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Single-Step PCR in Molecular Diagnosis of Hepatitis C Virus Infection

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The diagnostic utility of two PCR systems and three PCR detection methods for hepatitis C virus (HCV) RNA was evaluated in serum samples. A nested PCR was considered the reference assay and was compared with two single-step PCR methods: the first is based on the detection of PCR products by liquid hybridization with a ^{32}P-end-labeled probe, and the second is the Roche Amplicor colorimetric assay using microwell plate hybridization with a specific nucleic acid probe. Using the Pelicheck HCV RNA Eurohep genotype 1 proficiency panel, our laboratory achieved medium-high levels of performance with all three methods. The highest sensitivity was, however, observed with the isotopic single-step PCR (ss-PCR) method. The analytical sensitivity of ss-PCR with isotopic detection and ss-PCR with colorimetric detection was identical to that of nested PCR, with a 100% result concordance. Comparison of ss-PCR with enzyme-linked immunosorbent and RIBA assays in the analysis of clinical samples showed a high concordance. ss-PCR methods appear more suitable for diagnostic application. Nevertheless, HCV RNA PCR cannot be considered a screening assay; it should be requested in the presence of reactive serology or specific clinical symptomatology with altered liver parameters, and it is a potential tool for the follow-up of patients with HCV infection.

Hepatitis C virus (HCV) is the major causative agent of parenterally transmitted non-A, non-B hepatitis (4). Detection of HCV infection has been facilitated by the development of an antibody detection assay, but the utility of this assay is limited by the window period of 2 to 6 months between infection and seroconversion (2, 5, 6). Reverse transcriptase PCR (RT-PCR) has been shown to be an effective means of detecting specific hepatitis C viral sequences. To increase assay sensitivity, several groups have used nested PCR for HCV RNA detection, in which, following reverse transcription, cDNA is amplified with an outer set of primers; the reaction products are then subjected to a second round of amplification with an internal set of primers, greatly increasing the amount of amplicons available for the detection by ethidium bromide staining (7, 17). With nested PCR there is a high risk of PCR product contamination by first-round PCR products during the sample transfer step, prior to the second round of amplification. Recently, the frequency of false-positive results due to carryover contamination was reported to range from 4 to 31% (10, 21). An alternative approach to nested PCR is the use of single-step PCR (ss-PCR), in which a single amplification step is performed, combined with sensitive detection methods such as liquid hybridization or Southern blotting with radiolabeled oligonucleotide probes (9, 10). A tool to avoid false-positive results caused by the presence of previously amplified PCR products is chemical sterilization with uracil-*N*-glycosylase (UNG). This enzyme is added to the PCR mixture together with dUTP instead of dTTP, resulting in the synthesis of dUTP-containing PCR products distinguishable from the dTTP-containing target DNA. A preliminary incubation step at 50°C allows UNG to recognize and catalyze the destruction of previously amplified dUTP-containing DNA fragments, thus destroying possible carryover contaminants, while dTTP-containing DNA target fragments remain intact for subsequent amplification (15).

In this study we have evaluated the diagnostic utility of different PCR and detection methods to reveal HCV RNA in serum. A nested PCR, considered the reference assay, was compared with two ss-PCR methods: the first is based on the detection of PCR products by liquid hybridization with a ^{32}P-end-labeled probe (isotopic ss-PCR), and the second is a colorimetric method using microwell plate hybridization with a specific nucleic acid probe (Amplicor HCV PCR; Roche Diagnostics System) (colorimetric ss-PCR).

MATERIALS AND METHODS

Samples. A total of 126 blood samples from 111 patients with suspected hepatitis C infection or altered liver parameters and from 15 blood donors repeatedly antibody negative was selected. Serum was separated by centrifugation (1,000 × *g* for 10 min) within 3 h of collection, and aliquots were stored at −20°C for serological tests and at −70°C for RNA analysis.

Enzyme-linked immunosorbent assay (ELISA). The HCV 3.0 ELISA Test System (Ortho Diagnostic Systems Inc., Raritan, N.J.) was used for the detection of antibodies to HCV (anti-HCV). It utilizes microwells coated with a combination of recombinant HCV-encoded antigens (c22-3, c200, and NS5). The assay was performed following the manufacturer's procedures.

