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Automated Quantitative Determination of Hepatitis C Virus Viremia by Reverse Transcription-PCR

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An automated reverse transcription-PCR was developed for the quantitative detection of hepatitis C virus. The quantitation is based on the coamplification and labelling with digoxigenin-dUTP during PCR of two similar templates, the viral genome and a modified RNA which acts as a mimic target. Known amounts of the mimic RNA sequence were introduced into the clinical samples. The automated quantitation of the two coamplified and labelled products depends on the use of two biotinylated capture probes which are complementary, respectively, to a deleted sequence and to an inserted sequence introduced by site-directed mutagenesis in a wild viral cloned cDNA. This method proved to be simple, reproducible, and useful for quantitate hepatitis C virus viremia in chronically infected patients. This easy-to-perform, automated assay could also be used for the accurate determination of human immunodeficiency virus viremia or other RNA molecules.

Hepatitis C virus (HCV) infection often induces chronic active hepatitis leading to liver fibrosis. Alpha interferon therapy proved to be an effective treatment in a subset of patients (11). Until recently, the response to the treatment was monitored by measuring the alanine aminotransferase level in the patient's serum (6). HCV viremia is correlated with viral replication and, probably, with the progression of hepatitis. The quantification of HCV RNA in serum could provide a promising marker of viral replication and, therefore, of the efficiency of the antiviral treatment (14, 15). Until now, the evaluation of HCV RNA in serum was performed by reverse transcription (RT)-PCR (RT-PCR). This technique, however, is fastidious and time-consuming and lacks standardization. So far, only a small number of patients have been included in studies for the quantification of HCV RNA (10).

In the last 2 years, several protocols for quantitative RT-PCR have been described and have been applied mainly to human immunodeficiency virus and HCV. Some protocols rely on the endpoint dilution of the sample to be tested or compare the amount of amplified products with an external standard curve (16). The reliabilities of these approaches are questionable because there are differential efficiencies and kinetics of the RT and PCR, depending on the abundance of the templates in the samples and the presence of internal inhibitors. These variable conditions generate large differences in the total amount of amplified products obtained after exponential amplification (7, 20).

The most elegant and accurate methods for controlling all of the variable parameters is the use in the sample of an internal positive RNA control that is largely similar to the viral target and that can be easily discriminated in the detection steps (12, 17). In the present study, we developed such a quantitative RT-PCR that mostly depends on automated procedures.

For this procedure, we designed a mimic viral HCV RNA which differs from the viral sequence by a deletion and an insertion within the target sequence. This mimic RNA was introduced at the very beginning of the extraction protocol. In the subsequent steps, the viral and competitive templates were coprocessed, giving reliable quantitative results. During the PCR, a modified nucleotide, digoxigenin-dUTP (dig-dUTP), was incorporated into the amplified products. The automated quantification of the labelled amplicons was achieved by inverse hybridization with 5′-biotinylated probes. The biotinylated probes were then complementary either to the inserted sequence (the mimic probe) or the deleted sequence (the viral probe). Hybrids were bound on plastic surfaces through streptavidin-biotin interaction and were revealed by enzyme-linked immunoassay. The automated method described here provides a simplified procedure for assaying viral replication during antiviral therapy. Moreover, the methodology, which is described in this report, could be applied to the quantitation of other RNA viruses.

MATERIALS AND METHODS

Serum samples. The serum samples used in the study were sent to the Virology Laboratory (Centre Hospitalo-Universitaire, Pontchaillou, Rennes, France) for routine HCV analysis and were kept at −80°C.

Extraction and RT. HCV RNA was isolated from 200 μl of serum by the guanidinium thiocyanate-phenol-chloroform (GuSCN) method described by Chomczynski and Sacchi (5). cDNA synthesis was performed in a 20-μl reaction volume at 37°C for 50 min after brief denaturation at 80°C by using RC_2 (1 μM) as the downstream primer, 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Bethesda, Md.), 40 U of RNase inhibitor (Boehringer, Mannheim, Germany), and 0.5 mM (each) desoxyribonucleotide. The reaction was stopped by heating the tube to 90°C for 5 min. After cooling on ice, 10 μl of cDNA was stored at −80°C and 10 μl was added to a PCR tube.

