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Comparison of a Competitive Combined Reverse Transcription-PCR Assay with a Branched-DNA Assay for Hepatitis C Virus RNA Quantitation

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We have developed a sensitive and reproducible one-step competitive reverse transcriptase (RT) PCR assay, which allows hepatitis C virus (HCV) RNA quantitation in plasma over a broad range of values. The RNA samples and a constant amount of an internal standard were reverse transcribed and coamplified with the same primers in the same tube. A standard curve was obtained from an additional series of tubes containing both the internal standard and known amounts of a wild-type HCV RNA transcript, thus eliminating the need for titrating samples with the competitor. Eighty-eight anti-HCV-positive samples were tested by RT-PCR and a branched-DNA (bDNA) assay which has a detection limit of 3.5×10^5 copies per ml. Fifty-five samples were quantifiable by both methods (correlation coefficient, 0.72), the ranges of values found by the RT-PCR and bDNA assays being, respectively, 0.127×10^6 to 18.4×10^6 and 0.44×10^6 to 38×10^6 copies per ml. Six samples that had indeterminate values by the bDNA assay had RT-PCR values between 0.37×10^5 and 9.6×10^5 copies per ml. Twenty-two samples that had values below the cutoff value by the bDNA assay had RT-PCR values between 2.5×10^3 and 10.4×10^5 (18 less than and 4 more than the limit of 3.5×10^5 copies per ml). The remaining five samples were negative by both assays. The level of RT-PCR interassay reproducibility was high (correlation coefficient between duplicate values, 0.94). Our method, with a detection limit of 2,500 copies per ml, was markedly more sensitive than the bDNA assay. This method is convenient for following up patients with low viremia, a common situation with alpha interferon treatment.

Hepatitis C virus (HCV) is a positive-strand RNA virus responsible for most cases of chronic hepatitis, which often leads to cirrhosis and liver cancer. Response to alpha interferon has been defined as a decrease of alanine aminotransferase to normal values (8, 16). Some studies have shown that such a decrease is usually, but not always, associated with loss of HCV viremia as detected by reverse transcriptase (RT) PCR. Disappearance of HCV RNA has also been observed without levels of alanine aminotransferase returning to normal (3, 19). Several recent studies have shown that the level of viremia correlates with the clinical stage of disease and may be a prognosis factor in the response to antiviral therapy (1, 10, 14, 17). In the absence of international standardization, tests for HCV RNA quantitation should be as accurate and reproducible as possible to permit follow-up of patients undergoing alpha interferon treatment.

The most sensitive tests for quantitation of HCV RNA are based on RT-PCR. Some investigators have performed RT-PCR without an internal standard (IS). Quantitation by RT-PCR without the use of an IS may lead to erroneous results, because several factors may affect the reaction in an uncontrolled way. First, the levels of efficiency of the RT step may differ from sample to sample. Second, small intersample and intercycle variations in levels of amplification efficiency in the first cycles may have a marked effect on the final amount of amplified material, because of the exponential PCR process. Third, the efficiency of the PCR decreases in later cycles because of reannealing of amplicons and the limited availability of reagents, and the yield of amplified products is no longer proportional to the initial number of molecules. Finally, various inhibitors possibly present in specimens can affect both RT and DNA polymerase activities, leading to underestimation of viremia or even to false-negative results. In order to circumvent such problems, we developed a competitive and sensitive one-step RT-PCR assay in which a constant amount of an IS is reverse transcribed and coamplified with the RNA specimen to be tested. In such an assay, intersample and intercycle efficiency variations are expected to affect the HCV target RNA and the IS in the same way. Furthermore, false-negative results due to complete inhibition of PCR should be detected by the simultaneous lack of the IS and HCV signals.

Quantitative RT-PCR results are compared here with those obtained with a widely used commercial branched-DNA (bDNA) assay based on signal amplification (the Quantiplex HCV bDNA assay).

