Subtyping of Hepatitis C Virus Isolates by a Line Probe Assay Using Hybridization

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Received 17 June 1994/Returned for modification 9 August 1994/Accepted 27 September 1994

A reverse hybridization test (Inno-LiPA HCV; Innogenetics, N.V., Zwijnaarde, Belgium) was used for typing hepatitis C virus. All 38 samples, typed by PCR with primers from core and NS5 genes, were also genotyped by this test. Of the samples, 33 (87%) had the same subtypes by both assays. The correlations between PCR and Inno-LiPA for individual types were 77% for type I (1a), 90% for type II (1b), 100% for type III (2a), 100% for type IV (2b), and 100% for type V (3a). One of the type III (2a) samples also reacted with type I (1a) probes in the Inno-LiPA test.

A number of genotypes of hepatitis C virus (HCV) have been reported, depending on the methods used, such as detailed sequence homology studies (3), restriction fragment length polymorphisms of amplified PCR products (7, 8), and PCR amplification with subtype-specific primers (1, 4, 9, 10). The genotyping of HCV isolates has become important for epidemiological studies (2, 13) and treatment with interferon (6, 14).

Recently, a commercial test for HCV subtyping has been developed by Innogenetics, N.V., Zwijnaarde, Belgium. In this assay, oligonucleotides derived from the 5' noncoding region (NCR) act as specific probes for each genotype. The specific probes are immobilized as parallel lines on nitrocellulose strips and are then hybridized with amplified PCR product. The published data suggest that this line probe assay (LiPA) is able to identify HCV subtypes (12).

In this report we have compared genotyping by PCR and Inno-LiPA (Innogenetics) for HCV isolates. A total of 38 serum samples positive for anti-HCV by enzyme immunoassay and RIBA-3 (Chiron Corporation, Emeryville, Calif.) and positive for HCV RNA by PCR were typed by specific primers from the core and NS5 regions (1). Of the samples, 13 were type I, 10 were type II, 7 were type III, 6 were type IV, and 2 were type V according to the typing scheme of Okamoto et al. (9). According to the HCV genotype classification proposed by Simmonds et al. (11) and recently adopted by consensus at the 2nd International Conference of HCV and Related Viruses (August 1994, San Diego, Calif.), these genotypes will be further referred to as 1a (I), 1b (II), 2a (III), 2b (IV), and 3a (V). These samples were further tested with the Inno-LiPA HCV amplification and hybridization kit. The HCV RNA was extracted from 50 μ l of serum by a previously described method (5). The PCR amplification was performed with biotinylated primers according to the manufacturer's protocol. The nucleotide sequences, orientations, and positions of the primers were as follows: CCCTGTGAGGAAGTCTGTCTTCACGC, sense, –299; GGTGCACGGTCTACGAGACCT, antisense, –1; TCTAGCCATGGCGTTAGTRYGAGTGT, sense, –264; and CACTCGCAAGCACCTATCAGGCA GT, antisense, –29 (12). The Inno-LiPA HCV typing is based on the reverse hybridization principle. Oligonucleotide probes, specific for each subtype, are hybridized with an amplified bio-

tinylated sample. Following hybridization and a washing step, streptavidin labelled with alkaline phosphatase is used to detect the biotinylated hybrid. Color development was achieved by addition of the substrate nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP). The test was performed according to the recommended protocol. The pattern of reactivity to different probes was noted, and the subtypes were interpreted by comparison of the patterns of the reactive lines with a template (Fig. 1). The Inno-LiPA kits were kindly provided by Innogenetics for trial. All samples for which results that were discordant between Inno-LiPA and PCR subtyping were observed were further subjected to direct PCR cycle sequencing with the AmpliTaq Cycle Sequencing Kit (Perkin-Elmer Cetus, Norwalk, Conn.). The sequencing primers for the 5' NCR were 5'-GTGCAGCCTCCAGGACCC-3' (position –223, sense), 5'-GGGCACTCGCAAGCACCC-3' (position –43, antisense), and 5'-TAGATTGGGTGTGCGC GCGA-3' (position –467, sense) for the core gene.

