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A Reliable Internally Controlled RT-Nested PCR Method for the Detection of Hepatitis C Virus RNA

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We have developed a reliable internally controlled RT-nested PCR method for the detection of hepatitis C virus (HCV) RNA using *in vitro* synthesized Renilla luciferase (Rluc) RNA as an internal control. Using this method, the 5'-noncoding region of HCV RNA (144 nucleotides) and Rluc RNA (276 nucleotides) were efficiently amplified in a single tube, and the sensitivity and specificity of this method were comparable to standard RT-nested PCR. This method was successfully performed on RNA specimens obtained from *in vitro* HCV-infected human hepatocyte PH5CH8 cells, which support HCV replication. In addition, we demonstrated that this method was useful for the evaluation of antiviral reagents by confirming the anti-HCV activity of bovine lactoferrin, which we previously found to be a new inhibitor of HCV infection. Therefore, this method may be useful for the studies of not only HCV but also of other viruses.

Key words: Hepatitis C virus, Reverse transcription-nested PCR (RT-nested PCR), internal control

Reverse transcription-nested polymerase chain reaction (RT-nested PCR), in which the second PCR is performed using an internal primer set, is a powerful strategy for detecting small amounts of viral RNA molecules. In our laboratory, RT-nested PCR is routinely used to detect the RNA genome of the hepatitis C virus (HCV) because RT-PCR is not enough to amplify the HCV RNA genome. Infection with HCV causes chronic hepatitis and persistent infection that frequently leads to liver cirrhosis and hepatocellular carcinoma (1, 2). The 5'-noncoding (5'-NC) region, which is the most conserved region of a positive-stranded HCV RNA genome with a 9.6 kilobase (3), is usually used for amplification by RT-nested PCR. Although this is a highly sensitive method, which allows for the detection of 10 copies of the HCV RNA genome, it is not possible to monitor the efficiency of RT-nested PCR due to the lack of an internal control. Therefore, when we did not detect the HCV RNA genome using RT-nested PCR, it was very difficult to conclude whether this was due to the absence of HCV RNA in a specimen or to the failure of RT-nested PCR. In a revised method (4), duplicate specimens were required to evaluate the results of RT-nested PCR, but this method is labor intensive and not economical. Although the expression of housekeeping genes has been used as an internal control in RT-PCR (5, 6), the copy number of these endogenous mRNAs is too high to effectively monitor the efficiency of RT-nested PCR (with more than 60 cycles in total) quantitatively.

In this report, we describe a reliable internally controlled RT-nested PCR for the detection of HCV RNA using an exogenous *in vitro* synthesized Renilla luciferase (Rluc) RNA.

Materials and Methods

In vitro synthesized RNA. HCV RNA containing positions 1 through 2,768 of the HCV genome (3) was synthesized by *in vitro* transcription with T7 RNA polymerase using the pBluescript KS(+)/HCV plasmid vector (7) as a template, as previously described (8, 9). The pRL-TK plasmid vector (Promega) encoding Rluc was used to synthesize Rluc RNA as an internal control. The pRL-TK plasmid DNA was linearized by

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being cut at the *Xba*I site and then used as a template for *in vitro* transcription with T7 RNA polymerase as described previously (8). The synthesized Rluc RNA (949 nts) was treated with DNase QR (Promega) and purified using an RNaid kit (Bio 101) after agarose gel electrophoresis. Once more, RNAs were treated with DNase I (Genhanter) and purified using an RNaid kit after agarose gel electrophoresis as described previously (7).

