Hepatitis C virus (HCV) is the principal cause of death from liver disease and the leading indication for transplantation in the United States (4). Diagnosis, management of antiviral therapy, and determination of virological response to treatment are largely based on the detection and quantification of HCV RNA. Baseline HCV RNA levels and continued monitoring of the viral load have allowed physicians to predict and tailor therapy and is the best predictor of long-term response (6). If the target is present, the net fluorescence will increase. If the target is absent, the net fluorescence will decrease. The presence of the product will result in quenching of fluorescence (6). The iso-C is located at the 5′ end of the primer adjacent to a reporter fluorophore. As the amplicon elongates, the iso-G with a covalently attached quencher molecule is incorporated on the 3′ end of the product. Once these bases are integrated adjacent to one another during the amplification cycle, the presence of the product will result in quenching of fluorescence (6). The iso-C is located at the 5′ end of the primer adjacent to a reporter fluorophore. As the amplicon elongates, the iso-G with a covalently attached quencher molecule is incorporated on the 3′ end of the product. Once these bases are integrated adjacent to one another during the amplification cycle, the presence of the product will result in quenching of fluorescence (6). The iso-C is located at the 5′ end of the primer adjacent to a reporter fluorophore. As the amplicon elongates, the iso-G with a covalently attached quencher molecule is incorporated on the 3′ end of the product. Once these bases are integrated adjacent to one another during the amplification cycle, the presence of the product will result in quenching of fluorescence (6). The iso-C is located at the 5′ end of the primer adjacent to a reporter fluorophore. 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genotype information was available for 140 specimens; the remaining 30 specimens were stock material was diluted with normal human plasma to a concentration of 7.0 log10 IU/ml. The panel included seven replicates of each concentration. The infected individual (genotype 1b) following an institutional review board-approved protocol. The expected concentration of the HCV RNA in the plasma sample was determined using the COBAS Amplicor HCV Monitor (HCV Monitor; Roche Diagnostics Corporation, Indianapolis, IN).

Viral quality assurance panels were produced by the Division of AIDS Viral Quality Assurance Laboratory (DAIDS VQA Laboratory) at Rush University. The clinical specimens were submitted to Emory University Hospital Molecular Diagnostics Laboratory for routine quantitative HCV load testing by using the TaqMan test. Remaining plasma was stored at −70°C until testing with the MultiCode assay. This study was approved by the institutional review board. A total of 170 positive specimens were selected to represent a range of HCV loads and genotypes as follows: 73 HCV-positive specimens, with viral loads of >1 million IU/ml; 67 HCV-positive specimens, with viral load ranges of >10,000 IU/ml and <1 million IU/ml; 30 HCV-positive specimens, with viral loads of <10,000 IU/ml; and 32 HCV-negative specimens. Genotype information was available for 140 specimens; the remaining 30 specimens had a viral load of <10,000 and could not be genotyped. The genotype composition was as follows: genotype 1, 76 samples; genotype 2, 37 samples; genotype 3, 22 samples; and genotype 4, five samples. HCV genotyping was performed using the TrueGene HCV 5′ NC genotyping system (TrueGene 5′ NC; Siemens Healthcare Diagnostics, Inc., Tarrytown, NY). An HCV genotype panel was purchased from SeraCare Life Sciences (Milford, MA) and contained nine members (1a, 1b, 2a, 2b, 3a, 4a, 4b, 5a, 6a) and one negative panel member with expected concentrations determined by COBAS Amplicor HCV Monitor (HCV Monitor; Roche Diagnostics Corporation, Indianapolis, IN).

Viral quality assurance panels were produced by the Division of AIDS Viral Quality Assurance Laboratory (DAIDS VQA Laboratory) at Rush University Medical Center, Chicago, IL. A plasma sample was obtained from an HCV-infected individual (genotype 1b) following an institutional review board-approved protocol. The expected concentration of the HCV RNA in the plasma sample was determined using the COBAS Amplicor HCV Monitor v2.0 test. The stock material was diluted with normal human plasma to a concentration of 7.0 to 1.0 log10 IU/ml. The panel included seven replicates of each concentration. Aliquots were stored at −70°C prior to testing.

