ANALYSIS OF HEPATITIS C VIRUS ISOLATES USING MOLECULAR AND SEROLOGICAL TYPING METHODS

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Summary. – This study comprised 100 persons with antibodies to hepatitis C virus (HCV), including 77 intravenous drug users (IVDUs). They were tested with serological HCV typing assays (Murex HCV serotyping 1-6 assay; Chiron RIBA HCV Serotyping SIA). Patients with a positive polymerase chain reaction (PCR) for HCV (n = 66) were tested with genotyping molecular assays (Inno-Lipa HCV II test; Sorin GEN-ETI-K HCV typing assay). Comparison of the results of these tests showed that (a) 92% of samples could be typed by one test at least; 44% could be typed by all four tests; 88% could be typed by one serological test at least and 66% by one molecular test at least; (b) 81% of the samples successfully tested with both serological tests gave comparable results; 95% of the samples successfully tested with both molecular tests gave comparable results; (c) when serological and molecular tests yielded different results, sequences in the 5'-non-coding (5' NC) or E1 regions always confirmed the results of the molecular tests; (d) in case of discrepancy between the results of the molecular tests the E1 region sequences confirmed the Sorin test results.

It is concluded that the molecular tests compared gave similar results. The fact that the Murex serological test gave comparable results in more than 80% of cases indicates that it is an alternative to the molecular tests for routine diagnosis. However, comparison of the results of this test with those obtained in patients consulting a hepatology department showed that it gave the best results in a population of patients not exposed repeatedly to HCV.

Key words: hepatitis C virus; genotype; polymerase chain reaction; serology; diagnosis

Introduction

The virological diagnosis and the follow-up of hepatitis C (HC) are mainly based at present on serological tests and on detection of HCV genome in peripheral blood (de Lamballerie *et al.*,, 1996a). Since the first diagnostic procedures introduced in 1989, attempts to establish relations between biological parameters of the infection and development of the disease with or without interferon (IFN) therapy have been made. In particular, the genotype and the virus load of the infecting HCV strain and their relations with development of hepatocellular carcinoma and with IFN treatment efficiency have been thoroughly studied (Dusheiko *et al.*, 1994; Feray *et al.*, 1995; Lai *et al.*, 1993; Nagata *et al.*, 1994; Pozatto *et al.*, 1991, 1994, 1995; Qu *et al.*, 1994; Saito *et al.*, 1990; Tawaraya *et al.*, 1995; Telfer *et al.*, 1995; Tsubota *et al.*, 1993, 1994; Yamada *et al.*, 1994).

In a recent work, Hawkins *et al.* (1997) showed that the methods that had previously been used for quantifying HC viremia provided variable results depending on viral genotype rather than on real virus loads. This point is important

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; HB = hepatitis B; HBc = HBV core antigen; HBV = hepatitis B virus; HBs = HBV surface antigen; HC = hepatitis C; HCV = hepatitis C virus; HIV-1 = human immunodeficiency virus type 1; IFN = interferon; IVDU = intraveneous drug user; 5'NC = 5'-non-coding; PCR = polymerase chain reaction; TV = threshold value

because it suggests that the relations described between viral load and clinical development of the disease were influenced by methodological bias and that the most important virological parameter for the prediction of the development of the disease could be the viral genotype.

HCV isolates can be classified in 6 types and more than 30 subtypes as demonstrated by phylogenetic analysis of their genomes (Simmonds *et al.*, 1994, 1996; de Lamballerie *et al.*, 1997). In clinical studies, the type of the viral infecting strain is usually determined by two sorts of methods: molecular and serological. Molecular genotyping protocols are based on the identification of type-specific sequences in PCR products obtained by amplification of viral genome; serological typing assays are based on the detection of antibodies to type-specific antigenic peptides in serum. The latter methods are an indirect approach to the determination of the infecting genotype. It can be used in the absence of viremia and can provide different results from molecular typing in cases of iterative HCV infections.

In this article, we have studied a population of 100 prisoners with antibodies to HCV using four commercial typing tests (two molecular and two serological tests). We compare and discuss the results of these experiments.

Materials and Methods

Population studied. This study included a sample of 100 persons with antibodies to HCV (mean age of 30.4 ± 5.9; sex ratio of 2.9). All of them were detainees in the Marseille's (France) penitentiary and tested voluntarily for HCV, hepatitis B virus (HBV) and human immunodeficiency virus type 1 (HIV-1). Seventy-seven % of these persons were IVDUs. As regarding the HBV infection status, 3% of them had a chronic hepatitis (with positive HBV surface (HBs) antigen), 55% had recovered from infection with HBV (with antibodies to HBV core antigen (HBc) in the absence of HBs), 42% had never been in contact with HBV (no serological markers) or had been vaccinated (with antibodies to HBs antigen only). Seventeen % of them were found to be infected with HIV-1 according to the results of two different specific enzyme-linked immunosorbent assays (ELISAs).

