Acta virol. **37**: 123–131, 1993

ANALYSIS OF ELISA HEPATITIS C VIRUS-POSITIVE BLOOD DONORS POPULATION BY POLYMERASE CHAIN REACTION AND RECOMBINANT IMMUNOBLOT ASSAY (RIBA). COMPARISON OF SECOND AND THIRD GENERATION RIBA

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Received May 27, 1992

Summary. – A new RIBA-3 (Chiron-Ortho Diagnostic System) was performed for discriminating uninterpretable results of RIBA-2. Recognition of antibodies to hepatitis C virus by RIBA-2 and RIBA-3 was compared among 95 ELISA-2 (second generation ELISA) positive blood donors and correlated with alanine-aminotransferase (ALAT) levels and viremia, using polymerase chain reaction (PCR). These studies led to three important conclusions. First, all ELISA-2-positive, RIBA-2-positive and ALAT-positive samples were found viremic compared with 73 % of ELISA-2-positive, RIBA-2-positive and ALAT-negative samples. Then, the comparison of the different RIBAs allowed to conclude that RIBA-3 was more sensitive but less specific than RIBA-2. RIBA-3 was interesting to discriminate undetermined RIBA-2, owing to an improved specificity of C100-3 antigen. In fact, most of the C100-3 positive, RIBA-2 undetermined samples became RIBA-3 negative whereas C22-3 positive, RIBA-2 undetermined samples became RIBA-3 positive or undetermined. Finally, a significant correlation was found between the presence of antibodies against C33-c antigen and viremia.

Key words: hepatitis C virus; recombinant immunoblot assay; reverse transcriptase-polymerase chain reaction

Introduction

In recent years, non-A, non-B hepatitis has become the most common form of post-transfusion hepatitis (Aach *et al.*, 1991). Its major etiologic agent, hepatitis C virus (HCV), was identified using a recombinant immunoscreenning approach (Choo *et al.*, 1989; Kuo *et al.*, 1989). Viral genome is a positive-sense, single stranded RNA, approximately 10 kb long with a single long open-reading frame, and is probably a member of the Flaviviridae family (Miller and Purcell, 1990). An immunodominant region, designated c100 and encoded by the putative

nonstructural protein 4 (NS4) gene, has been expressed, purified, and incorporated into ELISA that is useful for detecting antibodies to HCV in infected blood (Kuo *et al.*, 1989; Miyamura *et al.*, 1990). It has become clear that this assay was neither sensitive nor specific (Hsu *et al.*, 1991; Van der Poel *et al.*, 1990; Weiner *et al.*, 1990). As with the first generation tests, the second generation ELISA-2 could yield false positive reactions, and specificity was improved by the development of RIBA (Boudart *et al.*, 1992; Follett *et al.*, 1991; Van der Poel *et al.*, 1991). This assay represented a preliminary approach but it does not provide definitive confirmation of HCV infection because of the presence of uninterpretable, undetermined results. A new four antigen RIBA-3, in which 5.1.1 antigen is replaced by NS5 antigen, and recombinant C100-3 and C22-3 antigens are replaced by synthetic C100-3 and C22-3 peptides, has been developed by Chiron-Ortho Diagnostic System to differentiate undetermined RIBA-2.

Purpose of our study was to compare, among HCV ELISA-2-positive transfusion samples, results obtained with second generation RIBA, ALAT levels and viremia using PCR. Then, sensitivity and specificity of second and third generation RIBA was checked, and the utility of RIBA-3 to discriminate undetermined RIBA-2 samples was examined.

Materials and Methods

Anti-HCV testing. Serum samples of 95 ELISA-2-positive donors were retested using RIBA-2 and RIBA-3 (Chiron-Ortho Diagnostic System, Roissy, France), according to manufacturer's instructions.

Oligonucleotides. PCR was performed as a two step reaction with a pair of "nested" primers (Féray *et al.*, 1992) localized in the 5' non-coding region and consisting of an outer primer pair, H-72 (5'-GCC-ATG-GCG-TTA-GTA-TGA-GT-3'), starting at map position 72 and H-327 (5'-TGC-ACG-GTC-TAC-GAG-ACC-T-3'), starting at map position 327, and an inner primer pair, H-93 (5'-GTG-CAG-CCT-CCA-GGA-CCC-CC-3'), starting at map position 93 and H-304 (5'-GGG-CAC-TCG-CAA-GCA-CCC-TAT-3'), starting at map position 304 (Kato *et al.*, 1990).

