JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 317–321 Vol. 35, No. 1
0095-1137/97/$04.00+0

Concordance of Hepatitis C Virus Typing Methods Based on Restriction Fragment Length Polymorphism Analysis in 5′ Noncoding Region and NS4 Serotyping, but Not in Core PCR or a Line Probe Assay

SONIA NAVAS, INMACULADA CASTILLO, JULIO MARTÍN, JUAN ANTONIO QUIROGA, JAVIER BARTOLOMÉ, AND VICENTE CARREÑO*

Hepatology Unit, Fundación Jiménez Díaz, and Fundación Estudio Hepatitis Virales, Madrid, Spain

Received 11 July 1996/Returned for modification 15 August 1996/Accepted 15 October 1996

Data from sequence analysis, genotyping by restriction fragment length polymorphism in the 5′ noncoding region, and NS4 serotyping of the sera of 20 patients with chronic hepatitis C virus infection have shown 100% concordance, while core-based methods and a line probe assay have shown several missing or wrong results.

Since the isolation and recognition in 1989 of the hepatitis C virus (HCV) as the agent responsible for most cases of non-A, non-B hepatitis (1, 5, 10), several studies have demonstrated a high nucleotide sequence variability in its RNA genome. At least six major HCV genotypes (designated 1 to 6) which differ in nucleotide sequence by more than 30% over the complete virus genome have been described, and a number of subtypes differing by more than 20% in sequence (designated 1a to 1c, 2a to 2c, 3a and 3b, 4a to 4f, 5a, and 6a) have been discovered (the discovery of new subtypes is expected) (16, 21, 22). Furthermore, the description and classification of new variants is under investigation. In addition, studies of possible correlations between different genotypes and both the evolution of liver damage and the response to antiviral treatment have shown that HCV 1b is more frequently found in patients with elevated aminotransferase levels and evidence of chronic liver disease (20, 24) and that infection by HCV 1b is less responsive to antiviral therapy than that by other subtypes (4, 9, 15, 25). However, there are still some controversies about the prognostic significance of genotypes for disease outcome (7).

In addition to HCV sequence analysis, several methods have been developed for HCV typing (6, 14, 17, 18, 23). Several authors have recently reported partial comparisons of some of these methods (8, 12, 13, 26), but some discrepancies have been observed in their results, and a more complete study is required. For this reason, the aim of this study was to investigate the accuracy of the methods currently most used (amplification with subtype-specific primers, hybridization with subtype-specific oligonucleotide probes, restriction fragment length polymorphism [RFLP] analysis, and serological response to synthetic peptides) for predicting HCV genotypes and subtypes in several regions of the HCV genome (5′ noncoding [5′NC], core, and NS4), as well as to compare the results of these methods with data obtained by sequence analysis in the 5′NC and core regions of HCV.

To this end, we have analyzed the sera of 20 patients with histologically proven chronic active hepatitis due to HCV infection. All of them had anti-HCV antibodies, as detected by ELISA III (Ortho Diagnostic Systems, Raritan, N.J.) and confirmed by RIBA III (Ortho). The criterion used to select these patients was the presence of HCV RNA in serum, as detected by reverse transcription (RT)-PCR after HCV RNA extraction by using the guanidinium isothiocyanate method (3) with primers from the 5′NC region of the HCV genome as previously described (14). In addition, quantitation of HCV RNA in copies per milliliter with the Amplicor HCV Monitor assay (Roche Diagnostic Systems, Inc., Branchburg, N.J.) was performed in duplicate. To avoid false-positive results, the contamination prevention measures of Kwok and Higuchi (11) were followed, and negative controls were included in all experiments. Genotyping by each method tested was performed in duplicate for all samples, and concordant results were obtained in each case.

Core genotyping. HCV genotyping by amplification in the core region of the HCV genome was performed in three ways: (i) as previously described by Okamoto et al. (named Core^{A}) (17), with a mixture of one universal sense primer and four antisense subtype-specific primers (for 1a, 1b, 2a, and 2b subtypes); (ii) according to the revised method described by Okamoto et al. (named Core^{B}) (18), improved to detect HCV subtypes 1a, 1b, 2a, 2b, and 3a; and (iii) by using a modification of this method which consists of the amplification of each HCV subtype with the same primers as those of the Core^{A} or Core^{B} method but in independent nested PCRs, one for each subtype. The latter modification was performed for the two core-based methods. All PCR products were visualized by polyacrylamide gel electrophoresis and ethidium bromide staining.