PCR conditions. Primers were defined in the 5′-noncoding region of the HCV genome. The comparison of 81 sequences of this region from strains isolated from individuals in different parts of the world (3) showed that some degree of variability could be mapped mainly at three different sites. The primers

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were chosen within the constant regions by a computer program that we have described previously (9). The computer program selects primers according to the low frequency of occurrence of their 3′ octamers in the human genome (9). RC_1 (5′-GTC TAG CCA TGG CGT TAG TA-3′), complementary to the positive RNA strand of the HCV genome, and RC_2 (5′-TCT CGC GGG GGC ACG CCC AA-3′), complementary to the negative RNA strand of the HCV genome, were thus selected. They corresponded, respectively, to nucleotides −265 to −246 and −96 to −115 on the HCV 1 strain sequence described previously (3).

Amplification of cDNA was carried out in a final volume of 25 or 50 μl of each 1 μM primer, *Taq* buffer (50 mM KCl, 10 mM Tris, 2 mM $MgCl_2$, 0.01% gelatin [pH 8.4]), 0.2 mM each deoxynucleoside triphosphate, and 2.5 U *Taq* polymerase (Perkin-Elmer Cetus, Saint Queutin, France). The samples were subjected to amplification in a DNA thermal cycler (Omnigene; Hybaid, Teddington, United Kingdom) for 35 cycles of denaturation (15 s at 93°C), annealing (1 min and 15 s at 55°C), and extension (1 min and 15 s at 72°C); this was followed by 10 min at 72°C.

Analysis by electrophoresis, Southern blotting, and hybridization. Aliquots of 10 μl of the amplification products were subjected to electrophoresis in a 2% agarose gel with ethidium bromide. After transfer to a Hybond N membrane (Amersham, Amersham, United Kingdom), hybridization was performed with a digoxigenin-labelled probe by methods that we have described previously (8). Briefly, the probe was synthesized by PCR. The template RNA was extracted from the serum of an HCV-positive patient and was reverse transcribed. The first amplification was performed with the RC_1 and RC_2 primers as described above. The second amplification included the first amplification product, a dTTP and digoxigenin-dUTP mixture instead of dTTP alone, and 15-base nested primers, which were immediately downstream of RC_1 and RC_2.

Site-directed mutagenesis. A deletion and an insertion were introduced within the target sequence defined for amplification. Serum from an HCV-positive patient provided the HCV sequence. Viral RNA was extracted, reverse transcribed into cDNA, and amplified with two primers, primers A and B, as described above. Primer A, with the *Bam*HI restriction site at the 5′ end, complementary to bases 11 to 25 (4), and primer B, with the *Eco*RI restriction site at the 5′ end, complementary to bases −7 to −21 (3), delimited the complete 5′-noncoding region of the HCV genome. After gel electrophoresis, the UV-visible bands of the amplified products were excised from the gel. DNA was purified by using the Geneclean II KIT (Bio 101, La Jolla, Calif.). The purified fragment was the template for two different amplifications. The first one was set up with primers A and D_1. As shown in Fig. 1a, primer D_1 was complementary to bases −229 to −220 and bases −179 to −170 and introduced a 40-base deletion in a very conserved region. The second one was defined by primers D_2 and B. Primer D_2 is antiparallel to primer D_1. After amplification, electrophoresis, and purification, the fragments AD_1 and D_2B were combined in a PCR vial and amplified with primers A and B to obtain one fragment with the deletion introduced by primer D_1. This fragment was cloned into the multiple cloning site of pBluescript SK (Stratagene, La Jolla, Calif.) by the procedure described by Sambrook et al. (18).

