JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2011, p. 3036–3039 Vol. 49, No. 8
0095-1137/11/$12.00 doi:10.1128/JCM.00577-11

Comparison of Serial Hepatitis C Virus Detection in Samples Submitted through Serology for Reflex Confirmation versus Samples Directly Submitted for Quantitation▽

Howard B. Gale,[1]* D. Robert Dufour,[2] Nazia N. Qazi,[3,4] and Virginia L. Kan[1,4]

Infectious Diseases Section, Medical Service, Veterans Affairs Medical Center,[1] Department of Pathology and Hepatology, Veterans Affairs Medical Center, and Department of Pathology, George Washington University,[2] GI-Hepatology Section, Medical Service, Veterans Affairs Medical Center,[3] and Department of Medicine, George Washington University,[4] Washington, DC

Received 22 March 2011/Returned for modification 19 May 2011/Accepted 25 May 2011

Using real-time technology, we reliably identified chronic hepatitis C virus (HCV) infection and quantified virus from reflex samples originally submitted for serologic testing. There was no need to process specimens obtained directly for quantitation separately. Whether the initial source is a reflex sample or one obtained directly, a repeat HCV RNA test is needed before starting treatment.

Hepatitis C virus (HCV) is a major public health problem in the United States. Acute infection is usually asymptomatic and remains undiagnosed, with 75 to 85% of patients developing chronic infection that decades later may manifest in cirrhosis and hepatocellular carcinoma (3, 14). Diagnosis is dependent on laboratory testing, typically beginning with detection of antibodies to HCV proteins, which can be observed due to current infection with or previous exposure to the virus, as well as to false-positive results. Confirmation of current infection requires detection of HCV RNA in the blood of persons who are anti-HCV positive. According to the most recent National Health and Nutrition Examination Survey published in 2006, there are an estimated 4.1 million anti-HCV-positive persons, of whom 3.2 million are also HCV RNA positive (3).

In most clinical settings, HCV RNA testing is done after a health care provider receives a positive anti-HCV result. A request is then made for measurement on a second sample from the same individual (direct testing). For various reasons, however, this often is not done, and individuals either are not correctly identified as being currently infected or are labeled as infected when they have actually cleared the virus. Reflex testing (HCV RNA testing done automatically on the same, positive anti-HCV sample) can significantly shorten the time to clarifying patient status and prevent diagnostic misclassification based on incomplete information.

The prevalence of chronic HCV infection is higher in certain populations, including those receiving care in the Veterans Health Administration (VHA) (6). The VHA responded to this dilemma in 1998 by implementing CDC guidelines to identify viremic, anti-HCV-positive veterans for appropriate counseling and management (1). To streamline the process, VHA Directive 2009-063 mandated reflex HCV RNA confirmatory testing after a reactive serologic screening. The application and subsequent clinical utility of this directive may be impacted by several laboratory issues. Prior studies analyzing reflex specimens (7, 16, 18) did not evaluate whether there are significant differences in the frequency of HCV RNA detection or in the HCV viral load compared to those in specimens treated more optimally from the time of collection (5, 13). In addition, the viral load is known to fluctuate over time (2, 8, 10, 12, 17).

Through automated, real-time PCR technology, our objective was to assess the reliability of using reflex samples received after serologic testing versus the reliability of using direct samples obtained for HCV quantitation in determining viral status and providing the baseline viral load for treatment at VA Medical Centers in Washington, DC, Baltimore, MD, and Martinsburg, WV. The period for our evaluation was from February 2008 through November 2010.

For reflex samples, peripheral blood was drawn by venipuncture into a serum separator tube and centrifuged within 6 h. The serum was stored at 2 to 8°C for 1 to 5 days before it was tested for the anti-HCV antibody on the Vitros ECiQ immunodiagnostic system (Ortho Clinical Diagnostics, Raritan, NJ) (7, 16, 18). Reactive sera, defined by a signal/cutoff ratio of >9.5 or, if the signal/cutoff ratio is <9.5, an indeterminate or positive recombinant immunoblot assay (RIBA) result, were frozen at −20°C for 1 to 3 days and then at −80°C until quantitative analysis. For direct samples, peripheral blood was drawn by venipuncture into EDTA or a serum separator tube and centrifuged within 6 h. The plasma/serum was frozen at −20°C for 0 to 3 days and then at −80°C until quantitative analysis.

Testing was performed by using the Abbott RealTime HCV assay (an analyte-specific reagent) with the *m*2000*sp* platform for sample preparation and the *m*2000*rt* for amplification and detection (Abbott Molecular, Inc., Des Plaines,

* Corresponding author. Mailing address: Infectious Diseases Section (151B), Veterans Affairs Medical Center, 50 Irving St. NW, Washington, DC 20422. Phone: (202) 745-8000, ext. 5506. Fax: (202) 745-8432. E-mail: howard.gale@va.gov.

