Novel, Ligation-Dependent PCR Assay for Detection of Hepatitis C Virus in Serum

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A simple, sensitive, and specific ligation-dependent PCR (LD-PCR) method for the detection of hepatitis C virus (HCV) RNA in serum is described. The assay uses two DNA capture probes for RNA isolation and two DNA hemiprobcs for subsequent PCR. Each capture probe has a 3' sequence complementary to the conserved 5' untranslated region of HCV RNA and a biotin moiety at the 5' end capable of interacting with streptavidin-coated paramagnetic beads. Each hemiprobe contains a sequence complementary to the 5' untranslated region in juxtaposition to one another and a common sequence for PCR primer binding. In guanidinium thiocyanate solutions, the capture probes and the hemiprobcs form a hybrid with their target, and the hybrid can be isolated from serum by the binding of the capture probes to the paramagnetic beads in the presence of a magnetic field. The hemiprobcs can then be linked to each other by incubation with T4 DNA ligase to form a full probe that serves as a template for a PCR. When serial 10-fold dilutions of synthetic HCV RNA (10⁷ to 10 molecules) were tested, there was a good correlation between the amount of PCR product and the initial number of RNA molecules, with a sensitivity of 100 HCV RNA molecules per reaction. Twenty-four specimens that had been tested by either a branched DNA probe (bDNA) assay (13 specimens) or a reverse transcription PCR (RT-PCR) assay (11 specimens) were also analyzed by LD-PCR. The results showed a good correlation among LD-PCR, RT-PCR, and the bDNA assay. However, both LD-PCR and RT-PCR were more sensitive than the bDNA assay when the HCV titer was low.

Many nucleic acid amplification techniques have been developed to meet the demands of laboratories for the rapid and accurate detection of infectious pathogens in clinical specimens (1, 21, 24). One such technique, PCR, is widely used in clinical laboratories. PCR allows one to detect infectious agents rapidly and sensitively by directly demonstrating the presence of their nucleic acid sequences in patients' specimens. Moreover, the quantitation of nucleic acids provides a means for monitoring the disease progression and response to therapy.

However, several problems hamper the use of PCR in clinical settings. First, the isolation of nucleic acids, either DNA or RNA, from clinical specimens involves laborious and time-consuming procedures that are impractical for processing large numbers of specimens in a clinical laboratory (4). Second, it is difficult to predict which PCR primers will be efficient for the detection of an organism, and selection usually requires the testing of several sets of primers in order to find a target region that can be efficiently amplified (16). This variability is due to the presence of secondary structures in the target region and to differences in the G+C contents and sequences among possible primer pairs (17). Third, it is difficult to detect multiple targets in a single reaction (multiplex PCR), since the efficiency of different primers varies. A small difference in priming efficiency will lead to a competition among primer pairs for substrates in a multiplex PCR (10). Therefore, a target sequence may not be fully and proportionally amplified in the presence of another target sequence. Similarly, quantitative measurements in a multiplex PCR are not likely to reflect the true titers

of different targets. Fourth, the species of an organism or a strain based on a single nucleotide difference is difficult to determine by target-directed PCR and usually requires target sequencing after PCR amplification.

We have developed a ligation-dependent PCR (LD-PCR) that addresses these problems. This system uses paramagnetic beads to simplify the target RNA isolation procedure and to facilitate the removal of PCR inhibitors that may be present in specimens. Target-specific assembly of nonamplifiable hemiprobcs into amplifiable functional probes by incubation with a DNA ligase increases assay specificity and allows discrimination among organisms on the basis of single nucleotide differences. Furthermore, the use of the same generic sequence as PCR primers to amplify all target sequences yields consistent results among different targets and allows uniform PCR conditions to be used (buffer condition, primer concentration, and temperature profile). This increases the accuracy and quantifiability of the multiplex PCR. In this report, we demonstrate the principle of the LD-PCR assay by describing a study in which we used hemiprobcs that are specific for HCV RNA in patients' specimens.

MATERIALS AND METHODS

Clinical specimens. Twenty-four serum specimens were obtained from the Molecular Diagnostic Laboratory of the Department of Pathology and the Liver Transplant Laboratory of the Department of Surgery at Mount Sinai Medical Center in New York City. Of the 24 specimens, 11 were submitted to the Molecular Diagnostic Laboratory for hepatitis C virus (HCV) testing by a reverse transcription PCR (RT-PCR) assay, and 13 were submitted to the Liver Transplant Laboratory for HCV testing by a branched DNA (bDNA) nucleic acid hybridization assay. All serum specimens were stored at -70°C until use.