The insertion sequence (Fig. 1b) was synthesized by PCR from the bacterial tetracycline resistance (TET) gene with the following primers. The I_1 primer sequence was complementary to the TET gene at its 3′ end and was complementary to bases −158 to −149 of the HCV noncoding region at its 5′ end. Similarly, the I_2 primer sequence was complementary to the TET gene at its 3′ end and complementary to bases −139 to −148 at its 5′ end. After electrophoresis and purification, the insertion fragment I_2I_1 was first combined in a PCR with the deleted fragment AB and was amplified with primers A and I_1. Similarly, a combined PCR with the same fragment was set up with the primers I_2 and B. After electrophoresis and purification, fragments AI_1 and I_2B were combined and amplified with primers A and B to get the final product, which included the insertion and the deletion.

This modified HCV sequence was cloned into pBluescript SK as described above. As shown on the pBluescript SK-HCV plasmid map (Fig. 1c), the HCV sequence is flanked by the T3 and T7 RNA polymerase recognition sites. The insert in the pBluescript SK-HCV plasmid was sequenced from linearized plasmid and primers A and B with an Applied Biosystems sequencer (Fig. 2). Except for the insertion and the deletion that were introduced, the plasmid sequence differed only slightly from the sequence of HCV 1 strain (3). Differences mainly mapped to the variable regions described above, and the sequences corresponding to primers RC_1 and RC_2 were conserved except for one nucleotide at the RC_1 5′ end.

Mimic RNA was transcribed from 1 μg of pBS-HCV *Eco*RI-digested plasmid with the Ampliscribe T3 transcription kit (Epicentre Technologies, Madison, Wis.) according to the manufacturer's procedure. A total of 50 μg of 424-base RNA was obtained; this RNA corresponded to the positive strand of the HCV genome. The integrity of the mimic RNA was checked by denaturing gel electrophoresis, and the mimic RNA was tested for contaminating DNA by PCR without the RT step (data not shown). Quantification of the RNA was done by recording the optical density (OD) at a wavelength of 260 nm. The mimic RNA was diluted in GuSCN buffer to obtain from 0.2 to 2×10^6 molecules per 600-μl aliquot. The aliquots could be stored at −20°C for several weeks without RNA degradation.

Quantitative PCR. For each serum sample, several RNA extractions were performed with aliquots of GuSCN buffer containing the mimic RNA (see above). After cDNA synthesis, PCRs were set up with the following modifications. The primer (RC_1 and RC_2) concentrations were 0.25 μM, and the labelling mixture was dig-dUTP (25 μM) and dTTP (175 μM). The final volume was 50 μl. Different concentrations of primers and nucleotides were tested, and these concentrations were selected for their specificities, sensitivities, and the optimum OD that was recorded.

Probes. Two 20-nucleotide probes were selected within the 40-base deletion and within the 90-base insertion sequences. The oligonucleotide corresponding to the deletion could only match the amplification products obtained from the viral RNA and was subsequently named the viral probe. Conversely, the oligonucleotide corresponding to the insertion was the mimic probe. The probes were labelled with biotin at their 5′ ends, and their sequences were as follows: viral probe, 5′-biotin-GAG AGC CAT AGT GGT CTG CG-3′; mimic probe, 5′-biotin-TGT TGG GAA GGG CGA TCG GT-3′.

Detection. Amplification products were detected by using the Enzymun-test DNA detection kit (Boehringer, Mannheim, Germany). Denaturation was performed in sample tubes containing 40 μl of the PCR mixture and 360 μl of 50 mM NaOH, and the tubes were vortexed and left for 60 min at room temperature. All of the subsequent steps were automatically done by the Boehringer ES 300 analyzer. Briefly, 100 μl was transferred with 400 μl of the virus-specific 5′-biotin-labelled capture probe into a streptavidin-coated tube (14 ng per tube), and the mixture was left for 120 min at 37°C. This transfer was repeated similarly with the mimic probe. Both probes were