Serological screening for HCV antibody was performed using two 3rd-generation-ELISAs: the Ortho test (Ortho Diagnostic Systems, Raritan, NJ) and the Murex test (Murex Diagnostics, Dartford, UK). A sample was considered positive if the two ELISAs were positive with ratios of more than 4, or if the ELISA positivity was confirmed by an immunoblot analysis, i.e the Murex HCV immunoblot assay or the RIBA-3 test (Chiron Co., Emeryville, CA).

HBs and antibodies to Hbc were detected using the related Abbott assays and the AXSYM automated ELISA reader (Abbott Laboratories, Chicago, IL).

Diagnosis of HIV infection was made with the Vidas HIV 1+2 (Biomérieux, La Balme Les Grottes, France) and the Serodia HIV (Bayer Diagnostics, Orcq, Belgium) assays.

Serological HCV typing was performed using two different commercial tests of the following characteristic.

The Murex HCV serotyping 1-6 assay (Murex Diagnostics, Dartford, UK) is a competitive ELISA test performed in a 96-well microplate format. It is based on the detection of serum antibodies to peptides specific for types 1-6 and located in the NS4 region of HCV genome (Bhattacherjee *et al.*, 1995; Simmonds *et al.*, 1993). In each well antibodies are allowed to react with a mixture of type-specific peptides in the presence of competitive peptides of all types tested except one. The assay does not allow the subtyping. According to the interpretation algorithm of absorbances (A) proposed by the manufacturer, possible results are as follows: non-typeable (NT); type 1, 2, 3, 4, 5, or 6; co-infection with different types. The Chiron RIBA HCV Serotyping SIA (Chiron Co., Emeryville, CA) is a qualitative non-competitive enzyme immunoassay in an strip immunoblot format. It is based on the detection of serum antibodies to eight type 1, 2 and 3-specific peptides located in the core and NS4 genome regions of HCV. Possible results are as follows: NT; type 1, 2, or 3; type "1 or 3 (core)" (because the type 1 core peptide shows significant cross reactivity with antibodies to type 3); co-infection with different types. All serological tests were performed according to the manufacturers' recommendations.

RNA was extracted from all sera (100 µl) using a guanidinium thiocyanate method (RNA Now, Ozyme, Montigny le Bretonneux, France).

Reverse transcription (with random hexanucleotides and *nested PCR* with outer primers 1 and 2 (Kato *et al.*, 1990; Stuyver *et al.*, 1993) and inner primers 4CH/ITS (Ravaggi *et al.*, 1992) were realized under standard conditions. A sample was considered positive for HCV RNA according to the result of the PCR protocol.

Molecular genotyping was performed using two different commercial assays of the following characteristic. The Inno-Lipa HCV II test (Innogenetics, Zwijndrecht, Belgium) is a line probe assay (Van Doorn *et al.*, 1994). After a nested PCR with two outer and two inner biotinylated primers for the 5'-untranslated region (5'UTR) of HCV the PCR products are hybridized with type- or subtype-specific probes fixed on a nitrocellulose strip. Then the streptavidin-labelled alkaline phosphatase and substrate are added, and a colored precipitate is produced. The test is qualitative, based on the presence of colored lines in case of perfect match between probes and PCR products. According to the interpretation algorithm proposed by the manufacturer, possible results are as follows: NT, type 1, 1a, 1b; type 2, 2a or 2c, 2b; type 3, 3a, 3b, 3c; type 4, 4a, 4b, 4c or 4d, 4e, 4f, 4h; type 5a; type 6a; coinfection with different types.

The Sorin GEN-ETI-K HCV typing assay (Sorin Biomedica, Saluggia, Italy) is based on the hybridization of PCR products in the core region of HCV with 9 type- or subtype-specific probes absorbed to separate microplate wells. A positive reaction is revealed by an antibody which specifically recognizes double stranded DNA, and a color reaction. Two outer and three inner primers for the PCR in the core region are supplied. Possible results are: NT, type 1a, 1b; type 2, 2a, 2b; type 3a; type 4; type 5; type 6; coinfection with different types. The Sorin assay was performed according to the manufacturer's recommendations but the interpretation algorithm of the test was modified. Using the recommended interpretation protocol, 57% of the samples

tested gave a result of co-infection with two different HCV types at least, including a large number of co-infections with genotype 5 (particularly associated with genotype 1b, see Discussion). These results were obviously incorrect. The threshold value (TV) of the Sorin method was calculated as proposed by the manufacturer (TV = mean negative control A value + 0.150). However, we considered positive only the samples with the A/TV ratio higher than 2, or higher than 5 in case of genotype 5. A simultaneous infection with another virus type was considered possible if the ratio of As of the corresponding wells was less than 4.

