

Improved Reflexive Testing Algorithm for Hepatitis C Infection Using Signal-to-Cutoff Ratios of a Hepatitis C Virus Antibody Assay

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BACKGROUND: Chemiluminescence immunoassay (CIA) is used to detect hepatitis C virus (HCV) antibody status on the basis of signal-to-cutoff (S/Co) ratios. Positive results of antibody to HCV (anti-HCV) are followed by either recombinant immunoblot assay (RIBA) to confirm anti-HCV positivity or reverse transcription (RT)-PCR to detect viremia. We hypothesized that by analyzing S/Co ratios, we could determine a strategy to reduce unnecessary supplementary testing in our population.

METHODS: CIA was performed to screen for anti-HCV, and positive results were followed up with RT-PCR testing. Negative RT-PCR results were followed up with RIBA, whereas positive RT-PCR results were assumed to be RIBA positive. ROC curves were analyzed to determine the optimal S/Co ratios to predict HCV infection.

RESULTS: We determined the S/Co ratios on 34 243 veteran patient samples. We found that with the CIA method 9.0% of patients had positive test results for anti-HCV. An S/Co ratio <3.0 ruled out active HCV infection and exposure with 100% negative predictive value. When the S/Co ratio was ≥ 20.0 , positive predictive values were 98.5% compared with RIBA results, and 81.0% compared with RT-PCR results.

CONCLUSIONS: RIBA is not necessary to confirm negative or positive CIA anti-HCV if the S/Co ratio is <3.0 or ≥ 20.0 , respectively. To confirm HCV exposure, samples with an S/Co ratio between 3.0 and 19.9 should be followed up with RIBA unless PCR testing has been performed and the result is positive. Samples with an

S/Co ratio ≥ 20.0 or positive RIBA results should be further tested by RT-PCR to determine HCV viremia status.

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Hepatitis C virus (HCV)⁹ is a common chronic viral infection in the US. An estimated 1.6% of the population test positive for antibody to HCV (anti-HCV), and 3.2 million (1.3%) are chronically infected (1). Patients who receive care at the Department of Veterans Affairs medical centers have been found to have a higher prevalence of anti-HCV antibodies (2). HCV infection also leads to end-stage liver disease, cirrhosis, and hepatocellular carcinoma, and is the leading indication for liver transplantation (3). Accurate, efficient, and cost-effective diagnosis of HCV infection through clinical laboratory testing is important for therapeutic decision-making.

The diagnosis of HCV infection is based on the detection of anti-HCV and HCV RNA. Detection of anti-HCV by immunoassay is the screening test used to evaluate HCV exposure. There are 2 main immunoassays for detecting anti-HCV, enzyme immunoassay (EIA) and chemiluminescence immunoassay (CIA). Unfortunately, both methods are limited by false-positive results, although results of a study by Dufour et al (4) showed that the CIA method demonstrated improved specificity over the EIA. The recombinant immunoblot assay (RIBA) for HCV antibodies is used as a supplementary or confirmatory test for EIA or CIA results by many clinical laboratories owing to its higher specificity. Detection of HCV RNA by reverse tran-

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⁹ Nonstandard abbreviations: HCV, hepatitis C virus; anti-HCV, antibody to HCV; EIA, enzyme immunoassay; CIA, chemiluminescence immunoassay; RIBA, recombinant immunoblot assay; RT, reverse transcription; S/Co, signal-to-cutoff; VAPSHCS, Veterans Affairs Puget Sound Health Care System; PPV, positive predictive value; NPV, negative predictive value.

scription (RT)-PCR is further used to confirm active HCV infection with viremia. However, compared to the EIA and CIA assays the RIBA and RNA assays may be more costly.

