Confirmation of second generation anti-hepatitis C virus enzyme immunoassays by antigenic cross-validation

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Abstract

Aim: To determine if a scheme for validating enzyme immunoassay (EIA) results could be devised that did not require costly and methodically elaborate supplemental assays.

Methods: Samples (n = 525) from patients with haemophilia A, leukaemia, and chronic liver disease and at increased risk of hepatitis C virus infection were tested by EIA-1 (Ortho Diagnostics), an assay which uses recombinant HCV fusion proteins as antigens, and by EIA-2 (United Biomedical), an assay based on synthetic HCV oligopeptide antigens.

Results: Samples (n = 193) were repeatedly reactive in both EIAs. Of these, 190 (98%) yielded reactivities in both of two supplemental assays used, one an immunoblot assay (RIBA) using recombinant HCV polypeptides similar to EIA-1 antigens, and the other a neutralisation EIA (EIA-2N) based on antigenic competition with HCV peptides similar to EIA-2 antigens. The three samples not reactive in supplemental tests exhibited low EIA optical density (OD) values (signal/cutoff ratios of less than 3). Hence, all specimens reactive and yielding high OD values in both EIAs were also reactive in supplemental assays. Twenty four samples were reactive in EIA-1 only and nine (38%) of these were reactive in RIBA. Fourteen of the 15 (93%) specimens reactive in EIA-1 but not RIBA were derived from patients with chronic liver dysfunction. Two samples were reactive in EIA-2 only, of which one was reactive in EIA-2N and none in RIBA.

Conclusions: Compared with EIA-2, EIA-1 yielded more validated reactive samples and resulted in more non-validated reactivities. It is therefore suggested that for clinical diagnosis: (i) EIA-1 be used for anti-HCV testing and EIA-2 for validation of EIA-1 reactivities; (ii) samples concordantly reactive in EIA-1 and EIA-2 and displaying high OD readings be considered HCV antibody positive without supplemental testing; (iii) supplemental testing by RIBA be limited to samples reactive in EIA-1 but equivocal or unreactive in EIA-2 and those concordantly reactive but exhibiting low absorbance readings.

Second generation commercial enzyme immunoassays (EIAs) for screening antibodies to the hepatitis C virus (HCV) have recently become available. They belong to two categories. One uses recombinant fusion proteins as antigens: c200, derived from putative non-structural (NS) regions of the virus; and c22, from the core region (Lee *et al* and Peterson *et al*, abstracts presented at the 2nd International Symposium on HCV, November 1990, Los Angeles). The other uses oligopeptides synthesised from presumed immunodominant regions of the viral polypeptide.¹ Because a greater variety of antigens is represented in these assays, they are more sensitive than first generation EIAs.^{1,2}

Current validation procedures are unsatisfactory because the virus has not been propagated in vitro and viral proteins representing the full antigenic repertoire are not available. Reactivities in EIA have to be confirmed by "supplemental" assays that use antigens from the same source and prepared in the same manner as those of the EIA. However, the availability of two antigen preparation modes, recombinant polypeptide and synthetic oligopeptide, potentially permits a cross-validation strategy in which an EIA or supplemental test incorporating one type of antigen is used to check the specificity of reactivity in an EIA incorporating the other antigen. With serum samples obtained from individuals at increased risk of HCV infection, we evaluated such a procedure for diagnostic use. In view of the much higher cost and greater complexity of supplemental assays compared with EIAs, our findings will be of interest to laboratories who need to devise, on a limited budget, validation strategies for HCV antibody assays.

Methods

Specimens were assembled from patients with haemophilia A (group A), leukaemia (group B), and chronic liver disease (CLD) (group C). As controls, sera were obtained from blood donors (group D). Samples of groups A, B, and C were consecutive referrals to the Virus Reference Laboratory for anti-HCV testing. Group C samples were studied only when referral forms indicated that they were taken from patients with abnormal liver functions tests of more than six months' duration. Group D sera were derived from successive donors attending the North London Blood Transfusion Centre.

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Table Reactivities of samples in anti-HCV EIA-1 and EIA-2

Assay result	Group				Total (n=702)
	A (n=164) (haemophiliacs)	B (n=140) (leukaemias)	C (n=221) (CLD)	D (n=177) (blood donors)	
EIA-1 ⁺	119 (73)	22 (16)	76 (34)	0 (0)	217 (31)
EIA-2 ⁺	115 (70)	20 (14)	60 (27)	2 (1)	97 (28)
EIA-1 ⁺ /EIA-2 ⁺	114 (70)	20 (14)	59 (34)	0 (0)	193 (37)
EIA-1 ⁺ /EIA-2 ⁻	5 (4)	2 (1)	17 (8)	0 (0)	24 (3)
EIA-1 ⁻ /EIA-2 ⁺	1 (1)	0 (0)	1 (0)	2 (1)	4 (1)

Figures in parentheses indicate percentage of cases within each group.