Sequencing of PCR products. For 10 samples, PCR products in the E1 region (nt 1783-1942) were subjected to direct sequencing as described elsewhere (de Lamballerie *et al.*, 1996b). Assignment of viral genotypes from RNA sequences in the E1 region was realized by alignment with a series of previously described sequences belonging to various genotypes and construction of phylogenetic trees. For 9 samples, PCR products in the 5'UTR were sequenced under comparable conditions. In the 5'UTR, we searched for previously described type-specific sequence patterns, particularly those used in the Inno-Lipa assay (Stuyver *et al.*, 1993).

Result analysis. As the nature of the information brought by each test was slightly different, we decided to use 5 codes to summarize the comparison of these results. "0" (zero) was used when one of the tests compared could not be realized (e.g. the genotyping assay in case of PCR-negative samples), or when one test did not provide any interpretable information (particularly in case of serological typing assays). "i" (identical) was used when all results were identical. If molecular and serological methods were compared, only the viral type (and not the subtype) was considered. "c" (compatible) was used in case of suspicion of coinfection when one common type at least was found in all considered tests. "dst" (different for subtype) was used when a difference was found only at the subtype level. "d" (different) was used when one of the results at least was completely different from the others.

Table 1. Results of HCV genotyping using the Murex and Chiron serological tests and the Inno-Lipa and Sorin molecular tests

HCV genotype	Test			
	Murex	Chiron	Inno-Lipa	Sorin
Non-typeable	18	20	34[a]	44 (41[a], 3[b])
1	41	41		
2	01	03		
3	20	25		
4	14		1	10
1 or 3 (core)		09		
1+3	03	02		
2+4	01			
3+2	01			
4+3	01			
1a			08	09
1a+1b			01	01
1a+3a				01
1a+4				01
1b			17	11
1b+3a			05	01
1b+4c or 4d			02	
2a				02
2a or 2c			02	
3a			22	18
3a+4				02
3a+4c or 4d			01	
4c or 4d			07	

[a]Non-amplifiable; [b]Amplifiable but non-typeable.

Results

Results of the different tests

Out of 100 sera with antibodies to HCV, 12 could not be typed by any serological test, 88 could be typed by one serological test at least and 74 could be typed by both the Murex and Chiron tests (Table 1). Sixty-six % of the samples tested had a positive PCR result and could therefore be subjected to molecular typing. All of them could be typed by one molecular test at least and 56 could be typed by both the Inno-Lipa and Sorin tests (Table 1).

Comparison of results of the different tests

This comparison is summarized in Table 2. It can be noted that a common viral type (corresponding to the "i or c or dst" pattern) could be found: in 66% of the interpretable results when all the tests were compared; in 81% when the serological tests were compared; in 95% when the molecular tests were compared; in 86% when the molecular tests were compared to the Murex serological test; in 70% when the molecular tests were compared to the Chiron serological test; in 81% when the Murex and Inno-Lipa tests were compared.

For 5 patients with discrepant results in the Inno-Lipa and Sorin tests, we analyzed the sequence of 5'UTR to check whether the sequence corresponding to the specific probe giving a positive result could be found or there was a non-specific hybridization of this probe. In all these cases such a specific sequence could be found.

For 4 samples with discrepant results between the molecular and serological assays, the 5'UTR was also sequenced. The result of the molecular typing was always confirmed.

For 10 samples corresponding to various patterns of discrepant results in the different tests, the sequence of the E1 genomic region was analyzed (Table 3).

