Arch. Pharm. Res. Vol.19, No.6, pp. 486-489, 1996

Rapid Detection of Serum HCV RNA by Combining Reverse Transcription and PCR without RNA Extraction

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(Received July 31, 1996)

A simple, rapid, specific and sensitive method for the detection of serum hepatitis C virus (HCV) RNA using the reverse transcription-polymerase chain reaction (RT-PCR) technique without conventional RNA extraction was developed. HCV template RNA from serum was obtained by boiling the serum at 95°C for 2 min, cooling rapidly in ice and removing the proteins by cetrifugation. RT-PCR amplifications including the reverse transcription and first PCR amplification were performed in one vessel containing both of reverse transcriptase and *Taq* DNA polymerase. The detection of HCV RNA from 10^{-3} μl serum was possible with this method. The suitability of this method for clinical analysis was evaluated by assaying HCV RNA in 225 patient samples including anti-HCV antibody negatives (13 samples) and positives (212 samples) by enzyme-linked immunosorbent assay test (ELISA). Detections of HCV RNA with this method were in 4 of 13 anti-HCV antibody negative samples (30.8%) and 95 of 212 positive samples (44.8%). The present method can be completed in 1 hr and has a wide range of application for the clinical utilities to determine the viral RNAs.

Key words : HCV RNA, RT-PCR, Boiling of serum

INTRODUCTION

Hepatitis C virus (HCV) is the principal causative agent for parentally transmitted non-A, non-B (NANB) hepatitis (Choo *et al.*, 1989). Two major techniques to detect HCV infections are currently in use. The first technique detects antibody produced in response to HCV infection (anti-HCV antibody) with immunoassay (Alter *et al.*, 1989). However, the detection with antibody is associated with both false-positive and negative reaction (Gary *et al.*, 1990; Aach *et al.*, 1991). Several weeks are required for development of anti-HCV antibody after HCV infection, which makes it impossible to detect HCV infection in early stage. Because anti-HCV antibody is an indirect index of HCV infection, this does not provide the means to differentiate the infection status between active infection, chronic infection and resolved infection. Direct detection of HCV RNA has been required to evaluate the infectivity. Since HCV genome is a single positive stranded RNA molecule, amplification of viral RNA by reverse transcription and nested polymerase chain reaction (RT-nested PCR) has been an adequate means for the direct detection of HCV (Garson and Tedder, 1990; Okamoto *et al.*, 1990; Weiner *et al.*, 1990). With RT-nested PCR method, HCV infections have been demonstrated in haemophiliacs treated with HCV contaminated factor VIII concentrates (Garson *et al.*, 1990), in liver biopsy specimens of chronic liver disease patients (Shieh *et al.*, 1991), and in sporadic acute NANBs hepatitis patients (Sakamoto *et al.*, 1993). However, the routine clinical usage of RT-nested PCR based detection of HCV RNA is seriously hampered by technical difficulties. Main restraints are the time and labor consuming step for extraction of viral RNA under RNase-free condition and the sequential reactions of reverse transcription (RT), 1st PCR and 2nd PCR, which raise the risks of contamination. To overcome the difficulties, direct slot hybridization of HCV RNA (Hu *et al.*, 1992), RT-one stage PCR with sensitive detection methods such as Southern blot or liquid hybridization with radioactive oligonucleotide probes (Gretch *et al.*, 1993), and combined RT-PCR assay using *Thermus thermophilus* DNA polymerase which possesses enhanced reverse transcriptase activity in the presence of manganese (Young *et al.*, 1993), have been demonstrated previously.

We have developed a simple and rapid procedure for RT-nested PCR amplification of HCV RNA from

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human serum with eliminating the RNA extraction step and performing the reverse transcription (RT) and 1st PCR amplification in one vessel containing both of reverse transcriptase and *Taq* DNA polymerase. These methods have been applied for the screening of HCV RNA in patient samples.

MATERIALS AND METHODS

RNA preparation from serum by boiling method

Normal and HCV infected sera were kindly provided by fellows in Korea Research Institute of Standards and Science and Dr. Y. G. Kim in Korea University Medical School. One volume of human serum boiled at 95°C for 2 min and cooled rapidly in ice was diluted with two volumes of diethylpyrocarbonate (0.01%) treated sterile water and returned to the ice bath until centrifugation. After centrifugation at 12,000 rpm for 5 min, the supernatant was used as RNA template or stored at -70°C.

