JOURNAL OF CLINICAL MICROBIOLOGY, May 2010, p. 1771–1774 Vol. 48, No. 5
0095-1137/10/$12.00 doi:10.1128/JCM.00142-10

Performance Characteristics of the MultiCode-RTx Hepatitis C Virus Load Test Targeting the 3′ Untranslated Region▽

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Received 22 January 2010/Returned for modification 5 March 2010/Accepted 13 March 2010

Viral load testing for hepatitis C virus (HCV) RNA has become a key parameter in the diagnosis of infection and treatment monitoring. This study evaluated the performance characteristics of the MultiCode-RTx HCV assay (MultiCode; EraGen Biosciences, Inc., Madison, WI), a real-time PCR test targeting the 3′ untranslated region (UTR) of the HCV genome, compared to the TaqMan HCV load ASR assay (TaqMan; Roche Diagnostics, Indianapolis, IN) that targets the 5′ UTR. For plasma specimens, the MultiCode assay had a limit of detection of 2.3 $\log_{10}$ IU/ml and a linear range of at least 6.7 $\log_{10}$ IU/ml. Comparison of plasma viral loads obtained by the MultiCode and TaqMan tests showed that they were in very close agreement (mean difference, −0.1 $\log_{10}$ IU/ml). No genotype bias was observed for genotypes 1, 2, and 3. When the MultiCode assay was evaluated with the MagNA Pure and easyMAG extraction methods, the viral loads for the easyMAG extraction were consistently higher for all specimens tested (mean difference, 0.45 $\log_{10}$ IU/ml). Aside from the limit of detection, the performance characteristics of the MultiCode assay were similar to the TaqMan assay for the clinical application of HCV load testing.

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 response to treatment with the current recommendation of combined pegylated interferon alfa and ribavirin (4). Viral load testing, however, does not predict the progression of HCV and is not associated with the severity of liver disease. Because the disease course is chronic and evolving over decades, treatment responses are defined by surrogate virological markers rather than clinical endpoints. The most important of these virological markers is the sustained virological response (SVR), which is defined as the absence of HCV RNA from serum by a sensitive PCR assay 24 weeks following the discontinuation of therapy and is the best predictor of long-term response to treatment (4, 8). A rapid virological response (RVR) in patients, characterized by undetectable HCV RNA by week 4 of treatment, predicts an increased likelihood of achieving SVR (4, 5). In contrast, those who fail to attain an early virological response (EVR), defined as a $\geq$2 $\log_{10}$ reduction or complete absence of HCV RNA at week 12 of therapy compared to baseline level, are unlikely to achieve SVR (4). Consequently, the ability to assess these virological markers has made quantitative viral load assays with sensitivities of 10 to 50 IU/ml essential for therapeutic monitoring (4).

A variety of tests are available for the detection and quantification of HCV RNA based on different nucleic acid amplification technologies. The current commercially available methods for the detection and quantification of HCV RNA viral load include transcription-mediated amplification, branched DNA, and real-time PCR assays, all of which target the highly conserved HCV 5′ untranslated region (UTR) (6, 7). However, a unique real-time PCR assay based on MultiCode-RTx HCV technology (MultiCode; EraGen Biosciences, Inc., Madison, WI) was developed for the detection and quantification of HCV RNA by targeting the similarly conserved 3′ UTR of the HCV genome (6).

The MultiCode assay is based on an expanded genetic library consisting of iso-C and iso-G nucleotide bases, which pair only with each other and are incorporated during amplification (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). If the target is present, the net fluorescence will decrease with each cycle. The greater the amount of target in the sample, the earlier this decrease is observed. The presence of HCV RNA is confirmed by melt curve analysis. The assay also contains an extractable internal control that has the ability to monitor isolation of nucleic acid, verify real-time PCR reagent performance, and confirm real-time PCR instrument function.

This study was designed to evaluate the performance of the MultiCode assay and to establish comparison data to determine its clinical utility. Performance of the MultiCode assay was measured by direct comparison to the TaqMan HCV load ASR (TaqMan; Roche Diagnostics, Indianapolis, IN) by use of

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▽ Published ahead of print on 24 March 2010.

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

Log_{10} IU/ml	No. of positive samples/ no. tested	% Positive
1.0	28/44	64
1.4	32/44	73
1.7	39/44	89
2.0	40/44	91
2.3[a]	42/44	95

[a] Limit of detection (95% of replicates) is 2.3 log_{10} IU/ml (200 IU/ml).

clinical specimens. The goal of this study was to determine the limit of detection, linear range, reproducibility, genotype variance, and agreement. In addition, the impact of extraction methods on viral loads was assessed using the MagNA Pure (Roche Diagnostics, Indianapolis, IN) and the easyMAG (bioMérieux, Durham, NC) methods for nucleic acid extraction.

MATERIALS AND METHODS

Standards and clinical samples. 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 TruGene HCV 5′NC genotyping system (TruGene 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, 2b, 3a, 4a, 4a, 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 log_{10} 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 (AcroMetrix, 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 log_{10} 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).

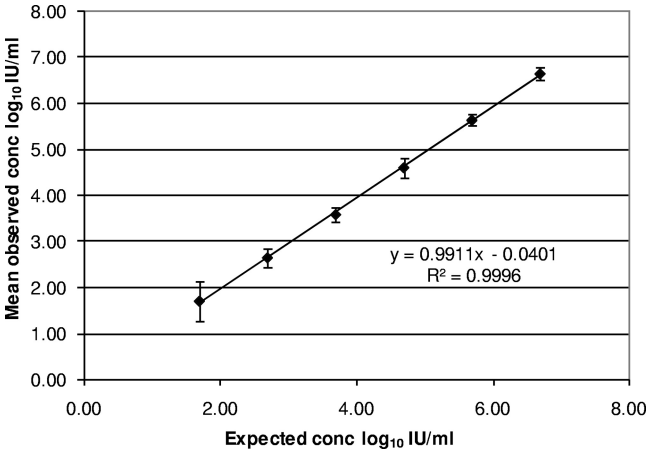

FIG. 1. Linear range of the MultiCode test using plasma samples. Data are means (±SD) for samples tested in duplicate in five separate runs (n = 10).