Internally controlled RT-nested PCR. RNA was prepared from cells using an ISOGEN extraction kit (Nippon Gene Co., Tokyo, Japan). 0.5 μg of the RNA was used for the detection of the 5'-NC region of HCV RNA. As an internal control, 0.5–50 pg of *in vitro* synthesized Rluc RNA was added to the RNA sample before RT. RT of HCV RNA with SuperScript II (Gibco BRL) was carried out using primer 319R (10), and RT of Rluc RNA was carried out using primer Rluc1R (5'-ATGGCAACATGGTTTCCACG-3'). HCV cDNA was amplified with *Taq* DNA polymerase (Sawady Tech., Tokyo, Japan) in first-round PCR using 319R and a sense outer primer 196 (10), and Rluc cDNA was amplified using Rluc1R and a sense outer primer Rluc1 (5'-TGTGCCACATATTGAGCCAG-3'). Rluc and/or HCV sequences were amplified (35 cycles) using the following thermal cycle: 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. Two microliters of the first-round PCR product were amplified in a second-round PCR (25 cycles) using the HCV primers 104 and 197R (10) and Rluc inner primers Rluc2 (5'-TTGGTATGGGCAAATCAGGC-3') and Rluc2R (5'-CTTCTTCAGATTTGATCAACGC-3') using the following thermal cycle: 94 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min. The ratios of HCV-to-Rluc primer concentrations were changed in order to find the best condition (see text). Amplified DNA (144 bp for HCV and 276 bp for Rluc) were detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis.

Standard RT-nested PCR. Standard RT-nested PCR for detection of the 5'-NC region of HCV RNA was performed as described previously (9). Briefly, antisense primer 319R was used to prime cDNA synthesis using SuperScript II. Amplification by PCR with *Taq* DNA polymerase was performed for 35 cycles using primer 319R and sense primer 196, and the internal primer pair 104 and 197R was used for the second round of PCR (25 cycles). Each PCR cycle was the same as that used for the internally controlled RT-nested PCR.

Virus inoculation. Virus inoculation using HCV-positive serum 1B-2 (10^7 HCV/ml) of human hepatocyte PH5CH8 cells was performed by a previously described method (11). Briefly, a total of 5 μl of undiluted serum 1B-2 was added to PH5CH8 cells (1×10^5), and they were incubated for 2 h at 37 °C. The cells were then washed with phosphate buffered saline (PBS) and were maintained in fresh medium (1 ml) at 32 °C. The cells were harvested at intervals during the culture period for the detection of HCV RNA.

Anti-HCV activity of bovine lactoferrin (LF). The assay for anti-HCV activity of bovine LF was performed by a method described previously (4) using PH5CH8 cells and inoculum 1B-2. Briefly, PH5CH8 cells (1×10^5) were plated in duplicate and cultured for 2 days before viral inoculation. 5 μl of HCV-positive serum 1B-2 and bovine LF (at a final concentration of 0.5 or 2.0 mg/ml) were preincubated in 500 μl of culture medium for 60 min at 4 °C, and then the mixture of HCV and bovine LF was added to the PH5CH8 cells cultured as described above, and this mixture was incubated for 90 min at 37 °C. The cells were washed with PBS and further cultured at 32 °C. The cells were harvested at 8 days post-infection for the detection of HCV RNA.

Results

To develop an internally controlled RT-nested PCR method, we chose the Rluc RNA as an internal indicator that can be used to monitor the efficiency of RT-nested PCR. The advantages of Rluc RNA are that the nucleotide sequence of Rluc RNA is not present in any animal genomes, and Rluc RNA is easily synthesized by *in vitro* transcription using commercially available plasmid vectors such as pRL-TK. Using *in vitro* synthesized HCV RNA and Rluc RNA, we first examined the conditions in which the efficiency of RT-nested PCR for detection of HCV ideally should not be decreased by co-amplification with Rluc RNA in a single tube. 10^3 copies of HCV RNA and 0.5, 5 and 50 pg of Rluc RNA were used with 2 (1 μM):1 (0.5 μM), 5 (1 μM):1 (0.2 μM) and 10 (1 μM):1 (0.1 μM) HCV-to-Rluc primer concentrations, respectively, in the RT reaction and in the first and second rounds of PCR. As shown in Fig. 1, co-amplification with 5:1 HCV-to-Rluc primer concentration and 5 pg of Rluc RNA yielded the best results, with no decrease in the band intensity of PCR products by RT-nested PCR for the detection of HCV RNA. Using

