Analysis of Hepatitis C Virus Isolates by Serotyping and Genotyping

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Hepatitis C virus (HCV)-positive sera from 106 chronically infected patients which had previously been genotyped were characterized by serotyping. Genotypes were determined by a first-generation line probe assay (INNO-LIIPA HCV) and by sequence analyses of the core, core-E1, and NS5B regions. HCV serotypes were determined by measuring type-specific antibodies to NS4-derived peptide antigens (Murex HCV serotyping 1-6 assay). Of 106 serum samples, serotype-specific antibodies were detected in 88 (sensitivity, 83.0%), and 77 (specificity, 87.5%) of these serotypeable samples revealed a corresponding serotype (total concordance, 72.6%). Eleven samples revealed discrepant results as follows. (i) Five serum samples in which only a single genotype was detected contained an additional serotype. (ii) In one sample with two genotypes only one serotype was detected. (iii) In five isolates the serotype (all serotype 1) was completely different from the genotype. Double infections, as determined by genotyping, were confirmed by serotyping in two of four cases. Of 11 serum samples from chronically infected hemodialysis patients, 7 (64%) were reactive in the serotyping assay. In conclusion, genotyping allows discrimination between (sub)types but requires the relatively complex reverse transcriptase PCR. The novel serotyping assay offers an alternative method to distinguish the major types of HCV, although the sensitivity of the assay may be limited by the immunocompetence of the infected host.

The detection of hepatitis C virus (HCV) in blood and blood-derived products is essential for the prevention of post-transfusion hepatitis. The first-generation serological assay detected antibodies against the recombinant c100-3 protein, predominantly derived from the NS4 region of the HCV genome. Novel generations of anti-HCV assays also allow detection of antibodies to multiple recombinant antigens or synthetic peptides derived from various parts of the HCV genome. The presence of HCV can be demonstrated more directly by the use of the reverse transcription-PCR (RT-PCR). The latter technique also allows detection of HCV in seronegative samples. Most HCV PCR assays use primers that are aimed at the well-conserved 5’ untranslated region. Other parts of the genome, such as the E1, NS4, and NS5 regions, are less well conserved and are therefore less suitable as targets for general RT-PCR.

Extensive sequence analyses and comparisons of numerous isolates from various geographic origins have resulted in the recognition of multiple HCV strains. A classification system for HCV isolates which differentiates between HCV types, subtypes, and individual isolates, depending on the degree of nucleotide sequence homology, has been proposed (26).

Several methods to determine the genotypes of HCV isolates, such as sequence analysis (36), restriction fragment length polymorphism (11), (sub)type-specific PCR (16), hybridization of PCR products with specific probes, and reverse hybridization (28, 35), have been developed.

HCV genotypes may have different clinical implications (5, 25). Firstly, the efficacy of serological screening assays may be different. Secondly, the epidemiology of, e.g., type 3 appears to be detected more often in relation to intravenous drug abuse (19). Whether the levels of viremia are really type dependent remains obscure (7). Furthermore, the severity as well as the progression rate of the liver disease may differ (6, 15, 21, 22, 24). Finally, there are indications that the efficacy of interferon treatment may also depend on the viral (sub)type (20, 23, 33). However, the exact relationships between HCV types and clinical parameters must be substantiated by future studies.

Sequence variation may influence the antigenic properties of specific epitopes. It has been shown that type 1-derived NS3 and NS4 antigens are not adequately recognized by antibodies from patients who are infected with non-type 1 HCV genotypes (3, 4, 10, 11, 13, 29, 35). Similarly, anti-E1 envelope antibodies to recombinant type 1b E1 protein were more prevalent in type 1a and 1b HCV sera than in type 2, 3, and 4 HCV sera (9). These findings initiated the exploration of possible serotyping assays. Several epitopes from the core and NS4 regions have been used for serological discrimination, but the use of these tests was limited (8, 14, 18, 31, 32, 34, 38). Detailed epitope mapping of the NS4 region revealed the presence of two major epitopes between amino acid residues 1691 and 1728, and although cross-reactivity has been observed, type-specific reactivity to several antigenic regions could be demonstrated (27). On the basis of branched type-specific oligopeptides, a serotyping assay has been developed (27). At first, this assay allowed detection of only types 1 to 3, but it has recently been extended to types 4, 5, and 6 (1).