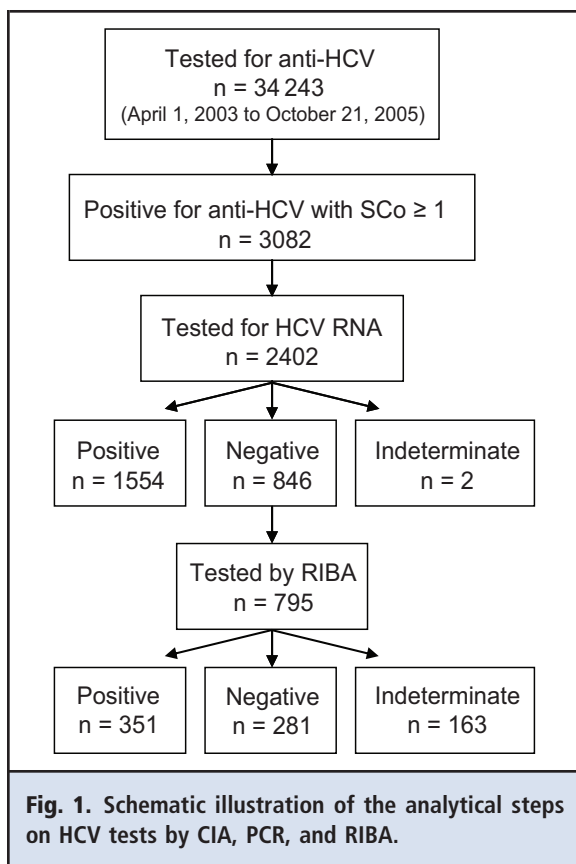
The strategies for detection of HCV infection by detection of anti-HCV and HCV RNA are variable. As many as 9 testing strategies have been analyzed (5). One recommended strategy is to first use EIA or CIA to test for HCV exposure (anti-HCV) and then use RT-PCR only if the immunoassay is positive. If the RT-PCR results are negative, then RIBA can be performed to determine if the antibody test result for EIA or CIA was false positive. Alternatively, samples with positive results by EIA/CIA can subsequently undergo RIBA testing, followed by RT-PCR only when the RIBA is positive. Contreras et al. (6, 7) used ROC curves to analyze the performance of the CIA test compared to the HCV RNA assay, using samples from Mexican blood donors. They showed that among the 856 samples positive for anti-HCV according to results of a third-generation amplified CIA, very low anti-HCV concentrations at a signal-to-cutoff (S/Co) ratio of <4.5 predicted lack of HCV exposure (6), and high anti-HCV concentrations (S/Co ratio of ≥ 20.0) predicted HCV viremia (7). In the present study, we used ROC curve analysis of CIA performance for samples from a population of patients evaluated at the Veterans Affairs Puget Sound Health Care System (VAPSHCS). We propose an algorithm for HCV testing based on anti-HCV S/Co ratios for the veteran population.

Materials and Methods

The clinical laboratory at the Seattle division of the VAPSHCS is responsible for performing anti-HCV and HCV RNA viral load testing for the Anchorage, Boise, Puget Sound, Spokane, and Walla Walla medical centers within the Veterans Integrated Service Network 20. Blood samples were collected into serum separator tubes, lavender (EDTA) tubes, or plasma preparation tubes (BD). Samples were processed by centrifugation at 800g–1600g for 20 min at ambient temperature. Samples for analysis were removed from cells within 6 h and either refrigerated at 2–8 °C for not more than 72 h or frozen at –70 °C before testing. We retrospectively reviewed results from a database of 34 243 patients who were tested at the clinical laboratory for anti-HCV by using the Vitros ECi CIA (Ortho Clinical Diagnostics) from April 1, 2003, to October 21, 2005. The manufacturer defines a positive anti-HCV result as any result with a sample S/Co ratio of ≥ 1.0 . The S/Co ratio for each specimen was obtained by measuring the signal strength produced by the sample divided by the signal strength used as the cutoff value for the specific analytical run. For all patients with positive anti-HCV