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 log_{10} IU/ml (Table 1). Based on detection of 95% of replicates, the limit of detection was 2.3 log_{10} 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 log_{10} 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 log_{10} IU/ml. The reproducibility varied through the linear range of the assay (0.12 to 0.43 log_{10} IU/ml), with the greatest precision at higher viral loads and lowest precision at low viral loads of 1.7 log_{10} IU/ml (Table 2).

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

TABLE 2. Reproducibility of the MultiCode test using plasma samples

Nominal concn (log_{10} IU/ml)	No. of samples tested	Mean viral load (log_{10} IU/ml)	SD (log_{10} IU/ml)	% CV[a]
1.70[b]	10	1.69	0.43	25.4
2.70	12	2.63	0.19	7.2
3.70	12	3.57	0.16	4.5
4.70	12	4.59	0.21	4.6
5.70	12	5.62	0.12	2.1
6.70	12	6.63	0.15	2.3

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

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

Samples	Mean viral load (SD) (log_{10} IU/ml)		Mean difference (MultiCode − TaqMan) (log_{10} IU/ml)
	MultiCode	TaqMan	
All	5.40 (1.40)	5.50 (1.34)	−0.10
Genotype 1 samples	5.88 (0.71)	5.98 (0.70)	−0.10
Genotype 2 samples	5.98 (1.00)	6.10 (1.02)	−0.12
Genotype 3 samples	6.05 (0.77)	6.10 (0.70)	−0.05
Genotype 4 samples	5.96 (0.49)	5.35 (0.53)	0.61

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 log_{10} 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 log_{10} IU/ml (1.4 log_{10} IU/ml) for the MultiCode test and 5.5 log_{10} IU/ml (1.3 log_{10} IU/ml) for the TaqMan assay. Based on the agreement analysis, the mean difference between the two tests was −0.10 log_{10} IU/ml (95% limits of agreement, −0.76 to 0.56 log_{10} 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 log_{10} 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 0.1 to 0.3 log_{10} IU/ml. Interestingly, all seven replicates of the 2.0 log_{10} 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 log_{10} IU/ml, with a mean difference of 0.45 log_{10} IU/ml (data not shown).

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

Genotype[a]	Expected concn (log_{10} IU/ml)[b]	MultiCode viral load (log_{10} IU/ml)	Difference (log_{10} IU/ml)
1a	4.05	3.81	−0.24
1b	4.11	3.83	−0.28
2b	4.11	3.41	−0.70
3a	4.48	4.32	−0.16
4a	4.60	4.36	−0.24
4a	4.76	4.48	−0.28
5a	4.08	3.76	−0.32
6a	4.20	4.04	−0.16

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

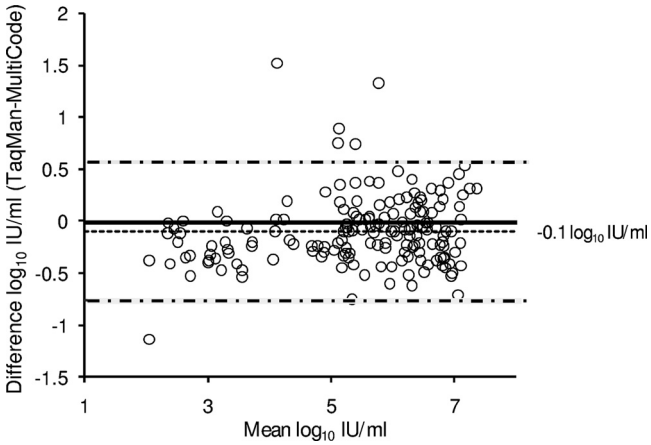

FIG. 2. Agreement plot for 170 plasma samples with a detectable viral load in the MultiCode and TaqMan tests. The dashed line indicates the mean difference of −0.10 log_{10} IU/ml. The solid line represents zero bias for the samples tested, and the dot-dashed line represents 95% limits of agreement of −0.76 to 0.56 log_{10} IU/ml (n = 170).

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

Nominal concn (log_{10} IU/ml)	No. of replicates	Mean observed concn (log_{10} IU/ml)	SD
0.0	7	—[a]	—
2.0	7	1.9	0.3
2.5	7	2.2	0.2
3.0	7	2.7	0.1
4.0	7	3.7	0.1
4.5	7	4.3	0.3
5.0	7	4.9	0.2
5.5	7	5.5	0.1
6[b]	6	6.1	0.2
6.5	7	6.6	0.1
7.0	7	7.3	0.1

[a] —, one of seven replicates was a false positive.
[b] One of the seven replicates (7.9 log_{10} IU/ml) was removed from the data set; if this sample was included, the mean concentration is 6.3 log_{10} IU/ml, with an SD of 0.7 log_{10} IU/ml.

The limit of detection of the MultiCode assay was 2.3 $\log_{10}$ 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 $\log_{10}$ IU/ml to at least 6.7 $\log_{10}$ 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 $\log_{10}$ IU/ml of those obtained with the TaqMan test. 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 $\log_{10}$ 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 $\log_{10}$ 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 (P30 AI050409).

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