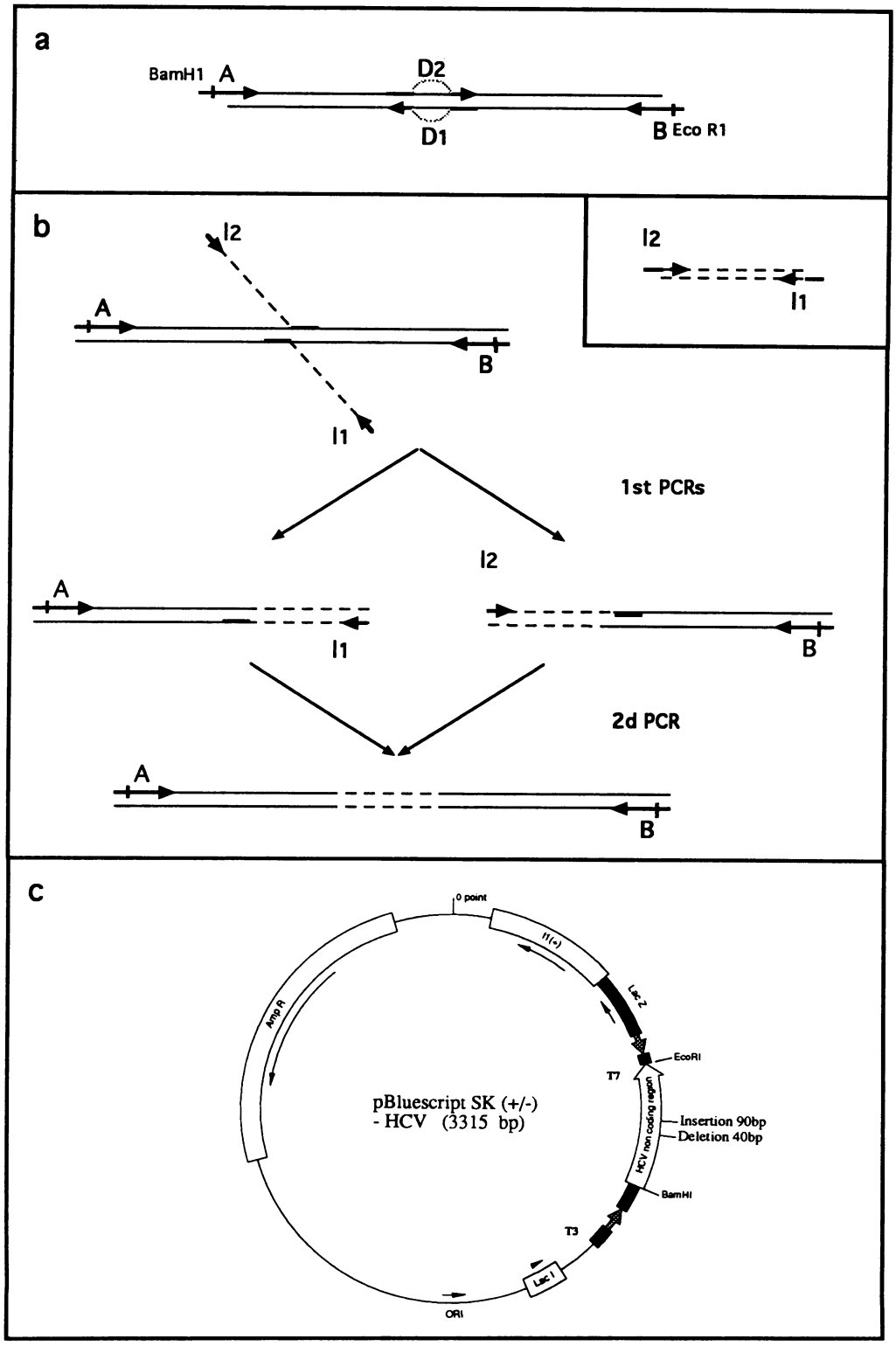

FIG. 1. Construction of the mimic template by site-directed mutagenesis. The bold segments of the primers represent the sequences complementary to the HCV genome. (a) A 40-bp deletion was introduced into the 5′-noncoding region of the HCV genome amplified by primers A and B by using the primers D_1 and D_2. (b) A 90-bp insertion was introduced into the deleted cloned 5′-noncoding region. The sequence to be inserted is depicted in the box, with the foreign sequence indicated by dashed lines. This sequence was used as a primer to generate two fragments by PCR in conjunction with primers AI_1 and BI_2. These two fragments were combined in a second PCR to synthesize the final product with primers A and B. (c) pBluescript SK-HCV map (c) (see text for details). ORI, origin.

