# Detection of HCV by PCR in Serum and PBMC of Patients with Hepatitis C After Treatment

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**Although hepatitis C is mainly hepatotropic, some studies suggest that hepatitis C virus (HCV) infects peripheral blood mononuclear cells (PBMC), using them as a reservoir, which might contribute to the development of resistance to treatment. Fifty-four hepatitis-C patients, who had been submitted to treatment, were selected. Blood samples were collected on the same day for the detection of HCV RNA in serum and PBMC by PCR, using the Amplicor HCV 2.0 assay (Roche Diagnostics). HCV genotyping was performed using the INNO-LiPA HCV kit (Versant, Bayer Diagnostics). HCV RNA was detected in both serum and PBMC in 35 (64%) patients and no RNA in 16 (29.6%). Disagreement between the serum and PBMC results was observed for three patients (5.6%), with HCV RNA being detected in PBMC but not in serum. Four months later, new serum and PBMC samples were collected from one of these patients and HCV RNA was detected in both samples, showing that PBMC can reveal signs of a lack of response to treatment. We conclude that the absence of HCV in the serum of patients with chronic hepatitis C by the end of treatment does not mean that there is no circulating virus. HCV in mononuclear cells may be an indicator of the persisting infection.**

**Key-Words: PCR, hepatitis C, PBMC, treatment.**

Considering that 170 million individuals are estimated to be infected with hepatitis C virus (HCV) worldwide, hepatitis C has a strong impact on public health. A vaccine protecting against infection is not available, and antiviral therapy offered is characterized by limited efficacy, high costs and substantial side effects [1,2]. Studies on the natural history of hepatitis C indicate that 55% to 85% of patients who develop acute disease remain infected, i.e., they acquire chronic hepatitis. Among these individuals, 5% to 20% are at risk of developing cirrhosis within a period of 20 to 25 years [2,3].

HCV is a member of the genus *Hepacivirus* and of the family Flaviviridae. It is an enveloped virus with an RNA genome of approximately 9,400 bp. Most of the genome forms a single open-reading frame that encodes three structural (core, E1, E2) and seven non-structural (p7, NS2-NS5) proteins. Short untranslated regions (UTRs) at each end of the genome are required for replication of the genome, a process that has recently been found to additionally require a cis-acting replication element in the coding sequence of NS5B [4]. In addition to tropism for hepatocytes, HCV presents other important extrahepatic sites for propagation, including peripheral blood mononuclear cells (PBMC) and the central nervous system [5-7]. Positive- and negative-strand HCV RNA have been detected in PBMC and bone marrow from chronically-infected patients [7,8].

Treatment for hepatitis C consists of the administration of pegylated or nonpegylated interferon-alpha (IFN-alpha) in combination with ribavirin; the duration of treatment adequate for each patient is determined according to the type of virus. At the end of treatment, patients are classified as responders if no HCV RNA is detected by PCR or as nonresponders if HCV-PCR is positive. The virological response is evaluated again after six months, and a sustained virological response (SVR) is defined as negative HCV-PCR and a nonsustained virological response is defined as positive HCV-PCR [9,10].

We determined if HCV was present in patients with hepatitis C at the end of treatment using the classical parameter for the assessment of the virological response, i.e. detection of HCV RNA in serum by PCR, in addition to investigation of HCV by PCR in PBMC.

We analyzed response to treatment of patients with hepatitis C based on the detection of HCV in serum and PBMC.

## Materials and Methods

### Patients and Samples

Serum and PMBC samples were collected from 54 patients prospectively seen at the Hepatitis Outpatient Clinic (LIM-47), Department of Infectious Diseases, Clinical Hospital, University of São Paulo, between March and December 2004. The samples were collected after the end of therapy.

Blood was collected from the patients by vacuum venipuncture, using a dry 10-mL tube. The serum was separated, centrifuged, aliquoted and stored at -20°C. For the separation of PBMC, blood was collected into 10-mL tubes containing heparin as anticoagulant. Immediately after collection, the cells were separated from whole blood by centrifugation on a Ficoll-Hypaque density gradient (density 1076). The plasma and ring of mononuclear cells were diluted, washed three times with RPMI culture medium, and adjusted to a final concentration of $1 \times 10^7$ cells. The PBMC pellet was aliquoted in Trizol LS and stored at -80°C [7].

### Detection of HCV RNA by the Polymerase Chain Reaction

The Amplicor HCV 2.0 assay (Roche Diagnostics, Mannheim, Germany) was used for the detection of HCV RNA.

