Quantification of serum hepatitis C virus core protein level in patients chronically infected with different hepatitis C virus genotypes

E Orito, M Mizokami, T Tanaka, J Y-N Lau, K Suzuki, M Yamauchi, Y Ohta, A Hasegawa, S Tanaka, M Kohara

Second Department of
Medicine,
Nagoya City University
Medical School,
Nayoya, Japan
E Orito
M Mizokami
K Suzuki
M Yamauchi

Liver Unit,
The Tokyo
Metropolitan
Komagome Hospital,
Tokyo, Japan
T Tanaka
S Tanaka

Section of
Hepatobiliary
Diseases,
Department of
Medicine,
University of Florida,
Gainesville,
Florida, USA
J Y-N Lau

International Reagents
Corporation,
Kobe, Japan
Y Ohta

Corporate Research &
Development
Laboratory,
Tonen Co,
Saitama, Japan
A Hasegawa

Department of
Microbiology,
The Tokyo
Metropolitan Institute
of Medical Science,
Japan
M Kohara

Correspondence to:
Dr M Mizokami,
Second Department of
Medicine,
Nagoya City University
Medical School,
1 Kawasumi,
Mizuho-ku,
Nagoya 467, Japan.

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Abstract

Background/Aim—A novel fluorescent enzyme immunoassay (FEIA) for the detection and quantification of serum hepatitis C virus (HCV) core protein was developed. The aim of this study was to evaluate the relation among serum HCV core protein level, HCV RNA level, and HCV genotype in patients with chronic HCV infection.

Patients and Methods—Serum HCV core protein, HCV RNA, HCV genotype were determined in 175 patients using the FEIA, branched DNA-assay (Quantiplex HCV RNA ver 1·0), and serologically defined genotyping assay, respectively. For the specificity, all 13 patients seronegative for anti-HCV were negative for serum core antigen and HCV RNA by FEIA and bDNA, respectively.

Results—FEIA assay seems to be more sensitive than bDNA for patients with HCV type 2 infection (detection: 83·4% *v* 63·4%, p<0·01). There was a good overall correlation between the FEIA and bDNA results. However, when the patients were stratified into their HCV types, a correlation was observed in HCV type 1 but not in type 2 infection. Patients with HCV type 2 infection had a lower serum HCV core protein level (median 56 RFI) compared with type 1 infection (median 149 RFI, p<0·01). Thirty seven patients subsequently received interferon α therapy, patients who showed a complete and sustained response had a lower pretreatment serum HCV core protein level compared with patients who had a relapse and non-responders (36 *v* 338 RFI, p<0·01).

Conclusions—This study showed that FEIA (1) is a good assay for the detection and quantification of serum HCV core protein level, (2) is also very sensitive in detecting HCV core protein in patients with HCV type 2 infection, and (3) may have a role as a predictor of subsequent response to interferon therapy.

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Hepatitis C virus (HCV) is believed to be a member of flavivirus family and has a single strand, positive-sense RNA genome, which is believed to couple with core protein to form the core particle covered by an envelope.[1-3] Because of the low level of circulating HCV particles and the lack of an easily available cell culture or small animal models, direct detection of HCV by conventional techniques was deemed difficult, if at all possible at the present time. So far, diagnosis of HCV infection has relied on the detection of antibody to HCV, an indirect evidence for exposure to this viral agent.[4-5] Detection of HCV genome was made possible with the highly sensitive reverse transcription polymerase chain reaction (RT-PCR),[6] and recently, by the branched DNA signal amplification assay (bDNA).[7] To elucidate the significance of HCV viraemia in relation to disease profile, pathobiology, and as a predictor of response to interferon α (IFN) therapy, quantification assays are necessary, and techniques based on PCR (for example, competitive PCR) or bDNA are used by various investigators to measure HCV viraemia level.[7 8] With these assays, the clinical and pathobiological implications of HCV viraemia have been extensively investigated.[8-11] However, with the availability of more HCV genomic sequences, it becomes obvious that even the highly conserved regions of HCV genome do vary. As a result, the quantitative bDNA assay was found to underestimate HCV type 2 and 3 RNAs.[12 13] Newer molecular biology based assays that can tackle this problem have been designed and hopefully may solve this technical issue.

