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Analysis of Sera Indeterminate by Ortho-HCV RIBA-2 by Using Three Confirmatory Assays for Anti-Hepatitis C Virus Antibody

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The diagnostic performances of three commercially available recombinant immunoblot assays (RIBAs) for anti-hepatitis C virus antibody were evaluated on 50 ORTHO-HCV RIBA-2 (RIBA-2)-indeterminate serum samples. Concordant interpretations were obtained with the three tests in 60% of the samples, with 56% positive, 2% indeterminate, and 2% negative results. Considering test performance in regard to the number of remaining indeterminate results, analyzing sera by RIBA-3, INNO-LIA HCV Ab III, and DECISCAN HCV reduced the number of samples reacting indeterminately to 40, 6, and 8%, respectively. The three serum samples classified as indeterminate in the INNO-LIA HCV Ab III as well as three of four serum samples interpreted as indeterminate in the DECISCAN HCV and 16 of 20 samples classified as indeterminate in the RIBA-3 were hepatitis C virus RNA positive by PCR. This study clearly shows the good performance of the three tests as confirmatory assays compared with that of the RIBA-2. However, according to the manufacturers' criteria of positivity, the INNO-LIA HCV Ab III and DECISCAN HCV appeared to be more suitable than the RIBA-3 for interpreting serum samples found indeterminate in the RIBA-2.

Since the development of the first enzyme-linked immunosorbent assay (ELISA) for the detection of anti-hepatitis C virus (HCV) antibody, the sensitivity and the specificity of subsequent assays (second and, recently, third generation) were progressively improved by the introduction of new HCV protein (1, 2, 7, 8). Recombinant immunoblot assays (RIBAs) were widely used as confirmatory tests. However, approximately 10% of patients (6) with suspected viral liver disease have an indeterminate result in the ORTHO-HCV RIBA-2 (RIBA-2). In these cases, HCV RNA was detectable by reverse transcriptase PCR in 50% of the patients. The commercial availability of several new confirmatory tests for HCV antibody detection provides the opportunity to evaluate serum samples previously considered indeterminate by a second-generation ORTHO RIBA (ORTHO Diagnostic Systems, Chiron Corp., Emeryville, Calif.). These new assay systems use various mixtures of recombinant HCV antigens and synthetic peptides and can detect a broader range of anti-HCV antibodies.

The aim of this study was to analyze, by three new immunoblot assays (RIBA-3, DECISCAN HCV, and INNO-LIA HCV Ab III), 50 samples indeterminate by RIBA-2 in order to determine if the use of these tests reduced the number of indeterminate results.

MATERIALS AND METHODS

Samples. Fifty serum samples reactive with the second-generation ORTHO HCV ELISA and classified as indeterminate in the second-generation RIBA-2 were included in this study. Of these, 42 displayed reactivity to c22 antigen only; 7, to c33c only; and 1, to c100 only. These serum samples were from 19 hemophilic patients, 15 dialysis or kidney transplant patients, 1 medullar allograft recipient, 6 patients with post-transfusion hepatitis, 5 intravenous drug users, and 4 patients without known risk factors for HCV infection. The human immunodeficiency virus status was positive in 18 patients (15 hemophilic patients and 3 intravenous drug users). Twelve patients had elevated alanine aminotransferase (ALT) activity levels. Liver biopsy had been performed in nine cases. Cirrhosis was found in six cases, and chronic persistent hepatitis was found in three cases. HCV-indeterminate sera from blood donors were excluded from this study. All sera were stored at −30°C until use.

Laboratory tests. All samples were tested for anti-HCV antibody by the following three assays: (i) the ORTHO RIBA-3 (Chiron Corp.), which contains synthetic peptides to NS4 (c100), core antigen (c22), and two recombinant antigens to NS3 and NS5 regions; (ii) the INNO-LIA HCV Ab III (Innogenetics NV, Ghent, Belgium), which contains six antigens representing core antigens (C1 and C2) and E2/NS1-, NS3-, NS4-, and NS5-derived proteins; and (iii) the DECISCAN HCV (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France), which is an immunoblotting assay based on two capsid antigens (one recombinant protein [C1] and one synthetic peptide [C2] mimicking immunodominant epitopes from the capsid), one NS3 antigen (recombinant protein), and one NS4 (peptide) antigen (Fig. 1). Interpretation of the assay was according to the manufacturer's instructions. Briefly, RIBA-3 and DECISCAN tests were positive if two or more visible bands were detected. With the RIBA-3, an intensity equal to or greater than a low immunoglobulin G control (rating of 1+) is required, while in the DECISCAN HCV assay a weak reactivity (rating of 0.5) is sufficient. Serum samples reacting with just one antigen were considered indeterminate. With the INNO-LIA HCV Ab III, a sample was considered positive if only one HCV antigen line had a reactivity rating of 2+ or higher or if at least two HCV antigen lines had a minimum reactivity of 1+. A sample is considered indeterminate if it is reactive with only one antigen (less than 2+).