California, USA, and Ortho Diagnostics Limited, Raritan, New Jersey, USA) incorporates c22 and c200 expressed in yeast (Lee *et al*, abstract presented at the 2nd International Symposium on HCV, November 1990, Los Angeles). EIA-2 (United Biomedical Incorporated, Lake Success, New York, USA) utilises oligopeptide antigens whose sequences were derived from putative capsid and NS regions.¹ Both are anti-globulin assays using antigens coated in microtitre plates. Results were expressed as optical density (OD) values related to a cutoff value derived from OD values of negative controls, calculated according to the manufacturers' instructions. OD values above cutoff indicated reactivity.

Two supplemental tests were used to validate EIA-1 reactive (EIA-1⁺) and EIA-2 reactive (EIA-2⁺) samples. The first, the recombinant immunoblot assay, RIBA (Ortho), is a second generation immunoblot assay using antigens c100-3, 5-1-1, c33c and c22 immobilised as individual bands on nitrocellulose strips. The first three antigens are shorter polypeptides of c200 derived from the NS3-NS4 region of the Chiron HCV strain. Reactivity of samples to each antigen was determined visually by comparing the intensity of each band with that of high and low IgG control bands incorporated in each strip. Results were expressed as reactive (RIBA⁺), when the intensity of two or more antigen bands was greater or equal to the low IgG control band; indeterminate (RIBAⁱ), when the intensity of one band was greater or equal to the low IgG control; and non-reactive (RIBA⁻), when antigen banding was not visible or of lesser intensity compared to the low IgG band. This nomenclature of RIBA reactivities follows that recommended by the manufacturers, but in this study RIBAⁱ as well as RIBA⁺ samples are considered to be reactive in RIBA.

The second supplemental assay EIA-2N (United Biomedical) is a competitive blocking assay in which samples were added to coated wells of EIA-2 after preincubation with neutralisation buffer containing soluble synthetic HCV peptides. Samples with OD values greater than or equal to 50% of the absorbance in EIA-2 were considered positive (EIA-2N⁺); those with values less than 50% were considered negative. In this study samples found to be negative by EIA-2N as well as those not reactive in EIA-2 are considered EIA-2N negative (EIA-2N⁻).

Results

Serum samples (n = 702) were tested by EIA-1 and by EIA-2: 219 and 198, respectively, were initially reactive and the repeat reactive rates were 99.5% and 99.6%. Only specimens with OD values greater than cutoff on repeat testing were considered reactive. Group A specimens had the highest rate of reactivities, either individually, in EIA-1 (EIA-1⁺) or EIA-2 (EIA-2⁺), and in both (EIA-1⁺/EIA-2⁺), followed by C, B, and D (table). Discrepancies arose in all groups and, especially in group C, more samples were reactive in EIA-1 only (EIA-1⁺/EIA-2⁻) than in EIA-2 only (EIA-1⁻/EIA-2⁺).

The outcome of RIBA and EIA-2N testing correlated with the intensity of sample reactivities in EIA-1 and EIA-2 (figure). Among 193 samples concordant in EIA-1 and EIA-2, 98 in A, 12 in B, and 56 in C were RIBA⁺/EIA-2N⁺; and 16 in A, seven in B, and one in C were RIBAⁱ/EIA-2N⁺ (all being reactive to c22 only). It is likely that these 190 specimens were positive for HCV antibodies. In contrast, there were three EIA-1⁺/EIA-2⁺ samples, one in B and two in C, that were RIBA⁻/EIA-2N⁻. The signal/cutoff ratios of these samples were less than 3 in both EIAs. Their lack of reactivity in supplemental tests and their very low OD readings suggest that results in EIA-1 and EIA-2 were falsely positive.

Among EIA-1⁺/EIA-2⁻ samples: in A two were RIBA⁺, two were RIBAⁱ (both being reactive to c22 only), and one was RIBA⁻; in B two were RIBAⁱ (both with reactivity to c33c only); and in C two were RIBA⁺, one was RIBAⁱ (being reactive to c22 only), and 14 were RIBA⁻ (figure). The RIBA reactive samples probably had HCV antibodies that were detectable by EIA-1 but not by EIA-2, while the 17 RIBA⁻ samples may be considered to have yielded false positive results in EIA-1.

