Evaluation of a New Assay in Comparison with Reverse Hybridization and Sequencing Methods for Hepatitis C Virus Genotyping Targeting Both 5′ Noncoding and Nonstructural 5b Genomic Regions

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We report the evaluation of a new real-time PCR assay for hepatitis C virus (HCV) genotyping. The assay design is such that genotype 1 isolates are typed by amplification targeting the nonstructural 5b (NS5b) subgenomic region. Non-genotype 1 isolates are typed by type-specific amplicon detection in the 5′ noncoding region (5′NC) (method 1; HCV genotyping analyte-specific reagent assay). This method was compared with 5′NC reverse hybridization (method 2; InnoLiPA HCV II) and 5′NC sequencing (method 3; Trugene HCV 5′NC). Two hundred ninety-five sera were tested by method 1; 223 of them were also typed by method 2 and 89 by method 3. Sequencing and phylogenetic analysis of an NS5b fragment were used to resolve discrepant results. Suspected multiple-genotype infections were confirmed by PCR cloning and pyrosequencing. Even though a 2% rate of indeterminates was obtained with method 1, concordance at the genotype level with results with methods 2 and 3 was high. Among eight discordant results, five mixed infections were confirmed. Genotype 1 subtyping efficiencies were 100%, 77%, and 74% for methods 1, 2, and 3, respectively; there were 11/101 discordants between methods 1 and 2 (method 1 was predominantly correct) and 2/34 between methods 2 and 3. Regarding genotype 2, subtyping efficiencies were 100%, 45%, and 92% by methods 1, 2, and 3, respectively; NS5b sequencing of discordants (16/17) revealed a putative new subtype within genotype 2 and that most subtype calls were not correct. Although only sequencing-based methods provide the possibility of identifying new variants, the real-time PCR method is rapid, straightforward, and simple to interpret, thus providing a good single-step alternative to more-time-consuming assays.

Hepatitis C virus (HCV) is the most important cause of chronic liver disease and is the leading indication for liver transplantation (2). It is estimated that HCV infects 3% (170 million people) of the world’s population (26). HCV possesses a positive-sense single-stranded RNA genome of approximately 9.5 kb, which is flanked by noncoding (NC) regions. The HCV genome encodes at least 11 proteins, which include both structural and nonstructural (NS) proteins (5, 7).

HCV is known to have a high rate of genetic heterogeneity. This has allowed HCV strains to be classified into a number of genetically distinct groups, known as genotypes, subtypes, isolates, and quasispecies (5). Genome sequence heterogeneity arises due to poor fidelity of the viral polymerase during replication. Sequence variability is not evenly distributed throughout the genome. The lowest sequence variability is found in the 5′NC region, which is the target of choice for many molecular diagnostics assays, including genotyping tests. Nevertheless, nucleotide sequencing coupled with phylogenetic analysis of more-variable genomic regions has been recommended for HCV genotyping in consensus proposals (27). As patients infected with different genotypes respond differently to antiviral drug therapy, identification of the infecting genotype has become important to guide the correct dose and duration of current combination therapy (pegylated alpha interferon plus ribavirin) (13, 19, 23).

A new HCV genotyping method (HCV genotyping analyte-specific reagent [ASR] assay; Abbott Molecular Inc., Des Plaines, IL) based on real-time PCR technology has recently been commercialized. This assay amplifies a portion of the HCV genome and assigns genotype in a single step using primers and probes targeting the NS5b region for genotypes 1a and 1b and the 5′NC region for genotypes 2a, 2b, 3, 4, 5, and 6. The aim of our study was to compare this new assay to two previously commercialized genotyping methods based on the 5′NC region: the TruGene HCV 5′NC genotyping kit (Bayer HealthCare, Berkeley, CA), based on semiautomated sequencing; and the Inno-LiPA HCV II assay (Innogenetics, Gent, Belgium), based on automated reverse hybridization. Discrepant results were resolved though NS5b sequencing and phylogenetic analysis, and suspected mixed-genotype infections were confirmed through PCR cloning and pyrosequencing methodologies.

