Automated RIBA Hepatitis C Virus (HCV) Strip Immunoblot Assay for Reproducible HCV Diagnosis

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A comparison between the CHIRON RIBA hepatitis C virus (HCV) processor and manual systems was performed by using 88 specimens repeatedly reactive by the second-generation HCV enzyme-linked immunosorbent assay (ELISA) (HCV 2.0 ELISA) and 111 random specimens from volunteer donors. For the second-generation RIBA HCV strip immunoblot assay (SIA) (RIBA HCV 2.0 SIA), test results correlated strongly between the manual and the automated runs (kappa value, 0.937). For the RIBA HCV 3.0 SIA, the correlation of the test results was also high (kappa value, 0.899). Among the specimens with positive results by RIBA HCV 2.0 and 3.0 SIAs, there was a very strong concordance of the test results between the manual and the automated runs with regard to the reactive bands. Nine samples had discordant results between the manual and the automated runs; this was probably attributable to increased variability in antigen scores close to the cutoff values for both tests. Run-to-run and within-run testing by the CHIRON RIBA HCV Processor System showed a very low rate of conflicting values. In conclusion, the CHIRON RIBA HCV Processor System is capable of performing RIBA HCV 2.0 and 3.0 SIAs accurately with minimal operator involvement. In addition, the CHIRON RIBA HCV Processor System shows excellent reproducibility, with the potential for operator-to-operator and site-to-site variability being greatly reduced. Our data indicate that this novel methodology may be very useful for supplemental anti-HCV testing of specimens repeatedly reactive by ELISA in routine clinical assessments and epidemiologic evaluations.

The discovery of the genome (4) sequence of hepatitis C virus (HCV), the causative agent of non-A, non-B hepatitis (NANBH) (1, 2), has resulted in the development of a variety of diagnostic assays for HCV antibodies (9). The earliest anti-HCV assays had important limitations, notably, a high rate of false-positive and false-negative results (12). To increase both sensitivity and specificity, in these diagnostic assays a greater number of HCV-encoded antigens are now included, allowing more specific antibody detection. Second-generation and now third-generation enzyme-linked immunosorbent assays (ELISAs) (ELISA-2 and ELISA-3, respectively) are also available. The anti-HCV ELISA-3 includes antigens coded by the putative core and NS3, NS4, and NS5 regions of the HCV genome. ELISA-3 is widely used to screen donor blood (11, 16), and despite its high specificity (99.7%), false-positive results may occur (14). The positive predictive value of the test depends on the prevalence of HCV antibodies in the donor population, which is very low. In view of this, anti-HCV ELISA reactivity should be tested with a supplemental assay. Recombinant immunoblot assays (15) (RIBA; Chiron Corporation) and synthetic peptide assays (Inno-Lia; Innogenetics) (7) have been developed as supplemental tests for discriminating between true- and false-positive results for samples repeatedly reactive by ELISAs (12). The second- and third-generation RIBA HCV strip immunoblot assays (SIAs) (HCV 2.0 and 3.0 SIAs, respectively) are established methods for supplemental testing of samples repeatedly reactive by HCV ELISAs. Since 1992, the third-generation RIBA system (RIBA HCV 3.0 SIA) has been widely used in Europe for supplementary testing of samples

repeatably reactive by HCV ELISA. The RIBA HCV 3.0 SIA has four significant differences from its predecessor (RIBA HCV 2.0 SIA): it includes a recombinant antigen from the NS5 region, the recombinant c22-3 antigen is replaced by a four-epitope core synthetic peptide c22(p), the 5-1-1 and c100-3 recombinant antigens are replaced by a mixture of synthetic peptides c100(p), and more c33-c recombinant antigen is used. Various investigators have shown that this new combination of antigens has greater specificity and sensitivity than its predecessor (3, 5, 8, 13, 17).

However, the current manual procedures in the RIBA HCV 2.0 and 3.0 SIAs are labor-intensive. Both band scoring and result interpretation are subjective. A CHIRON RIBA HCV Processor System has now been developed to automate supplemental testing for HCV. The bench-top instrument provides objective strip scoring and result interpretation; it can process up to 28 specimens plus controls per run and offers walkaway operational capability.

The purpose of this study was to evaluate the accuracy of the CHIRON RIBA HCV Processor System. We compared the RIBA HCV 2.0 and .0 SIAs performed with the CHIRON RIBA HCV Processor System with those processed manually for a large cohort of specimens. Additionally, the run-to-run and within-run precisions of the CHIRON RIBA HCV Processor System were tested by using a specimen yielding weak to medium reactivities for all RIBA antigens.