results, we reviewed S/Co ratios. For all patients with positive anti-HCV results we also reviewed the results of supplemental HCV RNA testing performed within 30 days of the initial positive anti-HCV testing. Before May 17, 2004, HCV RNA confirmatory testing was performed using the COBAS Amplicor (Roche Diagnostics) with a detection limit of 60 000 IU/L. For the remaining period of study, confirmatory nucleic acid testing was performed by using a quantitative PCR method, for research use only, performed on the COBAS TaqMan (Roche Diagnostics). The reportable range was 60 000 to 10×10^9 IU/L. Assay performance was validated by the VAPSHCS microbiology laboratory. For a period of time after May 2004, results of HCV that were detectable but $<60\,000$ IU/L (below the quantifiable linear range) were reported as indeterminate for clinicians because results at this concentration were considered potentially false positive. Currently, such results would be reported as “HCV RNA detected, $<60\,000$ IU/L.” For all patients with positive anti-HCV results but negative HCV RNA results, we reviewed the results of an additional RIBA HCV 3.0 strip immunoblot assay (Chiron Corporation) performed within 30 days of the initial positive anti-HCV testing. This process of screening HCV antibodies with reflex to HCV RNA by PCR and confirmation of anti-HCV by RIBA is our routine testing approach. However, because of insufficient sample quantity, sample deterioration, sample-processing errors, loss of patients to follow-up, and miscommunication between clinicians and patients, 22% of the projected number of PCR tests and 6% of the RIBA tests were not performed. For patients who had positive anti-HCV screening test results but negative HCV RNA results and negative or indeterminate RIBA results, the positive anti-HCV results were categorized as falsely positive according to the report by Alter et al. (8). In addition, we assumed that all patients whose results were positive according to the PCR assay results would have been positive by RIBA.

Because the S/Co ratio of positive results did not have a normal distribution, we determined the 2.5th, 25th, 50th (median), 75th, and 97.5th percentile values. Diagnostic sensitivity, diagnostic specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for S/Co ratios, and ROC curves were constructed by plotting sensitivity vs 1 – specificity. Optimal S/Co ratios were identified from analyses of ROC curves and associated data. The lower limits of the 95% CI for diagnostic sensitivity, diagnostic specificity, PPV, and NPV were calculated by using the classic Clopper–Pearson binomial CI method (9). For comparison of S/Co ratio with HCV RNA status, diagnostic sensitivity and specificity relate to the ability of CIA to detect all patients who are HCV RNA positive or negative, respectively. For comparison of S/Co ratio



with RIBA status, diagnostic sensitivity and specificity relate to the ability of CIA to detect all patients who are RIBA positive or RIBA negative, respectively.

The human subjects and research and development committees of the VAPSHCS, Seattle, Washington, approved this retrospective study.

Results

Of 34 243 patients tested for anti-HCV using the CIA during a consecutive 30-month period, 3082 patients (9.0%) had positive results (Fig. 1). Of these 3082 patients, 2402 patients had confirmatory HCV RNA testing performed within 30 days of the initial positive anti-HCV testing. Of these 2402 patients, 1554 patients (64.7%), 846 patients (35.2%), and 2 patients (0.1%) tested positive, negative, and indeterminate, respectively, for HCV RNA. Of the 846 patients with negative HCV RNA results, 795 patients had additional RIBA testing performed, with 351 patients (44.2%), 281 patients (35.3%), and 163 patients (20.5%) testing positive, negative, and indeterminate, respectively (Fig. 1). The 2.5th, 25th, 50th (median), 75th, and 97.5th percentile values for the S/Co ratio of positive results were 1, 20, 28, 33, and 41 respectively.

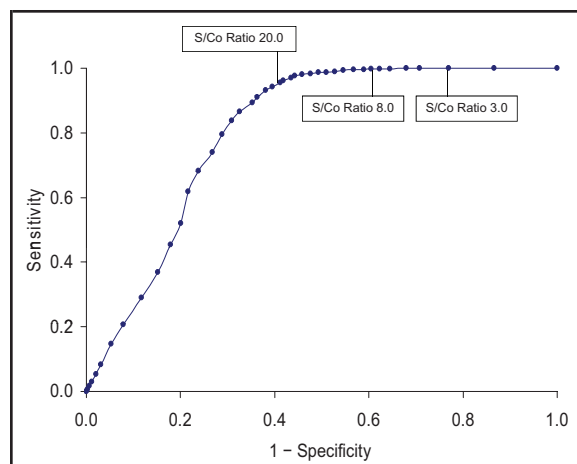


Fig. 2. PCR test ROC curve for different cutoff levels on the CIA for anti-HCV.