By using the Core^{A} assay, a high proportion of untyped samples was found (7 of 20; 35%) (Table 1). HCV 1b was detected in 40% (8 of 20), whereas HCV 1a was detected in only 15% (3 of 20). Mixed infections (1a plus 1b or 1b plus 2a) were detected in 10% (2 of 20). When the Core^{B} assay was applied, the percentage of unknown genotypes decreased to only 5% (1 of 20) (Table 1). In contrast to the Core^{A} results, apparently mixed infections were found in the rest of the samples (19 of 20). These were the following: HCV 1a plus 1b (13 of 20), HCV 1a plus 1b plus 3a (4 of 20), HCV 1a plus 1b plus 2a (1 of 20), and HCV 1a plus 3a (1 of 20).

By using independent reactions for each genotype, two of seven samples negative by Core^{A} assay were typed as HCV 1a (samples 1 and 14), the other Core^{A} results having been confirmed in the rest of the samples. When HCV genotyping was performed by independent reactions for each genotype in the Core^{B} assay, the same results as those for one-tube genotyping

* Corresponding author. Mailing address: Hepatology Unit, Fundación Jiménez Díaz, Av. Reyes Católicos, 2, 28040 Madrid, Spain. Phone: 34 1 543.19.64. Fax: 34 1 544.92.28.

TABLE 1. Results of HCV genotyping and serotyping by all methods and HCV RNA loads

Sample	HCV genotype or serotype by indicated method						
	Core^{A} (mixture/independent[a])	Core^{B} (mixture/independent[b])	5′NC RFLP	LIPA II	NS4 serotyping	Sequence analysis	HCV RNA load (copies/ml)
1	Negative/1a	1a+1b+3a/1a+1b+3a	1a	1a	1	N.D.[c]	1.2×10^4
2	Negative/negative	1a+1b/1a+1b	1b	Unknown	1	1b	1.9×10^3
3	1b/1b	1a+1b+3a/1a+1b+3a	3a	1	3	3a	3.8×10^3
4	1a/1a	1a+1b/1a+1b	1a	1a	1	N.D.	1.4×10^4
5	1b/1b	1a+1b+2a/1a+1b+2a	1b	1b	1	1b	2.3×10^5
6	1b/1b	1a+1b/1a+1b	1b	1	1	1b	2.6×10^4
7	Negative/negative	Negative/negative	4	1b	4	4d	1.0×10^4
8	1b/1b	1a+1b/1a+1b	1b	1b	1	1b	1.1×10^5
9	1b/1b	1a+1b/1a+1b	1b	1b	1	N.D.	5.0×10^5
10	1b/1b	1a+1b/1a+1b	1b	1b	1	N.D.	8.0×10^4
11	1a/1a	1a+1b/1a+1b	1b	1b	1	1b	5.3×10^4
12	Negative/negative	1a+3a/1a+3a	3a	3	3	3a	0.8×10^3
13	Negative/negative	1a+1b/1a+1b	1a	1a	1	1a	9.1×10^3
14	Negative/1a	1a+1b/1a+1b	1a	1b	1	1a	2.2×10^3
15	1b/1b	1a+1b/1a+1b	1b	1b	1	N.D.	1.1×10^5
16	Negative/negative	1a+1b/1a+1b	1b(+1a?)[d,e]	1b	1	1a+1b	5.5×10^3
17	1a/1a	1a+1b/1a+1b	1b(+1a?)[e]	Unknown	1	1a+1b	6.0×10^3
18	1a+1b/1a+1b	1a+1b+3a/1a+1b+3a	3a	3	3	3a	2.0×10^3
19	1b/1b	1a+1b/1a+1b	2a	2a	2	2a	7.8×10^3
20	1b+2a/1b+2a	1a+1b+3a/1a+1b+3a	1b	1b	1	1b	7.8×10^3

[a] Results obtained with the mixture of primers as previously described by Okamoto et al. (17) and with independent reactions for each subtype.
[b] Results obtained with the mixture of primers as previously described by Okamoto et al. (18) and with independent reactions for each subtype.
[c] N.D., not done.
[d] This coinfection was detected by the 5′NC RFLP analysis only when using the enzyme *Alw*26I instead of *Bst*UI in order to differentiate between 1a and 1b subtypes.
[e] ?, inconclusive result.

with the Core^{B} assay were obtained. Thus, by Core^{B} assay, 95% of the samples had mixed infections.