Recently, a novel assay for the quantification of serum HCV core protein was described. As core proteins are contained within the viral particle, serum levels of HCV core protein should reflect HCV viraemia. This fluorescent enzyme immunoassay (FEIA) is based on the capture and detection of HCV core protein by two highly affinity monoclonal antibodies.[14] Based on nucleotide sequence comparison, these two antibodies were directed to the conserved regions of the HCV core protein. The aim of this study was to determine the applicability of this assay in patients infected with different HCV genotypes. In those patients who subsequently received IFN therapy, pretreatment HCV core protein level in relation to subsequent response to IFN was also assessed.

Methods

Patients

A total of 175 patients (male:female=100:75, age=(mean (SD)) 56·7 (12·4) years) seropositive for antibody to HCV (anti-HCV, second generation enzyme immunoassay, Ortho Diagnostics, Raritan, NJ) were prospectively studied. All patients were seronegative for hepatitis B virus surface antigen (radioimmunoassay, AUSRIA 2, Abbott Laboratories, North Chicago, IL). Liver histology confirmed chronic hepatitis (CH) in 84, liver cirrhosis (LC) in 37, and the remaining 54 patients were found to have hepatocellular carcinoma (HCC) based on clinical and biochemical information, serum α fetoprotein levels, radiological findings and in 41 patients, also confirmed by liver histology. In all samples seropositive for anti-HCV, HCV RNA were detected by RT-'nested' PCR with primers derived from the highly conserved 5'-untranslated region.[6] Thirteen patients seropositive for HBsAg and seronegative for anti-HCV (male:female=9:4; age 45·8 (11·9) years) were recruited as liver disease controls (liver histology: CH in five, LC in five, and HCC in three).

All serum samples were prospectively collected at outpatient clinics and were prepared, aliquoted, and stored at -80°C within two hours. This method has been previously shown to best preserve HCV RNA, and possibly, viral particles.[15]

Of the 84 patients with CH, 37 were subsequently treated with lymphoblastoid IFN (Sumiferon, Sumitomo Pharmaceuticals, Japan), six million unit (MU) daily for two weeks followed by six MU thrice weekly for 22 weeks (468 MU in total). The use of IFN was approved by our local institutional review board and a written informed consent was obtained from all patients according to standard guidelines. Complete and sustained response was defined as sustained normalisation of serum alanine aminotransferase (ALT) during treatment and for the entire follow up period (at least 12 months) after cessation of IFN therapy. The other patients, including patients who relapsed after a complete response and patients showing no or partial response, were defined as non-responders.

Quantification of serum HCV core protein level by FEIA

Serum HCV core protein level was measured by sandwich FEIA using the monoclonal antibody 5F11 (recognising an epitope within amino acid 41–60) for solid phase capture and another monoclonal antibody 5E3 (epitope within 21–40) conjugated with β-D-galactosidase as a reporter.[14] Briefly, to 200 µl serum sample, 40 µl of 25% polyethylene glycol 4000 (PEG4000) was added and incubated for one hour at 4°C. The precipitant was obtained by centrifugation, dissolved in 50 µl of 0·5 M NaCl and 0·5 M sodium citrate, and denatured with 50 µl 0·5 M NaOH for 30 minutes at 37°C. These pretreated samples were added and incubated for 10 minutes in the EIA well coated with the monoclonal antibody 5F11 for 10 minutes. After washing with wash buffer, monoclonal antibody 5E3 conjugated to β-D-galactosidase was added for nine minutes at 37°C to trace HCV core protein. The substrate used was 0·1 mM 4-methylumbelliferyl β-D-galactopyranoside and this was incubated for nine minutes at 37°C. The relative fluorescence intensity (RFI) was measured by a fluorometer at 360 nm for excitation and 450 nm for emission. The cut off value of this assay was previously determined to be less than 20 RFI.

Serum HCV RNA level and HCV genotype determination

Serum HCV RNA level was measured quantitatively by bDNA (Quantiplex-HCV RNA, Ver 1·0, Chiron, Emeryville, CA).[8] The cut off value of this assay was 0·35 million genome equivalent/ml (Meq/ml). Recently, it was shown that HCV RNA level in patients with HCV type 2 infection requires a correction factor of 3 to give a better estimation of HCV RNA.[13] Accordingly, the viraemia level in these patients were adjusted according to this recommendation.