RT-PCR and bDNA assays. HCV RNA was isolated from 200 µl of serum by the acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction method described by Chomczynski and Sacchi (5). The purified RNA was resuspended in 100 µl of TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA). RT-PCR was

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performed by a modification of the method of Dilworth et al. (7a) by using Gene Amp RNA PCR kits (Perkin-Elmer, Norwalk, Conn.). RT and the first PCR were performed in 100 μ l of solution containing 50 μ l of HCV RNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 mM dCTP, 8 U of RNase inhibitor, 50 μ M murine leukemia virus reverse transcriptase, 2.5 U of *Taq* polymerase, 0.4 μ M outer primer pair NF5 (sense; 5'-GTGAGGAAGTACTGTCTTCACGCAG-3'; positions -295 to -271 [2]) and NR5 (antisense; 5'-TGCTCATGGTGCACGGTCTACGAGA-3'; positions 7 to -18). RT was carried out by incubating the reaction mixtures at 42°C for 15 min; this was followed by inactivation of the reverse transcriptase by incubating the mixture at 99°C for 5 min and then cooling it to 5°C for 5 min. The first PCR was carried out for 35 temperature cycles of 95°C for 15 s and 60°C for 30 s in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer). The PCR was completed by chain extension at 72°C for 7 min. For the second PCR amplification, 5 μ l of the PCR products from the first amplification was added to a 45- μ l mixture containing the same components as the first PCR except for the primers, which were 0.2 μ M inner primer pair NF2 (sense; 5'-TTCACGCAGA AAGCGTCTAG-3'; positions -279 to -260) and NR4 (antisense; 5'-CTATCA GGCAGTACCACAAGG-3'; -43 to -63). The same temperature profile used for the first PCR was used for the second PCR. A total of 20 μ l of the final PCR products was analyzed by electrophoresis through a 2% agarose gel. The bDNA assay was performed at the Division of Liver Diseases according to the manufacturer's instruction (Quantiplex HCV-RNA assay; Chiron Corporation, Emeryville, Calif.) (22, 23).

Preparation of synthetic HCV RNA transcripts. Synthetic HCV RNA was derived from a plasmid containing the 5' untranslated region (5'-UTR) of the HCV genome. Plasmid HCV-324 was generously provided by Chao-Hung Lee of the Indiana University School of Medicine (14). We selected the target sequence from the 5'-UTR of the HCV genome, since this nucleotide sequence is highly conserved among HCV isolates (14, 18) and since PCR of this region has been proven to be more sensitive and specific than PCR of other regions of the HCV genome (11, 19). A 271-nucleotide DNA fragment containing the HCV 5'-UTR (positions -324 to -74 [2]) was generated from plasmid HCV-324 by PCR with primers PsPcrPm1 (sense; 5'-GACTAATACGACTCACTATAGGCGACACT CCACCAT-3'; positions -324 to -309) and PsPcrPm2 (antisense; 5'-CCCAAC ACTACTCGGCTA-3'; positions -74 to -91). PsPcrPm1 contained a bacteriophage T7 promoter sequence (underlined region) at its 5' end. The T7 promoter sequence was incorporated into each DNA product as a result of PCR. The PCR was carried out in 50 μ l containing 1.0 ng of plasmid HCV-324, 0.1 μ M (each) primer (PsPcrPm1 and PsPcrPm2), 1.5 U of *Taq* DNA polymerase, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. The reaction mixture was incubated at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min for 35 cycles. A 5- μ l aliquot of the reaction mixture was analyzed by electrophoresis through a 6% polyacrylamide gel, and the DNA product in the sample was visualized by staining the gel with ethidium bromide. The DNA concentration was determined by comparing the density of the band with the densities of DNA markers of known concentration (pBR322 digested with *Msp*I; New England Biolabs). The DNA fragments were then purified by phenol-chloroform-isoamyl alcohol extraction; this was followed by precipitation with ethanol. The DNA fragments were then suspended in TE buffer to a final concentration of 50 ng/ μ l.

