Brazilian Journal of Medical and Biological Research (2002) 35: 589-598
ISSN 0100-879X

Hepatitis C virus infection among Brazilian hemophiliacs: a virological, clinical and epidemiological study

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Abstract

We determined and analyzed risk factors of hepatitis C virus (HCV)-infected Brazilian hemophiliacs according to their virological, clinical and epidemiological characteristics. A cross-sectional and retrospective study of 469 hemophiliacs was carried out at a Brazilian blood center starting in October 1997. The prevalence of HCV infection, HCV genotypes and factors associated with HCV RNA detection was determined. The seroprevalence of anti-HCV antibodies (ELISA-3.0) was 44.6% (209/469). Virological, clinical and epidemiological assessments were completed for 162 positive patients. There were seven (4.3%) anti-HCV seroconversions between October 1992 and October 1997. During the same period, 40.8% of the positive anti-HCV hemophiliacs had abnormal alanine transaminase (ALT) levels. Plasma HCV RNA was detected by nested-RT-PCR in 116 patients (71.6%). RFLP analysis showed the following genotype distribution: HCV-1 in 98 hemophiliacs (84.5%), HCV-3 in ten (8.6%), HCV-4 in three (2.6%), HCV-2 in one (0.9%), and not typeable in four cases (3.4%). Univariate analysis indicated that older age (P = 0.017) and abnormal ALT levels (P = 0.010) were associated with HCV viremia, while the presence of inhibitor antibodies (P = 0.024) and HBsAg (P = 0.007) represented a protective factor against the presence of HCV RNA. These findings may contribute to a better understanding of the relationship between HCV infection and hemophilia.

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Research partially supported by FAPEMIG (Nos. CBS 219896 and CBS 67497).

Received August 8, 2001
Accepted March 15, 2002

Key words
- Hepatitis C virus
- HCV genotypes
- Hemophilia
- Chronic liver disease
- Transfusion
- Brazil

Introduction

Since its discovery in 1989 (1), hepatitis C virus (HCV) has emerged as a major cause of chronic liver disease worldwide. It is transmitted primarily by the parenteral route, and sources of infection include blood transfusions or use of blood-derived products, injectable drug use and needle-stick accidents. Sexual and perinatal transmission have been demonstrated but are less frequent. More than 60% of the individuals exposed to HCV develop chronic infection. Approximately 20 to 30% of the chronically infected individuals will develop liver cirrhosis and/or hepatocellular carcinoma when followed for 20 to 30 years (2).

Since 1990, screening of blood donors

for antibodies to HCV has reduced the incidence of transfusion-associated hepatitis C (3). Patients with hemophilia were at high risk of post-transfusion hepatitis because of widespread use of plasma-derived products prior to the introduction of virucidal treatment. As a consequence, hepatitis C is by far the most common cause of chronic liver disease among hemophiliacs.

The frequency of detectable antibodies to hepatitis C (anti-HCV) in hemophiliac patients has been investigated in many countries. It has varied according to the sensitivity of the assay employed and, particularly, with the severity of the deficiency and hence the extent of need for and use of factor replacement. Almost all hemophiliacs treated with non-virus-inactivated factor concentrates have anti-HCV antibodies and up to 90% show evidence of persistent viremia and elevated alanine transaminase (ALT) levels (4). The use of expensive lyophilized hemoderivatives was restricted almost exclusively to developed countries until the late eighties. In Brazil, imported lyophilized concentrates of factors VIII and IX became systematically available to be used for coagulation factor replacement therapy only in the nineties, after the implementation of virucidal methods in their manufacturing.

HCV has been classified into six major genotypes on the basis of variations in the nonstructural NS-5 region of the viral genome (5). It has been suggested that genotype may influence progression of chronic liver disease. Some studies have indicated that HCV genotype 1 is associated with more aggressive liver disease (6,7), but this has not been a consistent finding. In addition to clinical differences, the various genotypes have distinct geographical distributions. Genotypes 1, 2 and 3 predominate in Northern Europe and North America, whereas type 4 is found in the Middle East and North Africa, type 5 in South Africa and type 6 in parts of the Far East (8). Few studies have been carried out in South America. The predominance of HCV genotype 1 among non-hemophiliac patients has been observed in this region, followed by genotype 3 and fewer cases of HCV genotypes 2, 4 and 5 (9-11). Mixed infection with several genotypes has also been documented in a small minority of patients. The only four studies which assessed HCV genotype distribution in Latin American hemophiliac patients have indicated the predominance of HCV genotype 1 (11-14).