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  1 GGATCCCACT CCACCATAGA TCACTCCCCT GTGAGGAACT TCTGTCTTCA
 51 CGCGGAAGCG CCTAGCCATG GCGTTAGTAC GTGTGTCGTG CAGCCACCAG
101 GACCCGTACA CCGGAATTGC CAGGACGACC GGGTCCGCGC GTCCCATTCG
151 CCATTCAGGC TGCGCAACTG TTGGGAAGGG CGATCGGTGC GGGCCTCTTC
201 GCTATTACGC CAGCTGGCGA AGGGGGTTTC TTGGATAAAA CGCGCTCAAT
251 GCCTGGAGAT TTGGGCGTGC CCCCGCGAGA CTGCTAGCCG AGTAGTGTTG
301 GGTCGCGAAA GGCCTTGTGG TACTGCCTGA TAGGGTGCTT GCGAGTGCCC
351 CGGGAGGTCT CGTAGACCGG AATTC
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FIG. 2. Sequence of the cloned mimic DNA. The inserted sequence is underlined. The sequences of primers RC_1 and RC_2 are boxed. Asterisks indicate the nucleotides which differ from the nucleotides in the HCV 1 strain sequence.

used at 2.7 nM in the hybridization buffer (pH 6.5). Extensive washing was performed with saline solution. The peroxidase-labelled anti-digoxigenin polyclonal antibody in Tris-HCl buffer (pH 7.5) was added. The mixture was incubated for 30 min at 37°C, and after washing, the enzyme substrate [1.9 mM 2,2′-aminobis(3-ethylbenzthiazoline sulfonic acid); ABTS] in 100 mM phosphate citrate buffer (pH 4.4) with 3.2 mM H_2O_2 (as sodium perborate) was added. The color was allowed to develop for 30 min at 37°C, and ODs were measured at a wavelength of 422 nm. Triplicate analysis of PCR products performed with the ES 300 analyzer showed a coefficient of variation of less than 1%. As a consequence, each analysis of the PCR product was performed only once.

Data analysis. Because the labelling of the amplification products by dig-dUTP is a direct function of the number of thymidine molecules in the sequence, a correction factor had to be introduced to compensate for the increased length of the mimic DNA. The correction factor is the ratio of the number of thymidine residues in the viral sequence to the number of thymidine residues in the mimic sequence and is equal to 0.78. All of the following data were corrected if not otherwise indicated.

The ODs from the mimic and viral probes were plotted as a function of the $\log_{10}$ of the number of mimic RNA copies. In the competitive assay, the point where the two curves crossed was the equivalence point. It was convenient to plot only one curve by calculating the ratio of the mimic OD by the viral OD. The equivalence point was then defined by a ratio equal to 1. The correlation of the ratios was analyzed by linear regression.

RESULTS

Sensitivity of the assay. The mimic RNA templates included in the GuSCN buffer were extracted, reverse transcribed, and amplified for 20, 25, 30, and 35 cycles. At 35 cycles, we obtained 0.25 OD unit with 20 copies of the targets in the reaction tube, while the mean of the negative controls was 0.06 OD unit. The sensitivities were lower for the other conditions. The plateau was reached at 35 cycles with 2×10^5 RNA copies in the sample. These conditions permitted a working copy number range of 10 to 10^5.

Detection of positive samples. To confirm that the assay in which the labelling was done during the PCR steps and the automated detection method could detect wild viral sequences, we compared the method with detection of the amplified products by electrophoresis, Southern blotting, and hybridization with the digoxigenin-labelled probe. Fifty-nine serum samples from patients sent to the laboratory for diagnostic purposes were tested in parallel. By Southern blotting and hybridization, 39 serum samples were positive and 20 were negative. For the automated reverse capture hybridization, we defined the cutoff as four times the OD obtained with negative controls; this was at OD 0.2 unit. The cutoff was below the OD recorded in the assay with 20 copies of mimic RNA. By using this criterion, 40 serum samples were positive. The mean OD values for positive samples was 4.3 ± 1.8. Nineteen serum samples were negative, with a mean OD of 0.09 ± 0.06. One serum sample was found to be negative by Southern blotting and weakly positive (OD = 0.6) by the automatic method.

