Detection of hepatitis C virus (HCV) core–specific antibody suggests occult HCV infection among blood donors

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BACKGROUND: Blood transfusion safety is based on reliable donor screening for transmissible infections such as the hepatitis C virus (HCV) infection.

STUDY DESIGN AND METHODS: A novel HCV core–specific antibody was assayed on random single donations from 2007 first-time blood donors who tested negative for anti-HCV and HCV RNA on routine screening. Sample collection broke the code between donations and donors for ethical reasons.

RESULTS: Forty-two donations (2.1%) displayed reactivity in the novel test. The specificity of the reactivity was evaluated by a peptide inhibition assay, and testing against additional nonoverlapping HCV core peptide epitopes and other HCV antigens was performed on these samples. Six donations (14.3%; 0.30% from the total) were considered to contain anti-HCV after such supplemental testing. HCV RNA detection was also performed in peripheral blood mononuclear cells (PBMNCs) and serum or plasma samples from reactive donors after virus concentration by ultracentrifugation. HCV RNA tested negative in all PBMNCs samples, and a very low amount of viral genome was detected in serum or plasma concentrates from three anti-HCV core–reactive donors (7.1%) but not among concentrates from 100 randomly selected nonreactive donors. Sequencing of these polymerase chain reaction products revealed differences between the isolates that excluded partially sample contamination from a common source.

CONCLUSION: These findings argue in favor of an ongoing occult HCV infection among these blood donors and account for some rather low, but perhaps not negligible, infection risk for such donations. Future studies involving larger samples of donations from traceable donors would enlighten the significance of these findings for the viral safety of the blood supply.

Chronic infection caused by hepatitis C virus (HCV) is characterized by the presence of both specific antibody to HCV (anti-HCV) and HCV RNA in serum or plasma.[1,2] Anti-HCV in the absence of viremia presumably reflects immune memory after a past, resolved infection. Anti-HCV and HCV RNA

ABBREVIATIONS: AI = absorbance index; CNM = Centro Nacional de Microbiología; Ct = threshold cycle; DEPC = diethylpyrocarbonate; FEHV = Fundación para el Estudio de las Hepatitis Virales; GGTP = γ-glutamyl transpeptidase; NC = noncoding; OCI = occult hepatitis C virus infection; PI = percentage of inhibition.

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testing are mandatory as part of blood donor screening, and both markers must test negative for the donor to be accepted. However, the risk of HCV transmission, although very low, is not zero.[3-5]

Occult HCV infection (OCI) has been reported and was characterized by the presence of HCV RNA in peripheral blood mononuclear cells (PBMNCs) and/or liver tissue among patients who tested negative for both anti-HCV and HCV RNA in serum or plasma.[6] HCV RNA was also detected in serum after concentrating viral particles by ultracentrifugation, which increases the sensitivity of the testing.[7] OCI has been found among patients with cryptogenic hepatitis and chronic renal diseases, but also in 3.3% of individuals from the general population displaying normal liver function tests.[6,8,9] A proportion of these cases tested in addition positive by a novel anti-HCV core–specific antibody test,[10] but this test has not been yet compared with conventional screening assays among blood donors. Actually, the occurrence of OCI among first time blood donors has never been communicated.

The aims of this prospective, collaborative study were: 1) to investigate the frequency and the significance of the finding of HCV core–specific antibody reactivity among blood donors testing negative for anti-HCV and HCV RNA in screening and 2) to assess whether such reactivity correlates with the finding of HCV RNA sequences in PBMNCs and/or serum or plasma concentrated by ultracentrifugation.