The objectives of the present study were to determine HCV prevalence, HCV genotypes and factors associated with HCV RNA detection among hemophiliac patients in the State of Minas Gerais, Brazil, as of October 1997.

Material and Methods

Patient selection

All male hemophiliac patients attending the Fundação Hemominas, the main blood center in the State of Minas Gerais, Brazil, from January 1985 (beginning of registration) through October 1997, were eligible for the study. Among 532 hemophiliacs registered at the center during this period, 469 (88.2%) were alive. Since October 1992, each hemophiliac registered at the center was tested for anti-HCV at least twice a year. All living male hemophiliac patients with positive anti-HCV-ELISA-3.0 as of October 1997 were included in the study in order to obtain serological confirmation and to complete virological, clinical and epidemiological assessment.

Clinical data

Clinical information was collected from patient treatment records and by clinical examination between October 1997 and October 1998. The protocol was approved by the institutional Ethics review board, and the participants or legal guardians gave

written informed consent.

Collected information included age, type of hemophilia (A or B), severity of hemophilia (classified as severe if factor VIII or IX blood levels were less than 1%, moderate if they were between 1 and 5% and mild if they were greater than 5%), presence of inhibitory antibodies (anti-factor VIII or anti-factor IX), time since diagnosis of hemophilia, intensity of treatment (measured as number of visits to the blood center to receive hemotherapy in the last two years before the study), and blood product type previously used. The previous occurrence of acute clinical hepatitis or jaundice and anti-HCV seroconversion status between October 1992 and October 1997 were investigated. After admission of the hemophiliacs to the blood center, serum ALT was measured at least twice a year. In this study, ALT status was classified according to the pattern over the preceding years (from 1 to 12 years, with a minimum of three determinations/patient): normal, if ALT levels were persistently below 40 IU/l, defined as the upper limit of normal (ULN); intermittently raised, if the levels were ever above the ULN, and persistently raised, if all values were above the ULN. The occurrence of other risk factors for liver disease, such as hepatitis B virus (HBV) infection (presence of HBsAg), alcohol abuse (intake of more than 40 g/day), history of schistosomal infection and/or hepatotoxic drug use, was investigated. Liver failure was characterized as hypoalbuminemia (<3.0 g%), hypoprothrombinemia (<60%) and occurrence of any clinical signs of severe liver disease (e.g., ascites, jaundice, encephalopathy, portal hypertension).

Exposure to other known risk factors associated with HCV infection was also assessed: injectable drug use, more than two sex partners/year, tattooing, presence of a household member with a history of hepatitis C, and needle-stick injury (health-care worker).

Serological data

The presence of anti-HCV antibodies was tested with a third-generation ELISA (HCV-ELISA-3.0) according to manufacturer instructions (Ortho-Clinical Diagnostics GmbH, Neckargemünd, Germany). Blood samples collected between October 1997 and October 1998 from the anti-HCV-ELISA-positive hemophiliacs were available for additional serological tests and HCV RNA analysis. The ELISA results were confirmed by recombinant immunoblot assay (anti-HCV-RIBA) according to manufacturer instructions (HCV-RIBA-3.0 SAI, Chiron Corporation, Emeryville, CA, USA). All patients were tested by standard techniques for other blood-borne infections such as HBV using anti-HBc-total and HBsAg, *Treponema pallidum* using ELISA or FTA-Abs, *Trypanosoma cruzi* using indirect immunofluorescence (IIF) plus indirect hemagglutination assay (IHA) plus ELISA, human immunodeficiency virus type 1 and type 2 (HIV-1/2) using ELISA-3.0 and Western blot for confirmation, and human T-lymphotropic virus type I and type II (HTLV-I/II) using ELISA and Western blot for confirmation.