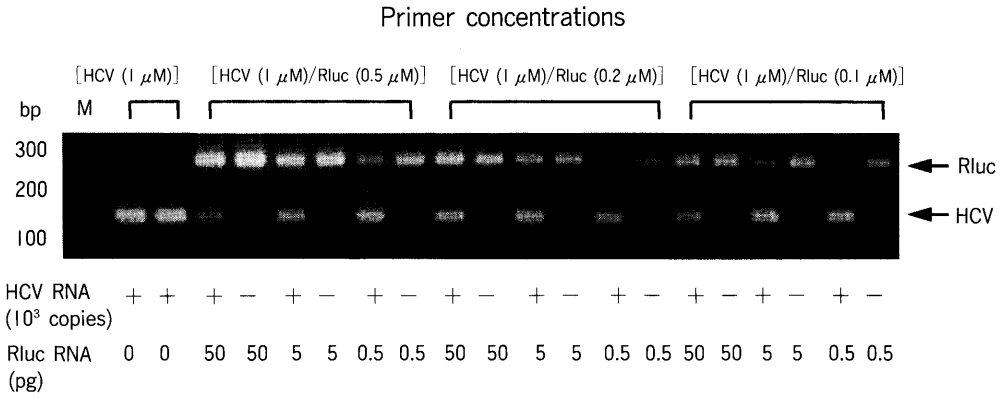

Fig. 1 Detection of *in vitro* synthesized HCV RNA and Rluc RNA by RT-nested PCR. 10^3 copies of HCV RNA and 0.5, 5 and 50 pg of Rluc RNA were used with 2 (1 μM): 1 (0.5 μM), 5 (1 μM): 1 (0.2 μM) and 10 (1 μM): 1 (0.1 μM) HCV-to-Rluc primer concentrations, respectively, in the RT reaction and in the first and second rounds of PCR. Cellular RNA (0.5 μg) from PH5CH8 cells was added to each reaction tube so as to be equivalent to the amount of RNA in the actual experimental specimens. PCR products (144 bp for HCV and 276 bp for Rluc, as indicated by arrows) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. Lane M, a 100 bp DNA ladder as a size marker.