RNA purification, reverse transcription, and PCR. RNA was prepared according to the guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987) with slight modifications. Briefly, 200 μl of the plasma sample was mixed with 500 μl of extraction buffer (4 mol/l guanidium thiocyanate, 25 mmol/l sodium citrate pH 7.0, 0.5 % sarkosyl and 7 % mercaptoethanol), 90 μl sodium acetate 2 mol/l pH 4.0, 500 μl phenol and 100 μl chloroform-isoamylacohol (24:1). Lysate was centrifugated, the water phase isopropanol precipitated and washed using 70 % ethanol. Dried pellets were resuspended in 40 μl DEPC-treated water and heated at 65 °C for 10 min to denature the RNA.

Synthesis of cDNA was carried out in a volume of 20 μl containing 12 μl of RNA, 20 units of ribonuclease inhibitor (Boehringer-Mannheim), 0.2 μg of random hexanucleotides (Boehringer-Mannheim), 1 mmol/l of each dNTPs (Pharmacia), 1 mmol/l $MgCl_2$, 2 μl of 10 × PCR buffer (Boehringer-Mannheim), and 10 units of M-MLV reverse transcriptase (Boehringer-Mannheim). The reaction proceeded at 20 °C for 10 min, followed by 45 min at 42 °C, boiling for 5 min and chilling on ice.

cDNA product was amplified in a 50 μl reaction containing 0.2 mol/l dNTP, 1.25 unit Taq polymerase (Amersham), 0.1 μg of each primer, and then overlaid with 50 μl of light mineral oil (Sigma). The reaction was performed for 30 cycles, including denaturation at 94 °C for 45 sec, primer annealing at 45 °C for 45 sec and primer extension at 72 °C for 1 min in a programmable Thermal Cycler (Perkin-Elmer Cetus). After the first amplification, 5 μl of the PCR product was amplified

with the corresponding inner primer pair and reaction was performed as above. After the second amplification, 10 μl of the PCR product was analyzed by electrophoresis in ethidium bromide stained Nusieve-agarose gel (FMC Bioproducts-TEBU) and visualized under ultraviolet light.

Controls. We use the contamination prevention measures of Kwok and Higuchi (1989). A result was considered as positive when a base pair product corresponding to the segment amplified by the inner primer pair (211 bp) was clearly visualized. Negative and positive controls were added during each step, and a result was considered valid if these controls were correct.

Results

HCV confirmatory PCR of RIBA-2-positive and ELISA-2-positive blood donors

HCV ELISA-2-positive plasma resulted from 95 volunteer blood donors in the Blood Transfusion Center in Marseille. We have used PCR to detect HCV RNA and have compared our results with positive-, undetermined-, and negative-RIBA-2 samples. HCV RNA detection was done using the reverse transcriptase-"nested" PCR (Garson *et al.*, 1990) with primers derived from the highly conserved 5' non-coding region (Féray *et al.*, 1992; Han *et al.*, 1991). The presence of HCV RNA was evaluated after 2–3 independent assays. In a population with ALAT $<$ n (n = 35 for women, and n = 45 for men), we found a good correlation between viremia and RIBA-2 reactivity; all RIBA positive samples were positive (25/25) for HCV RNA (Table 1) while, in the population with ALAT $<$ n, only 73.5 % (25/34) of RIBA reactive samples were viremic (Table 1). All C33-c non-reactive samples were negative (3/3) for HCV RNA.

20 of 21 (95.2 %) undetermined RIBA-2 samples did not react with PCR. The one that did exhibited a single band of the core protein (C22) and became positive with the RIBA-3. In our study, all RIBA-2 non-reactive samples were negative for HCV RNA.

RIBA-2 and RIBA-3 reactivity

RIBA-2 and RIBA-3 comparison, according to the viremia, showed on one hand that all PCR-reactive samples were found positive in RIBA-3 and that the number of undetermined samples decreased (21 to 8) (Table 2). On the other hand, we found that three negative RIBA-2 samples became undetermined and that the number of RIBA-3-reactive and PCR-negative samples increased (9 to 15). One RIBA-2-positive sample was RIBA-3-indeterminate, because of the substitution of the 5.1.1 antigen by NS5 antigen and, another RIBA-2-positive sample was RIBA-3-negative because of the substitution of the 5.1.1 antigen and a better specificity of C100-3 antigen.

Then, we have studied sensitivity and specificity of the RIBA-3 antigens concerning to RIBA-2 antigens on ELISA-2-positive blood samples (Table 3). We have found that C100-3 antigen provided in RIBA-3 was more sensitive and specific than the one from RIBA-2 (45 compared with 24 in the viremic population; 13 compared with 16 in the non-viremic population). C33-c RIBA-3 antigen seems more sensitive (100 % compared with 98 % reactivity in the viremic population) but less specific (13.6 % compared with 22.7 % reactivity in

Table 1. Distribution of results of RIBA-2 and PCR in 95 samples positive by ELISA-2 for HCV