MATERIALS AND METHODS

Construction of RNA standards. The 5′ untranslated region of HCV genotype 3a (HCV-3a) RNA from one patient with chronic hepatitis C was amplified following reverse transcription with the primer pair NCR1-NCR2 (9). The PCR fragment was cloned into the expression vector pBluescript KS after *Xho*I digestion. The orientation of the HCV-3a insert was determined by digesting the plasmid with the restriction endonucleases *Pvu*II and *Acc*I and analyzing the fragments in agarose gel. The HCV-3a plasmid was linearized with *Hin*dIII, and a positive HCV RNA strand 383 bases long, referred to as the wild-type (wt) standard, was synthesized in vitro with an Ampliscribe T3 transcription kit (Epicentre, Madison, Wis.) according to the manufacturer's instructions. The amount of RNA produced was measured spectrophotometrically after DNase I treatment and removal of unincorporated nucleotide triphosphates by gel filtration in a Chroma-Spin 100 column (Clontech, Palo Alto, Calif.). RNAs in the standards were, in addition, measured by a modified phosphate determination method (7) based on ammonium phosphomolybdate complex formation.

The IS was created by deleting an 83-bp segment internal to the primer binding sites and delineated with the *Asp*I and *Nru*I recognition sites on the

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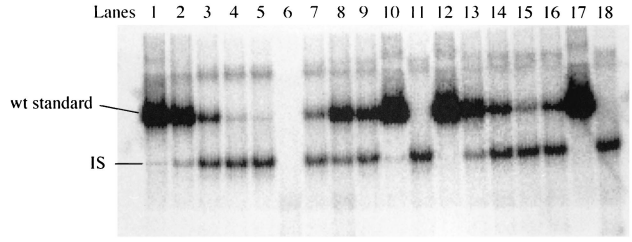

FIG. 1. Coamplification of various amounts of HCV RNA together with 10^3 copies of the IS. Lanes 1 to 5, wt standard at, respectively, 10^5, 10^4, 10^3, 3×10^2, and 10^2 copies; lane 6, negative control, which included all reagents except the IS; lanes 7 to 10 and 12 to 17, RNA from 10 different HCV antibody-positive specimens; lanes 11 and 18, negative control specimens. The sizes of amplified products were 244 bp for HCV and 161 bp for the IS.

HCV-3a plasmid. The Klenow DNA polymerase from *Escherichia coli* was used for filling in the 3′ termini of the DNA fragment, allowing ligation with the T4 DNA ligase under standard conditions (18). The IS was synthesized and quantified in the same way as the wt standard. The integrity of both RNAs was checked in a 5% polyacrylamide–8 M urea gel.

Competitive combined RT-PCR. RNA present in 150 μl of patient serum was extracted by an acid guanidium-phenol-chloroform method (5) and resuspended in 11 μl of sterile distilled water. Three microliters was used in a combined one-step RT-PCR assay in a final volume of 20 μl. This HCV RNA sample, or a known amount of the wt standard, was coamplified with 10^3 copies of the IS, with the same primers in the same tube, by sequential use of the RT and DNA polymerase activities of *Thermus thermophilus* polymerase, without reopening the tube.

The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 0.85 mM $MnCl_2$, 200 μM (each) deoxynucleotide triphosphates, 150 nM (each) primers KY78 and KY80, 5 U of *Thermus thermophilus* DNA polymerase (Perkin-Elmer Corporation, Norwalk, Conn.), and 5 μCi of [α-^{33}P]dCTP (specific activity, 1,000 to 3,000 Ci/mmol). Thus, the molar ratio of labeled to unlabeled dCTP was 1 to 4,000. The reaction was performed in a GeneAmp PCR system, model 9600 thermocycler (Perkin-Elmer). Reverse transcription was performed for 30 min at 70°C and was followed by a 1-min incubation at 95°C to denature the RNA-DNA heteroduplexes. PCR amplification started with 2 cycles at 95°C for 15 s and 60°C for 20 s and was followed by 38 cycles at 90°C for 15 s and 60°C for 20 s. The final extension step was at 60°C for 4 min (21).

