Revista da Sociedade Brasileira de Medicina Tropical 38(6):496-502,nov-dez, 2005

Prevalence of hepatitis C virus infection and HCV genotypes among hemophiliacs in the State of Bahia, Northeastern Brazil: analysis of serological and virological parameters

Prevalência da infecção pelo vírus da hepatite C e genótipos entre hemofílicos no Estado da Bahia, nordeste do Brasil: análise de parâmetros sorológicos e virológicos

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ABSTRACT

The objective of the present study was to analyze HCV serological and virological parameters from hemophiliacs in the State of Bahia. Anti-HCV was investigated by ELISA in a cohort of 268 hemophiliacs A/B who were followed-up in a reference unit for hemotherapy in the State of Bahia. HCV viremia and genotypes were also determined from a subset of 66 anti-HCV seropositive hemophiliacs. Seroprevalence among hemophiliacs was 42.2% (95% CI 36.5-48.1) and was significantly higher ($p<0.05$) according to age ≥10 years, presence of factor VIII/IX inhibitory antibodies and other infection markers. None of the hemophiliacs less than 5 years of age were anti-HCV seropositive. Viremia was detectable in 77.3% (51/66). HCV genotype 1 (74%) was the most prevalent followed by genotype 3 (22%) and genotype 2 (4%). Our results indicate that HCV prevalence is still high among hemophiliacs, although HCV transmission was not observed in young hemophiliacs.

Key-words: *Hepatitis C virus. Hemophilia. Prevalence. Genotype. Bahia.*

RESUMO

O objetivo deste estudo foi analisar parâmetros sorológicos e virológicos em hemofílicos no Estado da Bahia. O anti-VHC foi investigado por ELISA em uma coorte de 268 hemofílicos A/B sob acompanhamento em uma unidade de referência do Estado da Bahia. A viremia do VHC e genótipos foram determinados em um subgrupo de 66 hemofílicos soropositivos para o anti-VHC. A soroprevalência do anti-VHC entre os hemofílicos foi de 42,2% (IC 95% 36,5-48,1) e foi associada significativamente ($p<0,05$) a idade ≥10 anos, presença de anticorpos antifator VIII/IX e outros marcadores sorológicos de infecção. Nenhum dos hemofílicos com idade inferior a 5 anos foram anti-VHC positivos. A viremia foi detectada em 77,3% (51/66), sendo o genótipo 1 do VHC (74%) o mais prevalente, seguido pelos genótipos 3 (22%) e 2 (4%). Nossos resultados indicam que a prevalência do VHC é ainda alta entre os hemofílicos, muito embora a transmissão não tenha sido observada entre os menores de 5 anos.

Palavras-chaves: *Vírus da hepatite C. Hemofilia. Prevalência. Genótipos. Bahia.*

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Informed consent was obtained from all subjects who participated in this study. Guidelines of the Ethical Committee of the Gonçalo Moniz Research Center, FIOCRUZ, were followed in the conduct of this research.

Financial support: This work was partially supported by research grants from CNPq, FIOCRUZ, FAPESB, and CAPES/FIOCRUZ (doctorate scholarship, between March 1999 to March 2003).

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Recebido para publicação em 28/9/2004
Aceito em 20/8/2005

Hepatitis C virus (HCV), a positive-stranded RNA virus, has been identified as the major causative agent of non-A non-B post transfusion hepatitis[8 17]. Prior to the introduction of screening candidate blood donors for antibody to hepatitis C virus (anti-HCV) and inactivation methods for pooled plasma products, nearly all hemophiliacs became infected with HCV soon after their first exposure[23]. In the early 1990s, data from around the world showed that the seroprevalence of anti-HCV antibodies in hemophiliacs could reach up to 90%[11 21 26 31 47 48], because virtually all clotting factor concentrates manufactured before the late 1980s were contaminated[22]. As a consequence, HCV is by far the most common cause of infection among hemophiliacs.

Since the mid 1970s, with evidence of the existence of non-A non-B hepatitis viruses and the early 1980s with the epidemic of human immunodeficiency virus (HIV), physicians involved in hemophiliac care and manufacturers of clotting factors have been aware of the risk of transmission of unknown viruses[12 13 15]. Consequently, methods have been developed to inactivate these viruses, which became accessible in the developed countries in the mid 1980s. It is now known that some of these methods such as pasteurization in liquid state (10 hr at 60°C), viral inactivation by the so-called solvent detergent (SD) and dry heat treatment up to 80°C were effective, while others were inappropriate[9 14 15 30 32 41]. Other risk factors for HCV infection in hemophiliacs include age, severity of disease, previous blood transfusion, annual quantity of clotting factor and infection with human immunodeficiency virus (HIV)[6 34].