MATERIALS AND METHODS

Patients and study design. Serum specimens from 295 patients with chronic hepatitis C submitted to the Microbiology Department for clinical HCV genotyping were included in the study. One hundred fifty-eight of them were retro-

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spectively selected from 2001 to 2004 and comprised similar numbers of commonly detected genotypes (1a, 1b, 2, 3, and 4), as well as all available genotype 5 and possible mixed-genotype infections. These specimens had already been genotyped by the TruGene HCV 5'NC genotyping kit (n = 86), the Inno-LiPA HCV II assay (n = 89), or both (n = 17). For this study, sera were typed using the real-time PCR HCV genotyping ASR method. Another 137 consecutive sera were prospectively typed both by the TruGene HCV 5'NC genotyping kit and by the HCV genotyping ASR assay. Seven quality control for molecular diagnostics (QCMD) specimens (QCMD: Glasgow, Scotland) were also included.

HCV RNA extraction and RT-PCR. For 5'NC sequencing and reverse hybridization, viral RNA extraction, reverse transcription (RT), and PCR amplification procedures were performed using 200 μl of serum with the Cobas Amplicor HCV test (Roche Molecular Systems, Pleasanton, CA) according to the manufacturer’s instructions. For the real-time PCR method, RNA was extracted by use of the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

HCV genotyping. (i) Inno-Lipa HCV II reverse hybridization assay. The Inno-Lipa HCV II reverse hybridization assay is based on the reverse hybridization principle. Briefly, biotinylated PCR product from the 5'NC region was hybridized to specific immobilized probes. Detection was performed through an enzymatic reaction resulting in a purple precipitate, which was visually interpreted.

Hybridization and detection steps were performed on the Auto-Lipa instrument (Tecan Group Ltd., Salzburg, Austria), following the manufacturer’s instructions. The probes in this assay allow the identification of the following types: 1a, 1b, 1ab, 2a, 2b, 2a/c, 3, 4, 5a, and 5b.

(ii) Inno-Lipa HCV II reverse sequencing assay. The Inno-Lipa HCV II reverse hybridization assay is based on the reverse hybridization principle. Briefly, biotinylated PCR product was from the 5'NC region was hybridized with two sequencing primers labeled with different fluorescent dyes, followed by electrophoresis and data analysis on the OpenGene DNA sequencing system (Bayer HealthCare). Each bidirectional sequence was automatically aligned to a panel of reference sequences with the GreenLibrarian module of the GeneObjects software (Bayer HealthCare), allowing genotype assignment based on percent sequence identity.

(iii) HCV genotyping ASR assay. The HCV genotyping ASR assay is based on real-time PCR technology and includes probes labeled with different fluorescent dyes specific for genotypes 1a and 1b targeting the NS5b region, as well as primers and probes for genotypes 2a, 2b, 3, 4, 5, and 6 targeting the 5'NC region. Genotype is determined with three reactions per specimen; the mixture for reaction A contains primers and probes that recognize all genotypes to confirm the presence of HCV RNA and primers and probes for genotypes 1a and 1b; the mixture for reaction B contains primers and probes for genotypes 2a, 2b, and 3; and the mixture for reaction C contains primers and probes to identify genotypes 4, 5, and 6. The assay was performed according to the manufacturer’s instructions. The PCR product was subjected to pyrosequencing on a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s protocol. Amplification and detection were performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

(v) PCR cloning and pyrosequencing. Suspected multiple-genotype infections were confirmed by pyrosequencing and genotype-specific restriction endonuclease digestion. For this study, sera were typed using the pyrosequencing system (Biotage, Uppsala, Sweden). Generated sequences from minority genotypes were identified by alignment to reference sequences and BLAST analysis.

RESULTS

No statistically significant differences in agreement among the three techniques (real-time PCR, reverse hybridization, and 5'NC sequencing) were found when the results from retrospective and prospective studies were analyzed separately (P = 0.518) (data not shown). Thus, subsequent analyses were based on the analysis of the whole sample (n = 295). Overall results are shown in Table 1.