Viral load assays. The MultiCode and the TaqMan tests were performed following the manufacturers’ recommendations with the exception of the nucleic acid extraction procedure. For both assays, the MagNA Pure extraction instrument was used with the MagNA Pure LC total nucleic acid isolation kit. A total of 200 μl of specimen or standard was extracted and eluted into 65 μl with 50 μl to be used in the final reaction. For the limit-of-detection studies, 500 μl of specimen was used and eluted into 65 μl with 50 μl in the final reaction. The MultiCode test includes an RNA sample processing reference (SPR) that is extracted with the specimens and standards (0.325 μl SPR per specimen). The assay was run on an ABI 7500 with a standard block, and the MultiCode test analysis software (provided by the manufacturer) was used to determine HCV load.

A subset of samples were extracted using the NucliSens easyMAG following the manufacturers’ recommendations; 200 μl of specimen was extracted and eluted into 65 μl with 50 μl to be used in the final reaction.

Study design. Linearity and reproducibility were determined with HCV OptiQuant RNA panels (AeroMetrix, Benicia, CA); 10 replicates each of seven panel members (0; 50; 500; 5,000; 50,000; 500,000; 5,000,000 IU/ml) were run in duplicate across five runs. Each run contained a high and low positive control and a negative control. Limits of detection were determined with 44 replicates of a five-member panel (10; 25; 50; 100; 200 IU/ml) which were diluted from OptiQuant RNA and run in quadruplicate across 11 runs. Agreement was determined by testing the 170 clinical specimens as described above.

Statistical analysis. HCV load values were log10 transformed for analysis. Descriptive statistics and regression line equations were calculated with the analysis tool pack of Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). Agreement between viral loads was assessed by the method of Bland and Altman (2).

RESULTS

Performance characteristics of the MultiCode HCV test. The limit of detection of the MultiCode assay was determined by testing multiple replicates of samples ranging in concentration from 1.0 to 2.3 log10 IU/ml (Table 1). Based on detection of 95% of replicates, the limit of detection was 2.3 log10 IU/ml (200 IU/ml). The linear range was assessed by testing aliquots of HCV RNA ranging in concentration from 1.7 to 6.7 log10 IU/ml. Data shown in Fig. 1 are a mean (± standard deviation [SD]) for samples tested in duplicate in five separate runs. The assay was linear from 1.7 to 6.7 log10 IU/ml. The reproducibility varied through the linear range of the assay (0.12 to 0.43 log10 IU/ml), with the greatest precision at higher viral loads and lowest precision at low viral loads of 1.7 log10 IU/ml (Table 2).

Genotype bias. To determine whether genotype-specific quantification bias occurs in the MultiCode test, a total of 140

<table>
<thead>
<tr>
<th>Nominal conc (log10 IU/ml)</th>
<th>No. of samples tested</th>
<th>Mean viral load (log10 IU/ml)</th>
<th>SD (log10 IU/ml)</th>
<th>% CVa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.70b</td>
<td>10</td>
<td>1.69</td>
<td>0.43</td>
<td>25.4</td>
</tr>
<tr>
<td>2.70</td>
<td>12</td>
<td>2.63</td>
<td>0.19</td>
<td>7.2</td>
</tr>
<tr>
<td>3.70</td>
<td>12</td>
<td>3.57</td>
<td>0.16</td>
<td>4.5</td>
</tr>
<tr>
<td>4.70</td>
<td>12</td>
<td>4.59</td>
<td>0.21</td>
<td>4.6</td>
</tr>
<tr>
<td>5.70</td>
<td>12</td>
<td>5.62</td>
<td>0.12</td>
<td>2.1</td>
</tr>
<tr>
<td>6.70</td>
<td>12</td>
<td>6.63</td>
<td>0.15</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a CV, coefficient of variation. b 1.70, mean viral load based on positive specimens only.

FIG. 1. Linear range of the MultiCode test using plasma samples. Data are means (±SD) for samples tested in duplicate in five separate runs (n = 10).

TABLE 1. Limit of detection of the MultiCode test using plasma samples

<table>
<thead>
<tr>
<th>Log10 IU/ml</th>
<th>No. of positive samples/ no. tested</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>28/44</td>
<td>64</td>
</tr>
<tr>
<td>1.4</td>
<td>32/44</td>
<td>73</td>
</tr>
<tr>
<td>1.7</td>
<td>39/44</td>
<td>89</td>
</tr>
<tr>
<td>2.0</td>
<td>40/44</td>
<td>91</td>
</tr>
<tr>
<td>2.3</td>
<td>42/44</td>
<td>95</td>
</tr>
</tbody>
</table>

a Limit of detection (95% of replicates) is 2.3 log10 IU/ml (200 IU/ml).

