Towards optimal treatment for chronic hepatitis C infection
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Clinical performance of the new Roche COBAS® TaqMan HCV Test and High Pure System for extraction, detection and quantitation of HCV RNA in plasma and serum

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Preliminary results of these studies were disclosed at the Therapies for Viral Hepatitis Workshop, Boston, MA, USA, 2–3 November 2004.

We evaluated the Roche COBAS® TaqMan HCV Test For Use With The High Pure System (TaqMan HPS; Roche Diagnostics), for the extraction, detection and quantitation of hepatitis C virus (HCV) RNA in serum or plasma of HCV-infected individuals. The TaqMan HPS is a real-time PCR assay with a reported linear dynamic range of $3.0 \times 10^1$ to $2.0 \times 10^8$ HCV RNA IU/ml, and a reported lower limit of detection (LLD) of 10 IU/ml. Calculation of the HCV RNA titre is based upon an external standard curve in the presence of an internal control. Intra-assay and inter-assay variation were small in reference panel members with HCV RNA $\geq 100$ IU/ml. Genotype performance and quantitative correlation between the TaqMan HPS and the bDNA (VERSANT® HCV 3.0 assay; Bayer Diagnostics), assessed in 59 patient samples, were good for HCV genotype 1 but poor for genotypes 2, 3 and 4. For genotypes 2, 3 and 4, values obtained from the TaqMan HPS were in general 0.5 log lower than those from the bDNA. Sensitivity was poor in low viral titre samples of genotypes 1, 2, 3 and 4. The LLD (95%) was estimated at 41 HCV RNA IU/ml for genotype 4. The TaqMan HPS underestimates HCV RNA at all levels in plasma and serum from HCV-infected individuals, and the LLD should be reconsidered. This is clinically relevant because underestimation of HCV RNA levels during therapy may lead physicians into making incorrect treatment decisions.

Introduction

Worldwide, more than 170 million individuals have been infected with hepatitis C virus (HCV). Considerable advances have been made in the therapy of HCV during recent years. The current antiviral therapy consists of administration of pegylated interferon alpha and ribavirin for 24 (genotype 2 and 3) or 48 weeks (genotype 1 and 4) and leads to sustained virological response (SVR) in approximately 40% (genotype 1 and 4) to 80% (genotype 2 and 3) of patients. The measurement of HCV RNA levels in plasma has become an important tool for monitoring individuals before and during antiviral therapy. The goal of early HCV RNA quantitation during therapy is to predict a negative treatment outcome as early as possible. Failure to achieve a significant decrease in HCV RNA after 12 weeks of treatment (2 log decrease from baseline HCV RNA) is highly predictive of a negative treatment outcome [1]. These treatment decisions rely on frequent assessment of HCV RNA, and require assays that are both sensitive in the detection of HCV RNA and accurate in the quantitation of HCV RNA over a wide dynamic range, irrespective of HCV genotype.

An ideal test for monitoring patients during treatment and prediction of treatment outcome would be a single quantitative test that matches the sensitivity of a qualitative test while accurately quantitating both low and high viral loads. At present, when HCV RNA drops below the detection limit of the quantitative assays during therapy (for example, 615–600 HCV RNA IU/ml), HCV RNA can only be detected by qualitative assays such as PCR [COBAS® Amplicor HCV Test v2.0; Roche Diagnostics, Branchburg, NJ, USA; lower limit of detection (LLD) 50 IU/ml] or TMA (Transcription-Mediated Amplification;
broad range in most genotypes [5–14]. The limitations of the current assays require testing of low viral titre clinical samples by two or three assays (quantitative, PCR and TMA) to estimate low viral load, resulting in increased costs and laboratory workload. In our laboratory, we use diagnostic algorithms before, during and after treatment. Based on previous test results (if available, for example, during antiviral therapy), the specimen is first tested by either TMA or bDNA (VERSANT® HCV 3.0 assay; Bayer Diagnostics; linear dynamic range of 615 to $7.7 \times 10^5$ IU/ml). Depending on the TMA or bDNA result, the specimen is then tested by bDNA or TMA, respectively. If the viral load is $\leq 50$ IU/ml or $\leq 15$ IU/ml, the specimen is then also tested by PCR to determine if the viral load is $\leq 0$ IU/ml or $\geq 10$ IU/ml. These algorithms are effective, but time-consuming and difficult to interpret for some clinicians. A number of real-time PCR assays based on TaqMan technology have been described. These assays were sensitive and detected HCV RNA over a broad range in most genotypes [5–14].

