Evaluation of Quantitative Real-Time PCR as a Hepatitis C Virus Supplementary Test After RIBA Discontinuation

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Laboratory testing plays a major role in hepatitis C virus (HCV) diagnosis and patient follow-up. The high false positive rates of HCV screening tests require confirmation through a supplementary test. According to the 2003 CDC guidelines, recombinant immunoblot assay (RIBA) is indispensable to confirm positive screening results and differentiate biologic false positivity from true HCV exposure. However, RIBA has been permanently discontinued since 2011. In the 2013 update of its guidelines, CDC called for further studies on HCV laboratory testing without RIBA. In this study, we analyzed the applicability of quantitative real-time PCR (qPCR) as a supplementary HCV diagnostic test. By comparing our HCV testing performances before and after

RIBA discontinuation, we found that omitting RIBA has no significant effect on the accurate and efficient identification of HCV infection, provided that HCV antibody signal-to-cutoff ratio is considered. Furthermore, we proposed a new HCV testing algorithm that incorporates semiquantitative assessment of HCV antibody positivity and HCV viral load measurement by qPCR. By following the algorithm, we were able to address confirmation of positive HCV screening results and to provide useful information generally required by clinicians, including the needs of further laboratory testing or clinical follow-up, as well as HCV viral titers. *J. Clin. Lab. Anal.* 30:418–423, 2016. © 2015 Wiley Periodicals, Inc.

Key words: hepatitis C virus; laboratory testing; supplementary test; quantitative real-time PCR; recombinant immunoblot assay

INTRODUCTION

Hepatitis C virus (HCV) is a blood borne pathogen, which imposes severe public health threats on global populations. The World Health Organization (WHO) estimates that 130–150 million people have chronic hepatitis C infection (WHO Fact sheet No. 164, updated April 2014, WHO Media Center). In the United States, there are approximately 2.7–3.9 million people (1.0%–1.5% of population) infected by HCV, with an estimated 17,000 people newly infected in 2010 (1). While 15–45% of people infected with HCV eventually clear the virus, the majority of them will develop sequelae of chronic infection, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (2).

Laboratory testing plays a major role in HCV diagnosis and patient follow-up. Since the cloning of the HCV genome in 1989 (3), laboratory diagnosis of hepatitis C has been developing rapidly and new testing reagents continue to emerge for clinical testing (4,5). In 2003, The Cen-

ter for Disease Control and Prevention (CDC) published guidelines for laboratory testing and result reporting of antibodies to HCV (6). According to the guidelines, all patients are screened by serologic tests using a recombinant antigen to detect anti-HCV antibody. Currently available screening tests include enzyme immunoassay (EIA) and chemiluminescent immunoassay (CIA). Although these tests are relatively inexpensive and have short turnaround time, a significant percentage of false positive results have precluded reporting them to clinicians without an independent supplementary test. The CDC reported that the false positive rates using various HCV

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EIA 2.0 or 3.0 reagents in immunocompetent populations are between 15% and 60% (average 35%), compared to approximately 15% among immunocompromised populations (6). Therefore, it is critical to confirm positive screening results by a supplementary test, which can be either anti-HCV confirmatory test or nucleic acid test (NAT).

At our own hospital and many other institutions, the traditional approach to HCV testing has been an initial evaluation of anti-HCV status by CIA followed by a supplementary test through recombinant immunoblot assay (RIBA, Chiron HCV 3.0 SIA; Chiron Corp., Emeryville, CA). Real-time PCR has been used for monitoring virologic responses to treatment. However, some institutions had adapted an alternate algorithm, which uses NAT to confirm HCV active infection in patients with positive screening results. Patients with negative NAT results will require RIBA to confirm anti-HCV positivity, such that patients who have likely cleared HCV could be followed or further evaluated. Therefore, per 2003 CDC guideline, RIBA was required no matter which approach is used. Because RIBA is more expensive and time consuming, several investigators recently proposed using signal-to-cutoff ratio (S/Co) as a useful tool to cut down the needs for RIBA testing (7–9). These authors found that including S/Co ratio in the testing approach for HCV has dramatically reduced the number of cases that require RIBA testing.

In the United States, the only Food and Drug Administration (FDA)-licensed supplemental anti-HCV test is the strip immunoblot assay (Chiron RIBA® HCV 3.0 SIA). However, the RIBA reagent has been permanently discontinued, which has made NAT the only available supplementary test to confirm HCV infection. In May of 2013, CDC issued an update on the HCV testing approach, in which it stated that future studies are needed to evaluate the performance of HCV testing without RIBA (10). Clinicians also need new testing information without RIBA to guide their HCV management strategy. However, such studies and data are lacking in the literature. In this article, we analyzed the clinical and laboratory testing performance data of two groups of patients who were HCV tested before and after RIBA discontinuation. Our data demonstrated that omitting RIBA has minimal or no effect on the efficiency and accuracy of HCV testing, provided that S/Co ratio was taken into consideration. Based on these results, we also proposed a new testing strategy specifically designed for the era of RIBA unavailability.

