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Evaluation of a New Serotyping Assay for Detection of Anti-Hepatitis C Virus Type-Specific Antibodies in Serum Samples

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The performance of a new version (HC03) of the hepatitis C virus (HCV) serotyping 1–6 assay (Abbott Murex Laboratories), a specific test for serological determination of HCV types, was evaluated using a selected panel of 180 HCV RNA-positive sera. HC03 was more sensitive than the current HC02 version, typing 53 (37.6%) of 141 samples which were not typable with HC02. Furthermore, the HC03 specificity was 94.1% as evaluated with a panel of 22 genotyped samples. This new version of the test improves the quality of the serological approach to HCV type determination.

Hepatitis C virus (HCV) is a major cause of chronic liver disease. The virus belongs to the *Flaviviridae* family and has a 9.5-kb single-stranded RNA molecule genome containing a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). The ORF codes for a 3,000-amino-acid polyprotein which is processed into structural and nonstructural proteins by cellular and viral enzymes. Nucleotide sequence analyses from various HCV genomes have indicated that the envelope (*E1* and *E2*) and the *NS4* genes are highly variable, in contrast to the UTRs, which are highly conserved (14). Phylogenetic analysis of nucleotide sequences of numerous isolates has enabled definition of six clades and many subtypes within them (4, 5, 17, 18, 20, 21). Type and subtype classification of HCV strains has enabled specific description of the geographic distribution of the viral types (reviewed in reference 23): HCV types 1, 2, and 3 have a worldwide distribution, while the other types are found mainly in North and Central Africa (type 4), South Africa (type 5), and Southeast Asia (type 6) (18, 19). However, local patterns might also occur. For example, phylogenetic analyses of the *NS5B* gene have recently indicated the emergence of HCV type 4 in the northeastern suburbs of Paris (France) (13).

In addition to the epidemiological relevance, viral typing might have a clinical impact. Numerous studies have reported a relationship between HCV type and the response to interferon or pegylated interferon therapy, given alone or in combination with Ribavirin (12). Patients infected with HCV type 2 or 3 have a better response to treatment than those infected with HCV type 1. Consequently, the recommended duration of 6 months of therapy for HCV type 2 or 3 infections is extended to 1 year for HCV type 1 infections (9, 12). Therefore, HCV type determination is now routinely performed when therapy is indicated.

Many molecular methods based on reverse transcription and PCR amplification of various regions of the viral RNA have been developed to assess HCV genotypes (4, 8, 15, 19–21). Genotyping methods are usually highly sensitive and specific but are expensive, require proper handling and storage of the samples, and can be performed only on HCV RNA-positive samples. Genotype-specific synthetic peptides derived from the NS4 amino acid sequence have been used in an enzyme-linked immunosorbent assay (ELISA) to detect the presence of type-specific antibodies in the sera of infected patients (1, 18). Such a serological typing approach for typing HCV is easy to perform and remains reliable even when HCV RNA is undetectable. The HCV Serotyping 1–6 assay (HC02; Abbott Murex) is currently used because it has a high specificity (97.6%) compared to genotyping results (7, 10). The sensitivity of the test ranges from 70 to 87% but can be lower for samples from patients either coinfected with human immunodeficiency virus (HIV) (and thus immunosuppressed) or with mixed cryoglobulinemia (10) (our unpublished results).

Using a collection of HCV RNA-positive samples for which the HC02 serotyping assay had failed to determine the virus type, we evaluated a new version of the test (HC03). From this panel, 37.6% of the samples not typeable with HC02 were typed using HC03.

MATERIALS AND METHODS

Samples. A total of 180 HCV-RNA positive samples (HCV Monitor 1.0; Roche Diagnostics) which had previously been tested in a pretherapeutic setting by using the current Abbott Murex HCV Serotyping 1–6 assay (HC02) version were selected and retrospectively analyzed with the new version of the test (HC03). Of these 180 samples, 39 sera previously serotyped with HC02 were used as controls. The six HCV types were represented and distributed as follows: type 1, $n = 5$; type 2, $n = 5$; type 3, $n = 7$; type 4, $n = 6$; type 5, $n = 8$; type 6, $n = 4$; and mixed types, $n = 4$ (types 1 plus 4, 4 plus 5, 3 plus 6, and 2 plus 6). The remaining 141 samples, which HC02 had failed to type, were tested with HC03 to assess the improvement of sensitivity of HC03 over HC02. Of these

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samples, 42 were positive for HIV antibodies, 44 were negative, and the HIV serological status was not known for 55.

Another panel of 22 HCV RNA-positive samples, which belonged to a multicenter quality control panel (ANRS; Action Concertée 11), were genotyped using INNO-LiPA HCV II (Innogenetics), which allows typing and subtyping (although subtype determination is not always accurate with this test), and the results were compared to those generated from serological typing using both HC02 and HC03.