In the present study, we examined the performance of a commercial serotyping assay (Murex HCV serotyping 1-6 assay, based on the experimental prototype) with a panel of well-characterized HCV isolates from chronically infected patients. All isolates had been previously genotyped by a line probe assay (INNO-LIIPA HCV) and sequence analysis, and the panel comprises a heterogeneous population of isolates belonging to types 1 to 6, including various subtypes. All samples were tested by the serotyping assay, and the results of genotyping and serotyping methods were evaluated.

MATERIALS AND METHODS

Patient sera. Ninety-five serum samples were obtained from patients with chronic HCV infection from various geographic origins. Eleven serum samples were obtained from hemodialysis patients who were chronically infected with...
Results

A total of 106 serum samples from chronically infected patients were analyzed by a new serotyping assay, based on serological discrimination between the major types 1 to 6, by measuring type-specific NS4 antibodies. All isolates had been previously genotyped by reverse hybridization of RT-PCR products from the 5′ untranslated region in the line probe assay and sequence analysis of various regions of the RNA genome. The results from the genotyping and serotyping assays are summarized in Table 1.

Type-specific antibodies were detected in 88 (83.0%) of the 106 isolates. Overall, 77 of 88 (87.5%) serotypeable samples (72.6% of the total 106) revealed a serotype which was concordant with the genotype. Eleven of the 88 serotypeable samples (12.5%) revealed a serotype which was different from the genotype. This includes five samples with a single genotype but a double serotype. In each case one of the two serotypes corresponded to the detected genotype. Conversely, in one sample that contained genotypes 1a and 3a, only serotype 3 was detected. In five isolates the serotype was completely different from the genotype. Repetition of serotyping for these samples yielded identical results. Remarkably, these samples were all diagnosed as serotype 1, although one contained genotype 2, one contained genotype 3, two contained genotype 4, and one contained genotype 5 sequences. Optical density values in the serotyping test obtained from these discrepant samples did not differ significantly from those obtained from other samples. Five of seven samples with mixed serotype reactivity contained only a single genotype. Of the 11 serum samples from chronically infected hemodialysis patients, 7 (64%) were reactive in the serotyping assay.

Discussion

Sequence analysis throughout the entire HCV genome has revealed four levels of heterogeneity, i.e., types, subtypes, isolates, and quasispecies. To assess this variability of HCV, genotyping and serotyping methods can be applied. Genotyping analysis of HCV isolates directly characterizes heterogeneity of the viral RNA genome. The different genotyping methods are able to distinguish at the level of types and/or subtypes. To analyze differences at the isolate and quasispecies levels, sequence analysis is necessary.

In the present study, detection of type-specific antibodies to HCV types 1 to 6 has been evaluated. The overall sensitivity of serotyping does not differ significantly between the major types. However, some specific subtypes, such as 1e and 1f, may be untypeable, but the prevalences of these subtypes are very low. The nature of the serotyping assay would allow addition of NS4-derived peptides from novel subtypes. Types 2 and 4 appeared to be highly heterogeneous, comprising multiple subtypes (30, 36), and yet the levels of sensitivity and concordance of the serotyping assay for these types are as high as those for the other types, indicating that sequence heterogeneity of the subtype-specific NS4 epitopes is sufficiently limited to allow reactivity with the type 2 and 4 peptides of the serotyping assay.

Seven isolates were scored as double infections, although only two had been characterized as mixed infections by genotyping. This result may be explained by serological detection of past infection with a different genotype. Whereas genotyping detects only present viral genomes, the indirect nature of the serotyping assay may allow it to also detect antibodies related to past infection. However, the presence of cross-reactive antibodies cannot be excluded. It has also been reported that antibodies to NS4 epitopes do not persist for a long time after clearance of the virus, rendering the hypothesis of detection of past infection less likely (37).

Genotyping analysis of HCV isolates directly types the viral RNA, but it requires RT-PCR, which is a complex and error-prone technique (39). The appropriate internal and external controls should be included in each test to ensure sensitivity and accuracy. Usually, only the predominant viral variant will be analyzed, although mixed infections can be detected. The 5′ untranslated region is an attractive target for diagnostic PCRs, but it does not allow specific recognition of some subtypes.

