

## Clinical Significance of Serum Hepatitis C Virus (HCV) RNA as Marker of HCV Infection

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**We have evaluated the clinical significance of hepatitis C virus (HCV) RNA determination by analyzing a group of 221 hospitalized patients with abnormal liver function tests. Serum HCV RNA was detected by "nested" PCR amplification followed by nonisotopic hybridization. Of the 200 (90.5%) patients with anti-HCV-positive enzyme-linked immunosorbent assay results, 152 (76%) were RIBA reactive, 47 (23.5%) had indeterminate results, and 1 (0.5%) was nonreactive. Of the 180 (90%) patients positive for anti-HCV and HCV RNA, 138 (76.7%) were RIBA reactive and 42 (23.3%) were RIBA indeterminate. The pattern of RIBA reactivity did not correlate with the presence of HCV RNA. Elevated alanine aminotransferase levels were associated neither with the presence of viremia nor with the RIBA pattern. Histological findings consistent with non-A non-B hepatitis correlated with the presence of HCV RNA but not with the RIBA pattern. HCV RNA was detected in 11 of 21 (52.4%) anti-HCV-negative patients. These 11 patients were either immunosuppressed or in the prodromic phase of acute hepatitis C. Circulating HCV RNA can therefore be described as being predictive of virus-induced liver damage in anti-HCV-positive patients and may be useful in the diagnosis of HCV infection in anti-HCV-negative immunosuppressed patients or in those with early acute infection.**

The cloning of the hepatitis C virus (HCV) genome (5) and the subsequent development of specific assays for circulating antibodies (15) have confirmed the etiological importance of HCV infection in chronic liver disease and the prevalence of the virus in different risk groups (9, 20).

Serological tests for the detection of anti-HCV antibodies have been available for the last few years. However, the diagnosis of active HCV infection is still hampered by the lack of a test for the detection of viral antigens.

Several studies have defined the incidence and the prevalence of hepatitis C antibodies in blood donors (2, 8), in blood transfusion recipients (3, 10, 12, 24, 25), and in patients with chronic liver disease (3, 17). The presence of anti-HCV usually implies chronic HCV infection (6). However, some patients with resolved hepatitis C may remain anti-HCV positive for many years (10). The detection of circulating HCV RNA could be a more reliable approach for discriminating between resolved and persistent HCV infection. Recent studies have shown that, in resolved hepatitis C, HCV RNA disappears from the serum before anti-HCV antibodies, whereas, in chronic hepatitis C, the viral nucleic acid remains detectable for prolonged periods (13–17, 22).

Although recent developments in PCR technique have made it possible to detect HCV RNA in serum samples, the clinical significance of this test has not yet been completely established. We developed a technique for reverse transcription amplification (with nested PCR) (RT-PCR) and detection of amplified products with a nonisotopic hybridization system (18). In this study, we evaluated the possible clinical indications of HCV RNA determination in a group of hospitalized patients by comparing HCV RNA with other clinical and laboratory

characteristics of patients (anti-HCV reactivity, alanine aminotransferase [ALT] levels, liver histology, and presence of non-organ-specific autoantibodies).

### MATERIALS AND METHODS

**Serum samples.** To confirm the specificity of the method for the detection of HCV RNA, we analyzed the specimens collected from 60 anti-HCV-negative blood donors.

The clinical significance of HCV RNA determination was analyzed in 221 patients (103 males and 118 females, age  $41 \pm 18$  years) with abnormal liver function tests, hospitalized for acute ( $n = 31$ ) or chronic ( $n = 94$ ) non-A non-B hepatitis (NANBH), cryoglobulinemia ( $n = 42$ ), or liver ( $n = 14$ ), bone marrow ( $n = 5$ ), or kidney ( $n = 35$ ) transplantation. Two out of 221 patients were hepatitis B surface antigen positive, and 65 were anti-hepatitis B core antigen positive. A group of subjects ( $n = 23$ ) was anti-human immunodeficiency virus positive: 3 with acute and 20 with chronic hepatitis C.

