Genotyping of Hepatitis C Virus Isolates using CLIP Sequencing

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Determination of hepatitis C virus (HCV) genotypes and subtypes has become increasingly important for the clinical management and prognosis of HCV infections. The aim of the present study was to assess the specificity and reliability of a newly developed, commercially available HCV genotyping kit (TRUGENE HCV 5′NC genotyping kit). This technique utilizes PCR fragments previously generated by the diagnostic Roche AMPLICOR HCV test, which are subsequently subjected to simultaneous PCR amplification and direct sequencing (CLIP sequencing) of the 5′ noncoding region (5′NCR). HCV isolates from 100 randomly chosen patients were genotyped by both the TRUGENE HCV 5′NC genotyping kit and DNA enzyme immunoassay (DEIA). Typing results obtained by both methods were in complete concordance in 91% of the cases. HCV RNA from the samples with discordant genotype assignment in both assays was additionally amplified with primers from the HCV core and NS5B regions. Phylogenetic analysis of the obtained sequences and comparison of these results obtained from DEIA in six cases and CLIP sequencing in two cases. In the former six cases, the TRUGENE HCV 5′NC genotyping kit could not correctly differentiate between subtypes of genotypes 1 and 2 due to the high conservation of the 5′NCR. However, since there was not any misclassification between HCV genotypes 1 and non-1 types, the results obtained with this system are, in general, reliable and can be used in clinical practice. The TRUGENE HCV 5′NC genotyping kit in our hands proved to be a fast and convenient technique that might be an attractive option for HCV genotyping in laboratories already using the Roche AMPLICOR HCV test for diagnostic reverse transcription-PCR.

The hepatitis C virus (HCV) is a positive-stranded RNA virus that was identified as the main cause of posttransfusion non-A, non-B hepatitis (2). The genome of this virus is highly variable. So far, six major genotypes and more than 100 subtypes have been described (8, 19). Many studies have indicated an association between HCV genotype and both the responsiveness to alpha-interferon treatment and the degree of clinical progression of chronic HCV infection (4, 19, 28). Therefore, during the last few years, HCV genotyping has been implemented in clinical laboratory settings and there is an ongoing demand for the development of new, automated typing techniques that provide reliable results within a reasonable time frame (8, 16, 19). The use of a single amplification reaction for diagnostic HCV RNA detection including the possibility of genotype determination from these PCR products would be the most efficient way of HCV genotyping. In this study, we utilized PCR fragments previously generated by the Roche AMPLICOR HCV assay to assess the HCV subtype by subsequent PCR amplification and direct sequencing of the 5′ noncoding region (5′NCR) with a recently developed, and now commercially available, automated sequencing system.

Materials and Methods

Specimens. Randomly chosen sera from 100 HCV infected in- and outpatients (72 men and 28 women; mean age, 46 years [range, 18 to 87 years]) attending Essen University Hospital were included in this study. All patients tested positive for HCV antibodies by immunoassay (Saniﬁc Diagnostik Pasteur, Freiburg, Germany) and for HCV RNA by diagnostic reverse transcription (RT)-PCR (AMPLICOR; Roche Diagnostics, Mannheim, Germany). Sera were aliquoted and stored at −80°C until HCV typing.

TRUGENE HCV 5′NC genotyping kit. The HCV subtype of all samples was determined by the newly developed TRUGENE HCV 5′NC genotyping kit (Visible Genetics Europe, Evry, France). This test uses the 244-bp fragment from the 5′NCR of HCV previously amplified by the diagnostic Roche AMPLICOR HCV kit (27). After puriﬁcation with Chroma Spin 100 columns (Clontech Laboratories, Palo Alto, Calif.), the Roche amplicons were subjected to simultaneous PCR ampliﬁcation and sequencing (CLIP sequencing), according to the manufacturer’s instructions (instruction manual for TRUGENE HCV 5′NC genotyping kit, Visible Genetics, Toronto, Canada). Two characteristics of the CLIP reaction as a modulation of the original coupled ampliﬁcation and sequencing method by Ruano and Kidd (17) are noteworthy. (i) An engineered mutant of thermostable DNA polymerase is used which lacks 5′-3′ exonuclease activity and therefore produces uniform band intensities. (ii) Different far-red ﬂuorescent dyes are linked to the two inward-facing CLIP primers, allowing a template to be sequenced in both directions in a single run (26). Automated sequencing of the 183-bp fragment (nucleotides [nt] 96 to 278 [numbering according to reference 2]) resulting from the CLIP reaction was performed on a MicroGene Clipper sequencer. This platform employs ultrathin (50-μm-thick) disposable gels (26). Each pair of forward and reverse sequences were combined and automatically aligned with reference sequences stored in the GeneBank database in order to determine the HCV subtype and the closest isolate.

DEIA. All samples were also typed by DNA enzyme immunoassay (DEIA) (Gen-Eti-K DEIA; Sorin Biomedica, Saluggia, Italy), as described previously in full detail (24). In brief, a fragment from the HCV core region was ampliﬁed by nested RT-PCR, and the resulting amplicons were hybridized to type- or subtype-speciﬁc oligonucleotide probes adsorbed to microwell plates. The double-stranded DNA hybrid thus formed was detected by DEIA.