Nested RT-PCR

Serum samples were stored at -20ºC. Serum RNA was extracted and HCV RNA was detected by reverse transcription followed by nested PCR using primers derived from the 5' noncoding region of the HCV genome as described by Oliveira et al. (12). Genotype was determined by RFLP analysis of the PCR product as previously described by Oliveira et al. (12). Negative control samples were always run in parallel (no-cDNA, anti-HCV negative controls and RNase-treated positive control).

Statistical analysis

Univariate analysis was carried out to

compare HCV RNA-positive and -negative hemophiliac populations. Categorical variables were assessed using the chi-square test (Yates corrected) or Fisher's exact test, and continuous variables using the Student *t*-test. Transaminase abnormality was treated as a categorical variable (persistent/intermittent/normal).

Results

Of the 469 live hemophiliac patients, 209 (44.6%) were anti-HCV-ELISA-3.0 positive as of October 1997. The serological, virological, clinical and epidemiological assessments were completed for 162 (77.5%) of them (N = 209). Of the 47 (22.5%) anti-HCV-positive hemophiliacs that did not participate in the study, one refused to collaborate with the study and the remaining 46 did not return to the blood center, even after mail and/or telephone contact. There were no significant differences between participants and nonparticipants in terms of age, type and severity of hemophilia, presence of inhibitory antibodies and pattern of ALT measurements.

The anti-HCV-ELISA-3.0 serologic status was confirmed with the anti-HCV-RIBA-3.0 antibody in 155 (95.7%) of the 162 hemophiliacs studied, while the remaining seven (4.3%) were indeterminate. There were no significant differences between groups (positive versus indeterminate anti-HCV-RIBA-3.0) in terms of HIV serologic status (OR, 1.32; 95%CI, 0.15-30.31; P = 0.634).

The median age of the anti-HCV-ELISA-3.0-positive hemophiliac population (N = 162) was 24 years. Most of the patients had type A hemophilia (87.0%), severe (55.6%) or moderate (38.9%) disease, no inhibitor antibodies (92.6%) and were diagnosed as hemophiliacs after one year of age (54.9%) (Table 1). Most of the anti-HCV-positive hemophiliac patients (53.7%) had visited the blood center to receive hemotherapy at least 26 times/year during the previous two years. In addition, all patients had received blood or blood products without viral inactivation in the past, mainly cryoprecipitate (92.0%), packed red blood cells (54.3%) and fresh frozen plasma (52.5%).

There were seven (4.3%) anti-HCV seroconversions between October 1992 and October 1997 and acute clinical hepatitis or jaundice was observed in 26 (16.0%) patients. During the same period, 40.8% of the anti-HCV-positive hemophiliacs had abnormal ALT levels (persistently raised in 13.0% and intermittently raised in 27.8%) (Table 1). Other main risk factors for liver disease observed in this HCV-infected population were alcoholism (13.0%), schistosomiasis (11.7%) and hepatotoxic drug use (4.9%) (Table 1). Prior HBV infection (positive anti-HBc-total antibody) was observed in 110 (67.9%) hemophiliacs, but only six (3.7%) had HBV-HCV co-infection (positive HBsAg). Five (3.1%) hemophiliacs had clinical and laboratory signs of liver failure. Other assessed HCV transmission factors were intra-familial hepatitis C in 34 hemophiliacs (21.0%), more than two sex partners/year in 12 (7.4%), tattooing in three (1.9%), injectable drug use in two (1.2%), and needlestick injury (health-care worker) in one (0.6%). Serologic markers for other infections transmitted by blood and blood products in this hemophiliac population were observed in 29 (17.9%) for HIV-1/2 (ELISA and Western blot), seven (4.3%) for HTLV-I/II (ELISA and Western blot), five (3.1%) for *Treponema pallidum* (ELISA or FTA-Abs), and four (2.5%) for *Trypanosoma cruzi* (IIF/IHA/ELISA) (Table 2).