PATIENTS AND METHODS

Study population

A total of 2012 consecutive, unselected, first-time unpaid blood donors admitted for screening of blood donation markers were prospectively enrolled from October 2014 to July 2015 at three transfusion centres: Red Cross Spain (n = 823) and the transfusion centers from the communities of Madrid (n = 1094) and Castilla y León (n = 95). The inclusion criteria were: 1) first-time blood donors who agreed to sign the informed consent and 2) availability of sufficient serum, plasma, and PBMNC samples collected at the time of study entry. Units testing positive for markers of HCV infection (anti-HCV and/or HCV RNA), human immunodeficiency virus (HIV; anti-HIV and/or HIV RNA), hepatitis B virus (HBV) infection (HBV surface antigen and/or DNA), or other infections (syphilis, malaria) were not excluded. Five donations were excluded because the lack of PBMNC samples, and the final population consisted of 2007 donors of whom 1104 were males and 903 females. No other demographic data were provided to the investigators, and the link between unit and donor identification was broken before sending the materials to the laboratories involved in the study. This procedure prevented communication of results to the donors, although also the obtaining of additional samples and the design of follow-up studies. The study was approved by the Ethic Committee of the Hospital Clínico San Carlos (Madrid, Spain) and was conducted according to the Helsinki Declaration of the World Medical Association.

Screening tests

Participating centers performed anti-HCV screening by either PRISM or ARCHITECT chemiluminescent immunoassays (Abbott Laboratories), and individual HCV nucleic acid test (NAT) by Procleix Ultrio Elite (Grifols Diagnostic Solutions Inc.) or Cobas MPX Multiplex on the Cobas 6800/8800 platform (Roche Molecular Diagnostics). Anti-HCV–reactive, HCV NAT–nonreactive donations were confirmed by immunoblot (INNO-LIA HCV-Score, Fujirebio Europe). Liver function tests (aspartic aminotransferase [AST], alanine aminotransferase [ALT], and γ-glutamyl transpeptidase [GGTP]) were determined centrally at the Fundación para el Estudio de las Hepatitis Virales (FEHV) upon donor inclusion in the study by the Roche Cobas Mira analyzer (normal values for AST, $\leq$34 IU/L; ALT, males $\leq$40, females $\leq$32 IU/L; GGTP, males $\leq$50, females $\leq$30 IU/L).

Testing of anti-HCV core–specific antibody

HCV core–specific antibody was tested by an immunoassay with enhanced sensitivity (anti-hcv core high-sensitivity enzyme-linked immunosorbent assa (ELISA kit, DIATER Laboratories) using as antigen a single peptide from a conserved HCV core region (Amino Acids 5-19). The test is based on an investigational anti-HCV core immunoassay.[10] According to current CE labeling regulations, calculation of the cutoff value was referred to the formula $CO = 0.5 \times (NC + 0.1 \times PC)$, where NC is the mean value for the negative control (six replicas) and PC is the mean value for the positive control (two replicas) after assaying more than 1000 positive, negative, and interfering samples in parallel with two CE-marked methods. Testing was performed in prediluted samples (1/10) according to supplier's instructions, and sample-to-cutoff absorbance ratios (absorbance index [AI]) of at least 1.2 were considered reactive. The assay has shown 100% diagnostic sensitivity in chronic infections by HCV Genotypes 1 to 6, and specificities of 100 and 99.7% among blood donors and clinical specimens, respectively.[11]

Anti-HCV core–reactive samples were further analyzed by: 1) peptide inhibition assay,[10] the percentage of inhibition (PI) being calculated by the formula PI = 1 – [(absorbance of sample with 100 μg/mL of peptide)/(absorbance of sample without peptide)] × 100; 2) a supplemental anti-HCV core peptide immunoassay based on peptides spanning Amino Acids 21 to 40 and 101 to 120 of the HCV core sequence (European Patent EP2258714B1), the testing procedure being exactly as described for Peptides 5 to 19;[10-12] and 3) conventional confirmatory testing by a commercial immunoblot assay (INNO-LIA HCV-Score, Fujirebio Europe).