The results showed that most of the samples genotyped by PCR were also subtyped by the Inno-LiPA system (Table 1). From the 13 samples genotyped as type 1a by PCR, 10 were characterized as type 1a by Inno-LiPA, 2 were characterized as type one prime (1'), and one was characterized as type 1b by a later test. This difference was not significant ($P = 0.22$, Fisher's exact test), although the statistical analysis was hampered by the small number of samples tested. According to the protocol, the type 1' samples belong to type 1 because they react with group probes (3 and 4); however, because of a point mutation in the region corresponding to subtyping probe 5, they do not hybridize to it and thus cannot be further subtyped as 1a. This was confirmed with one of the two type 1' samples. Upon sequencing of the 5' NCR of sample 223, a single nucleotide substitution (T instead of C at position –94) was observed in the region corresponding to subtyping probe 5.

Sequencing of the 5' NCR of the second type 1' sample (1571) did not show mutations that could abolish the hybridization with subtype-specific probes for type 1. However, we found an A-to-G transition at position –99, suggesting that this sample should have reacted in the first place with subtype-specific probe 6 (genotype 1b). To further clarify this observation, we sequenced a PCR-amplified fragment of the core gene, in which a significant variation between type 1a and 1b is usually found (nucleotides 481 to 642), and complete sequence homology with previously published HCV genotype 1a was

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FIG. 1. (A) Location of different probes: 1, conjugate control; 2, amplification control. Lanes, 1a, 1b, 2a, 2b, and 3a, reactivity patterns of the respective subtypes; lane 1', type 1 mutant. The interpretation of the reactivity pattern for 3a is line 11 and/or line 12 and/or line 13; that for 3b is lines 11, 14, and 15.

observed. This suggests that this sample is type 1a, although it is not clear why it did not react with the subtype-specific probe.

The third discrepant sample (559), which was characterized as type 1b by Inno-LiPA and type 1a by PCR genotyping with both core and NS5 gene-specific primers, also had G at position -99. The sequencing of the above-mentioned region in the core gene also confirmed that it was type 1a of HCV.

Of samples genotyped as type 1b by PCR, 90% were also characterized as subtype 1b by Inno-LiPA. Again, one sample reacted only with group probes 3 and 4 but not with 1b-specific probe 6. Sequencing of part of the 5' NCR revealed a single mutation at position -94 (a T-to-C transition) which renders it nonreactive with probe 6. In this group, two samples (720 and 748) had very poor staining reactions with subtyping probe 6. This happened in spite of the fact that we used extended conjugate incubation of 45 min and prewarmed the substrate buffer to 37°C.

All of the type 2a isolates were characterized as 2a by Inno-LiPA except one in which a double infection of 1a and 2a

was observed. The same sample was repeatedly characterized as negative for type 1a by PCR with core and NS5 gene-specific primers. Sequencing of the nested PCR products from the 5' NCR and core gene of this sample showed that it belongs to type 2a only, as no bands with identical mobilities in two different lanes of the gel could be found. It is possible, however, that PCR cycle sequencing may fail if the starting concentration of HCV RNA type 1a is significantly lower than that of HCV RNA type 2a in the same sample. We have shown previously (1) that not all samples positive for HCV RNA in the 5' NCR were amplified with core- and NS5-specific primers for types 1 to 3. A possible explanation may be that the sensitivity of 5' NCR nested PCR is higher than that of core or NS5 nested PCR.

All type 2b isolates were also characterized as 2b by Inno-LiPA. Two samples were characterized as type 3a by PCR (10) and as type 3a by the Inno-LiPA test.

The positions of the type-specific probes in Inno-LiPA are properly chosen in the 5' NCR motifs consisting of nucleotides -170 to -155 (-170/-155), -140/-117, and -116/-70, with little similarity between types. Our results show that the line probe assay discriminates very well between HCV types 1, 2, and 3.

No problems were encountered with subtypes 2a and 2b. Also, the majority of type 1 HCV isolates were subtyped correctly as 1a and 1b. The sequence homology between these two subtypes in the 5' NCR is very high (98.8% identity between HCV type 1 [1a] and HCV type J [1b] [12]). The only possible sequence motif to discriminate between subtypes 1a and 1b was defined as probe 5 or 6. The difference between them is a single nucleotide change, an A-to-G transition at position -99 which obviously occurred in all of the subtype 1b HCV isolates in this study. However, at least in two HCV samples which were subtyped as 1a by core and NS5 PCR genotyping as well as sequencing of part of the core gene, we have observed an A-to-G substitution at position -99. This indicates that subtyping of HCV type 1 with probes 5 and 6 relying only on a single A-to-G transition may not be applicable to all HCV isolates.

The Inno-LiPA is easy to perform, and it could be used by most of the laboratories working with HCV subtyping. Subtyping by sequencing is a time-consuming process, and most laboratories do not have the facilities. The availability of the Roche Diagnostic Systems commercial HCV PCR assay (Branchburg, N.J.) will further simplify Inno-LiPA genotyping because the same amplified product can be directly used for subtyping.

