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Mixed Genotypes in Hepatitis C Virus Infection

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1. Introduction

Before the existence of commercial clotting factor concentrates, bleeding was the number one cause of death in persons with hemophilia and the only alternative treatment was cryoprecipitate. During the 1970s human freeze-dried (lyophilized) FVIII and FIX became available. The life of individuals with hemophilia was revolutionized because patients were able to treat themselves conveniently at home, as soon as spontaneous bleeds occurred. The commercial blood-derived products had a tremendous positive impact on physical, psychological and social lives. Unfortunately, they also carried an increased risk of blood-borne viral infections, largely due to their preparation from pools of plasma collected from thousands of donors. Consequently, the use of clotting factor concentrates resulted in human immunodeficiency virus (HIV) and hepatitis C virus (HCV) epidemics in this population (Lee C, 1995, 2009; Eyster, 2008; Ragni et al., 2010).

As reported in different cohorts around the world, many patients with hemophilia became infected with HIV between 1982 and 1985. In some hemophilic populations, subsequent testing of stored frozen plasma samples revealed that the first infections with HIV occurred in 1978-79, that the bulk of patients were infected in 1981-82, and that there were very few new infections by the end of 1984 (Eyster, 2008; Goedert et al., 1985).

The HCV epidemic was a much longer one, occurring between 1961 and 1985. The first patients became infected from the first large pool plasma-derived FIX concentrates and the epidemic ended with the dry heating of concentrates in 1985 (Lee C, 2009).

Particularly in Argentina, commercial factor concentrates were not accessible until 1975. As a result of the economic situation of the country, the non-availability of more expensive products led to a lower rate of HIV-infected people that reached 17% of our hemophilic population. Heat-inactivated factor concentrates were not available until November 1985.

Virtually, all hemophiliacs who received clotting factor concentrates prior to implementation of viral inactivation techniques became infected with hepatitis C virus at the time of the first infusion (Morfini et al, 1994; Lee C et al, 2002; Ragni et al, 2010). Prevalence rates of HCV infection up to 100% were reported in hemophilia patients treated with concentrates before 1985 (Yee et al., 2000; Lee C, 2009; Manucci, 2008; Arnold et al., 2006). Even though the introduction of heat-treated factor concentrates progressively decreased HCV transmission, the true risk ended when new regulations in blood donor screening together with the implementation of second and third generation immunoassays for the detection of antibodies against HCV was introduced in 1991 in Europe, in 1992 in the US and 1993 in

Argentina (Morfini et al., 1994; Franchini et al., 2001; Lee and Dusheiko, 2002; Tagliaferri et al., 2010; Argentinean Ministry of Health resolution #1077, 1993). However, it is important to notice that clotting factor concentrates used for Argentinean hemophilic patient treatments were never manufactured in the country but brought from the US or Europe.

Nowadays, viral inactivation and recombinant technologies have effectively prevented transfusion-transmitted viral pathogens in hemophilia. Though, due to the past chronic infections that occurred before viral inactivation procedures, transmissible agents continue to affect hemophilic population and hepatitis C represents a leading cause of morbidity and mortality in patients with hemophilia (Plug et al., 2006; Ragni et al., 2010).

2. HCV mixed genotype infections in hemophilic patients

HCV genotypes in individuals with hemophilia originate from variants within the infected donations used to manufacture factor concentrates or cryoprecipitate. Their HCV genotypes reflect the geographic distribution of genotypes from the blood donor population where the commercial products were manufactured (Jarvis et al., 1995, 1996; Fujimura et al., 1996; Preston et al., 1995; Toyoda et al., 1998; Tuveri R et al., 1997). Studies conducted within different groups provided no evidence that HCV genotypes differ significantly from each other in replication rate, transmissibility, or infectivity. However, selective transmission of HCV isolates during experimental chimpanzee infections and among humans exposed to commercially prepared factor VIII concentrate (lot DO56) containing multiple HCV species has also been suggested (Nainan et al., 2006).

The analysis of genotype distribution showed that several HCV genotypes might be circulating simultaneously in adults with hemophilia who received clotting factor concentrates before 1985 (Jarvis et al., 1995; Eyster et al., 1999; Schröter et al., 2003). Genotyping studies of multitransfused haemophiliacs suggested that the frequency of HCV mixed infection is high in this group but dissimilar rates of mixed infections were reported by different groups (Table 1).