MATERIALS AND METHODS

Patients

During the study period, April 6–29 of 2011, 656 patients were tested for anti-HCV by CIA at University

Hospitals Case Medical Center, Cleveland, OH. Forty-three of them were positive for CIA and reflexed to PCR test after RIBA was unavailable. These patients constituted our study group. To compare the HCV testing performances before and after RIBA discontinuation, we searched our database and identified 121 CIA-positive patients before RIBA discontinuation who were tested within 3 months of our study period, November 1 of 2010 to January 31 of 2011, as our database group. Patients in the database group were all reflexed to RIBA. Sixty-eight of them were positive or indeterminate for RIBA, and additionally had PCR performed. To avoid selection bias, we included all patients tested within the specified periods in both groups.

Serum Anti-HCV CIA

Serum samples were tested for anti-HCV on the Siemens Advia Centaur (Siemens Diagnostics, Deerfield, IL), following manufacturer's instruction manual. Advia Centaur HCV assay (Bayer HealthCare LLC, Tarrytown, NY) was used. As a CIA, the system reports an S/Co ratio. S/Co ratio <1 is defined as nonreactive, whereas S/Co ratio of 1–11 or >11 is reported as reactive. Samples tested reactive by CIA were reflexed to either RIBA (database group) or PCR (study group).

Serum Anti-HCV RIBA

For database group patients, Chiron RIBA® HCV 3.0 SIA was used to confirm serum samples tested reactive by CIA. RIBA uses recombinant c33c and NS5 antigens and synthetic 5-1-1, c100, and c22 peptides immobilized on test strips to detect specific binding of anti-HCV in human blood. The results are reported as indeterminate (one band with 1+ or above intensity), positive (at least two bands with 1+ or above intensity), or negative (no band with 1+ or above intensity).

Serum Real-Time PCR to Detect HCV Viremia

The serum samples with positive or indeterminate RIBA results in the database group, and those tested reactive by CIA in the study group, were subjected to HCV viremia detection using real-time PCR (Roche Cobas Ampliprep/Taqman, Indianapolis, IN) on COBAS® AmpliPrep and COBAS® TaqMan® Analyzer. FDA has approved this test for monitoring the virologic response to treatment. As a HCV RNA quantification test, it can detect genotypes 1 through 6 HCV viral RNA, and with a linear range of 43–69,000,000 IU/ml.

Data Collection and Analysis

Antibody S/Co ratios were evaluated for the patients in both groups with negative PCR. Clinical history related to HCV infection status and risk factors was also reviewed, whenever available in our electronic medical record system. The distributions of different test results in both groups were analyzed and compared. Using Fisher's exact test, we also analyzed whether antibody S/Co ratios are significantly associated with PCR results by pooling the data from database and study groups.

RESULTS

HCV Testing Without RIBA

Among 43 CIA-positive patients in the study group, 31 (72%) were PCR positive and 12 (28%) were PCR negative. In the group of 12 patients with negative PCR, three of four patients with S/Co ratios greater than 11 had documented diagnosis of HCV infection; the fourth had no medical history available for HCV status. Two patients with S/Co ratios <3 were likely CIA false positive. Six patients' antibody S/Co ratios were between 3 and 10, with no relevant medical information available for review (Table 1. Importantly, all 31 PCR positive patients had S/Co ratios greater than 11.

HCV Testing With RIBA

The data base group was composed of 121 CIA-positive patients who had RIBA reflex test with the following results: 104 RIBA positive (86%), 12 RIBA indeterminate (10%), and 5 RIBA negative (4%) (Fig. 1. Among the 116 RIBA positive or indeterminate patients, 68 had PCR performed to determine HCV viremia. Forty-nine patients (72%) were PCR positive, all of which had S/Co ratios greater than 11. For the 19 PCR-negative patients, high S/Co ratios (>11) were detected in 11 patients (16%), three patients (5%) had low S/Co ratios (<3), and five patients (7%) had S/Co ratios 3–11 (Table 1.

We further stratified these 68 patients to RIBA-positive (60 patients) and RIBA-indeterminate (eight patients) subgroups (Table 2. Among 60 patients with positive RIBA, 48 (80%) were PCR positive and 12 (20%) were PCR negative. High S/Co ratios (>11) were detected in 11 patients, who likely had cleared HCV infection. One patient with low S/Co ratio (1.61) could be RIBA false positive. For the eight RIBA indeterminate patients, one patient had positive PCR and S/Co ratio greater than 11 (active HCV infection). The other seven patients were PCR negative, including two patients with S/Co ratios <3 (likely CIA false positive), and five patients with S/Co ratios 3–11 (CIA false positive or had cleared infection).