HCV RNA transcripts were prepared by incubating 0.1 μ g of purified PCR DNA product with 200 U of T7 RNA polymerase (New England Biolabs) in 100 μ l of 40 mM Tris-HCl (pH 8.0)-20 mM MgCl₂-5 mM dithiothreitol-1 mM spermidine-0.05 μ g of bovine serum albumin, 25 U of RNasin (Boehringer Mannheim)-2 mM ATP-2 mM UTP or 0.2 mM [α -³²P]CTP (New England Nuclear)-2 mM GTP-2 mM CTP for 1 h at 37°C. A total of 15 U of ribonuclease-free DNase I (Boehringer Mannheim) was then added to the reaction mixture, and the mixture was incubated at 37°C for 15 min to digest the template DNA. A 2- μ l aliquot was removed from the reaction mixture and was diluted with 18 μ l of gel loading buffer (0.86 ml of formamide per ml, 100 mM Tris-borate [pH 8.3], 3 mM EDTA, 0.4 mg of bromophenol blue per ml, 0.4 mg of xylene cyanol per ml). The sizes and homogeneities of the RNA transcripts were determined by electrophoresis through a 6% polyacrylamide gel (containing 8 M urea) that was visualized by staining with ethidium bromide or autoradiography. A total of 2 μ l of diluted sample was blotted onto a 3MM filter paper disk (Whatmann), the RNA was precipitated on the filter paper under vacuum, and unincorporated [α -³²P]CTP was removed by washing the filter with 30 ml of ice-cold precipitation solution (300 mM phosphoric acid, 20 mM sodium PP_i, 1 mM EDTA). The amount of [α -³²P]RNA present in each sample was determined by measuring the radioactivity of each filter in an LKB 1209 Rackbeta scintillation counter in the presence of 3 ml of Aquasol-2 scintillation fluid (New England Nuclear). The transcripts were then purified by phenol-chloroform-isoamyl alcohol extraction; this was followed by precipitation with ethanol. The transcript was resuspended in TE buffer to a final concentration of 10¹¹ molecules per μ l. Six dilutions containing 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10², and 10 molecules of HCV target RNA per μ l were prepared.

LD-PCR assay. Hemiprobe 1 was pretreated by phosphorylation of the 5'-hydroxyl group for a subsequent ligation reaction. The phosphorylation reaction was carried out by incubating 10¹³ molecules of hemiprobe 1 in a 10- μ l reaction mixture containing 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM dithiothreitol, 10 mM ATP, and 10 U of T4 polynucleotide kinase (New England

Biolabs). After a 1-h incubation at 37°C, the reaction mixture was heated at 100°C for 5 min to inactivate the enzyme. The phosphorylated probe was diluted to a final concentration of 10¹¹ molecules per μ l for subsequent use.

The LD-PCR assay was started by adding 180 μ l of serum to concentrated lysis buffer (prepared by condensing 250 μ l of the lysis solution containing 5 M GTC [Fluka], 0.5% bovine serum albumin [Sigma], 80 mM EDTA, 400 mM Tris-HCl [pH 7.5], and 0.5% Nonidet P-40 [Sigma] overnight on a hot plate at 65°C) to give a final GTC concentration of 5 M. The serum and lysis buffer were mixed well by repeated pipetting and vortexing. The mixture was incubated for 1 h at 37°C to release the target RNA from HCV particles. A total of 80 μ l of the lysis mixture was then transferred to 120 μ l of hybridization buffer (0.5% bovine serum albumin, 80 mM EDTA, 400 mM Tris-HCl [pH 7.5], and 0.5% Nonidet P-40), which contained 10¹⁰ molecules of each hemiprobe (hemiprobe 1 and 2) and 10¹¹ molecules of each capture probe (capture probes 1 and 2). The addition of the hybridization buffer reduced the GTC concentration from 5 to 2 M to allow the hybridization to occur. This mixture was incubated for 1 h to allow the formation of hybrids. Hybrids consisted of two DNA capture probes and two DNA hemiprobe bound to their RNA targets. A total of 30 μ l of streptavidin-coated paramagnetic beads (Promega) was then added to the mixture, and the mixture was incubated at 37°C for 20 min to allow the hybrids to bind to the surfaces of the beads. The beads were then washed with 150 μ l of 2 M GTC to remove nonhybridized probes, as well as proteins, nucleic acids, and any potential PCR inhibitors. GTC was then removed by washing twice with 150 μ l of ligase buffer (66 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 1 mM ATP, 0.5% Nonidet P-40, 1 mM MnCl₂). During each wash, the beads were drawn to the wall of the assay tube by placing the tube on a magnetic separation stand (Promega), enabling the supernatant to be removed by aspiration. The hybrids were then resuspended in 20 μ l of ligase solution (66 mM Tris HCl [pH 7.5], 1 mM dithiothreitol, 1 mM ATP, 1 mM MnCl₂, 5 U of T4 DNA ligase [Boehringer Mannheim]), and the mixture was incubated at 37°C for 1 h to covalently link the hemiprobe that are hybridized to adjacent positions on the RNA target. A total of 10 μ l of the ligation reaction mixture (including beads) was then transferred to 20 μ l of a PCR mixture containing 0.06 μ M PCR primer 1 and 0.06 μ M PCR primer 2, 1.5 U of *Taq* DNA polymerase, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. The first PCR mixture was incubated at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles. After the first PCR, 5 μ l of each reaction mixture was transferred to 30 μ l of a second PCR mixture (seminested PCR) containing the same components as the first PCR mixture except that 0.66 μ M PCR primer 1 and 0.66 μ M PCR primer 3 were used. The second PCR was performed by the same protocol as the first PCR. Ten microliters of the second PCR mixture was analyzed by electrophoresis through a 6% polyacrylamide gel, and the products were visualized by UV fluorescence after staining with ethidium bromide.