HCV RNA was detected by nested RT-PCR in 116 patients (71.6%), including one patient with indeterminate anti-HCV-RIBA-3.0 antibody. RFLP analysis showed that the genotype distribution was: 98 hemophiliacs with genotype 1 (84.5%), 10 with genotype 3 (8.6%), three with genotype 4 (2.6%), and one with genotype 2 (0.9%). HCV was not typeable in four cases (3.4%).

Table 1. Univariate analysis of positive anti-hepatitis C virus (HCV) hemophiliacs in terms of positivity to HCV-nested-RT-PCR and other characteristics.

Characteristics	Number of patients					
	PCR+	PCR-	Total	Prevalent OR	(95% CI)	P values[d]
Type of hemophilia						
A	99	42	141	0.55	(0.15-1.90)	0.448
B	17	4	21	1.00		
Severity of hemophilia[a]						
Severe (<1%)	65	25	90	1.07	(0.51-2.25)	0.984
Mild/Moderate (≥1%)	51	21	72	1.00		
Age at time of diagnosis						
One year old	53	20	73	1.15	(0.55-2.42)	0.813
>1 year old	62	27	89	1.00		
Inhibitory antibodies						
Present	5	7	12	0.25	(0.06-0.95)	0.024*
Absent	111	39	150	1.00		
History of acute clinical hepatitis or jaundice						
Present	17	9	26	0.71	(0.27-1.89)	0.596
Absent	99	37	136	1.00		
ALT level						
Raised	55	11	66	2.87	(1.25-6.67)	0.010*
Normal	61	35	96	1.00		
Pattern of ALT elevation[b]						
Persistently	19	2	21	2.38	(0.41-17.78)	0.245
Intermittently	36	9	45	1.00		
Liver failure						
Present	4	1	5	1.61	(0.16-38.81)	0.561
Absent	112	45	157	1.00		
Schistosomiasis						
Yes	15	4	19	1.56	(0.45-5.94)	0.628
No	101	42	143	1.00		
HBsAg						
Positive	1	5	6	0.07	(0.00-0.66)	0.007*
Negative	115	41	156	1.00		
Alcoholism[c]						
Yes	17	4	21	1.80	(0.53-6.77)	0.448
No	99	42	141	1.00		
Regular use of hepatotoxic drug						
Yes	6	2	8	1.20	(0.21-8.97)	0.593
No	110	44	154	1.00		

OR = odds ratio, CI = confidence interval, RT-PCR = reverse transcription-polymerase chain reaction, ALT = alanine transaminase.
[a]According to lowest blood concentration of factor VIII or IX.
[b]Analysis restricted to those with raised ALT levels (total = 66).
[c]Intake of more than 40 g/day of alcohol.
[d]Chi-square test (χ^2), Yates corrected or Fisher's exact result.
*Statistically significant.

Univariate analysis of HCV viremia (PCR-positive versus PCR-negative) indicated that older age (P = 0.017) and abnormal ALT levels (P = 0.010) were associated with HCV viremia, while the presence of inhibitor antibodies (P = 0.024) and HBsAg (P = 0.007) were protective factors against the detection of HCV RNA (Tables 1 and 2).

Discussion

Patients with hemophilia were at high risk of post-transfusion hepatitis because of the large use of plasma-derived products before the mid to late 1980s, when effective methods of concentrate inactivation were introduced (4). At that time, this therapy was associated with a virtual certainty of transmission of viral hepatitis. Hepatitis C is still a major complication of coagulation factor replacement. The high prevalence of HCV infection observed in this hemophiliac population (44.6%) indicates that it is also a problem in Brazil. However, our study reports a lower prevalence compared to developed countries (8,15,16), where the widespread use of clotting factor concentrates in replacement therapy began before the availability of inactivated products.

In this study, the use of the anti-HCV-RIBA-3.0 as a supplemental test to the anti-HCV-ELISA-3.0 confirmed the high positive predictive value of the screening test for a population at high risk for HCV infection. There was confirmation of anti-HCV antibodies in 95.7% of the studied hemophiliacs, without any negative anti-HCV-RIBA. The occurrence of indeterminate results with this supplemental assay may be a consequence of: a) immunosuppression (i.e., HIV infection, immunosuppressive drug use, organ transplantation), b) window period in a

Table 2. Univariate analysis of positive anti-hepatitis C virus (HCV) hemophiliacs by their serological markers for other blood-borne infections and positivity of HCV-nested-RT-PCR.