PCR yield with viral and mimic RNAs. We compared the quantity of viral and mimic amplicons obtained with increasing numbers of amplification cycles. To do so, we started with about 10^4 of either viral or mimic RNA targets in the samples. After RT, cDNA was included in different PCR vials and placed in the thermocycler. After 25, 27, 29, 31, 33, and 35 cycles of amplification, one vial was removed, left for 10 min at 72°C, and stored until assayed. The PCR amplification products from the mimic RNA were captured by the mimic probe and the products from the viral RNA were captured by the viral probe. The curves drawn with the corrected values for mimic RNA and the viral data were similar, with identical slopes, showing that the assay yields with the two targets were identical (data not shown).

Quantitative assay. Figure 3a illustrates the principle of the quantitative automated reverse capture assay with the two different probes. HCV RNA was extracted from one HCV-positive serum sample with an unknown viral copy number, reverse transcribed into cDNA, and amplified five times along with an increasing number of mimic RNAs ranging from 50 to 5×10^5 copies. As expected, the OD of the mimic RNA increased with an increasing number of mimic templates. At the same time, the OD of the viral RNA was constant at first and then decreased when high mimic RNA copy numbers were present. As a result, there was a crossing point between the two curves, which is the graphic representation of the equivalence point at which the mimic and viral RNAs were present at equal concentrations. A competition between the two targets was observed only at the two ends of the curves, when the starting concentrations of each template were highly different. Around the crossing point, no obvious competition was recorded.

This was more graphically obvious by plotting the ratio of the OD of the mimic RNA by the OD of the viral RNA over the log of the mimic RNA copy number (Fig. 3c). The curve is sigmoidal, with a linear central region around the equivalence

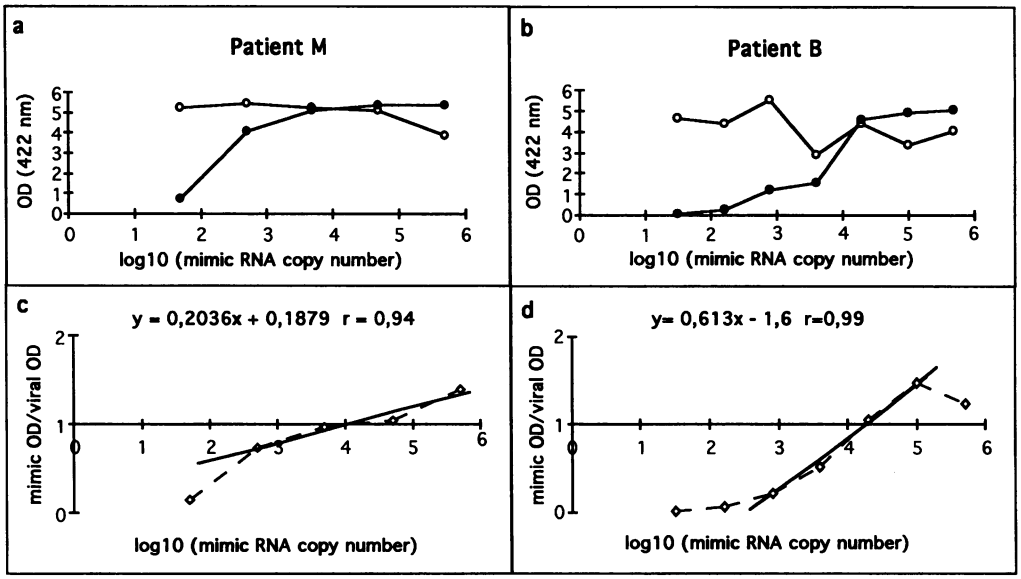

FIG. 3. Representative results of the quantitative RT-PCR assay. (a and b) ODs obtained with either the mimic or the viral probe. (c and d) OD ratios were plotted over the log of the mimic RNA copy numbers, and a linear regression was calculated for the equivalence. The viral copy number is equal to 9,727 copies · ml^{-1} of serum for patient M and to 17,742 copies · ml^{-1} of serum for patient B. ●, OD of mimic RNA; ○, OD of viral RNA. *r* indicates the correlation coefficient.