Serologically defined HCV genotype (HCV type) was determined by enzyme immunoassay into HCV type 1, 2, mixed type (type 1 and 2) and indeterminate type as previously described.[16] The nomenclature of HCV genotype was based on the system proposed by Simmonds et al.[17]

Statistics

Categorical data were analysed with Fisher's exact test. The Mann-Whitney non-parametric rank sum test and Student's t test were used where appropriate. Discriminant analysis was used for multivariate analysis.

Results

Of the 175 patients seropositive for anti-HCV, 129 (73·7%) had HCV type 1, 31 (17·7%) type 2, 3 (1·7%) mixed type (type 1 and 2), and 12 (6·9%) indeterminate HCV genotype. There was no significant difference in sex, age, history of transfusion, estimated duration of infection, serum ALT level between patients with different HCV types (Table I).

To evaluate the reproducibility of this HCV core protein assay, three positive standard samples with the low, middle and high HCV core protein level were measured, respectively (Table II). The intra-assay and the inter-assay coefficients of variation (CV) for the standards were calculated to be within the range of 4·20~11·05% and 4·14~9·52%, respectively. For the sensitivity of the assays, among the 175 patients, 146 (83·4%) were positive by FEIA, compared with 111 (63·4%) by bDNA assay (p<0·01). For the specificity, all 13 controls were negative for both HCV RNA by bDNA and HCV core protein by FEIA.

In patients infected with HCV type 1, the median serum HCV core protein level was 149

TABLE I   *Clinical and virological characteristics of the 175 patients with chronic hepatitis C virus infection and 13 controls*

| Characteristics | Serologically defined HCV genotype | | | | |
	Type 1	Type 2	Mixed type (type 1+2)	Indeterminate	Controls*
No of patients	n=129	n=31	n=3	n=12	n=13
Sex (male:female)	75:54	18:13	1:2	6:6	9:4
Age (year, mean (SD))	57·3 (12·5)	55·3 (12·3)	55·0 (10·8)	54·2 (13·4)	45·85 (11·9)
History of blood transfusion (%)	49 (38·0)	8 (25·8)	1 (33·3)	4 (33·3)	0
Duration of infection† (year) (mean (SD))	20·7 (14·2)	21·6 (13·2)	36	21·8 (16·0)	–
ALT (IU/l, mean (SD))	92·3 (90·4)	83·8 (62·0)	79·0 (62·0)	72·4 (48·4)	53·2 (46·0)
Disease (CH:LC:HCC)	61:25:43	16:7:8	2:1:0	5:4:3	5:5:3
HCV core protein level (relative fluorescence intensity (RFI)) by fluorescent enzyme immunoassay (FEIA)					
% positive	86·8	71·0	100	75·0	0
median	149	56	395	49	<20
range	<20~3708	<20~1460	157~594	<20~1262	<20
HCV RNA level (million eq/ml) by bDNA‡					
% positive	71·3	38·7	100	33·3	0
median	2·90	<1·05	3·88	<0·35	<0·35
range	<0·35~40·00	<1·05~20·07	1·10~10·00	<0·35~3·10	<0·35

*Controls were seropositive for HBsAg and seronegative for anti-HCV by 2nd EIA. †Duration of infection was estimated in patients with known history of blood transfusion only. ‡The values of HCV RNA level in patients with HCV type 2 infection were corrected by multiplying the value by 3.

RFI (range: <20 to 3780 RFI) compared with 56 RFI (range: <20 to 1460 RFI, p<0·01) in patients with HCV type 2 infection (Table I). The median serum HCV core protein level in the patients with HCV mixed type infection was 395 RFI (range: 157 to 594 RFI), and for the indeterminate group, the median was 49 RFI (range: <20 to 1262). There was no correlation between serum HCV core protein level and the demographic (sex, age, history of transfusion) and biochemical (serum ALT) parameters. However, the median serum HCV core protein level in patients with CH was 227 RFI (range: <20 to 3708 RFI), compared with 94 RFI (range: <20 to 2070 RFI, p=NS) with LC, and 59 RFI (range: <20 to 1513 RFI, p=0·01) with HCC.

