Discordant Results from Hepatitis C Virus Genotyping by Procedures Based on Amplification of Different Genomic Regions

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We compared the results of genotyping hepatitis C virus (HCV) either by PCR amplification of the core region or by hybridization of PCR-amplified products of the 5' untranslated region (5'UTR assay). Serum samples from 144 Italian anti-HCV-positive patients (106 drug abusers and 38 patients with chronic viral liver disease but no history of drug abuse) were studied. The original core region assay described by Okamoto et al. (H. Y. Okamoto, Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi, *J. Gen. Virol.* 73:673–679, 1992) allowed genotyping of 75 of 144 samples. A modified version of Widell et al. (A. Widell, S. Shev, S. Månsson, Y.-Y. Zhang, U. Foberg, G. Norkrans, A. Frydén, O. Weiland, J. Kurkus, and E. Nordenfelt, *J. Med. Virol.* 44:272–279, 1994) allowed genotyping of 11 of 79 samples (50 of 79 samples remained unclassified by the method of Okamoto et al. In contrast, all 144 samples were genotyped by the 5'UTR assay. Forty-six of 75 (61 percent) of the samples genotyped by the method of Okamoto et al. and 10 of 11 (91 percent) of the samples genotyped by the method of Widell et al. had results consistent with those obtained by the 5'UTR assay. According to the results of direct sequencing, the method of Okamoto et al. erroneously classified seven samples as having mixed infections. In conclusion, HCV genotyping seems more reliable when it is performed by the 5'UTR assay than by either of two core region assays. The major advantage provided by the 5'UTR assay is a much lower proportion of negative or indeterminate results in younger patients with histories of drug abuse or infection by genotypes other than HCV type 1.

Hepatitis C virus (HCV), the causative agent of most cases of non-A, non-B hepatitis (1, 7), exists as a heterogeneous group of viruses sharing approximately 70% homology overall (14). Comparative sequence analysis of HCV isolates has shown that a noncoding region of 341 nucleotides, the 5' untranslated region (5'UTR), is the best preserved of the entire HCV genome; this is followed by the domain encoding the putative core protein. On the other hand the regions of the HCV genome coding for putative envelope proteins are highly variable (14). Indeed, HCV has a high spontaneous mutation rate which is believed to play a major role in its strategy of persistence in the host (6).

In recent years, it has been suggested that an association might exist between infection with specific HCV strains and a poor response to interferon treatment (4, 16). If this were true, the clinical value of genotyping would be much increased, since the therapeutic approach for patients with HCV-related liver disease might be tailored according to the probability of a favorable response. Unfortunately, genotyping results obtained by different laboratories have generated a considerable amount of confusion because of the use of different methods. Recently, a panel of experts has agreed to classify HCV strains according to a new, unifying nomenclature (15). The logic behind the new classification system is that although different methodologies and genomic regions are used, comparable genotyping results can be expected. However, since most laboratories adopt a single method of genotyping, this hypothesis

has remained largely unproven, at least as far as European patients are concerned. The aim of the present study, therefore, was to compare, in serum samples obtained from Italian patients, the results of HCV RNA typing obtained by PCR amplification of the core region with the results obtained by hybridization of PCR-amplified products of the 5'UTR of the HCV genome.

MATERIALS AND METHODS

Patients. We studied 144 serum samples obtained from two groups of patients. The first group of patients comprised 106 drug abusers (78 males and 28 females; mean $\pm$ standard deviation age, 29.7 ± 5.1 years; age range, 18 to 43 years) attending a clinic for the prevention and treatment of drug dependence; the second group included 38 patients with no history of drug abuse (25 males and 13 females; mean $\pm$ standard deviation age, 48.2 ± 11.2 years; age range, 27 to 67 years) and biopsy-proven chronic liver disease (31 with chronic hepatitis and 7 with chronic hepatitis and cirrhosis). All patients had been proven to be positive for anti-HCV antibodies, detected by a second-generation enzyme-linked immunosorbent assay (Ortho Diagnostics, Raritan, N.J.), and for HCV RNA, as determined by PCR amplification of 5'UTR with nested primers. Serum samples, which had been separated from blood clot prepared in aliquots, and frozen within 2 h, were prospectively collected for a period of 4 months and were kept at -80°C until use, approximately 12 months from the time of collection of the first blood sample. All patients had given informed consent to participate in the study.

