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Detection of Hepatitis C Virus RNA: Comparison of One-Stage Polymerase Chain Reaction (PCR) with Nested-Set PCR

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We evaluated a new hepatitis C virus RNA assay based on one-stage PCR followed by liquid hybridization with an oligonucleotide probe and compared it with nested-set PCR. The one-stage and nested-set PCR assays had identical sensitivities in analytical experiments and showed 100% concordance when clinical specimens were used. One-stage PCR may be less prone to contamination than nested-set PCR.

The RNA polymerase chain reaction (PCR) assay has been used to identify persons with hepatitis C at greatest risk for developing direct complications of this infection (6, 16, 17). To increase assay sensitivity, several groups have used the "nested-set" approach to PCR for hepatitis C virus (HCV) RNA, in which first-round amplification products are subjected to a second round of PCR with an internal set of oligonucleotide primers, greatly increasing the amount of product DNA to enable detection by ethidium bromide staining (1, 2, 4, 6, 9–13). A pitfall of nested-set PCR is that the second-round PCR is set up in the presence of first-round PCR amplification products, which increases the risks of assay contamination by product DNA. An alternative approach to nested-set PCR is the use of one-stage PCR amplification combined with sensitive detection methods, such as Southern blot (8, 17) or liquid hybridization with radioactive oligonucleotide probes (7, 16). We therefore compared the analytical and clinical sensitivities of a one-stage PCR assay for HCV RNA with the nested-set PCR assay by using a battery of specimens obtained either from patients with suspected hepatitis C or from our hepatitis serology laboratory.

MATERIALS AND METHODS

Sera from patients evaluated for suspected hepatitis C at our university-based hepatitis clinic were selected for study. We also analyzed 20 consecutive HCV-seropositive specimens and 20 random seronegative blood donor specimens obtained from the Puget Sound Blood Center, Seattle, Wash.

Figure 1A is a schematic of the HCV RNA genome with expansion of the highly conserved 5′ noncoding region. We previously described the sensitivity and specificity of our one-stage PCR (Fig. 1A, primers 1 and 2) plus liquid hybridization assay for HCV RNA in our Diagnostic Virology Laboratory (7). Nested-set PCR (second-round amplification) was performed by using the internal primer pair 33 and 48 (Fig. 1A, primers 3 and 4) described by Okamato et al. (10). Detection of PCR amplification products by either liquid hybridization (7) or Southern blot (14, 15) with HCV-specific ^{32}P-labeled oligonucleotide probe Alx89 (8) has been described. Strict precautions were taken to avoid contamination by PCR products (7).

The analytical sensitivities of one-stage PCR and nested-set PCR were compared by serial-dilution assays. HCV cDNAs were synthesized from 100 μl of sera obtained from patients with documented active hepatitis C and were subjected to either 10-fold or 2-fold serial dilution with 1× cDNA assay buffer (50 mM Tris-HCl [pH 7.5], 75 mM KCl, 3 mM $MgCl_2$, 1 mM 4× deoxynucleoside triphosphate mix, 6 pmol of primer JHC51 [8] per μl). The cDNA dilutions were subjected to sequential one-stage and nested-set PCR amplifications. Amplification products were analyzed by the liquid hybridization technique or by Southern blot. Quantitation of cDNAs was estimated by fluorometry with the DNA-binding fluorochrome H33258 (3) and recombinant single-strand M13 DNA as the standard.

RESULTS

Figure 1B demonstrates one-stage and nested-set PCR amplification of HCV cDNAs, analyzed by agarose gel electrophoresis plus ethidium bromide staining. The cDNA template was either undiluted or diluted 10-fold prior to amplification. Lanes 4 and 5 demonstrate nested-set (second-round) PCR amplification of the first-round product DNAs shown in lanes 2 and 3, respectively. Following nested-set PCR, strong positive results were observed for both the undiluted (lane 4) and diluted (lane 5) cDNA templates.