5′NC region genotyping. HCV genotyping was also performed in the 5′NC region of the HCV genome in three ways: (i) by using the line probe assay (INNO-LIPA; Innogenetics NV, Zwijnaarde, Belgium), version II, in which oligonucleotide probes derived from the 5′NC region are hybridized with the biotinylated nested-PCR products (23); (ii) by amplification with universal primers followed by RFLP analysis of the radiolabelled [^{32}P]dATP nested-PCR products, as previously described by McOmish et al. (14); and (iii) by an alternative RFLP-typing scheme with the same PCR products as in (ii) above in two consecutive enzymatic reactions in order to discriminate among HCV types 1 to 6, the first reaction being performed with the enzyme *Mbo*I and the second, if needed, being performed with *Fok*I plus *Alw*26I or with *Hae*III plus *Alw*26I (electrophoretic patterns are shown in Fig. 1). Since HCV types 5 and 6 were not found in our population, their electrophoretic patterns were studied by computer analysis of theoretical digestions in published sequences obtained from the GenBank database. In the enzymatic digestions, 4 μl of nested-PCR products was incubated overnight at 37°C with 10 U of the corresponding restriction enzymes (Promega Corp., Madison, Wis.). The differences in the sizes of digested DNA were resolved by 12% polyacrylamide gel electrophoresis followed by autoradiography.

By using the line probe assay (LIPA), the detection of HCV genotypes and subtypes was as follows: 1b, 10 of 20 (50%); 1a, 3 of 20 (15%); type 1 without defined subtype, 2 of 20 (10%); 2a, 1 of 20 (5%); type 3 without defined subtype, 2 of 20 (10%); and undetermined, 2 of 20 (10%) (Table 1). Genotyping by 5′NC RFLP analysis with the two alternative RFLP-typing schemes showed 100% concordance between them. Genotype determinations were as follows: HCV type 1, 15 of 20 (75%); HCV type 2, 1 of 20 (5%); HCV type 3, 3 of 20 (15%); and HCV type 4, 1 of 20 (5%). Mixed infections (1a plus 1b) were suspected in two cases. Genotyping by 5′NC RFLP analysis and LIPA methods differed in 20% of the samples (4 of 20), and the differences observed were as follows: for two of the four samples, HCV type 1 detected by RFLP assays was not recognized by LIPA (unknown genotype); the two other samples (one HCV type 3 and one HCV type 4 by 5′NC RFLP methods) were recognized as HCV 1 by LIPA (Table 1).

Subtyping by 5′NC RFLP. Subtyping of samples with genotype 2 or 3 was performed as described by Davidson et al. (6), giving subtype 2a for a sample with genotype 2 and 3a for the three samples with type 3. HCV 1a and 1b subtyping was carried out using three restriction enzymes: *Bst*UI (New England Biolabs, Beverly, Mass.) as previously described (6), *Mvn*I (isoschizomer of *Bst*UI) (Boehringer GmbH, Mannheim, Germany), and *Alw*26I (Promega Corp.) (generating two fragments for HCV 1b [175 and 76 bp] but no digestion of HCV 1a).

HCV 1a and 1b subtyping of the 15 samples having type 1 with the enzyme *Bst*UI gave clear electrophoretic patterns in all but one (sample 17), in which the digestion showed an inconclusive 1a-1b electropherotype (presence of electrophoretic bands of 210, 180, 41, and 30 bp). When HCV 1a and 1b subtyping was performed with *Mvn*I, almost all of the samples gave inconclusive results. In contrast, when *Alw*26I was used to differentiate between 1a and 1b subtypes, inconclusive electropherotypes were observed in only two samples (16 and 17; presence of electrophoretic bands of 251, 175, and 76 bp). These inconclusive electropherotypes might reflect, for *Bst*UI and *Alw*26I, the existence of an undigested PCR product of the 1b subtype or a real mixed infection (HCV 1a plus 1b).