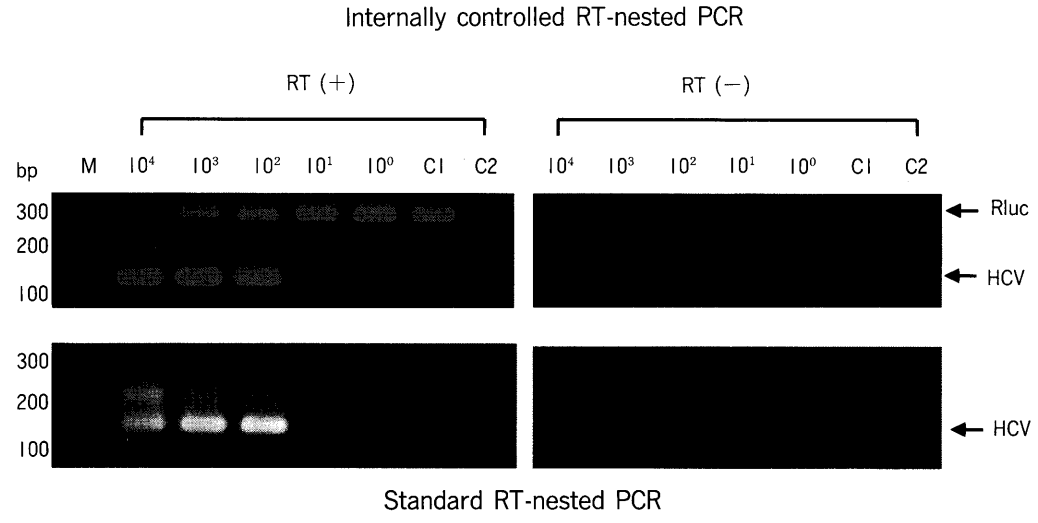

Fig. 2 Sensitivity and specificity of RT-nested PCR using *in vitro* synthesized HCV RNA and Rluc RNA. 1–10^4 copies of HCV RNA were subjected to internally controlled RT-nested PCR and standard RT-nested PCR. Nested PCR without an RT reaction was also performed. Five pg of Rluc RNA was used with 5 (1 μM): 1 (0.2 μM) HCV-to-Rluc primer concentrations in internally controlled RT-nested PCR. Cellular RNA (0.5 μg) was added as shown in Fig. 1. PCR products were detected as shown in Fig. 1. Lane M, a 100 bp DNA ladder as a size marker; lane C1, H_2O with Rluc RNA (only upper panel); lane C2, H_2O.

5:1 HCV-to-Rluc primer concentration and 5 pg of Rluc RNA, we next examined the sensitivity and specificity of this new method for the detection of HCV. As shown in Fig. 2, 100 copies of HCV RNA were detected by this method, indicating that the sensitivity of this method was comparable to standard RT-nested PCR. The detection limits (100 copies of HCV RNA) of both RT-nested PCR methods were the same as those of our previous report (9). We confirmed no amplification of Rluc RNA and HCV RNA without an RT reaction, indicating that the plasmid DNAs used as the templates were absolutely excluded with DNase treatment. Hybridization using

HCV-specific and Rluc-specific probes confirmed that PCR bands (144 and 276 bp) were derived from HCV RNA (144 bp) and Rluc RNA (276 bp), respectively, (data not shown).

To demonstrate the usefulness of this method, we used it to detect HCV RNA from HCV-infected PH5CH8 cells, which are cloned human non-neoplastic hepatocytes that support HCV replication (11, 12). PH5CH8 cells were inoculated with HCV-positive serum 1B-2 as previously described (11), and cellular RNA was obtained from the cells at 2, 4, 6, 10 and 16 days postinoculation, respectively, for the detection of HCV RNA. As shown in Fig. 3, by means of the internally controlled RT-nested PCR, we obtained distinct PCR bands indicating the presence of HCV RNAs in PH5CH8 cells, together with co-amplified bands derived from Rluc RNA. The intensities of the bands amplified with HCV primers were the same regardless of the presence of the internal control. This result indicates that our new method is highly reliable and potentially useful for the detection of HCV RNA.

Previously, we found that bovine LF, a milk glycoprotein belonging to the iron transporter family, prevented HCV infection in PH5CH8 cells (4). At that time, the anti-HCV activity of bovine LF was inferred from data obtained using RT-nested PCR without an internal control, although we performed RT-nested PCR in duplicate (4). To reevaluate our previous finding concerning the anti-HCV activity of bovine LF, we performed the same experiment (4) using PH5CH8 cells and serum 1B-2 as an inoculum, and we detected HCV RNA by both the standard RT-nested PCR and the internally controlled RT-nested PCR method. As shown in Fig. 4, we obtained the same results with both methods, indicating that bovine LF blocks HCV infection at a concentration of 0.5 mg/ml. Therefore, our previous finding was clearly confirmed by this internally controlled RT-nested PCR method.

Discussion

As shown in the experiments regarding the sensitivity, specificity and reproducibility of HCV RNA detection, our internally controlled RT-nested PCR method provides a reliable means of detecting small amounts of a virus genome such as HCV RNA and is especially useful for the evaluation of candidates for anti-HCV reagents such as bovine LF.