The COBAS® TaqMan HCV Test (TaqMan; Roche Diagnostics) is a real-time PCR assay for the detection and quantitation of HCV RNA in serum or plasma of HCV-infected individuals. Reverse transcription and PCR amplification and detection are performed in a sealed reaction tube, minimizing the risk of carry-over contamination. In real-time PCR, amplification products are detected as they are formed, allowing for detection over a broad range during a number of PCR cycles; the earlier a sample is detected, the higher the viral load. Calculation of the HCV RNA titre is based upon an external standard curve in the presence of an internal control. The TaqMan assay has been developed for use with the COBAS® TaqMan 48 Analyzer (Roche Diagnostics). The High Pure System (HPS; Roche Diagnostics) is a method for manual extraction of HCV RNA and is currently the recommended method for specimen preparation for the TaqMan assay. It is designed for parallel processing of 12 specimens and reduced handling time, facilitating high specimen throughput.

In the present study, we evaluated the TaqMan HPS using well-defined reference panels and clinical samples. We compared the TaqMan HPS with the bDNA, which is a robust assay for HCV RNA quantitation [15–20]. In addition, a comparison was made between extraction of HCV RNA using the HPS and the method described by Boom et al. (Boom extraction) [21].

**Materials and methods**

**Patient population and specimen collection**

A total of 142 blood specimens were collected from HCV-infected individuals monitored at the AMC Liver Centre, Department of Gastroenterology and Hepatology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands. Blood specimens were drawn as part of the usual follow-up and treatment monitoring, and approximately 25% of individuals were treatment-naive. EDTA-treated plasma samples were processed and frozen at $–70°C$ within 24 h of collection.

**Genotyping**

HCV genotypes of clinical samples were determined by direct sequencing using the TruGene® HCV genotyping assay and the OpenGene® automated DNA sequencing system (Bayer Diagnostics).

**Extraction and quantitation of HCV RNA**

Extraction and quantitation of HCV RNA were performed according to the manufacturer’s instructions.

**HPS and HCV quantitation standard**

The HPS is a method for manual extraction of viral RNA based on nucleic acid binding to glass fibres. The HCV virus particles are lysed by incubation with a protease and chaotropic lysis/binding buffer at an elevated temperature. The HCV RNA is released and protected from RNases in plasma and serum. Along with the lysis reagent, a known number of internal control (IC) RNA molecules (HCV Quantitation Standard RNA; Roche Diagnostics) are introduced into each specimen and are carried through the specimen preparation, reverse transcription, and PCR amplification and detection steps along with the HCV target. The IC is an armoured RNA construct that contains the HCV sequences, with identical primer binding sites as the HCV RNA target, together with a unique probe-binding region that allows the IC amplicon to be distinguished from the HCV target amplicon. After addition of isopropanol, the lysis mixture is centrifuged through a column with a glass fibre filter insert. During centrifugation, the HCV RNA and IC RNA are bound to the surface of the glass fibres. Unbound substances such as salts, proteins and other cellular remnants are removed by centrifugation. The absorbed nucleic acids are washed and eluted with an aqueous solution.

**TaqMan assay**

The TaqMan assay uses 500 μl of plasma or serum and has a reported linear dynamic range of $3.0 \times 10^3$ to $2.0 \times 10^9$ HCV RNA IU/ml, and a reported LLD of 10 IU/ml, according to the manufacturer. The TaqMan
technology is a real-time PCR platform based on simultaneous PCR amplification of target cDNA, using HCV-specific complementary primers, and detection of cleaved dual fluorescent dye-labelled oligonucleotide detection probes that permit quantitation of HCV target amplified product (amplicon) and detection of IC. The primers for reverse transcription and PCR amplification of the TaqMan assay define a sequence within the highly conserved 5′-untranslated region of the HCV genome. The whole process is performed in an automated Roche COBAS TaqMan 48 Analyzer (CTM 48; Roche Diagnostics), which automatically performs reverse transcription, PCR amplification of target cDNA, detection of cleaved dual fluorescent dye-labelled oligonucleotide probes that permit quantitation of HCV amplicon and IC, and data processing. The CTM 48 can process 48 specimens per run.

