DETERMINATION OF HEPATITIS C VIRUS GENOTYPES BY MELTING-CURVE ANALYSIS OF QUANTITATIVE POLYMERASE CHAIN REACTION PRODUCTS

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Abstract

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis.1,2 Acute HCV infection is often asymptomatic, and approximately 70% of cases progress to chronic hepatitis. This may lead to progressive liver disease, cirrhosis, liver failure, and hepatocellular carcinoma within 20 to 30 years. Factors associated with disease progression following HCV infection include viral genotype, the patient’s alcohol consumption, and viral load.3

A large number of methods have been developed for HCV genotyping. However, these methods are laborious and expensive. In this study, the COBAS AMPLICOR HCV MONITOR test, a commercially available quantitative assay for HCV RNA, was used. Amplification products obtained from 100 HCV-positive cases were subjected to real-time polymerase chain reaction (PCR) typing using a single pair of fluorescence resonance energy transfer (FRET) probes and melting-curve analysis. Of 100 samples tested, two inhibited the PCR, two samples yielded discrepancies between our results and the reference laboratory results, and the remaining samples provided correct typing. The present report suggests that HCV genotypes can be determined rapidly with FRET probes directly from COBAS AMPLICOR MONITOR test PCR products.

Key words: Hepatitis C virus genotyping, real-time polymerase chain reaction

Materials and Methods

Clinical samples

Serum samples from 98 seropositive hepatitis C patients were analyzed for HCV RNA levels between January 2003 and December 2004. Serum was separated from whole blood collected in serum separation tubes and immediately stored at –80°C.

Quantification of HCV RNA in serum

A commercial assay for the quantification of serum HCV, COBAS HCM-2, was used. This assay was carried out strictly in accordance with the manufacturer’s instructions. COBAS HCM-2 is based on reverse transcription (RT) and HCV RNA amplification with the oligonucleotide primers KY80 (5′GCAGAAAGCGTCTAGCCATGGCGT) and KY78 (5′CTCGCAAGCACCCTATCAGGCAGT), which target the 244 bp region located within the highly conserved 5′ noncoding region of the HCM genome. An internal quantitation standard (HCM QS) was diluted in lysis buffer, added to each sample, and was co amplified with the HCM RNA target. HCM QS is a noninfectious RNA transcript that has primer-binding regions identical to those of the HCV...
target RNA but a probe-binding region different from that of the target amplicon. Both the amplified products of the internal standard and sample RNA were serially diluted and detected by probe hybridization. Final PCR products were purified using the Roche purification kit to eliminate excess deoxyribonucleotides and amplification primers and were then frozen in aliquots for further real-time genotyping.

**Primers and probes**

Primers and probes for seminested PCR and HCV genotype determination in the LightCycler consisted of the forward primer HCV SF2 (5′-GTGCGACGCTCCAGGAGCCGCCG), the reverse primer NAR3 (5′-CCCTATCACGCGATCCACAA), the FRET anchor probe HCVG-fluorescein isothiocyanate (5′-GCCATAGTGCTGCGAGACGGT); 5′-LCRed640-GTACACCGGAATTGCAGGA-phosphate-3′). Both primers and the FRET probes have been published previously and were purchased from TIB MOLBIOL, Germany.

**Real-time genotyping**

Seminested, “hot start” PCR reactions were performed in a final volume of 10 μL, using the LightCycler-FastStart DNA master hybridization probes reaction kit. We combined 2.5 μL of the COBAS AMPLICOR reaction product (after purification) with 7.5 μL master mix in 20 μL LightCycler glass capillaries and seminested PCR was performed in the Roche LightCycler. Each 7.5 μL reaction contained 1 mM MgCl₂, 0.25 μM forward primer HCV SF2, 0.25 μM reverse primer NAR3, 0.2 μM HCVG-FITC probe, 0.2 μM RED-HCVG probe, and 1x LightCycler FastStart DNA master hybridization probes mix (contributing an additional 1 μM so that the final MgCl₂ concentration was 2 μM per reaction). After a preincubation step at 95°C for 10 minutes to activate the FastStart polymerase, PCR amplification (50 cycles) consisted of denaturation at 95°C for 3 seconds and a temperature transition rate of 20°C s⁻¹ annealing at 56°C for 10 seconds with a temperature transition rate of 0.5°C s⁻¹. After preincubation at 95°C for 10 minutes to activate the FastStart polymerase, PCR amplification (50 cycles) consisted of denaturation at 95°C for 3 seconds and a temperature transition rate of 20°C s⁻¹ annealing at 56°C for 10 seconds with a temperature transition rate of 0.5°C s⁻¹. After amplification, melting curve analysis was performed by heating to 95°C for 5 seconds with a temperature transition rate of 20°C s⁻¹, cooling to 40°C with a temperature transition rate of 20°C s⁻¹, holding at 40°C for 30 seconds and then heating the sample to 80°C at 0.1°C s⁻¹. Fluorescence data were collected continuously during this heating step to monitor the dissociation of the RED-HCVG sensor probe. The derivative melting curves were obtained using the LightCycler 4.0 data analysis software (Roche).