Among the 295 specimens tested by real-time PCR, a total of 13 indeterminate results were obtained, including eight of genotype 1, three of genotype 2, and two of genotype 3, despite the presence of high viral loads (69,200 IU/ml to >700,000 IU/ml). Genotype 1 specimens were retested by the real-time PCR 5'NC region assay, and a genotype was assigned to seven of them, while one sample remained indeterminate (subtyped as 1b by NS5b sequencing). Thus, a genotype call was obtained in 289 (97.9%) cases. Due to our study design, we could not...
compare this rate to those of the other methods in the retrospective study, since only specimens with a previous genotype call by the other methods were selected. In the prospective study, none of the 137 specimens tested by 5'NC semiautomated sequencing were indeterminate.

Concordance at the genotype level. For the analysis of genotype concordance between techniques, only sera with a genotype call by both methods (n = 289) were included in our analyses (indeterminate results were excluded). Results obtained by real-time PCR agreed well with those obtained by 5'NC sequencing (214/220 concordant results; Cramer’s V = 0.849; P < 0.05) and reverse hybridization (85/86 concordant results; κ = 0.982; P < 0.05). Correlation coefficients were also high (Spearman’s ρ values of 0.916 and 0.956, respectively). Although the number of specimens processed both by 5'NC sequencing and reverse hybridization was small, correlation was total (17/17 concordant results; κ = 1; ρ = 1).

Among eight (2.8%) discordant results at the genotype level (Table 2), five mixed-genotype infections were confirmed by cloning and pyrosequencing and/or QCMD. Two of them were detected by reverse hybridization, two by 5'NC sequencing, and one by real-time PCR. When specimen 2 (genotype 3/5 confirmed mixed infection shown in Fig. 1) was tested by real-time PCR, genotype 5 amplified strongly, while genotype 3 amplified 5.8 cycles later than the dominant genotype; therefore, mixed infection was not identified by the software. Among the three remaining discordant specimens, mixed-genotype infection could not be confirmed for two 1/4 mixed infections detected by real-time PCR, and specimen 8 had insufficient volume for further analysis. However, when the latter specimen was tested by real-time PCR, genotype 1b amplified nine cycles later than genotype 4, and NS5b sequencing confirmed the presence of genotype 1b in this specimen. The other five QCMD specimens were single-genotype infections and were correctly genotyped by both 5'NC sequencing and real-time PCR (these specimens were not tested by reverse hybridization since they belonged to the prospective study).

Concordance at the subtype level. Regarding genotype 1 infections, the subtyping efficiency for the real-time PCR

<table>
<thead>
<tr>
<th>Method</th>
<th>Genotype</th>
<th>5'NC and NS5b real-time PCR</th>
<th>5'NC sequencing</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
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<td>5'NC reverse hybridization</td>
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<td>1/2</td>
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<td>1/4</td>
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* Results in boldface represent consensus between the two methods.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Genotype by a:</th>
<th>Compared method</th>
<th>Confirmatory method</th>
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<td></td>
<td>5'NC reverse hybridization</td>
<td>5'NC sequencing</td>
<td>5'NC and NS5b real-time PCR</td>
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<tr>
<td>Specimen</td>
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<td>NS5b sequencing</td>
<td>Cloning and pyrosequencing</td>
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<td>8</td>
<td>-</td>
<td>1b</td>
<td>4c</td>
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a, not performed.  
b Genotype 3 amplified 5.8 cycles later than genotype 5.  
c Genotype 1b amplified nine cycles later than genotype 4. None of these results was given as a mixed-genotype infection by SGS v2.0 software.  
d NA, not applicable.
One of them was correctly typed as 1b by 5'NC sequencing and typed as 1a/1b by real-time PCR. Among the other specimens, the real-time PCR method was predominantly correct (9/10 cases) according to the NS5b sequencing. Similarly, one of the two discordant results obtained for the 34 specimens subtyped both by real-time PCR and reverse hybridization was typed as 1a/1b by the former assay. The other one was identified as a 1a/4 mixed infection by cloning and pyrosequencing, but genotype 1 in that specimen actually belonged to subtype 1b instead of 1a according to NS5b sequencing and real-time PCR.