RIBA-2 pattern				PCR results	% positivity
5.1.1	C100-3	C33-c	C-22		
RIBA-2 positive, ALAT > n				25/25	100
+	+	+	+	13/13	100
-	+	+	+	1/1	100
-	-	+	+	8/8	100
+	-	+	+	3/3	100
RIBA-2 positive, ALAT < n				25/34	73.5
+	+	+	+	7/10	70
-	+	+	+	3/4	75
-	-	+	+	13/14	92.6
+	-	+	+	2/2	100
-	+	-	+	0/2	0
+	+	-	-	0/2	0
RIBA-2, "indeterminate"				1/21	4.8
-	+	-	-	0/8	0
-	-	-	+	1/12	8.3
-	-	+	-	0/1	0

(+) positive; (-) negative

Table 2. Comparison of RIBA-2 and RIBA-3 results in respect to viremia

PCR	RIBA-2			RIBA-3		
	Pos.	Ind.	Neg.	Pos.	Ind.	Neg.
Positive (51)	50	1	0	51	0	0
Negative (44)	9	20	15	15	8	21

(Pos.) positive; (Ind.) indeterminate; (Neg.) negative

Table 3. Comparison of RIBA-2 and RIBA-3 antigen's distributions with regard to viremia

PCR	RIBA-2				RIBA-3			
	5.1.1	C100-3	C33-c	C22-3	NS5	C100-3	C33-c	C22-3
Positive (51)	25	24	50	51	30	45	51	51
Negative (44)	5	16	6	18	6	13	10	17

Table 4. Distribution of RIBA-2 and RIBA-3 antigens reactivity in 21 indeterminate RIBA-2 samples

Code	PCR	RIBA-2					RIBA-3				
		5.1.1	C100-3	C33-c	C22-3	R.	NS5	C100-3	C33-c	C22-3	R.
DH	Pos.	-	-	-	4	Ind.	3	-	4	4	Pos.
AW	Neg.	-	-	-	3	Ind.	-	2	2	4	Pos.
BA	Neg.	-	-	-	2	Ind.	-	1	-	3	Pos.
BI	Neg.	-	-	-	3	Ind.	1	-	-	4	Pos.
BP	Neg.	-	-	-	3	Ind.	-	2	2	4	Pos.
BX	Neg.	-	-	-	4	Ind.	-	-	1	4	Pos.
CF	Neg.	-	-	-	4	Ind.	2	2	2	2	Pos.
CI	Neg.	-	-	1	-	Ind.	1	4	4	4	Pos.
AI	Neg.	-	-	-	1	Ind.	-	-	-	4	Ind.
AY	Neg.	-	-	-	1	Ind.	-	-	-	2	Ind.
BN	Neg.	-	-	-	1	Ind.	-	-	-	3	Ind.
CX	Neg.	-	1	-	-	Inf.	-	1	-	-	Ind.
DC	Neg.	-	-	-	1	Ind.	-	-	-	3	Ind.
AT	Neg.	-	1	-	-	Ind.	-	-	-	-	Neg.
AV	Neg.	-	1	-	-	Ind.	-	-	-	-	Neg.
BE	Neg.	-	2	-	-	Ind.	-	-	-	-	Neg.
BR	Neg.	-	2	-	-	Ind.	-	-	-	-	Neg.
CY	Neg.	-	1	-	-	Ind.	-	-	-	-	Neg.
DD	Neg.	-	-	-	1	Ind.	-	-	-	-	Neg.
DF	Neg.	-	2	-	-	Ind.	-	-	-	-	Neg.
DG	Neg.	-	1	-	-	Ind.	-	-	-	-	Neg.

(R.) RIBA result; (Pos.) positive; (Ind.) indeterminate; (Neg.) negative; (1 to 4) blot signal intensity

the nonviremic population), whereas NS5 and 5.1.1 antigens possess equal sensitivity and specificity. We have also found an increased intensity of signals with all RIBA-3 antigens (data not shown). On the other hand, only C22-3 and chiefly C33-c antigen (χ^2, $p < 0.01$) correlated well with viremia (Table 3), 84 % to 89 % and 74 % to 75 % of samples with C33-c and C22-3 being viremic, respectively.

Confirmation of undetermined RIBA-2 by RIBA-3

Among 21 undetermined RIBA-2 samples, RIBA-3 were positive for 8, undetermined for 5 and negative for 8 samples (Table 4). Interestingly, 91.6 % (11/12) of RIBA-2 undetermined samples that became RIBA-3-positive or undetermined, were anti-C22-3 antigen reactive (χ^2, $p < 0.01$); whereas 87.5 % (7/8) of undetermined RIBA-2 samples that became RIBA-3 negative were anti-C100-3 reactive (χ^2, $p < 0.01$). The single anti-C33-c reactive, RIBA-2 indeterminate sample was RIBA-3-positive. PCR-positive, undetermined RIBA-2 sample was C22-3 antigen reactive and became positive with RIBA-3. In our study, RIBA-3 allowed the discrimination of 75 % of RIBA-2 undetermined samples.