▽ Published ahead of print on 8 June 2011.

TABLE 1. Variability of HCV RNA viral loads in serial clinical samples[a]

Serial specimen types used (*n*)	No. of days between sample collection	HCV RNA range (log_{10} IU/ml)	ΔHCV RNA (log_{10} IU/ml)		*P* value	|ΔHCV RNA|		
			Mean	Range		Mean log_{10} IU/ml	% (no.) of samples with result of >0.5	% (no.) of samples with result of >1.0
Reflex, direct (198)	6–941	2.228–7.127	0.030	−2.310–2.365	0.382	0.306	19 (37)	5 (10)
Direct, direct (493)	6–887	2.079–7.301	−0.061	−1.914–1.727	0.002	0.319	19 (95)	4 (21)

[a] Patients were treatment naïve with viral loads within the quantitative range. If there were more than two results for one individual, only the first and last were compared.

IL). The quantitative range was 20 to 20,000,000 international units per milliliter (IU/ml), or 1.301 to 7.301 log_{10} IU/ml. For quality control, five RNA levels from pooled patient sera/plasma were assayed on each run. From November 2009 to November 2010, the mean log_{10} IU/ml values (percent coefficient of variation [CV]) were 1.509 (11.4%), 2.445 (3.0%), 3.772 (2.5%), 5.249 (1.3%), and 6.723 (1.5%) on 38 assays. The slope, intercept, and r^2 were determined for statistical analysis by a least-squares linear regression analysis of the log_{10} IU/ml, and the *P* value was determined by a two-tailed, paired sample *t* test.

Quantitative HCV RNA testing of anti-HCV-positive patients is important for two main reasons. The absence of the detectable virus identifies individuals who do not require treatment. Based on 1,435 reflex samples, our frequency of detection of 80.5% was within the expected range (3, 14). Of the remaining 280 veterans, 67 had subsequent direct tests and 64 (96%) were again not viremic. In addition, the rate of HCV RNA decrease is a predictor of the sustained viral response to antiviral treatment (4, 9). For that reason, we wanted to explain the larger differences, of >0.5 log_{10} IU/ml, between the reflex sample results and the direct sample results for 19% of the chronically infected population (Table 1; Fig. 1A). Based on four small experiments, preanalytical factors did not account for this variation (Table 2). The most likely cause was biological fluctuation, matching the pattern observed in a parallel analysis of serial direct specimens (Table 1; Fig. 2A).

Utilizing a real-time assay with high sensitivity and broad dynamic range, the variability of serial HCV RNA that we observed was consistent with earlier findings (2, 8, 10, 12, 17), with no clinically significant differences between values from an initial reflex and subsequent direct sample and from two direct samples (Table 1; Fig. 1A and 2A). Viral loads remained stable at levels of <0.5 log change for most patients, but fluctuations of >1.0 log did occur. These differences did not correlate with the initial test result (Fig. 1B and 2B) or the number of days between samples (Fig. 1C and 2C). Spontaneous loss of virus from circulation is virtually never seen beyond the first 6 months (19), and the level of viremia is not an indicator of disease activity (17) or progression (11, 15). Based on our findings, whether an initial value is obtained from reflex or direct samples, a repeat HCV RNA test is needed just before starting treatment to reduce the impact of random fluctuation on the evaluation of treatment effectiveness.

We were able to reliably identify chronic hepatitis C virus infection and quantify virus from reflex samples originally submitted to the serology laboratory for anti-HCV antibody testing. Separate handling for quantitation was not necessary. Our results are applicable to the methods used here. They may not pertain to other anti-HCV assays, since the Vitros system uses disposable tips to prevent carryover. We

