

Quantitation of Hepatitis C Virus Genome Molecules in Plasma Samples

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A competitive reverse transcription PCR (cRT-PCR)-based assay for the quantitative detection of hepatitis C virus (HCV) viremia was developed, optimized, and applied to the direct molecular analysis of clinical samples from nine patients with persistent HCV infection. As for other competitive PCR-based applications, this method consists of the reverse transcription and subsequent amplification of two RNA species in the same tube: the wild-type template (to be quantified) and a known amount of a modified synthetic template. These templates have identical primer recognition sites and very similar (but not identical) sizes, thus allowing direct detection of both template species after gel electrophoresis and ethidium bromide staining. The results obtained by this cRT-PCR application for testing clinical samples from HCV-infected patients mainly indicate that the competitive approach reaches the degree of sensitivity (fewer than 5 HCV RNA molecules per 100 μ l) necessary to evaluate viral load in all HCV-infected patients, independently of clinical conditions, and that this technique is flexible enough to quantify highly divergent levels of cell-free HCV genome copy numbers in biological samples. Interestingly, we observed a sample-to-sample variation in the loss of detectable HCV genome molecules in serum in comparison with that in plasma from the same patient, thus indicating that serum specimens, although widely used in the past few years for qualitative molecular investigation of HCV-infected patients, cannot be used to obtain reliable quantitative data on HCV viremia from these patients.

Hepatitis C virus (HCV) is the major etiological agent of parenterally transmitted (acute and chronic) non-A, non-B hepatitis (3, 7); this virus has recently been associated with the pathogenesis of autoimmune chronic hepatitis (24, 25, 29), cirrhosis, and primary hepatocellular carcinoma (11, 37). Furthermore, a variety of extrahepatic disorders (such as glomerulonephritis, polyarteritis, and cryoglobulinemia) have been described in a subgroup of HCV-infected patients (1, 32).

HCV infection is routinely diagnosed by assaying serum-specific antibodies to products of complementary viral DNA sequences cloned in expression vectors (21) or to synthetic oligopeptides (6). In anti-HCV-positive patients, testing for cell-free HCV RNA sequences in blood is necessary to evaluate the viral load precisely. Extensive analysis of HCV activity during persistence has been performed over the past few years by using qualitative PCR-based methods (13, 17, 23, 39, 40, 42), thus showing that the great majority of anti-HCV-positive patients with chronic hepatitis (as shown by elevation of alanine aminotransferase levels or histologically determined liver damage) are also viremic. In these viremic patients, HCV RNA is also detectable in the form of positive and negative strands in liver cells (14) and, in several cases, in peripheral blood mononuclear cells (5, 26, 30, 43) and bone marrow cells (26).

Most pathogenic and clinical aspects of HCV infection call (directly or indirectly) for the absolute quantitation of the viral load, including the intermittent (and progressive) features of the clinical signs of chronic liver disease observed in most

HCV-infected patients (2) and the molecular monitoring of HCV-infected patients during and after treatments (2, 39).

A competitive strategy for quantitative PCR has recently been described (15, 16). The general concept of competitive PCR (cPCR) and competitive reverse transcription-PCR (cRT-PCR) (8, 10) consists of carrying out reverse transcription and amplification in the presence of a known number of competitor template molecules that recognize the same primers. The standard template is either shorter or longer than the target sequence in the sample. After amplification, the PCR products are separated by gel electrophoresis, in order to allow densitometric comparison of the relative band intensities for both species. Finally, the ratio of PCR products may be related to the initial template concentration.

In the study described here, we planned and developed a cRT-PCR assay for the absolute quantitation of HCV RNA molecules in plasma samples; subsequently, the technique was optimized by using reference samples and clinical material.

MATERIALS AND METHODS

Positive and negative controls and clinical samples. Two different positive controls were used in the study; first, a pool of HCV RNA-positive plasma samples (from 10 viremic HCV-infected patients) was collected in our laboratory and stored in 30- μ l aliquots; second, a reference plasma sample (a kind gift from R. H. Purcell) (13) was obtained from an infected patient whose inoculum had been shown to contain $10^{6.5}$ HCV 50% chimpanzee-infective doses (CID₅₀s) per milliliter. Negative control samples were taken from patients who tested negative for antibodies to HCV and were assayed in parallel with positive samples.