Serological markers	Number of patients					
	PCR+	PCR-	Total	Prevalent OR	(95% CI)	P values[a]
Anti-HBc-total (ELISA)						
Positive	75	35	110	0.57	(0.25-1.33)	0.223
Negative	41	11	52	1.00		
Anti-HIV (ELISA and WB)						
Positive	22	7	29	1.30	(0.48-3.68)	0.738
Negative	94	39	133		1.00	
Anti-HTLV-I/II (ELISA and WB)						
Positive	4	3	7	0.51	(0.09-3.03)	0.315
Negative	112	43	155	1.00		
Syphilis (FTA-Abs or ELISA)						
Positive	5	0	5	-	-	0.184
Negative	111	46	157			
Trypanosoma cruzi (IHA/IIF/ELISA)						
Positive	3	1	4	1.19	(0.11-30.62)	0.681
Negative	113	45	158	1.00		

OR = odds ratio, CI = confidence interval, ELISA = enzyme-linked immunosorbent assay, WB = Western blot, IHA = indirect hemagglutination assay, IIF = indirect immunofluorescence, RT-PCR = reverse transcription-polymerase chain reaction, HIV = human immunodeficiency virus, HTLV-I/II = human T-lymphotropic virus type I or II, FTA-Abs = fluorescent treponemal antibody-absorption.
[a]Chi-square test (χ^2), Yates corrected or Fisher's exact test.

recent HCV infection, or, c) a false-positive screening test. We observed that HIV infection did not influence the results and that an indeterminate anti-HCV-RIBA-3.0 test did not exclude active infection, since one of the seven patients with an indeterminate test had detectable circulating HCV-RNA.

Descriptive analysis of the positive anti-HCV hemophiliacs revealed a young population (median age, 24 years old) compatible with a group with shorter life expectancy when compared to the general population. The predominance of severe hemophilia was expected in a population infected by HCV with such a high exposure. Similar to the general hemophiliac population, hemophilia A was the predominant type (87.0%). It should be noted that more than half (54.9%) of the studied hemophiliac population had the coagulation disorder diagnosed only after one year of age. This could be the result of difficult access to the health care system by this population. The high frequency of visits to the blood center demonstrated how often this population is exposed to transfusions and, consequently, to the large pool of blood-borne infections. We detected a high proportion of past exposure to HBV (67.9%), with 3.7% of active HBV infection (positive HBsAg), and important frequency of co-infection with HIV (17.9%). This latter association has been growing in importance because these patients are at a higher risk of progression to chronic liver disease than those infected with HCV alone (17-19). However, little is known about the effect of co-infection on progression of HIV disease, with some authors defending the idea of a more rapid progression to AIDS (19-21). Positivity for other serologic markers of blood-borne pathogens such as HTLV-I/II (4.5%), *T. pallidum* (3.1%) and *T. cruzi* (2.5%) demonstrated that these must always be investigated, mainly in endemic areas. All hemophiliacs have received whole blood or some blood products without any viral inactivation in their lifetime. The wide use of cryoprecipitate (92.0%), packed red blood cells (54.3%) and fresh frozen plasma (52.5%) in such young hemophiliac population demonstrated how precarious replacement therapy was in Brazil until a few years ago. This can explain the low rate of seroconversion to HCV infection (4.3%) during the study period (from October 1992 through October 1997), since a high number of hemophiliacs was already infected with HCV by past exposure to multiple transfusions. On the other hand, and paradoxically, this was the cause of a smaller prevalence of HCV, HIV, and other blood-borne infections in this Brazilian hemophiliac population when compared with hemophiliac populations in developed countries. In Brazil and in other developing countries, hemophiliacs were mostly treated with locally produced cryoprecipitate and fresh frozen plasma without viral inactivation treatment, but manufactured from fewer donors when compared to the imported lyophilized concentrates. Nowadays, only inactivated clotting factors are used in the replacement therapy of Brazilian hemophiliacs at blood centers.