HCV genotyping by hybridization of PCR-amplified products of 5'UTR (5'UTR assay). HCV RNA was detected in serum as described previously (16). Briefly, 3 μl of serum was mixed with 17 μl of buffer containing 10 mmol of Tris-HCl (pH 8.4) per liter, 50 mmol of KCl per liter, 2.5 mmol of MgCl_2 per liter, 1 mmol of the four deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden) per liter, and 50 pmol of an antisense outer primer (5'-GTCCACGG TCTACGAGACCT-3') per liter, and the mixture was heated at 92°C for 1 min. After quick chilling in ice, 10 U of RNasin (Boehringer Mannheim, Mannheim, Germany) and 10 U of murine myeloblastosis virus reverse transcriptase (GIBCO BRL, Milan, Italy) were added, and the mixture was incubated for 1 h at 37°C . PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus,

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Norwalk, Conn.) in a reaction volume of 100 μ l containing 10 mmol of Tris-HCl (pH 8.4) per liter, 2 mmol of $MgCl_2$ per liter, 50 mmol of KCl per liter, 0.2 mmol of each deoxynucleoside triphosphate per liter, 2.5 U of *Taq* polymerase (Boehringer Mannheim), and 50 pmol of both antisense and sense outer primer (5'-G CCGTGGCGTATGATGAGT-3') per liter. The reaction mixtures were overlaid with 100 μ l of mineral oil (Sigma Chemicals, Milan, Italy) and were incubated at 94°C for 1 min, at 45°C for 1 min, and at 72°C for 1 min. Then, 29 further PCR cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s were run. For the second round of PCR, a 3- μ l aliquot of the first PCR mixture was amplified with 50 pmol of the inner sense (5'-GTGCAGCCTCCAGGACCC-3') and inner antisense (3'-CCGTGAGCGTTCGTGGGATA-5') primers per liter as described above. The amplified products were visualized by electrophoresis in a 1.5% agarose gel with ethidium bromide staining. Both negative (deionized ultrapure water and serum from anti-HCV-negative healthy blood donors) and positive controls were used in each run.

For HCV genotyping, 10 μ l of the PCR products from each sample was denatured at 95°C for 5 min and incubated in a prehybridization solution containing 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.25% powdered milk at 42°C for 1 h. Then, after the addition of 40 μ l of 20 $\times$ SSC (3 mol of NaCl per liter and 0.3 mol of sodium citrate per liter), they were spotted onto five nitrocellulose filters and hybridized with genotype-specific ^{32}P -labeled oligonucleotide probes, as follows: type I, 5'-CGCTCAATGCCTGGAGAT-3'; type 2a, 5'-CACTCTATGCCCCGCCAT-3'; type 2b, 5'-CACTCTATGTCCG GTCAT-3'; type 3, 5'-CGCTCAATACCCAGAAAT-3'; and type 4, 5'-CGCT CAATAGCCCGGAAAT-3'. Hybridization was performed for each filter at 42°C for 2 h. The filters were washed twice with two changes of 6 $\times$ SSC with 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min, twice in 3 $\times$ SSC with 0.1% SDS at room temperature for 10 min, and twice in 1 $\times$ SSC with 0.1% SDS at 55°C for 15 min. Autoradiography was performed with Kodak X-OMAT AR films, with overnight exposure at -80°C. Finally, to distinguish subtype 1a from subtype 1b, PCR products were digested with *Bsr*U-I endonuclease, which recognizes two restriction sites within HCV type 1b (HCV-1b) products and one restriction site in HCV-1a products. Therefore, for samples obtained from patients infected with HCV-1b, digestion with *Bsr*U-I endonuclease generates three fragments (of 137, 44, and 30 bp), whereas for samples obtained from patients infected with HCV-1a, two fragments are generated (of 167 and 44 bp). Digestion products can then be visualized by electrophoresis on 12% acrylamide gels with ethidium bromide staining. For 20 randomly chosen samples, the results of HCV genotyping by this method were confirmed by direct sequencing by the dideoxy chain termination method with T7 DNA polymerase (Sequenase; United States Biochemicals, Denver, Colo.), according to the manufacturer's instructions.