(This study was presented in part at the 46th Annual Meeting of the American Association for the Study of Liver Disease, Chicago, Ill. November 1995.)

MATERIALS AND METHODS

Enzyme immunoassays. The ELISA-2 Ortho HCV ELISA system (Ortho Diagnostic Systems, Raritan, N.J.) detects antibodies to a structural antigen (core antigen) and a fusion of the c100-3 and c33-c antigens (c200) of HCV. It

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TABLE 1. Specimens with discordant results by RIBA HCV 2.0 SIA for RIBA Processor System versus manual assay[a]

Specimen	RIBA Processor System						RIBA manual assay					
	Rating (score)					Interp	Rating (score)					Interp
	5-1-1	c100-3	c33-c	c22-3	SOD		5-1-1	c100-3	c33-c	c22-3	SOD	
B	± (0.36)	− (0.05)	± (0.66)	− (0.05)	− (0.00)	NEG	± (0.78)	− (0.07)	2+ (1.18)	− (0.07)	− (0.01)	IND
A	− (0.01)	− (0.01)	− (0.05)	1+ (0.85)	− (0.01)	IND	− (0.04)	− (0.05)	− (0.03)	± (0.59)	− (0.06)	NEG

[a] Ratings: −, 0.00 to 0.09; ±, 0.10 to 0.84; 1+ 0.85 to 1.14; 2+, 1.15 to 2.69. Abbreviations: SOD, superoxide dismutase; Interp, interpretation; NEG, negative; IND, indeterminate.

uses recombinant antigens derived from three regions of the viral genome (core, NS3, and NS4).

RIBA HCV SIAs. The RIBA HCV 2.0 strip consists of a nitrocellulose solid support on which four bands of recombinant HCV proteins (bands 2 to 5), two bands of high-level and low-level immunoglobulin G (IgG) (bands 1 and 7, respectively), and a superoxide dismutase band (band 6) are immobilized. Band 2 contains the 5-1-1 antigen derived from the NS4 region of the HCV genome. Band 3 contains the c100-3 recombinant antigen derived from the NS4 region. Band 4 contains c33-c recombinant antigen derived from the NS3 region of the HCV genome. Band 5 contains a recombinant antigen (c22-3) derived from the core region of the HCV genome. The RIBA HCV 3.0 strip is different from the RIBA HCV 2.0 strip in that in addition to recombinant HCV antigens it contains peptide segments of the HCV genome. In the RIBA HCV 3.0 strip the distribution of the bands is as follows. Band 2 contains two synthetic peptides, 5-1-1(p) and c100(p), from the NS4 region of the HCV genome. Band 3 contains c33-c recombinant antigen derived from the NS3 region. Band 4 contains c22(p) from the core portion of HCV. Band 5 contains NS5 region of the HCV genome.

The procedures for manual and automated RIBA HCV SIAs were as follows: 20 microliters of each specimen in which the presence or absence of HCV is to be confirmed was added to a tube containing a RIBA HCV 2.0 or 3.0 strip and 1 ml of RIBA HCV 2.0 or 3.0 SIA specimen diluent. The next step was a 4-h incubation with the specimen at room temperature, decantation, a 30-min incubation in specimen diluent, two washes in wash buffer, a 10-min incubation in conjugate (peroxidase-labeled goat anti-human IgG [heavy and light chains]) followed by three washes in wash buffer, and a 15-min incubation in substrate (4-chloro-1-naphthol) followed by two washes with deionized water.

Algorithm for supplemental testing of HCV by RIBA HCV SIA. Anti-HCV reactivity in a specimen is determined by comparing the intensity of each band to the intensity of the human IgG (level I and level II) internal control band on each strip. The identities of the antibodies are defined by the specified location of the antigen band on the strip in the kit. The intensity of the antigen and peptide bands is scored in relation to the intensities of the internal IgG controls according to the manufacturer's recommendations. A negative, indeterminate, or positive result is based on the reaction pattern present on the strip. Interpretation of the results was performed according to the manufacturer's instructions.

CHIRON RIBA HCV Processor System. The CHIRON RIBA HCV Processor System measures the intensities of the control and antigen bands of an immunoblot strip on which there are four bands of immobilized recombinant antigens and/or synthetic peptides. It illuminates the strip and creates a density of reflectance, measuring the light differentially reflected from the developed bands and white background. An algorithm interpolates relative intensity values for antigen band reactivity, with constants assigned to the control bands.

