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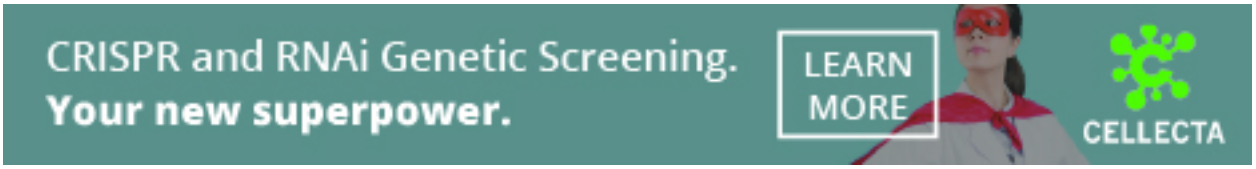

Direct PCR Amplification of HCV RNA from Human Serum

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Hepatitis C virus (HCV) is a positive-stranded RNA virus responsible for most cases of parenterally transmitted non-A, non-B hepatitis (NANBH).[1,2] The polymerase chain reaction (PCR) is, at present, the only method allowing the detection of HCV RNA in biological specimens, thus providing the direct appraisal of infectivity.[3] Therefore, serum HCV RNA determination ideally represents the method of choice for the prevention of HCV transmission. The routine clinical use of PCR-based technology, however, is seriously hampered by technical difficulties. In the case of HCV infection, one of the main obstacles is the necessity of extracting viral RNA before amplification, a time-consuming step that requires RNase-free conditions that are difficult to obtain in diagnostic laboratories.

Here we describe a simple and rapid method for reverse transcription (RT)-PCR amplification of HCV RNA from human serum that does not require the RNA extraction step. After denaturation (30 sec at 92°C), 3 μl of serum was directly added to the RT mix (50 mM Tris HCl, pH 8.2, 70 mM KCl, 10 mM $MgCl_2$, 4 mM DTT, 12 units of human placental ribonuclease inhibitor (HP-RI), 0.4% Nonidet P-40, 50 pmoles of a specific antisense primer (A1), 250 μM dNTPs, 6 units of AMV-RT, in a final volume of 25 μl). RT was carried out at 42°C for 60 min, and the cDNA was added directly, after denaturation (5 min at 100°C), to the PCR mix. Amplification was performed following the nested primers protocol,[4] as previously described,[5] with two sets of primers located in the 5′ untranslated region (UTR) of the viral genome. The first round of PCR (35 cycles) was carried out using primers A1 (5′-GATGCACGGTCTACGAGACCTC-3′) and S1 (5′-AACTACTGTCTTCACGCAGAA-3′), generating a PCR product of 289 bp. For the second round (25 cycles), we used primers A2 (5′-GCGACCCAACACTACTCGGCT-3′) and S2 (5′-ATGGCGTTAGTATGAGTG-3′), generating a PCR product of 187 bp. PCR cycles were as follows: denaturation at 94°C for 1 min, annealing of primers at 45°C for 1 min, and elongation at 72°C for 2 min.

This method was used to screen serial serum samples collected from a patient with post-transfusion NANBH. In this patient, positivity for the anti-HCV ELISA assay (Ortho Diagnostics), detecting antibodies against the non-structural viral antigen C-100, was first observed 13 weeks after transfusion. By contrast, the detection of the viral nucleic acid was possible as soon as 3 weeks after transfusion (Fig. 1, lane b), confirming the superior sensitivity of PCR for early diagnosis of acute NANBH.[3,4] Further prelevments, collected 5–56 weeks after transfusion, confirmed persistent positivity for HCV RNA.

Comparison of direct amplification from serum (Fig. 1, part 1) with the standard procedure (RNA extraction[6], RT-PCR) (Fig. 1, part 2), performed on equivalent amounts of template, showed a perfectly comparable sensitivity. By both methods, we obtained amplification products easily visible on agarose gel from all samples. The very low circulating HCV titers present in most of patients represent a major problem for the determination of the viral nucleic acids in serum samples. The present method for direct HCV RNA determination was successfully performed using up to 12 μl and down to 1 μl of a $1:10^4$ dilution of a positive control serum. This indicates that the high sensitivity of this PCR protocol allows the detection of circulating HCV particles in a wide range of concentration.