RESULTS

Design of the LD-PCR assay. The LD-PCR assay used two DNA capture probes for the isolation of HCV RNA-hemiprobe hybrids (Fig. 1). Each capture probe had a 3' hybridization region of 41 nucleotides complementary to the target RNA, a 5' biotin moiety, and a four-nucleotide linker between the two regions (Table 1). In the presence of 5 M GTC, a chaotropic agent, HCV RNA was released from the viral particles. The GTC concentration was then reduced to 2 M and the HCV RNA was hybridized to the hemiprobe and capture probes. The hybrids were captured onto the paramagnetic beads through the 3' regions of the capture probes that hybridize to the target and their 5' biotin moieties that bind to streptavidin coated on the surfaces of the beads. Two separate capture probes were used to increase the efficiency of capture. They hybridized to the target RNA on both sides of the sequence to which the hemiprobe was bound (Fig. 1). In the presence of a magnetic field provided by a magnetic separation stand, the HCV RNA-hemiprobe hybrids were drawn to the side of the assay tube. Unhybridized probes and other components, including all proteins and nontarget nucleic acids, were removed by aspiration of the supernatant (Fig. 2). Further washing with 2 M GTC completed the removal of unbound components. Washing twice with ligase buffer removed residual GTC, which could potentially inhibit subsequent ligation and PCR.

The unique approach of the assay was the assembly of an amplifiable full probe from nonamplifiable hemiprobe in a target-specific manner (Fig. 1). In our design, amplifiable full

FIG. 1. Schematic representation of the capture of HCV RNA and hemiprobcs. The HCV RNA is captured on paramagnetic beads (a) through the binding of the biotin moiety (c) of a capture probe (d) to streptavidin (b) coated on the beads. Hemiprobe 1 and hemiprobe 2 are also captured by binding to the HCV RNA through complementary regions (g and h). Then, hemiprobe 1 and hemiprobe 2 are covalently linked together by incubation with T4 DNA ligase to form a full probe. Sequences at noncomplementary regions (f and i) of hemiprobe 1 and 2 are generic for the binding of PCR primers.

probes were divided into two separate hemiprobcs in the middle of the target hybridization region. Hemiprobe 1 and hemiprobe 2 contained a 21-nucleotide and a 39-nucleotide PCR primer hybridization region, respectively (Table 1). Both hemiprobcs also contained a 30-nucleotide target hybridization region. Neither hemiprobe by itself could be amplified exponentially, because each of the PCR primers bound to only one hemiprobe. When these hemiprobcs were hybridized to their target RNAs, the 5' end of hemiprobe 1 was brought into juxtaposition with the 3' end of hemiprobe 2. Incubation of this hybrid with T4 DNA ligase covalently linked the two probes in a target-specific manner, generating a full probe. After the ligation of the hemiprobcs, the full probes were amplified by PCR by using PCR primer 1 and PCR primer 2 to generate a 120-bp PCR product (Table 1). These products were amplified further with PCR primer 1 and PCR primer 3 (i.e., seminested PCR) to generate a 102-bp fragment. Seminested PCR increased the sensitivity of the assay. With magnetic hybrid isolation, target-specific ligation, and powerful PCR amplification, the assay generated a signal with extreme simplicity, specificity, and sensitivity.