Reactivity for anti-HCV core was thought specific when the PI was at least 50%. Concurrent reactivity in the

TABLE 1. Primers and probes used for HCV RNA amplification*

Name	Nucleotide sequence (5′ to 3′)	Position
Real-time PCR		
Forward primer	CTGTGAGGAACTACTGTCTT	36-55
Reverse primer	CTCGCAAGCACCCTATCAGG	283-302
Anchor probe	GCAGCCTCCAGGACCCCCC-Fl	98-116
Sensor probe	LC640-CCCGGGAGAGCCATAGTGGTCTG-ph	119-141
Nested PCR		
Outer forward primer	CCCTGTGAGGAACTWCTGTCTTCACGC	38-62
Outer reverse primer	GCTCATGRTGCACGGTCTACGAGACCT	312-338
Inner forward primer	TCTAGCCATGGCGTTAGTAYGAGTG	71-95
Inner reverse primer	CACTCGCAAGCACCCTATCAGGCAGT	279-304

* Nucleotide designation according to Takamizawa et al.[14]

supplemental anti-HCV core peptide test and the finding of reactivity (positive or indeterminate results) by immunoblot were also taken into account for the final interpretation. Samples testing positive by at least two of the three supplemental tests were considered positive for anti-HCV.

HCV RNA detection in PBMNCs and in ultracentrifuged serum and plasma samples

Nonrefrigerated blood samples were received at FEHV within 24 hours after extraction. Upon arrival, serum samples were obtained from coagulated blood, made into aliquots, and stored at −30°C. PBMNCs and plasma were isolated from anticoagulated blood by density gradient centrifugation (Biocoll, Biochrom). Plasma was aliquoted and stored at −30°C, while cells were washed three times in phosphate-buffered saline and thereafter stored in solution (RNAlater, Ambion) also at −30°C.

HCV RNA detection was performed by laboratory personnel who were blinded to the serologic status of the donors. Each polymerase chain reaction (PCR) run included a maximum number of 10 samples along with negative controls (repeatedly HCV RNA–negative sera and PBMNCs samples) and reagent blanks in which total RNA was replaced with PCR-grade water. Negative controls and blanks were coprepared with the samples and accompanied them through the entire PCR process. As positive controls, HCV RNA–positive sera and PBMNCs from patients with chronic HCV infection were used. The guidelines of Kwok and Higuchi[13] were strictly observed for avoiding contaminations.

Total RNA was isolated from PBMNCs with an RNA isolation system (SV Total, Promega). After precipitation, pellets were dissolved in diethylpyrocarbonate (DEPC)-treated water and RNA concentration was determined by spectrophotometry. Total RNA was extracted from 250 μL of serum using reagent (Trizol LS, Invitrogen), and the pellet was dissolved in 10 μL of DEPC-water. In addition, 2 mL of serum and 2 mL of plasma were ultracentrifuged[7] over a 10% sucrose cushion at 100,000 × g for 17 hours at 4°C. Pellets were dissolved in 250 μL of TE buffer (10 mmol/L tris(hydroxymethyl)aminomethane-HCl, 10 mmol/L ethylenediaminetetraacetic acid; pH 7.5) and total RNA was isolated with reagent (Trizol LS, Invitrogen) and precipitated, and the pellet was dissolved in 10 μL of DEPC-water.

Amplification of the HCV RNA 5′ noncoding (NC) region (226 bp) was performed by quantitative real-time reverse transcription (RT)-PCR with fluorescence resonance energy transfer probes. Two microliters of total RNA isolated from 250 μL of serum or from 2 mL of ultracentrifuged serum or plasma, or 0.5 μg of total RNA from PBMNCs was retrotranscribed and amplified in a single-tube reaction containing RNA reaction mix (LightCycler Master Hybprobe, Roche Diagnostics), 3.25 μmol/L $Mn(OAc)_2$, 0.5 μmol/L of each primer, 0.2 μmol/L of the anchor probe, and 0.4 μmol/L of the sensor probe. RT was performed at 61°C for 30 minutes; the mixture was then heated at 95°C for 2 minutes; and then PCR was performed for 50 cycles of 95°C for 0 seconds (denaturation), 55°C for 12 seconds (annealing), and 72°C for 10 seconds (extension). The fluorescence was measured at the end of each annealing step. A standard curve constructed with 10-fold dilutions of a synthetic HCV RNA was used for quantification. The lower limit of HCV RNA detection of the assay was 10 IU/mL (mean threshold cycle [Ct], 39.32) with a lower limit of quantification of 100 IU/mL (mean Ct, 35.64), as determined by testing serial dilutions of HCV RNA–positive serum sample, in which HCV RNA quantification was previously assessed by an HCV test (Cobas TaqMan, Roche Diagnostics). PCR testing was considered positive if the Ct level was obtained at no more than 39 cycles, and quantification was achieved by reference to the standard curve.