Subtyping by both RFLP methods and LIPA differed in 2 of the 16 samples with identical genotypes. For one sample, the

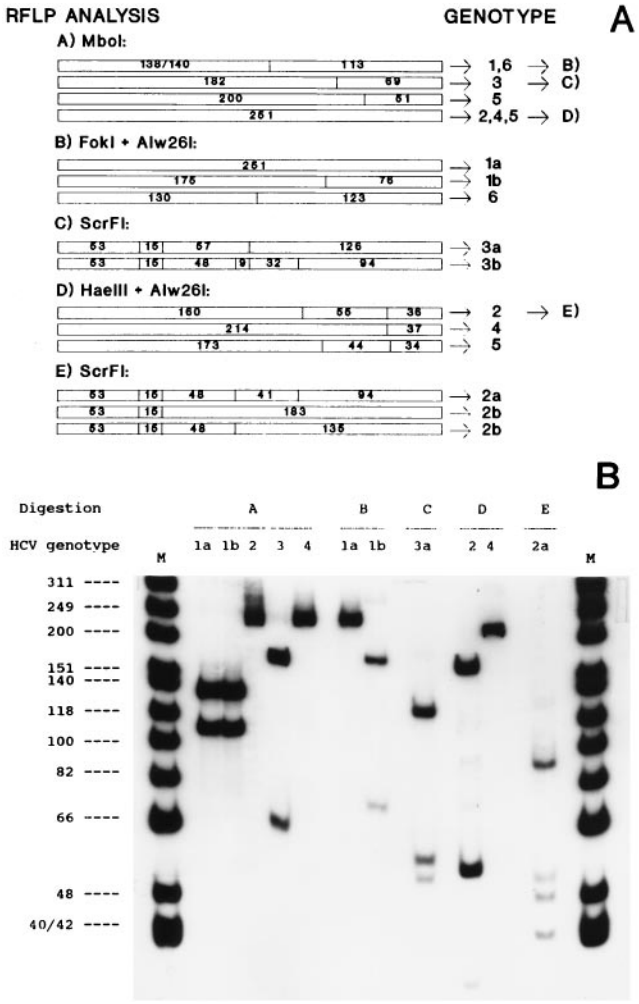

FIG. 1. (A) Schematic representation of predicted electrophoretic patterns in the 5′NC regions of HCV types 1 to 6. The patterns were determined by cleavage with *Mbo*I (digestion A) followed, if needed, by cleavage with *Fok*I plus *Alw*26I (digestion B), *Scr*FI (digestion C), or *Hae*III plus *Alw*26I (digestion D) and finally, if needed, by cleavage with *Scr*FI again (digestion E). The expected sizes of fragments are given in base pairs. (B) Autoradiography of electrophoretic patterns obtained for HCV types 1 to 4 by using the above-described scheme of enzymatic digestions. Lane M, PhiX174-RF DNA *Hae*III-digested molecular size markers (sizes are expressed in base pairs).

HCV subtype was 1a by 5′NC RFLP but was subtype 1b by LIPA; for another, the HCV was 1a plus 1b by 5′NC RFLP, but was HCV 1b alone by LIPA (Table 1).

In summary, excluding the Core^B assay (which appears to be almost useless for typing since it detected mixed infections and could not identify 19 of 20 specimens), only 8 of 20 (40%) serum samples analyzed gave concordant results with all the methods applied (Core^A assay, LIPA, 5′NC RFLP as described by Davidson et al. [6], and modified 5′NC RFLP as described in this paper).

Sequence analysis. As the definitive approach to the investigation of discrepant results among different methods in HCV genotyping, sequence analysis was performed after amplification on the 5′NC region and the core gene (nucleotides 259 to 492) of the HCV genome (outer primers: sense, 5′-CTGTGA GGAACTACTGTCTT-3′, and antisense, 5′-RAAGATAGA RAARGAGCAACCKGG-3′; inner primers: sense, 5′-TTCA CGCAGAAAGCGKCTAG-3′, and antisense, 5′-CCRGGNA RRTTCCCYGTTGC-3′; nucleotide symbols correspond to the International Union of Pure and Applied Chemistry-International Union of Biochemistry biochemical nomenclature). Nested-PCR products were cloned (pMOS Blue T-vector kit, Amersham International, Little Chalfont, United Kingdom) and sequenced (Sequenase version 2.0; U.S. Biochemicals, Cleveland, Ohio) for the 12 samples with discrepant results (12 of 20 [60%]; samples 2, 3, 6, 7, 11, 13, 14, 16, 17, 18, 19, and 20) among the four methods taken into consideration (Core^A, both 5′NC RFLP typing schemes, and LIPA), as well as for 3 samples with concordant results (samples 5, 8, and 12). In the three samples with concordant results, the HCV sequence analysis consistently confirmed the previously obtained genotype result by the four methods studied.