Possible explanations for mixed infections in this population include exposure to multiple viruses from receipt of clotting factor concentrates prepared from multiple donors but also, combined with the absence of protective immunity following initial exposure to the virus (Farci et al., 1992), from exposure to multiple viruses over time among repeated infusions, prior to HCV testing and inactivation.

When individuals are infected with more than one genotype, changes in the predominant genotype over time could be observed. As a result of immunologic pressure, genetic interaction between virus and host, or treatment intervention, the establishment of the dominant genotype may change over time (Jarvis et al., 1995; Eyster et al., 1999; Schröter et al., 2003).

Genotype changes were noted more frequently in the HIV-positive subjects than in the HIV-negative subjects. Some reports provided evidence of association between HIV status and the likelihood of HCV genotype to change, possibly related to loss of immune function and a consequent greater susceptibility to the new predominant viral variant (Jarvis et al., 1995; Eyster et al., 1999).

In the hemophilic population, changes in HCV genotype could be due to reactivation or reinfection but depending on which period is being evaluated is more likely to find one or the other. If the genotype shift precedes the implementation of heat-inactivated concentrates (1985), it is not easy to attribute the genotype change to HCV reactivation or reinfection.

Though, if the change in predominant genotype occurs after 1991-1992, when the true risk of HCV transmission through blood products ended, it is more likely to be due to reactivation rather than reinfection, assuming the absence of other patient's risk factors.

<i>Author</i>	<i>Year</i>	<i>% HCV mixed infections</i>	<i>Number of patients</i>	<i>Country</i>
Jarvis LM et al	1994	31	29	UK
Isobe K et al	1995	31	63	Japan
Tagariello G et al	1995	11	36	Italy
Preston FE et al	1995	7	96	UK
Fujimura Y et al	1996	12	74	Japan
Takayama S et al	1997	46.7	80	Japan
Tuveri R et al	1997	4 (5'NC region) 18 (core region)	45	France
Toyoda H et al	1999	20.8	53	Japan
Eyster E et al	1999	6 (1985-87) 3 (1988-95)	32	US
Toyoda H et al	1998	22	63	Japan
Schroter M et al	2003	2.2	600	Germany
Buckton et al	2006	19	37	UK
Samini Rad et al	2007	27.6	34	Iran

Table 1. Rates of HCV mixed infections in haemophilia

Some groups investigated the dynamics of the change in genotype in more detail, analyzing series of samples from different time points. There was no obvious trend towards replacement with any particular variant (Jarvis et al., 1995; Eyster et al., 1999). Regardless the origin of the genotype shift (reinfection or reactivation), it could be revealing the presence of a mixed infection in the hemophilic patient.

3. Complications in assessing HCV mixed-genotype infections

A well-recognized deficiency of most genotyping assays is their limited sensitivity for detecting HCV mixed-genotype infections. The rate of HCV mixed infections is extremely dissimilar in the same type of patients described by different scientific groups. As seen in Table 1, the reported prevalence is variable depending on the genotyping methodology used. The difficulties of assessing the true prevalence of mixed infections in patients with multiple exposures remain a matter of concern (Bowden et al., 2005).

Although the prevalence and the mechanisms of HCV compartmentalization are still unknown, the presence of different HCV viral variants and different genotypes in different

tissues or compartments has been observed and could be associated with the existence of extrahepatic replication sites. This fact might result in additional complications to the analysis of HCV genotype in hemophilic patients.

3.1 Methods for HCV genotyping and limitations with mixed infections

The currently available assays, including direct DNA sequencing are designed to identify only the HCV genotype dominant in the population. Most of these techniques share the requirement of an initial target amplification step to generate suitable amounts of template for genotypic analysis. The PCR-resulting sequence for genotypic analysis may be generated by the viral isolate with the highest concentration in the serum or the one that preferentially binds to the primers used for the PCR (polymerase chain reaction). As a result, genotypes present at lower proportional levels in a mix or with lower affinity could be missed or mistyped (Hnatyszyn, 2005).