Quantitation
Calculation of the HCV RNA titre is based on an external standard curve. The IC compensates for effects of inhibition and controls for the preparation and amplification processes to allow accurate quantitation of HCV RNA in each specimen. The use of an IC enables the verification of the sensitivity of the assay and avoids false-negative results. Results are reported as ‘target not detected’ when the result is valid but below the detectable limit; ‘HCV RNA detected, less than 10 HCV RNA IU/ml’ when the result is valid but the titre is below the defined LLD of the assay; ‘number of detected HCV RNA IU/ml’ (for example, 3.45X10^6 IU/ml) when the result is valid and the titre is within the defined range of the assay (that is, between 1.0X10^6 and 2.0X10^6 HCV RNA IU/ml); and ‘greater than 2.0X10^6 HCV RNA IU/ml’ when the result is valid but the titre is above the defined range of the assay. Results are considered true-negative when no HCV RNA is detected and the IC is positive.

Test panels
The performance of the TaqMan HPS was assessed in six different panels: (i) a genotype 1 HCV RNA quantitation panel (containing 0, 2.5, 5, 10, 25, 35, 50, 100, 1000, 500 000 and 7 000 000 IU/ml, respectively), calibrated against the WHO International Standard for HCV RNA) for intra-assay variation (in triplicate), and inter-assay variation (in three runs). This panel was prepared by AcroMetrix for validation of the TaqMan HPS [Lot numbers AX02315N05 (negative control) and AX02319C01 to AX02319C11; AcroMetrix, Benicia, CA, USA]; (ii) 100 uninfected clinical samples for specificity; (iii) 59 clinical samples for linearity, genotype performance and quantitative correlation in IU/ml between the TaqMan HPS and the bDNA assay, according to the dynamic range and the thresholds of both assays; (iv) 58 clinical samples with HCV RNA detectable by TMA but undetectable by bDNA for sensitivity (HCV RNA > 15 IU/ml but ≤15 IU/ml); (v) 25–27 replicates of a six-member serial dilution panel (containing 5, 10, 15, 20, 33 and 50 IU/ml, respectively) derived from a single clinical HCV genotype 4 sample diluted in HCV-seronegative S/D plasma (ESDEP®; Sanquin, Amsterdam, The Netherlands) to assess the LLD of the TaqMan HPS.

Analytical performance of the TaqMan HPS
Reproducibility, specificity, genotype performance, quantitative correlation to the bDNA assay, linearity, sensitivity, LLD and extraction were assessed using the test panels and clinical samples. All assessments were performed by the same operator. For assessment of intra-assay variation, three replicates of all panel members of the Acrometrix panel were tested. All three replicates of each panel member were tested in the same run. For assessment of inter-assay variation, three replicates of all panel members of the Acrometrix panel were tested in three separate runs over a period of 3 days. All three replicates of each panel member were tested in different runs. Specificity was assessed using 100 clinical samples that were HCV antibody negative and HCV RNA negative (assessed by TMA). Genotype performance and quantitative correlation to the bDNA assay were assessed in 59 clinical samples of genotypes 1 to 5. All 59 samples contained levels of HCV RNA that were within the dynamic ranges of both assays. Linearity was assessed in serial 10-fold dilutions (using HCV-seronegative plasma) made of 10 high-titre clinical samples of genotypes 1, 2 and 3.