RIBA. The Chiron RIBA 2.0 Strip Immunoblot Assay (Ortho Diagnostic Systems) was used as a confirmatory test for antibody specificity to individual proteins encoded by HCV. It utilizes four recombinant HCV-encoded antigens (5-1-1, c100-3, 33c, and c22-3) immobilized as individual bands onto test strips. The assay was performed following the manufacturer's procedures, and the results were evaluated following the suggested interpretation criteria.

Nested PCR. Total RNA was obtained from 100 μl of serum by the procedure of Chomczynski and Sacchi (guanidinium thiocyanate-phenol-chloroform) and was resuspended in 33 μl of double-distilled H_2O (3). RT-PCRs were performed with 5 μl of RNA using a set of primers described by Novati et al. (19) for the 5′ noncoding region of the HCV RNA genome, which has been shown to be the most conserved region among different HCV strains (11, 12, 20). To avoid false-positive results, we applied the methods to prevent contamination described by Kwok and Higuchi (14). cDNA was synthesized with 50 pmol of outer antisense primer (HCVOA 5′-TGCACGGTCTACGAGACCTC-3′ [nucleotides {nt} 320 to 339]), 1 mM deoxynucleoside triphosphates (dNTPs), 20 U of RNase inhibitor (Promega Corp., Madison, Wis.), and 40 U of Moloney murine leukemia virus RT (Gibco BRL, Life Technologies Inc., Gaithersburg, Md.). Following reverse transcription, PCR mixture (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 50 pmol of outer sense primer [HCVOS, 5′-GCCATG GCGTTAGTATGAGT-3′ {nt 82 to 101}], and 2.5 U of *Taq* polymerase [Perkin

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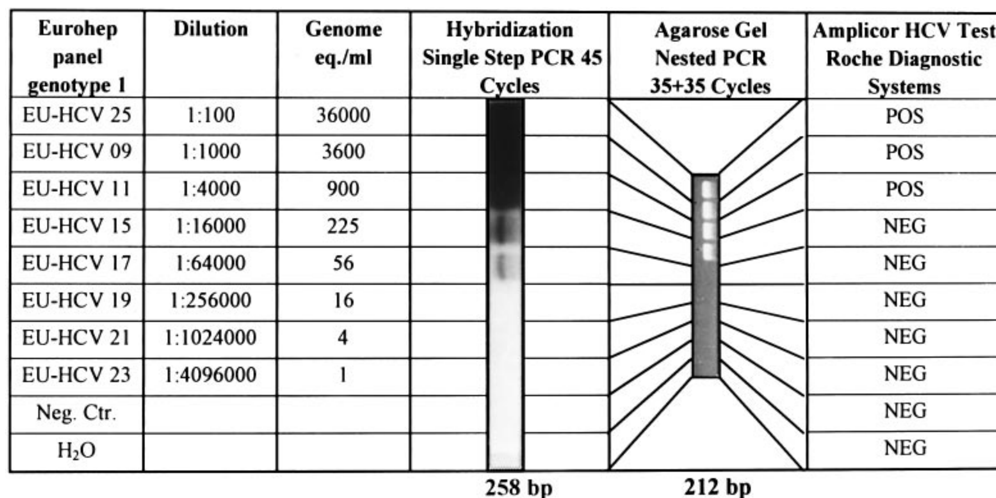

Eurohep panel genotype 1	Dilution	Genome eq./ml	Hybridization Single Step PCR 45 Cycles	Agarose Gel Nested PCR 35+35 Cycles	Amplicor HCV Test Roche Diagnostic Systems
EU-HCV 25	1:100	36000			POS
EU-HCV 09	1:1000	3600			POS
EU-HCV 11	1:4000	900			POS
EU-HCV 15	1:16000	225			NEG
EU-HCV 17	1:64000	56			NEG
EU-HCV 19	1:256000	16			NEG
EU-HCV 21	1:1024000	4			NEG
EU-HCV 23	1:4096000	1			NEG
Neg. Ctr.					NEG
H_2O					NEG

FIG. 1. Evaluation of the analytical sensitivity of ss-PCR and nested PCR in the Pelicheck HCV RNA Eurohep genotype 1 plasma standards. POS, positive; NEG, negative; Neg. Ctr., negative control.