An important drawback for the clinical use of HCV RNA amplification is the extreme variability of the viral genome, especially in the regions encoding the envelope viral proteins. The observation of conserved mutations among American and Japanese isolates suggested the existence of HCV subtypes segregated in different geographic areas.[3] The choice of oligonucleotide primers located in highly conserved genomic regions, such as the 5′ UTR,[3] is therefore critical for a clinically useful PCR protocol. By comparative nucleotide sequence analysis of variable regions, we recently detected the common distribution in our geographic area of HCV genomes belonging to the two main subtypes (HCV-US and HCV-J) (unpublished results). Despite the presence of extensive sequence divergence in the envelope genes, HCV genomes belonging to

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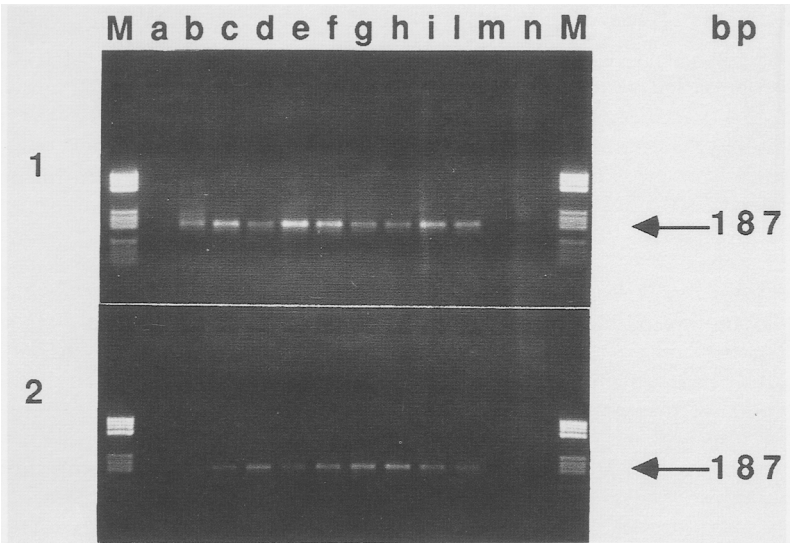

Figure 1 Detection of HCV RNA in serum samples collected from a NANBH patient. (*1*) RT-PCR performed directly on 3-μl serum samples. (*2*) RT-PCR performed on RNA extracted from equivalent amounts of the same serum samples. Twenty microliters of each PCR reaction (carried out in a 100-μl volume) was loaded onto a 1.5% agarose gel. (Lanes *a–l*) Serial samples from a subject with post-transfusion NANBH. Time after transfusion: (lane *a*) 2 weeks; (lane *b*) 3 weeks; (lane *c*) 5 weeks; (lane *d*) 7 weeks; (lane *e*) 10 weeks; (lane *f*) 13 weeks; (lane *g*) 15 weeks; (lane *h*) 18 weeks; (lane *i*) 25 weeks; (lane *l*) 56 weeks. (Lane *m*) Serum from a healthy blood donor; (lane *n*) PCR mix without cDNA; (lane *M*) molecular weight marker obtained from cleavage of plasmid pBR322 DNA with *Hae* III.

both subtypes could be amplified by the primers used in this study (data not shown), confirming the high conservation of the 5′ UTR sequence among different HCV isolates.[3]

In conclusion, we have described a simple and rapid RT-PCR protocol for the determination of HCV RNA that enables a bypass of the RNA extraction step. The possibility of performing RT-PCR amplification directly on serum samples significantly facilitates HCV RNA determination on large series and should contribute to a wider application of this method in clinical laboratories.

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