point defined by a ratio equal to 1. It was then possible to perform a linear regression analysis with the points close to the equivalence point, which permitted us to calculate the viral copy number for $y = 1$.

The linear regression analysis of the OD ratio was also convenient for correcting the ectopic OD recorded from one assay tube (Fig. 3b and d). The low OD observed for the vial containing 5×10^3 mimic RNA copies gave a ratio which fit in the ratio curve well.

Three quantitative assays were performed on 3 different days starting with one sample to test the reproducibilities of the assays. Within the equivalence zone, the three curves showed very similar shapes (Fig. 4a, b, and c) and the linear regression analysis displayed very close intersections for a ratio of 1 (Fig. 4d). The mean viral copy number in the sample volume tested was 15,133 ± 2,049.

DISCUSSION

A number of difficulties are associated with accurate quantitation by PCR technologies. The yield of the amplified products can be modified by inhibitors present in patient serum, the variability in RNA extraction, and the efficiency of RT (7, 20). Introduction of a mutated template at the very beginning of the procedure enabled us to internally control the variable parameters associated with RNA quantitation. Unfortunately, the addition of a positive control increased the complexity of the detection step and therefore decreased the feasibility of using the assay on a routine basis unless automated procedures can be used. With the quantitative RT-PCR with a positive internal control, such as the one that we described here, automation was made possible because (i) the amplified products are labelled during the amplification step with a nonradioactive modified nucleotide, and (ii) two site-directed mutations, a deletion and an insertion, permit the use of two specific probes. Therefore, simple automated quantification of the amplified products can be performed.

The deleted sequence was chosen in a conserved region. The ability of the viral probe to recognize wild viral sequences from clinical samples was assessed by comparing the results obtained by PCR labelling and then automated detection and the results obtained by classical PCR and then Southern blotting and hybridization. It was confirmed that the viral probe recognized all of the samples that emitted a positive hybridization signal, showing the stability of the chosen sequence. Very recently, Kleter et al. (13) analyzed the sequence of the 5′-noncoding region of 62 isolates made of at least four genotypes of HCV. That analysis showed that the sequence of the viral probe is indeed identical among the four genotypes; therefore, quantitation is not restricted to the quantitation of the HCV genotype of the isolate used to construct the mimic RNA sequence.

The inserted sequence was chosen for its similar percent GC content and GC stretches as those of the deleted viral sequence. Sequence analysis of the mimic target confirmed that there were no major changes in the cDNA used for the plasmid construction. The sequences around the primers were very close to those of the HCV 1 strain (3). Furthermore, we demonstrated that in spite of the differences introduced between the two targets, the PCR yields were identical. Quantification of the yield of amplified products through increasing cycle numbers is the only method that can be used to show that two different targets behave similarly in a PCR (1). These identical yields indicate that the sequence of the mimic RNA was indeed a mimic target which could be used as an internal positive control.

The sensitivity of the RT-PCR could then be assayed with the mimic RNA. Under the conditions that we defined, with the labelling during the PCR step and with 35 amplification cycles, 20 RNA copies in the processed serum could be detected. The plateau was reached for 10^5 RNA copies in the sample. These conditions give a 4-log-unit working range of RNA copy number with increasing ODs and a good sensitivity.