Ten microliters of labeled amplicons were run through an 8% nondenaturing polyacrylamide gel. Samples and wt standard amplicons were 244-bp long. The deletion-containing IS was only 161-bp long, thus allowing easy separation (Fig. 1). The dried gel was exposed for 3 h in an InstantImager apparatus (Packard, Chicago, Ill.). The radioactivity emitted by each specific band was measured, and their ratio, referred to as HCV (or wt) standard/IS, was calculated.

HCV genotyping. HCV genotypes were established by the line probe assay HCV INNO-LIPA kit (Innogenetics, Zwijndrecht, Belgium) according to the manufacturer's instructions.

Standard curve. In order to test whether the wt standard and the IS were amplified with the same efficiency, decreasing amounts of the wt standard or the IS, ranging from 10^5 to 10 copies, were separately amplified for 40 cycles. The curves obtained were compared. For the quantitation of HCV RNA in patient specimens, a standard curve was obtained by reverse transcribing and amplifying in an additional six tubes 10^3 copies of the IS together with known amounts of the wt standard ranging from 10^5 to 10^2 copies.

Comparison method. Results obtained by our RT-PCR were compared with those obtained by the bDNA assay (Quantiplex HCV bDNA assay, version 1.0; Chiron Corporation, Emeryville, Calif.). This assay is based on signal amplification with a bDNA (2, 20). Serum samples were tested in duplicate. Differences between duplicate measurements did not exceed 20%, as recommended by the manufacturer.

The detection limit of the assay is 3.5×10^5 equivalents (copies) per ml. Results were considered below the cutoff value when the number of RNA molecules was lower than 3.5×10^5 equivalents per ml and indeterminate if only one of the duplicates was below the cutoff.

Validation for clinical samples. To ensure that the wt standard RNA transcript constituted an adequate standard for the quantitation of HCV RNA in samples, one patient's serum sample was serially diluted 1:5 in normal human serum. The amount of HCV RNA in this sample was first measured by the HCV bDNA assay, and the value was found to be close to that obtained by the RT-PCR assay. RNA from each dilution was extracted, and 3 μl was coamplified with 10^3 copies of the IS. The curve obtained was compared with that established with the wt standard in the same run.

Serum samples. A total of 330 serum samples positive for anti-HCV were tested by our quantitative RT-PCR. These samples were obtained from patients with chronic hepatitis C, before or during treatment with alpha interferon.

Eighty-eight samples, selected for covering a broad range of viremia values in the quantitative RT-PCR assay, were further tested by the Quantiplex HCV bDNA assay. Of these 88 samples, 83 were known to be positive and 5 were known to be negative by the qualitative test Amplicor HCV RNA (Hoffmann-La Roche, Basel, Switzerland).

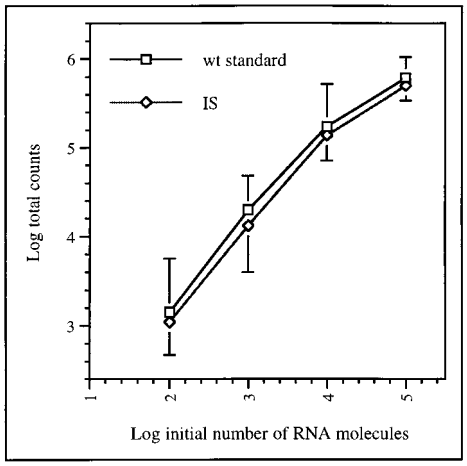

FIG. 2. Separate amplification of the wt standard or the IS, in the absence of competitor for 40 cycles (1:10 dilutions from 10^5 down to 10 copies). Average values ± 95% confidence intervals ($n = 4$) are noted. In these assays, no signal was obtained with the 10-copy dilution from each standard.

RESULTS

RNA standards. Both RNA transcripts, when controlled on ethidium bromide-stained polyacrylamide gel, showed single bands of the expected size. No additional band was seen, which indicates that the transcripts consisted of full-length RNA. The ratios of the optical density at 260 nm to the optical density at 280 nm were 1.91 (wt standard) and 1.93 (IS), which suggests that no significant amounts of contaminants, such as proteins or phenol, were present in the preparations. Moreover, RNA concentrations, when measured by phosphate analysis, were found to be the same as when measured by spectrophotometry.