For assessment of sensitivity for low viral titre specimens we tested (i) 42 clinical samples with HCV RNA < 15 IU/ml but ≥15 IU/ml (bDNA negative, TMA positive); (ii) eight clinical samples with HCV RNA ≤0 IU/ml but > 15 IU/ml (TMA positive, PCR negative); (iii) eight clinical samples with HCV RNA < 15 IU/ml but ≥0 IU/ml (PCR positive, bDNA negative). For assessment of the LLD, 25–27 replicates of a dilution panel derived from a single clinical HCV genotype 4 sample (containing 5, 10, 15, 20, 33 and 50 IU/ml, respectively) were tested. In addition, for comparison of the HPS to the Boom extraction and quantitative correlation to the bDNA assay, HCV RNA was extracted and quantitated in 14 clinical samples of genotypes 1 to 4 using both extraction methods. Assessment of the upper limits of the dynamic range was not performed.

Statistical analysis
Variability between replicate tests and runs was described as the mean HCV RNA levels obtained with the TaqMan HPS, the standard deviation (SD) and the coefficient of variation (CV) by using the statistical
functions of SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Linear regression analysis was done in scatter plots for log-transformed HCV RNA levels using GraphPad Prism 4 (GraphPad Software, Inc, San Diego, CA, USA). The correlation coefficient, slope and intercept were obtained by least-squares linear regression analysis.

Results

Reproducibility of the TaqMan HPS
Reproducibility was evaluated with the AcroMetrix genotype 1 panel without retesting any panel member. The intra- and inter-run reproducibilities were assessed by testing each panel member in replicates of three. Both intra-assay (Figure 1A) and inter-assay (Figure 1B) variation in the AcroMetrix panel were small in panel members with HCV RNA levels of 100 IU/ml and above, with CVs for quantitation of 1–6%. At 50 HCV RNA IU/ml, intra- and interrun CVs for quantitation were 20%. In panel members with HCV RNA levels below 50 IU/ml, reproducibility decreased with CVs for quantitation of 4–102%.

Specificity of the TaqMan HPS
A total of 100 unique seronegative and TMA-negative serum and plasma samples were tested to assess the specificity relative to the lower limit of 10 HCV RNA IU/ml of the TaqMan HPS. No target was detected in 99 samples, whereas one sample had an invalid result, revealing a specificity of 99% (data not shown).

Genotype performance, and correlation between the TaqMan HPS and the bDNA assay
Correlation between the TaqMan HPS and the bDNA assay, and genotype performance were assessed using 59 clinical samples of genotype 1 (n=25), genotype 2 (n=9), genotype 3 (n=17), genotype 4 (n=6) and genotype 5 (n=2). All 59 clinical samples contained levels of HCV RNA that were within the dynamic ranges of both assays (Figure 2). In 24 out of 59 samples (41%), equivalent quantitation (that is, less than a 0.3-log-unit difference in quantitation values between methods) was found with both quantitative assays. Regression analysis per genotype showed a good correlation for the 25 genotype 1 samples in this panel (Table 1 and Figure 2; r^2, 0.9567; slope, 1.001; intercept, 0.1231). Regression analysis for genotype 2 (Table 1 and Figure 2; r^2, 0.8923; slope, 0.8579; intercept, 0.1489), genotype 3 (Table 1 and Figure 2; r^2, 0.8929; slope, 0.9681; intercept, −0.1174), and genotype 4 (Table 1 and Figure 2; r^2, 0.7748; slope, 0.9253; intercept, −0.3433) showed poor correlation. Regression analysis for genotype 5 samples was not performed due to the small number of samples. Differences in quantitation values of 0.3 log unit or more for the two methods were found in 35 out of the 59 clinical samples: 11 of the 25 genotype 1 samples, all nine genotype 2 samples, 10 out of the 17 genotype 3 samples, five out of the six genotype 4 samples, and none of the two genotype 5 samples (Table 1 and Figure 3A). Differences of more than 0.5 log unit in quantitation values for the two methods were found in 19 out of the 59 clinical samples: two of the 25 genotype 1 samples, six out of the nine genotype 2 samples, seven out of the 17 genotype 3 samples, four out of the six genotype 4, and none of the two genotype 5 samples (Table 1 and Figure 3A). For genotype 1 it appeared...
that values obtained from the TaqMan HPS were in
general 0.2 log higher than those from the bDNA
assay (Figure 3B). However, for genotypes 2, 3 and 4,
it appeared that values obtained from the TaqMan
HPS were in general 0.5 log lower than those from the
bDNA assay (Figure 3B).