Fig. 1. Distribution of RIBA results in database group patients. Before RIBA discontinuation, 121 anti-HCV CIA positive cases were reflexed to RIBA test; 104 patients were RIBA positive (86%), 12 were RIBA indeterminate (10%), and 5 were RIBA negative (4%).

Comparison of HCV Testing Results With and Without RIBA

Since patient populations of these two groups are comparable based on the number of patients identified per month, we then compared their distribution of test results directly. Both groups had 72% positive PCR results. CIA false positive (defined as PCR negative and S/Co ratio <3) rates were both 5% (Table 1, although the numbers of patients were low for both groups (three for the database group and two for the study group, respectively). Of note, in the database group, only five patients (4%) who were RIBA negative did not have PCR performed (Fig. 1, whereas in the study group, all CIA-positive patients were reflexed to have PCR test. In summary, our analysis of these two strategies, one incorporating RIBA and then NAT following positive screening result and one reflexing to NAT directly, indicates that the latter has almost identical performance outcome as the former does.

DISCUSSION

HCV screening tests, including EIA and CIA, were initially developed for detecting blood donors who have been exposed to HCV. Therefore, ensuring superb sensitivity is the top priority for the test manufacturers (5). While this

TABLE 1. Comparison of PCR and Antibody Results of Database Group and Study Group

	PCR positive, Ab S/Co >11	PCR negative, Ab S/Co >11	PCR negative, Ab S/Co 3–11	PCR negative, Ab S/Co <3	N
Database group	49 (72%)	11 (16%)	5 (7%)	3 (5%)	68 (100%)
Study group	31 (72%)	4 (9%)	6 (14%)	2 (5%)	43 (100%)

strategy has succeeded tremendously in reducing HCV transmission through blood transfusion, it is also problematic by creating very high false positive rates for HCV screening tests. In a population with low HCV prevalence, the false positive rates have been shown to exceed the true positive rates (poor positive predictive values) (5). In the 2003 guidelines, CDC mandated that confirmation of positive screening results through supplementary serum test or NAT is necessary before reporting these results (6). As the only FDA-approved serum HCV supplementary test, RIBA was indispensable in the CDC-recommended HCV testing algorithm released in 2003. Nonetheless, some investigators have questioned that RIBA tests have minimal or no added value, especially when the S/Co ratios of HCV screening tests is in place (9, 11). A significant change to the HCV testing field is the permanent discontinuation of RIBA reagents since 2011. Therefore, the only available FDA-approved supplementary tests in the United States are now NATs. Facing this critical change, CDC has published an update on HCV testing practice in May of 2013 (10). If a patient is positive on HCV screening test but negative by NAT, he or she does not have active HCV infection. However, in order to differentiate past exposure from false positivity, the new recommendation suggests the same sample should be retested using another FDA-approved serum HCV antibody assay, or HCV RNA assay is repeated. CDC clearly stated that more studies are needed to inform the best practices in this field (10), indicating that we are lacking sufficient data to make a definitive conclusion as to which strategy is superior. From a patient management point of view, a positive result by CIA screening but a negative result by NAT confuses health care providers as to the next appropriate step that needs to be taken.

In this article, we analyzed the HCV testing performances before and after RIBA discontinuation in our hospital-based patient population. The patient

populations for our study group and database group were highly comparable. The only difference was that all CIA-positive patients had PCR tests performed in the study group, while 4% of CIA-positive/RIBA-negative patients in the database group had PCR tests waived (Fig. 1). However, in both groups, PCR positivity rates in CIA-positive patients and the CIA false positive rates are almost identical, although the numbers of patients with false positive CIA are very small (less than five patients) for both groups. We concluded that RIBA discontinuation had minimal effect on HCV testing except that there is slightly lower number of patients who will be tested by NAT following a positive screening result if RIBA result is available, that is, 4% of CIA-positive patients, or approximately five patients each quarter.

Overall, our results showed that after RIBA discontinuation, HCV RNA real-time PCR serves as an excellent substitute supplementary test. Based on our results, we proposed a testing algorithm for HCV diagnosis to be used in the era of RIBA unavailability (Fig. 2). According to our algorithm, all HCV antibody screen positive patients will be reflexed to HCV NATs. A positive PCR result not only confirms the presence of HCV antibody, but also supports active HCV infection. For patients with negative PCR results, the reported S/Co ratios of CIA might provide valuable information. S/Co ratio <3 in CIA-positive/PCR-negative patients usually indicates false positive reaction, as has been suggested by other studies showing that very low antibody titers reliably indicate false positivity (7–9), and hence patients need not be further tested. On the other hand, a negative PCR result with S/Co ratio >11 likely means clearance of past HCV infection. Lai et al. reported that 95.5% of HCV viremic patients had S/Co ratio >20 by CIA assay (9). In our study, 100% of PCR-positive patients from both the study group and database group ($n = 80$) had S/Co ratio >11, and none of those with S/Co

TABLE 2. Comparison of PCR and Antibody Results of RIBA positive and RIBA Indeterminate Patients in the Database Group

	PCR positive, Ab S/Co >11	PCR negative, Ab S/Co >11	PCR negative, Ab S/Co 3–11	PCR negative, Ab S/Co <3	N
RIBA positive	48 (80%)	11 (18%)	n.a.	1 (2%)	60 (100%)
RIBA indeterminate	1 (12%)	n.a.	5 (63%)	2 (25%)	8 (100%)

n.a.: not applicable.