Rapid RT-PCR method

The combined RT-1st PCR amplifications were carried out in 10 μl reaction mixtures containing 3 μl of template, 50 μM each dNTP, 0.5 μM each 5-noncoding region outer primer 1CH, 5'-GATGC ACGGT CTACG AGACC TC-3', and 2CH, 5'-AACTA CTGTC TTCAC GCAGA A-3' obtained from Oligo *et al.* (Lazizi *et al.*, 1992), 5 U of cloned Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Inc.), 0.2 U of *Taq* DNA polymerase and 1x RT-PCR buffer (10x buffer consists of 500 mM Tris-HCl (pH 8.3), 200 mM KCl, 30 mM $MgCl_2$, 5% dimethylsulfoxide and 2.5 mg/ml bovine serum albumin). The reactions were performed in FTC 2000 capillary thermal cycler (Daehan Medical Co., Korea) which was developed for usage of PCR in clinical application because of its fast PCR reaction (30 min for 30 cycles) using glass capillary containing 10 μl reaction volume without a mineral oil overlay with air flow system. RT reaction was allowed to proceed for 5 min at 37°C and was followed by 1st PCR reaction proceeded with 20 cycles at 94°C for 5 s, 50°C for 5 s and 72°C for 10 s. The 1st PCR reaction product was stored in refrigerator for 5 min to minimize the contamination. The 2nd PCR amplifications were carried out in 10 μl reaction mixtures containing 1 μl of 1st PCR product, 0.5 μM each inner primer (1TS, 5'-GAGCA CCAAC ACTAC TCGCT-3', and 4CH, 5'-ATGGC GTTAG TATGA GTG-3') (Lazizi *et al.*, 1992), 200 μM each dNTP and 0.4 U of Taq DNA polymerase. The 2nd PCR reaction was allowed to proceed 30 cycles at 94°C for 5 s, 50°C for 10 s and 72°C for 15 s. Amplified samples (188 bp product) were separated by electrophoresis on 2% agarose gel and detected by ethidium bromide staining.

Conventional RNA preparation method

RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1992) from 100 μl of serum. Extracted RNA was suspended in 10 μl of diethylpyrocarbonate (0.01%) treated sterile water. First strand cDNA was synthesized from 5 μl of the RNA at 37°C for 1 hr with 50 U of cloned Moloney murine leukemia virus reverse transcriptase in 10 μl buffer containing 50 mM Tris-HCl (pH 8.3), 20 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 500 μM each dNTP, 2 μM primer (1CH) and 20 U of RNasin (Promega, USA). The 1st PCR reactions were carried out in 10 μl reaction mixtures containing 5 μl of cDNA, 200 μM each dNTP, 2 μM each outer primer, 0.4 U of Taq DNA polymerase and 1x PCR buffer same as described in above boiling method. The reactions were performed in FTC 2000 capillary thermal cycler over 30 cycles at 94°C for 5s, 50°C for 5s and 72°C for 10s. The 2nd PCR was carried out with same condition as described in above boiling method.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the difference in signal intensity of amplification products after combined RT-nested PCR depending on the RNA preparation methods. Amplification efficiencies from 1 μl of boiled serum without RNA extraction (Fig. 1B) are higher than those from extracted RNA of 50 μl serum with conventional method (Fig. 1A). Because these results were reproducible, positive band in anti-HCV antibody negative sample (lane 5 of Fig. 1B) could be detected as HCV-RNA positive by lowering the detection limit with this boiled method without RNA extraction. When boiled HCV positive serum was serially diluted 10-fold, HCV RNA was successfully determined in 2 μl~0.001 μl of serum (Fig. 2). This indicates that the sensitivity of present method allows

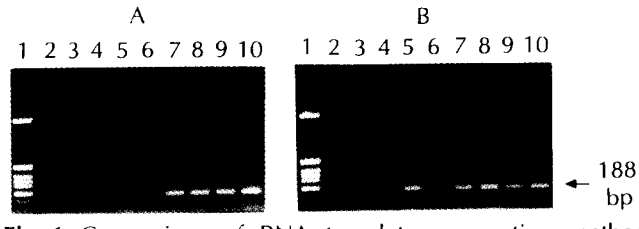

Fig. 1. Comparison of RNA template preparation method for RT-nested PCR as RNA template between (A) conventional RNA preparation method and (B) boiling method. Lane 1, pBR322/*Hinf*I digest; lane 2-6, anti-HCV antibody negative sera; lane 7-10, anti-HCV antibody positive sera. 1CH/2CH and 1TS/4CH primer sets were employed.