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Briefly, viral RNA was extracted from plasma by lysis of viral particles with a chaotropic agent (guanidinium thiocyanate), followed by alcohol precipitation of RNA. A shortened RNA fragment was introduced as an internal control with the lysis buffer and served as an extraction and amplification control for each sample. HCV RNA was retrotranscribed to cDNA and amplified by a single tube RT-PCR. Primers KY78 and KY80 were used in the reaction, as previously described, to amplify a sequence of 244 nucleotides within the conserved 5'UTR of the HCV genome. Amplified DNA was detected using target-specific oligonucleotide probes that permitted independent identification of HCV amplicons and internal-control amplicons [11,12].

HCV Genotyping by the Line Probe Assay (LiPA)

RNA was isolated from serum, and 5'-UTR genotyping was performed with the INNO-LiPA HCV II kit (Versant, Bayer Diagnostics, Tarrytown, NY, USA), according to manufacturer's instructions. Briefly, the 5'-UTR was amplified with biotinylated primers. Biotin-labeled PCR products were reverse hybridized to specific probes attached to nitrocellulose strips. Incubation with a chromogen then results in a purple precipitate that forms a line on the strip. The HCV type is deduced on the basis of the patterns of hybridizing bands, using the LiPA-interpretation chart [13].

**Results**

Fifty-four patients were evaluated after the end of treatment with IFN-alpha and ribavirin. Twenty-seven (50%) patients were males and 27 (50%) were females, with a mean age of 47.7 years.

Serum and PBMC samples were collected 7 to 2,730 days (mean 1,170 days) after the end of treatment.

**Discussion**

Researchers have long sought to determine whether HCV replicates outside the liver, because finding HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment. Nonetheless, a definitive demonstration of extrahepatic HCV replication has been limited by several biological and technical considerations. Foremost, the lack of a robust cell-culture system has made it exceedingly difficult to compare HCV replication in different cell populations. To date, the dynamics of HCV replication is typically investigated by intensive study of serum-specific or liver-specific HCV RNA; however, viral replication in extrahepatic reservoirs, such as PBMC, may not mean replication in these other compartments [14]. Furthermore, the PBMC compartment may be a privileged site for HCV, which is able to reinitiate viral replication after termination of HCV treatment when conditions again become more favorable. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, reinfection from extrahepatic sites, such as the PBMC compartment, may occur. Future studies of HCV quasispecies diversification in serum and PBMC may provide additional evidence that HCV replication and evolution are distinct in these compartments [14].

The prevalence of the HCV genotypes that we observed was similar to that of other studies in Brazil, with a prevalence of type 1, followed by type 3 and, at a lower frequency, type 2 (Table 1) [15,16]. When we examined the probable forms of acquisition of HCV, analysis of the patient records and interviews revealed blood transfusion as the most frequent, suggesting that most patients contracted the disease before 1992, when serological screening of blood banks for HCV became obligatory in Brazil (Table 2) [17].

At the end of treatment with IFN-alpha in combination with ribavirin, 28 (51.9%) patients were considered to be responders, i.e., HCV-PCR negative, based on the data from the patient records; however, parallel analysis of PBMC revealed a lower percentage of responders (N = 23, 42.6%). Among three patients considered to be nonresponders by analysis of PBMC, one patient tested positive for HCV in serum when reassessed after four months. Analysis of the records of the other two patients showed that one patient continued to be HCV-PCR negative and was considered to present an SVR, whereas the other patient was classified as a nonresponder. These data indicate that PBMC may be an important reservoir for HCV in the human host.

The lack of detection of HCV RNA in PBMC in our study could be questioned because of the low sensitivity of the HCV-PCR techniques used and because virus volumes in this reservoir may be below the lower detection limit of the method we used. Although we used efficient techniques for the detection of HCV in serum, which are employed worldwide for the routine follow-up of patients infected with HCV, the qualitative Roche Amplicor HCV 2.0 PCR kit presents a lower detection limit of 50 IU/mL to detect HCV virus in PBMC.

PBMC may carry a small number of viruses that require other laboratory methods to increase the sensitivity of detection of these particles. Pham et al. [7] evaluated PBMC samples from 12 patients considered to be responders to IFN-alpha treatment and from five patients who presented spontaneous cure after a period of up to five years with negative viremia; the authors detected HCV RNA by PCR in these cells. They used mitogen-stimulated lymphocyte cultures to consistently increase the number of cells and, consequently, the number of viral particles in PBMC. All samples tested were positive for both, positive and negative-strand HCV RNA, which could indicate viral replication at these extrahepatic sites [18].