Both direct sequencing and restriction fragment length polymorphism assays seem to require 10% to 30% prevalence of minor strains before they can detect them (Qian et al., 2000; Frederick, 2010).

Hybridization based genotyping methods represent an attractive genotyping option compared to sequencing methods. The potential advantage of hybridization assays is their ability to detect mixed populations of virus with a prevalence as low as 2% (Qian et al., 2000). However similar to most genotyping methods, it involves PCR amplification of target regions of the viral genome and is confined by the advantages and disadvantages associated with PCR (Hnatyszyn, 2006; Frederick, 2010). It was reported that the latest version of the LiPA (Versant LiPA v2.0, Bayer HealthCare-Diagnostics), which targets both the 5' UTR and the core protein, seems to have improved on the problem of subtyping and has performed better than a direct sequencing assay (Trugene) as well as an earlier version of the hybridization assay (Versant LiPA v1.0). The addition of sequences from the core protein has allowed better distinction between subtypes 1a and 1b as well as subtypes c to l of genotype 6 (Bouchardeau et al., 2007).

Population-based DNA sequencing (i.e., cloning and sequencing of HCV cDNA) is so far, an accurate method for detection of mixed-genotype infections; however, this is not routinely feasible because it is time consuming and expensive. In addition, several clones would need to be completely sequenced in the presence of multiple coexisting genotypes (Frederick, 2010).

3.2 Different genotypes in different reservoirs

Compartmentalization between different tissues of the same patient (serum-brain, serum-saliva, serum-peripheral blood mononuclear cells) was described in several studies (Radkowski et al., 2002; Roque Afonso et al., 2005). Furthermore, the existence of different genotypes and/or viral variants in different tissues of the same patient has been also demonstrated (Radkowski et al., 2002; Roy et al., 1998) and particularly, peripheral blood mononuclear cells can harbor distinct HCV variants that are not detected in plasma samples (Di Liberto et al., 2006, Roque-Afonso et al, 2005); adding a potential complication to the assessment of the correct HCV genotype.

As a consequence of unknown and multiple host-viral factors, different HCV genotypes between compartments (liver and extrahepatic reservoirs) could be reflecting true different viral variant proportions. Alternatively, they could be the consequence of technical inability to detect minor components with the same efficiency in a mixed population.

4. Importance of HCV genotype analysis

Hepatitis C virus genotype has been described as an independent response predictor for antiviral therapy. Its analysis, in combination with viral load, serves to optimize the therapeutic regimen (Zeuzem et al., 2004). Considering the fact some genotypes could be more resistant than others (Hayashi et al., 2006), most treatment protocols require the correct identification of the infecting HCV genotype to provide the dose and duration of antiviral therapy.

Revealing the occult genotypes might be necessary to choose the adequate antiviral therapy because strains that are not detected could have an unexpected impact on treatment. Furthermore, recurrence from reservoirs has been suggested (de Felipe B et al., 2008; Lee WM, et al., 2005).

5. HCV mixed-genotype infections in a population with hemophilia – Experience in Argentina

Our studies rely in a group of individuals with hemophilia extensively evaluated who is assisted at the Fundación Argentina de la Hemofilia through clinical visits and periodic diagnostic studies.

Using a cell culture system that allows the detection of the HCV genome during prolonged time periods (Baré et al., 2005), we investigated the presence of HCV in peripheral blood mononuclear cell (PBMC) cultures and the HCV genotypes associated to the lymphoid cells. Although peripheral blood mononuclear cells (PBMC) are not the primary site of HCV replication, previous reports emphasize their role as viral reservoirs (Radkowski et al., 2005; Pham et al., 2004). Furthermore, different genotypes have been reported both in plasma and PBMC (Roque Afonso et al., 2005) and it has also been speculated that extrahepatic HCV diversity may be an important determinant of treatment response (Blackard et al., 2007).

The final purpose of our study was to investigate the existence of unapparent HCV mixed infections in the hemophilic population in Argentina, analyzing the HCV genotypes detected in plasma or serum samples and comparing them to viral strains emerging under long-term PBMC cultures.