HCV genotyping by PCR amplification of HCV core region (core region assays). PCR amplification of the core region was performed both by the method described by Okamoto et al. (10) (referred to as the Okamoto method) and by a modified version of this original method described by Widell et al. (18) (referred to as the Widell method). Briefly, by the Okamoto method 200 μ l of each of the serum samples was diluted with 1 ml of ice-cold RNA extraction solution (RNA-ZOL; GIBCO BRL, Milan, Italy) and thoroughly mixed. Then, serum proteins were removed by phenol-chloroform extraction. RNA was precipitated by keeping the solution on ice for 10 min after the addition of 1 volume of ice-cold isopropanol. After pelleting by centrifugation in a microcentrifuge, the RNA was washed once with 75% ethanol, vacuum dried, and then resuspended in 50 μ l of diethylpyrocarbonate (DEP)-H₂O. The RNA suspension (15 μ l diluted with 5 μ l of DEP-H₂O) was heated at 65°C for 2 min to denature the RNA. The RNA was maintained at 55°C for 10 min in the presence of 50 pmol of the antisense outer primer (5'-ATGTACCCCATGAGGTCGGC-3') per liter. cDNA synthesis was carried out in a final volume of 25 μ l at 37°C for 60 min in the presence of 2.5 mmol of each deoxyribonucleotide per liter, 40 U of RNAsin (Boehringer Mannheim), and 40 U of murine myeloblastosis virus reverse transcriptase (GIBCO BRL). The cDNA was amplified in the presence of 50 pmol of both outer sense (5'-CGCGCGACTAGGAAGACTTC-3') and outer antisense primers per liter in a final volume of 100 μ l with 2.5 U of *Taq* polymerase (Boehringer Mannheim). Two sequential amplifications were performed. For the first amplification, 35 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min were run. For the second amplification, 30 further cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min were run. A total of 10 μ l of the first amplification was added to 90 μ l of the reaction mixture for the second PCR containing 50 pmol of the universal inner sense primer (5'-AGGAAGACTTC CGAGCGGTC-3') per liter and 50 pmol of each four type-specific inner antisense primers (type I, 5'-TGCCTTGGGGATAGGCTGAC-3'; type II, 5'-GAG CCATCCTGCCACCCCA-3'; type III, 5'-CCAAGAGGGACGGGAACTTC-3'; and type IV, 5'-ACCCTCGTTTCCTGACAGAG-3') per liter. The products of the PCR were identified in acrylamide gel as bands of 57 (type I), 144 (type II), 174 (type III), and 123 (type IV) bp by direct comparison with molecular mass markers. Finally, for the identification of HCV type V (11), which must be carried out in a separate tube, we used 50 pmol of specific sense primers (5'-CGCGCGACGCGTAAACTTC-3') per liter for the first PCR and 50 pmol of antisense primers (5'-GCTGAGCCAGGACCGGTCT-3') per liter and 50 pmol of sense primers (5'-CGTAAACTTCTGAACGGTC-3') per liter for the second PCR. With these primers genotype V appears in the acrylamide gel as a band of 88 bp.