**Linearity of the TaqMan HPS**
Linearity was determined in dilution series of 10 clin-
cal samples of genotypes 1, 2 and 3, in a range of
3.0 \( \times 10^1 \) to 1.23 \( \times 10^7 \) IU/ml. Linear regression analysis
showed a good correlation for the dilution series of
clinical genotype 1 samples \( (n=6; r^2, 0.9940; \text{slope},
0.9520; \text{intercept}, 0.2007; \text{data not shown}), \text{and clini-
cal genotype 2 and 3 samples} \ (n=4; r^2, 0.9932; \text{slope},
0.9340; \text{intercept}, 0.3728; \text{data not shown}).

**Sensitivity of the TaqMan HPS**
The sensitivity of the TaqMan HPS was assessed by
analysis of 58 clinical samples of genotypes 1, 2, 3
and 4 with HCV RNA \(<15 \text{ IU/ml but } \geq 5 \text{ IU/ml}. In
13 samples, HCV RNA was not detected by the
TaqMan HPS, in 14 samples HCV RNA was detected
but lower than 10 IU/ml, and eight samples had HCV
RNA \( \geq 15 \text{ IU/ml by the TaqMan HPS (Table 2).}

**LLD of the TaqMan HPS**
The LLD was assessed using 25–27 replicates of a
dilution panel derived from a single clinical HCV
genotype 4 sample (containing 5, 10, 15, 20, 33 and
50 IU/ml). HCV RNA levels of 50 IU/ml, 33 IU/ml
and 15 IU/ml were detected with a sensitivity of
100%, 88% and 50%, respectively. The LLD (95%)
of HCV RNA was estimated at 41 IU/ml. At the
reported LLD of 10 IU/ml (according to Roche), no
target was detected in 19 out of 25 replicates. In the
majority of positive samples, viral titres were under-
estimated and reported as HCV RNA below 10 IU/ml
by the TaqMan HPS (Table 3).

**Extraction of HCV RNA**
In addition, for comparison of the HPS with the
Boom extraction, and quantitative correlation to the
bDNA assay, HCV RNA was extracted and
quantitated in 14 clinical samples of genotypes 1 to 4
using both extraction methods and the TaqMan
assay. Results obtained by both extraction methods
and the TaqMan assay were compared with results
obtained by the bDNA assay. The differences in
quantitation between the TaqMan assay and bDNA
assay were smaller in 11 out of 14 clinical samples
when the Boom extraction method was used. Quantitation differences in 10 out of 14 samples were
within 0.5 log of the bDNA assay with Boom extrac-
tion whereas quantitation differences in 6 out of 14
samples were within 0.5 log of the bDNA assay with
the HPS (Figure 4).

**Table 1. Correlation coefficients and differences in quantitation for HCV RNA obtained by the TaqMan HPS and bDNA assays per genotype**

<table>
<thead>
<tr>
<th>Methods compared</th>
<th>Total</th>
<th>Genotype 1</th>
<th>Genotype 2</th>
<th>Genotype 3</th>
<th>Genotype 4</th>
<th>Genotype 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan HPS versus bDNA</td>
<td>59</td>
<td>25</td>
<td>9</td>
<td>17</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>( r^2 ) value</td>
<td>0.8758*</td>
<td>0.9567*</td>
<td>0.8923*</td>
<td>0.8929*</td>
<td>0.7748*</td>
<td></td>
</tr>
<tr>
<td>Difference in quantitation &gt;0.3 log</td>
<td>35/59</td>
<td>11/25</td>
<td>9/9</td>
<td>10/17</td>
<td>5/6</td>
<td>0/2</td>
</tr>
<tr>
<td>Difference in quantitation &gt;0.5 log</td>
<td>19/59</td>
<td>2/25</td>
<td>6/9</td>
<td>7/17</td>
<td>4/6</td>
<td>0/2</td>
</tr>
</tbody>
</table>