HCV serological typing. The HCV Serotyping 1–6 assay is an ELISA which distinguishes type-specific antibodies to the six major HCV types. Both HC02 and HC03 use a 96-well microtiter plate as the solid phase, with wells coated with synthetic HCV NS4 peptides of each type. Type-specific antibodies present in the serum are neutralized by type-specific HCV NS4 peptides in a liquid phase. A sample is tested using eight wells of one strip. There are two control wells: in the first ("No competition"), there are no soluble peptides to adsorb the serum antibodies, and the maximum signal is generated. In the second, antibodies are neutralized with the "Competing solution—All", which contains HCV NS4 peptides of all types, generating the minimum signal (background signal). Each of the six remaining wells represents one of the six major HCV types, and type-specific signals are generated due to the addition of competing solutions consisting of HCV NS4 peptides of all types but one. For example, "Competing solution 1" contains all peptides except for those specific for type 1, and so forth. In each well, HCV antibodies present in the serum interact with the type-specific soluble competing peptides which are in excess of those coated on the wells and therefore block any cross-reaction. Thus, type-specific antibodies which have not been neutralized by the competing solution because the corresponding peptides were missing from the mix are captured onto peptides coated on the well surface and produce a signal.

The new version of the assay (HC03) has been modified to improve the sensitivity of the assay, particularly to types 1 and 3, and to reduce the number of samples which are not typeable. The raw data interpretation criteria have also been improved. To summarize, any $A_{450/690}$ (where 450 is 450 nm, the absorbance at which the reading should be processed, and 690 is 690 nm, the reference wavelength, given a microplate reader with a dual wavelength capability) optical densities (ODs) below 0.150 are regarded as not interpretable for type specificity. The "No competition" well and the six typing wells are considered positive if the ODs exceed the cutoff value. The cutoff is the greater value of 0.2× the "No competition" OD and 1.5× the "Competing solution—All" OD. If more than one typing well passes the cutoff, the "Competing solution—All" OD is subtracted from each of the other ODs and the new value for 0.2× the "No competition" OD is used as a new cutoff value. If two wells still pass the cutoff, a mixed reactivity is considered, but if more than two wells pass, the sample is considered to be not typeable. A sample is considered nontypeable (NT) if only the "No competition" well passes the cutoff value, and a sample is considered nonreactive (NR) if none of the wells pass the cutoff value. Along with the improved sensitivity of the assay, these new criteria are thought to contribute to increase the number of typeable samples concordant with genotyping and to decrease the number of typeable samples discordant with genotyping.

Genotyping analyses. Genotyping analyses were performed using either restriction fragment length polymorphism (RFLP) or phylogenetic analysis of the *NS5B* gene sequence. The RFLP method was described previously (8). Briefly, viral RNA was amplified by RT-PCR using 5′ UTR-specific primers (6). Amplified products were digested with either *Rsa*I and *Hae*III, *Hin*fI and *Mva*I, *Scr*Fi, or *Bst*UI, and the digested products were visualized after gel electrophoresis.

Phylogenetic analysis of the *NS5B* region was performed as previously reported (13), with some modifications. Briefly, after TRIzol (Life Technology) extraction of the viral RNA and reverse transcription with Superscript II (Life Technology), cDNA was amplified with 0.25 mM each degenerated primer (Sn755 and Asn1121) using 2 IU of AmpliTaQ Gold (Applied Biosystems) in a mixture containing the AmpliTaq Gold buffer, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 2 mM $MgCl_2$. A touch-down PCR was performed including one denaturation step of 5 min at 93°C and five cycles comprising 30 s of denaturation at 93°C, 45 s of annealing at 60°C, and 1 min of elongation at 72°C. During the 35 following cycles, the annealing temperature was reduced by 0.3°C per cycle, and for the 5 final cycles, the annealing temperature was 49.5°C. The reaction was ended by a 4-min 15-s elongation step. The amplification products were sequenced and analyzed as previously described (13).

Statistical analysis. Fisher's exact test was used for statistical comparisons. Differences were considered significant at $P \leq 0.05$.

TABLE 1. Comparison of HC02 and HC03 serotyping assays with a panel of 22 control samples previously genotyped with InnoLiPA HCVII

Assay	No. of samples positive for serotype[a]:				Total no. of samples ($n = 22$)	Sen/Spe (%)[b]
	1 ($n = 11$)	2 ($n = 3$)	3 ($n = 7$)	2a-c/4 ($n = 1$)		
HC02	9	0	5	(1)[c]	15	68.2/93.3
HC03	9	1	6	(1)[c]	17	77.3/94.1

[a] *n*, number positive for serotype by INNO-LiPA HCVII genotyping.
[b] Sensitivity (Sen) and specificity (Spe) of HC02 or HC03 compared to genotyping.
[c] Sample with a mixed genotype 2a-c plus 4 was detected as serotype 2 by both HC02 and HC03.

