Papers

Clinical application of the Quantiplex HCV RNA 2.0 and Amplicor HCV Monitor assays for quantifying serum hepatitis C virus RNA

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Abstract

Aim—To compare the performance characteristics and clinical application of two different technologies for quantifying serum hepatitis C virus (HCV) RNA levels.

Methods—HCV RNA was quantified by Amplicor HCV Monitor assay (Amplicor) and Quantiplex HCV RNA 2.0 assay (bDNA-2) in 119 sera from 107 HCV infected patients.

Results—Both assays had similar sensitivity (79.4% for Amplicor; 86.0% for bDNA-2), acceptable coefficients of variation (5.3% in Amplicor; 2.6% in bDNA-2), and good linearity ($r^2 \geq 0.98$). There was a positive correlation between quantification values of both methods ($r = 0.683$, $p < 0.001$). The Amplicor values were on an average 1.76 log lower than bDNA-2 results. Male subjects and HCV genotype 1b were significantly associated with higher viral load determined by Amplicor, but not with viral load measured by bDNA-2. In 70 chronic HCV infected patients treated with interferon alfa, mean (SD) pretreatment viral load in 27 complete responders (3.47 (0.84) logs for Amplicor, 5.63 (0.58) for bDNA-2) was significantly lower than in non-responders (4.43 (1.01) logs for Amplicor, 6.10 (0.67) logs for bDNA-2; $p < 0.001$). Cut off points of 3.9 logs for Amplicor and 5.8 logs for bDNA-2 were determined to be the best for predicting response to interferon alfa, giving acceptable sensitivity (70.4%, 74.1%), specificity (72.1%, 65.1%), and accuracy (71.4%, 68.6%), respectively.

Conclusions—Both the Amplicor and bDNA-2 assays are clinically useful methods for HCV RNA quantification and are reliable for predicting the outcome of treatment, despite differences in absolute quantification values and in the correlation between HCV genotypes and viral load.

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Hepatitis C virus (HCV), a highly heterogeneous, single stranded RNA virus, is the major aetiological agent in parenterally transmitted non-A, non-B hepatitis and often causes persistent infection leading to chronic liver disease and primary hepatocellular carcinoma.¹⁻³ Serum HCV RNA (viral load) may correlate with the clinical stage of disease⁴ and HCV genotype.⁵ A low HCV viral load has also been observed to be one of the important predictive markers of successful treatment with interferon alfa.⁶⁻⁸ Assays for serum HCV RNA quantification should therefore be accurate and reproducible to permit careful clinical assessment of different HCV patient populations. Competitive reverse transcription polymerase chain reaction (RT-PCR) is useful for evaluating the state of viral replication.^{4,5} However, the method is costly, time consuming, and technically challenging, as reflected by large variations in sensitivity among different laboratories. Two commercial assays for HCV RNA quantification have become available and are widely used: the branched DNA assay (Chiron Corporation, Emeryville, California, USA) and the Amplicor HCV Monitor assay (Roche, Nutley, New Jersey, USA).^{9,10} There are relatively few studies comparing the current version of bDNA assay¹¹ (Quantiplex HCV RNA 2.0; bDNA-2) and the original version of the Amplicor assay, particularly in assessing

Figure 1 Analysis of HCV RNA in serial dilutions of high titre serum by two different quantitative assays, the Quantiplex branched-DNA 2.0 (bDNA-2) assay and the Roche Amplicor HCV Monitor assay.

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Table 1 Correlation between HCV genotypes and quantification values by the Amplicor and bDNA-2 assays

Genotype	No	HCV RNA concentration*		Spearman's rank correlation coefficient	p Value
		Amplicor	bDNA-2		
All types	119	4.16 (1.09)	5.93 (0.69)	0.683	<0.001
1b	40	4.81 (0.99)	5.99 (0.73)	0.843	<0.001
2a	46	3.77 (0.87)	6.06 (0.63)	0.784	<0.001
2b	7	4.15 (1.57)	5.88 (1.05)	0.785	<0.05
3a	1	4.11	5.46		
Mixed	9	3.60 (1.06)	5.78 (0.57)	0.801	0.01
Unclassified	16	3.95 (1.07)	5.57 (0.53)	0.758	0.001

* Expressed as mean (SD) of logarithmic transformation (log copy or equivalent/ml).