TaqMan HPS, COBAS® TaqMan HCV Test, For Use With The High Pure System (Roche Diagnostics); bDNA, VERSANT® HCV 3.0 assay (Bayer Diagnostics); *\( P<0.0001; \)
†\( P=0.0001; \) ‡\( P<0.05. \)
Discussion

In the present study, we evaluated the TaqMan HPS for the detection and quantitation of HCV RNA in plasma and serum of HCV-infected individuals. The performance characteristics were established using well-standardized panels and clinical samples. At HCV RNA levels of 100 IU/ml and higher, reproducibility was good with CVs of less than 10% for intra- and inter-assay run variability. From 10 to 50 HCV RNA IU/ml, reproducibility was poor with CVs of 20% at 50 IU/ml for both intra-assay and inter-assay run variability. Excellent specificity (≥99%) relative to the lower detection limit of 10 IU/ml was found.

In only a minority of clinical samples nearly equivalent quantitation (less than a 0.3-log-unit difference) was found with both assays for all HCV genotypes. Only for genotype 1 did quantitation appear reliable.

In clinical samples with genotypes 2, 3 and 4, quantitation differences were inconsistent and large in comparison with the bDNA assay. To our knowledge this is the first study in which a substantial number of non-1 genotypes were analysed using the TaqMan HPS assay. In other studies where the TaqMan but not the HPS, was evaluated, the number of non-1 genotype specimens was either too low to determine bias in quantitation [5,9], no genotype bias was observed [6,10] or detailed analysis was not performed [7].

To examine whether the HPS extraction of HCV RNA influenced the poor performance of the TaqMan assay in non-1 HCV genotypes, we compared it with the Boom extraction [21] in 14 clinical samples. Comparison with the Boom extraction showed that extraction of HCV RNA by the HPS was inferior, but even when a better method for extraction of viral RNA was used, the differences in quantitation between the TaqMan and bDNA remained (Figure 4). Apparently, other components in the TaqMan assay are also involved in its poor performance.

The HPS is based on specific binding of nucleic acids to glass fibres, purification by washing and centrifugation and subsequent elution. If binding of HCV RNA to the surface of the glass fibres is compromised in any way, part of the HCV RNA present in the specimen will be lost. The Boom extraction method is based on specific binding of nucleic acids to silica particles, and the lysing and nuclease-inactivating properties of guanidine thio-cyanate (GuSCN). Nucleic acid–silica complexes are sedimented by centrifugation and washed five times, dried and then the nucleic acids are eluted and ‘released’ into the supernatant during a final centrifugation step. The main differences between the HPS and the Boom extraction are (i) the nucleic acids have to ‘hang on’ to the surface of the glass fibres in a column during centrifugation steps in the HPS extraction, whereas they are spun down in the pellet during centrifugation steps in the Boom extraction, and (ii) the first two washing steps in the Boom extraction are performed in the presence of GuSCN, optimizing binding of nucleic acids to the silica particles. The genotype difference may be caused by components (for example, buffer solutions) of the HPS system that might influence the unfolding of the HCV RNA. Although certain parts of the 5’UTR are highly conserved among genotypes [22], other parts of the 5’UTR and flanking regions are not. The tertiary and quaternary structures of the HCV RNA may differ among genotypes, and sequence differences outside the 5’UTR may influence its folding and unfolding. Incomplete unfolding may (i) compromise binding of HCV RNA to glass fibres and cause loss of HCV RNA during the HPS extraction, and/or (ii) reduce binding of
Chapter 2

Table 2. Analytical sensitivity of the TaqMan HPS in clinical samples with HCV RNA >5 IU/ml but <615 IU/ml