All samples were stored at  $-80^{\circ}\text{C}$  until used.

**RNA standards.** Standard RNA preparations, synthesized for the evaluation of the HCV RNA detection method, were obtained as described below. The amplification product derived from HCV-infected serum with external primers 1A and 1B (19) was cloned into the *Sma*I-cut Bluescript plasmid vector (Stratagene, La Jolla, Calif.). The nucleotide sequence of both strands was determined by the dideoxy-chain termination method. The cloned fragment was transcribed in vitro from linearized plasmid by using the RNA transcription kit (Stratagene), and the RNA concentration was determined spectrophotometrically by UV  $A_{260}$ . Serial dilutions of the RNA template were reverse transcribed and amplified following the same protocol used for RT-PCR of serum samples.

**Anti-HCV testing.** All serum samples were tested by a second-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostics, Inc., Raritan, N.J.), based on two distinct

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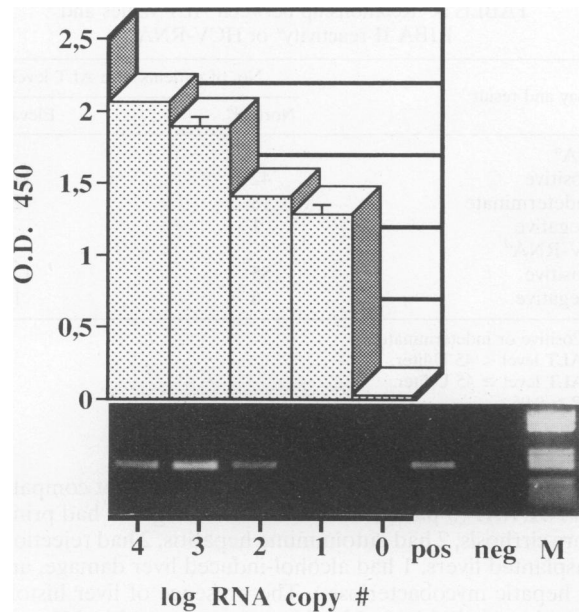


FIG. 1. Lower detection limit of RT-PCR-DEIA assay. A dilution series of HCV RNA transcribed in vitro from a recombinant vector was reverse transcribed, amplified, and detected by DEIA and gel electrophoresis to assess the analytical sensitivity of the assay (pos, positive control; neg, negative control; M, molecular weight marker).

HCV-encoded antigens (C200 plus C22) derived from non-structural and structural HCV regions, respectively.

All ELISA reactive samples were tested by RIBA-II assay (Chiron RIBA HCV Test System; Chiron Corp., Emeryville, Calif.) according to the manufacturer's instructions.

**Autoantibody testing.** Sera diluted 1/10 were screened by indirect immunofluorescence on rat tissue (liver, kidney, stomach) sections to detect antibodies to nucleus (ANA), smooth muscle (SMA), liver or kidney microsomes (ALKM1A), and mitochondria (AMA).

**HCV RNA extraction, reverse transcription, and amplification.** Serum samples derived from all patients, tested by ELISA and RIBA assays, were analyzed for the presence of HCV RNA.

Three microliters of serum sample was heated to 92°C for 2 min to destroy secondary structures and then reverse transcribed and amplified by RT-PCR with primers located in the conserved (21) 5' untranslated region, as previously described (19, 23). Pre-PCR and post-PCR reactions were physically

separated to prevent contamination in the different steps of HCV-RNA amplification. Negative controls (reaction mixture without nucleic acids or with nucleic acids extracted from normal control sera) were performed in each experiment, and results were only considered if consistent in at least two independent experiments.

