ABSTRACT

BACKGROUND: Clinically, both viral load and genotypes have been found to be major predictors of antiviral therapy outcome regarding chronic hepatitis C and they are, under normal circumstances, performed as separate assays.

DESIGN AND METHODS: In order to improve the diagnostic strategy and subsequently reduce the reagent costs we have developed and established the simultaneous quantification and genotyping of hepatitis C virus RNA by a two-step real-time PCR on the LightCycler Instrument (Roche Diagnostics).

RESULTS: The quantification assay was calibrated against WHO Standard 96/790. The detection limit was 30 IU/ml, the dynamic range up to 500,000,000 IU/ml. Intra- and inter-assay imprecisions were 1.2% and 1.9% (n = 10), respectively. The HCV RNA values obtained by real-time PCR assay were highly correlated with those obtained by the Cobas Amplicor HCV monitor test (r = 0.992; p < 0.001).

CONCLUSIONS: The genotyping was performed by means of the melting temperature analysis. The concordance between our new genotyping method and the Trugene HCV 5'NC Kit was at the level of genotypes 100%. This rapid (3 h) and convenient assay is suitable for HCV genotyping, HCV detection and disease monitoring.

Keywords: HCV, real-time PCR, genotyping

INTRODUCTION

Affecting nearly 300 million people worldwide, hepatitis C virus (HCV) infection is considered to be the most frequent cause of post-transfusion non-A, non-B hepatitis. HCV, a member of the Flaviviridae family, is a single-stranded RNA virus, containing a genome of 9,400 nucleotides. The HCV genome demonstrates considerable diversity of nucleotide sequences. Based on the identification of these genomic differences, HCV has been classified into multiple strains. It is thought that genetic heterogeneity of HCV may account for some of the differences in disease outcome and response to treatment observed in HCV infected persons. HCV genotype 1, which is the most prevalent worldwide, is associated with more severe clinical manifestations, higher levels of HCV viremia and less amenable to treatments such as interferon alpha or PEG-interferon alpha / ribavirin therapy than HCV genotypes 2 or 3. Therefore, clinical trials of antiviral therapies require both HCV viral load and genotype information for an appropriate strategy of treatment.

Assays for HCV genotyping are generally based on sequencing technology or line-probe test, but these assays are time-consuming, labour intensive, expensive and require significant expertise and additional devices such as an autosequencer. Recently, several other methods have been published, e.g. heteroduplex mobility analysis using temperature gradient capillary electrophoresis, genotyping with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), genotyping with genaturing high-performance liquid chromatography, melting curve analysis with a single set of fluorescence resonance energy transfer probes with
Because we already quantify the HCV RNA viral load by means of the LightCycler, we have tried to combine the quantification and genotyping of HCV on the same instrument using hybridisation probes. After several rounds of optimisation, the simultaneous quantification and genotyping of hepatitis C virus RNA by a two-step real-time PCR on the LightCycler instrument was successfully established.

MATERIALS AND METHODS

CLINICAL SPECIMENS

EDTA whole blood samples were obtained from 76 patients of the Klinikum Stuttgart, Katharinenhospital, Germany. All samples were sent to the laboratory for routine analysis of HCV viral load. Samples were centrifuged for 2 hours and divided into aliquots, which were stored at – 80°C. All samples were first measured with the Cobas Amplicor HCV Monitor test (Roche Diagnostics, Mannheim, Germany) and then examined with our new method on the LightCycler instrument (Roche Diagnostic, Mannheim, Germany).