Discussion

HCV ELISAs have a limited role in the diagnosis of infected patients due to significant false positivity rate in low risk blood donor population (Hsu *et al.*, 1991; Van der Poel *et al.*, 1990; Weiner *et al.*, 1990), and supplementary tests, such as RIBA-1 and RIBA-2 were purchased to aid in discriminating between the true and false positive results. Now, a new four antigens RIBA-3 has been developed by Chiron-Ortho Diagnostic System to characterize undetermined RIBA-2, using synthetic C100 and C22 peptides and by replacing 5.1.1 antigen (NS4 antigen) by NS5 antigen. Another approach to confirmation is the detection of circulating HCV RNA by PCR applied to HCV cDNA obtained by reverse transcription. This method is technically difficult but allows direct detection of the viral genome in clinical samples and is very useful in cases of presumed HCV infection where antibodies against HCV antigens are not detected (Alter *et al.*, 1989), for undetermined RIBA (Follett *et al.*, 1991), and for monitoring HCV viremia (Farci *et al.*, 1991) or interferon treatment (Chayama *et al.*, 1991).

Purposes of this study were, first, to evaluate, in 95 ELISA-2-positive plasma samples the correlation between RIBA-2 and PCR in comparison with ALAT levels and, then, to evaluate the sensitivity and the specificity of RIBA-2 and RIBA-3. Our PCR results suggest that almost all RIBA-2-positive donors have HCV infection. All samples with ALAT > n are viremic compared with 73 % of samples with ALAT < n. The 9 PCR-negative, RIBA-2-positive donors are present in the population with ALAT < n and may reflect a resolved hepatitis or an absence of replicating virus, as suggested by previous PCR studies (Farci *et al.*, 1991).

The proportion of HCV RNA positive plasma samples according to reactivity toward 5.1.1, C100-3, C33-c and C22-3 RIBA-2 antigens are 83 %, 60 %, 89 % and 74 %, respectively (Table 3). Compared with RIBA-2, NS5, C100-3 and C22-3 RIBA-3 antigens have identical or more specific reactivity (83 %, 76 % and 75 %, respectively), whereas RIBA-3 C33-c antigen (84 % reactivity) correlated less with viremia. Yet, we find a good correlation, particularly, between RIBA-2 C33-c antigen reactivity and HCV RNA positivity (χ^2, $p < 0.01$). C33-c seems to predict infectivity with high specificity. These data are consistent with a hypothesis that C33-c antigen, which is encoded by the NS3 region of HCV genome and is associated with helicase activity necessary for HCV multiplication (Houghton *et al.*, 1991) is a good marker of HCV viremia (Martinot-Peignoux *et al.*, 1992).

In our study, the third generation RIBA-3 decreases the number of undetermined patterns present with the second generation RIBA-2, allows the recognition of all viremic samples, but correlates less with the detection of HCV genomic material (Table 2). Indeed, we find 6 additional RIBA-3-positive, PCR-negative samples. In addition, due to the replacement of 5.1.1 antigen by NS5 antigen and the better specificity of C100-3 antigen, two NS5-positive, C100-3-positive RIBA-2 samples become RIBA-3 indeterminate and RIBA-3-negative, respectively; these two samples are PCR-negative. Fifty-nine percent of viremic plasma are found NS5 antigen reactive; this fact may probably result from a greater heterogeneity or from a poor immunogenecity of this antigen.

According to manufacturer's instructions, RIBA-3 may be used to discriminate undetermined RIBA-2. Among undetermined assays, RIBA-3 detects eight additional positive samples (Table 4), one being viremic. These samples possess C22-3 antigen reactivity. Five samples remain undetermined, and eight become negative. Interestingly, we find a very good correlation between RIBA-2 reactivity and RIBA-3 results. Almost all undetermined-RIBA-2, positive- or undetermined-RIBA-3 samples (11/12) possess RIBA-2 C22-3 antigen reactivity whereas almost all undetermined-RIBA-2 and negative-RIBA-3 samples (7/8) possess RIBA-2 C100-3 antigen. In RIBA-2, anti C100/3 reactivity can reflect a false positivity in contrary to C22-3 reactivity. Among undetermined RIBA-2, only one sample is C33-c reactive and becomes RIBA-3-positive. In our study, RIBA-3 allows the discrimination of 75 % of undetermined RIBA-2.

Briefly, several points emerge from the present study. Our data indicate that almost all ELISA-2-positive and RIBA-2-positive samples are viremic when ALAT > n. Then, comparison of different RIBA allows to conclude that RIBA-3 is more sensitive but less specific than RIBA-2; RIBA-3 is interesting to discriminate undetermined RIBA-2. In addition, we find that C33-c antigen is a good prognostic marker of viremia.

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