Elmer Corp., Norwalk, Conn.]) was added to the same tube and the first PCR (step I) was performed for 35 cycles in a GeneAmp PCR Cetus 9600 thermocycler (Perkin-Elmer Corp.). One microliter of step I PCR product was further amplified with the inner nested primer set (antisense [HCVIA], 5′-GGGCACT CGCAAGCACCCTAT-3′ [nt 296 to 316]; sense [HCVIS], 5′-GTGCAGCCTC CAGGACCCCC-3′ [nt 105 to 124]) for an additional 35 cycles under the same reaction conditions. Nested PCR products (10 μl) were analyzed by electrophoresis in 2% agarose gels and visualized by UV fluorescence after ethidium bromide staining.

ss-PCR with isotopic detection. HCV RNA extraction conditions and PCRs were as described above for the first step of nested PCR. Amplification was performed for 45 cycles. ss-PCR products were detected by liquid hybridization (8, 13): 30 μl of amplification product was directly mixed with 1 μl of ^{32}P-end-labeled HCV-specific oligonucleotide probe (HCV, 5′-ACACCGGAATTGCC AGGACGACCGGGTCCTTTCTTG-3′ [nt 165 to 200]) in 66 mM NaCl and 44 mM EDTA. At least 100,000 cpm of probe for each sample was used for optimal results. The hybridization mixture was heated at 95°C for 4 min to dissociate the double-stranded DNA product and then at 55°C for 10 min to allow DNA product-probe annealing. The hybridization mixtures were immediately analyzed by electrophoresis in 10% polyacrylamide gels and then autoradiographed (8).

ss-PCR with colorimetric detection. The Amplicor HCV PCR kit (Roche Diagnostics System Inc., Branchburg, N.J.) includes a protocol that allows simplified RNA extraction from 100 μl of serum. HCV RNA was extracted with guanidinium thiocyanate and then recovered by isopropanol precipitation. The RNA was resuspended in 1 ml. Fifty microliters of the HCV target RNA was reverse transcribed and amplified in a single-tube reaction by recombinant thermostable DNA polymerase (r*Tth*) in the presence of Mn^{2+} ions. The PCR mixture (50 μl) contains dNTPs with dUTP instead of dTTP, 5′ biotinylated antisense primer (KY 78, 5′-CTCGCAAGCACCCTATCAGGCAGT-3′ [nt 276 to 299]), sense primer (KY 80, 5′-GCAGAAAGCGTCTAGCCATGGCGT-3′ [nt 56 to 79]), and UNG (18). The biotinylated RT-PCR products were chemically denatured, hybridized to a specific HCV probe (KY 88, 5′-GTTGGGTCG CGAAGGCCTTGTGGT-3′ [nt 251 to 275]), and immobilized to the surface of the microplate well (20). The addition of an avidin-horseradish peroxidase conjugate and then of substrate allowed development of color. The colorimetric reaction was detected with an automated microplate reader at 450 nm according to the manufacturer's instructions (16).

Proficiency panel. The Eurohep genotype 1 serial dilution panel of the Pelicheck HCV RNA sensitivity panel, provided by the Central Laboratory of The Netherlands Red Cross (Amsterdam, The Netherlands) was used to assess the analytical sensitivity of the RT-PCR methods. This set has been designed by the Eurohep Collaborative Study Group to standardize HCV RNA assays for the diagnosis of HCV infection and is composed of HCV RNA genotype 1 plasma standard dilutions prepared in a pool of plasma units negative for hepatitis B surface antigen, anti-hepatitis B core, anti-HCV, anti-human immunodeficiency virus types 1 and 2, anti-human T-cell leukemia virus type 1, syphilis, HCV RNA, hepatitis B virus DNA, and human immunodeficiency virus RNA (23). In a second worldwide collaborative study, the panel has been tested by 86 laboratories, 44 of which have been successively selected for their high-quality performance. These 44 laboratories generated 56 data sets from which, with a statistical program (probit analysis), have been calculated the detection end points of 50% of the laboratories with different PCR protocols (4a).

