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A refined long RT-PCR technique to amplify complete viral RNA genome sequences from clinical samples: Application to a novel hepatitis C virus variant of genotype 6☆

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Abstract

The goal of this study was to adapt a long RT-PCR technique to amplify large PCR fragments from the genome of hepatitis C virus (HCV) isolates using clinical samples. This was done by using a reverse transcriptase devoid of RNase H activity and a mixture of two antibody-bound thermostable polymerases to combine the high processivity of *Taq* and the high fidelity of *Pwo* with its $3' \rightarrow 5'$ exonuclease activity. Other modifications included gentle handling during RNA extraction, the absence of tRNA and random primers, a two-step reverse transcription procedure to optimize cDNA synthesis, and increasing the annealing temperature for primers. With this approach, the HCV-1 genome (nucleotides 35–9282) was amplified consistently as two overlapping fragments of 5344 and 4675 bp from a pooled chimpanzee plasma sample containing approximately 10^6 genome copies of HCV RNA/ml. Using the conditions that we identified, 96% of the complete genomic sequence of a distinct HCV genotype 6 variant (km45) was determined from less than 300 μl of serum. This method should prove useful for molecular, epidemiological and clinical studies of hepatitis C where samples are limited but complete virus sequence is required, for example, identifying mutational hot spots of HCV under specific clinical conditions.

Keywords: HCV genome; Long RT-PCR; Genotype

1. Introduction

HCV is an enveloped virus with a single-stranded positive-sense RNA genome of approximately 9.6 kb and is classified in the *Hepacivirus* genus of the *Flaviviridae* (Reed and Rice, 2000). HCV chronically infects an estimated 180 million people worldwide (Anonymous, 1997), often leads to fibrosis and cirrhosis, and is associated with hepatocellular carcinoma (Choo et al., 1989; Alter and Seeff, 2000).

Phylogenetically, HCV is divided into six major genotypes, and within each genotype the closely related isolates are grouped into different subtypes (Simmonds et al., 1994; Simmonds, 1999; Robertson et al., 1998). Genotype identification is generally performed by direct sequencing of PCR amplicons of specific genomic regions such as the core, E1, HVR1 and NS5B regions (Abid et al., 2000; Farci et al., 2000, 2002; Jeannel et al., 1998; Simmonds, 2001; Zein et al., 1996). Analysis of longer fragments of the HCV genome would allow more sequence information to be obtained with less effort and provide more detailed information about HCV.

☆ The complete HCV genome sequence determined in this study has been submitted into GenBank accession number is AY878650.

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However, technical limitations have made it difficult to amplify large fragments, and previous studies have amplified up to 20 fragments, each less than 1 kb in length (Chamberlain et al., 1997), or used a mixture of PCR fragments and cloned cDNA libraries prepared from clinical specimens (Sakamoto et al., 1994) to determine the entire HCV genome. This is time-consuming and has been done only under specific circumstances.

Since DNA templates up to 40 kb were able to be amplified (Cheng et al., 1994), long RT-PCR techniques were reported to amplify the viral RNA genomes of HAV and HCV (Tellier et al., 1996) and in several cases long (5 kb) or close to genome length HCV amplicons have been generated from 10^4 to 10^6 genome copies (Tellier et al., 1996; Wang et al., 1997; Rispeter et al., 1997). However, these procedures are not reproducible and may reflect that these methods were developed with samples containing a very high HCV titer, such as purified RNA from liver explants or RNA concentrated from a large serum or plasma sample. These techniques have not been used with specimens obtained from clinical and epidemiological studies which have relatively lower virus titers, in the range of 10^6 to 10^7 genome copies/ml (Puig et al., 2002; Shiffman et al., 2003).

A refined long RT-PCR technique is described that amplifies nearly the complete genome sequence of HCV-1 in two overlapping PCR fragments from pooled chimpanzee plasma which contained an estimated HCV titer of 10^5 CID_{50}/ml or 10^6 genome copies/ml (Bradley, 1991). This is equivalent to the HCV concentration present in most clinical samples. In addition, this approach was used to amplify and sequence the complete genome of an HCV genotype 6 variant (Lu et al., 2005) from less than 300 μl of serum.