TABLE 1. HCV genotyping by 5'UTR assay and core region assay with serum samples from 106 drug abusers

Core region assay result ^a	No. of samples with the following result by the 5'UTR assay:					
	Type 1a (n = 29)	Type 1b (n = 20)	Type 2a (n = 22)	Type 2b (n = 5)	Type 3a (n = 27)	Type 4a (n = 3)
Core negative (n = 50)	6	9	16	4	12	3
Not classified (n = 11)	2	2	2	1	4	
Type I (n = 25)	16	4	2		3	
Type II (n = 4)		4				
Type III (n = 0)						
Type IV (n = 1)					1	
Type V (n = 8)			2		6	
Mixed infections (n = 7)	5	1			1	

^a Types in roman numerals refer to the classification system by Okamoto et al. (9, 10). In the proposed nomenclature (15), type I corresponds to type 1a, type II corresponds to type 1b, type III corresponds to type 2a, type IV corresponds to type 2b, and type V corresponds to type 3a. Core negative, PCR-negative samples with core universal primers; not classified, samples PCR positive with core universal primers but PCR negative with type-specific primers; mixed infections, infection with more than one type.

Seventy-nine of the 106 serum samples from drug abusers were also tested by the Widell method, which uses the following newly designed primers: first amplification, sense primer 5'-TAGATTGGGTGTGCGCGCA-3' and antisense primer 5'-ATGTACCCCATGAGGTCGGC-3'; second amplification, sense primer 5'-AGGAAGACTTCGAGCGGTC-3', antisense type I-specific primer 5'-TGGGGATAGGCTGACGTCTACCT-3', antisense type II-specific primer 5'-CCATCCTGCCACCCCA-3', antisense type III-specific primer 5'-CCAAG AGGGACGGGAACCTC-3', and antisense type IV-specific primer 5'-GCAAC CCTCGTTTCC(GA)TACAGAG-3'. Type V requires distinct sense (5'-GTGT GCGCGACGCGTAAA-3') and antisense (5'-TAGATTGGGTGTGCGCGCA-3') primers. For further details regarding this method, see the report by Widell et al. (18).

Statistical analysis. In order to detect differences among categorical variables, Pearson chi-square tests were applied, as implemented in program 4F of the BMDP statistical software package, release 7.0.

RESULTS

The Okamoto method was much less efficient than the 5'UTR assay, allowing genotyping of only 75 of 144 samples; in contrast, no genotype remained indeterminate when the 5'UTR assay was used. Concordant results between the two methods were found for 46 of 75 samples (61.3%). In detail, for the group of drug abusers (Table 1), PCR amplification of the core region was unsuccessful for 50 of 106 (47.2%) samples (core-negative samples). Moreover, for 11 patients amplification of core HCV RNA could not be followed by amplification with type-specific primers (indeterminate samples). Among the remaining 45 samples, fully concordant results between the two genotyping methods were found for only 26 samples (57.8%). According to the results of the Okamoto method, samples from seven patients showed mixed infections (type 1a plus type 1b in five patients, type 1a plus type 2b in one patient, and type 2b plus type 3a in one patient); one of the two genotypes was also identified by the 5'UTR assay and was confirmed in each case by direct sequencing of PCR products. If the results of genotyping of these serum samples are considered concordant, the overall concordance for the samples from drug abusers increased to 73.3%. Among the genotypes identified by the Okamoto method, type 1b infections were the only ones consistently confirmed by the 5'UTR assay; on the other hand, the Okamoto method identified only 5 of the 20 patients (25.0%) who, according to the results of the 5'UTR assay, were infected

TABLE 2. Comparison of HCV genotyping by PCR amplification of core HCV RNA by the original Okamoto method and the Widell method