Table 2 Background of HCV infected patients and their serum HCV RNA levels measured by the Amplicor and bDNA-2 assays

Characteristics	No	Amplicor*	bDNA-2*
All patients	107	4.17 (1.10)	5.93 (0.65)
Age (years)			
Below 40	32	4.03 (1.09)	5.88 (0.65)
40 to 60	54	4.34 (1.07)	5.97 (0.64)
Above 60	21	3.96 (1.18)	5.92 (0.72)
Sex			
Male	59	4.40 (1.08)†	6.03 (0.61)
Female	48	3.90 (1.07)†	5.82 (0.68)
Mode of transmission			
Transfusion	41	3.96 (1.13)	5.88 (0.60)
Percutaneous procedure	35	4.20 (1.07)	5.93 (0.62)
Unknown	31	4.31 (1.09)	6.01 (0.67)
Alanine aminotransferase			
Normal (≤ 25 IU/l)	6	3.40 (0.84)	5.46 (0.55)
Abnormal (> 25 IU/l)	101	4.22 (1.10)	5.96 (0.65)
Histology			
Acute hepatitis	4	4.45 (1.24)	5.80 (0.81)
Chronic persistent hepatitis	17	4.52 (0.89)	6.11 (0.76)
Chronic active hepatitis	53	3.99 (1.08)	5.94 (0.59)
Liver cirrhosis	22	4.28 (1.16)	5.91 (0.58)
Hepatocellular carcinoma	11	4.19 (1.34)	5.74 (0.86)
HCV subtype			
1b	40	4.81 (0.99)‡	5.99 (0.73)
Non-1b	67	3.79 (0.98)‡	5.90 (0.60)
2a	41	3.72 (0.86)	6.05 (0.58)
2b	4	4.45 (1.58)	5.92 (0.91)
3a	1	4.11	5.46
Unclassified	12	3.94 (1.17)	5.54 (0.50)
Mixed infection	9	3.60 (1.06)	5.78 (0.57)

* Mean (SD) deviation of logarithmic transformation of log copies or equivalents/ml.

†Probability of difference in sex and viral load by the Amplicor assay ($p < 0.05$).

‡Probability of differences in HCV genotypes and viral load by the Amplicor assay ($p < 0.001$).

their ability to predict response to interferon alfa treatment in patients with chronic hepatitis C. Objectives of the current study were: (1) to compare the clinical sensitivity, reproducibility, linearity, and correlation of the Amplicor and bDNA-2 assays in patients of different HCV genotypes and stages of infection; and (2) to

Table 3 Stepwise multiple linear regression analysis of factors associated with serum HCV RNA levels

Assay variables	Coefficients	Standard error of coefficient	Adjusted multiple r^2	p Value
Amplicor assay				
/ Intercept (log copies/ml)	4.38			
/ Sex (male = 1, female = 2)	0.39	0.19	0.23	<0.05
/ HCV genotype (genotype 1 = 1, non-genotype 1b = 0)	0.97	0.20	0.20	<0.01
bDNA-2 assay				
/ Intercept (log eq/ml)	5.93			

Factors such as age, sex, mode of transmission, aminotransferase, histology, and HCV genotype were analysed by stepwise multiple linear regression. Males and patients infected with genotype 1b virus had significantly higher HCV RNA levels, as determined with the Amplicor assay. No factors were significantly associated with viral load measured by the bDNA-2 assay.

determine the clinical value of these two commercial HCV viral load assays for predicting response to interferon alfa treatment.

Methods

PATIENTS

One hundred and seven Taiwanese patients were enrolled in the study. Seventy patients with chronic hepatitis C were treated with 6 MU of interferon alfa given intramuscularly three times a week for 24 weeks. Complete responders were defined as patients showing normal alanine aminotransferase (ALT) levels and clearance of HCV RNA by nested RT-PCR at the end of the treatment and for six months after the cessation of treatment. All other patients were classified as non-responders. All serum samples were positive for HCV RNA, including 107 samples collected at the time of liver biopsy or aspiration cytology for measurement of baseline viral load, and an additional 12 samples from 12 patients at the third month of interferon alfa treatment.

DETECTION/QUANTIFICATION OF SERUM HCV RNA AND GENOTYPING

HCV RNA was detected qualitatively by nested RT-PCR using 5' non-coding region (5'NCR) specific primers.¹² The detection limit of the nested RT-PCR is 50 copies/ml. HCV genotyping was determined by method described by Okamoto *et al.*¹³ Levels of serum HCV RNA were measured with the bDNA-2 and Amplicor assays according to the manufacturer's instructions, which were followed exactly. HCV RNA was detected directly in the bDNA-2 assay by a series of probe hybridisations to boost the signal coming from each HCV RNA. The relative intensity of the signal was compared against an external standard curve, giving a quantification of 0.2–120 million equivalents (Meq)/ml. The Amplicor assay is based on reverse transcription and amplification of HCV RNA with primers targeting an xbp region within the 5' NCR of the HCV genome. An internal quantitation standard (an RNA molecule derived from genotype 1 sequence of the HCV) was coamplified with the HCV RNA target. Both the amplified products of the internal standard and sample RNA were serially diluted and detected by hybridization in a colorimetric assay. Results were calculated manually according to the manufacturer's instructions, giving a quantification range of 10^{3-6} copies/ml.