The quantitative method was also optimized by using serum and plasma samples from nine patients (six females and three

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TABLE 1. Oligonucleotides used as primers, their positions on the HCV-1 prototype genome, and sequence of the pC71AN cloned competitor^a

Oligonucleotide	No. of bases (nucleotide position)	Sequence
HCV4	21 (-91 to -68 5'-UTR)	5'-TAGCCGAGTAGTGTGGGTGCGCA-3'
HCV5	24 (-6 to -29 5'-UTR)	5'-ACGGTCTACGAGACCTCCCGGGGC-3'
HCV4 Δ XbaI	51	5'-TCTCTAGATAGCCGAGTAGTGTGGGTGCGGATGCCTGATAGGGTGC ^b TTC-3'
HCV5BamHI	32	5'-CGGGATCCACGGTCTACGAGACCTCCCGGGGC-3'
pC71AN sequence	71 (-91 to -6/ Δ 15 ^b)	5'-TAGCCGAGTAGTGTGGGTGCGGATGCCTGATAGGGTGC ^b TTCCTAGTGCCCGGGAGGTCTCGTAGACCGT-3'

^a See reference 31.

^b Δ 15, deletion of 15 bases.

males; ages, 23 to 68 years; mean age, 41 years) with persistent HCV infection. Five of these patients had a histological diagnosis of chronic persistent hepatitis, three of chronic active hepatitis, and one of cirrhosis. Serum and plasma samples were collected simultaneously from these subjects.

Synthetic oligonucleotides. The oligonucleotides used as primers in this work are listed in Table 1. They were synthesized in our laboratory by using phosphoramidite chemistry and a Beckman DNA-sm synthesizer (Beckman Instruments Inc., Fullerton, Calif.).

Strategy for HCV competitor synthesis and cloning. For competitive analysis, we chose the 5'-UTR (untranslated re-

gion) fragment of the HCV genome (from positions -91 to -6), which is highly conserved among the different HCV genotypes described. The 86-bp fragment was specifically amplified by the HCV4-HCV5 primer set (Table 1). A modified primer, named HCV4 Δ XbaI, was synthesized; it has the following sequence pattern on the HCV genome: positions -91 to -34, including the 15-base deletion from positions -67 to -53 and the XbaI restriction site, in addition to the original sequence at the 5'-end, thus resulting in a 51-base oligonucleotide (Table 1 and Fig. 1). HCV RNA from a clinical sample was reverse transcribed and amplified with the HCV4 Δ XbaI and the modified HCV5BamHI primer pair, thus yielding a