Regarding the genotype 2 infections, subtyping efficiencies were 9/20 (45%) for 5'NC sequencing, 12/13 (92.3%) for reverse hybridization, and 27/27 for real-time PCR. Three out of the 30 specimens genotyped as genotype 2 by either reverse hybridization or 5'NC sequencing were indeterminate by real-time PCR. NS5b sequencing and phylogenetic analysis showed that two of these specimens clustered together and did not group with any of the confirmed genotype 2 subtypes. This potentially reveals that they may belong to a putative new subtype within genotype 2. Further investigations are ongoing. For the other specimen, there was an insufficient volume of serum remaining to permit further analyses. Comparison of subtype discrimination across all specimens showed that 16 were discordant (Table 4). NS5b sequencing revealed that probes 2a and 2ac from the real-time PCR and the reverse hybridization assays, respectively, lead to misclassification of subtypes 2c, 2i, and 2j.

**DISCUSSION**

Since HCV genotype is one of the most important factors determining the outcome of antiviral therapy (13), most clinical laboratories routinely perform HCV genotyping. HCV...
genotyping methods, such as automated reverse hybridization (29) and semiautomated sequencing (10, 11), need a previously amplified genomic product as starting material. Since 5′NC amplification is regularly performed for HCV molecular diagnosis and viral load quantitation, genotyping methods based on this highly conserved region are convenient. However, discrimination among subtypes (especially 1a versus 1b and 2a versus 2c) and certain genotypes (genotype 6 isolates have been identified as genotype 1) is not always reliable using this region, and this often leads to subtyping errors (6, 27, 30, 32). Nucleotide sequencing followed by phylogenetic analysis of more-variable genomic regions, such as NS5b or core, has been recommended for HCV genotyping in consensus proposals (27). Nevertheless, this procedure is considered impractical for most clinical laboratories because it is time-consuming and techni- cally challenging and does not readily permit detection of mixed-genotype infections. The purpose of this study was to compare a novel commercial assay based on real-time PCR, targeting both the 5′NC and NS5b regions, with two commonly used genotyping assays in the clinical setting (reverse hybridization and semiautomated sequencing, both based on the 5′NC region).

Overall, the real-time assay performed well compared to results obtained by the two other methods. Among the 295 specimens tested by real-time PCR assay, 13 (4.4%) were positive with the universal HCV probe but not reactive to any genotype/subtype-specific probes and were therefore classified as indeterminates. Upon the retesting of genotype 1 specimens with the HCV genotyping ASR 5′NC region assay, the rate of indeterminates decreased to 2%. This is lower than previously reported for this and other real-time-based techniques (8, 18). The amount of HCV RNA did not apparently account for the discordant results at the genotype level (eight specimens/NC region-based techniques, as expected. Among the discordant results at the genotype level (eight specimens [2.8%]), five were confirmed as mixed-genotype infections by cloning and pyrosequencing and/or QCMD. Mixed-genotype infections have been widely reported, particularly in those multiply exposed to HCV infection (1, 24, 31). Detection rates vary depending on the patient population studied and the HCV genotyping method used (9, 12, 15). In our study, not all confirmed mixed infections could be detected by all the methods used. These results may reflect different sensitivities of the three compared methods at detecting minority types or different sensitivities of each method according to genotype. The criterion used by the SGS v2.0 software to interpret results obtained by the real-time PCR method as mixed infections (the cycle thresholds of the amplified genotypes must be within three cycles of one another) should be reoptimized at least for some of the primer/probe sets. We found this software was too restrictive, and this limited the detection of mixed infections; in a specimen with a 3/5 infection according to QCMD and cloning, the weaker amplification signal was not detected as a mixed-genotype infection. Currently, the most widely accepted methodology to confirm mixed-genotype infection is cloning RT-PCR products and sequencing individual clones. In the method we used, the predominant type was cleaved by genotype-specific restriction endonuclease previous to cloning and pyrosequencing. Thus, this method allowed an effective identification of minority variants without the need for screening a large number of clones. Moreover, pyrosequencing is faster and simpler than standard sequencing. However, two 1/4 mixed infections in our study could not be confirmed by this method. This fact does not completely exclude the presence of a true mixed infection because of several limitations (4): (i) this method is not able to detect a mixed infection when the viral load of the minority genotype is less than 1:10,000 of that of the dominant genotype; (ii) in some cases, the number of clones obtained to screen by pyrosequencing is limited; and (iii) 1/4 mixed infections are more difficult to confirm with this methodology because there is no appropriate restriction endonuclease to specifically cleave genotype 4. Conversely, the two 1/4 mixed infections detected by real-time PCR that could not be confirmed could have been due to cross-reactivity between genotypes 1 and 4 in the real-time assay, as suggested by Cook et al. (8). Our studies indicate that SGS v2.0 software results should be manually reviewed in search of mixed-genotype infections.