5.1 Cell culture system – Methodology details

Patient's PBMC derived from EDTA anti-coagulated blood samples, obtained at different time points, were cultured following the culture methodology (Ruibal et al., 2001). Briefly, PBMC were obtained by Ficoll-Paque PLUS density gradient (GE Healthcare, Bio- Sciences, USA). Cells were washed three times with Dulbecco's phosphate buffered saline 10X (Sigma Aldrich, USA) (PBS) and suspended to one million cells per milliliter in RPMI 1640 tissue culture medium (Hyclone RPMI-1640 medium 1X, Hyclone laboratories, Utah, USA) containing 10% fetal bovine serum and antibiotics (penicillin/streptomycin, 10 mg/ml) (RPMI-FBS). Two million cells were suspended in 2 ml RPMI-FBS using round-bottom 5 ml polystyrene tubes and left undisturbed in a 5% CO₂ incubator. For each patient, 4 to 10 different tubes were set up. Beginning on day 5 - 6 of culture, half of the supernatant (SN) was replaced twice a week with fresh medium by gentle aspiration, avoiding cell pellet disturbance. The SNs collected were frozen for future studies.

From our previous experience, using non-stimulated prolonged PBMC culture as described here, the virus was released spontaneously from the in vivo infected cells and increased the

chance of finding minor HCV genotypes not detected in the bulk of PBMCs at day 0 (before culturing the cells) (data not shown).

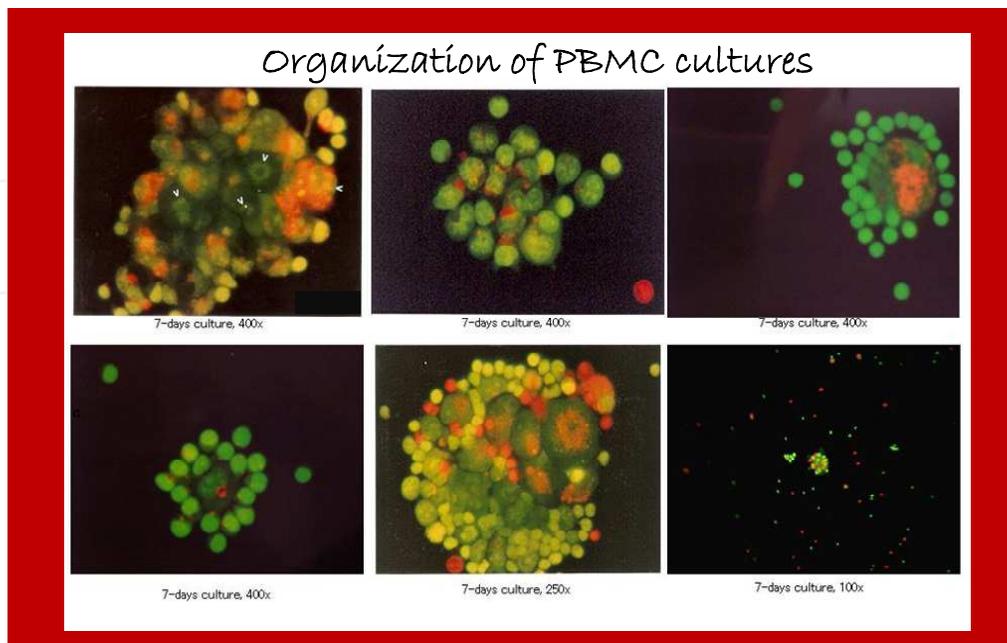


Fig. 1. Cell culture methodology (Ruibal et al.,1997): Organization of peripheral blood mononuclear cell cultures (PBMC). Aggregation of monocytes and lymphocytes is observed soon after the culture is started. Clumps containing T and B lymphocytes, dendritic cells and monocyte/macrophages are formed. Throughout the days, cell aggregates increase their size; monocyte/macrophages proliferate in close contact with lymphocytes and fuse into giant multinucleated cells. Apoptotic cells and debris are removed by activated macrophages.