Reverse transcriptase PCR testing was carried out with 0.5

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TABLE 1. Analysis of 50 RIBA-2-indeterminate serum samples by three serological assays and PCR

RIBA-2		ALT level[a]	C1		C2		c22 (RIBA)	E2/NS1 (INNO)	NS3		c33 (RIBA)	NS4		c100 (RIBA)	NS5		PCR[c]
Antigen	Sample no.		DECI	INNO	DECI	INNO			DECI	INNO		DECI	INNO		INNO	RIBA	
c100	1	N															Neg
c33	2	N		±	1+	2+	2+		1+	1+	2+		1+	1+		1+	Neg
	3	N		1+	1+		3+		3+	2+	4+			1+			Pos
	4	4.5N							3+	2+	4+						Pos
	5	N		2+	2+		3+	3+	1+	1+	4+	1+	1+	4+	4+	4+	Pos
	6	N							2+	3+	4+	±		4+			Pos
	7	N							3+	1+	4+		±	1+			Pos
	8	N							1+	1+	4+			±			Pos
c22	9	N	1+	1+	2+	1+	3+		±	±	1+	±	±	±		1+	Pos
	10	1.5N	±	3+	3+	2+	4+		±	±	2+	1+	1+	3+			Pos
	11	N	2+	1+	3+	1+	4+	2+			1+						Pos
	12	1.5N	1+	2+	2+	1+	3+										Neg
	13	N	2+	2+	3+	2+	4+	1+	±		3+	±	±	1+			Pos
	14	N	2+	3+	3+	2+	4+			±	±						Pos
	15	N	2+	3+	3+	3+	4+			±	2+		1+	2+			Pos
	16	nd	1+	2+	2+		3+		±	±	3+		2+	2+			Neg
	17	N	1+	3+	3+	3+	4+				±						Pos
	18	1.5N	±	3+	2+	1+	3+				±		±				Pos
	19	N	2+	2+	3+	2+	4+				2+			2+	±		Pos
	20	N	1+	1+	3+		4+	2+					1+				Pos
	21	N	2+	2+	3+	3+	4+				1+		±	±			Pos
	22	N	1+	2+	3+	2+	4+				1+		±	±			Pos
	23	N	1+		2+	2+	4+		1+		1+	±	±	1+			Pos
	24	1.5N	2+	3+	3+	1+	4+	±			±						Pos
	25	2N	2+	2+	3+	2+	4+				±			±			Pos
	26	1.5N	3+	2+	3+	2+	4+	1+	±		1+		1+	1+	±	±	Neg
	27	N		3+	1+	±	3+										Neg
	28	N	2+	3+	3+	2+	4+	1+			1+			±	1+	±	Neg
	29	1.5N	2+	2+	3+	2+	4+				1+			±			Pos
	30	N	2+	2+	3+	2+	4+				±		±	±			Pos
	31	N	3+	3+	3+	2+	4+	1+		±	3+		1+	3+	3+	3+	Pos
	32	N	2+	3+	3+	2+	4+				4+	1+	±	2+	1+		Neg
	33	N	1+	2+	2+	2+	4+				±			±			Neg
	34	N	2+	2+	3+	2+	4+				3+		±	1+			Pos
	35	N	3+	3+	3+	3+	4+		±	±	2+		±	1+	1+	±	Neg
	36	N	1+	3+	3+	±	4+	2+									Pos
	37	2N	1+	3+	2+	2+	4+				±				1+		Neg
	38	N	2+	3+	3+	2+	4+	2+			1+		±	±			Pos
	39	N	2+	3+	3+	3+	4+		±	2+	1+						Pos
	40	N	1+	±	2+		4+										Pos
	41	N	3+	4+	3+	4+	4+	±	1+	1+	2+	2+	2+	3+		1+	Neg
	42	N	2+	4+	3+	3+	4+	1+					±				Pos
	43	4.5N	2+	1+	3+	1+	4+		±	±	1+	±	1+	1+		±	Pos
	44	N	3+	2+	3+	2+	4+		1+	1+	2+						Pos
	45	2N	3+	2+	3+	2+	4+										Pos
	46	2N	2+	2+	3+	2+	4+		1+	1+	±		±	1+	1+		Neg
	47	N	2+	2+	3+	3+	3+		1+	±	±			±	4+		Pos
	48	N	2+	3+	3+	3+	4+	3+	±		2+			±			Pos
	49	N	3+	2+	3+	2+	4+				±			±			Pos
	50	N	2+	3+	3+	2+	4+	1+			±						Pos