To further confirm the results, the remaining total RNA extracted from positive samples was sent to a second laboratory (Centro Nacional de Microbiología [CNM]) and used to perform an additional amplification of the 5′ NC region of the HCV genome by nested RT-PCR.[14] The PCR products obtained (235 bp) were also purified and sequenced as described below. The sequences of the primers used in these tests are given in Table 1.

Sequencing and sequence analysis

PCR products from positive samples obtained either at the FEHV or at the CNM were purified by PCR clean-up

technology (Illustra Exoprostar 1 step, VWR International Eurolab), and both strands were directly sequenced using Sanger sequencing. Sequence analysis was performed on the fragment comprised between Nucleotide 104 and Nucleotide 287, which were present in all PCR products.

Statistical analysis

Categorical variables were compared using the chi-square test (or Fisher's exact test when applicable). Continuous variables were compared using the nonparametric Mann-Whitney's U test. All p values reported are two-tailed.

RESULTS

Infectious testing at the transfusion centers

Anti-HCV reactivity was recorded in two donors (0.1%), but none of them coud be confirmed on supplemental immunoblot testing. Therefore, all donors finally tested negative for anti-HCV. Markers of other infectious diseases were detected in six donors (0.3%): HBV DNA in two (0.1%), malaria in two (0.1%), syphilis in one, and cytomegalovirus in one. None of the 2007 donors gave a HCV NAT yield.

Anti-HCV testing

Forty-two samples (2.1%) displayed reactivity in the anti-HCV core test, and the eight samples reacting for any infectious marker in the blood bank screening did not account among them. Table 2 shows the AI values obtained for each sample (mean, 1.88; range, 1.20-6.20) and the individual results obtained from supplemental testing. Twelve reactive samples (28.6%) rendered PI of more than 50% in the inhibition test, which led us to consider the reactivity as due to a specific reaction involving some antibody species and some epitope represented in the linear peptide spanning Amino Acids 5 to 19 of the HCV core antigen. Reactivity to epitopes spanning Amino Acids 21 to 40 or 101 to 120 was found in 15 samples (35.7%). Seven samples (16.7%) reacted with at least one antigen in immunoblot: six samples were indeterminate (C1, E2, or NS4 lines; Samples 2, 19, 21, 26, 30, and 33) and one was positive (C1 and C2 lines; Sample 40). Criteria for anti-HCV positivity were satisfied by six samples (14.3%, 0.30% from the total; Samples 2, 19, 21, 30, 33, and 40), and one sample was considered indeterminate because of isolated reactivity to NS4 antigen in immunoblot (Sample 26).

HCV RNA detection

First, HCV RNA was tested in 250 μL of serum, in 2 mL of ultracentrifuged serum, and in PBMNCs from the 42 anti-HCV core–reactive donors and from 100 anti-HCV core–negative donors. RNA testing was negative in all sera (250 μL) and PBMNCs, but was positive (Ct value, 37.11; 10-100 IU/mL) in the 2 mL of ultracentrifuged serum from one donor who tested indeterminate for anti-HCV by immunoblot (Donor 26, Table 2). Viral RNA was also then tested by a different laboratory worker, who was unaware of the previous results, in 2-mL ultracentrifuged plasma samples from the 142 donors mentioned above. Donor 26 tested also positive in plasma (Ct value, 36.84: 10-100 IU/mL), as well as two additional donors (Donors 14 and 19; Ct values, 37.48 and 37.73, respectively) who had tested negative in ultracentrifuged serum. Positive results were obtained in different runs on different days, excluding intersample cross-contamination. Donor 14 did not meet the tentative criteria for anti-HCV positivity, but Donor 19 met them and tested indeterminate (single E2 line) in immunoblot. The 5′ NC region of these three donors was again amplified from the remaining FEHV-concentrated plasma in CNM, by nested RT-PCR, with positive results.