RESULTS

Evaluation of sensitivity. In order to assess the analytical sensitivity of the two ss-PCR methods versus that of the nested PCR, we repeatedly tested in parallel the serial dilution panel of the Pelicheck HCV RNA Eurohep genotype 1 plasma standards. In each experiment we included two reagents and three negative controls. The results obtained with nested PCR (225 genome equivalents per ml) and colorimetric ss-PCR (900 genome equivalents per ml) methods qualified the performance of our laboratory in the medium-high quality level, on the basis of the results obtained with the same proficiency panel in the second international collaborative study. Fifty percent of the laboratories, in fact, were able to detect 488 HCV genome equivalents per ml by nested PCR, 606 genome equivalents per ml by colorimetric ss-PCR (Amplicor), and 1,096 genome equivalents per ml by isotopic ss-PCR. The extinction signal for our isotopic ss-PCR was repeatedly observed at the 1:64,000 dilution, corresponding to 56 genome equivalents per ml. A representative experiment is shown in Fig. 1.

Concordance between PCR methods. The comparative analysis of the three PCR methods was performed by parallel testing of a total of 71 serum samples, 17 of them collected from 17 patients with a diagnosis of suspected HCV infection, 39 that were antibody positive or antibody indeterminate, and the remaining 15 of which were from blood donors repeatedly antibody negative. One of the nested PCR experiments for 20 samples had to be repeated because of carryover contamination observed in the test negative controls. The analytical sensitivity of ss-PCR with isotopic detection and ss-PCR with colorimetric detection was identical to that of nested PCR, with a 100% result concordance (36 samples were positive, and 35 were negative).

Comparison of ELISA, RIBA assay, and ss-PCR assay. Fifty-five additional samples collected from patients with suspected hepatitis C infection were tested for the presence of HCV RNA by our isotopic ss-PCR method. In order to understand the diagnostic significance of PCR, a comparative analysis of ELISA, RIBA, and ss-PCR results was done on a total of 126 samples, as shown in Fig. 2.