For serum HCV RNA levels, the median levels for the patients with HCV type 1, 2, mixed type, and indeterminate type were 2·90 Meq/ml (range: <0·35 to 40·00 Meq/ml), <1·05 Meq/ml (range: <1·05 (the converted cut off for HCV type 2) to 20·07 Meq/ml), 3·88 Meq/ml (range: 1·10 to 10·00 Meq/ml) and <0·35 Meq/ml (range: <0·35 to 3·10 Meq/ml), respectively. There was a significant difference in serum HCV RNA levels between patients with HCV type 1 and 2 infection

TABLE II   *The reproducibility of HCV core protein assay by fluorescent enzyme immunoassay*

Number	Low level	Middle level	High level
Intra-assay			
1	30·5	150·2	255·2
2	30·6	143·4	228·6
3	37·0	163·4	233·5
4	26·8	162·7	249·7
5	36·4	157·3	265·5
6	31·0	161·4	283·1
7	37·5	157·9	244·8
8	35·2	155·9	238·3
9	37·5	149·3	221·4
10	33·1	152·0	247·6
Mean (SD)	33·56 (3·71)	155·35 (6·53)	246·77 (18·19)
CV (%)	11·05	4·20	7·37
Inter-assay			
1	30·5	149·8	244·5
2	33·5	150·6	250·3
3	29·7	162·8	226·5
4	34·8	163·4	265·0
5	37·4	155·7	231·9
Mean (SD)	33·18 (3·16)	156·46 (6·47)	243·64 (15·27)
CV (%)	9·52	4·14	6·27

CV: coefficient of variation.

(p=0·02) (Fig 1). In patients with HCV type 1 infection, 17 (13·2%) were below the cut off value by FEIA, compared with 37 (28·7%) by bDNA (p<0·01) (Table III). In patients with HCV type 2 infection, nine (29·0%) were below cut off by FEIA, compared with 19 (61·3%) by bDNA (p=0·01).

Overall, there was a correlation between serum HCV core protein level by FEIA and serum HCV RNA level as determined by bDNA (r=0·47, p<0·01). When only patients with detectable HCV core protein and HCV RNA were analysed (n=108, type 1: type 2: mixed type: indeterminate=89: 12: 3: 4), there was a correlation between HCV core protein level and HCV RNA level in patients with HCV type 1 infection (r=0·53, p<0·01), but no correlation was seen in patients with HCV type 2 infection (r=0·25, p=0·44) (Fig 2).