The diagnostic sensitivity and specificity (95% CIs) are 100% (99.8%–100%) and 23.0% (20.3%–26.0%) for an S/Co ratio of 3.0, 99.7% (99.3%–99.9%) and 39.5% (36.2%–42.9%) for an S/Co ratio of 8.0, and 95.5% (94.3%–96.5%) and 58.8% (55.5%–62.2%) for an S/Co ratio of 20.0. The area under the curve (95% CI) is 0.806 (0.785–0.827).

We next used ROC-curve analysis for HCV PCR testing to determine optimal S/Co ratios for the prediction of HCV viremia in veteran patients. From the RT-PCR ROC curve (Fig. 2), we identified an S/Co ratio of 20.0 as being optimal on the basis of examination of the curve and associated diagnostic sensitivity, diagnostic specificity, and PPV (see Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol57/issue7>). This S/Co ratio of 20.0 corresponded to a diagnostic sensitivity, diagnostic specificity, PPV, and NPV for HCV viremia of 95.5%, 58.8%, 81.0%, and 87.7%, respectively (Table 1) in our population of veterans. In addition, our data showed an HCV RNA positivity of 81% at an S/Co ratio ≥ 20 , (see online Supplemental Table 2). The diagnostic specificity of 58.8% at an S/Co ratio of 20.0 indicates that among the PCR-negative samples, 58.8% samples had an S/Co ratio < 20.0 and 41.2% had an S/Co ratio ≥ 20.0 . Of the total 846 samples negative according to PCR, 795 samples (474 with S/Co ratio < 20.0 and 321 with S/Co ratio ≥ 20.0) were further tested by RIBA. As shown in online Supplemental Table 3, there were 55 positive and 419 negative or indeterminate RIBA samples with an S/Co ratio < 20.0 , whereas there were 296 positive and 25 negative or indeterminate RIBA samples with an S/Co ratio ≥ 20.0 . We determined the diagnostic sensitivity, diag-

Table 1. Diagnostic performance of CIA in the prediction of viremia by RT-PCR.^a

S/Co ratio	3.0	8.0	20.0
Diagnostic sensitivity, %	100 (99.8–100)	99.7 (99.3–99.9)	95.5 (94.3–96.5)
Diagnostic specificity, %	23.0 (20.3–26.0)	39.5 (36.2–42.9)	58.8 (55.5–62.2)
PPV, %	70.4 (68.5–72.3)	75.1 (73.2–77.0)	81.0 (79.1–82.7)
NPV, %	100 (98.1–100)	98.8 (97.0–99.7)	87.7 (84.7–90.1)

^a Values in parentheses are the limits of 95% CI.

nostic specificity, PPV, and NPV at an S/Co ratio of 8.0. Our values were 99.7%, 39.5%, 75.1%, and 98.8%, respectively for HCV viremia (Table 1).

We also used ROC curve analysis for RIBA testing to determine an S/Co ratio cutoff point for the diagnosis of HCV exposure. From the RIBA ROC curve (Fig. 3) and associated diagnostic sensitivity, diagnostic specificity, and PPV (see online Supplemental Table 4) and taking into account the data for PCR (see online Supplemental Table 1), we decided on an S/Co ratio of 20.0 as the optimal cutoff point for further investigation. An S/Co ratio of 20.0 or higher corresponded to a diagnostic sensitivity, diagnostic specificity, PPV, and NPV of 93.3%, 94.4%, 98.5%, and 77.6%, respectively, for anti-HCV as confirmed by the RIBA (Table 2).