RESULTS

To evaluate the sensitivity and specificity of the test, HC03 serotyping results were compared to the genotypes obtained with the commercial assay INNO-LiPA HCVII for 22 sera from a genotyping quality control panel. Of the 22 samples, 17 were positive with HC03, accounting for a sensitivity of 77.3% compared to genotyping. The remaining five samples (HCV genotype 1b, $n = 2$; type 2, $n = 2$; type 3, $n = 1$) were either NT or NR, and a suspected mixed infection, 2a-c/4, was detected as serotype 2. Except for this mixed infection, all the HC03 results were concordant with genotyping, accounting for a specificity of 94.1%. When these samples were serotyped using HC02, similar results were obtained except for two samples, which were NT with HC02 and types 2 and 3 with HC03. Thus, in this panel, HC02 displayed a sensitivity of 68.2% and a specificity of 93% (Table 1). These results might suggest that HC03 is more sensitive than HC02 (77.3 and 68.2% respectively), although the difference is not statistically significant for such a small cohort ($P = 0.36$).

The sensitivity and specificity of HC03 were further investigated and compared to those of HC02 by testing a panel of 180 sera. This panel contained 39 sera previously typed with HC02 and used as controls and 141 samples for which HC02 had failed to detect a type. Among these, 88 were NT and 53 were NR as defined by the manufacturer for HC02. Of the 39 samples that had well-defined HCV types as previously determined by HC02, 35 (89.7%) were typeable with HC03. The four HC03-NT samples (HC02 type 3, $n = 1$; type 5, $n = 2$; type 4/5, $n = 1$), were genotyped, and the genotype was concordant for only two of them (Table 2). Two of four HC02 mixed-type samples had, with HC03, a single type which corresponded to one of the types detected with HC02 (Table 2).

Of 141 samples, 53 (37.6%) which were either NT or NR with HC02 were typeable with HC03. These 53 HC03-positive samples contained 35 type 1 (66%), 5 type 3 (9.4%), 6 type 4 (11.3%), 2 type 5 (3.8%), 2 type 6 (3.8%), and 3 mixed infections (1 plus 2, 1 plus 3, 1 plus 4 [5.7%]). No serotype 2 was found. None of the eight samples that had mixed serotypes with either HC02 or HC03 displayed a mixed genotype as determined by RFLP (Table 3).

The ability of HC03 to recover HC02-negative samples was dependant on whether these samples were NT or NR by HC02, with 51% recovery when the samples were NT but only 15% when they were NR ($P = 0.0002$), indicating that in some cases the antibody response to HCV infection cannot be detected

TABLE 2. Number of samples of each serotype obtained with HC03 from a control panel of 39 samples previously serotyped with HC02

HC03 serotype	No. of samples positive by HC02 for serotype[a]:									
	1 (n = 5)	2 (n = 5)	3 (n = 7)	4 (n = 6)	5 (n = 8)	6 (n = 4)	3 + 6 (n = 1)	4 + 5 (n = 1)	1 + 4 (n = 1)	2 + 6 (n = 1)
1	5									
2		5								
3			6				1			
4				5						
5					6					
6						4				1
1 plus 4				1 (4a)[a]					1	
NT			1 (3a)		2 (3a, 5)			1 (1a, b)		

[a] n, number positive for serotype by HC02.
[b] The genotypes determined by INNO-LiPA HCVII assay are indicated in parentheses.

with HC03 type-specific peptides. Moreover, the serotyping efficiency of HC03 depended on the HIV serological status of the samples: 21 (47.7%) of 44 anti-HIV-negative samples were typeable with HC03, whereas only 7 (16.7%) of 42 anti-HIV-positive samples were typeable (P = 0.002).

To determine the HCV type distribution among 14 of the 88 samples that were NT using either version of the serotyping assay, the samples were genotyped using an RFLP method. The HCV genotype distribution among these samples was as follows: genotype 1, n = 3; genotype 2, n = 3; genotype 3, n = 3; genotype 4, n = 5.

DISCUSSION

The aim of this study was to evaluate the new version of the Murex HCV Serotyping 1–6 assay (HC03) in relation to the former version (HC02). These serotyping ELISAs use synthetic peptides derived from the NS4 region of the HCV genome to detect type-specific antibodies (1, 18, 22). Previous studies have shown that compared to genotyping, HC02 has a high specificity (90 to 98%) and a moderate sensitivity (70 to 75%) (10, 16). The main reason for this lack of sensitivity was the high prevalence of patients coinfected with HIV in the studied population. Because of its practicability, the HC02 assay is a very useful alternative to molecular methods of HCV type determination (7). The new version (HC03) has been redeveloped to improve sensitivity, particularly to types 1 and 3, which are currently the most prevalent in Western Europe and the United States, and to reduce the number of NT and NR results. The interpretation criteria have also been improved to increase the number of typeable samples concordant with genotyping and to decrease the number of typeable samples discordant with genotyping.