Thirty seven of 84 patients with chronic active hepatitis C were subsequently treated with IFN. Eleven (29·7%) of them showed sustained response, and 26 (70·3%) were non-responders. There was no difference in sex (male:female=4:7 v 8:18), age (52·1 (9·2) v 53·1 (10·4)), history of blood transfusion (2 of 11 v 8 of 26) and pretreatment ALT (mean (SD)) (90·9 (45·6) v 120·7 (65·5) IU/l) between the patients showing sustained response and no response. However, the median HCV core protein level in the sustained response group (median: 36 RFI, range; <20 to 418 RFI) was significantly lower than the no response patients (338 RFI, range; 57 to 1580 RFI) (p<0·01) (Fig 3). For the role of genotype in the response to IFN, two (8·7%) of the 23 patients with HCV type 1 developed sustained response, compared with seven (63·6%) of 11 patients with HCV type 2 (p<0·01). To further analyse factors (sex, age, pretreatment ALT level, HCV genotype, serum HCV RNA level, and serum HCV core protein level) that predicted the subsequent response to IFN therapy, multivariate analysis was performed using a stepwise forward approach. Only HCV genotype (p=0·001) and HCV core protein level (p=0·026) were shown to be significant predicting factors by this analysis.
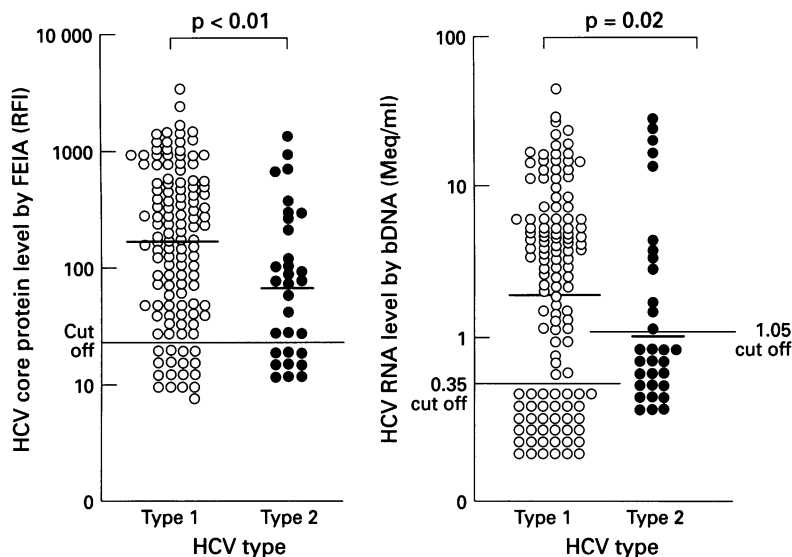
Figure 1: HCV core protein levels (left), and HCV RNA levels (right) in patients with HCV types 1 and 2 infection. The median level of HCV core protein in patients with HCV type 1 was 149 relative fluorescence intensity (RFI) (range; <20 to 3708 RFI), compared with 56 RFI (range; <20 to 1460) in those with HCV type 2 (p<0·01). As the values of serum HCV RNA level by bDNA assay were underestimated in patients with HCV type 2 infection, the values were multiplied by 3 as recommended.[12] The median levels of HCV RNA in patients with HCV type 1 was 2·90 million eq/ml (Meq/ml) (range; <0·35 to 40·0), compared with <1·05 Meq/ml in patients with HCV type 2 (range; <1·05 to 20·07 Meq/ml) (p<0·01). The solid bars represent the medians.

Discussion

This study illustrates four important points. Firstly, this FEIA assay was shown to be a sensitive assay with good reproducibility for the quantification of serum HCV core protein

TABLE III   Relation between serum HCV core protein level as determined by FEIA and HCV RNA level as evaluated by bDNA in patients with chronic HCV types 1 and 2 infection

HCV RNA level by bDNA	HCV core protein level by FEIA		
	≤20 RFI	>20 RFI	Total
HCV type 1 infection (n=129)			
≤0·35 Meq/ml	15 (11·6)	22 (17·1)	37 (28·7)
>0·35 Meq/ml	2 (1·6)	90 (69·8)	92 (71·4)
Total	17 (13·2)	112 (86·9)	129 (100)
HCV type 2 infection (n=31)			
≤1·05 Meq/ml	9 (29·0)	10 (32·3)	19 (61·3)
>1·05 Meq/ml	0 (0)	12 (38·7)	12 (38·7)
Total	9 (29·0)	22 (71·0)	31 (100)

Figures in parentheses are percentages.

irrespective of HCV genotypes. Secondly, there was a correlation between serum HCV core protein level and HCV RNA level as determined by FEIA and bDNA, respectively, in patients with HCV type 1 but not in type 2 infection. Thirdly, patients with HCV type 2 infection had lower level of serum HCV core protein compared with HCV type 1. Finally, low serum HCV core protein level is also an indicator of a subsequent favourable response to IFN.

The finding that this FEIA assay is a sensitive assay for patients with different HCV genotypes emphasise the usefulness of this assay. In an analysis of the conservation of the HCV core amino acid sequences from all known isolates available through GenBanks, the region recognised by the monoclonal antibodies (amino acid 21–60) was shown to be highly conserved (data not shown).

There are a few possible explanations to account for the lack of correlation between serum HCV core protein and HCV RNA in patients infected with HCV type 2. Firstly, the number of patients with HCV type 2 infection was small in this study and may miss a weak correlation. Secondly, the number of HCV core proteins in HCV type 2 may be more variable or low compared with HCV type 1. As most other viruses contain a comparatively stable number of core molecules per viral particle, it is fairly safe to assume that this is the case for HCV, arguing that the quantification of HCV RNA by bDNA version 1·0 on HCV type 2 may be more variable than HCV type 1. Further studies based on large number of patients comparing FEIA and bDNA version 2·0 are required to further define the concordance between these two assays.