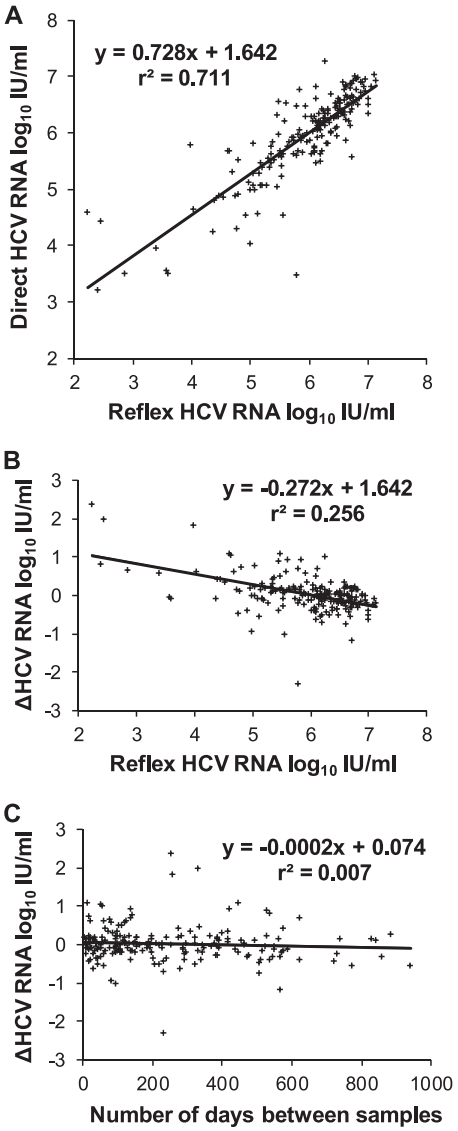

FIG. 1. Comparison of HCV RNA assay results for 198 pairs of serial clinical samples, reflex and direct. Patients were treatment naïve. If there were more than two results for one individual, only the first and last were compared.

TABLE 2. Interassay comparisons: effects of preanalytical factors on HCV RNA

Specimen type (*n*) comparison	HCV RNA range (log_{10} IU/ml)	Linearity			ΔHCV RNA (log_{10} IU/ml)		*P* value	Mean |ΔHCV RNA| (log_{10} IU/ml)
		Slope	Intercept	r^2	Mean	Range		
Sample 1 vs. sample 2[a] (29)	1.477[b]–7.053	1.002	−0.034	0.992	−0.025	−0.415[b]–0.222	0.352	0.103
Serum 1 vs. serum 2[c] (10)	5.013–6.586	1.151	−0.910	0.973	−0.010	−0.205–0.205	0.782	0.103
Serum vs. plasma[d] (10)	4.783–6.911	0.968	0.103	0.976	−0.089	−0.320–0.054	0.102	0.106
Reflex vs. direct[e] (10)	5.182–6.848	0.991	0.137	0.875[f]	0.080	−0.202–0.487[f]	0.203	0.139

[a] Following analysis, plasma/serum was refrozen at −80°C, thawed, and analyzed again in January 2008.
[b] An initial viral load of 1.892 was followed by 1.477 log_{10} IU/ml (78 → 30 IU/ml); the next greatest decrease was −0.307 log_{10} IU/ml.
[c] Following analysis, serum was refrozen at −80°C, thawed, stored for 5 days at 2 to 8°C, and analyzed again in November 2010.
[d] Serum and EDTA-plasma were drawn at the same time, handled as direct samples (serum sample centrifuged 2 to 4 h before plasma), and analyzed separately from November 2010 to February 2011.
[e] Reflex sera and direct EDTA-plasma samples were drawn at the same time, processed, and analyzed separately in November 2010 and January 2011.
[f] The next greatest increase in ΔHCV RNA log_{10} IU/ml after 0.487 was 0.183. Excluding this outlier, the r^2 was 0.946.

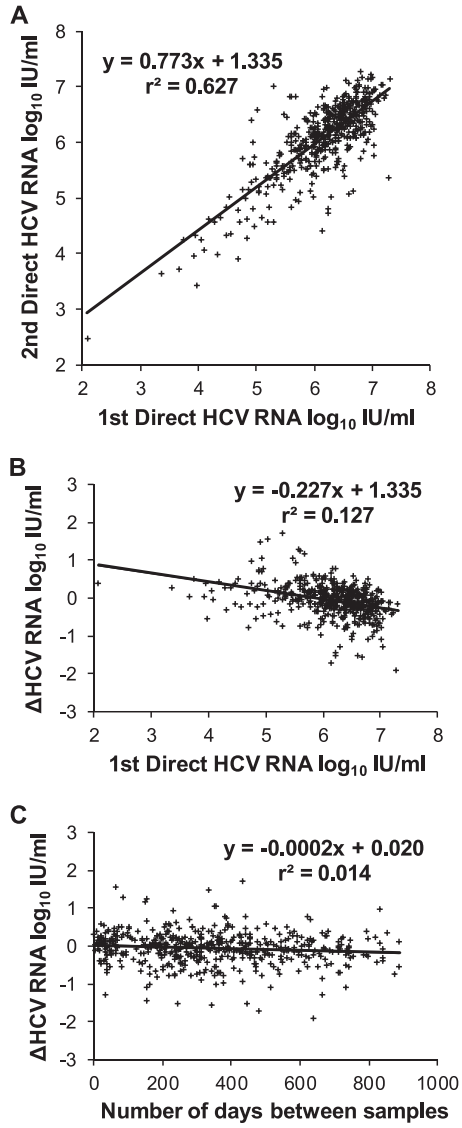

FIG. 2. Comparison of HCV RNA assay results for 493 pairs of serial clinical samples, both direct. Patients were treatment naïve. If there were more than two results for one individual, only the first and last were compared.

believe that our results do apply to alternative HCV RNA testing methods, because the Abbott RealTime results are similar to those of other studies. The convenience and reliability of reflex testing make it a preferred option for confirming HCV infection and determining viral load.