<table>
<thead>
<tr>
<th>HCV RNA concentration, IU/ml</th>
<th>Number of samples tested</th>
<th>Number of HCV RNA detected</th>
<th>HCV RNA detected, &lt;10 HCV RNA IU/ml</th>
<th>HCV RNA detected, &gt;615 IU/ml</th>
<th>Target not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;615 but &gt;5</td>
<td>42</td>
<td>33/42</td>
<td>9/42</td>
<td>8/42</td>
<td>9/42</td>
</tr>
<tr>
<td>Genotype 1: n=6</td>
<td>Genotype 1: n=5</td>
<td>Genotype 3: n=3</td>
<td>Genotype 3: n=3</td>
<td>Genotype 4: n=1</td>
<td></td>
</tr>
<tr>
<td>&lt;615 but &gt;50</td>
<td>8</td>
<td>8/8</td>
<td>1/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>Genotype 3: n=1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 but &gt;5</td>
<td>8</td>
<td>4/8</td>
<td>4/8</td>
<td>4*/8</td>
<td></td>
</tr>
<tr>
<td>Genotype 1: n=3</td>
<td>Genotype 3: n=1</td>
<td>Genotype 3: n=2</td>
<td>Genotype 4: n=1</td>
<td>Genotype 4: n=1</td>
<td></td>
</tr>
</tbody>
</table>

*In one sample the genotype could not be determined.

Table 3. Analytical sensitivity of the TaqMan HPS in a dilution panel prepared from a single HCV genotype 4 clinical sample

<table>
<thead>
<tr>
<th>HCV RNA concentration, IU/ml</th>
<th>Number of replicates tested</th>
<th>Number of HCV RNA detected</th>
<th>% positive</th>
<th>HCV RNA detected, &lt;10 HCV RNA IU/ml (range in IU/ml)</th>
<th>HCV RNA detected, &gt;10 IU/ml</th>
<th>Target not detected</th>
<th>Invalid results</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>25</td>
<td>3</td>
<td>12</td>
<td>2</td>
<td>1 (15)</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>6</td>
<td>24</td>
<td>6</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>13</td>
<td>52</td>
<td>11</td>
<td>2 (20–91)</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>27</td>
<td>19</td>
<td>70</td>
<td>15</td>
<td>4 (11–15)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>33</td>
<td>25</td>
<td>22</td>
<td>88</td>
<td>17</td>
<td>5 (10–24)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>17</td>
<td>8 (11–75)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

the primers and probe, both resulting in underestimation of HCV RNA in the specimen.

Poor genotype performance is an old problem in HCV RNA quantitation [23]. We found poor correlation between the TaqMan and bDNA using two different extraction methods. Although, according to Roche, the TaqMan assay is equipped with similar primers and probes as the COBAS® Amplicor HCV Monitor Test, v2.0 (Roche Diagnostics), which has excellent genotype performance [23], the poor correlation between the TaqMan and bDNA using two different extraction methods suggests that reaction mixtures, primer and/or probe design of the TaqMan are suboptimal for non-1 genotypes.

Overall, the TaqMan HPS displayed good linearity within the dynamic range of the dilution series of clinical samples of genotype 1, because the slope was close to 1 and the intercept approached zero (with a 95% confidence interval). For genotypes 2 and 3 the slope was close to 1 but the intercept was 0.3728.

In our study, out of 58 clinical samples with viral titers below the lower limit of the bDNA assay (<15 IU/ml) but detectable by TMA (≥10 IU/ml), 13 samples were not detected and two samples were clearly underestimated by the TaqMan HPS, both genotype 1 and genotype non-1. Among 42 samples with an expected HCV RNA load <15 but >10 IU/ml that were quantified by the TaqMan, eight samples were detected but too low to quantify, and eight samples were not detected. Surprisingly, eight samples had >15 HCV RNA IU/ml according to the TaqMan HPS. Out of eight samples with an expected HCV RNA load <10 but ≥5 IU/ml were detected by the TaqMan assay; in one sample HCV RNA was detected but reported as <10 IU/ml. In both detected and undetected samples, genotypes 1 to 4 were distributed equally.