TABLE 2. Reproducibility of the MultiCode test using plasma samples
clinical specimens were tested, and the results were compared to concentrations determined by the TaqMan test. Mean viral loads (±SD) for each genotype are shown in Table 3. There did not appear to be any genotype bias (genotypes 1, 2, and 3) for the MultiCode test compared to the TaqMan test. There were too few genotype 4 specimens (total of five) to draw any conclusions; however, it is interesting that four of five specimens had higher values with the MultiCode test than with the TaqMan test. The results of the HCV RNA genotype panel are shown in Table 4. The viral load values obtained with the MultiCode test were consistently lower than the expected concentrations which were determined by the Monitor HCV test, with differences ranging from 0.16 to 0.70 log₁₀ IU/ml.

Agreement between the MultiCode test and the TaqMan assay. A total of 170 clinical specimens that had detectable viral loads by the TaqMan test were run with the MultiCode test (Fig. 2). For these samples, the population mean (±SD) was 5.4 log₁₀ IU/ml (1.4 log₁₀ IU/ml) for the MultiCode test and 5.5 log₁₀ IU/ml (1.3 log₁₀ IU/ml) for the TaqMan assay. Based on the agreement analysis, the mean difference between the two tests was −0.10 log₁₀ IU/ml (95% limits of agreement, −0.76 to 0.56 log₁₀ IU/ml) (Fig. 2).

Viral quality assurance panel. The 77-member panel obtained from the VQA laboratory had specimens ranging in concentration from 0 to 7.0 log₁₀ IU/ml. One of seven negative specimens tested positive (Table 5). The mean values obtained with the MultiCode test were in good agreement with the expected concentrations, with mean differences ranging from 0.11 to 1.14 log₁₀ IU/ml. Interestingly, all seven replicates of the 2.0 log₁₀ IU/ml sample were detected.

Comparison of extraction methods. To evaluate the effect of different extraction methods on viral loads, plasma specimens were tested by the MagNA Pure and easyMAG extraction methods. Twenty-one positive specimens were extracted. For all 21 specimens, the viral load attained with the easyMAG was higher than that observed for the MagNA Pure. The differences ranged from 0.11 to 1.14 log₁₀ IU/ml, with a mean difference of 0.45 log₁₀ IU/ml (data not shown).

TABLE 3. Mean viral load and standard deviation for MultiCode and TaqMan tests by genotype

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean viral load (SD) (log₁₀ IU/ml)</th>
<th>Mean difference (MultiCode - TaqMan) (log₁₀ IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MultiCode</td>
<td>TaqMan</td>
</tr>
<tr>
<td>All</td>
<td>5.40 (1.40)</td>
<td>5.50 (1.34)</td>
</tr>
<tr>
<td>Genotype 1 samples</td>
<td>5.88 (0.71)</td>
<td>5.98 (0.70)</td>
</tr>
<tr>
<td>Genotype 2 samples</td>
<td>5.98 (1.00)</td>
<td>6.10 (1.02)</td>
</tr>
<tr>
<td>Genotype 3 samples</td>
<td>6.05 (0.77)</td>
<td>6.10 (0.70)</td>
</tr>
<tr>
<td>Genotype 4 samples</td>
<td>5.96 (0.49)</td>
<td>5.35 (0.53)</td>
</tr>
</tbody>
</table>

TABLE 4. HCV RNA genotype performance panel for the MultiCode test

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected concn (log₁₀ IU/ml)</th>
<th>MultiCode viral load (log₁₀ IU/ml)</th>
<th>Difference (log₁₀ IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>4.05</td>
<td>3.81</td>
<td>-0.24</td>
</tr>
<tr>
<td>1b</td>
<td>4.11</td>
<td>3.83</td>
<td>-0.28</td>
</tr>
<tr>
<td>2b</td>
<td>4.11</td>
<td>3.41</td>
<td>-0.70</td>
</tr>
<tr>
<td>3a</td>
<td>4.48</td>
<td>4.32</td>
<td>-0.16</td>
</tr>
<tr>
<td>4a</td>
<td>4.60</td>
<td>4.36</td>
<td>-0.24</td>
</tr>
<tr>
<td>4a</td>
<td>4.76</td>
<td>4.48</td>
<td>-0.28</td>
</tr>
<tr>
<td>5a</td>
<td>4.08</td>
<td>3.76</td>
<td>-0.32</td>
</tr>
<tr>
<td>6a</td>
<td>4.20</td>
<td>4.04</td>
<td>-0.16</td>
</tr>
</tbody>
</table>

a Genotype determined by Bayer TruGene HCV NS5b test.
b Determined by COBAS Amplicor HCV Monitor test.