The fact that 37.6% of HC02-negative samples gave a positive result with HC03 indicated that HC03 is more sensitive. However, the clinical background of the patients appeared to be critical for HC03 serotyping efficiency because the recovery of the HC02 NT and NR samples was significantly higher in HIV-negative than in HIV-positive patients. This might be due to an impaired antibody response during the course of HIV disease. Another hypothesis can be raised for patients with a high probability of multiple reexposure to HCV, for example intravenous drug users. The high genetic variability in viral envelope glycoproteins may account for a possible inefficiency of the neutralizing antibodies in preventing some reinfections (18). Multiple infections might generate a high background cross-reactivity, which could impede the type-specific reactivity. Thus, for HIV-HCV-coinfected patients, HCV type determination might be more efficiently done by a genotyping method.

TABLE 3. Comparison of mixed typing results as detected by HC02, HC03, and RFLP genotyping

Method	Serotype							
HC02	4	4+5	3+6	1+4	2+6	NT	NT	NT
HC03	1+4	NT	3	1+4	6	1+2	1+3	1+4
RFLP	4	1a	ND[a]	1a	1a	4	3a	4

[a] ND, not done due to insufficient material.

The 53 serotypes recovered with HC03 displayed an unusual distribution regarding the classical prevalence observed in the population studied (10; our unpublished results). One would have expected a similar type distribution between the serotypes obtained with HC02 and those recovered with HC03. Interestingly, two main differences were observed in the type distributions obtained with the two tests. First, with HC03, no samples of serotype 2 and only five samples of serotype 3 were observed. This might suggest that the ability of HC03 antigens to react with anti-NS4 type 2 and 3 antibodies was not significantly (type 2) or only moderately (type 3) improved compared to that of HC02. Second, the large number of HCV type 4 samples recovered with HC03 (n = 6) and with RFLP genotyping (n = 5), accounting for 16.4% in the studied population, confirmed the emergence of these strains in the northern Parisian suburbs (3, 10). These type 4 viruses are encountered mainly in two distinct patient populations, as confirmed by the results of the phylogenetic analysis of the nucleotide sequences of the *NS5B* and *E1* regions of the genome (13). Possible reasons for this are that African migrants who live in this area have imported viral isolates of genotypes 4a(B), 4f, 4h, and 4k and also that HCV types 4a and 4d are nowadays diffusing among HCV-infected intravenous drug users. The possible emergence of type 4 viruses in some areas of northern Europe needs to be further evaluated (11, 17). However, if this was confirmed, it should encourage the use of typing assays (genotyping or serotyping) able to detect all types of viruses, not just the three most prevalent (HCV types 1, 2, and 3).

When HC03 was used on samples that had already been typed with HC02, 4 of 39 samples were NT with HC03. This apparent lack of sensitivity of HC03 versus HC02 might indicate that HC03 is more type specific than HC02. Indeed, one

of the two HC02 type 5 samples that were NT with HC03 was genotyped as 3a, indicating a false result in HC02 serotyping that was avoided with HC03. Furthermore, a sample displaying a mixed reactivity (types 4 plus 5) with HC02 and found to be NT with HC03 had genotype 1a using RFLP and 1b by analysis of the nucleotide sequence of the NS5B region.

No concordance was observed between serotyping and genotyping results concerning mixed infections. Indeed, for the five mixed reactivities detected by HC03, genotyping detected only one type, and a mixed infection with types 2a-2c plus 4 which was detected in a sample genotyped with INNO-LiPA was monoreactive (type 2) with both HC02 and HC03. These results can be explained by the fact that genotyping identifies the current dominant HCV population(s) that can be detected through PCR in a patient's circulation while serotyping detects antibodies representing a viral population(s) either detectable or undetectable by PCR. A mixed infection may be underestimated either by genotyping (because of the possibility of variable ratios of genotypes) or by serotyping (because type-specific NS4 antibodies may be detected only months after the onset of infection).

However, although the number of mixed infections detected by HC03 (3 [5.7%] of 53) was in agreement with previous reports (2), the fact that no concordance was found between serotypes and genotypes encourages a cautious interpretation of the mixed serotypes detected with HC03.

In summary, this evaluation indicates that HC03 is as easy to perform as the former HC02 but is more specific and more sensitive. Further evaluations, including comparisons of serotypes obtained by HC03 and genotyping, are still needed to specify the usefulness of HC03 in medical procedures each time HCV typing is required. However, genotyping procedures remain required when HC03 fails to identify the type or in cases of mixed reactivity.

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