Overall, three of 42 (7.1%) anti-HCV core–reactive donors displayed low amounts of circulating viral RNA, while none of the 100 anti-HCV core–negative donors tested positive ($p = 0.025$). HCV RNA was also tested in ultracentrifuged serum, plasma, and PBMNC samples from the two donors yielding reactivity for anti-HCV in the screening tests at the blood banks, and all samples tested negative.

Sequencing and sequence analysis

Analysis of the sequences of the PCR products identified all isolates as HCV Genotype 1b (BLAST analysis, NCBI). Donors 14 and 19 shared an identical nucleotide sequence for the genome fragments amplified independently in two laboratories. Identity reached 100% in the BLAST analysis performed with 94 sequences of the NCBI database, including the sequence used as reference in the alignment (Fig. 1). In contrast, the sequence from Donor 26 displayed six nucleotide changes (C114G, A116C, G253C, G256C, G271C, and A276G) in comparison with the former. Substitutions C114G and G256C were detected in fragments amplified from serum but not from plasma, and substitutions A116C and G253C were found only in the product amplified at one of the two laboratories (CNM).

Liver function tests

Abnormal liver function tests were recorded in 306 of 2007 (15.3%) donors. Altered transaminase levels were detected in one of five (20%) anti-HCV core–positive donors and in five of 37 (13.5%) among the remaining anti-HCV core–reactive donors (four males and two females). The frequency of anti-HCV core reactivity did not display significant differences in regard to this feature (6/306 with altered values vs. 36/1701 with normal values).

DISCUSSION

The prevalence of anti-HCV among blood donors declined dramatically in Spain during the past decade and was

TABLE 2. Results obtained on individual samples from anti-HCV core–reactive donors