In the majority of the developing countries, these inactivated products were introduced only in the mid 1990s, substituting locally produced cryoprecipitate and fresh-frozen plasma. In Brazil, seroprevalence of HCV infection in hemophiliacs can range from 44.6% to 60% according to studies conducted in Southeastern and Central areas of Brazil[6 24 34]. Carmo et al[6] published a follow-up study from the State of Minas Gerais, Southeastern Brazil, revealing a tendency towards a decrease in the general seroprevalence of HCV infection among hemophiliacs. However, the majority of seropositive hemophiliacs identified were viremic, indicating the necessity of specialized health care and treatment.

The study of the genetic variability of HCV strains have led to a consensus classification of six major genotypes, many of which include a number of closely related subtypes[7 44]. Studies suggest that the clinical features of liver disease depend on HCV genotypes[20 33 42 49]. It is also noteworthy that the success of interferon treatment seems to be genotype related[16]. These observations make the identification of infecting HCV genotypes from different geographical regions and groups under risk of great interest. Furthermore, genotypes can be a very useful tool for molecular epidemiology purposes.

Thus, the primary objective of the present study was to determine the hepatitis C virus (HCV) seroprevalence, viremia and genotypes isolated from a cohort of hemophiliacs in the State of Bahia, Northeastern Brazil.

PATIENTS AND METHODS

Patients. Between November 1999 and August 2000, the Hemotransfusion and Hemotherapy Foundation of Bahia (HEMOBA) attended a total of 339 patients with hemophilia A or B. However only 268 were tested in HEMOBA for the presence of anti-HCV by ELISA (3rd Generation, Abbott Murex, IL, USA) and became eligible for the study.

Laboratory data. Clinical and laboratory data were collected from patients' records and are summarized in Table 1. Serological tests for anti-HCV (Abbott Murex, IL, USA), HBsAg/anti-HBc (Biorad, CA, USA), anti-HIV (Biorad, CA, USA), anti-HTLV I/II (Abbott Murex, IL, USA), Chagas' disease (Embrabio, SP, Brazil) and Syphilis (Weiner Lab, Argentine) were based on ELISA according to each manufacturer's instructions. To test the coagulation factors and the presence of inhibitor antibody activity, the functional method based on the comparative activity assays against a factor deficient plasma was used according to the kit instructions (Biomérieux, NC, USA).

Samples for molecular assays. Of the total of anti-HCV positive hemophiliacs, only 66 patients returned to HEMOBA to receive treatment during the period of this study and were interviewed and had their serum collected for molecular assays. The Institution Ethical Committee approved this study and informed consent was obtained from all subjects enrolled in the study. Within 2 hours after venopuncture all samples were aliquoted and stored immediately at -70ºC until use. Aliquots were not thawed more than once prior to analysis.

Extraction of HCV RNA and cDNA synthesis. Two hundred microliters of serum were used for HCV RNA extraction using Trizol LS reagent (Invitrogen Life Technologies, CA, USA) following the manufacturer's instructions and were precipitated with ethanol and then dried[36]. HCV RNA was immediately transcribed into cDNA using random primers (Amersham Biosciences, NJ, USA). Samples with HCV RNA undetectable by nested-PCR described below were extracted twice in different experiments. Even if they were confirmed negative, all these patients were recalled to repeat the blood collection within six months after the first examination in order to avoid false negative results.

HCV RNA detection and genotyping. cDNA was targeted by a nested-PCR directed to the 5'UTR using specific primers 939, 209, 940 and 211 as described previously[7]. The size of the nested-PCR product was 251 bp. Positivity was confirmed by identification of this fragment after electrophoresis on a 1.5% routine agarose gel and ethidium bromide staining under UV light. Positive samples were genotyped by RFLP[10 28]. Briefly, restriction digests were carried out on the 251 bp PCR products for 4-16h after adjustment with 10x enzyme reaction buffer as appropriate. Reactions were at 37°C in the presence of 10 units each of (a) *Rsa*I and *Hae*III, and (b) *Hin*fI and *Mva*I. Digestion products were visualized under UV light after electrophoresis through a 4% Metaphor agarose gel (BMA, ME, USA) in 1 x Tris-borate buffer containing 0.5mg/ml ethidium bromide. Figure 1 illustrates the band pattern consistently produced by RFLP in different genotypes. Genotypes were determined according to Simmond's classification[44].