Result by Okamoto method ^a	No. of samples with the following result by the Widell method:					
	Core negative (n = 67)	Not classified (n = 1)	Type 1a (n = 1)	Type 1b (n = 4)	Type 2b (n = 1)	Type 3a (n = 5)
Core negative (n = 39)	36			1		2
Not classified (n = 11)	10				1	
Type I (n = 13)	12			1		
Type II (n = 6)	4			2		
Type IV (n = 1)						1
Type V (n = 7)	5					2
Mixed infections (n = 2)		1	1			

^a Types in roman numerals refer to the classification system by Okamoto et al. (9, 10). In the proposed nomenclature (15), type I corresponds to type 1a, type II corresponds to type 1b, type III corresponds to type 2a, type IV corresponds to type 2b, and type V corresponds to type 3a. Core negative, PCR-negative samples with core universal primers; not classified, samples PCR positive with core universal primers but PCR negative with type-specific primers; mixed infections, infection with more than one type.

with HCV-1b (4 patients were infected with HCV-1b and 1 patient had a mixed infection).

The results of genotyping by the Widell method, performed with a subsample of serum samples including most core-negative and indeterminate serum samples, are presented in Table 2. HCV RNA, however, could be amplified from only 12 of 79 samples (including 3 core-negative samples and 1 indeterminate sample) by this technique. Ten of these 12 samples gave results concordant with those of the 5'UTR assay; 4 of 10 samples had a result concordant with that obtained by the Okamoto method. No mixed infections were detected.

Genotyping of serum samples obtained from patients with chronic liver disease (Table 3) confirmed a disappointingly low rate of concordant results, with the partial exception of genotype 1b, although core-negative samples were rarer in this group (3 of 38 versus 50 of 106 samples; Pearson chi-square test, 18.5; $P < 0.0001$).

A core-negative sample was associated with identification by

TABLE 3. HCV genotyping by 5'UTR assay and core region assay with sera from 38 patients with chronic liver disease

Core region assay result ^a	5'UTR assay				
	Type 1a (n = 5)	Type 1b (n = 17)	Type 2a (n = 14)	Type 2b (n = 0)	Type 3a (n = 2)
Core negative (n = 3)	1	1	1		
Not classified (n = 5)		2	3		
Type I (n = 6)	2	2	2		
Type II (n = 21)	2	11	7		1
Type III (n = 1)					1
Type IV (n = 2)		1	1		

^a Types in roman numerals refer to the classification system by Okamoto et al. (9, 10). In the proposed nomenclature (15), type I corresponds to type 1a, type II corresponds to type 1b, type III corresponds to type 2a, type IV corresponds to type 2b, and type V corresponds to type 3a. Core negative, PCR-negative samples with core universal primers; not classified, samples PCR positive with core universal primers but PCR negative with type-specific primers.

TABLE 4. Factors associated with unsuccessful PCR amplification of core HCV RNA^a

Factor	No. of samples with the following core HCV RNA result/total no. of samples:		<i>P</i>
	Positive	Negative	
Type other than type 1	37/91	36/53	<0.005
Age ≤30 yr	31/91	31/53	<0.005

^a Data were analyzed by the Pearson chi-square test.

the 5'UTR assay of genotypes other than type 1a or 1b and age ≤30 years (Table 4). Among drug abusers, a pattern of drug abuse involving more than 30 injections per week was also significantly associated with unsuccessful amplification of core HCV RNA (15 of 50 versus 8 of 56 samples; Pearson chi-square test, 3.8; $P = 0.05$).

DISCUSSION

HCV genotyping is a time-consuming, expensive procedure that is usually performed in research laboratories. One of the issues that needs to be clarified before using the procedure in clinical practice is to determine whether different genotyping methods are comparable. In the present study, we showed that this is not necessarily true. For reasons which are not entirely clear, our results are at variance with those of the single multicentric study addressing this issue published to date (8), in which the degree of concordance between six different systems of genotyping HCV strains was estimated to be >90%. One explanation for these differences might be the different kinds of patients whom we studied. All our patients were from north-east Italy, whereas Lau et al. (8) studied 139 patients from three areas of North America (Florida, Missouri, and California). These latter patients were comparable in age to the subgroup of patients with chronic liver disease whom we studied. However, we also had a second group, made up of younger subjects, who were drug abusers and who did not necessarily have altered liver function. Finally, our patients had a much higher prevalence of infection with HCV genotypes 2a (25.0 versus 2.2%) and 3a (20.1 versus 5.8%). Indeed, nearly 80% of the population studied by Lau et al. (8) was infected with HCV genotype 1, in comparison with less than 50% in the population group whom we studied.