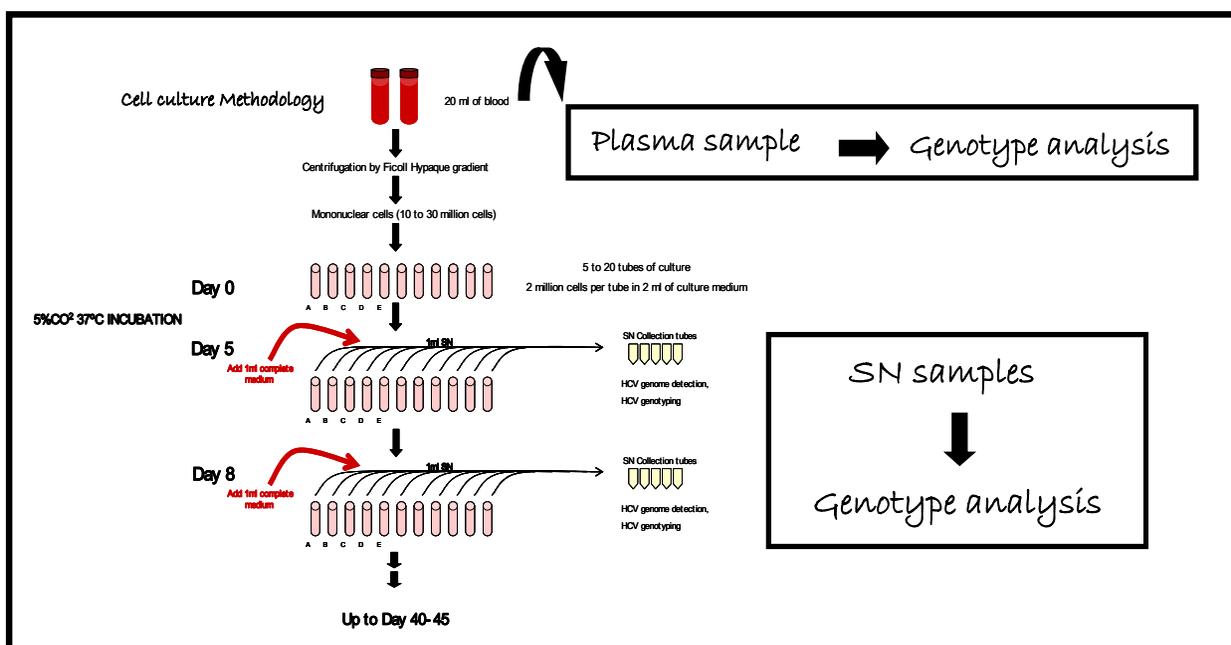


Fig. 2. Cell culture methodology scheme. SN: supernatant

5.2 HCV+ results in supernatants during cell culture

In our first report that was carried out studying only one culture per patient, HCV+ results were observed in 12 of 21 (57%) patients for the HCV monoinfected group and 23 of 31 (74%) of the coinfecting group had HCV positive cultures (Baré et al., 2005). The difference between the populations did not reach statistical differences. However, in our longitudinal study involving 50 patients (25 HCV monoinfected and 25 HIV/HCV coinfecting) and after analyzing 2 or 3 cell cultures per patient throughout 10 years of infection, almost every patient demonstrated at least one HCV+ PBMC culture. All of the HIV/HCV coinfecting patients (25 of 25) and 92% (23 of 25) of the monoinfected individuals had HCV+ PBMC cultures. During different weeks of culture, the rate of HCV+ SN for both groups was similar reaching 50 to 70 % during the first week. As the culture progressed, the percentage of HCV+ results in monoinfected population decreased gradually and generally, disappeared after the 4th week. Conversely, in the coinfecting population HCV positivity remained longer.