[a] ALT, serum ALT levels in time of upper limits of normal (N).
[b] DECI, DECISCAN HCV; INNO, = INNO-LIA HCV Ab III; RIBA, RIBA-3.
[c] Pos, positive; Neg, negative.

ml of serum, using primers directed against the 5′-noncoding region of the HCV genome, as previously described (4). We modified our previous method by using only one amplification step with our outer primers. We used the same annealing temperature (58°C), but we increased the number of cycles to 40. The antisense primer was biotinylated, and the biotinylated amplified product was captured on the surface of streptavidin-coated microplates, alkaline denatured, and hybridized with a digoxigenin-labeled probe (5′-TAC TAA CgC CAT ggC TAg-3′). The hybridized digoxigenin-labeled probe was detected by an anti-digoxigenin alkaline phosphatase-coupled antibody (obtained from Boehringer), chromogenic substrate, and optical density (OD) reading at 405 nm. The sensitivity and specificity of this PCR technique have been validated in a quality control study (5).

Statistical analysis. Student's *t* test was used to compare the

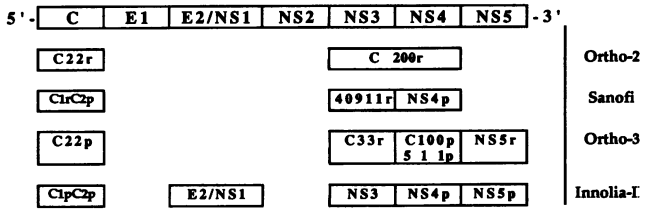

FIG. 1. Epitope map of the recombinant (r) and synthetic peptide (p) antigens tested in the different confirmatory anti-HCV antibody assays used in this study.

differences among the results. A P value of <0.05 was considered significant.

RESULTS

First, we compared the results obtained with the three confirmatory HCV antibody assays and interpreted them as recommended by the manufacturers (Table 1). For thirty (60%) serum samples concordant results by the three confirmatory tests were obtained, with 28 positive, 1 negative, and 1 indeterminate serum sample. For the remaining 20 (40%) samples, discordant results were obtained (Table 2). Among them, 16 (80%) serum samples were found positive by both INNO-LIA HCV Ab III and DECISCAN HCV tests but indeterminate by the RIBA-3. The remaining four serum samples were found positive in only one test and indeterminate by the other two tests. We tried to evaluated the performance of each test in regard to the indeterminate results. Of the 50 RIBA-2-indeterminate samples tested in the RIBA-3 (Table 3), 29 (58%) were classified as positive, 20 (40%) were indeterminate, and 1 (2%) was negative. All sera reactive to c33 (7 of 50 samples) or c22 (42 of 50 samples) in the RIBA-2 were also reactive to these antigens in the RIBA-3. Five of seven serum samples which displayed reactivity against c33 only in the RIBA-2 became positive in the RIBA-3 because of c22 and/or c100 reactivity. Among the 42 serum samples which displayed reactivity against c22 only in the RIBA-2, 23 reacted also with c33 and 14 (33%) exhibited a reactivity against c100 in the RIBA-3. Only five samples reacted with NS5 in the RIBA-3. All 50 RIBA-2-indeterminate samples were tested in the INNO-LIA HCV Ab III, which resulted in 46 positive, 3 indeterminate (6%), and 1 negative sample. Comparable results were obtained in the DECISCAN HCV (45 positive, 4 indeterminate, and 1 negative sample). There were no serum samples classified as positive by the RIBA-3 and the INNO-LIA HCV Ab III because of NS5 band reactivity or, in the INNO-LIA HCV Ab III, because of E2/NS1-band-only reactivity. The seven samples which displayed reactivity against c33 only in the RIBA-2 also exhibited NS3 band reactivity in the INNO-LIA HCV Ab III and DECISCAN HCV. Also, the 42 serum samples with c22 reactivity alone in the RIBA-2 were found reactive with at least one of the two antigens from the core region (C1 and/or C2).