We detected HCV genotype 4 with a sensitivity of 100% at 50 IU/ml. But even then, 17 out of the 25 results (68%) were reported as: ‘HCV RNA detected, less than 10 IU/ml’, meaning that the result was valid but the titre was below the LLD of the assay defined by
Roche at 10 HCV RNA IU/ml. At 41 HCV RNA IU/ml, 95% detection of HCV genotype 4 was estimated. In a Roche evaluation, detection of HCV RNA with a sensitivity of 100% was at 9.6 IU/ml for genotype 1, at 17.0 IU/ml for genotype 2, at 97.3 IU/ml for genotype 3, at 282.6 IU/ml for genotype 4, at 226.4 IU/ml for genotype 5 and at 15.9 IU/ml for genotype 6 (Roche, personal communication, data not shown). In another recent evaluation of the TaqMan assay, the LLD was higher than 10 IU/ml for genotype 2 (between 25 and 50 IU/ml) and genotype 3 (25 IU/ml) [9]. Considering the LLD at 41 IU/ml for genotype 4, the poor reproducibility below 100 HCV RNA IU/ml for the Acrometrix genotype 1 dilution panel, and the poor sensitivity in samples of genotypes 1, 2, 3 and 4 with low viral titres, the TaqMan assay should be regarded as qualitative below 100 IU/ml for genotype 1 and possibly other genotypes.

The way that results for low viral titres are reported by the TaqMan adds to the confusion. When HCV RNA is detected at levels below 10 HCV RNA IU/ml (the 95% LLD of the assay defined by Roche) they are reported as: ‘HCV RNA detected, less than lower limit of quantitation’; (ii) a defined lower limit of quantitation (to be determined, probably between 50–100 IU/ml for HCV genotype 1) to be reported as ‘number of detected HCV RNA IU/ml’ (for example, 3.45X10^0 IU/ml). The main difference between this formulation and the way Roche presents the results, is the distinction between a LLD (qualitative) and a lower limit of quantitation.

In summary, this study describes the performance of the new Roche COBAS® TaqMan HCV Test For Use With The High Pure System for the extraction, detection and quantitation of HCV RNA in plasma and serum. The assay uses a 500-μl sample volume and has a run size of up to 48 specimens. The TaqMan HPS is reproducible, fast and specific. The comparison of the TaqMan HPS with the bDNA assay showed a poor correlation within the dynamic ranges of both assays. Detailed analysis revealed a quantitation difference depending on the HCV genotype. Compared with the bDNA assay, the TaqMan HPS underestimates HCV RNA at all levels in plasma and serum from individuals infected with HCV genotypes 2, 3 and 4. Furthermore, the LLD seems higher than previously reported; in the Acrometrix genotype 1 panel, a titre of 10 IU/ml was detected in five out of six replicates, but in three of these five replicates the titre was reported as below the reported LLD of the assay (10 IU/ml, according to Roche); consistent detection of HCV RNA was achieved at levels above 4.1X10^0 IU/ml for genotype 4; these observations imply that the lower limit of the TaqMan HPS should be reconsidered. The nature of the genotype difference in quantification is unclear. Use of the Boom extraction compared with HPS to extract HCV RNA reduces the difference, but does not resolve it. Primer design and composition of reaction mixtures may be of influence. If treatment decisions are based on quantitation of HCV RNA, accurate quantitation, irrespective of genotype, is mandatory. The underestimation of HCV RNA levels using the TaqMan HPS is clinically relevant as it may result in failure to identify non-responders early during therapy. Failure to qualitatively detect low levels of HCV RNA may also lead to erroneous conclusions regarding treatment outcome and may lead to falsely assigning patients infected with genotype 2 and 3 to shorter 12-week treatment regimens when they test negative for HCV RNA at week 4 [24]. If the TaqMan HPS was also used to assess HCV RNA at start of therapy, following the NIH recommendation to use the same assay for patient monitoring during treatment, the recommended stopping rule (a ≥ log decrease in HCV RNA after 12 to 24 weeks of antiviral therapy) could still be applied [1,25]. However, we would recommend using another assay for HCV RNA quantitation. A decision to stop or...
continue antiviral treatment must be based on reliable quantitation and detection of HCV RNA. At present, the TaqMan HPS is unable to provide this.

While the data were being analysed, Roche Diagnostics warned the customers to use the TaqMan HPS only for quantitation of HCV RNA in individuals infected with genotypes 1 and 6. The German Paul-Ehrlich-Institut prohibited use of the TaqMan HPS for screening and manufacturing of blood products in August 2004 [26].

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