Table1, Figure 5). All the RHDV samples which had been kept at -20°C for 2, 4, 12 months could be detected RHDV positive

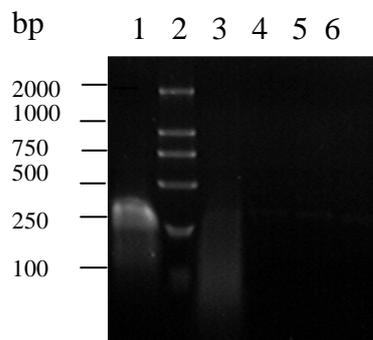


Figure 3: Results of specificity assay for RT-PCR; 1, RHD positive liver sample; 2, DNA marker DL2000; 3, dd H₂O control; 4, Lapine rotavirus cell virus; 5, rabbit vesicular stomatitis virus; 6, Negative control (SPF rabbit liver)

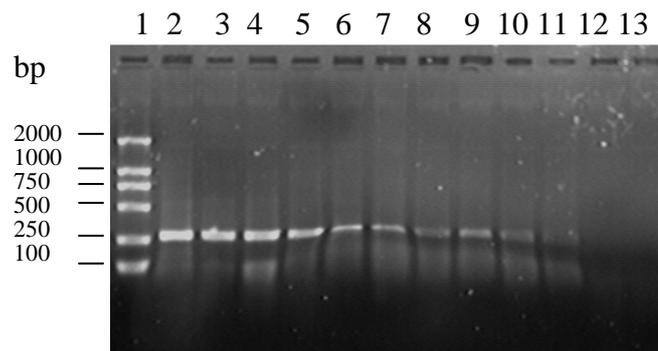


Figure 4: Results of Sensitivity Assay of RT-PCR 1, DNA marker DL2000; 2, positive liver sample of HA value 3; 3-6, Different dilution (2^{-1} - 2^{-4}) of RHD positive liver; 7-12, Different dilution (10^{-1} - 10^{-6}) of RHD positive liver; 13, negative control (SPF rabbit liver)

Table 1: RHDV Distribution of organs of RHD rabbits

Organs	HA	RT-PCR
Liver	5+	+
Lymph node	-	+
Thymus	-	+
Lung	1+	+
Blood	1+	+
Feces	-	-
Muscle	-	+
Spleen	4+	+
Kidney	3+	+
Brain	-	+
Heart	1+	+
Spinal cord	-	+
Marrow	-	+

Note: "+", HA or RT-PCR positive; "-", HA or RT-PCR negative

DISCUSSION

Reverse transcriptase-PCR (RT-PCR) based detection of viral RHDV RNA has been shown to be rapid, sensitive, special, and efficient. RT-PCR has been successfully used to analyze the RHDV genome [16-18]. In this study, a little of sample was needed for the detection of RHDV by RT-PCR method. In sensitivity experiment, HA was chosen as a control and the results showed that the sensitivity was 4×10^4 times higher than that of HA. The minimum RNA concentration that could be detected was 3.32 ng/ μ l. In virus distribution experiment, the RT-PCR method showed significant sensitivity. All the samples of RHD rabbits could be detected RHDV positive by RT-PCR, except for feces. Therefore, the method owns an important significance to investigation of the epidemiology, diagnosis and the detection of rabbits' products

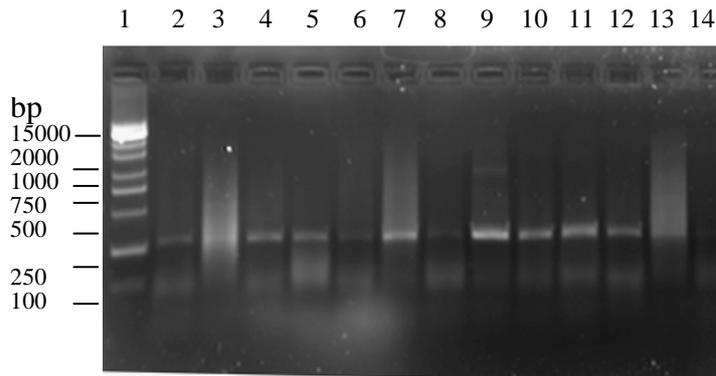


Figure 5: Results of RHDV organs distribution. 1, Wide range DNA ladder Marker; 2, spinal cord; 3, marrow; 4, lymph node; 5, lung; 6, muscle of anterior limb; 7, heart; 8, thymus; 9, muscles of abdomen; 10, blood; 11, brain; 12, RHD positive liver; 13, thymus; 14, feces

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