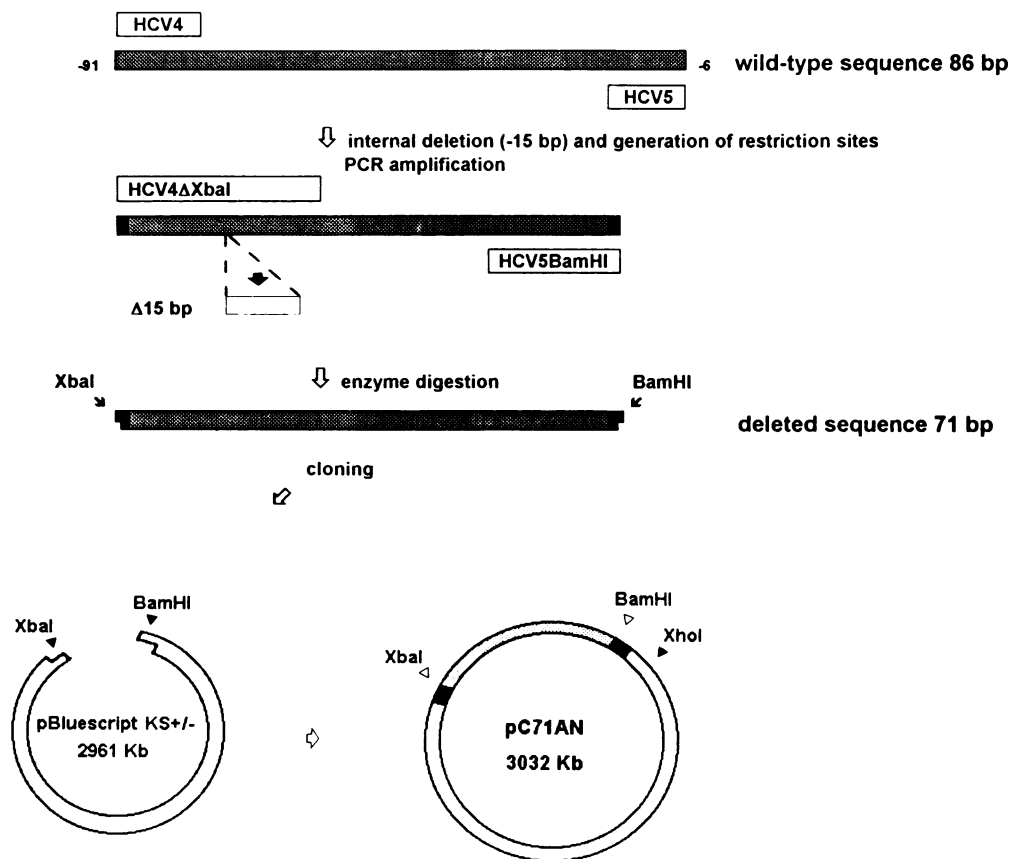


FIG. 1. Synthesis and cloning strategy of the deleted competitor. The 86-bp wild-type sequence of HCV (positions -91 to -6) was amplified with primers HCV4 and HCV5 and was reamplified with the primer HCV4 Δ XbaI (including a 15-bp internal deletion) and the primer HCV5BamHI bearing the respective restriction sites. The deleted amplified products and the plasmid vector pBluescript KS+/- were digested with these enzymes and ligated together. Upon transformation of *Escherichia coli* dH5 α , bacteria from one colony were grown and large amounts of the recombinant plasmid pC71AN were recovered on a CsCl gradient.

shorter version of the original wild-type sequence with the respective restriction sites at the 5' ends. This PCR product was then purified and cleaved with both restriction endonucleases and was ligated in the pBluescript II KS plasmid vector (Stratagene, La Jolla, Calif.) cloning box, 3' of the T7 RNA polymerase promoter sequence. Large amounts of this plasmid (called pC71AN) were purified on a cesium chloride gradient (Centrikon T2060 centrifuge; Kontron Instruments, Zurich, Switzerland) and were carefully quantitated by spectrophotometric analysis and gel electrophoresis. The estimated copy number was also confirmed by endpoint dilution amplification and the Poisson distributions of positive results in two independent assays, with the sensitivity approaching one molecule. To confirm the specificity of the 15-bp deletion (positions -67 to -53), the 5'-UTR fragment inserted into pC71AN was bidirectionally sequenced by using the chain termination method (Sequenase; Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Synthesis of the competitor RNA. One microgram of the pC71AN plasmid was cleaved with *Xho*I (64 bp downstream of the insert) in order to synthesize a 161-nucleotide transcript. The linearized plasmid was precipitated and resuspended in 100 μ l of transcription buffer (Bethesda Research Laboratories, Bethesda, Md.) containing 10 mM dithiothreitol, 0.5 mM (each) ribonucleotide triphosphates, and 100 U of RNasin (Promega, Madison, Wis.). The T7 RNA polymerase (Bethesda Research Laboratories) was added (50 U) to start the transcription, which was carried out at 37°C for a total of 4 h. Aliquots from the reaction mixture were collected after 1, 2, 3, and 4 h and were run on a 6% denaturing polyacrylamide gel for kinetic analysis. After 4 h the reaction entered the linear phase and the molar yield was 1,450 RNA molecules per DNA molecule. After DNase treatment, the residual DNA (calculated by endpoint dilution PCR amplification of RNA suspensions with no reverse transcription) was calculated to be approximately 1 molecule per 100,000 RNA molecules; thus, it did not interfere in the competitive reactions. Finally, the sensitivity of cRT-PCR was calculated by amplification of endpoint dilutions of the competitor RNA and by determining the Poisson distribution of positive results; approximately four target molecules were detected in two independent assays.

Sample preparation and nucleic acid purification. Fresh plasma samples (EDTA treated) and serum samples from HCV-infected patients were centrifuged at $9,200 \times g$ for 15 min soon after collection in order to remove platelets and cell debris. RNA was directly extracted from 100 μ l of the supernatant by the guanidinium-thiocyanate method described previously (4).