We thank Rebecca Shinol, Karen Rexroth, Maria Earp, and Barbara Harris for their technical support and Judith Myers and Bonnie Charon for their clinical perspectives.

The IRB and R & D Committee at this VA Medical Center reviewed and approved the manuscript. The views expressed are those of the authors and do not reflect the views or policies of the Department of Veterans Affairs. The authors report no conflict of interest.

REFERENCES

1. **Alter, M. J., W. L. Kuhnert, and L. Finelli.** 2003. Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. MMWR Recommend. Rep. **52**(RR03)**:**1–16.
2. **Arase, Y., et al.** 2000. Fluctuation patterns of HCV-RNA serum level in patients with chronic hepatitis C. J. Gastroenterol. **35:**221–225.
3. **Armstrong, G., et al.** 2006. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Ann. Intern. Med. **144:**705–714.
4. **Davis, G., et al.** 2003. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology **38:**645–652.
5. **de Moreau de Gerbehaye, A., M. Bodéus, A. Robert, Y. Horsmans, and P. Goubau.** 2002. Stable hepatitis C virus RNA detection by RT-PCR during four days storage. BMC Infect. Dis. **2:**22.
6. **Dominitz, J., et al.** 2005. Elevated prevalence of hepatitis C infection in users of United States veterans medical centers. Hepatology **41:**88–96.
7. **Dufour, D., M. Talastas, M. Fernandez, and B. Harris.** 2003. Chemiluminescence assay improves specificity of hepatitis C antibody detection. Clin. Chem. **49:**940–944.
8. **Fanning, L., et al.** 2000. Natural fluctuations of hepatitis C viral load in a homogeneous patient population: a prospective study. Hepatology **31:**225–229.
9. **Fried, M. W., et al.** 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. **347:**975–982.
10. **Halfon, P., et al.** 1998. Assessment of spontaneous fluctuations of viral load in untreated patients with chronic hepatitis C by two standardized quantitation methods: branched DNA and Amplicor Monitor. J. Clin. Microbiol. **36:**2073–2075.
11. **Hu, S., N. Kyulo, V. Xia, D. Hillebrand, and K. Hu.** 2009. Factors associated with hepatic fibrosis in patients with chronic hepatitis C: a retrospective study of a large cohort of U.S. patients. J. Clin. Gastroenterol. **43:**758–764.
12. **Kuramoto, I., T. Moriya, V. Schoening, and P. Holland.** 2002. Fluctuation of serum HCV-RNA levels in untreated blood donors with chronic hepatitis C virus infection. J. Viral Hepat. **9:**36–42.
13. **Leckie, G., et al.** 2004. Performance attributes of the LCx HCV RNA quantitative assay. J. Virol. Methods **115:**207–215.
14. **Lim J.** 2001. Natural history of hepatitis C infection: a concise review. Yale J. Biol. Med. **74:**229–237.
15. **McCormick, S., Z. Goodman, C. Maydonovitch, and M. Sjogren.** 1996. Evaluation of liver histology, ALT elevation, and HCV RNA titer in patients with chronic hepatitis C. Am. J. Gastroenterol. **91:**1516–1522.
16. **Oethinger, M., D. Mayo, J. Falcone, P. Barua, and B. Griffith.** 2005. Effi-

ciency of the Ortho VITROS assay for detection of hepatitis C virus-specific antibodies increased by elimination of supplemental testing of samples with very low sample-to-cutoff ratios. J. Clin. Microbiol. **43:**2477–2480.
17. **Pontisso, P., et al.** 1999. Hepatitis C virus RNA profiles in chronically infected individuals: do they relate to disease activity? Hepatology **29:**585–589.
18. **Seo, Y. S., et al.** 2009. Significance of anti-HCV signal-to-cutoff ratio in predicting Hepatitis C viremia. Korean J. Intern. Med. **24:**302–308.
19. **Yokosuka, O., et al.** 1999. Spontaneous negativation of serum hepatitis C virus RNA is a rare event in type C chronic liver diseases: analysis of HCV RNA in 320 patients who were followed for more than 3 years. J. Hepatol. **31:**394–399.