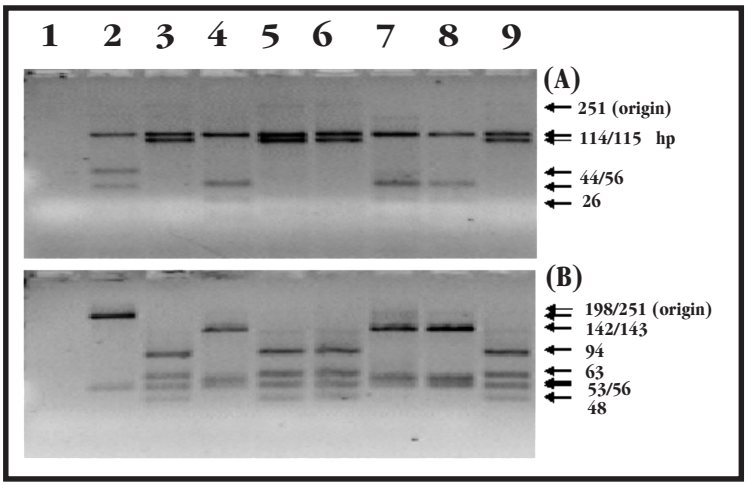

Figure 1- Electrophoresis through a 4% Metaphor agarose of restriction digests carried out on the 251 bp PCR fragment. Reactions were at 37°C in the presence of 10 units each of (A) RsaI and HaeIII, (B) HinfI and MvaI as described by McOmish et al[28] and Davidson et al[10 28]. Lane 1 - blank control; lane 2 - genotype 2 control; lane 3 - genotype 1 control; lane 4 - genotype 3 control; lanes 5, 6, 9 - genotype 1 samples and lanes 7, 8 - genotype 3 samples. Genotype was deduced from the banding patterns produced by the two restriction enzyme combinations.

Statistical analysis. Data were entered into two statistical database packages: Epi Info 6.04d (Center for Disease Control, GA, USA) and SPSS v 10 (IL, USA). Estimates for 95% confidence intervals (95% CI) of prevalence were calculated using the Mid-p algorithmic. Fisher's exact test and χ^2 test (Yates corrected) were used to compare frequencies between groups when appropriate. ANOVA was used to compare means or Kruskal-Wallis test when the variances were heterogeneous. In all tests, p values < 0.05 were considered statistically significant.

RESULTS

Baseline characteristics of the 268 hemophiliacs participating in this study are shown in Table 1. The mean age for all hemophiliacs was 19.5 ± 12.1 years old, age range from <1 to 66 years old, 238 (88.8%) were hemophilia A and 30 (11.2%) were hemophilia B, most live in the interior of the State (66.8%), the severity of disease was predominately mild to moderate (89.6%) and there was a high prevalence of factor VIII/IX inhibitory antibodies (35.7%).

Of the 268 hemophiliacs, 113 were found to be anti-HCV antibody positive resulting in an overall seroprevalence of 42.2% (95% CI 36.5-48.1). Other serological markers were also determined: HBV in 22.5%, HIV in 10.4%, HTLV I/II in 3.7%, Chagas' disease in 4.1% and syphilis in 1.1%. Almost half of the patients (128) presented at least one marker and 50% (64/128) of these presented multiple markers.

Seroprevalence for anti-HCV according to age is presented in Figure 2. Seroprevalence decreased significantly during the last decade. There were no seropositive cases among hemophiliacs younger than 5 years old. By bivariate analysis, anti-HCV antibody positivity was associated with age older than 10 years, residence in Salvador, the capital of the State,

Table 1 - Baseline characteristics of hemophiliacs participating in this study.