After completing these preliminary steps, we proceeded to define a low S/Co ratio cutoff point with high

diagnostic sensitivity and NPV, such that S/Co ratios below this would most likely represent false positives. From both the RT-PCR (Fig. 2) and the RIBA (Fig. 3) ROC curves, we identified an S/Co ratio of 3.0 to be the highest value with a diagnostic sensitivity of 100% (Table 1 and Table 2). The NPV for negative result of RT-PCR (Table 1) or RIBA (Table 2) at an S/Co ratio of 3.0 was also 100%. These data demonstrate that in our patient population of veterans, who were predominantly male, all patients with an anti-HCV result indicated by an S/Co ratio <3.0 were negative for both HCV viremia and HCV exposure.

Discussion

Accurate and efficient diagnosis of HCV infection among veterans is important. In our current study, we analyzed HCV S/Co ratios by using ROC curves to develop an improved algorithm for HCV testing in our veteran population. Applying ROC curve analyses as previously described by Contreras et al. (6), we determined that the optimal high S/Co ratio cutoff for confirmation of HCV exposure by RIBA testing is 20.0 (Fig. 3). This cutoff has both high diagnostic sensitivity and a specificity of >93% and a very high PPV of nearly 99% (Table 2). The high diagnostic specificity and PPV values suggest that S/Co ratios of 20.0 or higher strongly indicate HCV exposure. In addition, when the S/Co ratio was ≥ 8.0 , the PPV was lower at 93.3% (Table 2). However, on the basis of the higher PPV when the S/Co ratio was ≥ 20.0 in our study, we recommend that, at least for our population of veterans, those with an anti-HCV test result with an S/Co ratio of ≥ 20.0 , rather than ≥ 8.0 , proceed directly to nucleic acid testing to assess HCV viremic status. Supplemental RIBA testing is unnecessary because such high S/Co ratios are confirmed by positive anti-HCV RIBA results $\geq 98\%$ of the time.

We recommend that samples from patients with an anti-HCV test result indicated by an S/Co ratio ≥ 20.0 proceed directly to PCR testing to assess HCV viremic status, particularly because Contreras et al. (7) previously used ROC curve analysis to define an S/Co

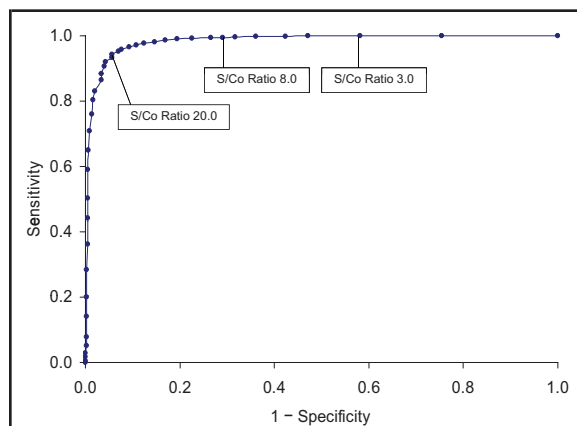


Fig. 3. The RIBA test ROC curve for different cutoff levels on the CIA for anti-HCV.

The diagnostic sensitivity and specificity (95% CIs) are 100% (99.8%–100%) and 41.9% (37.3%–46.6%) for an S/Co ratio of 3.0, 99.5% (99.1%–99.8%) and 70.9% (66.5%–75.1%) for an S/Co ratio of 8.0, and 93.3% (92.1%–94.4%) and 94.4% (91.8%–96.2%) for an S/Co ratio of 20.0. The area under the curve (95% CI) is 0.983 (0.977–0.990).