We compared the analytical sensitivity of one-stage PCR with that of nested-set PCR by using serially diluted HCV cDNAs as the template and either ethidium bromide staining, liquid hybridization, or Southern blot as the detection method (Fig. 2). HCV cDNAs were quantitated, subjected to serial twofold dilutions, and sequentially amplified by one-stage and nested-set PCR. Nested-set PCR was clearly more sensitive than one-stage PCR when ethidium bromide staining was used as the detection method (Fig. 2A). However, when either liquid hybridization (Fig. 2B) or Southern blot (Fig. 2C) was used as the detection method, the analytical sensitivity of one-stage PCR was identical to that of nested-

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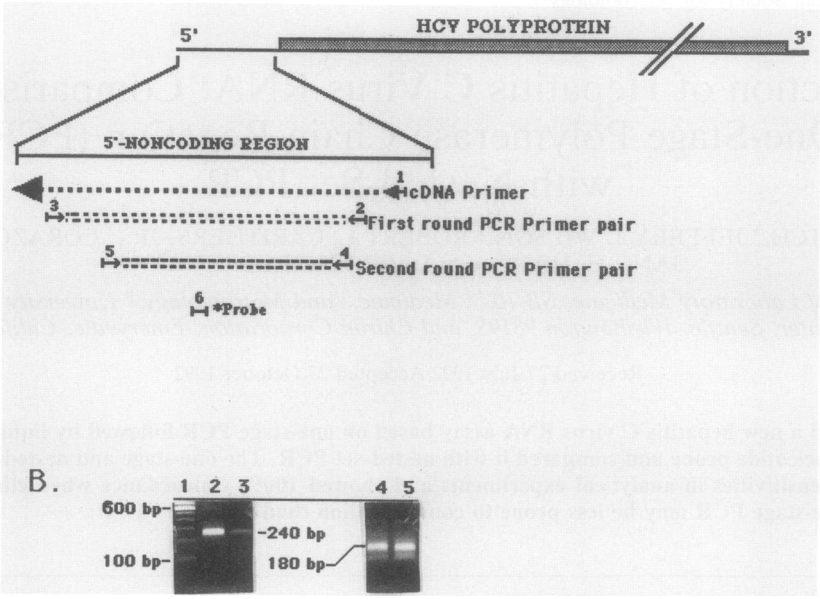

FIG. 1. Amplification of genomic sequences from the 5′ noncoding region of HCV by either one-stage or two-stage (nested-set) PCR. (A) Diagram of the HCV RNA genome with enlargement of the highly conserved 5′ noncoding region. The approximate locations of oligonucleotide primers used for cDNA synthesis (JHC51; arrow 1), first-round PCR amplification (JHC52 and JHC93; arrows 2 and 3), second-round PCR amplification (33 and 48; arrows 4 and 5), and the oligonucleotide probe (Alx89; arrow 6) are shown. (B) Agarose gel electrophoresis and ethidium bromide staining of one-stage and nested-set PCR amplification products derived from HCV cDNAs as described in the text. Lanes: 1, DNA size standards; 2 and 3, first-round PCR amplification products; 4 and 5, second-round PCR amplification products. Approximate sizes of DNA standards and first-round and second-round PCR amplification products are given in base pairs.

set PCR. The results were repeated in independent experiments with sera from four different patients. The doublet observed following liquid hybridization (Fig. 2B) is an artifact occasionally observed with this method; it was not observed following Southern blot analysis of the same PCR products (Fig. 2C).

The analytical sensitivities of the two methods can be best interpreted as 10 molecules, because of the stochastic variation which occurs when quantitating small numbers of molecules. These sensitivities were confirmed by using purified synthetic HCV RNA as the template (data not shown). Thus, both one-stage PCR plus liquid hybridization and nested-set PCR allow detection of HCV cDNAs or synthetic HCV RNA templates near the theoretical limits of analytical sensitivity.

We next compared the sensitivity of our one-stage PCR assay with that of nested-set PCR by using the following clinical specimens: serum samples from 20 consecutive hepatitis clinic patients, 20 random anti-HCV positive specimens, and 20 random anti-HCV-negative blood donor specimens. cDNAs synthesized from all sera were amplified sequentially by both one-stage PCR and nested-set PCR, and amplification products were analyzed by liquid hybridization. We observed 100% concordance between the results of the one-stage PCR and nested-set PCR assays for the clinical specimens we tested.