Clearance of HCV from serum and from PBMC occurs less frequently in patients previously resistant to IFN-alpha. Children with chronic hepatitis C require longitudinal observation after successful antiviral treatment, since HCV RNA in PBMC has been detected in 37% of patients considered to be free of the virus by ordinary measures [5]. However, our *in vitro* study suggests that HCV-RNA positivity in PBMC may be due to binding of the virus to blood cells and

**Table 1.** Distribution of HCV genotypes in the patients

| Genotype | Total |
|---|---|
| 1 | 31 (57%) |
| 2 | 2 (3.7%) |
| 3 | 11 (20%) |
| ND | 10 (19%) |
| Total | 54 (100%) |

ND: not determined.

**Table 2.** Distribution of patients according to risk factors for the acquisition of HCV

| Epidemiology | Women | Men | Total |
|---|---|---|---|
| Unknown | 9 (32%) | 10 (36%) | 19 (34%) |
| Transfusion | 13 (46%) | 8 (29%) | 21 (38%) |
| Drug use | 1 (3.6%) | 6 (22%) | 7 (13%) |
| Perforating and cutting wound | 2 (7.1%) | 2 (7.1%) | 4 (7.1%) |
| Surgery | 2 (7.1%) | 2 (7.1%) | 4 (7.1%) |
| Tattoo | 1 (3.6%) | 0 (0%) | 1 (1.8%) |

**Table 3.** Detection of HCV in serum and PBMC of infected patients after the end of treatment

| Serum | PBMC | No. of patients | % |
|---|---|---|---|
| + | + | 35 | 65 |
| + | - | 0 | 0 |
| - | + | 3 | 5.6 |
| - | - | 16 | 30 |

**Table 4.** Distribution of HCV patients according to treatment response

| Serum | PBMC | R (%) | NR (%) | NS (%) | SVR (%) |
|---|---|---|---|---|---|
| + | + | 10(20) | 21 (42) | 33 (66) | 0 (0) |
| + | - | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| - | + | 2 (4) | 1 (2) | 2 (4) | 1 (2) |
| - | - | 16 (32) | 0 (0) | 0 (0) | 14 (28) |

R: responders; NR: nonresponders; NS: nonsustained response; SVR: sustained virological response.

not to true virus production, indicating that PBMC do not function as an extrahepatic reservoir for HCV. Small amounts of HCV RNA may still be present in serum or attached to blood cells and may be responsible for reinfection of the graft. This may even occur in patients who are complete responders to IFN-alpha therapy immediately before transplantation [19].

HCV RNA in PBMC at the end of IFN treatment is a predictor of a lasting response to antiviral therapy in patients with chronic hepatitis C [6]. HCV RNA in PBMC is detectable years after the patient has been considered a responder to antiviral therapy [6], suggesting that the predictive effect of HCV RNA in PBMC after IFN treatment is more important than other factors [6,18,20]. These data suggest differential regulation of HCV RNA in the serum and PBMC compartments and may partially explain the limited HCV antiviral response rates observed in HIV-coinfected individuals [14]. Laskus et al. [21] observed recurrence of the disease in transplant patients with no detectable HCV RNA in serum but positive in PBMC; analysis of HCV genome sequences in the two reservoirs showed that they were similar [21].

An SVR to treatment with pegylated or nonpegylated IFN-alpha combined with ribavirin is observed in 40% to 50% of cases, and factors that negatively interfere with the response to treatment include HCV genotype 1, high viral load, male gender, advanced age, high degree of fibrosis, steatosis, and poor treatment compliance [22,23]. In our study, 27.7% of the patients presented an SVR. This low SVR rate might be explained by the small number of participants, most of them carrying genotype 1. A nonsustained virological response was observed in 64.8% of the patients. In these cases, viremia was reevaluated six months or more after the end of treatment by HCV-PCR, and the treatment response was also evaluated based on clinical and biochemical parameters. In our study, investigation of HCV in PBMC contributed to the evaluation of treatment outcomes. If PBMC indeed is an extrahepatic reservoir for HCV, this approach may provide additional data for a reliable assessment of treated patients or patients presenting spontaneous cure of hepatitis C.

Our results support the view that PBMC are an important extrahepatic reservoir for HCV. In addition, the lack of detection of viremia at the end of treatment alone does not indicate absence of circulating virus. The finding of HCV in PBMC may also contribute to our understanding of the persistence or recurrence of HCV infection.

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