**Detection of amplification products.** The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in Tris-borate-EDTA buffer (pH 8) and ethidium bromide staining. The specificity of the amplified fragments was confirmed by a nonradioactive hybridization assay (DNA Enzyme Immunoassay [DEIA], GEN-ETI-K DEIA, Sorin Biomedica, Saluggia, Italy) as previously described (18). The DEIA relies on an anti-double-stranded DNA monoclonal antibody capable of revealing the hybridization event. The test is similar to a standard immunoassay, and the results are expressed as units of optical density at 450 nm.

**Statistical analysis.** Statistical analysis was conducted by the chi-square test and Fisher's exact test. A *P* value of <0.05 was considered significant.

**RESULTS**

**Analytical evaluation of the method.** The specificity of the method was determined by testing 60 serum samples, obtained from anti-HCV-negative blood donors, using the RT-PCR-DEIA assay. The sera were interspersed among the samples under investigation. No false-positive results were obtained (data not shown).

The sensitivity of RT-PCR-DEIA was evaluated by using known amounts of RNA transcript as templates in RT-PCR reactions. Hybridization with an internal probe immobilized on microtiter plates (DEIA) showed a lower detection limit of 10 RNA copies contained in the initial sample prior to amplification (Fig. 1). Interassay reproducibility was tested by comparing the optical density values obtained for negative and positive controls included in each amplification run (25 runs). Intra-assay reproducibility was evaluated by using RT-PCR-DEIA for testing 10 replicates of a sample containing 10<sup>5</sup> RNA copies. All interassay and intra-assay replicates of the HCV-infected sample gave positive results with coefficients of variation of 13 and 1.2%, respectively.

**Clinical evaluation of HCV RNA determination.** The clinical characteristics of the analyzed patients are reported in Table 1. Seventy-two percent of serum samples had elevated levels of ALT. Circulating viral RNA was found in 25 of 31 (80.6%) patients with acute NANBH, while only 18 (58%) of these developed anti-HCV antibodies at the moment of HCV RNA testing. Seroconversion to anti-HCV antibodies was observed

TABLE 1. Characteristics of 221 patients tested for HCV infection markers

Hospitalization diagnosis	No. of patients	Age (yr) (mean ± SD)	No. of male patients	No. with ALT level		No. with liver histology		Anti-HCV test result				No. (%) positive for HCV RNA
				Normal (<45 U/liter)	High (≥45 U/liter)	Viral hepatitis	Other	No. (%) positive by ELISA	No. by RIBA			
									Reactive	Indeterminate	Nonreactive	
Chronic hepatitis	94	42 ± 15	44	14	80	50	7	94 (100)	79	15	0	86 (91.5)
Acute hepatitis	31	35 ± 16	18	0	31			18 (58)	12	6	0	25 (80.6)
Transplantation (liver, kidney, bone marrow)	54	30 ± 17	34	26	28	24	5	46 (85)	30	15	1	43 (80)
Cryoglobulinemia	42	59 ± 9	6	21	21	26	0	42 (100)	31	11	0	37 (89)
Total	221	41 ± 18	102	61	160	100	12	200 (90.5)	152	47	1	191 (86.4)

TABLE 2. Correlation between HCV RNA and anti-HCV antibodies tested by ELISA II and RIBA II assays<sup>a</sup>

Assay and result	No. of serum samples with HCV RNA result	
	Positive	Negative
<b>ELISA<sup>b</sup></b>		
Positive	180	20
Negative	11	10
<b>RIBA<sup>c</sup></b>		
Positive	138	14
Indeterminate	42	5
Negative	0	1

<sup>a</sup> Positive or indeterminate.<sup>b</sup>  $P < 0.001$ .<sup>c</sup>  $P > 0.05$ .

in all these patients in subsequent samples. Viral sequences were found in 80% of transplanted patients, four (9.3%) of which were anti-HCV negative. Of the 180 of 200 (90%) anti-HCV-positive patients who demonstrated circulating viral sequences, 138 (76.7%) were RIBA reactive and 42 (23.3%) were RIBA indeterminate (Table 2). HCV RNA prevalence in RIBA indeterminate or reactive samples was not significantly different in any group of patients.