Efficiency of capturing the target RNA and hemiprobcs with paramagnetic beads. The ability to form an HCV RNA-capture probe hybrid in the presence of GTC was analyzed by using ³²P-labeled target RNA. Initially, we tested the formation of the hybrids in the presence of various concentrations of GTC (1 to 5 M) and observed that 2 M GTC allowed the best formation of hybrids. Subsequent hybridization reactions were carried out in the presence of 2 M GTC. The efficiency of capturing ³²P-labeled HCV RNA was determined by measuring the radioactivity remaining on the paramagnetic beads

after each wash. The results showed that in the absence of capture probes, 99.6% of the counts were removed after washing with 2 M GTC, and further washing with ligase buffer removed the remaining counts (Table 2). These results indicate that washing was extremely efficient and eliminated almost all components present in the hybridization reaction. Our results also showed that HCV RNA was efficiently captured on the paramagnetic beads, even after extensive washing. A total of 25.7% of the ³²P-labeled HCV RNA was retained on the paramagnetic beads by using capture probe 1, 35.8% was retained by using capture probe 2, and 41.5% was retained by using capture probes 1 and 2. These results indicate that the use of two capture probes is more efficient than the use of a single capture probe. Further analysis of the efficiency of capturing hemiprobcs by using ³²P-labeled hemiprobe 2 showed that of the 41.5% HCV RNA that was captured, 60% of the RNA formed hybrids with the hemiprobcs and was retained on the paramagnetic beads after stringent washings.

Ligation of the hemiprobcs with T4 DNA ligase. Double-stranded DNA is the natural substrate of T4 DNA ligase, and DNA-RNA hybrids are poor substrates (9). In order to overcome this inefficiency, we replaced Mg²⁺ with Mn²⁺ as a divalent ion for the enzyme. The hybridization reaction was carried out in the presence of 10¹⁰ molecules of HCV RNA, 10¹¹ molecules of hemiprobe 1, 10¹¹ molecules of ³²P-labeled hemiprobe 2, and 10¹² molecules of each capture probe. A large number of HCV RNA molecules was used for hybridization to ensure the visibility of signals by autoradiography. After three washes, the hybrids were incubated with T4 DNA ligase at various concentrations of MnCl₂. Figure 3 shows that there was a slight increase in the amount of ligated full probe with

TABLE 1. Sequences of PCR primers, capture probes, and hemiprobcs

Probes (no. of nucleotides) ^a	Sequence (5' to 3') ^b
PCR primer 1 (18).....	GTTAGCAGATACACAGAC (sense)
PCR primer 2 (18).....	CAAGAGCAACTACACGAA (antisense)
PCR primer 3 (18).....	TTCTCGATTAGGTTACTG (antisense)
Capture probe 1 (45).....	Biotin-AAGAGCGTGAAGACAGTAGTTCCTCACAGGGGAGTGATTCATGGT
Capture probe 2 (45).....	Biotin-AAGACCCCAACTACTCGGCTAGCAGTCTTGCGGGGACGCCCA
Hemiprobe 1 (51)	ACTCACCAGTTCGCGAGACCACTATGGCTCGTTGTCCTGTATCTGCTAAC
Hemiprobe 2 (69)	CAAGAGCAACTACACGAA TTCTCGATTAGGTTACTG CAGAGGACCCGGTCGTCCTGGCAATTCGGTGT
Full probe (120)	CAAGAGCAACTACACGAA TTCTCGATTAGGTTACTG CAGAGGACCCGGTCGTCCTGGCAATTCGGTGT ACTCACCAGTTCGCGAGACCACTATGGCTCGTTGTCCTGTATCTGCTAAC

^a PCR primer 1 and PCR primer 2 were used for the first PCR with ligated full probes, and PCR primer 1 and PCR primer 3 were used for the second PCR.
^b Underscores indicate sequences complementary to the 5'-UTR of HCV, outlined letters indicate the binding regions for PCR primer 1, boldface letters indicate the binding region for PCR primer 2, and italic letters indicate the binding region for PCR primer 3.