			Anti-HCV core test and inhibition assay			Supplemental anti-HCV core ELISA*						
						21-40 test		101-120 test		Supplemental anti-HCV immune blot	Interpretation of anti-HCV testing	HCV RNA in plasma (2 mL), IU/mL
Donor	Sex	AST/ALT/ GGTP (IU/L)†	Abs.	AI	PI (%)	Abs.	AI	Abs.	AI			
1	Female	8/9/18	0.206	1.67	9.8	0.394	**1.76‡**	0.299	**1.77‡**	Negative	Negative	0
2‡	Male	10/17/35	0.310	2.53	**75.7‡**	0.060	0.44	0.073	0.43	**C1 (1+)‡**	**Positive‡**	0
3	Female	11/18/21	0.766	6.20	**90.5‡**	0.106	0.77	0.112	0.66	Negative	Negative	0
4	Female	6/6/14	0.348	2.63	**79.0‡**	0.075	0.55	0.100	0.59	Negative	Negative	0
5	Male	11/14/22	0.476	3.59	**80.1‡**	0.068	0.50	0.092	0.54	Negative	Negative	0
6	Male	25/54/29	0.176	1.39	20.1	0.134	0.60	0.120	0.71	Negative	Negative	0
7	Female	18/20/25	0.142	1.21	<5	0.073	0.53	0.146	0.82	Negative	Negative	0
8	Male	14/27/65	0.167	1.42	<5	0.262	1.17	0.244	**1.44‡**	Negative	Negative	0
9	Female	14/11/11	0.229	1.78	12.5	0.105	0.77	0.126	0.74	Negative	Negative	0
10	Male	24/22/16	0.202	1.79	<5	0.075	0.55	0.101	0.59	Negative	Negative	0
11	Female	17/13/27	0.150	1.33	<5	0.304	**1.67‡**	0.424	**2.39‡**	Negative	Negative	0
12	**Male‡**	**36§**/36/24	0.175	1.53	11.3	0.109	0.48	0.102	0.60	Negative	Negative	0
13	Male	12/14/18	0.250	2.18	**70.0‡**	0.064	0.47	0.080	0.47	Negative	Negative	0
14‡	Male	16/19/20	0.139	1.21	<5	0.085	0.62	0.164	0.92	Negative	Negative	**Positive (<100)‡**
15	Male	19/17/18	0.192	1.49	**52.2‡**	0.087	0.47	0.157	0.88	Negative	Negative	0
16	Female	14/15/13	0.155	1.20	<5	0.082	0.60	0.240	**1.36‡**	Negative	Negative	0
17	Female	29/26/14	0.157	1.21	10.4	0.199	1.09	0.306	**1.73‡**	Negative	Negative	0
18	Male	18/28/24	0.171	1.23	11.2	0.076	0.41	0.130	0.73	Negative	Negative	0
19‡	Male	17/19/23	0.220	1.81	**67.5‡**	0.069	0.51	0.096	0.57	**E2 (1+)‡**	Positive	**Positive (<100)‡**
20	Female	16/15/23	0.225	1.86	32.3	0.068	0.50	0.092	0.54	Negative	Negative	0
21‡	Female	30/22/21	0.202	1.66	**66.9‡**	0.082	0.36	0.082	0.49	C1 (1+)	Positive	0
22	Female	14/18/16	0.218	1.82	<5	0.273	**1.22‡**	0.210	**1.24‡**	Negative	Negative	0
23	Female	20/17/18	0.181	1.51	10.1	0.268	**1.20‡**	0.286	**1.69‡**	Negative	Negative	0
24	Male	26/39/31	0.147	1.22	<5	0.061	0.33	0.160	0.90	Negative	Negative	0
25	Male	32/33/40	0.166	1.38	<5	0.105	0.57	0.195	1.10	Negative	Negative	0
26‡	Female	17/24/12	0.643	5.35	<5	0.059	0.43	0.075	0.44	NS4 (1+)	Negative	**Positive (<100)**
27	Female	18/21/18	0.189	1.40	<5	0.287	**1.57‡**	0.419	**2.37‡**	Negative	Negative	0
28	Female	19/28/**40‡**	0.224	1.66	38.1	0.206	0.92	0.129	0.76	Negative	Negative	0
29	Male	29/**53§**/21	0.171	1.44	**60.5‡**	0.131	0.59	0.127	0.75	Negative	Negative	0
30‡	Male	32/28/20	0.202	1.70	**51.0‡**	0.099	0.44	0.092	0.54	**E2 (1+)‡**	**Positive‡**	0
31	Male	30/40/39	0.167	1.46	7.8	0.122	0.67	0.175	0.99	Negative	Negative	0
32	Male	25/28/26	0.190	1.60	<5	0.533	**2.92‡**	0.675	**3.81‡**	Negative	Negative	0
33‡	Female	17/15/20	0.238	1.99	**67.3‡**	0.072	0.39	0.142	0.80	**C1 (1+)‡**	Positive‡	0
34	Male	21/22/18	0.154	1.25	8.0	0.446	**2.43‡**	0.343	**1.82‡**	Negative	Negative	0
35	Female	25/28/**118‡**	0.165	1.40	6.2	0.250	**1.36‡**	0.271	**1.44‡**	Negative	Negative	0
36	Female	23/18/24	0.165	1.40	<5	0.524	**2.86‡**	0.551	**2.92‡**	Negative	Negative	0
37	Male	13/16/20	0.224	1.88	**67.6‡**	0.089	0.49	0.101	0.54	Negative	Negative	0
38	Male	24/16/19	0.188	1.56	6.7	0.320	**1.75‡**	0.326	1.72‡	Negative	Negative	0
39	Male	19/19/30	0.262	2.02	<5	0.166	0.90	0.161	0.85	Negative	Negative	0
40‡	Female	17/16/16	0.176	1.51	<5	0.416	2.27‡	0.370	**1.96‡**	**C1 (1+), C2 (1+)‡**	**Positive‡**	0
41	Male	22/28/47	0.261	2.10	<5	0.620	**3.38‡**	0.674	**3.57‡**	Negative	Negative	0
42	Female	12/13/23	0.293	2.52	<5	0.075	0.41	0.078	0.41	Negative	Negative	0