Standard curve. When the wt standard and the IS were amplified separately for 40 cycles in the absence of competitor, similar levels of efficiency for the PCR amplifications were obtained. In the range of 10^5 to 10 copies, the yields of amplification were nearly the same for both standards (Fig. 2). However, total counts in the IS were slightly lower than those in the wt standard because the shorter IS incorporated fewer ^{33}P-labeled nucleotides than did the wt standard. In this particular experiment, no signal was obtained with the 10-copy dilution from each standard.

Results obtained from coamplifying increasing amounts of the wt standard with a constant number of IS copies (10^3) for 40 cycles are depicted in Fig. 3. As expected, the competition resulted in decreased amplification of the IS, particularly when the number of wt standard molecules was higher than that of IS molecules. When equimolar amounts of wt and IS were amplified together, the ratio of their levels of amplification was close to one.

Figure 4 shows the linear relationship between the initial number of wt standard molecules and the wt standard/IS ratio found after amplification. Such a standard curve was established in each run to allow for the quantitation of HCV RNA in patients' samples. The amounts of HCV RNA in the samples were calculated by relating the amplicon ratios, HCV sample/IS, to the corresponding ratios obtained for the standard curve.

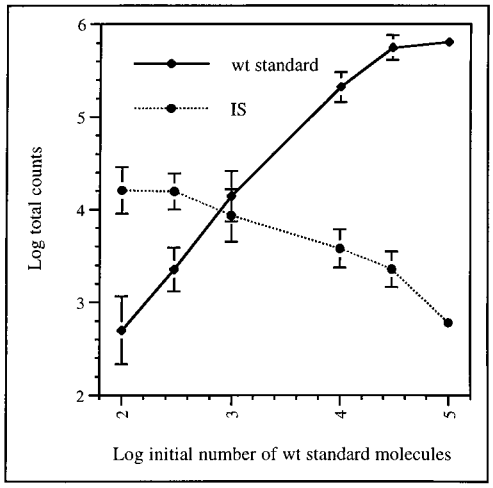

FIG. 3. Coamplification of a constant amount of the IS (10^3 molecules) with various numbers of wt standard molecules ranging from 10^5 to 10^2. PCR was performed for 40 cycles. Fragments were separated by polyacrylamide gel electrophoresis with 8% polyacrylamide gel, and the levels of radioactivity present in both amplicons were measured in an InstantImager. Average values ± 95% confidence intervals (n = 16) are noted.

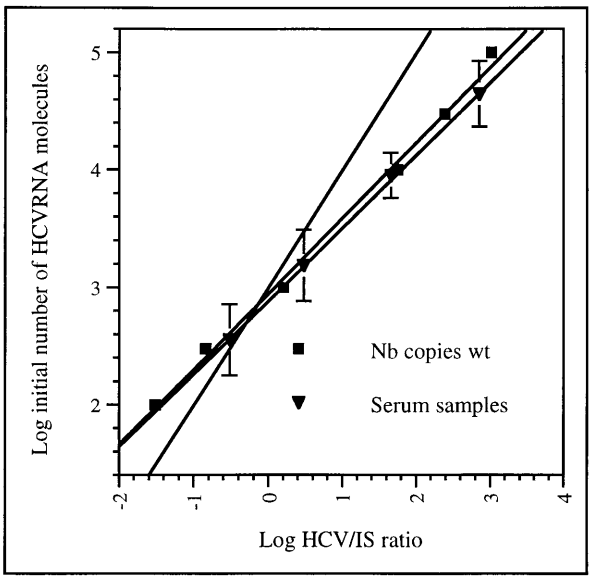

FIG. 5. Comparison of the standard curve obtained with serial 1:5 dilutions of one HCV RNA-positive sample (average values ± 95% confidence intervals; n = 5) and the standard curve obtained with the wt standard RNA transcript. The line without symbols is the one which would be expected if the IS and HCV RNA were amplified with equal levels of efficiency.