HCV RNA was detected in 80 of the 126 serum samples

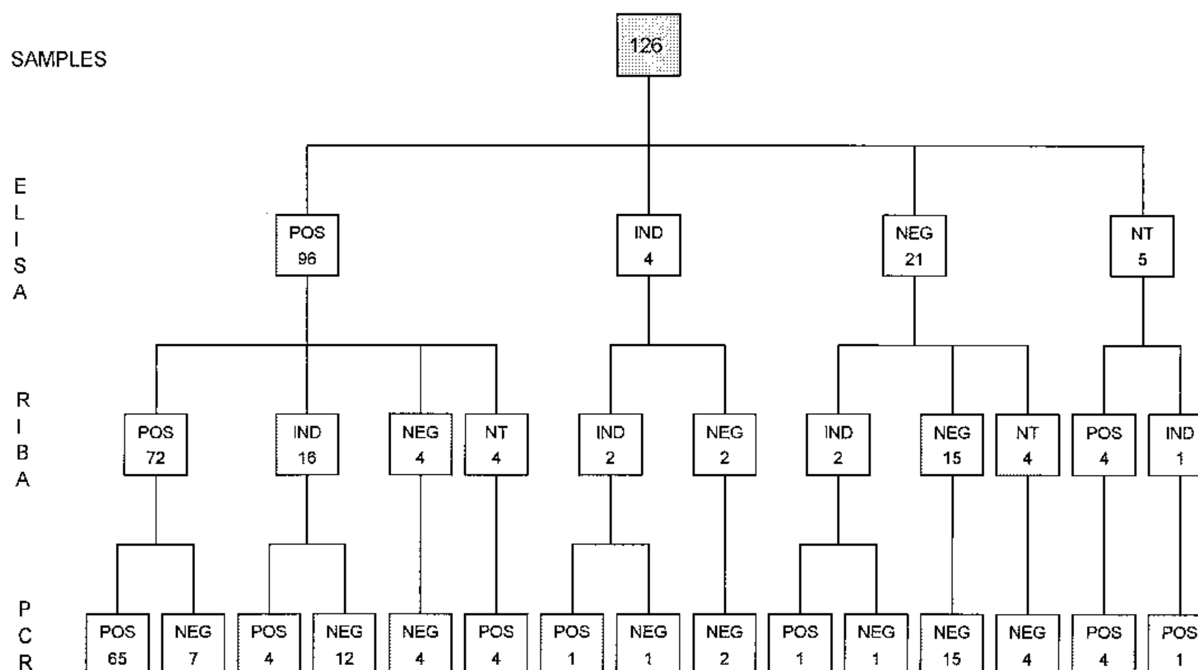

FIG. 2. Comparison of HCV ELISA, RIBA assay, and ss-PCR RNA assay. POS, positive; NEG, negative; IND, indeterminate; NT, not tested.

tested. HCV viremia was associated with RIBA positivity in 95% of samples, while all the 21 RIBA-negative samples were also PCR negative. Of RIBA-indeterminate samples (17% of total samples) 33% were PCR positive. Two PCR-positive samples with indeterminate serology (one RIBA indeterminate only and the other both ELISA low reactive and RIBA indeterminate) had clinical evidence of hepatitis C infection. The first was from a hepatitis B surface antigen-positive patient with cirrhosis, and the second was from a child born to a seropositive mother.

DISCUSSION

In this study we have evaluated two ss-PCR assays for HCV RNA and compared them with the nested PCR, which is used by several research laboratories (6, 7).

The analytical sensitivity was assessed with the HCV RNA Eurohep proficiency panel (23). The results obtained with nested and colorimetric ss-PCR methods qualified the performances of our laboratory in the medium-high level. The isotopic ss-PCR showed a greater analytical sensitivity, with 4- and 16-fold differences in the limit of detection of HCV RNA compared with those of nested PCR and colorimetric ss-PCR, respectively. The last positive dilution in the colorimetric ss-PCR contained only 900 HCV RNA genome equivalents per ml. This can be partially explained by the different input volume of extracted sample used in the colorimetric ss-PCR.

ss-PCR methods appear more suitable for diagnostic application. Specificity is guaranteed by the use of a specific probe for HCV nucleic acid detection, and UNG chemical sterilization obviates the possibility of PCR product contamination, which can occur during nested PCR (9, 10, 17, 21), as also observed in this study. In particular the Amplicor HCV PCR assay eliminates an aliquot transfer step between the reverse transcription and amplification steps and reduces handling time, thus minimizing exposure to contamination and sample management problems (8, 15, 22).

The specificity of our ss-PCR has been estimated to be good, as assessed by the analysis of the primers and probe sequence. It is nevertheless possible that the sensitivity for certain variants would be altered, since a small proportion of sequenced HCV isolates does not perfectly match the primers or the probe used in these experiments. We are in process of optimizing our ss-PCR using the KY78-KY80 primers and KY88 probe (22) for diagnostic purposes.

The detection of HCV RNA in serum is an issue of direct clinical and therapeutic importance: it can be used to identify persons at risk of transmitting HCV infection, monitor responses to interferon therapy, and resolve the status of RIBA-indeterminate patients (1, 6, 9, 22). Our data show a high rate of PCR positivity with RIBA-positive specimens (90.2%) and PCR negativity among all the RIBA-negative samples. Thus, it appears that confirmation of serological results (ELISA and RIBA) constitutes a reasonable method for the follow-up of patients with HCV infections. Such patients are candidates for potential referral for antiviral chemotherapy. However, it should be noted that RIBA-indeterminate patients may be HCV RNA PCR-positive; the clinical course and response to therapy of this group need further study. In conclusion, HCV RNA PCR is not a screening assay and should be requested in the presence of reactive serology or specific clinical symptomatology with altered liver parameters or in the evaluation of interferon therapy efficacy.

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