* Additional HCV core peptide antibody reactivity against epitopes spanning Amino Acids 21-40 and/or 101-120 of the HCV core sequence (European Patent EP2258714B1).
† Normal values for AST, ≤34 IU/L; ALT, males ≤40 IU/L, females ≤32 IU/L; normal values for GGTP, males ≤50 IU/L, females ≤30 IU/L).
‡ Values above the normal level.
Bold highlight means significant reactivity in the test.

recently reported 0.013% from 1.7 million first-time donors.[15] Among the general population of adults, the overall prevalence is more than 100-fold higher (1.8%).[16] The small size of the sample studied might account for the lack of anti-VHC–positive donors resulting in this study from the screening performed at the transfusion centers.

In contrast, anti-HCV core testing selected 42 donors to investigate, and supplemental studies could identify, according to the criteria explained, five donors displaying prior contact with HCV but not actual infection and three donors displaying OCI (Table 3). The yield of this alternative testing was 0.39% (8/2007). Most of the donors (34/42, 81%) lacked, therefore, any evidence of prior contact with

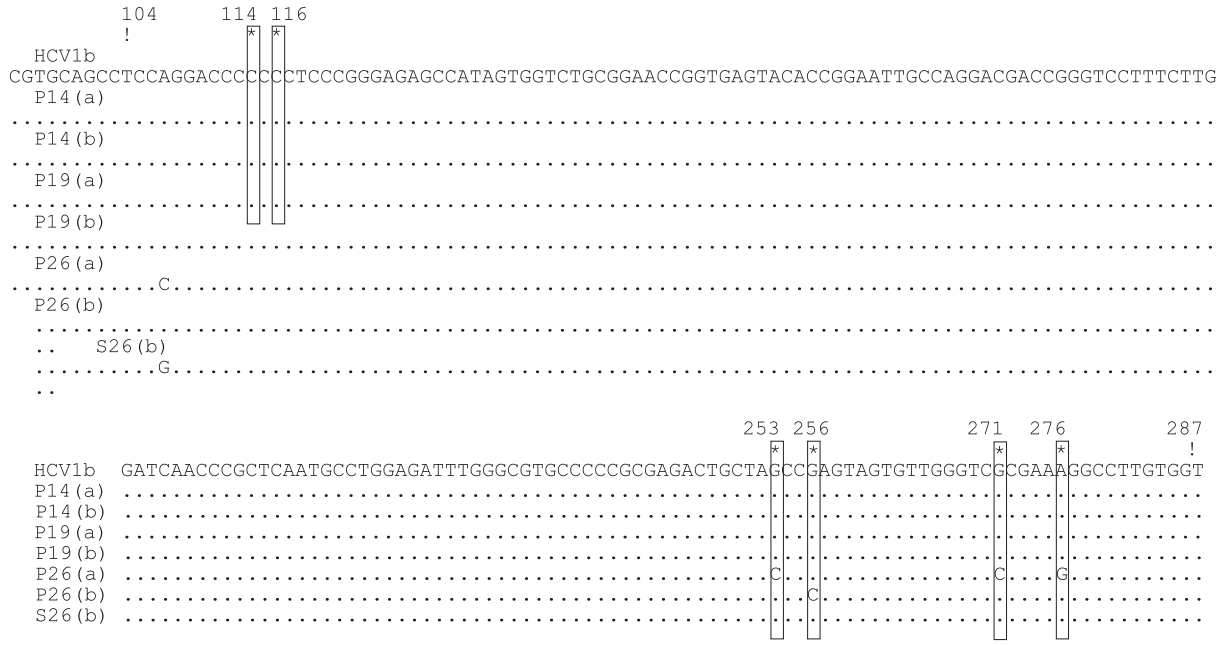

Fig. 1. Sequence comparison of PCR products amplified from plasma and serum of the three positive donors. Nucleotide positions and sequence of HCV 1b were taken from AB249644. (A) PCR products obtained at the CNM; (B) PCR products obtained at the FEHV. P = plasma sample; S = serum sample.