TABLE 2. Discordant results obtained with the three confirmatory assays

Sample no.	Result[a] with given test			
	DECISCAN HCV	RIBA-3	INNO-LIA HCV Ab III	HCV RNA
4	Ind	Ind	+	+
7	Ind	+	Ind	+
12	+	Ind	+	−
14	+	Ind	+	+
17	+	Ind	+	+
18	+	Ind	+	+
20	+	Ind	+	+
24	+	Ind	+	+
25	+	Ind	+	+
27	Ind	Ind	+	−
30	+	Ind	+	+
33	+	Ind	+	−
36	+	Ind	+	+
37	+	Ind	+	−
40	+	Ind	Ind	+
42	+	Ind	+	+
45	+	Ind	+	+
47	+	Ind	+	+
49	+	Ind	+	+
50	+	Ind	+	+

[a] Ind, indeterminate; +, positive.

TABLE 3. RIBA-3 patterns in 50 RIBA-2 indeterminate serum samples

Antigen	Sample no.	Reactivity pattern				Result
		c100-3	c33	c22	NS5	
c100-3	1	−	−	−	−	Negative
c33	2	+	+	+	+	Positive
	1	+	+	+	−	Positive
	2	+	+	−	−	Positive
	2	−	+	−	−	Indeterminate
c22	2	+	+	+	+	Positive
	11	+	+	+	−	Positive
	1	−	+	+	+	Positive
	9	−	+	+	−	Positive
	1	+	−	+	−	Positive
	18	−	−	+	−	Indeterminate

All of the 50 serum samples were further tested for the presence of HCV RNA by reverse transcriptase PCR (Table 4). Thirty-seven (74%) samples were found positive. Twenty PCR-positive serum samples were confirmed anti-HCV positive and one sample (no. 8) was found indeterminate in all three confirmation assays. The 16 remaining PCR-positive serum samples gave either positive (RIBA-3 = 1; INNO-LIA HCV Ab III = 14; DECISCAN = 14) or indeterminate (RIBA-3 = 15; INNO-LIA HCV Ab III = 2; DECISCAN = 2) results in the confirmation assays. Among the 16 serum samples indeterminate by the RIBA-3 and positive by the two other tests, 13 also were shown to be HCV RNA positive by PCR. The remaining three serum samples indeterminate by the RIBA-3, of which two (no. 4 and 27) were also indeterminate by DECISCAN HCV and the other (no. 40) was indeterminate by the INNO-LIA HCV Ab III, were PCR positive, negative, and positive, respectively. Thus, the 3 samples classified as indeterminate in the INNO-LIA HCV Ab III, as well as 3 of 4 serum samples interpreted as indeterminate in the DECISCAN HCV and 16 of 20 serum samples classified as indeterminate in the RIBA-3, were HCV RNA positive by PCR.

The only sample exhibiting a c100 band alone in the RIBA-2 (no. 1) and found negative by the three confirmatory tests was also shown to be HCV RNA negative by PCR; in addition, the patient had no clinical or biological evidence of hepatitis. Among the remaining 12 HCV RNA PCR-negative samples, 8

TABLE 4. HCV antibody testing by the three confirmatory assays and HCV RNA testing by PCR in 50 RIBA-2-indeterminate serum samples

PCR result	No. of samples with given result by:									Total
	RIBA-3			DECISCAN HCV			INNO-LIA HCV Ab III			
	Positive	Negative	Indeterminate	Positive	Negative	Indeterminate	Positive	Negative	Indeterminate	
Positive	21 (20)[a]	0	16 (1)	34 (20)	0	3 (1)	34 (20)	0	3 (1)	37 (21)
Negative	8 (8)	1 (1)	4	11 (8)	1 (1)	1	12 (8)	1 (1)	0	13 (9)

[a] Number in parentheses is the number of concordant results in the three tests.

were classified as anti-HCV positive by all confirmatory tests and 4 were either positive (RIBA-3 = 0; INNO-LIA HCV Ab III = 4; DECISCAN = 3) or indeterminate (RIBA-3 = 4; INNO-LIA HCV Ab III = 0; DECISCAN = 1) in all three confirmatory tests.