Discussion

HC is a public health problem from an epidemiological point of view: in many developed countries the seropreva-

Table 2. Comparison of the results obtained with four different typing tests

Tests	Codes of results				
	"0"	"i"	"c"	"dst"	"d"
Murex/Chiron/Inno-Lipa/Sorin	56	23 (52%)	06 (14%)	–	15 (34%)
Murex/Chiron	26	51 (69%)	09 (12%)	–	14 (19%)
Inno-Lipa/Sorin	44	37 (66%)	10 (18%)	06 (11%)	03 (05%)
Murex/Inno-Lipa/Sorin	51	30 (61%)	12 (25%)	–	07 (14%)
Chiron/Inno-Lipa/Sorin	53	27 (57%)	6 (13%)	–	14 (30%)
Murex/Inno-Lipa	43	33 (58%)	13 (23%)	–	11 (19%)

"0" = non-interpretable results; "i" = identical results; "c" = compatible results; "dst" = results different for the subtype only; "d" = different results. For further information see Materials and Methods. The results are given in absolute numbers and % values (of interpretable results).

Table 3. Result of the assignment of HCV genotype using the E1 genomic sequence in ten cases of discrepant results

Test				
Murex	Chiron	Inno-Lipa	Sorin	E1 sequence
1	1	4	4	4
NT	3	1b	1a	1a
3	1 or 3	1b	A/NT	1a
4	1 or 3	3a+1b	3a+4	3a
4	1 or 3	3a+4	3a+4	3a
1	NT	1b	3a+1a	3a
3+1	1	3a	3a	3a
1	2	3a	3a	3a
1	1	1b	1a	1a
1	1	3a	3a	3a

NT = non-typeable; A/NT = amplifiable but non-typeable.

lence of the illness lies between 0.5 and 2% (Garson *et al.*, 1992; Yuki *et al.*, 1994). Most of the epidemiological studies were conducted on blood donors and there are a few data on general population. In certain African and Asian populations a high prevalence has been reported (Fretz *et al.*, 1995; Sheu *et al.*, 1993; Wu *et al.*, 1992; Wu *et al.*, 1995; Xu *et al.*, 1994). It is probable that more than 50% of the persons with antibodies to the virus are suffering from chronic HCV infection (Cuthbert 1994). Moreover, according to the World Health Organization estimate, 3% of the world population has been infected with HCV and there are more than 170 million chronic carriers who are at risk of developing liver cirrhosis and/or liver cancer. The discovery of biological markers predictive of development of the illness that could be used to improve the protocols of treatment proved to be difficult. The influence of the genotype of the infecting strain on development of the disease (with or without IFN treatment) has been investigated in many studies (Hino *et al.*, 1994; Kanai *et al.*, 1992; Pozatto *et al.*, 1994; Saito *et al.*, 1990; Silini *et al.*, 1995; Takada *et al.*, 1992; Tsubota *et al.*, 1993; Yoshioka *et al.*, 1992). Recent data suggest that this parameter is more reliable than the virus load measurement (Hawkins *et al.*, 1997). It is therefore important to evaluate the typing assays that could be used routinely for clinical follow-up of HCV-infected patients.

In this study, we have used four different commercial typing kits to test a population of 100 prisoners with antibodies to HCV, including a high proportion of persons (77%) known to be IVDUs. These patients have probably underwent repeated inoculations of HCV and could therefore have been infected with strains of different types, simultaneously or sequentially. Two typing methods were based on the detection of type-specific serum antibodies, and two on the identification of type-specific sequences in the viral genome.

Ninety-two % of the patients could by typed by one test at least. Eighty-eight % could be typed by at least one serological test, and 66% by at least one molecular test. This difference may be explained by the fact that 66% only of the patients had a positive PCR result and could therefore be typed using a molecular assay. When the results of the two serological tests were compared, 19% of the samples had clearly different results in the two methods. In all cases of discrepancy but one, the Murex test found antibodies to type 4. As the Chiron test does not include type 4-specific peptides it should have found all the type 4 samples to be "non-typeable". In 4 cases, the "type 4" result of the Murex test could be confirmed by the two molecular assays: the result of the Chiron test was "1" in one case, "1 or 3" in one case, "3" in one case and "non-typeable" in one case only. It is therefore certain that this test can provide false results when type 4 samples are tested. Moreover, the interpretation of all type 4 results using the serological test in the NS4 region should be very careful since point mutations have been described that would transform type 1-specific NS4 peptides into peptides recognized as belonging to type 4 (Pawlotsky, personal communication). It should be noted too that in 9 cases the Chiron test result was "1 or 3 (core)", corresponding in 6 cases to "4" in the Murex test.

When the results of each serological test were compared to those of the molecular tests, identical or compatible re-

sults were found in 86% of cases for the Murex test and 68% only for the Chiron test, indicating that the former test provides apparently more reliable data.