SEQUENCES OF PRIMERS AND PROBES

Out of different primers tested we have selected the established forward primer NC1 and reverse primer SR2\(^\text{15-19}\) (Fig. 1). With this primer pair the best detection limit was achieved without the need of semi-nested PCR. For genotyping, the hybridization probes published by Bullock et al. were used.\(^\text{13}\)

INTERNAL CONTROL

Clinical specimens may contain inhibitors of PCR amplification, which can be monitored by using internal controls (IC).\(^\text{20}\) These controls can be heterologous PCR systems or modified target variants, for example those containing a mutated probe-binding site. DNA based controls monitor the performance of the DNA PCR only, whereas RNA based controls enable to examine in addition the extraction of the sample and the reverse transcription step. We used an internal control containing the target sequence of HCV genotype 1 cloned into a plasmid, replacing the probe-binding site by a fragment from Lambda DNA (GenExpress, Berlin, Germany). Detection of the control derived PCR product was achieved using the hybridization probes HCV-IC-FL (5’- GGT GCC GTT CAC TTC CCG AAT AAC-fluorescein, J02439/2331-2354) and HCV-IC-LC (Red705-LC705-CGG ATA TTT TTG ATC TGA CCG AAG CG-phosphate, J02439/2356-2381).

IN VITRO TRANSCRIPTION OF THE INTERNAL DNA CONTROL INTO RNA USING THE T7 PROMOTER

The plasmid containing the internal control sequence was linearized prior to in vitro transcription to generate RNA transcripts of defined length. In vitro transcription was performed with T7 RiboMax Express Large Scale RNA Production System (Promega, Mannheim, Germany) according to the instructions of the manufacturer. 500 copies of the RNA transcripts were added to each plasma sample as internal control, enabling to monitor the RNA extraction, reverse transcription, and PCR efficiency.

EXTERNAL CONTROL

As external controls HCV reference standards (#75/98) calibrated against the WHO Standard 96/790 were obtained from the Paul-Ehrlich-Institute, Langen, Germany. Each vial was reconstituted with 0.5 ml of distilled water to yield 25,000 IU/ml of HCV genotype 1a. This concentration is considered equivalent to 75,000 copies/ml according to the data sheet provided by the PEI.

SAMPLES

RNA from 500 µl EDTA plasma samples was extracted and purified on the QIAamp DSP viral vacuum kit (Qiagen, Hilden, Germany). Reverse transcription reactions (20 µl) were performed in 200 µl-vessels using a block thermocycler (Peqlab, Erlangen, Germany) and contained the following components at the indicated final concentrations or amounts in sterile molecular-grade water: Sample-RNA (14 µl), reverse primer SR2 (1.4 µM), dNTPs (each 1.13 mM), superscript II reverse transcriptase (30 U), RNAse Out (10 U), 5x-RT-buffer (Invitrogen). The sample RNA primer dNTP mixture was first denatured by 65°C for 2 min, followed by an annealing step from 65°C to 25°C with 0.3°C/sec. Thereafter, the RT mixture (4.4 µl) was added to the vessels followed by incubation at 40°C for 35 min. The reverse transcriptase was inactivated at 95°C for 1 min.

LIGHTCYCLER REAL-TIME PCR

After the completion of the reverse transcription the LightCycler cDNA PCR was performed with the following components at the indicated final concentrations or amounts in sterile molecular-grade water: PCR reaction mixture (8 µl) contains 1 µM HCV forward primer NC1 (5’-CCC TGT GAG GAA CTG TCT TCA CCG AAT AAC-fluorescein, J02439/2331-2354) and HCV-IC-LC (Red705-LC705-CGG ATA TTT TTG ATC TGA CCG AAG CG-phosphate, J02439/2356-2381).
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Figure 1. Alignment of the HCV genotype sequences showing overlapping and conserved regions of the different HCV genotypes and the locations of the used primers and probes. HCV genotype 1b/2-fw: additional forward primer for the differentiation of genotypes, NC1 common fw primer: forward primer for the quantification, HCV FL anchor: Detection probe with fluorescent colour fluorescein at the 5’end and at the 3’end phosphate, HCV LC sensor: Detection probe with fluorescent colour LC640 at the 3’end, SR2 reverse primer: reverse primer for the quantification and genotyping.