Validation for clinical samples. The standard curve established with the RNA extracted from serial dilutions of the patient serum containing a known amount of HCV RNA was found to run almost parallel to that obtained with the wt standard RNA transcript (Fig. 5). The standard curve could therefore be used directly for patient serum quantitation, provided that the number of RNA molecules contained in 40 μl of serum (or 3 μl of the RNA solution) was between 10^5 and 10^2. The lowest amount of wt standard that was consistently detected was 10^2 copies. This amount, therefore, allowed us to reach a sensitivity of 2,500 copies per ml (i.e., 10^2 copies in 40 μl equals 2,500 copies in 1 ml). In practice, samples with more than 3×10^4 RNA molecules per 40 μl of serum were diluted to avoid inaccuracies resulting from high ratios of samples to IS. Figure 5 also depicts the theoretical curve that would be expected if the two competing RNAs (HCV and IS) were amplified with equal efficiencies.

Reproducibility. To test the reproducibility of our results, 73 serum samples with HCV RNA values between 0 and 2.5×10^7 copies per ml were analyzed twice in separate runs and with different RNA extracts (Fig. 6). The nonparametric Spearman correlation coefficient obtained between duplicates was 0.94 ($P < 0.0001$), and the coefficient of variation was 32.7%.

Comparison of the Quantiplex HCV bDNA assay with quantitative RT-PCR. Of the 88 serum samples compared, 55 were

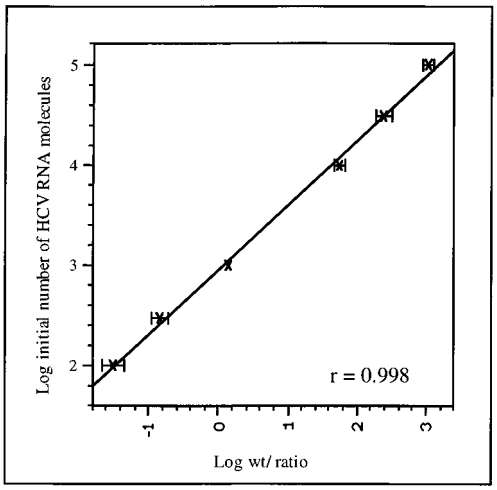

FIG. 4. Standard curve for HCV RNA quantitation established with the same data used to obtain the results shown in Fig. 3. The ratio of the wt standard to the IS (wt/IS ratio) found after amplification is shown to be proportional to the number of wt standard transcript molecules introduced before amplification. Average values ± 95% confidence intervals (n = 16) are shown.

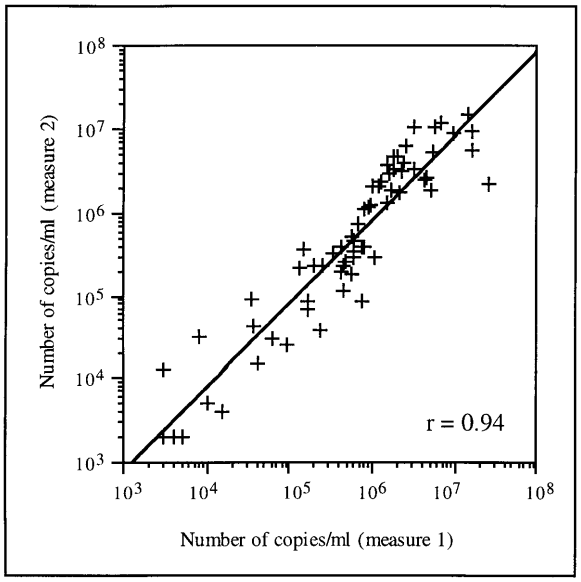

FIG. 6. HCV RNA values determined by RT-PCR. Reproducibility was calculated with pairs of measurements taken of separate RNA extracts and in separate runs (n = 73).