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TABLE 1. Comparison of HCV typing results obtained by GEN-ETI-K DEIA and TRUGENE HCV 5′NCR genotyping kit

<table>
<thead>
<tr>
<th>DEIA HCV subtype</th>
<th>No. of isolates with HCV subtype according to TRUGENE HCV 5′NCR genotyping kit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>1a</td>
<td>14</td>
</tr>
<tr>
<td>1b</td>
<td>2</td>
</tr>
<tr>
<td>2a</td>
<td>5</td>
</tr>
<tr>
<td>2b</td>
<td>2</td>
</tr>
<tr>
<td>2c</td>
<td>4</td>
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<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Not typeable</td>
<td>1</td>
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</table>

Discrepant genotype and subtype assignments are indicated in boldface type.

RESULTS

An HCV type and subtype could be determined for all 100 samples with the TRUGENE HCV 5′NCR genotyping kit, whereas one sample was not typeable by DEIA. The results obtained with the two tests were in complete agreement in 91% of the cases. A different genotype assignment was achieved in two cases (1b versus 3a and 1b versus 4) and divergent HCV 1 and 2 subtypes were determined for six isolates (Table 1).

For those samples that gave discrepant results with the TRUGENE HCV 5′NCR genotyping kit and DEIA, a phylogenetic analysis of the 5′NCR sequences was performed to check for the reliability of HCV subtype determinations by means of the GeneLibrarian database. Identical results were obtained in six cases. Samples S3, S7, and S8, however, turned out to be HCV subtypes 1b and 2b in phylogenetic analysis, whereas typing by the GeneLibrarian database indicated the presence of subtypes 1a and 2a/c, respectively (Fig. 1; Table 2). Direct sequencing and analysis of the less-conserved HCVC core and NS5B fragments revealed that the TRUGENE HCV 5′NCR genotyping kit provided the correct genotype assessment (genotypes 3a and 4) for samples S5 and S6. Regarding the discrepant subtyping results (samples S1 to S4, S7, and S8), DEIA was correct in all six cases. Sample S9, which could not be typed by DEIA and yielded subtype 2a/c by the TRUGENE HCV 5′NCR genotyping kit, belonged to the rare subtype 2i (Fig. 1; Table 2).

DISCUSSION

Nucleotide sequence analysis is the current “gold standard” for identifying different HCV genotypes and subtypes but is generally regarded as not practical for routine clinical laboratory settings (8, 19). Therefore, a variety of surrogate HCV typing procedures has been proposed during recent years, mainly based upon amplification of the viral sequences by PCR, using either type-specific primers (15), analysis of PCR products by hybridization with genotype-specific probes (21, 25), or restriction fragment length polymorphism (1, 22). The underlying assumption of all these assays that the region (e.g., 5′NCR, core, or NS5B) analyzed is representative of the whole HCV genome is generally supported by the very consistent typing results which have been obtained so far using assays.
TABLE 2. Final HCV subtype assignment based on results of HCV core and NS5B sequencing for samples that yielded discrepancies in other assays

<table>
<thead>
<tr>
<th>Sample</th>
<th>DEIA</th>
<th>TRUGENE 5’NCR HCV genotyping kit</th>
<th>Core 5’NCR</th>
<th>Direct sequencing</th>
<th>Interpretation</th>
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<tbody>
<tr>
<td>S1</td>
<td>1a</td>
<td>1b</td>
<td>1a</td>
<td>1a</td>
<td>1a</td>
</tr>
<tr>
<td>S2</td>
<td>1a</td>
<td>1c</td>
<td>1a</td>
<td>1a</td>
<td>1a</td>
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<tr>
<td>S3</td>
<td>1b</td>
<td>1a</td>
<td>1b</td>
<td>1b</td>
<td>1b</td>
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<tr>
<td>S4</td>
<td>1b</td>
<td>1a</td>
<td>1b</td>
<td>1b</td>
<td>1b</td>
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<tr>
<td>S5</td>
<td>1b</td>
<td>3a</td>
<td>3a</td>
<td>No amplicon</td>
<td>3a</td>
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<td>1b</td>
<td>4</td>
<td>4</td>
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<tr>
<td>S7</td>
<td>2b</td>
<td>2a/c</td>
<td>2b</td>
<td>2b</td>
<td>2b</td>
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<tr>
<td>S8</td>
<td>2b</td>
<td>2a/c</td>
<td>2b</td>
<td>2b</td>
<td>2b</td>
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<tr>
<td>S9</td>
<td>Not typeable</td>
<td>2a/c</td>
<td>2i</td>
<td>Not done</td>
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</tbody>
</table>

Taken together, the newly developed TRUGENE HCV 5’NCR genotyping kit and the MicroGene Clipper sequencing platform turned out to be a convenient analytical system that provides clinically valid HCV typing results in about 5 h. The assay might be an attractive option for HCV genotyping in laboratories that already use the Roche AMPLICOR HCV test for diagnostic RT-PCR.

ACKNOWLEDGMENTS

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REFERENCES