**Comparative testing**

To compare this novel method with a standard genotyping method, the same 98 serum samples were genotyped by hybridization with sequence-specific oligonucleotides.

**Results**

For optimal discrimination, the FITC-labeled anchor probe was designed to anneal to an invariant region of the 5′-UTR. The fluorescence resonance energy transfer (FRET) sensor probe was designed to allow discrimination of HCV genotypes 1a/b, 2a/c, 2b, 3a, and 4 during melting curve analysis because it hybridizes with different affinities to a region of the 5′-UTR that varies among the different HCV genotypes. Table 1 shows the genotype-specific Tm determined using purified HCV monitor PCR product. Figure shows the melting curve analysis of the various genotypes obtained from the sera of different patients.

Discrepancies between our method and the original method of hybridization with sequence-specific oligonucleotides occurred in two of the samples (Table 2). Two samples were typed as type 2b by our method, while they were identified as type 4 by the reference laboratory standard method. All other samples were typed correctly and were concordant with the reference laboratory standard method.

**Discussion**

Nucleotide sequence analysis may be regarded as the gold standard for identification of different HCV genotypes

<table>
<thead>
<tr>
<th>Table 1: Hepatitis C virus genotype Tm as determined by melting-curve analysis</th>
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<tbody>
<tr>
<td>Hepatitis C virus genotype</td>
</tr>
<tr>
<td>1a/b</td>
</tr>
<tr>
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<tr>
<td>4</td>
</tr>
<tr>
<td>2b</td>
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<td>3a</td>
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Figure: Melting curve analysis of different HCV genotypes
The present study further demonstrates that high-directly with COBAS AMPLICOR MONITOR test PCR genotypes can be determined rapidly with FRET probes between-run variation of the genotype Tm. Accomplished with a single sensor probe. More samples need those assays because of insufficient sample material. We were not able to repeat hybridization method occurred in two of the samples (Table 2).

In this study, we combined a COBAS AMPLICOR HCV MONITOR test with real-time PCR genotyping and compared the results with those yielded by hybridization with genotype-specific oligonucleotides. With this approach, HCV genotyping was possible in about one hour.

The present study demonstrates that melting-curve analysis can be used to identify and classify HCV types/subtypes with considerable confidence. Type 1 HCV is treated differently from other common types of HCV and has a poorer prognosis. Thus, our primary goal in designing this study was to identify type 1 HCV. To meet this goal, the sensor probe chosen was identical in sequence to the type 1 sequence so that DNA from type 1 virus associated with a high Tm. We took advantage of the ability of melting-curve analysis to detect sequence variation(s) with respect to the probe as revealed by an altered Tm. A lower Tm may represent a different genotype or perhaps a minor sequence variation in the type 1 genome. A high Tm indicates target sequence identity and thus the presence of the common type 1 virus associated with a high Tm. We took advantage of the ability of melting-curve analysis to detect sequence variation(s) with respect to the probe as revealed by an altered Tm. A lower Tm may represent a different genotype or perhaps a minor sequence variation in the type 1 genome. A high Tm indicates target sequence identity and thus the presence of the common type 1 virus associated with a high Tm.

Discrepancies between our method and the oligonucleotide hybridization method occurred in two of the samples (Table 2). However, no discrepancies occurred with type 1—the main target of our study. The discrepancies might be due to sample confusion or primer mismatch. We were not able to repeat those assays because of insufficient sample material.

In conclusion, the present report suggests that HCV genotypes can be determined rapidly with FRET probes directly with COBAS AMPLICOR MONITOR test PCR product. The present study further demonstrates that high-specificity identification of the common type 1 virus can be accomplished with a single sensor probe. More samples need to be analyzed by this method to determine the within- and between-run variation of the genotype Tm.

### Table 2: Concordance of the genotypes determined by melting-curve analysis and by the reference laboratory

<table>
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<tr>
<th>Type</th>
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<th>Reference</th>
<th>Laboratory</th>
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<tr>
<td>Total</td>
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<td>20</td>
<td>10</td>
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</table>

References

14. Ross RS, Viazov SO, Holtzer CD, Beyou A, Monnet A, Mazure...


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