We tested contamination rates in our one-stage and nested-set PCR assays as follows. We intermixed 18 high-titer-positive HCV cDNAs with 72 negative specimens containing buffer plus *Taq* polymerase. The specimens were amplified by sequential one-stage and nested-set PCR and analyzed. No contamination events were observed when ethidium bromide staining was used as the detection method. However, when liquid hybridization was used, 3 of the 72 negative samples (4%) showed evidence of contamination following nested-set PCR only.

DISCUSSION

It has been proposed that nested-set priming is essential for clinical implementation of the RNA PCR assay for HCV because of the assay sensitivity (11). We describe a one-stage PCR assay for HCV RNA which, when combined with either liquid hybridization or Southern blot analysis, is equal in sensitivity to the nested-set PCR assay. Therefore, we found no evidence to support contentions that nested-set PCR is more sensitive than one-stage PCR.

In addition to sensitivity, the major concern about PCR testing in the clinical diagnostic laboratory is accuracy. Contamination is a widely recognized problem with PCR. We report that some contamination events associated with nested-set PCR are not obvious unless sensitive detection methods such as liquid hybridization are used. In our experience, strong positive results following nested-set PCR and ethidium bromide staining are rarely due to contamination. However, the use of nested-set PCR plus a radioactive probe to detect low-titer infections increases the risk of false-positive results. The current study establishes that one round of PCR plus liquid hybridization offers sufficient sensitivity and less potential for contamination.

The detection of HCV RNA in serum is an issue of direct clinical and therapeutic importance (5, 18). We believe that one-step RNA PCR plus liquid hybridization offers a useful method for identifying persons at risk of transmitting HCV infection and for monitoring responses to interferon therapy. Liquid hybridization not only increases assay sensitivity but also increases the confidence level of a positive result since the oligonucleotide probe is specific for HCV nucleic acid. In

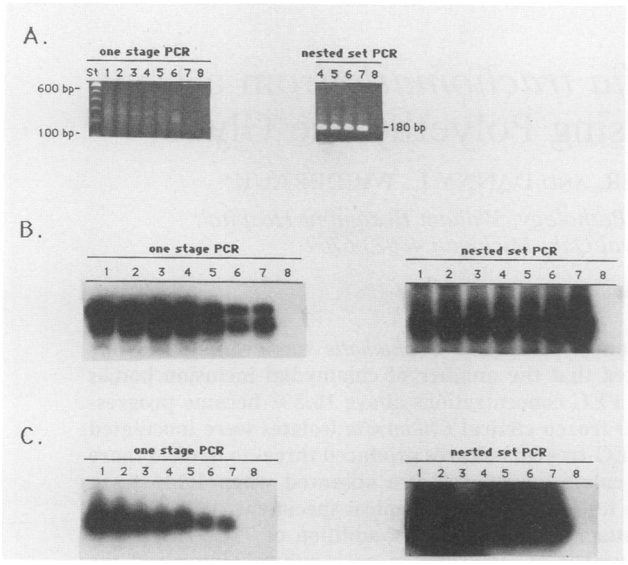

FIG. 2. Comparison of one-stage and nested-set PCR in a cDNA dilution assay. HCV cDNAs were initially diluted to a low concentration (approximately 100 copies per 10 μl) and then subjected to serial twofold dilution, as described in the text. The dilute cDNAs were amplified by sequential one-stage and nested-set PCR. Amplification products were analyzed by either agarose gel electrophoresis (A), liquid hybridization (B), or Southern blot (C). Autoradiogram exposures were equal in each experiment. Lane numbers correspond to the following cDNA dilutions: lane 1, approximately 100 copies of HCV cDNA; lane 2, 2-fold dilution of lane 1 cDNA; lane 3, 4-fold dilution of lane 1 cDNA; lane 4, 8-fold dilution of lane 1 cDNA; lane 5, 16-fold dilution of lane 1 cDNA; lane 6, 32-fold dilution of lane 1 cDNA; lane 7, 64-fold dilution of lane 1 cDNA; lane 8, 128-fold dilution of lane 1 cDNA. Sizes of DNA standards (lane St) and second-round PCR product DNA are designated in base pairs (bp).

the current study the incidence of HCV viremia in patients with clinical manifestations of active hepatitis C was 100%, whereas it was 75% for random seropositive specimens. The correlation between HCV RNA titer and stage of disease or response to therapy requires further study.

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