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Concerning the evaluation of the accuracy of the HCV genotyping assay a panel of HCV genotypes consisting of types 1a, 1b, 2a, 2b, 2c, 2i, 3a, 4, 5a and 6 obtained from the National Reference Center for hepatitis C (Institute of Virology, Essen, Germany) and 30 clinical samples previously determined on the Trugene HCV 5'NC Kit as the reference method were compared.

In order to analyze the impacts of sequence variability we produced the quantification standard for each genotype and created the standard curves.

**Statistics**

Method comparison was performed using Passing-Bablok regression analysis. With a p value below 0.05 the correlation coefficient was considered statistically significant showing excellent agreement between the two data sets investigated.

**Results**

**Quantification**

The detection limit of the HCV RT-LC-PCR assay was, in the first step, determined by diluting the HCV reference standard with HCV negative plasma. It was found to be 30 IU/ml (Table 1). As a second step, the dynamic quantification range of the HCV LC-PCR assay was examined by using a log-fold dilution series of a HCV quantification standard (GenExpress, Berlin, Germany) containing 10^{10} HCV cDNA. The upper quantification limit extends up to 500,000,000 IU/ml. In a third step, the precision of the HCV LC-PCR assay was determined by assessing the intra- and inter-assay coefficients of variation (CV). For the intra-assay, the crossing-point values of the 10 measurements of an HCV sample with approximately 150,000 copies/ml in a single run were used. The CV of intra-assay was 1.2%. For the interassay CV, the crossing-point values for amplification of HCV from the aforementioned sample of 10 different PCR runs performed on 10 different days were used. The CV of interassay was 1.9%. For evaluation of the accuracy of the HCV quantification assay a panel of HCV genotypes consisting of types 1a, 1b, 2a, 2b, 2c, 2i, 3a, 4, 5a and 6 obtained from the National Reference Center for hepatitis C (Institute of Virology, Essen, Germany) and 76 clinical samples previously determined on the Cobas Amplicor HCV Monitor Test as the reference method were compared. It could be shown that results generated with our new protocol were in overall good agreement with the Cobas Amplicor. Using Passing Bablok statistics, correlation coefficients were \( r = 0.992 \) (Y = 0.972X – 553.4, p < 0.001, n = 76) between our new assay and target values (Fig. 2).
Table 1. Percentage of HCV genotype 1a RNA positive replicates in dilutions (HCV negative plasma) of the PEI HCV-RNA reference standard (#75/98)

<table>
<thead>
<tr>
<th>HCV RNA (IU/ml)</th>
<th>No. of positive tests/No. of tests</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>7/10</td>
<td>70</td>
</tr>
</tbody>
</table>

Concerning the differences of PCR efficiencies there was no significant deviation among the various genotypes.

Regarding the influences of the variability of target sequences we performed the multiple measurement series with a panel of HCV genotypes consisting of types 1a, 1b, 2a, 2b, 2c, 2i, 3a, 4, 5a and 6. The coefficient of correlation was $r = 0.907$ ($Y = 1.428X - 148.8$, $p < 0.001$, $n = 30$) between our new assay and target values (Fig. 3).

In order to externally verify our new method we attend twice a year the external quality assessment scheme for virus genome detection of HCV RNA (Instand e.V., Düsseldorf, Germany) and pass, so far each time, the examination.

The ICs were amplified in a competitive fashion together with the samples. The optimal concentration of ICs was 200 IU per capillary. In this concentration there was no inhibition of HCV PCR. When the samples contained more than approximately 2,000 HCV RNA IU/ml, the IC was competitively inhibited.
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By means of the melting curve analysis we could differentiate four genotypes (Table 2) (Fig. 4).

The disadvantage of the melting curve method previously published by Bullock et al.\(^{13}\) is the inability to differentiate between genotypes 2 and 5 (the same melting temperature: 60° ± 1°C), between genotypes 1 and 6 (the same melting temperature: 60° ± 1°C) and also between genotypes 3b and 4 (the same melting temperature: 55° ± 1°C).