In order to investigate the inconclusive HCV 1a-1b electropherotype of samples 16 and 17, 30 clones from each sample with an insert of 791 bp corresponding to the 5′NC and core regions of HCV (nucleotides 279 to 512) were sequenced. The sequencing results demonstrated the presence of 1a plus 1b coinfections with different 1a to 1b proportions (sample 16: 1a, 1 clone [3%]; 1b, 29 clones [97%]; sample 17: 1a, 17 clones [57%]; 1b, 13 clones [43%]). It is of note that Simmonds et al. (21) have reported that approximately 2 to 5% of the 5′NC sequences of HCV genotype 1 may have a mutation in nucleotide 99 which could induce a false 1a-1b subtyping. In our study, this change was not observed. Figure 2 shows the alignment of the consensus sequences of the 5′NC and core regions of subtypes 1a and 1b (2), with the consensus sequences obtained for each subtype in the two samples with coinfection 1a plus 1b. The sequence divergences in the core region (nucleotides 1 to 492) among consensus sequences of subtypes 1a and 1b (2) and sequences obtained of subtypes 1a and 1b in samples 16 and 17 were studied (Table 2). As expected, the divergences observed between sequences of the same subtype were smaller than those observed between sequences of different subtypes.

Because RFLP with *Bst*UI subtyped sample 16 as infected only with HCV 1b (not revealing the presence of HCV 1a), all the 801-bp clones obtained from this sample were subjected to amplification of the 5′NC region (6) and the PCR products were subtyped again with *Bst*UI. In all cases, the enzyme gave the expected electrophoretic pattern according to the subtypes deduced by sequencing. Thus, we do not have an explanation for the lack of detection of a mixed infection by *Bst*UI subtyping in the original PCR product of this patient.

In the five samples with discrepancies in genotyping or subtyping results between the 5′NC RFLP and LIPA methods (samples 2, 3, 6, 7, and 14), the sequencing results demonstrated that these five samples (one with subtype 3a, one with genotype 4, two with subtype 1b, and one with subtype 1a) were erroneously recognized as HCV 1b, type 1, or not identified by LIPA, while both 5′NC RFLP analyses gave the correct results. The 5′NC sequences of these samples presented the corresponding consensus type motifs of each genotype or subtype, without any mutation which could justify theoretical hybridization with LIPA HCV 1b probes or the lack of hybridization with the corresponding oligonucleotide probes.

Sequence analysis of those samples with discrepancies between the 5′NC RFLP, LIPA, and Core^A methods showed 100% concordance between the HCV type demonstrated by sequencing and the type assigned by the 5′NC RFLP and LIPA methods. Thus, HCV types 2a and 3a were mistyped by Core^A as HCV 1b (samples 19 and 3) or HCV 1a plus 1b (sample 18). Furthermore, in several samples, the Core^A assay failed to recognize HCV 1b (sample 2), HCV 1a (sample 13), or HCV 1a plus 1b (sample 16) and mistyped HCV 1b (samples 11 and