cRT-PCR. The cRT-PCR was performed in two steps by a method previously optimized in our laboratory for the quantitative analysis of human immunodeficiency virus type 1 viremia (4, 30). In particular, cDNA was synthesized by using the antisense HCV5 primer, which is specific for the genomic HCV RNA strand, in the presence of HCV genomic sequences purified from plasma (or serum) samples and the RNA competitor. The copy number of RNA competitor molecules was adjusted to 250,000, 50,000, 10,000, and 2,000 (and, in some assays, 1,250,000, or 400 and 80 competitor RNA molecules; see Results) in the respective tubes against a constant amount of wild-type viral RNA extract. After reverse transcription at 42°C for 30 min, the amplification reaction was carried out for 50 cycles by using an automated thermal cycler (model 9600; Perkin-Elmer Cetus, Norwalk, Conn.) in a mixture (final volume, 100 μ l) containing $1 \times$ PCR buffer (50 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 2.5 U of *Taq* DNA polymerase, and a final concentration of 50 pmol each of the

HCV4 and HCV5 primers. The amplification profile was as follows: denaturation at 94°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 45 s. Following PCR amplification, 5- μ l aliquots of the reaction mixture were run on a 10% polyacrylamide minigel at 180 V for 50 min in order to obtain complete separation of the 86-bp wild type from the deleted 71-bp fragment.

Competition analysis. Analysis of competitive PCR amplification was carried out after ethidium bromide staining of electrophoresed PCR products. Gels were scanned by using a video densitometer (Ultra Violet Products Ltd., Cambridge, United Kingdom) by positive fluorescence emission on the transilluminator. Peak areas (W_a = wild-type area; C_a = competitor area) of both amplified products were calculated by the machine's software (Gel Analysis Program; Ultra Violet Products) for each lane. The C_a value was corrected (C_{ac}) for its lower molar level of ethidium bromide incorporation by the following equation: $C_{ac} = C_a \times \text{wild-type length/deleted length} = C_a \times 1.2112$. The C_{ac}/W_a ratio was calculated for each sample and was plotted on the y axis against the copy number of the deleted RNA competitor (C). A simple regression curve was fitted for positive controls and for each sample. The copy number of the wild-type template (W) could be calculated from the curve expression for $C_{ac}/W_a = 1$.

RESULTS

Effect of RNA purification on template recovery. In cPCR-based methods, RNA purification from biological samples is the only step which is not under competitive control. However, a method developed for the absolute quantitation of the nucleic acid copy numbers present in low amounts in biological samples allows the mean loss of target molecules (dependent on the particular purification procedure adopted) to be determined. In order to evaluate the effect of RNA purification on template recovery and to further control the reliability of the quantitative results obtained by cRT-PCR, HCV RNA copy numbers were calculated in four plasma samples by using cRT-PCR; subsequently, the samples were reextracted by the complete procedure and were challenged against a competitor series in four independent experiments (data not shown). The mean loss of HCV RNA molecules by the RNA extraction method used in the present study was calculated to be 31.5% (standard deviation, 5.1%). This principally indicates that all absolute quantitative data regarding RNA should be corrected for a percentage loss determined specifically for the extraction method used. However, the quantitative data presented in this report are not corrected so as to allow for a direct approach to the cRT-PCR results.

Optimization of cRT-PCR for quantitative detection of HCV RNA sequences in biological samples. In a first series of experiments, a reference serum sample tested for infectivity in chimpanzees (see Materials and Methods) was used to evaluate the quantitative features of the cRT-PCR assay. Conventional qualitative RT-PCR was used in our laboratory to detect HCV RNA sequences in this serum sample at a dilution of 10^{-6} , but not at a dilution of 10^{-7} . According to the data on the HCV infectivity of this serum sample supplied previously ($10^{6.5}$ CID_{50} /ml) (13), this corresponds to almost 5 CID_{50} s, which is close to the results (positive at a dilution of 10^{-7} , corresponding to less than 1 CID_{50}) originally reported. In this case, the minimal loss of titer was probably due to storage and transport. When tested against different competitor copy numbers in cRT-PCR experiments, the infectious serum exhibited a constant amount of about 5,000,000 HCV genome molecules