DISCUSSION

HCV RNA testing has allowed for the capability to not only diagnose HCV infection, but also guide management of antiviral therapy and determine treatment response. Current commercially available assays target the 5′ UTR of the HCV genome; however, the MultiCode test was developed for the detection and quantification of HCV RNA by targeting the similarly conserved 3′ UTR. In this study, we evaluated the performance characteristics of the MultiCode test compared to those of the TaqMan test. Furthermore, we assessed the impact of different extraction methods on viral loads obtained by the MultiCode test.

TABLE 5. Data for viral quality assurance panel

<table>
<thead>
<tr>
<th>Nominal concn (log₁₀ IU/ml)</th>
<th>No. of replicates</th>
<th>Mean observed concn (log₁₀ IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>2.0</td>
<td>7</td>
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<td>7.3</td>
</tr>
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</table>

* — one of seven replicates was a false positive.

b One of the seven replicates (7.9 log₁₀ IU/ml) was removed from the data set; if this sample was included, the mean concentration is 6.3 log₁₀ IU/ml, with an SD of 0.7 log₁₀ IU/ml.
The limit of detection of the MultiCode assay was 2.3 log10 IU/ml (200 IU/ml) which is on average less sensitive than both the TaqMan test (limit of detection between approximately 10 and 20 IU/ml depending on the genotype [9]) and the Abbott real-time PCR assay (limit of detection of 10 IU/ml; Abbott Laboratories, Abbott Park, IL) [3]. This decrease in sensitivity may limit the utility of the MultiCode test for use in identifying patients in SVR or RVR. The MultiCode test demonstrates an acceptable linear range from 1.7 log10 IU/ml to at least 6.7 log10 IU/ml; however, we were unable to obtain specimens with adequate viral loads to establish the upper end of the linear range. In addition, the reproducibility is similar to that observed with other real-time PCR tests (1, 3), with greatest variability observed near the limit of detection. There is remarkably close agreement between the MultiCode and TaqMan tests considering the TaqMan test targets the 5’ UTR and the MultiCode targets the 3’ UTR. Based on these data, it appears that the MultiCode test has clinical utility as a therapeutic monitoring tool for HCV.

In genotype bias experiments, no difference in quantification was observed for genotypes 1, 2, and 3 with the MultiCode test; mean viral load values were within 0.12 log10 IU/ml of those obtained with the TaqMan assay. In contrast, it is noteworthy that four of five genotype 4 specimens had higher viral load values with the MultiCode test than with the TaqMan test. Due to the low sample number, it is difficult to draw any conclusions, but these data may preliminarily suggest that the TaqMan test may underquantify some genotype 4 specimens relative to those of genotypes 1 to 3. Similar results were seen in another study of the TaqMan test (3). When testing the SeraCare genotype panel, the viral load obtained with the MultiCode test for the genotype 2b sample was 0.7 log10 IU/ml lower than that obtained with the COBAS Amplicor HCV Monitor test. It is unlikely that this reflects a problem with underquantification of genotype 2b, as the viral load values obtained with the MultiCode and TaqMan tests were within 0.12 log10 IU/ml for the 37 samples tested.

The easyMAG and MagNA Pure tests are acceptable extraction methods for the MultiCode test; however, the easyMAG more efficiently extracts RNA from plasma samples compared to the MagNA Pure. Further evaluation is needed to determine if extraction with the easyMAG would improve the sensitivity of the MultiCode test. The extraction data were not differentiated for genotype.

Although there are no real-time PCR royalty fees associated with the use of the MultiCode test, as it targets the 3’ UTR, the assay is not fully automated and requires hands-on time for master mix preparation and assay setup. When comparing the costs of the two tests, it is important to note that the extraction reagents are not included in the MultiCode test kit. In summary, the MultiCode test is less sensitive than the TaqMan test but, overall, shows acceptable performance characteristics for use in monitoring HCV antiviral drug responses. Further studies are needed to assess the performance with regard to genotypes 4 to 6.

ACKNOWLEDGMENTS

We thank Nell Lurain and the VQA laboratory for generously providing the panels, EraGen Biosciences for providing reagents, and Nabila Sharifi from the Emory Molecular Diagnostics Laboratory for her assistance in this project. This work was supported in part by the Emory Center for AIDS Research (F30 AI050409).

REFERENCES