FIG. 2. Schematic representation of magnetic isolation, target-specific ligation, and PCR amplification, leading to the detection of HCV RNA. A patient's serum is mixed with lysis buffer to release HCV RNA. Hemiprobe, capture probes, and paramagnetic beads are added to the lysis mixture to allow the formation of a hybrid composed of HCV RNA, hemiprobe, and capture probes. The hybrid is captured on a paramagnetic bead, allowing extensive washing to remove unbound hemiprobe and other components of the serum. The hemiprobe aligned on the HCV target is linked together by incubation with T4 DNA ligase. PCR amplification is then carried out by the addition of primers and *Taq* polymerase.

the decrease in $MnCl_2$ concentrations, with an optimal concentration of 1 mM, with which ligation was 60% efficient (Fig. 3, lane B). Subsequent PCR amplification of the ligation products confirmed that maximal production of PCR product oc-

TABLE 2. Efficiency of capturing HCV RNA

Capture probe ^a	Count (cpm)				% Retained ^b
	Total ^c	2 M GTC ^d	First ligase buffer ^e	Second ligase buffer ^e	
1	533,124	17,252	10,853	9,984	25.7
2	536,095	21,843	14,991	13,991	35.8
1 and 2	536,853	24,092	17,096	16,261	41.5
None	535,781	3,360	515	235	0.6

^a ^{32}P -labeled HCV RNA transcripts were incubated in a hybridization reaction in the presence of capture probe 1, capture probe 2, or both capture probes or in the absence of capture probes.

^b Efficiency of capturing HCV RNA transcripts on paramagnetic beads was determined by calculating the percentage of the counts per minute incorporated into HCV RNA transcripts that were still present after the second wash with ligase buffer.

^c Total counts per minute added to each hybridization reaction. A total of 7.28% counts were incorporated into HCV RNA transcripts.

^d Counts per minute retained on paramagnetic beads after washing with 2 M GTC.

^e Counts per minute retained on paramagnetic beads after the first and second washes with ligase buffer.

curred with 1 mM $MnCl_2$. In the absence of $MnCl_2$, no full-sized probes were observed (Fig. 3, lane A), indicating that divalent ion is required for ligation. Furthermore, when no target RNA was present in the hybridization reaction, neither hemiprobe 1 nor hemiprobe 2 was retained on the paramagnetic beads (Fig. 3, lane F). These results confirmed that through successive washes with GTC solution and ligase buffer, HCV RNA was retained and so were the hybridized hemiprobe. The results also indicated that in the presence of $MnCl_2$, the DNA-RNA hybrid can be used as a substrate for T4 DNA ligase and can efficiently link two hemiprobe when they are properly aligned on the target RNA.

Sensitivity of the LD-PCR assay. The sensitivity of the LD-PCR assay was determined by amplifying 10-fold serial dilutions of synthetic HCV RNA in HCV-negative serum (the amount of HCV RNA ranged from 10^7 to 10 molecules). Figure 4 shows that in the absence of target RNA, there was no background signal. This finding demonstrates the specificity of the assay and confirms that target RNA must be present for hemiprobe to be captured. By seminested PCR, as few as 100 molecules of HCV RNA could be detected in each reaction mixture, indicating that the sensitivity of LD-PCR is comparable to that of conventional RT-PCR (7). Furthermore, there was a decrement in the density of the expected bands on the

FIG. 3. Autoradiograph showing the ligation of hemiprobe to form full probe after incubation with T4 DNA ligase. A total of 10^{10} molecules of HCV RNA targets were used to initiate hybridization in the presence of 10^{11} molecules of hemiprobe 1 and 10^{11} molecules of ^{32}P -labeled hemiprobe 2. After three washes, the hybrids were incubated in ligation buffer in the presence of T4 DNA ligase. Lanes A through E, hybridization in the presence of 0, 1, 2, 3, and 4 mM $MnCl_2$, respectively; lane F, no HCV RNA present in the hybridization reaction.

FIG. 4. LD-PCR detection of various amounts of HCV RNA (the number of HCV RNA molecules is listed above each lane). HCV RNA transcripts were serially diluted (from 10^7 to 10 molecules per reaction mixture) in HCV-negative serum. Capture, ligation, and amplification were performed, and the LD-PCR products were examined by electrophoresis through a 6% polyacrylamide gel. The results show that as few as 100 molecules of HCV RNA can be detected in a sample. Furthermore, there is a decrement in the density of the product band which was proportional to the decrease in the number of HCV RNA molecules that were present in each sample.

polyacrylamide gel which was proportional to the concentration of the target RNA in each sample, indicating that the amount of PCR product synthesized depends upon the initial target concentration. The results also indicate that the paramagnetic beads did not interfere with the ligation and PCR steps. Therefore, all ligation reactions and PCR amplifications were carried out in the presence of paramagnetic beads.