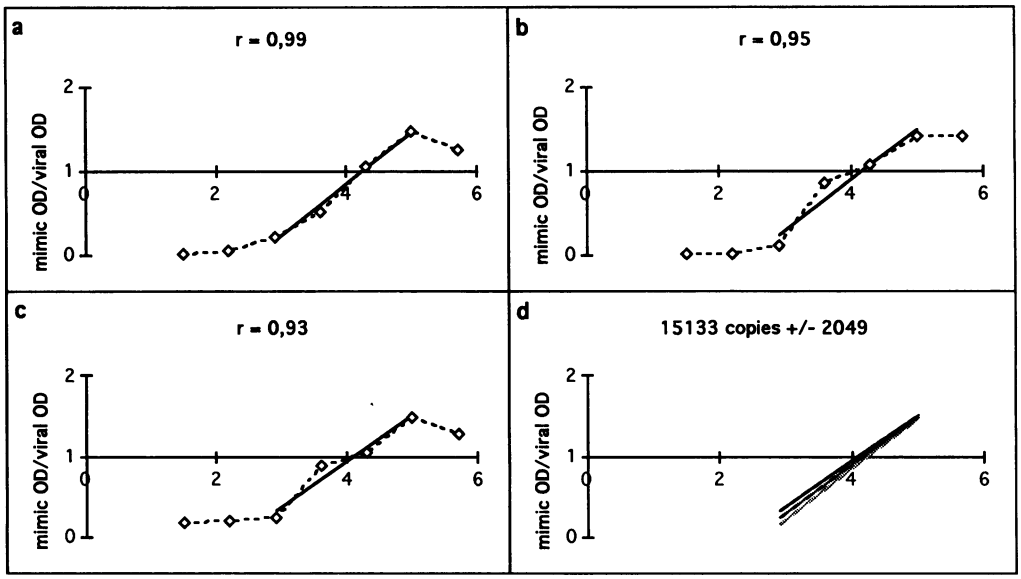

FIG. 4. Reproducibility of the quantitative RT-PCR assay. (a, b, and c) The same serum sample processed in three independent assays; (d) superposition of the three linear regression curves. *r* indicates the correlation coefficient.

When the two RNA targets were amplified separately, the yields of the amplified products were identical. When the two targets were introduced into one PCR vial and after 35 cycles of amplification, the OD ratio curve (mimic OD/viral OD, plotted over the log of the mimic RNA copy number) was linear for a large range of the mimic concentration. Within a 2-log-unit range of mimic template around the equivalence point, the OD ratio curve is linear, meaning that both targets are coamplified with a constant ratio of mimic/viral copy number through the whole procedure. A similar linearity over 2 log units of mimic target number was observed by a different competitive RT-PCR by using nonautomated quantification of PCR products (1).

Linear regression analysis performed within the linear region of the OD ratio curve allows calculation of the actual number of viral templates for the equivalence point corresponding to a ratio of 1. Conversely, a large excess of one template over the others leads to variation in the initial ratio of both targets during amplification. The two different slopes observed at the ends of the curve expressed these variations graphically.

The OD ratio curves were linear within the equivalence zone for all of the samples studied, even if different ODs were sometimes recorded between aliquots of one serum sample because of variations in the efficiencies of the three assay steps. In these cases, the ODs of the mimic and the viral RNAs were proportionally affected, indicating again that the internal positive control was a mimic target and that the OD ratio bypassed the efficiency variability. This sample-to-sample variability highlights the difficulty of quantifying RNA by RT-PCR by referring to an external standard curve or by endpoint dilution analysis (2, 19).

The main feature of the assay described here is the possible automation of amplicon quantitation because of the use of two capture probes. In the present study, we used an automated analyzer which was initially designed for protein quantitation (ES 300; Boehringer). Nevertheless, other analyzers that rely on the interaction between streptavidin and biotin could be used. For example, this assay could be performed with streptavidin-coated microplates and similar automated procedures.

Automated quantitation of both species of amplified products not only greatly simplifies the assay but also enhances the reproducibility and the accuracy of the data. This method provides a good indicator of the level of HCV viremia in chronically HCV-infected patients (data not shown). To our knowledge, no other quantitative RT-PCR is easier to perform than this automated assay. Similar mimic designs coupled with automated quantitation could also be useful for other RNA determinations such as the HIV genome.

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