In order to improve the method we investigated the variations in sequences of the primer target region (Fig. 1). According to the sequence analysis we added the two new forward primers, which made it possible to differentiate the genotypes 1, 6 and genotypes 2, 5. The additional HCV type 1b specific forward primer shows a perfect match with the genotypes 1b, 3a and 3b. This primer has five mismatches with genotypes 4 and 6. The second additional HCV type 2 specific forward primer has 100% concordance with genotype 2 but three mismatches with genotype 5. By means of these additional primers the method can separate the rare genotypes (3b, 5 and 6) from the main genotypes (Table 3). For this improved differentiation only one more PCR is required.

In order to validate the accuracy of genotyping we used a panel of various HCV genotypes (1a, 1b, 2a, 2b, 2c, 2i, 3a, 4, 5a and 6 and 30 patient samples, which were at first genotyped with the Trugene HCV 5'NC Kit as the reference method. The results were thereafter compared. The con-
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**Table 3. Percent amplification efficiency of HCV genotypes**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Slope</th>
<th>Interception</th>
<th>Efficiency</th>
<th>Cp of 10^5 IU/ml (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.493</td>
<td>39.65</td>
<td>93.3%</td>
<td>20.1 (CV: 2.1)</td>
</tr>
<tr>
<td>2</td>
<td>-3.459</td>
<td>40.22</td>
<td>94.6%</td>
<td>20.9 (CV: 2.3)</td>
</tr>
<tr>
<td>3a</td>
<td>-3.610</td>
<td>41.37</td>
<td>89.2%</td>
<td>21.7 (CV: 2.4)</td>
</tr>
<tr>
<td>4</td>
<td>-3.543</td>
<td>40.67</td>
<td>91.5%</td>
<td>21.0 (CV: 3.1)</td>
</tr>
<tr>
<td>5</td>
<td>-3.576</td>
<td>40.57</td>
<td>90.4%</td>
<td>21.6 (CV: 1.6)</td>
</tr>
<tr>
<td>6</td>
<td>-3.519</td>
<td>39.86</td>
<td>92.4%</td>
<td>20.5 (CV: 2.1)</td>
</tr>
</tbody>
</table>

The percent amplification efficiency was calculated as \[10(-1/slope) - 1\] X 100.

Cp = crossing point = threshold cycle (CT): The parameter Cp is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (baseline). A plot of the log of initial target copy number for a set of standards versus Cp is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring Cp and using the standard curve to determine starting copy number.

CV: coefficient of variation.

**DISCUSSION**

**QUANTIFICATION**

The highly conserved 5’ UTR of HCV is the most suitable target sequence for the quantification of HCV RNA. Concerning the PCR efficiency among the various genotypes (1-3 mismatches in the region for the detection probes according the genotype) there was no significant deviation. We have similar observations with a real-time PCR quantification of all subtypes of the human immunodeficiency virus type 1 using locked nucleic acid-based probes. We assume that the perfect match of primer sequences is the most important factor regarding the PCR efficiency. When there are 1-3 mismatches in the primer targeting sequences the PCR efficiency reduces correspondingly. We utilise this fact for further differentiation of genotypes 1, 6 and 2, 5, respectively by means of additional forward primers (Fig. 1).

**Table 4. Scheme of interpretations**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>primer: NC1 + SR2</th>
<th>forward primer: HCV type 1b specific</th>
<th>forward primer: HCV type 2 specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>positive (65°C)</td>
<td>positive (65°C)</td>
<td>positive (65°C)</td>
</tr>
<tr>
<td>2</td>
<td>positive (60°C)</td>
<td>no amplification</td>
<td>positive (60°C)</td>
</tr>
<tr>
<td>3a</td>
<td>positive (51°C)</td>
<td>positive (51°C)</td>
<td>positive (51°C)</td>
</tr>
<tr>
<td>3b</td>
<td>positive (55°C)</td>
<td>positive (55°C)</td>
<td>positive (55°C)</td>
</tr>
<tr>
<td>4</td>
<td>positive (55°C)</td>
<td>no amplification</td>
<td>no amplification</td>
</tr>
<tr>
<td>5</td>
<td>positive (60°C)</td>
<td>no amplification</td>
<td>no amplification</td>
</tr>
<tr>
<td>6</td>
<td>positive (65°C)</td>
<td>no amplification</td>
<td>no amplification</td>
</tr>
</tbody>
</table>