	Type of disease		
	hemophilia A	hemophilia B	Total
Characteristics	nº = 238 (88.8%)	nº = 30 (11.2%)	nº (%)§
Age (yr)			
mean ± σ	19.6 ± 12.3	18.5 ± 11.1	19.5 ± 12.1
range	<1 - 66	2 - 40	<1 - 66
Residence			
capital	79 (33.2)	10 (33.3)	89 (33.2)
other cities	159 (66.8)	20 (66.7)	179 (66.8)
Severity of disease			
severe (<1%)	18 (10.5)	2 (9.1)	20 (10.4)
mild/moderate (≥ 1%)	153 (89.5)	20 (90.9)	173 (89.6)
Factor VIII/IX inhibitory antibodies			
present	15 (41.7)	0 (0)	15 (35.7)
absent	21 (58.3)	6 (100)	27 (64.3)
Seropositivity			
anti-HCV	105 (44.1)	8 (26.7)	113 (42.2)
AgHBs/anti-HBc	53 (22.3)	7 (23.3)	60 (22.5)
anti-HIV	24 (10.1)	4 (13.3)	28 (10.4)
anti-HTLV I/II	10 (4.2)	0 (0)	10 (3.7)
Chagas' disease	9 (3.8)	2 (6.7)	11 (4.1)
syphilis	2 (0.8)	1 (3.3)	3 (1.1)

§ Totals vary according to the availability of data.

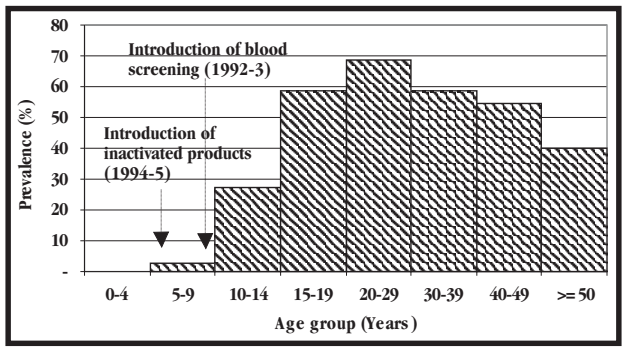

Figure 2 - Seroprevalence of anti-HCV antibody according to age in hemophiliacs in the State of Bahia, northeastern Brazil, 2000.

presence of inhibitory antibodies, and positivity to HBV, HIV, HTLV-I/II and Chagas' disease (*T. cruzi* infection) but not with the type of hemophilia, the severity of the disease or positivity to syphilis (Table 2).

From a subset of 66 hemophiliacs, HCV-RNA could be detected in 77.3% (51/66). The prevalence of HCV infection confirmed by the detection of HCV-RNA in hemophiliacs in Salvador is therefore estimated to be 32.6% (95% CI 24.3-42.6). However, this sample may not be representative, since the presence of HCV-RNA could not confirm any of the associations with potential risk factors found for anti-HCV+ hemophiliacs (Table 2).

Specimens from 50 hemophiliacs could be genotyped. Despite several attempts, one sample from a hemophilia patient could not be genotyped successfully. The distribution of HCV genotypes is shown in Table 3. HCV genotype distribution was similar to the distribution from local candidate blood donors (data not shown), with predominance of genotype 1 (74%), followed by genotype 3 (22%) and the rare genotype 2 (4%). As for viremia, the genotype could not be associated with any of the characteristics analyzed previously (Table 4).

Table 2 - Potential risk factors associated with the prevalence of anti-HCV and viremia of hemophiliacs in Salvador, BA, Brazil.

Characteristics	anti-HCV antibodies positivity				HCV-RNA detectable			
	nº tested§	% positive	OR (95% CI)	p values	nº tested §	% detectable	OR (95% CI)	p values
All subjects	268	42.2	-	-	66	77.3	-	-
Age, years								
≥10	198	56.1	43.48 (9.94-266.79)	<0.01	65	78.5	7.43 (0.47-224.72) ¥	NS¶
< 10†	70	2.9	1.00		1	0.0	1.00	
Residence								
capital	89	59.6	2.92 (1.73-4.94)	<0.01	38	76.3	0.88 (0.23-3.30)	NS¶
other cities†	179	33.5	1.00		28	78.6	1.00	
Type of disease								
hemophilia A	238	44.1	2.17 (0.93-5.07)	NS	60	78.3	1.81 (0.20-13.90)	NS¶
hemophilia B†	30	26.7	1.00		6	66.7	1.00	
Severity of hemophilia								
severe (<1%)	20	55.0	2.36 (0.85-6.62)	NS	6	66.7	0.47 (0.05-4.60)	NS¶
mild/moderate (≥1%)†	173	34.1	1.00		37	81.1	1.00	
Inhibitory antibodies								
present	15	60.0	4.29 (0.93-20.88)	0.06‡	7	85.7	4.00 (0.15-186.31)	NS¶
absent†	27	25.9	1.00		5	60.0	1.00	
Other viral markers								
AgHBs/anti-HBc positive	60	81.7	9.95 (4.60-21.97)	0.01	28	85.7	2.44 (0.59-10.75	NS¶
negative†	207	30.9	1.00		38	71.1	1.00	
anti-HIV positive	28	85.7	10.18 (3.18-36.24)	<0.01¶	15	80.0	0.81 (0.15-3.95)	NS¶
negative†	240	37.1	1.00		51	76.5	1.00	
anti-HTLV I/II positive	10	100.0	16.50 (2.16-347.00)¥	<0.01¶	7	71.4	0.71 (0.10-6.08)	NS¶
negative†	258	39.9	1.00		59	78.0	1.00	