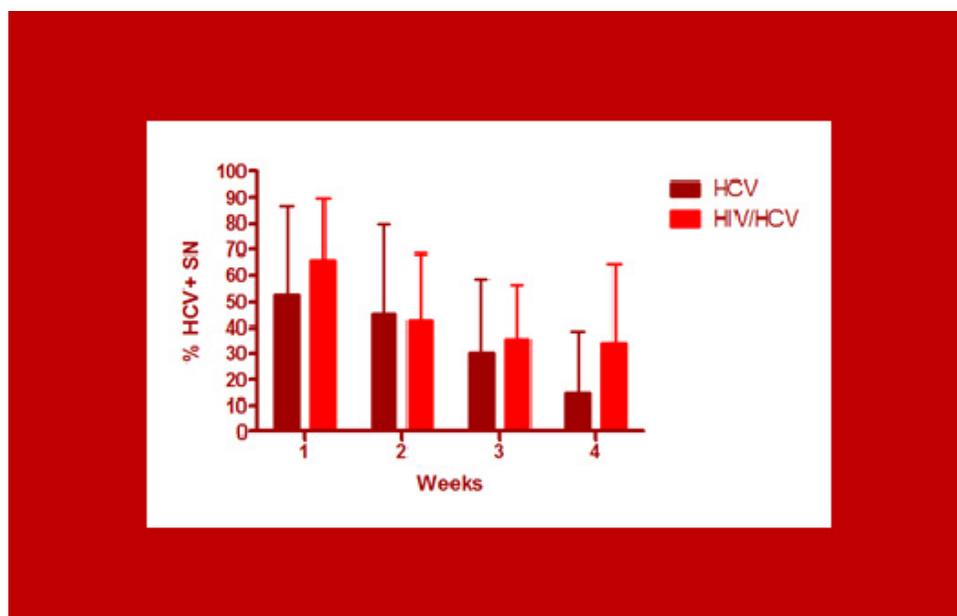


Fig. 3. HCV positivity in culture supernatants. Detection of HCV genome in cell culture supernatants during 4 weeks of culture. The percentage of HCV+ cell cultures for HIV/HCV coinfecting and HCV monoinfected group were similar. HCV positivity decreased along the days of culture in both groups.

HCV recovery in supernatants appeared intermittently along the days of culture. HCV+ PCR results alternated with HCV negative results. The rate of HCV genome recovery in cell-free supernatant was used as an estimation of viral burden in the cells. Frequencies of HCV+ results out of the total SNs were analyzed.

$$\% \text{ HCV+} = \frac{\text{number of HCV RNA+ SNs}}{\text{total number of SN examined}} \times 100$$

The percentage of HCV+ SN in cell culture from the whole population ranged from 3 to 79% with a median value of 41.4% and a mean of 38.6%. The analysis of HCV+ percentages in different cultures from the same individual demonstrated that HCV positivity remained stable when considering only cultures from patients without HCV treatment.

The HCV genome was present at very low level with a mean value for coinfecting patients of $3.39 \pm 0.34 \log$ (IU/ml) and $3.37 \pm 0.26 \log$ (IU/ml) for the HCV group (as reported in our first study). That could be the reason for the observed intermittent signal in the PCR results. Although there were no statistical differences between populations, the duration of the positive signal for more than 4 weeks in the coinfecting group was remarkable.

Trying to find any clinical or viral related factor to the chance of originating an HCV+ culture, we found that the frequency of HCV+ results did not correlate with HCV or HIV viremia, neither HCV nor HIV viral loads in plasma samples. However, the patients with CD4+ counts lower than 250 cells/mm³ were associated to higher frequencies of HCV+ results in cell cultures. It could be postulated that the low CD4+ cell counts that is a consequence of HIV infection could be related to a deficient control of HCV in culture.

5.3 HCV genotypes in plasma samples and culture supernatants

HCV genotype distribution was assessed in a group of 288 HCV chronically infected patients with hemophilia. Most of them were evaluated with one single plasma sample. All patients included in the study had a positive test for HCV antibodies detected by EIA assay (3rd generation) and RIBA 2.0 or 3.0 (both Ortho Clinical Diagnostics, Raritan, NJ, USA). Plasma samples were analyzed through genotyping techniques currently used for diagnosis (restriction fragment length polymorphisms and/or LIPA techniques).