Serotyping analysis of HCV isolates provides an indirect typing method based on the production of type-specific antibodies by the infected host. Therefore, the typeability of HCV depends on the immunocompetence of the infected host. It is well-known that a seronegative window of variable length exists after infection. During that period, it is impossible to detect any antibodies to HCV. In many cases, production of antibodies to the core and NS3 proteins precedes production of NS4

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**TABLE 1. Comparison of serotyping and genotyping results**

<table>
<thead>
<tr>
<th>Genotype(\text{a})</th>
<th>No. of samples with the following serotype:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>NT(\text{b})</th>
<th>NR(\text{c})</th>
<th>Mixed</th>
<th>Total</th>
<th>Sens (%)(\text{d})</th>
<th>Spec Conc (%)(\text{e})</th>
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<td>24</td>
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<td>92.3</td>
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<td>1</td>
<td>13</td>
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<tr>
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<td></td>
<td>10</td>
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<td>1</td>
<td>2</td>
<td>15</td>
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<td>64.3</td>
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<tr>
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<td>100</td>
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<td>9</td>
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<td>83.0</td>
<td>87.5</td>
<td>72.6</td>
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\(\text{a}\) As determined by the INNO-LiPA HCV assay and by sequencing of core, core-E1, and NS5B regions.  
\(\text{b}\) NT, no type-specific antibodies.  
\(\text{c}\) NR, nonreactive.  
\(\text{d}\) Sens, sensitivity (number of correct serotypes/total number of samples).  
\(\text{e}\) Spec, specificity (number of correct serotypes/total number of serotypeable samples).  
\(\text{f}\) Conc, concordance (number of correct serotypes/total number of samples).
antibodies. Since HCV RNA is usually detectable very soon after infection, genotyping analysis is the only method available during this window period. Moreover, humoral immune responses may vary considerably between patients, recognizing different spectra of viral epitopes. Several of the eight serum samples that were nonreactive in the serotyping test did contain anti-NS4 antibodies (data not shown) as measured by a line immunoassay (INNO-LIA HCV Ab III; Innogenetics) or a recombinant immunoblot assay (Ortho, Ratarin, N.J.). Apparently, these sera did not contain any antibodies to the NS4 epitopes presented in the serotyping assay. On the other hand, a number of sera showed type-specific antibodies, although anti-NS4, anti-5-1-1, and anti-c100-3 were not detectable by line immunoassay or recombinant immunoblot assay (data not shown). These data indicate that the absence or presence of anti-NS4 antibodies, as detected by confirmation assays, is not indicative of the typeability by the serotyping assay. Also, patients may have impaired immunoreactivity, as do patients infected with human immunodeficiency virus, transplant recipients undergoing immunosuppressive therapy, and dialysis patients. The limited immunocompetence of dialysis patients may lead to delayed seroconversion (12, 17) and may even completely prevent diagnosis of HCV infection by serological screening assays in some cases (2). In our panel, samples from 11 dialysis patients with chronic HCV infection were included. Although this number is too limited to allow us to draw any firm conclusions, the fact that samples from 4 of these 11 patients were nonreactive may indicate the limited applicability of the serotyping assay for these particular patient groups, whereas only 7 of the 95 nondialysis isolates were nonreactive.

Serotyping of a larger number of HCV-infected dialysis patients will be the subject of future studies.

Genotyping methods can distinguish HCV variants at the subtype level, but they are more difficult to perform because of the complexity of RT-PCR. Genotyping may be the only available method for analysis of seronegative samples or HCV-infected immunocompromised hosts. On the other hand, serotyping may detect antibodies to past infection, whereas genotyping detects present HCV viremia.

In conclusion, the present study indicates that the serotyping assay is useful for characterization of HCV isolates, especially in laboratories that lack the specific expertise to perform genotyping methods. Serotyping results correlated well with genotyping results, although some isolates were discrepant. The serotyping test did contain type-specific differences in geographic origin and disease. The typing methods correlated well with genotyping. Serotyping of a larger number of HCV-infected dialysis patients will be the subject of future studies.

**REFERENCES**


