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Predictive markers for hepatitis C antibody ELISA specificity in Australian blood donors

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SUMMARY. The hepatitis C antibody reactivity rate in 91,748 blood donors tested using the ORTHO HCV C-100 ELISA system was 0·51%. Specificity of ELISA positive reactions was measured using a recombinant immunoblot assay (RIBA). The aim of this study was to identify markers in ELISA positive donors which were predictive of a RIBA positive result.

Samples from 430 ELISA positive donors were tested by the first generation RIBA, RIBA-1, which incorporates two HCV peptides C-100 and 5-1-1. Fifty-five per cent (236) were positive and 19% (83) indeterminate. Multivariate analysis of gender, age, HCV ELISA OD ratio, alanine aminotransferase (ALT) status and hepatitis B core antibody (anti-HBc) status identified age, magnitude of HCV ELISA OD ratio and anti-HBc status as the only independent predictors of a positive RIBA-1 result. The relative odds of being RIBA-1 positive were 4·6-fold (95% CI 1·3–16·4) higher among donors aged 25–34 years compared with donors less than 25 or greater than 44; 6·1-fold (2·1–17·9) higher if the donor was anti-HBc positive and 273·4-fold (30·9–2417) higher if the HCV ELISA OD ratio was greater than 5·98 compared to those with a ratio less than 1·77. Seventy-eight of the 83 RIBA-1 indeterminates were tested on the second generation RIBA, RIBA-2, which includes two additional HCV peptides, C22 and C33c. Thirty-one per cent (24) were positive and 41% (32) were negative. Multivariate analysis showed that the presence of an elevated ALT level and anti-HBc were the only independent predictors of a RIBA-1 indeterminate result resolving as positive on RIBA-2.

Key words: alanine aminotransferase, blood donors, hepatitis B core antibody, hepatitis C virus, multivariate analysis, non-A, non B hepatitis.

The genome for a blood-borne non-A, non-B (NANB) hepatitis agent, designated hepatitis C virus (HCV), has been cloned using molecular biology techniques (Choo *et al.*, 1989). By inserting portions of the genome in expression vectors, HCV peptides can be synthesized. Such peptides have been used to develop enzyme-linked immunosorbent assays (ELISA) for the detection of HCV antibodies (anti-HCV, Kuo *et al.*, 1989). The first generation ELISA, currently licensed for diagnostic use, incorporates the yeast-derived C100-3 HCV peptide, a product of the non-structural HCV NS-3 and NS-4 regions of the viral genome. The specificity of this assay appears highest among groups such as post-transfusion NANB hepatitis patients, intravenous drug users and haemophiliacs (Colombo *et al.*, 1990). In healthy populations, such as blood donors, the specificity is reduced (Skidmore, 1990), therefore the number of false-positive results creates potential problems with respect to donor deferral, counselling and follow-up investigations.

The development of a first-generation recombinant immunoblot assay (RIBA-1) by Chiron (Emeryville, U.S.A.) has allowed some control of specificity of the ELISA result (Ebeling *et al.*, 1990; Van der Poel *et al.*, 1990a). RIBA-1 incorporates the HCV C-100 peptide plus a smaller product from the same gene region, derived from *E. coli* and designated 5-1-1. RIBA-1 is considered a supplementary rather than a confirmatory test. A second generation RIBA (RIBA-2) incorporates two additional recombinant antigens, C33c and C22, which are protein products from the non-structural gene region, NS-3, and the structural core gene, respectively. This test may be considered closer to a true confirmatory assay (Van der Poel *et al.*, 1991).

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RIBA positive patterns have been shown to correlate with the presence of circulating HCV RNA and with HCV transmission (Van der Poel *et al.*, 1991). Other factors, such as raised ALT levels (indicative of abnormal liver function), persistent HCV ELISA positivity and high HCV ELISA OD ratios have also been shown to correlate with infectivity when blood donor–recipient studies have been performed (Van der Poel *et al.*, 1990b).

This study of Australian blood donors identifies factors present in ELISA anti-HCV positive donors which correlate with RIBA positivity. As supplementary or confirmatory tests are not always available, this information is important for blood transfusion services and diagnostic laboratories that are concerned with the identification of potentially infectious donors who require a specialist referral.