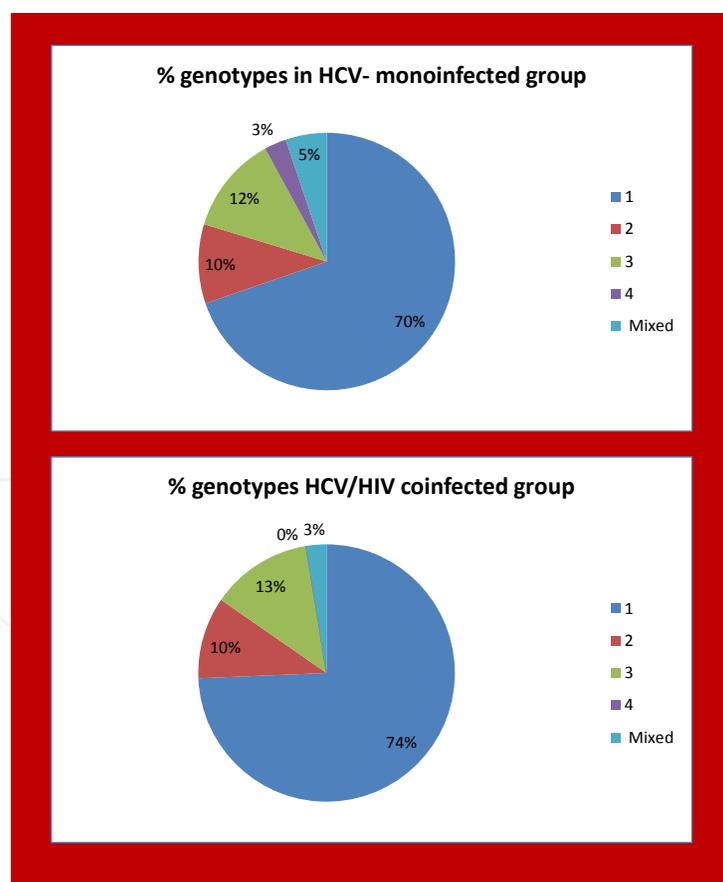


Fig. 4. HCV genotype distribution in HCV monoinfected and HIV/HCV coinfecting group

The distribution of HCV genotypes in the hemophilic population in Argentina reflects the plasma donors in Europe and United States and was reported previously by Picchio et al, in 59

individuals, finding that HCV genotype 1 was the predominant viral variant detected among HIV-negative (76%) and HIV-positive (82.5%) patients, followed by genotypes 3 (10.4%), 2 (2%) and a small proportion of multiply co-infected patients including genotypes 4 and 5 (6.25%). In the group of 288 individuals, the HCV genotype distribution for the entire population was 72 % of genotype 1, 10% of genotype 2, 12% genotype 3 and 2.5 % of genotype 4. Similar distribution was observed when considering HCV mono-infected or HIV/HCV co-infected population ($p>0.05$). Taking into account different HCV genotypes (and not HCV subtypes) as shown in fig 4, mixed-genotype infections were observed in 5.6 % of the patients (Parodi et al., 2008).

Analysis of HCV genotypes associated to lymphoid cells found along cultures have previously demonstrated the presence of occult HCV mixed-genotype infections in 62% of 16 patients with hemophilia (Parodi et al., 2008).

In the present study, plasma and cell culture samples were longitudinally analyzed in 25 HCV monoinfected and 25 HIV/HCV coinfecting individuals in a period of time between 1993 and 2010. Different time point cultures were evaluated. HCV genome presence and genotyping were analyzed as described previously (Parodi et al., 2008) on an average of 15 culture supernatants per time point. Plasma samples were also studied at different dates along the time period and for at least 3 time points for each individual. Genotypes found in plasma samples were compared to the correspondent samples obtained under culture.

Analyzing 3 or more sequential plasma samples included in the studied period, the 69% (33 of 48) of the patients showed genotype 1, 4% genotype 2 and 4% genotype 3 (2 of 48), 2% (1 of 48) genotype 4, while 21% (10 of 48) had mixed-genotyped HCV infections. Results in culture samples performed during the same time period showed the presence of genotype 1 in 46% (21 of 46) of the patients and mixed infections in 50% (23 of 46) of the subjects. The remaining 4% (2 of 46) was genotype 2. Using the cell culture system the chance of discovering mixed-HCV genotype infections was 3.8 times greater than with longitudinal analysis of plasma samples ($OR=3.8$, $p= 0.005$, $95\%CI =1.54 - 9.4$). However, also the analysis of serial plasma samples instead of one single plasma sample increased the chance of discovering mixed infections in 5.2 times ($OR=5.2$, $p= 0.0006$, $95\%CI =2.15 - 12.5$).