Detection of HCV RNA in clinical specimens. A total of 24 serum specimens were tested for the presence of HCV RNA by the LD-PCR assay; 11 of the specimens had been tested by RT-PCR (9 positive and 2 negative specimens) and 13 of the specimens had been tested by the bDNA method (13 positive specimens) (Table 3). All nine RT-PCR-positive specimens tested positive by LD-PCR and 2 RT-PCR-negative specimens were negative by LD-PCR, indicating a good correlation between the two PCR tests. Six of the 11 specimens tested by RT-PCR had previously been tested by the bDNA assay; 3 of the specimens were weakly positive (ranging from 5.31×10^5 to 81.73×10^5 eq/ml) and 3 were negative (below 3.5×10^5 eq/ml). These six specimens tested by the bDNA assay were later sent to the Molecular Diagnostic Laboratory for further confirmation by the RT-PCR test. All three bDNA assay-positive specimens tested positive by RT-PCR as well as by LD-PCR. One of the three bDNA assay-negative specimens was also negative by both PCR tests. However, the other two bDNA assay-negative specimens tested positive by both PCR tests, indicating that the bDNA test gave a false-negative result. This is probably due to the low level of sensitivity of the bDNA assay (cutoff value, 3.5×10^5 eq/ml).

TABLE 3. Comparison of LD-PCR with RT-PCR and the bDNA assay

Test (no. of specimens)	No. of specimens					
	LD-PCR		bDNA		RT-PCR	
	+	-	+	-	+	-
RT-PCR (11)	9	2	— ^a	—	9	2
bDNA assay (13)	13	0	13	0	ND ^b	ND

^a —, of 11 specimens submitted for RT-PCR testing, 6 were previously tested by the bDNA assay, with 3 testing weakly positive and 3 testing negative. RT-PCR as well as LD-PCR confirmed the three positive specimens. However, of the three bDNA assay-negative specimens, two tested positive by RT-PCR and LD-PCR and one tested negative by both PCR tests, indicating that the two bDNA tests gave false-negative results.

^b ND, not done.

FIG. 5. Detection of HCV RNA in clinical specimens. The LD-PCR products amplified from five positive serum specimens (lanes 1 to 5, respectively) are shown. All specimens show the expected band (102 bp in length) on the polyacrylamide gel. The low band density of the specimen in lane 4 may represent a low HCV RNA titer.

All 13 bDNA assay-positive specimens also tested positive by LD-PCR (Table 3). Since the bDNA assay-positive specimens were strongly positive (ranging from 238×10^5 to 620×10^5 eq/ml), no confirmatory RT-PCR test was performed on these specimens. These results show that there is a good correlation among LD-PCR, RT-PCR, and the bDNA assay. However, the correlation between both PCR tests and the bDNA assay becomes poor when the number of HCV RNA in the sample approaches the cutoff value (3.5×10^5 eq/ml) of the bDNA assay. These results suggest that both LD-PCR and RT-PCR are more sensitive than the bDNA assay. Figure 5 shows an example of the LD-PCR results for five clinical specimens.

DISCUSSION

We have described a novel strategy that combines magnetic isolation of RNA-hemiprobe hybrids, target-specific ligation of hemiprobe, and PCR amplification of full probes. One-step isolation of HCV RNA with paramagnetic beads offers several advantages. First, dissolution of serum in 5 M GTC lyses viral particles, denatures proteins, inactivates nucleases, and relaxes RNA secondary structures. Subsequent reduction of the GTC concentration to 2 M promotes the rapid formation of hybrids between target RNA and probes. Second, the capture of RNA-probe hybrids on paramagnetic beads allows for extensive washing to remove all other components in serum that may inhibit ligation and PCR (3). In addition, removal of nontarget nucleic acids present in the specimen (e.g., serum and tissue) and unbound hemiprobe significantly reduces the probability of false-positive signals. This is especially important for the detection of low genome copy numbers in the presence of a large amount of nonspecific nucleic acid (12, 20). Although the capture of HCV RNA was only 41% efficient, LD-PCR was able to detect as few as 100 molecules of HCV RNA per reaction mixture, indicating that the sensitivity of the assay was not compromised. Third, the use of magnetic isolation eliminates complicated, laborious RNA extraction procedures and enables the processing of a large number of specimens in a clinical laboratory setting (8). Furthermore, in the future, automation of specimen handling will enable the processing of multiple specimens simultaneously, thus reducing the variability due to the handling of each specimen and increasing the reliability of quantitative assays. Lastly, since paramagnetic beads do not inhibit the enzymatic reactions, the entire assay (from specimen processing to PCR amplification to detection) can be carried out in the same vessel in a contained environment, thus eliminating the possibility of contaminating other samples (8).