Table 2. Diagnostic performance of CIA in the prediction of the presence of anti-HCV by RIBA.^a

S/Co ratio	3.0	8.0	20.0
Diagnostic sensitivity, %	100 (99.8–100)	99.5 (99.1–99.8)	93.3 (92.1–94.4)
Diagnostic specificity, %	41.9 (37.3–46.6)	70.9 (66.5–75.1)	94.4 (91.8–96.2)
PPV, %	87.5 (86.0–88.9)	93.3 (92.1–94.4)	98.5 (97.9–99.1)
NPV, %	100 (98.0–100)	97.3 (94.8–98.7)	77.6 (73.8–81.0)

^a Values in parentheses are the limits of 95% CI.

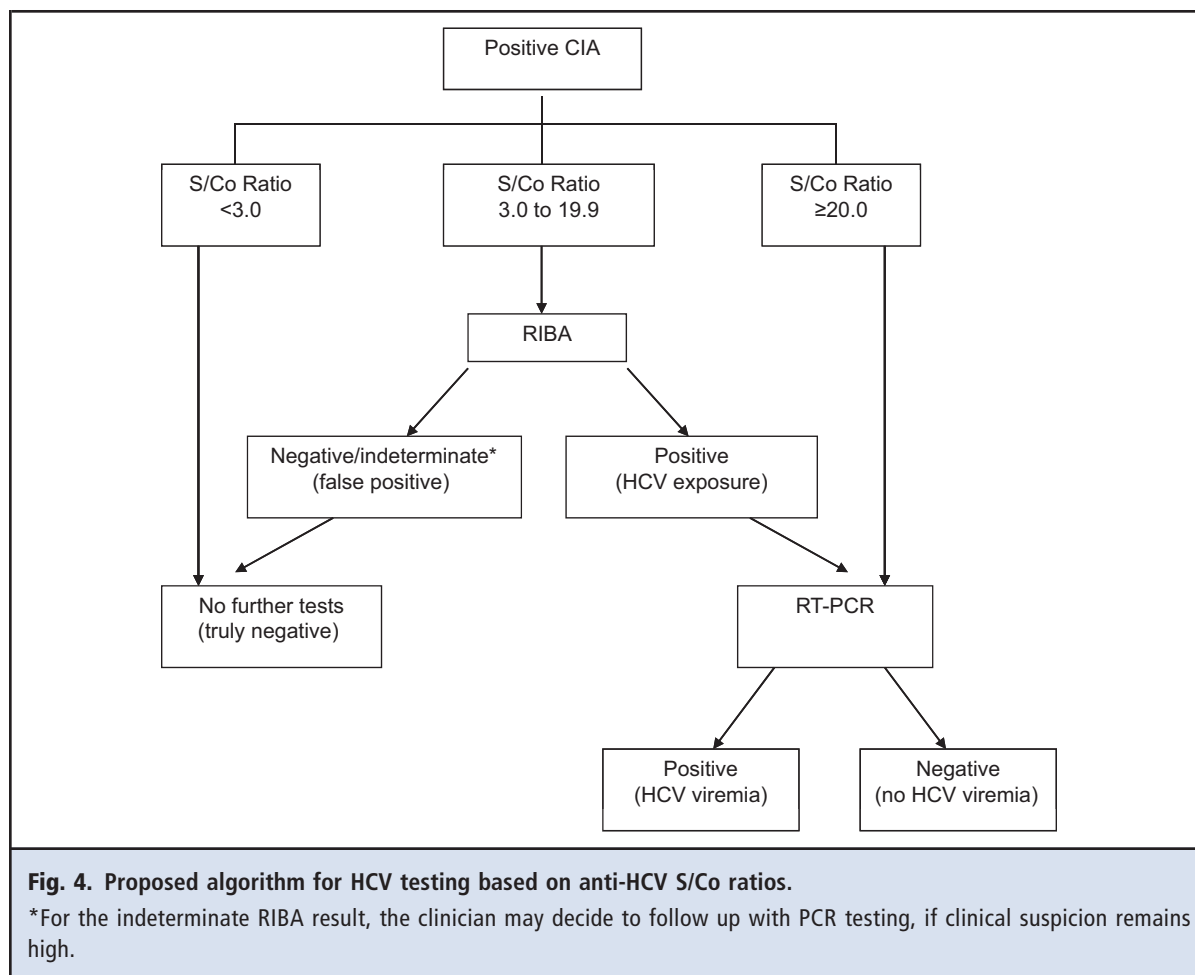
ratio of 20.0 as an optimal predictor of HCV viremia in blood donors. Of note, our values for diagnostic specificity and PPV in predicting HCV viremia at an S/Co ratio of 20 were much lower than the >90% values reported by Contreras et al. (7). The lower diagnostic specificity and PPV in our study suggest that we are identifying a greater proportion of our population who have an S/Co ratio of ≥ 20.0 but do not have HCV viremia as assessed by RT-PCR testing. In a manner similar to Contreras et al. (7), we determined the diagnostic sensitivity, diagnostic specificity, PPV, and NPV in predicting HCV viremia at an S/Co ratio of 8.0. At this S/Co ratio of 8.0, comparable diagnostic sensitivity of 99.7% and NPV of 98.8% were achieved between the Contreras study population (7) and our groups. However, our values for diagnostic specificity and PPV were lower than their values of 85.3% and 77.9%, respectively, which suggests that we identify a greater proportion of our population who have an S/Co ratio of ≥ 8.0 but do not have HCV viremia.