Study samples. A comparison of RIBA HCV 2.0 and 3.0 SIA results between the processor and the manual runs was performed with a cohort of 199 specimens. There were 111 random serum samples from presumably healthy blood donors and 88 specimens repeatedly reactive by anti-HCV ELISA-2. Among the anti-HCV ELISA-2-reactive specimens, 4 serum samples were from patients with acute NANBH, 33 serum samples were from patients with chronic NANBH, 26 samples were from 12 patients with posttransfusion hepatitis C, 19 serum samples were from chronic hemodialysis patients, and 6 serum samples were from 4 chronic hemodialysis patients showing seroconversion for HCV infection.

CHIRON RIBA HCV Processor System and RIBA HCV SIA reproducibility testing. RIBA HCV 2.0 SIA reproducibility testing was performed with 109 samples; each specimen was tested by the CHIRON RIBA HCV Processor System in four runs. RIBA HCV 3.0 SIA reproducibility testing was performed with 168 specimens; each sample was tested by the CHIRON RIBA HCV Processor System in six runs. Material of the same lot was used for the RIBA HCV 2.0 and 3.0 SIA reproducibility testing.

Statistical analysis. The agreement between the reactivity to HCV antigens shown by the CHIRON RIBA HCV Processor System versus that shown by the RIBA manual assay was measured by using kappa statistics (10). The statistical package JMP IN, version 3.1.7 for MacIntosh (SAS Institute Inc.), was used.

RESULTS

RIBA HCV 2.0 SIA: automated versus manual testing. For the RIBA HCV 2.0 SIA the correlation of all test results between the manual and the automated assays was 98.9% (197 of 199). The kappa value was very high (0.937). Forty-nine were specimens positive by the manual and the automated assays; 22 samples showed indeterminate results by the manual technology and by testing with the RIBA Processor System. One-hundred twenty-six samples gave negative results. Two samples had discordant results between the two procedures. Both of them tested negative by PCR. The first sample (Table 1, specimen A) was negative by the manual RIBA HCV 2.0 SIA but indeterminate by the automated RIBA HCV 2.0 SIA. The conflicting result concerned band c22-3. The second sample (Table 1, specimen B) was indeterminate by the RIBA manual assay and negative by testing with the CHIRON RIBA HCV Processor System, with band c33-c having conflicting results. The discordant results were due to increased variability of antigen scores close to the cutoff values for both tests.

Among the specimens positive by RIBA HCV 2.0 SIA, there was a strong correlation between manual and automated runs with regard to the number of reactive bands: 96% (46 of 49) of samples positive by RIBA HCV 2.0 SIA showed bands with concordant reactivities. Two specimens (specimens C and D) showed two reactive bands by the manual procedure and three reactive bands by the automated methodology. One sample (specimen E) had three reactive bands by the manual protocol and two reactive bands by testing with the CHIRON RIBA HCV Processor System.

RIBA HCV 3.0 SIA: automated versus manual testing. For the RIBA HCV 3.0 SIA the correlation of test results was 96.5% (192 of 199) between runs performed with the CHIRON RIBA HCV Processor System and manually. The kappa value was high (0.899). Sixty-five samples were positive by the manual assay and by testing with the CHIRON RIBA HCV Processor System; nine specimens gave indeterminate reactivities by the manual and the automated technologies. One hundred eighteen specimens had negative results. Seven samples generated conflicting results (Table 2). Four samples were indeterminate by the manual RIBA HCV 3.0 SIA but tested positive by testing with the CHIRON RIBA HCV Processor System. The discordant samples concerned 5-1-1(p) c100(p) bands (specimen F), the c22(p) band (specimens G and H), and the c33-c band (specimen I). Specimens G and I were positive by PCR, and specimens F and H were negative by PCR. Two samples were negative by manual RIBA HCV 3.0 SIA and gave indeterminate reactivities by testing with the CHIRON RIBA HCV Processor System. The conflicting results involved the c33-c (specimen L) and the c22(p) (specimen M) bands. One specimen (specimen N) was positive by the RIBA manual assay and gave indeterminate results by testing with the CHIRON RIBA HCV Processor System. The discord-

TABLE 2. Specimens with discordant results by RIBA HCV 3.0 SIA for RIBA Processor System versus manual assay[a]