**GENOTYPING**

Worldwide, 6 major genotypes, and more than one hundred different subtypes of the virus have been identified by nucleotide sequencing. The genotypic diversity of HCV, due to the mutation rate of the virus, interferes with effective humoral immunity against it. Although neutralizing antibodies to HCV have been detected in the serum of infected patients, these are, at best, short-lived. Moreover, HCV infection has not been shown to induce lasting immunity against re-infection with different virus isolates, or even the same isolate. Thus, neither homologous
nor heterologous immunity appears to develop after acute HCV infection.4

Some HCV genotypes are distributed worldwide, while others are more geographically confined. Genotypes 1a, 1b, 2a, 2b, 2c, and 3a account for more than 90% of the HCV infections in North and South America, Europe, Russia, China, Japan, Australia, and New Zealand. According to data obtained from the Clinic of Gastroenterology, Sofia University Hospital [personal communication Dr. A. Ivanova] genotype 1 dominates in Bulgaria (84.7%) followed by genotype 3a (12.5%). Genotype 3a is more common among younger populations. Other subtypes of genotype 3 are highly prevalent in Nepal, Bangladesh (3b), India, and Pakistan. Most infections in Egypt are genotype 4a, and this and other subtypes of genotype 4 are found in Central Africa. Genotype 5a accounts for about 50% of infections in South Africa. Genotypes 4 and 5 are found only sporadically outside Africa. Genotype 6 isolates are primarily found in Southeast Asia.3,4

It should be noted that the genotype distribution could vary significantly among different population groups in the same geographical area. Associations between genotype and mode of transmission also exist. Genotype 3, for instance, is more prevalent among intravenous drug users.3

The HCV genotype may be an important factor influencing the severity of liver disease. Infection with genotype 1 has been associated with more advanced liver disease and the development of both liver cirrhosis as well as hepatocellular carcinoma.4 Observations have also shown that patients with genotype 1 typically respond weakly to interferon therapy. However, while differences in pathogenicity and responsiveness to antiretroviral therapy have been reported among the genotypes, the biological impact of these differences still remains incompletely defined. Nevertheless, the current limited knowledge base does not undermine the fact that HCV genotyping is an important factor to consider in the management of treatment for HCV infection.3,5

The highly conserved 5' UTR of HCV can be routinely used to identify HCV RNA in sera and to determine HCV genotypes. Some commercial kits are based on the analysis of the sequence variability of the 5' UTR of HCV for genotyping (InnoLiPA and TRUGENE). The current limitations of using the 5' UTR for HCV genotyping are related to the low discriminating power of the 5' UTR sequence for determination of the particular subtype.22,23

The high level of conservation found in this region does not allow accurate distinction of the HCV genotypes at the subtype level (e.g. subtypes 1a, 1b, 1c).

However, these inherent disadvantages of using the 5' UTR for HCV genotyping are not crucial for actual clinical use. As practice shows, any HCV genotyping system based on the 5' UTR sequences...
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CONCLUSIONS
The presented new HCV PCR method proved to be reliable, reproducible and accurate. It combines the adequate lower detection limit of 30 IU/ml plasma with an extended upper detection limit of 500,000,000 IU/ml plasma including the genotyping of HCV within 3 hours and is suitable for both disease detection, HCV therapy monitoring and genotyping. Furthermore our new method reduces the reagent costs immensely because of the simultaneous quantification and genotyping in the single run.

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