OR = odds ratio and CI confidence interval. †Subjects in this category served as reference group. § Totals vary according to the availability of data. ‡Inhibitory antibodies were significantly associated with anti-HCV positivity by Mantel-Haenszel. ¥OR was estimated by adding 1 in each cell. ¶Fisher e-test.

Table 3 - Prevalence of HCV genotypes in hemophiliacs in State of Bahia, Northeastern Brazil.

	Nº (%) of isolates of the genotype			
Exposure category	All	Type 1	Type 2	Type 3
Hemophiliacs	50 (100.0)	37 (74.0)	2 (4.0)	11 (22.0)

Note: Genotyping by RFLP analysis of the PCR product from the 5'UTR as described previously[10 28]

Table 4 - HCV genotype and potential risk factors in hemophiliacs in Salvador-BA, Brazil.

	HCV genotypes				
Characteristics	Nº tested §	Type 1	Type not-1	OR (95% CI)	p values
All subjects	50	37 (74.0)	13 (26.0)	-	-
Age, years					
≥10	50	37 (74.0)	13 (26.0)	undefined	NS
< 10†	0				
Residence					
capital	28	20 (71.4)	8 (28.6)	0.74 (0.16-3.20)	NS
other cities†	22	17 (77.3)	5 (22.7)	1.00	

continue...

Table 4 - Continuation.

Characteristics	HCV genotypes				
	No tested §	Type 1	Type not-1	OR (95% CI)	p values
Type of disease					
hemophilia A	46	35 (76.1)	11 (23.9)	3.18 (0.27-37.96)	NS¶
hemophilia B†	4	2 (50.0)	2 (50.0)	1.00	
Severity of hemophilia					
severe (<1%)	5	4 (80.0)	1 (20.0)	1.33 (0.10-37.59)	NS¶
mild/moderate (≥ 1%)†	28	21 (75.0)	7 (25.0)	1.00	
Inhibitory antibodies					
present	5	4 (80.0)	1 (20.0)	8.00 (0.16-1,826.20)	NS¶
absent†	3	1 (33.3)	2 (66.7)	1.00	
Other viral markers					
AgHBs/anti-HBc positive	25	20 (80.0)	5 (20.0)	1.88 (0.43-8.42)	NS
negative†	25	17 (68.0)	8 (32.0)	1.00	
anti-HIV positive	12	8 (66.7)	4 (33.3)	0.62 (0.12-3.23)	NS¶
negative†	38	29 (76.3)	9 (23.7)	1.00	
anti-HTLV I/II positive	4	4 (100.0)	0 (0.0)	1.97 (0.19-48.99) ¥	NS¶
negative†	46	33 (71.7)	13 (28.3)	1.00	

OR = odds ratio and CI confidence interval. †Subjects in this category served as reference group. § Totals vary according to the availability of data. ¥OR was estimated by adding 1 in each cell. ¶Fisher e-test.

DISCUSSION

The overall prevalence of anti-HCV in our population of hemophiliacs (42.2%) was similar to other studies performed in different regions of Brazil[4 6 34]. However, because most of our patients were also infected with HIV and HTLV I/II, it cannot be excluded that the detection rate of anti-HCV in this group may underestimate the real seroprevalence, as has been demonstrated previously[18].