SUBJECTS AND METHODS

Between 19 February and 31 December 1990, 159,598 donations were collected from 91,748 donors by the Queensland Division of the Australian Red Cross Blood Transfusion Service. Blood was collected only from donors who had signed a declaration form indicating that they were not at risk for HIV infection. Citrated plasma samples were screened within 24 h of collection for hepatitis B surface antigen (HBsAg, Auszyme Monoclonal ELISA, Abbott Diagnostic Systems Chicago, Ill), antibody to HIV-1 (Vironostika anti-HTLV-111 Microelisa, Organon Teknika, Boxtel, Holland), antibody to HCV C100-3 (Ortho HCV ELISA TEST System, Raritan, NJ) and elevated plasma ALT levels (Hyland *et al.*, 1988). Samples reactive on initial testing were retested in duplicate and categorized as positive if one or both retests were positive. ELISA-positive HBsAg reactives were confirmed using the Abbott Antibody to Hepatitis B Surface Antigen Confirmatory Assay. ELISA positive HIV-1 antibody reactions were confirmed using a Western Blot assay (Biorad).

The HCV ELISA OD ratio was obtained from the absorbance of the sample divided by cut-off value established by the manufacturer's criteria. Samples with OD ratios equal to or greater than 1 were designated as positive. These were tested for core antibody to hepatitis B virus (anti-HBc, Abbott Corzyme) and stored at −30°C for a maximum of 9 months before testing on the first generation Chiron RIBA [RIBA-1] HCV Test System (Emeryville, CA). Samples were interpreted as either positive (1+ or greater reactivity against both HCV C-100 and 5-1-1 peptides), negative (no reactivity) or indeterminate (1+ or greater reactivity against either C-100 or 5-1-1 but not both) according to the manufacturer's instructions. RIBA-1 indeterminate samples from 78 donors and RIBA-1 positive and negative samples from 100 donors were tested by the second generation RIBA [RIBA-2] assay. A positive RIBA-2 result was indicated by reactivity against any two of the four HCV peptides (5-1-1, C-100, C33c and C22); a negative by no reactivity and an indeterminate by reactivity against one peptide only.

Statistical methods

The following data were available for statistical analysis among 430 HCV ELISA-positive subjects: gender ($n=427$), age ($n=426$), RIBA-1 results ($n=430$), anti-HBc status ($n=395$) and ALT levels ($n=427$). The mean donor age was 35·8 years (SD 9·7 years) and the mean HCV ELISA OD ratio was 4·1 (2·1).

The frequency distributions of the continuous variables, age, HCV ELISA OD ratios and ALT levels were considered for skewness. As a result, log-transformed ALT values were used in analysis to reduce the effects of the significant skewness towards larger values. Age was classified in 10-year age-groups of <25, 25–34, 35–44, 45–54, >54 years. The ELISA OD ratio was arbitrarily classified by quartiles of the distribution. These were $<1{\cdot}77$, 1·77–4·77, 4·77–5·98 and $>5{\cdot}98$. ALT levels were grouped as normal (<46 IU/l) and abnormal ($\geqslant 46$ IU/l).

Student's *t*-test and analysis of variance were used to compare means. Associations between categorical variables were investigated using the chi-squared test, with Yates' correction for comparisons with 1 d.f. Multiple linear regression modelling was used to investigate the independent and joint effects of variables on absolute OD/COV fraction. Relationships between these variables and RIBA response were investigated using two sets of logistic regression analyses, comparing the characteristics of negative and positive RIBA responses, and secondly negative and indeterminate RIBA responses (Schlesselman, 1982).

Ninety-five per cent confidence intervals for the true odds ratios were calculated using estimates and corresponding standard errors from the logistic regression models.

RESULTS

Serological markers in blood donors

In this donor population the seroprevalences for HBsAg and anti-HIV were 0·038% (35/91,748) and 0·001% (1/91,748), respectively. These donors include

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both first time ($n=35,622$) and repeat donors ($n=56,126$). The HBsAg detection rate amongst first time donors was 0·067% (24/35,622). The anti-HCV ELISA reactivity was 0·51% (464/91,748). Donors had not previously been screened for the HCV antibody.

RIBA reactivity among anti-HCV ELISA positive samples

Samples from 430 ELISA positive donors were available for RIBA-1 testing. Fifty-five per cent (236) were positive, 26% (111) were negative and 19% (83) were indeterminate. Among the indeterminates, 76% reacted with C-100 only, 14% with 5-1-1 only and 10% with superoxide dismutase (SOD, a protein fused with the HCV peptides to enhance expression in vectors).

RIBA-2 tests were performed on 78 of the 83 subjects for whom the RIBA-1 response was indeterminate. This test resolved the status of 72% of subjects; 31% (24) were positive and 41% (32) were negative.