2. Materials and methods

2.1. Specimens

A plasma pool from a chimpanzee chronically infected with HCV-1 (M62321), which had been quantitated for infectivity using chimpanzee inoculation studies, was found to contain 1×10^5 CID_{50}/ml of HCV (Bradley, 1991). Samples of this pooled plasma were used as the starting material to determine the optimal conditions for long RT-PCR amplification. A human serum sample containing a distinct HCV variant of genotype 6 (China, km45, Lu et al., 2005) was used to address the ability of this technique to determine a complete HCV genome sequence using the long RT-PCR technique we describe.

2.2. Refined long RT-PCR

Total RNA was extracted from 100 μl plasma or serum using Tripure (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's protocol. Special care was taken to avoid shearing the long RNA template by inverting the tubes gently and slowly 4–5 times for all mixing steps after chloroform was added. The precipitated RNA was resuspended in 9.5 μl of DEPC treated water and cDNA synthesized in a 20-μl reaction volume containing 4 μl of 5× first-strand buffer (Invitrogen, Carlsbad, CA), 10 mM DTT, 1 mM dNTPs (Roche Applied Sciences), 200 units of Superscript II RNase H^- reverse transcriptase (Invitrogen), 20 units of RNasin (Roche Applied Sciences), and 0.2 pmol of an appropriate anti-sense PCR primer (Table 1). RNA was denatured at 95 °C for 3 min, and the reaction mixtures were placed on ice for 3 min. Reverse transcription was started by adding reverse transcriptase plus RNasin and incubating at 43 °C for 60 min. This was followed by an increase to 53–55 °C over 45 min using a temperature gradient rising at 2 °C/min. This was done to overcome secondary structures in RNA templates that decrease the efficiency of reverse transcription. Incubations were terminated at 70 °C for 15 min, followed by the addition of 2.2 units of RNase H (Invitrogen), and incubating at 37 °C for 20 min.

The first round of PCR was carried out in 50 μl reaction volumes consisting of 2 μl cDNA, 10 pmol of each outer primer (Table 1), 5 μl 10× PCR buffer (Clontech, Palo Alto, CA), 200 μM dNTPs (Roche Applied Sciences), 1.5 units Advantage DNA polymerase (Clontech), and 2 μl DMSO (Invitrogen). The second round of PCRs were also in 50 μl volumes containing 2 μl of the first-round PCR product, 10 pmol of each inner primer (Table 1), 5 μl 10× PCR buffer (Clontech), 200 μM dNTPs (Roche Applied Sciences), 1.5 units Advantage DNA polymerase (Clontech), and 2 μl of DMSO (Invitrogen). Both rounds of PCR were performed with one cycle at 94 °C for 1 min followed by 38 cycles of 94 °C for 25 s and 65–68 °C for 2.5–10 min (time varied with the length of templates, approximately 1 kb/min). The final PCR step was at 68 °C for 10 min. PCR products were resolved on 1% agarose gels and stained with ethidium bromide.

2.3. Long RT-PCR with other reagents

2.3.1. Amplification of TMV RNA

Tobacco mosaic virus (TMV) RNA was purchased previously from Boehringer/Mannheim (Indianapolis, IN). (Its catalogue number was 1120387 and this product was no longer available after the company was merged as Roche Applied Science.) After dilution, it was applied directly to reverse transcription or extraction with Tripure (Roche Applied Sciences) or QIAamp Virus RNA Kit (QIAgen, Valencia, CA) followed by reverse transcription. The resulted cDNA was amplified using High Fidelity PCR System (Roche Applied Sciences) or Expand Long Template PCR System (Roche Applied Sciences) or GeneAmp XL PCR System (Applied Biosystems, Foster City, CA) with primers TMV1 and TMV2 as described previously (Tellier et al., 1996).

2.3.2. Amplification of HCV

Total RNA was extracted from 100 μl chimpanzee plasma using Tripure (Roche Applied Sciences). Extracted RNA