TABLE 3. Interpretation of the results shown in Table 2

Anti-HCV present	Conventional HCV infection	OCI	Number of donors (donor number)
No	No	No	34
Yes	No	No	5 (2, 21, 30, 33, 40)
Yes	No	Yes	1 (19)
No	No	Yes	2 (14, 26)

HCV, and these false-positive cases represented 1.7% of the sample studied.

The novel assay could actually detect anti-HCV where some current assays did not, including some assays using a sample dilution lower than 1:10. These findings would suggest that minute amounts of antibody directed toward an epitope present in the single peptide used could be more prone to be detected when the specific activity of the target is not diluted in the multiantigen design of the solid phase of a conventional immunoassay (European Patent EP2258714B1).[10] The results obtained by immunoblot supported the interpretation for seven samples, and recording of isolated reactivity to core antigens by INNOLIA among four of them was of interest.[17] This advantage was, however, jeopardized by the finding of a large number of false-positive results, which might be elicited by some human protein displaying cross-reactivity with epitopes of this region of the HCV core protein.[18-20] Alternatively, presence of maturation-altered or low-avidity anti-HCV[21-23] could explain the results, as suggested previously for OCI.[10]

Although sample concentration by ultracentrifugation does not account among the procedures standardized for HCV diagnosis by PCR, it offers a valuable research tool for improving the knowledge of the natural history of the HCV infection. Sample contamination, either from external sources before testing or from sample to sample during testing, must be carefully considered to discuss the results obtained with this approach. Three facts would support the results of this study in regard to this important issue: 1) positive results were obtained in different runs on different days; 2) negative controls, the rest of the samples included in each PCR run, and 100 anti-HCV–negative donors tested negative; and 3) results were reproduced from the RNA extracts by a second laboratory using a different set of primers and a totally independent aliquot of sample, which is of special significance for supporting the results obtained on Sample 14 (presence of HCV RNA in absence of anti-HCV). Sequencing confirmed the identity of the products as corresponding to the expected region of HCV Genotype 1b, a viral genotype common in Spain.[24] The fragments displayed, in addition, sequence diversity, which excludes contamination from a common source. Given that the 5′ NC region of the HCV genome is highly conserved,[25] the sequence identity found for two of the three isolates would fall within the expected and should not be necessarily interpreted as reflecting sample-to-sample contamination. In regard to the interlaboratory discrepancy found in the sequencing of the third isolate, it

might represent an artifact of the amplification procedures or could alternatively reflect differential amplification of viral quasispecies present in the sample.

In conclusion, these findings reinforce the notion of OCI as part of the natural history of the HCV infection,[26-30] although a couple of points remain obscure. First, the discrepancy between the results obtained by PCR on samples of serum and plasma taken at once from the same donor, which perhaps reflects unknown technical issues that could be critical when the viral load is extremely low. Second, the differences found in the sequence of the fragments amplified from samples from Donor 26 at the two participating laboratories, which can respond either to technical or biologic reasons. Third, the weak antibody response and the very low viral load found at once in these donors, which is a feature characteristic of OCI that awaits satisfactory explanation in terms of the putative pathogenic mechanisms leading to it. Although not conclusive yet, the findings of this report suggest that the safety of the blood supply might be improved by the anti-HCV core test used in the study and would justify future studies with larger samples of traceable donors who can be fully characterized and submitted to follow-up studies after selection by the novel test. In regard to such future investigations, collecting and storing PBMNCs for performing cellular recall HCV antigen studies would be of interest, and the evaluation of the outcome of anti HCV core–positive units among their recipients must be regarded in these studies as an ethical commitment.

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CONFLICT OF INTEREST

Fundación para el Estudio de las Hepatitis Virales holds patent grants related to occult HCV detection. RP is scientific founder and stockholder of DIATER S.A., and JA is an employee of the company. The remaining authors have disclosed no conflicts of interest.

AUTHOR CONTRIBUTIONS

JAQ, AA, JB, IC, and JA performed experiments, analyzed results, and prepared tables and figures; MAA, EF, MIG, RG, SP, and LAR performed mandatory testing at transfusion centers and managed the sample delivery logistics; and RP, VC, and JME designed the study and wrote the manuscript.

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