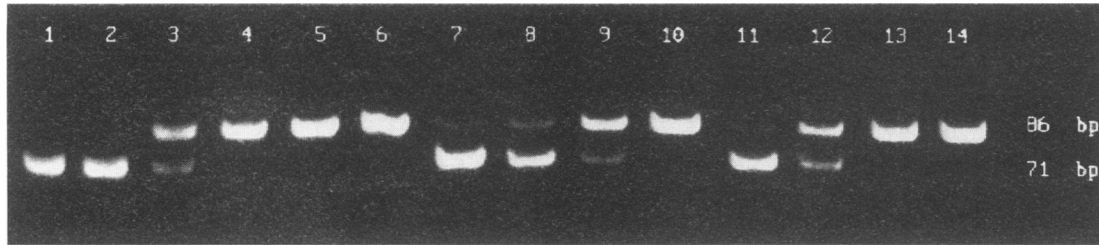


FIG. 2. Quantitation of HCV genome molecules in plasma samples by cRT-PCR. HCV RNA equivalents corresponding to 100 μ l of plasma from two HCV-infected patients (lanes 7 to 10 and 11 to 14, respectively) were challenged against 50,000 (lanes 7 and 11), 10,000 (lanes 8 and 12), 2,000 (lanes 9 and 13), and 400 (lanes 10 and 14) copies of the deleted competitor RNA. A pool of plasma samples from HCV-infected patients (lanes 1 to 6) was also tested: HCV RNA equivalents corresponding to 100 μ l of plasma were assayed against 250,000, 50,000, 10,000, 2,000, 400, and 80 copies of competitor RNA (lanes 1 to 6, respectively). The results obtained by gel densitometric analysis allowed the *Cac/Wa* ratio (see text) to be calculated, and this value was plotted against the competitor copy number in the respective graphs. The copy number of the wild-type template could be calculated from the curve expression for *Cac/Wa* = 1.

per ml in three independent assays (4,780,000, 4,960,000, and 5,150,500; average, 4,963,500).

Furthermore, since the method described here was planned for application to a large number of clinical samples, we defined a standard set of competitor concentrations. In preliminary experiments with a set of clinical samples, we observed that viremia may vary with the individual with persistent HCV infection, ranging from a few hundred molecules per milliliter of plasma to 1,000,000 genome copy numbers per ml of plasma or more. Accordingly, four dilutions of competitor HCV RNA (ranging from 250,000 to 2,000 molecules per tube) were routinely used for cRT-PCR assays against a constant amount of HCV RNA extracted from 100- μ l clinical samples (Fig. 2). Use of different competitor RNA concentrations was necessary under particular conditions (in the case of very high viremia levels, 1,250,000 competitor RNA molecules; in the case of low viremia levels, 400 and 80 competitor RNA molecules).

Quantitation of HCV RNA molecules in plasma samples by cRT-PCR. Serum and plasma samples were collected simultaneously from nine consecutive patients testing positive for anti-HCV antibodies with and without clinical symptoms of chronic liver disease. The comparative analysis of HCV RNA molecules from these clinical samples was carried out to identify reliable samples for evaluating viral load in HCV-infected patients. The data obtained are reported in Table 2. A highly variable (and unpredictable) drop in HCV RNA copy numbers was documented in serum samples, thus indicating that these specimens cannot be used for the precise quantitation of viremia in infected subjects. In fact, these results demonstrate not only that quantitation from serum is inaccurate in terms of the absolute values detected but also that the relative semiquantitative evaluation may be totally wrong.

The data obtained from plasma samples from nine consecutive patients with persistent HCV infection (Table 2) show that cRT-PCR also allows for the quantitation of very low viremia levels (as documented for patient 8).

DISCUSSION

In the last few decades, HCV infection has been a serious complication of blood transfusion (3). This infection also occurs in sporadic form (35), whose major routes of transmission are currently under evaluation (22, 27, 36, 41). While generally mild in its acute form, HCV frequently persists, and all natural history studies conducted over the past few years (2, 13, 19) have confirmed the serious long-term consequences of this infection. In this context, parallel clinical, molecular, and

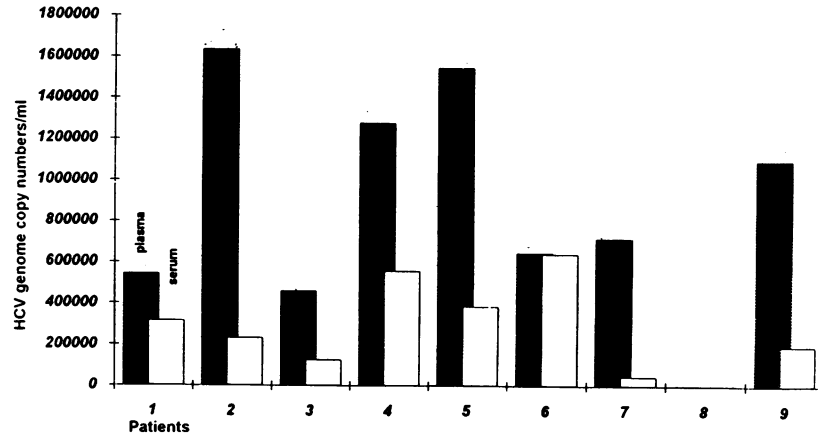
histopathological investigations are necessary for a complete understanding of both the pathophysiology of HCV infection and the virus-host relationship. From this point of view, the diagnostic characterization of infected patients by quantitative molecular methods for investigating HCV viremia may supply important second-level information, thus being a concrete virological complement to other diagnostic and prognostic indices of liver disease. Furthermore, quantitation of HCV load unquestionably plays a key role in evaluating the real efficacy of treatments at any time during and after long-term therapies with specific anti-HCV compounds; although highly sensitive and specific, qualitative PCR-based techniques are generally inefficient for such assessments.