Concordance at the subtype level was studied for genotypes 1 and 2, since the real-time method does not include subtype-specific probes for genotypes 3 to 5. Regarding genotype 1, subtyping efficiencies were 100%, 77.2%, and 73.9% for the real-time PCR method and the 5′NC sequencing and reverse hybridization assays, respectively. These data are consistent with other studies that have compared HCV genotyping results based on the 5′NC region (14, 33). The real-time PCR method agreed more frequently with NS5b sequencing than 5′NC region-based methods, as we expected, since discrimination between subtypes within genotype 1 is based on the NS5b region. There were two specimens in which both 1a and 1b subtypes gave a positive signal by real-time PCR. These results could point to the presence of a mixed infection comprising both subtypes, which could not be confirmed by the methods used, recombination, or a low percentage of unspecific binding of primers to their respective subtypes, as previously suggested (8). Regarding genotype 2, most specimens were subtype discordant between techniques, and none of the genotyping techniques assigned a reliable subtype call. Only methods based on the use of more-variable regions, such as NS5b sequencing and phylogenetic analysis, should be relied upon for accurate subtyping of genotype 2 specimens. This method also offered the opportunity to identify new genotype 2 variants. Studies to confirm these results are in progress. Concerning genotypes 3 to 5, no comparison was performed at the subtype level because the real-time PCR assay has only a single probe for each of these genotypes regardless of subtype. Regarding reverse hybridization and 5′NC sequencing, only two specimens belonging to genotype 3 and none belonging to genotypes 4 and 5 were tested by both methods.

The limitations of reverse hybridization and 5′NC sequencing methods for discrimination among subtypes are related to the high degree of conservation of the 5′NC region. Genotypes 1a and 1b differ by a single nucleotide (A/G) at position −99 within this region, and subtypes 2a and 2b differ by only two nucleotides at positions 124 and 164. Moreover, a reliable discrimination of subtype 2a and 2c isolates is not possible based upon sequence analysis of the 5′NC region alone (27, 28). While the clinical significance of the infecting HCV sub-
type remains unknown, accurate subtyping is necessary for epidemiological purposes, such as outbreak studies, and vaccine trials. Our results confirm that NS5b-based genotyping methods are preferable to 5’NC region-based tests, as previously reported (17, 21, 25). Next-generation HCV genotyping techniques are attempting to investigate these more-informative regions. Indeed, a new version of the reverse hybridization assay used in this study (Versant HCV genotype 2.0 assay; Siemens Medical Solutions Diagnostics) has recently been commercialized; this assay comprises probes targeting core and 5’NC regions to improve genotypic precision (22).

In conclusion, although only sequencing-based methods provide the possibility of identifying new HCV variants, the real-time PCR method is suitable for genotyping in routine clinical laboratories. The test is rapid (results available within 2.5 h excluding RNA extraction), straightforward, and simple to interpret, thus providing a good single-step alternative to more-complex assays. Moreover, correlation with the other methods at the genotype level was high, and being based on the NS5b complex assays. Moreover, correlation with the other methods for useful support in statistical analysis.

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