Among the 78 RIBA-1 indeterminate samples, approximately one-third responded positively to bands C33c (31%) and C22 (32%) on the RIBA-2. Nearly all subjects (61/62), whose response was negative for 5-1-1 on the RIBA-1 remained so on RIBA-2. Similarly, nearly all subjects (11/13), whose response was negative on band C-100 remained so on RIBA-2.

Eighteen of the 78 donors with RIBA-1 indeterminate results were tested by RIBA-2 on multiple occasions (range 2–4). Results were consistent in 17 donors. The other donor gave an indeterminate result on three occasions and a negative result between the second and fourth donation.

An additional 50 RIBA-1 positive and 50 RIBA-1 negative samples were also tested on the RIBA-2. Forty-nine of the 50 RIBA-1 positive samples were RIBA-2 positive. The remaining sample showed reactivity against C-100 and 5-1-1 but at a level below that required for interpretation as a positive. No bands were present against the C22 or C33. A second sample collected at a 5-month interval was available from this donor. Results were indeterminate on RIBA-1 and negative on RIBA-2 when tested in parallel. Reactivity occurred against C-100 and 5-1-1 in both tests but only the reactivity against 5-1-1 on the RIBA-1 was above the level required for a positive interpretation. Reactivity against C22 and C33c remained absent. The donor had a normal ALT level and was negative for anti-HBc. Forty-six of the 50 RIBA-1 negative samples remained negative by RIBA-2. Four samples became RIBA-2 positive due to reactivity against C22 and C33c. All four had raised ALT levels and one showed anti-HBc reactivity.

Characteristics that affect the magnitude of the anti-HCV ELISA OD ratio

In univariate comparisons of means, anti-HCV ELISA OD ratios were significantly higher for males than for females ($P=0·002$) and peaked among subjects aged 25–34 years ($P<0·001$) compared with other ages. Similarly, mean ELISA OD ratios were significantly higher among subjects with abnormal ALT levels ($\geq$46 IU/l) compared with normal levels (<46 IU/l, $P<0·001$), and among those with either a positive anti-HBc ($P=0·016$) or positive RIBA-1 response ($P<0·001$, Table 1).

Multivariate regression analyses were performed to determine whether these identified variables were independently associated with the magnitude of a positive ELISA OD ratio. Age, abnormal ALT level and RIBA-1 result were the only three independent factors significantly contributing to variation in OD ratios. The predicted magnitude of the OD ratio from the final model is estimated as a base-line mean of 4.4206 (SE=0·2647), to which is added 1·1023 (0·2275) for an abnormal ALT level, 0·5515 (0·1410) for a positive RIBA result, and finally subtracting 0·0007 (0·0001) times the square of the donor's age.

Characteristics that affect the RIBA-1 response

In univariate comparisons (Table 2), RIBA-1 positive responses were significantly higher with male gender ($P<0·001$), subjects aged 25–44 years ($P<0·001$), those with abnormal ALT levels ($P<0·001$) and those with a positive anti-HBc result ($P<0·001$). RIBA-1 positive responses were also significantly associated with higher anti-HCV ELISA OD ratios ($P<0·001$).

In multivariate logistic regression analyses age, anti-HBc results and magnitude of anti-HCV ELISA OD ratio remained independent predictors of RIBA-1 positivity. Table 2 represents the relative odds of being RIBA-1 positive, based on the estimates from a model containing all variables. For a donor with an ELISA OD ratio greater than 5·98 the odds were 273·4 (95% CI 30·9–2,417) times greater than for a donor with an ELISA OD less than 1·77. The odds were 40·5 (12·7–130) greater, with the OD between 4·77 and 5·98, than for a donor with an OD less than 1·77. The presence of anti-HBc increased the chance of being RIBA-1 positive 6·1 times (2·1–17·9). The odds for a donor in the 25–34 age group were 4·6 (1·3–16·4) times greater than for a donor below 25 or between 45 and 54 years.

