Evaluation of Two Hepatitis C Virus Genotyping Assays Based on the 5′ Untranslated Region (UTR): the Limitations of 5′ UTR-Based Assays and the Need for a Supplementary Sequencing-Based Approach

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We evaluated two genotyping methodologies that characterize the 5′ untranslated region (5′ UTR) of the hepatitis C virus (HCV) genome. The limitations of these genotype assays need to be thoroughly evaluated, and sequencing-based approaches may be needed to complement these methods in clinical settings.

Characterization of the hepatitis C virus (HCV) genotype is crucial for determination of the proper course and duration of treatment in the affected patient (3, 4, 12). Here, we evaluated two genotyping assays that target the HCV 5′ untranslated region (5′ UTR): (i) the restriction fragment mass polymorphism (RFMP) assay, which is one of the most common assays in South Korea, and (ii) the Abbott RealTime HCV genotype II (GT II) assay (Abbott Molecular, Inc., Des Plaines, IL), which targets the 5′ UTR and NS5B region, which has recently been introduced as a diagnostic tool.

This study consisted of 100 South Korean patients. The RFMP assay was carried out as previously described (3). The GT II assay was performed according to the manufacturer’s protocol. The 5′ UTR and NS5B fragments were analyzed by nested PCR and sequenced using the ABI PRISM 3730 genetic analyzer (Applied Biosystems, Foster City, CA) (1). Nucleotide sequences were aligned with reference panels provided by the Los Alamos National Laboratory (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html) and the RefSeq database. A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 in accordance with the neighbor-joining method.

HCV genotypes were determined in 91 cases, and HCV was not detected in 9 cases. The discordances between the GT II and RFMP assays were 94.5% (86/91) at the genotype level and 82.4% (75/91) at the subtype level (Table 1). Three discrepant cases at the genotype level were reported as genotypes 1b, 2 and 3 (2 + 3), and 2 + 4 in the GT II assay and as 6c, 2, and 2a/c in the RFMP assay. Two additional discrepant cases were untypeable at the genotype level in the GT II assay but were characterized as genotype 2 in the RFMP assay. Sequence analysis revealed that one case of genotype 2a was mistyped as a mixed genotype of 2 and 4 (2 + 4), and two cases of genotype 2a were untypeable by the GT II assay but correctly typed by the RFMP assay. Conversely, sequence analysis revealed that one case of the mixed genotype 2a + 3a was mistyped as genotype 2 and genotype 1b was mistyped as genotype 6c in the RFMP assay but correctly typed in the GT II assay (Table 2). Eleven cases were discrepant at the subtype level. All except one (insufficient amount of viral RNA) were resolved into genotype 1b. However, seven cases were mistyped as genotype 1 (subtypes other than 1a and 1b) in the GT II assay and two cases were mistyped as genotype 1a and genotype 1 in the RFMP assay, respectively. The distribution of HCV in this study was as follows: genotype 1b, 50% (45/91); genotype 2, 47% (43/91); genotype 3, 1% (1/91); genotype 4, 1% (1/91); and a mixed infection of genotype 2 + 3, 1% (1/91). The distribution of HCV genotypes in this study was similar to that of others conducted within the South Korean population (6, 11).

The 5′ UTR is the most conserved region throughout the HCV genome (9), and the majority of commercial assays used to identify the HCV strain target only this region. However, the known variants of HCV are classified on the basis of the partial sequences from subgenomic regions, such as core/E1 or NS5B (10), so the HCV genotype could be misclassified by commercial assays.

As shown in previous studies, two HCV genotyping assays utilized to determine the HCV 5′ UTR show a high level of concordance at the genotype level (2, 11). However, our data indicate substantial discordance at the subtype level, indicating that certain considerations are needed when applying 5′ UTR-based genotyp-
ing assays in a clinical setting. True mixed HCV genotype infections can be missed, and false mixed HCV genotype infections can arise using these assays. In this study, one case was interpreted as a false mixed genotype in the GT II assay, which is likely due to cross-reactivity. Conversely, the RFMP assay missed one true mixed genotype.

In this study, two cases of genotype 2a were interpreted as of indeterminate genotype in the GT II assay and were interpreted as genotype 2 and not 2a or 2c in the RFMP assay. Sequence analysis revealed that these cases were genotype 2a. These cases had variations at positions $-139$ and $-140$—the probe binding site for genotype 2 of the GT II assay (Fig. 1). The RFMP assay mistyped genotype 1b as 6c in this study. We analyzed two additional cases that had previously been identified as genotype 6c in the RFMP assay. Sequence analysis of the 5’ UTR revealed that these cases were actually genotype 1b. These cases had a common adenine duplication variation at position $-138$ (Fig. 1)—the cleavage site of the FokI enzyme used in the RFMP assay (5). Therefore, this variation changes the molecular weights of the digested fragments when using the Mmel and AvaII enzymes. The molecular weights of no. 38 and no. 72 are 5,378.8 to 5,891.8 and 5,353.8 to 5,876.8, which were misidentified as genotype 1a in the RFMP assay (8).

The distribution of HCV genotypes as determined in the RFMP assay may differ from the actual number of cases as it pertains to genotype 1a, genotype 6, and mixed infection in South Korea. Therefore, to accurately characterize the distribution of genotype 1a, genotype 6, and mixed infection cases, studies utilizing technologies such as next-generation sequencing and molecular cloning are needed to validate previously published data.

Genotyping assays targeting the 5’ UTR generally exhibit a high level of reliability at the genotype level. However, because of minor 5’ UTR sequence variations, the current assays cannot distinguish between closely related HCV strains from various geographic regions. Therefore, the limitations of these genotype assays need to be thoroughly evaluated, and sequencing-based

### Table 2: Discordant results at the genotype level between the GT II assay and the RFMP assay

<table>
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<tr>
<th>Patient no.</th>
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<th>RFMP</th>
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<th>NSSB</th>
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<td>6c</td>
<td>1b</td>
<td>1b</td>
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<td>2a + 3a</td>
<td>2a + 3a</td>
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<td>2a/c</td>
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<tr>
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<td>Indeterminate</td>
<td>2a</td>
<td>2a</td>
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</tr>
</tbody>
</table>

![Fig 1](A) 5’ UTR-based sequence alignment of two genotype 2a cases that were interpreted as indeterminate in the GT II assay. These cases had variations at positions $-139$ and $-140$, which are the estimated binding sites for the probe used in the GT II assay to validate genotype 2. (B) 5’ UTR-based sequence alignment of three genotype 1b cases that were misidentified as genotype 6 in the RFMP assay. Three cases have a common variation of adenine duplication at position $-138$ similar to the reference strains of genotype 6 but show close homology to the reference strain of genotype 1b. (C) 5’ UTR-based sequence alignment of 2 genotype 1b cases that were misidentified as genotype 1a in the RFMP assay. Three cases have variations at positions $-94$ and $-99$, which alter the predicted molecular weights of the digested fragments with the Mmel and AvaII restriction enzymes used in the RFMP assay.
approaches may be needed to complement these methods in clinical settings.

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REFERENCES