The finding that serum HCV core protein level in patients with HCV type 2 infection was lower than those with HCV type 1 is also in accord with previous studies using competitive RT-PCR or bDNA assays, which also showed lower serum HCV RNA level in patients with HCV type 2 compared with type 1.[10] [11] [18] The recent finding that bDNA version 1·0 assay underestimates HCV RNA derived from HCV
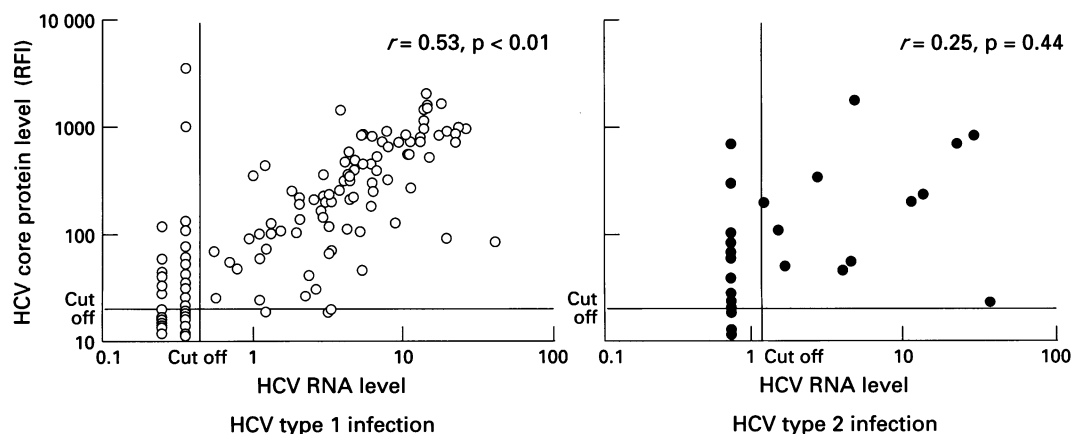

HCV type 1 infection

HCV type 2 infection

Figure 2: The correlation between serum HCV core protein level by FEIA and HCV RNA level by bDNA in patients with HCV types 1 (left) and type 2 (right) infection. In all patients, there was a significant correlation between serum HCV core protein and HCV RNA levels (n=175, r=0·48, p<0·01). When only patients with detectable HCV core protein and HCV RNA were analysed, HCV core protein level correlated to HCV RNA level in patients with HCV type 1 infection (n=89, r=0·53, p<0·01), but not in patients with HCV type 2 infection (n=12, r=0·25, p=0·44).
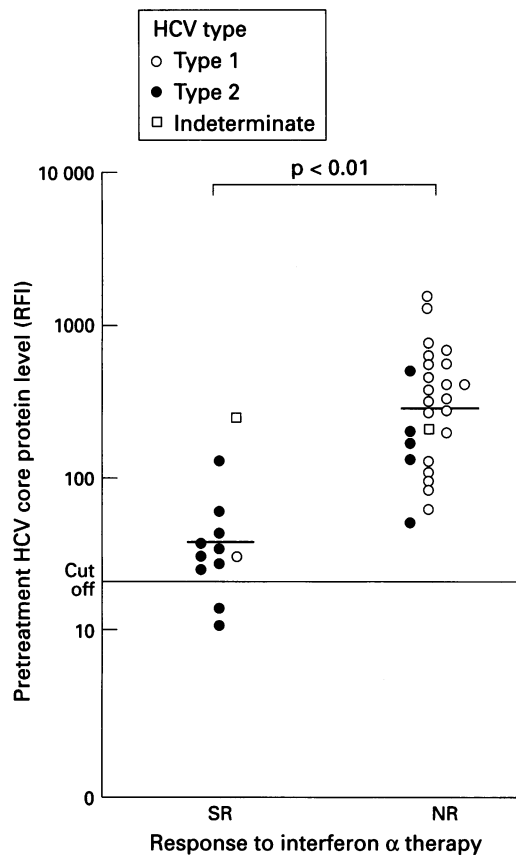
Figure 3: Pretreatment HCV core protein level in lymphoblastoid interferon α therapy. The median level (36 RFI) of HCV core protein in the complete and sustained responders (SR) was significantly lower compared with the non-responders (NR) (338 RFI, p<0·01). The solid bars represent the medians.