Twenty-three of 221 (10.4%) patients had HIV infection with CD4<sup>+</sup> cell counts of  $<200/\text{mm}^3$  and 54 (24.4%) were undergoing immunosuppressive therapy. The possible influence of immunosuppression on anti-HCV response was evaluated by comparing the prevalence of HCV RNA and the pattern of RIBA reactivity in immunocompetent and immunosuppressed patients. No significant difference in HCV-RNA prevalence was observed between RIBA positive or indeterminate patients belonging to both groups (Table 3).

The patients were divided into two groups on the basis of ALT values (normal or elevated). ALT values did not correlate with the antibody pattern, determined by RIBA II assay, or with the presence of HCV RNA (Table 4).

Twenty-three (24.5%) patients with chronic hepatitis and 6 (14.3%) patients with cryoglobulinemia were positive for non-organ-specific autoantibodies (ALKM1A was detected in 9 patients, ANA was detected in 11 patients, AMA was detected in 2 patients, SMA was detected in 5 patients, and both ANA and SMA were detected in 2 patients). HCV RNA was detected in 24 of 29 (82.6%) autoantibody positive and 87% of autoantibody negative patients; there was no significant difference in the prevalence of the two groups.

Liver histology was available for 112 anti-HCV positive patients. Seventy had chronic active hepatitis, 18 had chronic persistent hepatitis, 1 had chronic lobular hepatitis, 11 had

TABLE 3. Relationship between RIBA II reactivity and HCV RNA in immunocompetent and immunosuppressed<sup>a</sup> patients

Patient group and HCV RNA result	No. of patients with RIBA result		
	Positive	Indeterminate	Negative
<b>Immunocompetent<sup>b</sup></b>			
Positive	96	16	0
Negative	7	1	1
<b>Immunosuppressed<sup>b</sup></b>			
Positive	42	26	0
Negative	7	4	0

<sup>a</sup> CD4<sup>+</sup> count  $< 200/\text{mm}^3$  or under immunosuppressive therapy.<sup>b</sup>  $P > 0.05$ .TABLE 4. Relationship between ALT values and RIBA II reactivity<sup>a</sup> or HCV-RNA

Assay and result	No. of patients with ALT level	
	Normal <sup>b</sup>	Elevated <sup>c</sup>
<b>RIBA<sup>d</sup></b>		
Positive	42	110
Indeterminate	18	29
Negative	1	0
<b>HCV-RNA<sup>d</sup></b>		
Positive	55	125
Negative	6	14

<sup>a</sup> Positive or indeterminate.<sup>b</sup> ALT level  $< 45$  U/liter.<sup>c</sup> ALT level  $\geq 45$  U/liter.<sup>d</sup>  $P > 0.05$ .

liver cirrhosis, and 12 had histological features not compatible with NANBH (3 patients had minimal changes, 3 had primary biliary cirrhosis, 2 had autoimmune hepatitis, 2 had rejection of transplanted livers, 1 had alcohol-induced liver damage, and 1 had hepatic mycobacteriosis). The presence of liver histology suggestive of viral hepatitis did not correlate with the pattern of RIBA reactivity (positive or indeterminate) but was strongly associated with the presence of HCV RNA (Table 5). It is worth noting that among patients negative for HCV RNA, three had histological diagnosis of primary biliary cirrhosis and were positive for AMA, two had evidence of autoimmune liver disease, one had concurrent chronic hepatitis B, one patient had histological signs of alcohol-induced liver damage, and one had hepatic mycobacteriosis.

## DISCUSSION

We have developed a RT-PCR-DEIA assay for the detection of HCV RNA in serum samples (23). Analytical evaluation of the method showed good sensitivity, reproducibility, and specificity. The clinical significance of HCV RNA determination was established by analyzing a group of hospitalized patients and comparing the PCR results with anti-HCV reactivity, ALT values, presence of autoantibodies, and liver histology.