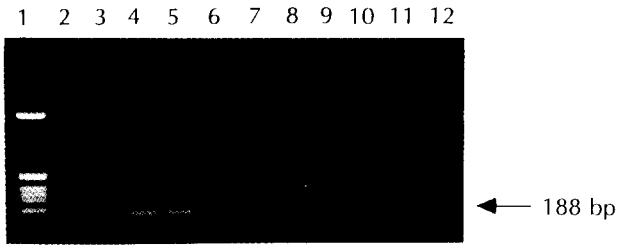

Fig. 2. Sensitivity of RT-PCR amplification for HCV RNA from boiled serum. The supernatants of boiled serum were serially diluted with diethylpyrocarbonate treated sterile water for using as RNA template. Lane 1, pBR322/*Hinf*I digest; lane 2, reagent control without RNA template; lane 3, negative control; lane 4, 2 μl serum; lane 5-11, serial 10-fold dilution of boiled serum ranging from 1 μl to 0.000001 μl, respectively; lane 12, 1 μl serum without reverse transcriptase

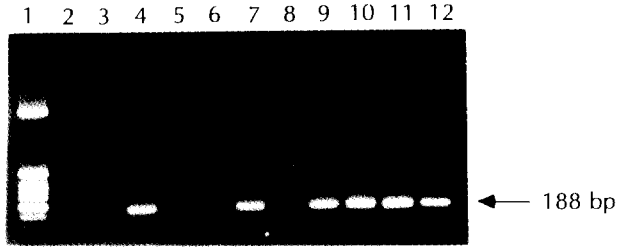

Fig. 3. Detection of combined RT-nested PCR products (188 bp) from 3 μl of boiled serum from anti-HCV antibody positive or negative patients using 1CH/2CH and 1TS/4CH primer sets. Lane 1, pBR322/*Hinf*I digest; lane 2, reagent control without RNA template; lane 3, negative control; lane 4, positive control; lane 5-8, anti-HCV antibody negative sera; lane 9-12, anti-HCV positive sera

the detection of HCV RNA in a wide range of concentration.

An important drawback for clinical use of RT-nested PCR for HCV RNA detection is time and labor consuming process, and sample-to-sample carryover or contamination. To overcome these problems, combined RT-1st PCR as described in MATERIALS AND METHODS makes the procedure be simple (it takes about 1 hr from RNA preparation to combined RT-nested PCR) and dramatically reduces the contamination possibilities. In this experiment, RT reaction was allowed to proceed for 5 min which was enough time to synthesize the cDNA. But there was no more increase of efficiency of cDNA synthesis when RT reaction was carried out for longer time than 5 min (data not shown). It caused rather increase the contamination possibilities because of the net increase of total RT-1st PCR reaction time. It is important to reduce the total RT-1st PCR reaction time (20 cycles in our experiments) and to minimize the concentrations of PCR reaction components (i.e. concentrations of dNTP, primer, Taq DNA polymerase, reverse transcriptase) for eliminating the contamination possibilities.

HCV RNA was screened by present method in 225 clinical samples from anti-HCV antibody negatives (13 samples) and positives (212 samples) sera detected by ELISA test (Innotest HCV kit, Green Cross Co., Korea). Negative control sample and reagent control (no nucleic acid) were included in each set of amplification reaction to test the contamination (Fig. 3). HCV RNA with this method was detected in 4 of 13 anti-HCV antibody negative samples (30.8%) and 95 of 212 positive samples (44.8%). When screening of clinical samples were repeated three times to find out false-positive, the results were exactly reproducible which means no contamination events during the amplification process. The results show that there are discrepancies between HCV-RNA positive and anti-HCV antibody positive as expected. Lane 7 in Fig. 3 is an example to illustrate the HCV RNA positive even it is anti-HCV antibody negative sample. It is possible to detect HCV RNA in negative anti-HCV antibody samples because of the high sensitivity of this method. The discrepancies of ELISA results using the various company ELISA kits for detecting the anti-HCV antibody were observed. Because 212 anti-HCV antibody positive samples were collected from various places, sample storage conditions and the detection method using various ELISA kits should be considered. It is possible to explain the negative detection with RT-PCR in anti-HCV antibody positive samples as causing from the RNA degradation by the careless transfer of samples or the discrepancies of ELISA data. With this consequence, it is more beneficial to combine the ELISA test in which several serological HCV markers have to be used simultaneously and RT-nested PCR technique in which the possibility of contamination should be eliminated for diagnosis of HCV infection. Combining RT-nested PCR technique using boiled serum as RNA template could facilitate diagnostic assays for detecting HCV RNA, confirm the discrepant results obtained by various ELISA kits and may have a wide range of application for the clinical utilities to determine other single (or double) stranded RNA viruses.

In conclusion, a simple and rapid combined RT-nested PCR protocol without RNA extraction has been described for the clinical utility to determine HCV RNA in human serum. HCV RNA detection in 225 clinical samples was very reproducible, which can exclude the possibility of contamination. In addition, the present assay protocol can be applied to the amplification of any RNA templates including RNA viruses, especially for the clinical samples which require the speedy analysis of large number of specimen.

ACKNOWLEDGMENT

We thank Dr. Y. G. Kim in Korea University Medical School for kindly providing anti HCV positive sera and Dr. Y. C. Sung in Pohang Institute of Science and Technology for generous gifts of Korean HCV recombinant cDNA. This work was sponsored by the fund from the Korea Ministry of Science and Technology.

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