ID	Serial plasma samples	n	Genotypes in culture SN	n cult	nSN
HIV/HCV coinfected group					
1	1+2	10	1+2	3	44
2	1	9	1+2	3	40
3	1	8	1+2	3	62
4	1+2	9	1+2	4	59
5	1+2+3	9	1+3	4	19
6	1	9	1+3	6	01
7	1	7	1+2+3	3	45
8	1+4	9	1+4	4	6
14	1	6	1	4	63
15	1	8	1+2+3	4	53
16	1	6	1	3	34
17	1	5	1	4	52
26	1+3	7	1+2	3	42
27	1+3	6	1+2	4	63
29	1	6	1+2	3	4
30	2	6	2	3	48
36	1+3	4	1+3	3	4
39	1	5	1	2	28
40	1	4	1+3	3	45
41	1	3	1	2	32
42	1	3	1+3	2	29
43	1	5	1	2	32
47	-	5	1	2	32
48	3	3	1+3	2	48
49	-	5	2	2	32
HCV monoinfected group					
8	4	3	1+2+3+4	2	54
10	1	7	1+2+3	2	34
11	1	5	1+2	2	38
12	1+3	6	1+3	3	44
13	1	4	-	3	29
18	1	4	-	2	26
19	1	3	-	2	22
20	1	3	-	2	9
21	1	4	-	2	24
22	1	5	-	2	29
23	1	4	-	3	40
24	1	5	1+3	2	28
25	1	3	1+2	2	29
28	1	3	-	1	14
31	3	8	-	2	30
32	1	4	-	2	29
33	1+2	4	-	2	22
34	1+3	6	-	2	30
36	1	3	-	2	31
37	1	4	-	2	29
38	1	4	-	2	21
44	2	5	1+2	2	32
45	1	6	-	2	31
46	1	3	-	1	15
60	1	5	-	1	16

Table 2. Genotypes in plasma and culture supernatants in 50 patients. SN: supernatant

From 23 subjects with HCV+ typeable supernatants in cell culture who showed mixed HCV genotypes, 16 were HIV/HCV coinfecting and 7 HCV mono-infected patients. HIV infection was found to be associated to the possibility of having mixed genotype infection ($p=0.02$, $OR=4.57$, $95\%CI =1.38 - 15.11$).

6. Conclusion

In our experiments, distinct HCV genotypes associated to PBMC and not present in serial plasma samples were verified. Therefore, mononuclear cells might be acting as an independent viral reservoir in this cohort whether or not HCV replicates inside these cells. Mononuclear cells might be involved in HCV persistence as an extrahepatic reservoir in this cohort. Even if HCV replication or cell adsorption were not explored in our experiments, the fact that we detected PBMC-associated genotypes throughout a long-time period is compatible with the existence of an extra-hepatic replication site. Otherwise, if extremely low or occult replication of lymphotropic variants was taking place in the liver, viral particles could be adsorbed preferentially to blood monocytes that continuously circulate through this tissue, and be detected thereafter in the PBMC cultures.

Techniques that involve cell culture and cloning methodologies are time-consuming, expensive and difficult to perform in a diagnostic facility. However, through the use of a reliable genotyping technique in combination with the analysis of multiple time points in an extended period of time, the chance to identify most of the infecting HCV genotypes, when mixed infections are present, could be increased.

The final goal of detecting the predominant together with minor HCV genotypes is to help to provide the proper dose and duration of antiviral therapy. Otherwise, changes in the predominant variant could have unexpected impact on the treatment response. The clinical and therapeutic implications of lymphotropic HCV variants related to their persistence requires further investigation, especially in hemophilic HIV/HCV coinfecting persons.

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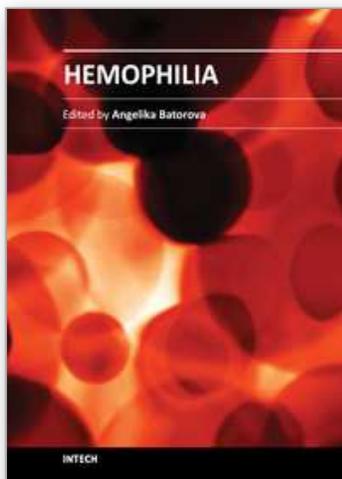
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