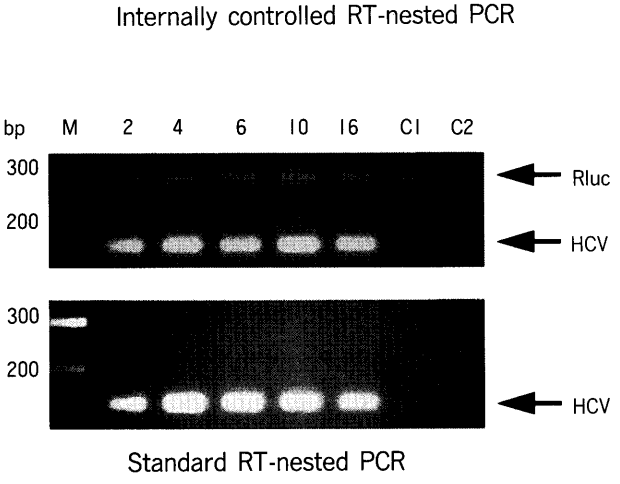

Fig. 3 Detection of HCV RNA and Rluc RNA in samples from HCV-infected PH5CH8 cells. PH5CH8 cells were inoculated with serum 1B-2 according to a previous method (11). The results of the internally controlled RT-nested PCR (upper panel) and standard RT-nested PCR (lower panel) are shown. PCR products were detected as shown in Fig. 1. Lane M, a 100 bp DNA ladder as a size marker; lane C1, H_2O with Rluc RNA (only upper panel); lane C2, H_2O. Numbers indicate days post-infection.

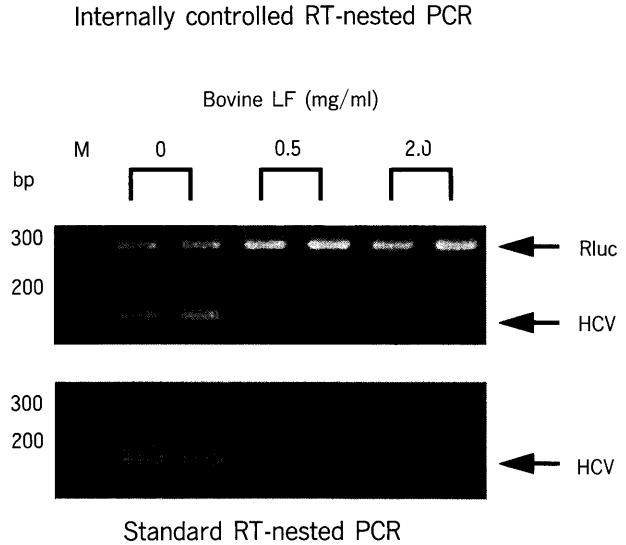

Fig. 4 Anti-HCV activity of bovine LF. PH5CH8 cells and inoculum 1B-2 were used for the assay of bovine LF anti-HCV activity, as described previously (4). The internally controlled RT-nested PCR (upper panel) and standard RT-nested PCR (lower panel) were performed using duplicate RNA samples. PCR products were detected as shown in Fig. 1. Lane M, a 100 bp DNA ladder as a size marker.

Recently, new RT-nested PCR for the detection of HCV RNA using bovine viral diarrhea virus (BVDV) as an internal control was reported (13). In this method, BVDV was added to the sample before RNA extraction; however, this method will not be widely used in many laboratories because a BVDV proliferation system must be maintained. In contrast, the Rluc RNA used as an internal control in our method is easily obtained by *in vitro* transcription, and synthesized Rluc RNA can be stored at −80 °C until use.

For the detection of HCV RNA in human sera, an Amplicor HCV PCR kit (Roche Diagnostics Inc., Tokyo, Japan) is widely used as an internally controlled single-step RT-PCR method (14). However, HCV RNAs in HCV-infected human cells were undetectable with the Amplicor HCV PCR kit (7), suggesting that the Amplicor HCV PCR kit does not maintain its high sensitivity for HCV RNA detection under the condition of coexisting cellular RNA. The RT-nested PCR method developed in this study is highly sensitive in and applicable to the detection of HCV RNA in cells. Therefore, our method may be useful for the detection of a viral genome derived from infected cells.

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