According to our results, the problem of HCV genotyping by methods based on the variability of the HCV core region is twofold and concerns both the efficiency and the accuracy of these methods. In our study, efficiency was unsatisfactory because of a very high rate of indeterminate results for the samples, particularly samples from drug abusers. Because degradation of the viral genome was excluded (all samples had detectable HCV RNA), indeterminate results were presumably the result of a mismatch between the primers used and the actual core nucleotide sequence of the HCV RNA. In this regard, it should be noted that the comparison of HCV core gene sequences among different isolates whose sequences have been published shows a remarkable degree of variability (3); indeed, genotyping by methods derived from the type-specific primers of the core region designed by Okamoto et al. (9, 10) has already been reported to result, in Italian patients, in a high rate of samples with indeterminate results (12). HCV genotype 3a, the transmission of which has occurred only for the past 20 years, is much more common in drug abusers than in patients with posttransfusion or sporadic HCV hepatitis

(13), who are more frequently infected with genotype 1b (11). It is worth mentioning that as a consequence of the HCV epidemics in drug abusers, the distribution of HCV genotypes is expected to change markedly in the future (11). Accordingly, in our study the highest prevalence of indeterminate results was found among samples from younger patients with high levels of intravenous drug abuse in whom infection with new HCV core variants is more likely.

As far as the accuracy of HCV genotyping is concerned, our study found a poor concordance between the Okamoto method of genotyping and the other two methods, particularly for genotypes other than type 1. Moreover, this time in agreement with Lau et al. (8), we found that the use of the Okamoto method might lead to an overestimation of the rate of mixed infections. Genotyping of PCR-amplified products does not rule out the presence of variants if they do not represent >10 to 20% of the genome amplified; therefore, one might suppose that the original Okamoto method might be highly sensitive in detecting this degree of viral heterogeneity. However, most mixed infections appeared to be with HCV-1b along with another genotype; hence, these results might possibly have been due to nonspecific binding of type 1b-specific primers. Overestimating the prevalence of type 1b, which has been associated with an unfavorable outcome of therapy, might therefore end with patients being unduly excluded from therapy with one of the therapeutic options. Moreover, it has been indicated that drug abusers have a high prevalence of mixed infections (13), probably because of repeated viral inocula, but according to our results, true mixed infection might be rare in this kind of patient and, presumably, exceedingly rare in the general population.

Although modifications to the original Okamoto method by the adoption of newly designed primers have been claimed to improve the efficiency of genotyping by type-specific PCR, in our hands this proved not to be true. The Widell method was more accurate than the Okamoto method, but at the cost of even further reduced efficiency. In other words, the problem with genotyping by core region assays appears more complex, and it is not clear whether it can be resolved simply by using a better selection of primers.

In conclusion, genotyping methods based on PCR amplification of the core region do not ensure the detection of all HCV strains, nor offer results fully comparable to those obtained by other methods, and might be responsible for the overestimation of mixed infections. Our results suggest that caution is needed before considering the results of genotyping obtained by different methodologies comparable and before adopting a unifying nomenclature. Indeed, one might hypothesize that some controversy (2, 17, 19) regarding the clinical value of genotyping might derive from this basic methodological issue. It is hoped that, in the future, laboratories might adopt a single method of HCV genotyping, which we propose might be based on 5'UTR, the best-preserved part of the HCV genome (5).

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