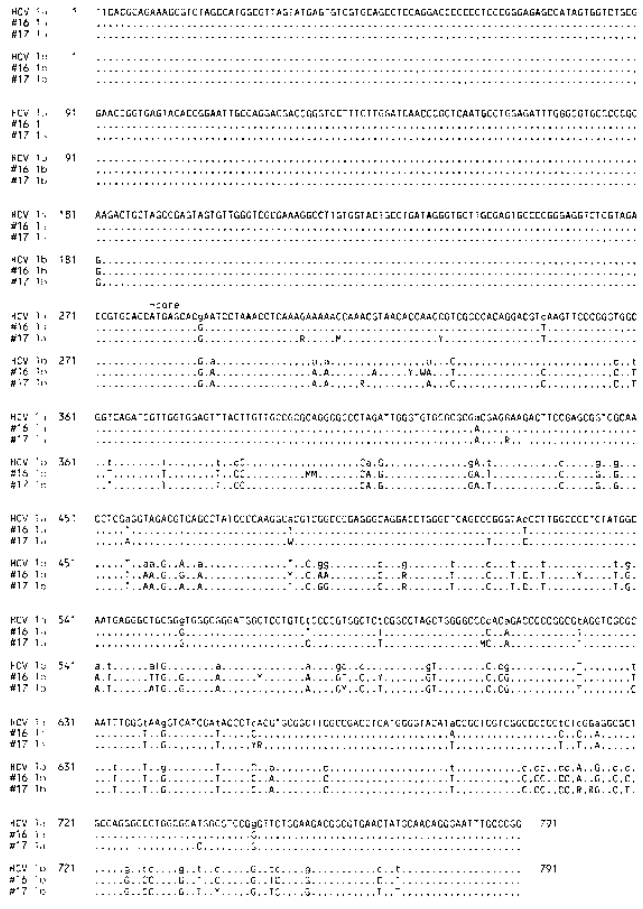

FIG. 2. Alignment of the consensus sequences of the 5′NC and core regions (nucleotides 279 to 512) of HCV subtypes 1a (HCV 1a) and 1b (HCV 1b) (2) with the consensus sequences obtained for each subtype in the two samples with coinfection (samples 16 and 17), after analyzing 30 clones (sample 16: 1a, 1 clone; 1b, 29 clones; sample 17: 1a, 17 clones; 1b, 13 clones). Invariant nucleotides within a consensus sequence are capitalized, and variable nucleotides are lowercase. Invariant nucleotides with respect to HCV 1a are denoted by dots. Nucleotide symbols correspond to the International Union of Pure and Applied Chemistry-International Union of Biochemistry biochemical nomenclature.

20) and HCV 1a plus 1b (sample 17) as HCV 1a and 1b plus 2a, respectively.

In addition to the lack of specificity reported above in the type-specific primer corresponding to 1a, it is worth noting that the Core[B] type primer used for detection of HCV 3a amplified nonspecifically both HCV 1a (sample 1) and HCV 1b (sample 20). This lack of type specificity was experimentally assessed by

TABLE 2. Sequence divergence in the core region of HCV genome (nucleotides 1 to 492) in samples 16 and 17[a]

Sample no. and subtype	% Sequence divergence from sequence of:				
	HCV 1a	HCV 1b	16 1a (n = 1)	16 1b (n = 29)	17 1a (n = 17)
HCV 1b	2–18				
16 1a (n = 1)	1.0	5.9			
16 1b (n = 29)	5.9–7.5	1.4–3.3	5.7–7.5		
17 1a (n = 17)	1.2–2.8	6.3–7.9	1.6–3.3	6.3–9.6	
17 1b (n = 13)	5.9–6.9	0.4–1.8	5.5–6.5	1.8–4.7	6.1–8.7

[a] HCV 1a and HCV 1b refer to the consensus sequences of the core regions of subtypes (2). n, number of clones typed as 1a or 1b in each sample analyzed.

TABLE 3. Correspondence between actual HCV types and the results obtained from the typing methods studied

Actual HCV type	No. of samples correctly identified (%) by indicated method			
	5′NC RFLP	LIPA II	NS4 serotyping[a]	Core[A]
1a (n = 4)	4 (100)	3 (75)		3 (75)
1b (n = 9)	9 (100)	7 (78)	15 (100)	6 (67)
1a + 1b (n = 2)	2 (100)[b]	0 (0)		0 (0)
2a (n = 1)	1 (100)	1 (100)	1 (100)	0 (0)
3a (n = 3)	3 (100)	2 (67)	3 (100)	
4 (n = 1)	1 (100)	0 (0)	1 (100)	

[a] NS4 serotypes can be related only to HCV genotypes, not to subtypes.

[b] One of these coinfections was detected by the 5′NC RFLP analysis only when using the enzyme *Alw*26I instead of *Bst*UI in order to differentiate between the 1a and 1b subtypes.

means of PCR with HCV 1a and HCV 1b plasmids (containing 5′NC and core regions of the HCV genome). In both cases, a false HCV 3a fragment (84 bp) was obtained.

NS4 serotyping. Serological response of the host to virus infection was also studied by using a commercial enzyme immunoassay which employed synthetic NS4 peptides (Murex Diagnostics Limited, Dartford, United Kingdom). The results obtained for 5′NC RFLP genotyping and for NS4 serotyping showed a concordance of 100%.

Relation between HCV genotypes and viremia. Some authors have reported a correlation between infection by virus with the HCV 1b genotype and the presence of high titers of virus in serum (19). In spite of the small number of samples analyzed in this study, we have evaluated the possible relationship between the viral load in each sample (expressed as the average of two determinations, in copies per milliliter) (Table 1) and the real HCV genotype. Statistical analysis has been performed with the nonparametric Wilcoxon's rank sum test for comparison of two groups of independent samples.