With the use of HCV-safe clotting products and the introduction of screening tests in the blood banks, there is a very important tendency towards decrease of seropositivity for anti-HCV antibodies among hemophiliac patients in Brazil and other countries[4 6 32]. In our study, HCV seroprevalence among hemophiliacs younger than 10 years old was quite similar to that found among candidate blood donors screened at HEMOBA (1.5%, personal communication) during the same period of time. However, it will need another five to ten years to confirm that young hemophiliacs are really protected from HCV infection.

Residence in Salvador, the state capital, was shown to be an independent risk factor for acquiring HCV but not for developing chronic infection. This association may be particularly influenced by age, access to the service, time of residence in the city and other confounding factors (data not shown), and more exploratory analysis is necessary. Although the risk of HCV infection from environmental exposure (not related to transfusion of blood or derivates) is low, it is not altogether absent. In Bahia, Silva et al (1995) studied the seroprevalence of anti-HCV in urban and rural populations and demonstrated that HCV was 1.5% prevalent in Salvador and absent in a city of the interior. This data suggests that HCV can be an endemic urban disease.

Even though the detection of anti-HCV could be used as a part of this study, these results should be interpreted with some care. Anti-HCV antibodies were associated with serological markers for other blood-borne infections (HBV, HIV and HTLV-I/II). These findings surely reflect the similar modes of transmission. However, it is still unclear whether HIV infection can interfere negatively by immune-suppression of some individuals, which could increase the uncertainty of our results and lead to underestimation of the prevalence[6]. Some investigators have reported HCV seronegative candidate blood donors or patients that were HCV-RNA positive by PCR, specially when associated with HIV and low CD4 cells counts ($<200x10^9/l$)[3 43 45]. On the other hand, the possibility of false positive results caused by unspecific reactions by the kits cannot be excluded. Therefore, HCV viremia and genotyping were also investigated in hemophiliacs.

HCV-RNA could be detected in the majority of anti-HCV positive hemophiliacs (77.3%) which is compatible to the chronicity rates reported for this infection[2]. Hence, about 20% of HCV-infected hemophiliacs seem to have resolved HCV infection. It cannot be ruled out that some hemophiliacs have viral replication below the detection limit of polymerase chain reaction (PCR). To increase the sensitivity of PCR some authors have suggested the application of the PCR to detect HCV viremia in whole blood instead of in serum[27 39 40]. Furthermore, a new promising methodology base on transcription-mediated amplification has become available for more accurate HCV-RNA detection[37 38].

While certain risk factors could be identified for anti-HCV hemophiliacs in Salvador, no significant association could be linked to the presence of HCV-RNA in serum or the HCV genotypes. Some studies have reported that HCV viremia was associated with older age and abnormal ALT levels, while the presence of inhibitory antibodies and HBsAg were protective factors against the detection of HCV-RNA. The mechanisms for this have been not explained[6], and we have not found significant evidence in favor.

In our study, HCV genotype 1 was most frequent (74%), followed by genotype 3 (22%) and genotype 2 (4%). Although heterotypic superinfection and mixed infections of hepatitis

C virus may be possible[46], the distribution of HCV genotypes among the hemophiliacs was similar to the candidate blood donors (data not shown). Similar results have already been reported for other regions in Brazil in the same exposure group[4 5 25]. Considering the dynamic behavior of HCV infection, a study has shown that a phenomenon of HCV superinfection and overgrowth can occur in chronically infected patients and suggests that HCV genotypes 1a and 1b may possess replicative advantages over other genotypes[19]. However, this has not been confirmed experimentally[29]. It is therefore probable that the high prevalence of HCV genotype 1 in this population reflects the origin of the blood and derivates.

It was not possible to study all the hemophiliacs in this region, which would have enabled us to draw firm conclusions. However, these findings clarify the status of HCV infection in hemophiliacs from a northeastern area of Brazil, and highlight the importance of studying the HCV genotypes due to their relevance in the management of patients under interferon therapy. Special consideration has to be taken since most of our patients are infected by HCV genotype 1 and are co-infected with HIV, which can lead to more rapidly developing progressive liver disease in infected hemophiliacs[1 35]. Therefore, this study demonstrates that hemophiliacs are a group at high risk for severe chronic hepatitis C disease in Bahia and will require hepatological assistance and longer antiviral therapy.

ACKNOWLEDGMENTS

We thank the patients for compliance in participating in this study, the Associação Bahiana dos Hemofílicos, and the staff from Hemotransfusion and Hemotherapy Foundation (HEMOBA/ SESAB) for efficient assistance during the field surveys.

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