However, a comparison between the results of all the serological and molecular tests showed that in a number of cases they were providing two distinct results. In such 10 cases, we sequenced the 5'UTR (4 cases) or E1 genomic regions (6 cases) of the viral strain and in all these cases we found the genotype predicted by the molecular tests as previously reported (Pawlotsky *et al.*, 1997). This phenomenon had previously been described in HIV-infected patients and is believed to be due to repeeated infections with isolates belonging to different viral types. During a period of time, these patients would have antibodies to one type, but a viremia with a predominant strain more recently acquired and belonging to another type. Moreover, in many cases the appearance of antibodies to the NS4 protein (mainly used in serological typing assays) follows that of antibodies to other viral proteins, such as core or NS3 proteins (Van Doorn *et al.*, 1996). It should be noted that in this study three patients only with such results were HIV-positive, suggesting that this situation could be encountered among immunocompetent patients exposed to multiple viral infections. Nine of these ten patients were known to be IVDUs; one patient was HIV-positive.

It was interesting to compare these results to those obtained from patients with no risk of repeated infections. We had previously studied a population of 67 patients (mean age of 44; sex ratio of 1.9) consulting a hepatology department following a positive result of HCV serology. None of these patients was known to be IVDU or infected with HIV or had a simultaneous infection with HBV (with a positive HBs antigen). None was haemodialyzed or was known to be immunodeficient. Thirty-seven of them had a positive result of PCR test for HCV and could be tested by the Murex and Inno-Lipa tests. The comparison of the results of both tests showed that cases of possible co-infection were less frequent (8%) in this population than in the population including IVDUs (23%, $p = 0.06$), and that a common type ("i or c" pattern) was found in 94.5% in the former group *versus* 81% in the latter group ($p = 0.05$). These results suggest that the molecular tests should be preferred in testing populations including IVDUs, but that the Murex serological test can provide reliable information concerning the infecting HCV in a population of patients with no risk of repeated HCV infections. It is a convenient method for routine analysis, requiring no molecular biology equipment or experience but an automated ELISA reader.

The comparison of the results of the Inno-Lipa and Sorin tests proved that they were similar in most of the cases. A common viral type was found in 95% of the cases. Differences in the determination of the subtype were more frequent (9%). In one case of different type result and in three cases of different subtype result, we sequenced a part of the E1 gene. Each time the result of the Sorin test was confirmed. This could be due to a higher variability of the core genome region compared with the 5'UTR, permitting a more precise assignment of subtypes. However, it is not certain that the subtype determination gives a relevant information for clinical follow-up of HCV-infected patients (Pawlotsky *et al.*, 1996). Our data are not consistent with those of Giannini *et al.* (1995) and Lau *et al.* (1995) who found that genotypes determined by analysis of the 5'UTR were more reliable than those determined by subtyping the core genomic region. However, these authors used a different methodology for the type determination in the core region, based on the use of type-specific primers (Okamoto *et al.*, 1992).

The diagnosis of co-infections by these genotyping tests remains difficult. For molecular tests, a positive hybridization signal for two probes of different specificity can be due to a real co-infection, but obviously, a non-specific hybridization can occur, particularly in case of very strong positivity for the predominant type. For this reason, we decided to modify the rules of interpretation of the Sorin test, which provides quantitative A values. Since the strong positivity of a sample was often associated with diagnosis of multiple infection (which is illogical), we introduced a link between the A value of the main type and that of the potential co-infecting type. We found that when the ratio of these values was less than four, a co-infection could be diagnozed by another typing method at least. In case of the type 5 probe, many non-specific hybridizations were observed, especially in case of infection due to a type 1b strain. This could be explained by the fact that the type 1b and 5 probes have 12 nt in common and led us to modify the threshold value for the type 5 positivity.

It can be concluded that both molecular tests used can provide reliable typing results, even in a population including a large proportion of IVDUs, the Sorin test giving a more precise information on subtypes (at least for type 1) than the Inno-Lipa test. The advantage of the Inno-Lipa test is that (a) it can be performed using the PCR diagnosis products if one PCR primer at least is biotinylated (e.g. in the Amplicor HCV commercial kit, Roche Diagnostic Systems, Bâle, Switzerland), (b) PCR protocols in the 5'UTR are at present well established (all diagnostic procedures use a PCR amplification in this region), and (c) this test includes probes for several subtypes which cannot be identified by the Sorin test (e.g. subtypes of types 3 and 4). The serological tests used were found to provide interesting results. Eighty-one % of the results of the Murex test were confirmed by the Inno-Lipa molecular test among samples with a positive PCR result. However, we believe that these tests, to give their best results, should not be used in populations including patients suffering from immunodeficiency or repeatedly exposed to HCV.

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