Thus, the viral load of samples with genotypes 2, 3, or 4 (taken together) showed a statistically significant lower value than those with genotype 1 ($P < 0.05$), and samples with genotypes 1a, 2, 3, or 4 (taken together) also showed a statistically significant lower viral load than those infected with genotype 1b only ($P < 0.05$). In addition, samples with genotype 3 showed a statistically significant lower viral load than those with genotype 1 or with genotype 1b only ($P < 0.05$ in both cases).

It is not likely that the statistically significant differences reported above are due to a possible difference in the efficiencies of amplification of the genotypes by the set of primers used in the Amplicor HCV Monitor assay, since these are almost completely conserved among the sequences reported for each HCV genotype, with the sole exception of two polymorphic sites in the sense primer KY80 (27).

Conclusions. Considering the genotyping and serotyping results as a group, it is remarkable that data from sequence analysis, genotyping by RFLP in the 5′NC region in the two typing schemes tested, and serotyping with peptides derived from the NS4 region showed a 100% concordance. Accordingly, 5′NC RFLP analysis seems to be the most accurate method for HCV genotyping (Table 3). In contrast, LIPA II failed to recognize the real genotype in one case each of samples with HCV types 1a, 1b, 3a, and 4 and was unable to detect the two cases with coinfection 1a plus 1b. Furthermore, the Core[A] typing method failed to recognize the real genotype in one sample with HCV 1a, in three samples with HCV 1b, in the

sample with HCV 2a, and in the two samples with coinfection HCV 1a plus 1b.

Finally, although there was a 100% concordance between 5′NC RFLP and NS4 serotyping, the fact that serotyping cannot discriminate between HCV subtypes makes this method less useful than RFLP analysis in the 5′NC region. With respect to the latter method, the typing scheme proposed in this study, based on a first digestion with the enzyme *Mbo*I, is as efficient as that previously reported by McOmish et al. (14), but its requirement of a lesser number of enzymatic reactions makes the *Mbo*I-based method more advisable.