The frequency of inhibitor antibodies, one of the main complications of replacement therapy, was similar to that observed in other hemophiliac populations (22). And the high frequency of intrafamilial HCV infection can be partially explained by the presence of other hemophiliac cases.

Only a small proportion of our hemophiliac population showed previous occurrence of clinical hepatitis or jaundice (16.0%), in agreement with other studies (8,23). The observation that most anti-HCV-positive hemophiliacs (59.9%) did not show abnormal ALT levels at any time during the study period was interesting, demonstrating the lack of a relationship between the degree of abnormality of serum transaminase levels and seropositivity for HCV. Most of the hemophiliacs with raised ALT levels (67.7%) showed an intermittent pattern, following the classical natural history of HCV infection. Liver failure was detected

in only 4.1% of the patients, probably due to the short period of observation in such a young population and the high mortality of those with advanced liver disease and AIDS before the beginning of this study. An important presence of alcoholism (13.0%) was observed. The association between schistosomiasis, hepatitis C and cirrhosis has been studied by other authors (24-26). The role of endemic schistosomal infection in this population (11.7%) needs to be better determined in further studies.

The finding of HCV-RNA positivity in 71.6% of this anti-HCV-positive hemophiliac population is in agreement with previous studies. Nevertheless, considering the possibility of transient HCV viremia, a higher rate of HCV-RNA detection might be observed if serial blood samples were obtained.

The distribution of HCV genotypes in the studied population did not significantly differ from the published data on hemophiliac and non-hemophiliac populations in Latin America, where genotype 1 infection is predominant (9-11,13,14). Actually, the distribution differs between Brazil and developed countries, where the distribution of HCV genotype among hemophiliacs usually reflects the origin of the blood donors used in the manufacture of pooled factor VIII and IX concentrates, with some of them imported from distant countries (27,28). In Brazil, the HCV genotype distribution would be more a reflex of the general Brazilian population from which hemoderivatives were manufactured before the introduction of viral inactivation, with a lack of exotic genotypes.

The association between HCV viremia and increased age found here in univariate analysis ($P = 0.017$) is controversial in the literature. Some authors (29) found a similar association, while others (30) did not. A longer time of exposure to HCV, with possible reinfections, and more advanced chronic liver disease could partially explain this association. The association between HCV viremia and raised ALT levels ($P = 0.010$) was described in hemophiliac populations by other authors (18,31). This information can be useful for clinicians who treat positive anti-HCV hemophiliacs but do not have any molecular method (e.g., PCR) available to determine the persistence of HCV infection. Raised ALT can be a clue of HCV viremia, an important piece of information for hemophiliacs, for whom there has been an obvious reluctance to perform liver biopsies due to the risk of bleeding complications (32).

The protective effect of chronic HBV infection (positive HBsAg) found in this study ($P = 0.007$) has also been described by other authors (33,34). It has been attributed to viral interference between HBV and HCV, with the mechanism still not elucidated. The finding of a protective effect of inhibitor antibodies to HCV viremia ($P = 0.024$) was of interest, although the numbers are very low and the statistical significance is consequently not at all robust. These antibodies are one of the most serious complications of hemophilia therapy and have been associated with other diseases such as chronic lymphocytic leukemia, Sjögren's syndrome and systemic lupus erythematosus (35). The role of the inhibitor antibodies in the host immunological response to HCV infection needs to be further studied.

In conclusion, this study provides evidence that HCV infection is emerging as a major problem for Brazilian hemophiliacs, especially when genotype 1 is involved, and shows that the detection of HCV viremia may be associated with some variables such as age, ALT level, active HBV co-infection, and inhibitor antibodies.

Acknowledgments

We are indebted to the members of the study staff at the participating institutions for careful data collection. The authors wish to thank Drs. Vanderson G. Rocha, Diana Taboada, Cláudia Maria Franco Ribeiro, Mitiko Murao, and Sônia Maria N. Silva, for their continuing support and helpful discussions.

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