Most of the anti-HCV-ELISA-positive samples (99.5%) were RIBA reactive or indeterminate. The only ELISA-positive, RIBA-negative patient was HCV RNA positive and developed RIBA reactivity in subsequent samples. The detection of HCV RNA was significantly associated with the pres-

TABLE 5. Relationship between histological damage<sup>a</sup> and RIBA II reactivity<sup>b</sup> or presence of HCV RNA

Assay and result	Histology	
	Compatible	Not compatible
<b>RIBA<sup>c</sup></b>		
Positive	90	9
Indeterminate	10	3
Negative	0	0
<b>HCV RNA<sup>d</sup></b>		
Positive	95	4
Negative	5	8

<sup>a</sup> Compatible or not compatible with NANBH.<sup>b</sup> Positive or indeterminate.<sup>c</sup>  $P > 0.05$ .<sup>d</sup>  $P < 0.001$ .

ence of anti-HCV ELISA reactivity. This suggests that anti-HCV can be considered an indirect marker of HCV replication in hospitalized patients. A high percentage (91.5%) of HCV RNA positivity was found in the group of patients with chronic hepatitis. Failure to detect HCV RNA in a small proportion (8 cases out of 94) of anti-HCV-positive patients may have been due to intermittent viremia during chronic infection or to other causes of liver disease.

HCV viremia in the absence of specific antibodies was observed in 5% of our patients. Considering that most of these patients later seroconverted to anti-HCV or were under immunosuppressive therapy, HCV RNA detection may be said to provide an early diagnosis of acute NANBH and may help in the diagnosis of HCV infection in immunosuppressed patients.

Recent studies (7) suggest a striking association of mixed cryoglobulinemia with HCV infection. HCV RNA was detected in 37 of 42 anti-HCV-positive patients with cryoglobulinemia. Cryoprecipitation of HCV RNA could explain the negative PCR results in 5 of 42 patients. Quantitative studies, in fact, showed that more than 99% of the HCV RNA in the serum of patients with cryoglobulinemia had cryoprecipitated (1). The analysis of cryoprecipitate in anti-HCV positive patients with cryoglobulinemia might therefore improve the detection of HCV RNA.

ALT levels on a single determination did not correlate with the pattern of RIBA reactivity nor with viremia, consistent with the presence of fluctuating ALT levels in patients with chronic hepatitis C (11).

The clinical implications, if any, of different profiles of anti-HCV RIBA seroreactivity in patients with HCV infection is still being debated. The RNA PCR assay positivity rates of 91% for RIBA-positive and 90% for RIBA-indeterminate clinical specimens are higher than those previously reported (4). The characteristics of the studied population could explain this difference. The high HCV RNA prevalence in RIBA-indeterminate samples reported in this study indicates that a RIBA-indeterminate result does not exclude ongoing HCV infection. In addition, our results show that the pattern of RIBA reactivity (indeterminate or positive) does not correlate with virus-induced liver damage or with viremia, independent of immunosuppression.

HCV infection may be associated with a wide spectrum of liver disease (4), and the presence of anti-HCV may be an index of past resolved infection. The association between anti-HCV and circulating HCV RNA in our series indicates, however, that a past resolved infection is infrequent in hospitalized patients with altered liver function tests.

In anti-HCV-positive subjects, we also observed a significant correlation between circulating HCV RNA and the histological lesions indicative of viral hepatitis, independent of the degree of activity. This suggests the importance of PCR assay as a predictor of HCV-induced liver damage. Furthermore, the presence of other causes of liver damage in anti-HCV-positive, HCV RNA-negative subjects might indicate nonspecific reactivity with second-generation ELISA and/or RIBA.

In conclusion, HCV RNA testing could be considered for both anti-HCV-positive and -negative (immunosuppressed or in the early phase of acute hepatitis) patients in order to obtain prognostic information and to consider antiviral treatment. Further experience using PCR techniques for the detection of HCV RNA in different clinical situations is needed before the clinical significance of this assay can be assessed.

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