The target-specific assembly of amplifiable DNA from nonamplifiable probes by ligation significantly increases assay

specificity, since the 5' end of one hemiprobe must align perfectly with the 3' end of the other hemiprobe on a target DNA or RNA in order for ligation to occur (13). Despite the presence of a DNA-RNA hybrid instead of the natural DNA-DNA substrate for T4 DNA ligase, our results demonstrate that improved ligation efficiency can be achieved by replacing $MgCl_2$ with $MnCl_2$ in the ligation reaction. Significantly, the use of T4 DNA ligase eliminates the RT step for the detection of an RNA target in PCR, thereby avoiding problems related to reverse transcriptase, such as interference with cDNA synthesis due to the presence of secondary structures in the RNA and stimulation of primer-dimer formation by reverse transcriptase (6). The possibility that nonspecific ligation will generate false-positive signals is not a concern, since our results show that no PCR product was generated in the absence of target. In order for ligation to occur, both hemiprobcs must hybridize with perfect matches in juxtaposition to each other (13). This stringent requirement makes detection of a single nucleotide mutation possible. The essence of the LD-PCR assay is that it combines the specificity of ligation-dependent gene detection (13) and the powerful amplification ability of PCR. This combination compensates for the inadequate sensitivity of the direct ligation-dependent detection method and the low level of specificity of the ligase chain reaction.

A significant advantage of using a generic amplifiable probe as a PCR template in the LD-PCR over target-directed PCR is that the primer binding region is common to all probes, irrespective of the embedded target binding sequences. Since PCR efficiency is largely primer dependent (12, 20), the generic primer binding region allows us to arbitrarily choose a primer sequence that achieves a maximal PCR efficiency. Careful selection of the primer binding sequence eliminates potential interference from secondary structures, optimizes the G+C content, reduces primer-dimer formation, and allows the reaction conditions (i.e., temperature profile, buffer components, and primer concentration) to be optimized. Once the optimal sequence has been established, it can be used with all probes. This is a fundamental improvement in PCR primer design. Conventionally, the testing of a number of different primer pairs is required for the selection of an efficient pair of primers because of the limitations imposed by the choice of target sequence (2, 19). Furthermore, the use of generic binding regions allows for the coamplification of different targets with the same primer pair in a single combined assay mixture (e.g., multiplex LD-PCR). Since the size of the probes and the size of the PCR primers is the same, PCR efficiencies for different probes, regardless of the targets, are the same. As a consequence, competition among different primer pairs, as seen in target-directed PCR, is eliminated. The number of different probes produced is directly proportional to the initial number of specific targets. After PCR amplification, each amplified probe can be sorted out by hybridizing a signature probe to the sequence embedded in each probe that uniquely identifies its target. The use of multiplex LD-PCR to detect human immunodeficiency virus (HIV) and HCV in a single reaction vessel is under development.

In the same multiplex LD-PCR format, use of the generic probe sequence also offers a simple method for competitive quantitation of HCV RNA. In addition to the patient's sample and HCV-specific capture probes and hemiprobcs, known amounts of HIV transcripts, HIV-specific capture probes, and hemiprobcs (which serve as competitors for competitive quantitation) can be added to the hybridization mixture. After capture and ligation, both the HIV-specific probes and the HCV-specific probes are coamplified proportionally with the same pair of PCR primers. The amount of HCV RNA present in the

patient's sample can be determined on the basis of the known amount of HIV transcript present. Therefore, with generic probe sequences, it is not necessary to construct each plasmid encoding specific competitive RNA, which is laborious (10). Consequently, the false signal obtained as a result of the incomplete digestion of the plasmid DNA can be avoided (15).

In summary, the LD-PCR assay combines all of the advantages of magnetic RNA isolation, target-dependent ligation, and powerful PCR amplification. Therefore, it offers an extremely simple, sensitive, and specific assay system that can readily be adopted for use in a clinical laboratory.

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