A commonly encountered drawback of PCR is the low tube-to-tube reproducibility of the amount of final product; this may occur even under the most tightly controlled assay conditions. Recently, it has been observed that cPCR and cRT-PCR methods share several advantages over the other approaches proposed for molecular quantitation (4, 9, 10, 12, 28, 33, 34).

The laboratory procedure for the quantitative detection of HCV RNA sequences in plasma samples described in this report has been developed in complete agreement with the general concept of cRT-PCR with competitor RNA. Additionally, several particular aspects of the competitive strategy provide the key for the development of reliable cRT-PCR applications and deserve careful attention. First, although correct in theory, the use of competitors bearing a new restriction site (18, 38) should be avoided since the efficiency of enzymatic digestion may vary unpredictably, thus biasing evaluation of quantitative results. Second, great diversity in sequence length between the wild-type sequence and the RNA competitor may give rise to differences in amplification efficiencies; accordingly, internal insertions or deletions must be reduced to the minimum necessary to obtain discrete bands corresponding to the different PCR products after gel electrophoresis. Third, in cRT-PCR, the wild-type sequence and the competitor RNA are reverse transcribed in the same tube, under identical reaction conditions, and with the same efficiency; accordingly, the use of DNA competitors after reverse transcription of the wild-type sequence (assuming a given efficiency for reverse transcription) (20) is incorrect methodologically and may be highly imprecise. For this reason, not only the concentration but also the integrity of the competitor RNA used in the assay must be carefully ascertained in cRT-PCR applications. In the assay described in this report, the plasmid pC71AN (containing a 15-bp deleted 5'-UTR

TABLE 2. Quantitation of genome molecules in plasma and serum samples from nine untreated, HCV-infected patients^a

Patients	HCV genome copy numbers per ml	
	plasma	serum
[1]	543,360	316,520
[2]	1,636,684	231,900
[3]	457,783	124,445
[4]	1,277,667	555,596
[5]	1,548,118	384,702
[6]	644,539	639,280
[7]	714,566	44,812
[8]	600	420
[9]	1,093,801	191,497



^a A total of 5,000,000 HCV genome molecules per ml were detected from a reference serum sample containing $10^{6.5}$ CID_{50} s. A total of 850,000 HCV genome molecules per ml were detected from a standard HCV RNA-positive pool arranged in our laboratory and used as a positive control in our experiments.

fragment of HCV cDNA) was used to obtain the synthetic competitor RNA. The competitor RNA copy numbers were assayed by different methods: first, by spectrophotometric analysis and gel electrophoresis, after which the estimated copy number was confirmed in two independent assays by endpoint dilution RT-PCR experiments and determination of the Poisson distribution of positive results.

In the present study, we comparatively evaluated serum and plasma samples from the same patients using cRT-PCR. Interestingly, the data indicated that a highly variable loss of HCV genome molecules occurs in all the serum samples (Table 2). As a consequence of the unpredictable features of the drop in viral genome copy numbers observed in serum, serum specimens appear to be totally inadequate for the correct quantitative detection of viral load in patients with HCV infection. Since almost all quantitative or semiquantitative investigations of HCV-infected patients reported so far have been carried out by using serum samples, this point deserves to be underlined clearly.

Finally, although no direct comparative evaluation was performed in the present study, our data also suggest that nested PCR amplification is not required for qualitative (and, in our case, quantitative) HCV RNA detection from clinical specimens of infected patients when (i) the amplified viral sequence is accurately selected, (ii) the PCR conditions are optimized, and (iii) more than 40 amplification cycles are used. However, according to the general concept of cPCR and cRT-PCR, any variable (predictable or unpredictable) influencing the efficiency of the amplification reaction will have the same effect on both template species; under these conditions, the quantitative results are not influenced by the number of PCR cycles by the cRT-PCR (10, 12).

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