Of the EIA-1⁻/EIA-2⁺ samples, one in A was EIA-2N⁺ but negative in RIBA; this sample probably contained viral antibodies detectable only by EIA-2 but not EIA-1. One sample, in C (figure) and two in D, were unreactive in either supplemental test; these three samples may be considered EIA-2 false positive.

Discussion

In this study of serum samples obtained from patients at increased risk through transfusion or sporadic exposure of HCV infection, we found that a recombinant antigen based EIA

Figure 1 Relation for three clinical groups; haemophiliacs (A), those with leukaemia (B) and patients with CLD (C), of intensities of sample reactivities in EIA-1 and EIA-2 to the outcome of RIBA and EIA-2N testing. Dotted vertical and horizontal lines at the signal/cutoff ratio of 1 in both axes indicate thresholds of reactivity in EIA-1 and EIA-2, respectively. ▲: RIBA⁺/EIA-2N⁺; ■: RIBA⁺/EIA-2N⁻; X: RIBA⁺/EIA-2N; □: RIBA⁻/EIA-2N; ×: RIBA⁻/EIA-2N⁺; ∴: RIBA⁻/EIA-2N⁻.

(EIA-1) for anti-HCV yielded more validated reactive results than an EIA based on synthetic peptides (EIA-2) (199 v 191), but that EIA-1

also led to more non-validated reactivities than EIA-2 (15 v 1). This shows that these two second generation anti-HCV EIAs are divergent in terms of specificity and sensitivity. Ninety three per cent of the samples with non-validated EIA-1 reactivity (14 of 15) were group 3 patients. This high incidence of false positive results probably stems from the inclusion in the heterogeneous group 3 of individuals with autoimmune chronic active hepatitis (AI-CAH). Sera from such patients were known to yield false positive reactivities in the anti-HCV EIA that incorporated first generation recombinant proteins,³ and these proteins continue to be used as antigens in EIA-1.

We also found that most specimens (190 of 193) yielding concordant reactivities in the two EIAs were true positive results, being reactive in supplemental assays. The three samples that, though concordant in EIAs and non-reactive in supplemental tests, exhibited OD readings were very near the cutoff. These findings are consistent with the view that when specimens are derived from groups at high risk of viral infection, they will very often react concordantly positive in different screening assays for viral antibody.

More importantly, the results from this study may be used to formulate an algorithm for anti-HCV validation that obviates the need for supplemental testing of every EIA reactive specimen, which would be a costly exercise. It is suggested that in testing samples for HCV antibodies from individuals at known risk of HCV infection, the more sensitive EIA-1 should be used to screen and the more specific EIA-2 to validate EIA-1 reactivities. Samples found to be concordantly reactive and exhibiting moderate to strong OD readings (OD/cutoff of more than 3) can be considered positive for HCV antibodies without requiring further tests. Supplemental testing by RIBA may then be confined to samples reactive in EIA-1, but equivocal or non-reactive in EIA-2 and those with weak OD values in both EIAs. In the last group, although it is expected that they will generally be EIA false positive, as found in this study, the possibility cannot be excluded that some may contain HCV antibodies at low titres detectable by both EIA and RIBA.

We have considered the possibility that the polymerase chain reaction for amplifying HCV RNA⁴ be used to validate EIA reactivities in place of the proposed scheme but regard it impractical at present. First, it will be even more costly than universal supplemental testing of EIA reactive samples. Second, only few laboratories will have the resources and expertise to perform the assay regularly and accurately; the approach suggested here can be adopted by all diagnostic laboratories. Third, at the time of writing, the assay and the necessary reagents are restricted by the patent holders to research use only.

The scheme proposed here is directly applicable in diagnostic settings where a substantial proportion of the samples requiring anti-HCV testing is drawn from individuals likely to have been exposed to the virus, such as blood

product and organ recipients, parenteral drug users, and patients with liver disease. Whether it can be applied to the testing of blood donors and others at low risk, such as samples in population prevalence studies, is not known and has to be investigated separately. It is expected that the positive predictive value of concordant reactivities in the two EIAs will be less in them than in high risk populations and a different validation approach may be required.

Other EIAs using recombinant and oligopeptide antigens from different HCV strains (Lee *et al* and Lunel *et al*, abstracts presented at the 3rd International Symposium on HCV, 1991, Strasborg, France) may soon become available. These should be evaluated in the same manner to determine, firstly, if even more sensitive and specific EIAs can be used as better screening or validation EIAs, and, sec-

ondly, if a third EIA, different in antigenic nature and assay configuration from the screening and validation EIAs, might be used for further investigation of discordant specimens instead of the more elaborate and expensive supplemental tests.

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