type 2 and 3 illustrates the difficulties in developing a reliable assay for the quantification of a viraemia,[12] given the pronounced genetic heterogeneous nature of the HCV genome. bDNA assay version 2·0 was designed to tackle these problems. As this FEIA is using monoclonal antibodies that recognised highly conserved epitopes across HCV type 1 and 2, it is likely that this assay will also be very useful in quantification of HCV viraemia. A proper head to head comparison between two assays in the future may resolve this issue.

It was reported that low pretreatment HCV RNA level is a good predictor of a subsequent favourable response to IFN therapy.[8 11] Our finding of a significant difference in serum HCV core protein level between patients showing different subsequent response to IFN suggests that measuring serum HCV core protein level is a good alternative.

1 Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244:** 1–3.

2 Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* 1991; **88:** 2451–5.

3 Kaito M, Watanabe S, Tsukiyama-Kohara K, Yamaguchi K, Kobayashi Y, Konishi M, et al. Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* 1994; **75:** 1755–60.

4 Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcel RH, et al. An assay for circulating antibodies to a major etiological virus of human non-A, non-B hepatitis. *Science* 1989; **244:** 362–4.

5 Van der Poel VCL, Cuypers HTM, Reesink HW, Weiner AJ, Quan S, DiNello R, et al. Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay. *Lancet* 1991; **337:** 317–9.

6 Okamoto H, Okada S, Sugiyama Y, Tanaka T, Sugai Y, Akahane Y, et al. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5'-noncoding region. *Jpn J Exp Med* 1990; **60:** 215–22.

7 Lau JYN, Davis GL, Knniffen J, Qian KP, Urdea MS, Chan CS, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* 1993; **341:** 1501–4.

8 Kaneko S, Murakami S, Unoura M, Kobayashi K. Quantitation of hepatitis C virus RNA by competitive polymerase chain reaction. *J Med Virol* 1992; **37:** 278–82.

9 Kato N, Yokosuka O, Hosoda K, Ito Y, Ohto M, Omata M. Quantification of hepatitis C virus by competitive reverse transcription-polymerase chain reaction: increase of the virus in advanced liver disease. *Hepatology* 1993; **18:** 16–20.

10 Yoshioka K, Kakumu S, Wakita T, Itoh Y, Takayanagi M, Higashi Y, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon alfa therapy: relationship to genotypes of hepatitis C virus. *Hepatology* 1992; **16:** 293–9.

11 Orito E, Mizokami M, Nakano T, Terashima H, Nojiri O, Sakakibara K, et al. Serum hepatitis C virus RNA level as a predictor of subsequent response to interferon-α therapy in Japanese patients with chronic hepatitis C. *J Med Virol* 1994; **44:** 410–4.

12 Collins ML, Zayati C, Detmer JJ, Daly B, Kolberg JA, Cha TA, et al. Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. *Anal Biochem* 1995; **226:** 120–9.

13 Lau JYN, Simmonds P, Urdea MS. Implications of variations of "conserved" regions of hepatitis C virus genome. *Lancet* 1995; **346:** 425–6.

14 Tanaka T, Lau JYN, Mizokami M, Orito E, Tanaka E, Koyosawa K, et al. Simple fluorescent enzyme immunoassay for detection and quantification of hepatitis C viremia. *J Hepatol* 1995; **23:** 742–5.

15 Davis GL, Lau JYN, Urdea MS, Neuwald PD, Wilber JC, Lindsay K, et al. Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* 1994; **19:** 1337–41.

16 Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, Yagi S, Tanaka S, Hasegawa A, et al. Significance of specific antibody assay for genotyping of hepatatis C virus. *Hepatology* 1994; **19:** 1347–53.

17 Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, et al. A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; **19:** 1321–4.

18 Lau JYN, Mizokami M, Kolberg JA, Davis GL, Prescott LE, Ohno T, et al. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. *J Infect Dis* 1995; **171:** 281–9.