Recently, Perrons and Garson (9) suggested scoring reactivities against 5-1-1 and c100 as indeterminate rather than anti-HCV antibody positive. In the same way, it does not seem logical to consider reactivity against C1 and C2 (which are included in the DECISCAN HCV and INNO-LIA HCV Ab III) as if it was a reactivity against two independent HCV antigens. Considering C1 plus C2 as one antigen, among the 50 RIBA-2-inderminate serum samples tested, 10 were found positive and 39 were found indeterminate (78%) in the DECISCAN HCV, while 45 remained persistently positive and 4 remained indeterminate (8%) in the INNO-LIA HCV Ab III.

The ELISA OD readings of all tested serum samples were correlated with PCR positivity ($P = 0.011$); in contrast, no relationship was found between OD reading and confirmatory assay positivity, ALT elevation, and human immunodeficiency virus or immune status. The results of samples from the immunocompromised patients ($n = 34$) were analyzed separately and compared with those of samples from the immunocompetent patients ($n = 15$). As shown in Table 5, there was no significant difference between the two groups.

DISCUSSION

This study shows clearly the good performance of the three confirmatory assays tested compared with that of the RIBA-2. As previously reported, the adjunction of either HCV-recombinant antigens or HCV-synthetic peptides has improved the specificity and sensitivity of new tests (1, 2). Despite the fact that these assays used various mixtures of HCV antigens, concordant interpretations were obtained in 60% (30 of 50) of the cases, with 56% (28 of 50) positive, 2% (1 of 50) indeterminate, and 2% (1 of 50) negative results. Serum 1, which was found negative by the three confirmatory assays, probably exhibited a false reactivity against c100 in the RIBA-2. In 40% (20 of 50) of the cases, discordant results were observed with the three tests. However, 16 of these serum samples were found positive by both DECISCAN HCV and INNO-LIA HCV Ab III tests but indeterminate by the RIBA-3. Thus, concordant results were obtained with the first two tests in 47 of 50 (94%) serum samples. Considering the performance of the tests in regard to the number of remaining indeterminate results, analyzing 50 RIBA-2-indeterminate serum samples by the RIBA-3, INNO-LIA HCV Ab III, and DECISCAN HCV reduced the number of samples reacting as indeterminate to 20 (40%), 3 (6%), and 4 (8%), respectively. Of the 21 serum samples which were indeterminate by at least one of the three tests, 17 (80.9%) were confirmed to be positive for HCV RNA by PCR. Among these, 14 were also found positive in the INNO-LIA HCV Ab III and 13 were positive in the DECISCAN HCV and could be regarded as true positives. The remaining four serum samples found to be HCV RNA negative by PCR (no. 12, 27, 33, and 37) were, respectively, indeterminate in the RIBA-3, positive in the INNO-LIA HCV Ab III, and positive in the DECISCAN HCV, except one sample also found indeterminate by the last assay. This serum sample was obtained from a hemophilic patient with a normal transaminase level. However, in the absence of true gold standards to define the HCV status of each patient (because of the possibility of virus variants), the question of the specificity of each test should be addressed with caution. Furthermore, we cannot avoid a sample bias since more than two-thirds of our patients were immunocompromised.

TABLE 5. Results of ELISA OD, ALT levels, HCV confirmatory assays, and PCR according to immunostatus of patients

Test	Results for:		*P* value
	Immunocompromised patients ($n = 34$)	Immunocompetent patients ($n = 15$)	
ELISA OD[a]	5.34 ± 0.31	4.38 ± 0.71	0.15
ALT level[b]	1.30 ± 0.14	1.07 ± 0.06	0.30
RIBA-3 positive	55.82%	66%	0.48
DECISCAN positive	91.17%	86%	0.63
INNO-LIA HCV Ab III positive	88.23%	100%	0.41
PCR positive	82.35%	60%	0.09

[a] ELISA OD in time of upper limits of the cutoff value.
[b] ALT levels in time of upper limits of the normal value.

Using the manufacturers' criteria of positivity, the INNO-LIA HCV Ab III and DECISCAN tests appeared in this study to be more suitable than the RIBA-3 for interpreting the serum samples found indeterminate in the RIBA-2. This could be due to a lower sensitivity of the RIBA-3 versus the INNO-LIA HCV Ab III and DECISCAN HCV. However, if RIBA-3 +/− bands were taken into account, the number of results indeterminate by the RIBA-3 was reduced to only 7 (14%). Among them, one was HCV RNA negative by PCR. In the same way, if the serum samples only positive on one line with a rating of 2+ or higher were considered positive in the RIBA-3, all of the 50 serum samples indeterminate by the RIBA-2 were found positive.

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