REFERENCES

1. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q. L. Choo, and G. Kuo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. **321:**1494–1500.
2. **Buck, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.
3. **Castillo, I., J. Bartolomé, J. A. Quiroga, and V. Carreño.** 1992. Comparison of several PCR procedures for detection of serum HCV-RNA using different regions of the HCV genome. J. Virol. Methods **38:**71–80.
4. **Chemello, L., A. Alberti, K. Rose, and P. Simmonds.** 1994. Hepatitis C serotype and response to interferon therapy. N. Engl. J. Med. **330:**143.
5. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a bloodborne non-A, non-B viral hepatitis genome. Science **244:**359–362.
6. **Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, G. A. Medgyesi, H. Kiyokawa, G. Olim, G. Duraisamy, T. Cuypers, A. A. Saeed, D. Teo, J. Conradie, M. C. Kew, M. Lin, C. Nuchaprayoon, O. K. Ndimbie, and P. L. Yap.** 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. J. Gen. Virol. **76:**1197–1204.
7. **Dusheiko, G., H. Schmilovitz-Weiss, D. Brown, F. McOmish, P. L. Yap, S. Sherlock, N. McIntyre, and P. Simmonds.** 1994. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology **19:**13–18.
8. **Giannini, C., V. Thiers, J.-B. Nousbaum, L. Stuyver, G. Maertens, and C. Brechot.** 1995. Comparative analysis of two assays for genotyping hepatitis C virus based on genotype-specific primers or probes. J. Hepatol. **23:**246–253.
9. **Hino, K., S. Sainokami, K. Shimoda, S. Iino, Y. Wang, H. Okamoto, Y. Miyakawa, and M. Mayumi.** 1994. Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. J. Med. Virol. **42:**299–305.
10. **Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnik, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, H. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W. S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244:**362–364.
11. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positive with PCR. Nature **339:**237–238.
12. **Lau, J. Y. N., M. Mizokami, J. A. Kolberg, G. L. Davis, L. E. Prescott, T. Ohno, R. P. Perrillo, K. L. Lindsay, R. G. Gish, K.-P. Qian, M. Kohara, P. Simmonds, and M. S. Urdea.** 1995. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. J. Infect. Dis. **171:**281–289.
13. **Mahaney, K., V. Tedeschi, G. Maertens, A. Di Bisceglie, J. Vergalla, J. Hoofnagle, and R. Sallie.** 1994. Genotypic analysis of hepatitis C virus in American patients. Hepatology **20:**1405–1411.
14. **McOmish, F., P. L. Yap, B. C. Dow, E. A. C. Follett, C. Seed, A. J. Keller, T. J. Cobain, T. Krusius, E. Kolho, R. Naukkarinen, C. Lin, C. Lai, S. Leong, G. A. Medgyesi, M. Hejjas, H. Kiyokawa, K. Fukada, T. Cuypers, A. A. Saeed, A. M. Alrasheed, M. Lin, and P. Simmonds.** 1994. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J. Clin. Microbiol. **32:**884–892.
15. **Mita, E., N. Hayashi, H. Hagiwara, K. Ueda, Y. Kanazawa, A. Kasahara, H. Fusamoto, and T. Kamada.** 1994. Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. Dig. Dis. Sci. **39:**977–982.
16. **Okamoto, H., K. Kurai, S. Okada, K. Yamamoto, H. Iizuka, T. Tanaka, S. Fukuda, F. Tsuda, and S. Mishiro.** 1992. Full-length sequence of hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology **188:**331–341.
17. **Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugay, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. **73:**673–679.
18. **Okamoto, H., H. Tokita, M. Sakamoto, M. Horikita, M. Kojima, H. Iizuka, and S. S. Mishiro.** 1993. Characterization of the genomic sequence of type V (3a) hepatitis C virus isolates and PCR primers for specific detection. J. Gen. Virol. **74:**2385–2390.
19. **Pozzato, G., S. Kaneko, M. Moretti, L. S. Croce, F. Franzin, M. Unoura, L. Bercich, C. Tiribelli, M. Crovatto, G. Santini, and K. Kobayashi.** 1994. Different genotypes of hepatitis C virus are associated with different severity of chronic liver disease. J. Med. Virol. **43:**291–296.
20. **Silini, E., F. Bono, A. Cividini, A. Cerino, S. Bruno, S. Rossi, G. Beloni, B. Brugnetti, E. Civardi, L. Salvaneschi, and M. U. Mondelli.** 1995. Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. Hepatology **21:**285–290.
21. **Simmonds, P., A. Alberti, H. J. Alter, F. Bonino, D. W. Bradley, C. Bréchot, J. T. Brouwer, S. W. Chan, K. Chayama, D. S. Chen, Q. L. Choo, M. Colombo, H. T. M. Cuypers, T. Date, G. M. Dusheiko, J. I. Esteban, O. Fay, S. J. Hadziyannis, J. Han, A. Hatzakis, E. C. Holmes, H. Hotta, M. Houghton, B. Irvine, M. Kohara, J. A. Kolberg, G. Kuo, J. Y. N. Lau, P. N. Lelie, G. Maertens, F. McOmish, T. Miyamura, M. Mizokami, A. Nomoto, A. M. Prince, H. W. Reesink, C. Rice, M. Roggendorf, S. W. Schalm, T. Shikata, K. Shimotohno, L. Stuyver, C. Trepo, A. J. Weiner, P. L. Yap, and M. S. Urdea.** 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology **19:**1321–1324. (Letter.)
22. **Simmonds, P., E. C. Holmes, T. A. Cha, S. W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. **74:**2391–2399.
23. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. **74:**1093–1102.
24. **Takada, N., S. Takase, N. Enomoto, A. Takada, and T. Date.** 1992. Clinical backgrounds of the patients having different types of hepatitis C virus genomes. J. Hepatol. **14:**35–40.
25. **Tsubota, A., K. Chayama, K. Ikeda, A. Yasuji, I. Koida, S. Saitoh, M. Hashimoto, S. Iwasaki, M. Kobayashi, and K. Hiromitsu.** 1994. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology **19:**1088–1094.
26. **Van Doorn, L.-J., B. Kleter, L. Stuyver, G. Maertens, H. Brouwer, S. Schalm, R. Heijtink, and W. Quint.** 1994. Analysis of hepatitis C virus genotypes by a line probe assay and correlation with antibodies profiles. J. Hepatol. **21:**122–129.
27. **Young, K. K. Y., R. M. Resnick, and T. W. Myers.** 1993. Detection of hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. J. Clin. Microbiol. **31:**882–886.