Specimen	RIBA Processor System						RIBA manual assay					
	Rating (score)					Interp	Rating (score)					Interp
	5-1-1(p) and c100 (p)	c33-c	c22(p)	NS5	SOD		5-1-1(p) and c100 (p)	c33-c	c22(p)	NS5	SOD	
F	1+ (0.88)	3+ (3.09)	− (0.06)	± (0.37)	− (0.01)	POS	± (0.34)	4+ (3.48)	− (0.02)	± (0.12)	− (0.00)	IND
H	± (0.72)	2+ (1.63)	1+ (1.05)	− (0.01)	− (0.00)	POS	± (0.58)	2+ (1.66)	± (0.80)	− (0.04)	− (0.02)	IND
G	± (0.35)	4+ (5.38)	1+ (0.95)	− (0.09)	− (0.00)	POS	± (0.23)	4+ (5.07)	± (0.59)	− (0.05)	− (0.02)	IND
L	− (0.03)	1+ (0.92)	± (0.36)	− (0.05)	− (0.06)	IND	− (0.00)	± (0.61)	± (0.11)	− (0.03)	− (0.02)	NEG
I	± (0.89)	1+ (0.85)	± (0.39)	1+ (1.07)	− (0.00)	POS	± (0.38)	± (0.53)	± (0.37)	1+ (0.95)	− (0.00)	IND
M	− (0.02)	± (0.57)	2+ (1.15)	− (0.02)	− (0.01)	IND	− (0.02)	± (0.36)	± (0.83)	− (0.00)	− (0.02)	NEG
N	− (0.04)	± (0.76)	4+ (5.61)	− (0.03)	− (0.02)	IND	− (0.02)	1+ (0.94)	4+ (4.84)	− (0.00)	− (0.01)	POS

[a] Ratings: −, 0.00 to 0.09; ±, 0.10 to 0.84; 1+, 0.85 to 1.14; 2+, 1.15 to 2.69; 3+, 2.70 to 3.29; 4+, >3.29. Abbreviations: SOD; superoxide dismutase; Interp, interpretation; POS, positive; IND, indeterminate; NEG, negative.

ant result involved the c33-c band. Specimens M and N were found to be PCR positive; sample L gave negative results by PCR. The discordant results between the two tests were related to increased variability of antigen scores close to the cutoff values for both tests.

Among specimens positive by RIBA HCV 3.0 SIA, there was a strong correlation between the manual and the automated runs with regard to the number of reactive bands: 92% (60 of 65) of positive samples gave bands with concordant reactivities. Three positive specimens (specimens O, P, and Q) showed three reactive bands with the automated methodology and two reactive bands by the manual procedure; one specimen (specimen R) showed four reactive bands by testing with the CHIRON RIBA HCV Processor System and two reactive bands by the manual assay; a fifth specimen (specimen S) had four reactive bands by testing with the CHIRON RIBA HCV Processor System and three bands by the manual methodology.

CHIRON RIBA HCV Processor System and RIBA HCV SIA reproducibility testing. For the RIBA HCV 2.0 SIA, the precisions of assays run on the CHIRON RIBA HCV Processor System for the reactive HCV antigen bands were <16% (range, 7 to 15.9%) for within-run and run-to-run testing of a single specimen. The mean relative intensity values, standard deviation, and coefficient of variation for each of four RIBA HCV 2.0 SIA-antigenic bands are reported in Table 3.

For the RIBA HCV 3.0 SIA, the precision of assays run on the CHIRON RIBA HCV Processor System for the reactive HCV antigen bands was <10% (range, 5.2 to 9.6%) for within-run and run-to-run testing of a single specimen. The mean relative intensity values, standard deviation, and coefficient of variation for each of four RIBA HCV 3.0 SIA antigenic bands are presented in Table 4.

DISCUSSION

RIBA HCV 2.0 and 3.0 SIAs have been established as supplemental assays for discriminating between true- and false-positive specimens which are repeatedly reactive in anti-HCV screening ELISAs. RIBA HCV SIAs are labor-intensive and require subjective scoring of strips and interpretation of results. However, the RIBA methodology lends itself to automation, as reported previously (6), and with automation, it can be used to test large numbers of patient specimens. Recently, a CHIRON RIBA HCV Processor System has been developed for automated supplemental testing for HCV.

We describe here the application of this automated technique for supplemental testing of samples for HCV infection.