Fig. 2. Proposed HCV test strategy after RIBA discontinuation. As per our algorithm, all HCV antibody screen positive patients are reflexed to HCV NATs. A positive PCR result confirms the presence of HCV antibody and active HCV infection. For patients with negative PCR results, the S/Co ratios of CIA need to be reviewed. S/Co ratio <3 indicates false positive reaction and patients need not be further tested. S/Co ratio >11 likely means clearance of past HCV infection, but PCR may need to be repeated to rule out rare situations that HCV RNA is not detectable. For patients with S/Co ratio 3–11, clinical correlation should be used to decide whether a repeat PCR is needed.

TABLE 3. Analysis of the Correlation of PCR Results and Anti-body S/Co Ratios by Fisher’s Exact Test

	PCR positive	PCR negative	Total
S/Co >11	80	15	95
S/Co <11	0	16	16
Total	80	31	111

ratio <11 ($n = 16$) had positive PCR results (Table 3. Our data strongly suggest that S/Co ratio >11 carries high risk of active HCV infection. However, a concern of nondetected HCV viremia exists in patients who have negative PCR results but S/Co ratio >11. It is known that HCV RNA may not be detectable in some patients during the acute phase of their diseases, and intermittent HCV RNA positivity has been observed among persons with chronic HCV infection (12, 13). Thus, we suggest that for patients with negative PCR and S/Co ratio >11, PCR should be repeated in 6 months to rule out active HCV infection. Lastly, patients with S/Co ratio 3–11 fall in the “gray-zone” group, as these patients could have cleared HCV infection, or the results represent false positivity. In our study, none of patients with S/Co ratio 3–11 had active HCV infection. We propose that clinical correlation should be used to decide whether a repeat PCR is needed for these patients. It must be pointed out, however, that

the use and interpretation of the cutoff values for anti-HCV are based on our data obtained through Siemens Centaur anti-HCV assay. Therefore, data obtained from other anti-HCV CIA or EIA assay must be evaluated to develop test-specific S/Co ratio or cutoff values for results interpretation. In this study, we used HCV testing data at our institution, before and after RIBA discontinuation, to compare two HCV testing approaches, one including RIBA and the other without RIBA. Although two different groups of patients were used for comparison, each group comprises all the patients undergoing HCV screening and follow-up within the specified periods to avoid selection bias. However, we cannot rule out potential bias resulting from differences in demographic features between these two groups of patients.

Currently, in addition to the FDA-approved COBAS® AmpliPrep® TaqMan® real-time PCR assay for monitoring HCV viral load, which we used in this study, there is also the FDA-approved diagnostic NAT for qualitative detection of HCV RNA, COBAS AMPLICOR® Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ). This is a diagnostic test for the qualitative detection of HCV RNA in human blood, with the lower limit of detection approximately 50 IU/ml in plasma and 60 IU/ml in serum. The quantitative real-time PCR assay has multiple advantages compared to the qualitative RT-PCR assay. First, it incorporates Taqman® probes to ensure higher specificity. Second, it reports viral

titers in addition to confirmation of positive or negative results. Third, its sensitivity is equivalent to, if not higher than that of qualitative PCR test. With the new version of the COBAS® AmpliPrep® TaqMan® real-time HCV PCR assay, the lower limit of detection is 20 IU/ml. The downside of real-time PCR assay is its relatively higher cost when compared to the qualitative PCR assay. However, the ability to report HCV titers, which is generally required by clinicians, outweighs the cost concern.

In summary, we compared the diagnostic accuracy and efficiency of HCV testing before and after RIBA discontinuation and found that omitting RIBA has minimal, if any, effect on HCV diagnosis, provided that HCV antibody S/Co ratio is included in the testing algorithm. In response to the CDC update on HCV testing in 2013, which called for more studies to clarify the HCV testing recommendations after RIBA discontinuation, we proposed a new HCV testing algorithm. Using HCV real-time PCR as a supplementary test, plus references from the antibody S/Co ratio, we were able to address confirmation of the positive antibody screening results and provide useful information generally required by clinicians, including the needs of further laboratory testing or clinical follow-up, as well as HCV viral titers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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