Characteristics that affect the RIBA-2 response in the RIBA-1 indeterminates

A positive RIBA-2 result for the RIBA-1 indetermi-

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Table 1. Mean hepatitis C antibody ELISA OD ratios by gender, age group, ALT level, Anti-HBc and RIBA-1 results

	Number of donors	Anti-HCV ELISA OD* MEAN	(SD)†	Significance level (*P*)
Gender				
Males	301	4·29	(2·09)	=0·002
Females	126	3·59	(1·99)	
Age (years)				
<25	42	4·03	(2·01)	<0·001
25–34	195	4·71	(1·90)	
35–44	116	4·05	(2·09)	
45–54	49	2·52	(1·78)	
55+	24	2·43	(1·58)	
ALT				
Normal	314	3·70	(2·05)	<0·001
Abnormal	113	5·22	(1·68)	
Anti-HBc				
Negative	301	3·91	(2·10)	=0·016
Positive	94	4·48	(2·05)	
RIBA-1				
Negative	111	2·36	(1·44)	<0·001
Positive	236	5·29	(1·56)	
Indeterminate	83	3·03	(1·87)	

* ELISA OD ratio = ELISA absorbance of sample divided by cut-off absorbance.
† SD = standard deviation.

nate subset was significantly associated with age ($P<0·001$), ALT level ($P<0·001$), anti-HBc ($P<0·001$) and anti-HCV ELISA OD ratio ($P<0·01$) by the univariate analysis, Table 3. However, after a multivariate analysis only an abnormal ALT level and anti-HBc positive result remained as independent predictors of a positive RIBA-2. In this RIBA-1 indeterminate subset, 10 out of 11 (91%) with raised ALT levels were RIBA-2 positive. Similarly, 12 out of 15 (80%) with anti-HBc positivity were RIBA-2 positive. However, the absence of these markers did not preclude the possibility of a RIBA-2 positive result. Of the samples which resolved as RIBA-2 positive, 58% (14/24) had normal ALT levels and 50% (12/24) were negative for anti-HBc.

DISCUSSION

The specificity of HCV C-100 ELISA positive reactions was measured in this donor population using first and second generation RIBA. This population is not biased by prior screening for the surrogate NANB hepatitis markers, anti-HBc and raised ALT levels. Anti-HBc testing is not performed in this Transfusion Service. ALT testing is implemented, although with the exception of donors with ALT levels greater than 100 IU/l, donors with raised values have not been deferred. However, their donations were discarded. By extrapolating from the results obtained using RIBA-1 and RIBA-2 assays, we estimate that about 0·3% of donors react specifically with the C-100 antibody on the first generation ELISA. The demonstration of viral RNA using the polymerase chain reaction (PCR) technique would corroborate the ELISA reactivity but was beyond the scope of this study. In general, however, a positive RIBA result has been shown to correlate with donor infectivity and with the presence of circulating viral RNA (Van der Poel *et al.*, 1990b, 1991).

Multivariate analysis in this study identified independent factors in anti-HCV (C-100) ELISA-positive blood donors, which were predictors of RIBA-2 positive results. These were donor age, anti-HBc status and magnitude of anti-HCV ELISA OD ratio. The odds of being RIBA-2 positive were greatest in the 25–34 year age group and higher for anti-HBc positive versus negative donors. The strongest predictor was the magnitude of the ELISA OD ratio. This suggests

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Table 2. Proportions (%) of donors in each RIBA-1 category classified by gender, age group, ALT and Anti-HBc status and Anti-HCV ELISA OD ratio

	Number of donors	RIBA-1 result (percentage of donors) Positive	Negative	Indeterminate	Relative odds* (95% CI)†
Gender‡					
Males	301	62	22	16	1·0
Females	126	38	34	28	0·5 (0·2, 1·2)
Age‡ (years)					
<25	42	41	38	21	1·0
25–34	195	72	14	14	4·6 (1·3, 16·4)
35–44	116	55	28	16	2·1 (0·6, 8·0)
45–54	49	18	47	35	1·0 (0·2, 4·7)
55+	24	12	46	42	0·4 (0·1, 3·6)
ALT‡					
Normal	314	48	30	22	1·0
Abnormal	113	76	12	12	0·8 (0·3, 2·3)
Anti-HBc‡					
Negative	301	48	31	21	1·0
Positive	94	74	9	17	6·1 (2·1, 17·9)
HCV ELISA‡ OD Ratio§					
<1·77	105	15	54	31	1·0
−4·77	108	30	44	27	2·4 (1·0, 5·7)
−5·98	108	83	7	10	40·5 (12·7, 130)
>5·98	109	90	1	9	273·4 (30·9, 2417)

* Relative odds based on multivariate models including all factors.
† 95% confidence intervals for true odds are presented in parentheses.
‡ At the univariate level the distribution of RIBA-1 results differs significantly ($P<0·001$) in each category.
§ ELISA OD ratio = ELISA absorbance of sample divided by cut-off absorbance

that a low positive OD ratio does not justify vigorous clinical investigation of an otherwise healthy donor.