Our results showed that for the 1833 of 2402 samples (76.3%) with an S/Co ratio ≥ 20.0 according to the CIA, 1484 had positive RT-PCR results (81%). Other groups have previously reported RNA positivity rates of 90% (4), 93% (7), 81% (10), and >60% (11), at an S/Co ratio of ≥ 20.0 . These differences might be due to lot-to-lot variation in S/Co ratio as conceptualized by Dufour (11), and it would be helpful for this issue to be addressed in future studies. Most current testing algorithms indicate that RIBA should be performed when RT-PCR is negative. However, if the RIBA tests can be omitted when the S/Co ratio is ≥ 20.0 , of the total of 34 243 samples from veteran patients included in this study, negative RT-PCR results for 348 patients indicated that they would not need further testing by RIBA.

From both the CIA vs RT-PCR (Fig. 2) and the CIA vs RIBA (Fig. 3) ROC curves, we also determined that an S/Co ratio of 3.0 was the highest value associated with a diagnostic sensitivity of 100%, and an NPV of 100% for using either PCR (Table 1) or RIBA (Table 2). Indeed, when the S/Co ratio was <3.0, we observed no positive RIBA or PCR test results. Previously, Oethinger et al. (10) and Contreras et al. (6) also sug-

gested that supplemental testing is not necessary for patients with S/Co ratios below 5.0 and 4.5, respectively, because such supplemental testing was demonstrated to yield 0 positive results for anti-HCV by RIBA testing in 163 samples and 0 positive results for HCV RNA by RT-PCR testing in 83 samples at an S/Co ratio of <5.0 (10), and only 7 positive results for anti-HCV by RIBA testing in 322 samples (2.2%) and 0 positive results for viremia at an S/Co ratio of <4.5 (6). However, such S/Co ratio cutoffs of 4.5 or 5 in our study of veteran patients would miss 3 in 78 CIA-positive patients with S/Co ratios ranging from 3.0 to 5.0 (3.8%), with either PCR positivity ($n = 1$) or RIBA positivity ($n = 2$). In contrast, we would not miss any cases with positive RIBA or RT-PCR if we used an S/Co ratio of 3.0 as the cutoff. For 195 of 2402 CIA results (8.1%) with an S/Co ratio <3.0, no supplemental testing was needed. Thus, we recommend that for patients with an anti-HCV test result with an S/Co ratio <3.0, no supplemental testing is required (Fig. 4). This recommendation will lead to a significant reduction in the number of false-positive results, as well as the number of HCV RNA and RIBA tests performed compared to the prior HCV testing approach in which a result is considered positive at an S/Co ratio of >1.0.