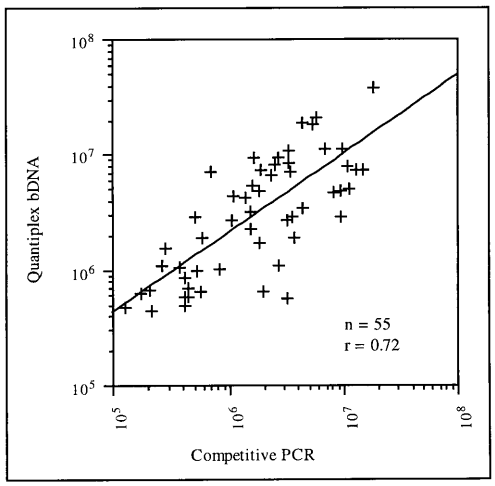

FIG. 7. Correlation between the number of copies obtained by the Quantiplex HCV bDNA assay and the competitive RT-PCR. Of these 55 samples, 37 had to be diluted to enter the linearity range of the RT-PCR assay.

quantifiable by both the bDNA assay and the quantitative RT-PCR (Fig. 7), 28 were quantifiable by RT-PCR only, and 5 were quantifiable by neither of these methods. In the group of 55, the Spearman correlation coefficient was 0.72 ($P < 0.0001$). The bDNA assay values ranged from 4.4×10^5 to 3.8×10^7 copies per ml, whereas quantitative RT-PCR values ranged from 1.27×10^5 to 1.84×10^7 (Table 1). Six of these 55 samples had quantitative RT-PCR values below 3.5×10^5 copies per ml.

The bDNA assay (version 1.0) is known to underestimate HCV genotype 2 by a factor of 3 and genotype 3 by a factor of 2 (15). When the bDNA assay values were corrected accordingly, the correlation coefficient increased to 0.84. On average, the bDNA assay gave uncorrected values 1.56-fold higher than those of the quantitative RT-PCR. The bDNA assay values were 1.99-fold higher when corrected by the factors mentioned above.

Six samples of the group of 28 were found to be indeterminate by the bDNA assay, with quantitative RT-PCR values between 0.37×10^5 and 9.65×10^5 copies per ml (3 samples contained less and 3 samples contained more than 3.5×10^5 copies per ml as shown by quantitative RT-PCR. The results with the other 22 samples were found to be below the cutoff of the bDNA assay, with RT-PCR values between 2.5×10^3 and 1.04×10^6 copies per ml (18 samples contained less and 4 samples contained more than 3.5×10^5 copies per ml). All of these 83 samples had previously tested positive by the Amplicor test. The remaining 5 samples, which were negative by both the quantitative RT-PCR and the bDNA assays, were also negative by the Amplicor test.

The reproducibility of the bDNA assay was found to be higher than that of RT-PCR, with a coefficient of variation of 8.8%.

TABLE 1. Comparison of the results obtained by the quantitative RT-PCR and those obtained by the Quantiplex HCV bDNA assay

No. of samples	Range of values (copies/ml)	
	Competitive RT-PCR assay	bDNA assay
55	1.27×10^5–1.84×10^7	4.4×10^5–3.8×10^7
3[a]	3.7×10^4–2.4×10^5	Indeterminate[b]
3	4.5×10^5–9.6×10^5	
18[a]	2.5×10^3–3.2×10^5	Below cutoff[c]
4	3.6×10^5–1.04×10^6	
5	0	Below cutoff[c]
Total 88		

[a] We have separated samples with more than 3.5×10^5 copies per ml from those with less than 3.5×10^5 copies per ml as measured by RT-PCR.

[b] Indeterminate indicates that one of the results of duplicate experiments was above and the other was below the cutoff value.

[c] Below cutoff indicates that the bDNA assay results were lower than 3.5×10^5 copies per ml.

DISCUSSION

In the competitive RT-PCR described here, HCV RNAs from patients or wt standard RNA was coamplified with the IS by using the same primers in the same tube. Thus, factors, such as depletion of reaction components or the presence of inhibitors, would be expected to affect the amplification efficiencies of HCV RNA and IS similarly. PCR inhibitors, possibly present in serum, may reduce assay sensitivity and even lead to complete inhibition of PCR. The test remains able to correctly quantify HCV RNA in spite of the potential presence of inhibitors, provided the latter do not fully block amplification. Indeed, relatively high interassay variations were found when absolute counts were considered, whereas ratios remained constant for the same samples.