Once the ELISA OD ratio was known, information on the ALT level for the donor was of no value in the assessment of the risk of being RIBA-1 positive. However, an abnormal ALT level was an independent predictor of the magnitude of the ELISA OD ratio. A high OD ratio probably reflects high titre HCV antibodies, whilst an abnormal ALT level represents hepatocellular damage which may accompany the HCV infection that produces this immunological response. A previous study showed that the ELISA anti-HCV detection rate rises from 0·6% in blood donors with normal ALT values to 24% in donors with ALT values greater than 100 IU/l (Morgan *et al.*, 1990). As a surrogate marker in the absence of the C-100 ELISA, ALT testing may identify a proportion of infected donors albeit at the expense of discarding blood unnecessarily. In this population only 8% of donors with abnormal ALT levels are HCV C-100 ELISA positive (Morgan *et al.*, 1990).

In contrast anti-HBc provided additional information in predicting RIBA-1 positivity. In this population, where hepatitis B prevalence is low (less than 0·1%), we suggest that anti-HBc identifies a population subset which has been exposed to blood-borne viruses via parenteral or sexual routes at some time in the past. However, the importance of anti-HBc as a predictive marker may vary in differing populations. Other studies have shown no association between anti-HBc status in donors and either anti-HCV or post-transfusion NANB hepatitis in donor recipient studies (Van der Poel *et al.*, 1990a; Esteban *et al.*, 1990).

The second generation RIBA-2 incorporates two additional HCV antigens, C22 and C33c, and was used in this study to resolve the status of the RIBA-1

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Table 3. Proportion of RIBA-1 indeterminates in each RIBA-2 category classified by gender, age group, ALT and Anti-HBc status and Anti-HCV ELISA OD ratio

	Number of Donors	RIBA-2 result (percentage of donors)			Significance level (*P*)
		Positive	Negative	Indeterminate	
Gender					
Males	46	30	50	20	=0·075
Females	32	31	28	41	
AGE (years)					
<25	9	11	56	33	<0·001
25–34	26	69	0	31	
35–44	17	29	53	18	
45–54	17	0	65	35	
55+	9	0	78	22	
ALT					
Normal	66	21	49	30	<0·001
Abnormal	11	91	0	9	
Anti-HBc					
Negative	58	21	45	34	0·001
Positive	15	80	13	7	
HCV ELISA OD ratio*					
<1·77	31	26	58	16	=0·010
−4·77	28	29	46	25	
−5·98	11	36	0	64	
>5·98	8	50	12	38	

* ELISA OD ratio = ELISA absorbance of sample divided by cut-off absorbance.

indeterminate samples. In this RIBA-1 indeterminate subset, elevated ALT levels and the presence of anti-HBc were identified as the only independent predictors of a positive RIBA-2 result. The magnitude of the ELISA HCV OD ratio was not an independent predictor and was less than 4·77 in 76% (58/78) of cases (Table 3). The breakdown in OD correlation results from the two additional HCV peptides into RIBA-2. These increase the sensitivity by detecting the respective HCV antibodies when C100 and 5-1-1 antibody levels are low or absent. It appears that in the RIBA-1 indeterminate subset an elevated ALT may act as a surrogate marker for C22 and/or C33c antibody.

Note that four RIBA-1 negative samples were RIBA-2 positive due to C22 and C33c peptide reactivity. All had elevated ALT levels and one was anti-HBc positive. Until the second generation ELISA and the RIBA-2 are introduced, care should be taken in interpreting an apparent RIBA-1 negative result. RIBA-1 indeterminate samples have been implicated in the transmission of the HCV virus, and both RIBA-1 indeterminate and negative samples may contain HCV RNA, although at a much lower frequency than RIBA-1 positive samples (Bellobuono *et al.*, 1990; Weiner *et al.*, 1990).

Finally, until supplementary HCV testing is performed, the age of the donor, the magnitude of the ELISA OD ratio and the presence of anti-HBc play a role in assessing whether the ELISA-positive reaction may be specific. As the sensitivity of both the ELISA and RIBA assays is increased, through the addition of peptides such as C22 and C33c, the role of ALT testing as a surrogate marker will diminish. In contrast, in some populations where hepatitis B is not endemic, anti-HBc will remain as an indicator of ELISA specificity and a marker for population subsets who have been exposed to parenteral and/or sexually transmitted diseases.

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