Confirmatory anti-HCV testing by RIBA is not necessary when the S/Co ratio is either <3.0 or ≥ 20.0 , because of the high rate of true negatives and true positives, respectively. For patients with an S/Co ratio ranging from 3.0 to 19.9, the RIBA confirmation test is needed owing to the possible false-positive results by CIA. There are 2 approaches for RIBA testing. The first approach is performing PCR first to detect the presence of HCV viral RNA associated with viremia, and then performing RIBA testing on all patients with negative PCR results to determine if the screening anti-HCV result was a false positive. Many clinical laboratories apply this approach. The second approach is performing RIBA first to confirm the CIA results, and then performing PCR to detect active HCV infection for the patients with positive RIBA results. The order of the PCR and RIBA tests depends on both PCR and RIBA assays with respect to the rates of positive results in a



given patient population, the relative testing costs, and logistical concerns such as in-house availability, shipping costs, and sample handling.

In our study of the veteran population, we found that the rate of PCR positivity was relatively low (19%) when the S/Co ratio was in the range of 3.0–19.9. We therefore recommend supplemental RIBA testing directly after the CIA test. If supplemental RIBA testing is positive, thus confirming HCV exposure, then additional PCR testing should be performed to assess the status of HCV viremia. However, if supplemental RIBA testing is negative or indeterminate, thus suggesting a lack of HCV exposure, then additional PCR testing may be unnecessary. Alternatively, with an indeterminate RIBA result, the clinician may decide to follow up with PCR testing, if clinical suspicion remains high. It is important to consider this testing alternative because in our study we made the assumption that in all patients with PCR-positive results the findings were confirmed as positive by RIBA.

Our fine-tuning of the HCV-testing algorithm with decision making based on the S/Co ratio of the

screening antibody test will not be possible with qualitative anti-HCV testing approaches. For example, in June 2010, the US Food and Drug Administration cleared the OraQuick HCV Rapid Antibody Test (OraSure Technologies) (12). The requirement for a positive result is confirmation of the OraQuick anti-HCV result with supplemental alternative anti-HCV (RIBA) or HCV RNA testing, as we have described in our analysis. Although this device has many advantages with respect to testing, a disadvantage is that this is a qualitative assay and does not provide a discriminating approach to testing as we have described based on the magnitude of the S/Co ratio.

Of note, our result of 65% for the proportion of patients who tested positive for RNA after initially testing positive for anti-HCV by CIA is between the 81% and 57% reported by Dufour et al. (4) and Oethinger et al. (10), respectively. It should be pointed out that in the study by Dufour et al. (4), RIBA was performed on all samples with S/Co ratios <8 , and HCV RNA was not performed if RIBA was negative, which increased

the proportion of samples that were RNA positive in the remaining patients tested.

It should be noted that all of our recommendations are appropriate only for the Vitros assay and are not applicable for other assays. The CDC, on its website (<http://www.cdc.gov/hepatitis/HCV/LabTesting.htm>), gives cutoff values for low positive for all of the currently available assays (13). Whereas the CDC guidelines identify a Vitros assay S/Co ratio of 8.0 to be predictive of a true antibody-positive result $\geq 95\%$ of the time, in our study we selected an S/Co ratio of 20 for a different purpose, namely, to define a cutoff above which RIBA would almost never be negative.

Our proposed algorithm for HCV testing based on anti-HCV S/Co ratios is summarized in Fig. 4. If the S/Co ratio is < 3.0 or ≥ 20.0 , then a supplementary RIBA test is not necessary to confirm the negative or positive results, respectively. If the S/Co ratio is within the range of 3.0–19.9, then the anti-HCV CIA results should be confirmed by the RIBA test unless PCR testing is performed and the results are positive. Anti-HCV results with an S/Co ratio of ≥ 20.0 on the CIA or confirmed by RIBA when the S/Co-ratio results are in the range of 3.0–19.9 should be further investigated with a PCR test to assess for the presence of HCV viremia. The use of this diagnostic approach should improve the accuracy and efficiency of HCV testing.

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