The RIBA HCV SIA methodology detects serum antibodies to individual recombinant antigens or synthetic peptides. Individual HCV proteins or peptides are immobilized on a nitrocellulose strip, where they react with anti-HCV antibodies if the antibodies are present in the patient's serum. If bound conjugate is present, a colorimetric enzyme detection system will produce an insoluble blue-black reaction product at each specific HCV antigen, peptide, or control band. For supplemental testing for HCV, the RIBA strip is then interpreted by the CHIRON RIBA HCV Processor System, an instrument which illuminates the strip, measures the light differentially reflected from the developed bands and the white background, and determines the intensities of the reactive bands in relation to the intensities of the internal control bands on each strip. The relative intensity values for antigen band reactivity are scored by the CHIRON RIBA HCV Processor System, which uses the value assigned to the control bands. The CHIRON RIBA HCV Processor System is capable of performing the RIBA HCV 2.0 and HCV 3.0 SIAs with minimal operator involvement. It provides an objective evaluation of band scoring and interpretation of results and offers a walkaway operational capability.

To evaluate the accuracy of the CHIRON RIBA HCV Processor System in performing supplemental testing by the RIBA HCV 2.0 and 3.0 SIAs, we compared the results obtained with the CHIRON RIBA HCV Processor System with those obtained by the manual procedure with specimens from presumably healthy blood donors and from patients serologically or clinically confirmed as having HCV infection. For the RIBA HCV 2.0 SIA, we observed a very high correlation between the manual and the automated procedures (kappa value, 0.937). For the RIBA HCV 3.0 SIA, the correlation of test results between the manual and automated tests was also high (kappa

TABLE 3. RIBA HCV 2.0 SIA precision study[a]

Antigen	Relative intensity value (mean ± SD)	Coefficient of variation (%)
5-1-1	1.54 ± 0.16	10.45
c100-3	1.65 ± 0.16	9.71
c33-c	2.48 ± 0.31	12.48
c22-3	1.35 ± 0.16	11.58
SOD[b]	0.01 ± 0.01	80.47

[a] A total of 109 specimens were studied.
[b] SOD, superoxide dismutase.

TABLE 4. RIBA HCV 3.0 SIA precision study[a]

Antigen(s)	Relative intensity value (mean ± SD)	Coefficient of variation (%)
c100(p) and 5-1-1(p)	1.90 ± 0.15	7.83
c33-c	2.54 ± 0.19	7.56
c22(p)	2.18 ± 0.15	6.71
NS5	1.90 ± 0.15	8.01
SOD[b]	0.02 ± 0.01	76.86

[a] A total of 168 specimens were studied.
[b] SOD, superoxide dismutase.

value, 0.899). The discordant results between the two procedures were likely related to the increased variability of antigen scores close to the cutoff values for both tests. Moreover, we found a very strong correlation between manual and automated runs regarding the number of reactive bands in samples positive by the RIBA HCV 2.0 and 3.0 SIAs. Ninety-three percent (111 of 119) of specimens positive by the RIBA HCV 2.0 and 3.0 SIAs showed bands with concordant reactivities between the manual and the automated procedures.

The run-to-run and within-run precisions offered by the CHIRON RIBA HCV Processor System were excellent. For reproducibility testing, a specimen was tested in several replicate runs, and the coefficients of variation were less than 16 and 10% for the RIBA HCV 2.0 and 3.0 SIAs, respectively. Thus, the CHIRON RIBA HCV Processor System's results were highly reproducible.

At present, the serological diagnosis of antibody against HCV remains the most common method of assessing HCV infection. Although a positive result by ELISA and RIBA HCV SIA indicates active HCV infection, it does not distinguish between current infection and previous exposure. Direct detection of HCV would be more useful. However, methodologies aimed at the direct detection of the HCV genome, such as PCR, are expensive and laborious and need appropriate standardization (18). Commercially available kits allowing HCV RNA detection have only recently been introduced.

In conclusion, (i) the CHIRON RIBA HCV Processor System proved to be capable of performing RIBA HCV 2.0 and HCV 3.0 SIAs with minimal operator involvement, was able to objectively interpret the results, and had a walkaway capability. (ii) The CHIRON RIBA HCV Processor System was substantially equivalent to the manually performed assay; for the RIBA HCV 2.0 and 3.0 SIAs, we found a very strong correlation between the results of the manual and the automated runs. The few discordant results between the two procedures were due to increased variability of antigen scores close to the cutoff values for both tests. (iii) The CHIRON RIBA HCV Processor System offers excellent reproducibility, and the potential for operator-to-operator and site-to-site variabilities is greatly reduced.

The CHIRON RIBA HCV Processor System is a very useful tool for confirming HCV-positive results obtained by ELISA in routine clinical assessments and in epidemiological surveys.

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