The KY primer set used (21), which is the same as in the HCV Amplicor assay, was selected in order to recognize sequences in the highly conserved 5′ untranslated region. However, Bukh et al. (4) found, of 44 nucleotide sequences representative of different geographical locations, four sequences with two base changes in the KY80 annealing site. These changes were A to G at position −272 and T to C at position −264, when compared with the genotype 1a prototype strain HCV-1 (6, 12). By looking at all sequences available in the GenBank database, we observed that these changes were characteristic of genotype 3a. Our wt standard and IS were created with a strain belonging to genotype 3a and probably contained the two mutations. The efficiencies obtained when using these standards in our RT-PCR indicate that, in the experimental conditions used, the two mismatches did not significantly destabilize the annealing of KY80 to its target. Indeed, the patient samples tested were satisfactorily amplified regardless of their genotype. Small differences in levels of amplification efficiency due to genotypes cannot be ruled out; however, the RT-PCR results correlated with those obtained by the bDNA assay to similar extents for each genotype.

As a principle, the differences in length and in base composition between target RNA and the IS should be as small as possible in order not to interfere with the reannealing rate of PCR products, RT efficiency, heteroduplex formation, or other events. Here, an appreciable difference between the target RNA and the IS was present (deletion of 83 of 244 bp in the IS), and one cannot exclude that the reannealing rate or other events might have slightly differed between target and IS RNAs. Such differences could perhaps explain why the standard curve slightly deviates from the theoretical curve (Fig. 5).

Nevertheless, the deviation from the theoretical curve did not appear to be a problem for the HCV RNA quantitation of patients' samples. Indeed, both the wt standard curve and that established with a serially diluted HCV RNA-positive sample ran parallel and showed the same deviation from the theoretical curve. This finding indicates that an HCV RNA transcript provided an adequate standard for the quantification of the HCV genome from patients and that the competitive RT-PCR

can be used for serum samples. The good correlation observed between RT-PCR and bDNA assay results ($r = 0.72$) also indicates that deviation from the theoretical curve does not prevent correct quantitation of patients' HCV RNA.

The good reproducibility of results from duplicates extracted and analyzed in different runs ($r = 0.94$), as well as the straight line obtained when serially diluted serum samples were used, shows that the efficiency of the extraction procedure was constant throughout all concentrations of HCV RNA tested. Thus, the variation in the efficiency of the extraction procedure does not appear to contribute substantially to the total variation, even though the IS was added after the extraction procedure.

Some investigators have compared quantitative RT-PCR and bDNA assays, obtaining correlation coefficients similar to that obtained by us (2, 11). However, the RT-PCR methods used in these studies were more time consuming than ours. In the first study, quantitation by RT-PCR was performed by using endpoint dilution (without IS), a method which may be inaccurate. In the second study, quantitation was made by coamplifying the specimen to be tested with various amounts of an IS, a procedure commonly used in competitive RT-PCR. In our test, the use of a standard curve eliminated the need for titrating each sample with the competitor (13) and allowed us to apply the assay to a large number of specimens. Results of the RT-PCR assay were found to correlate well with those obtained by the bDNA assay, especially when the lower sensitivity of the bDNA assay for genotypes 2 and 3 was taken into account. Only four samples found to be below the cutoff value in the bDNA assay were found to be discrepant, having over 3.5×10^5 copies per ml in the RT-PCR but being only slightly above this limit (Table 1).

As far as comparisons with the bDNA method are concerned, we must admit that this RT-PCR remains more time consuming and that its level of reproducibility is lower.

The competitive RT-PCR method described here provides a sensitive tool with reproducible results for quantitation of HCV particles in serum samples. Our test, with a detection limit of 2,500 copies per ml, is well suited for the follow-up of patients with low viremia, a situation commonly observed during alpha interferon treatment.

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