The Inno-LiPA is available in kit form, and this will facilitate worldwide epidemiological studies of HCV. To the best of our knowledge, this is the first report comparing PCR-based genotyping with a commercial hybridization probe assay for HCV subtyping.

In conclusion, the line probe assay can discriminate between different subtypes of HCV in most of the samples tested.

We thank L. Stuyver of Innogenetics for the research kits and M. Kanabe for providing the secretarial help. We also thank Wilf Cuff for statistical analysis.

REFERENCES

- Andonov, A., and R. K. Chaudhary. 1994. Genotyping of Canadian hepatitis C virus isolates by PCR. *J. Clin. Microbiol.* **32**:2031-2034.
- Bukh, J., R. H. Purcell, and R. H. Miller. 1993. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. *Proc. Natl. Acad. Sci. USA* **90**:8234-8238.
- Cha, T., E. Brall, B. Irvine, B. Kolberg, J. Chien, D. Kuo, and M. Urdea. 1992. At least five related, but distinct, hepatitis C viral genotypes exist. *Proc. Natl. Acad. Sci. USA* **89**:7144-7148.

TABLE 1. Subtyping of HCV isolates by specific PCR primers and a line probe assay

HCV types by PCR ^a	No. of isolates typed by PCR	Commercial test Inno-LiPA reactivity pattern ^b	Inno-LiPA subtype	No. of isolates typed by Inno-LiPA (%)
Type I (1a)	13	3, 4, 5	1a	10 (77.0)
		3, 4	1	2 (15.0)
		3, 4, 6	1b	1 (7.7)
Type II (1b)	10	3, 4, 6	1b	9 (90.0)
		3, 4	1	1 (10.0)
Type III (2a)	7	7, 8, 9	2a	7 (100.0)
		3, 4, 5 and 7, 8, 9	1a and 2a	1 (14.0)
Type IV (2b)	6	5, 7, 8, 10	2b	6 (100.0)
Type V (3a)	2	11, 12, 13	3a	2 (100.0)

^a All HCV samples were characterized as the same genotype with specific primers from both the core and NS5 genes.

^b Numbers refer to lines in Fig. 1 (A).

4. Chayama, K., A. Tsubota, Y. Arase, S. Saitoh, I. Koida, K. Ikeda, T. Matsumoto, M. Kobayashi, S. Iwasaki, S. Koyama, T. Morimaga, and H. Kumada. 1993. Genotypic subtyping of hepatitis C virus. *J. Gastroenterol. Hepatol.* **8**:150–156.
5. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
6. Kaanai, K., M. Kako, and H. Okamoto. 1992. HCV genotypes in chronic hepatitis C and response to interferon. *Lancet* **339**:1543.
7. McOmish, F., S.-W. Chan, B. C. Dow, J. Gillon, W. Frame, R. Crawford, P. Yap, E. Follett, and P. Simmonds. 1992. Detection of three types of hepatitis C virus in blood donors: investigation of type specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. *Transfusion (Bethesda)* **33**:7–13.
8. Nakao, T., N. Enomoto, N. Takada, A. Takada, and T. Date. 1991. Typing of hepatitis C virus genomes by restriction fragment length polymorphism. *J. Gen. Virol.* **72**:2105–2112.
9. Okamoto, H., Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, 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.
10. Okamoto, H., H. Tokita, M. Sakamoto, M. Horikata, M. Kojima, H. Izuka, and S. Mishiro. 1993. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. *J. Gen. Virol.* **74**:2385–2390.
11. Simmonds, P., A. Alberti, H. Alter, F. Bonino, D. W. Bradley, C. Brechot, 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. Trépo, A. Weiner, P. L. Yap, and M. S. Urdea. 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* **19**:1321–1324.
12. Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Hensvyn, 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.
13. Takada, N., S. Takase, A. Takada, and T. Date. 1993. Differences in the hepatitis C virus genotypes in different countries. *J. Hepatol.* **17**:277–283.
14. Tsubota, A., K. Chayama, Y. Arase, K. Ikeda, A. Yasuji, I. Koida, S. Saitoh, M. Hashimoto, S. Iwasaki, M. Kobayashi, and K. Hiromitsu. 1993. Factors useful in predicting the response to interferon therapy in chronic hepatitis C. *J. Gastroenterol. Hepatol.* **8**:535–539.