STATISTICAL ANALYSIS

Data are expressed as mean (SD) after logarithmic transformation of original values. The χ^2 test, Student's *t* test, analysis of variance, Spearman's rank correlation coefficient, simple linear regression, and stepwise multiple linear regression were used. We compared the area under the curve with the receiver operating characteristics (ROC) analysis^{14,15} and made an attempt to derive a suitable clinical cut off for each assay that would best predict the response to interferon alfa treatment. For the purpose of analysing the

Figure 2 Pretreatment levels of serum hepatitis C virus RNA in responders (CR) and non-responders (NR) to interferon alfa treatment at 6 MU thrice weekly for 24 weeks assessed by the Roche Amplicor HCV Monitor assay (Amplicor) and Quantiplex branched-DNA 2.0 assay (bDNA-2). Of 70 patients, 27 were responders. The bars represent mean pretreatment level of serum hepatitis C virus RNA in each group.

data by suitable statistical methods, we assigned a nominal value of 0.1 Meq/ml to samples that were negative by bDNA-2 but positive for HCV RNA by nested RT-PCR, and a nominal value of 500 copies/ml to samples that were Amplicor negative but positive by nested RT-PCR.

Results

PERFORMANCE CHARACTERISTICS OF THE AMPLICOR AND bDNA-2 ASSAYS

Of 107 samples for baseline viral load, HCV RNA was quantifiable in 85 patients (79.4%) by Amplicor and in 92 (86.0%) by bDNA-2. Eighty (74.8%) samples were quantifiable by both assays, while 10 (9.4%) were under the detection limit of both assays. For 12 PCR positive samples collected at the third month of interferon alfa treatment, the percentage of quantifiable samples by Amplicor and bDNA-2 was similar (41.7% and 58.3%, respectively).

To investigate assay reproducibility, each assay was tested on two occasions using different lots of reagents with separate aliquots of a

panel of 16 samples. The mean per cent coefficient of variation based upon repeat testing was 5.3% (range 0.8% to 10.4%) for Amplicor and 2.6% (range 1.3% to 7.1%) for bDNA-2. Linearity of quantification was examined with a dilution panel made by six threefold serial dilutions of a high titre serum sample into normal human sera. As shown in fig 1, both bDNA-2 and Amplicor had good linearity within the linear range ($r^2 = 0.99$ and 0.98, respectively).

HCV RNA quantification values measured by the Amplicor and bDNA-2 assays were positively correlated (Spearman's rank correlation coefficient, $r = 0.683$, $p < 0.001$). The Amplicor values were on average 1.76 log lower than the bDNA-2 results. Non-parametric tests of correlation gave a high correlation coefficient upon pairwise comparison of samples with different genotypes (table 1). However, the mean difference in quantification values between these two assays was 1.1 log for genotype 1 and 2.1 logs for non-genotype 1 ($p < 0.05$).

HCV VIRAL LOAD AND CLINICAL MANIFESTATIONS

Patient age, sex, mode of transmission, liver biochemistry, liver histology, and viral genotype were analysed to evaluate the relation between HCV viral load and clinical manifestation of HCV infection. By univariate analysis, viral load determined with Amplicor did not correlate with patient's age, mode of transmission, and severity of liver disease (table 2). HCV viral load was significantly different between male and female patients ($p < 0.05$), and among different HCV genotypes ($p < 0.001$). In further analysis using stepwise multiple linear regression, sex and HCV genotype remained as significant factors affecting serum HCV RNA levels measured by Amplicor (table 3). For the bDNA-2 quantification results, none of the factors analysed was found to be correlated with viral load by either univariate or multivariate analyses.

PRETREATMENT HCV RNA LEVELS AND RESPONSE TO INTERFERON ALFA TREATMENT

In 70 interferon alfa treated patients, pretreatment viral load in 27 complete responders (38.6%) (3.47 (0.84) logs for Amplicor, 5.63 (0.58) for bDNA-2) was significantly lower than that in non-responders (4.43 (1.01) logs for Amplicor, 6.10 (0.67) logs for bDNA-2; $p < 0.001$, fig 2). There was no association between sex, age, pretreatment ALT levels, liver histology, HCV genotype, kind of interferon, or history of blood transfusion with the interferon response. After dividing patients into three groups with low, medium, and high viral load, the percentage of complete responders was observed to decrease with higher pretreatment serum HCV RNA concentrations ($p < 0.05$, χ^2 test with linear correlation) (table 4). In an attempt to accurately predict interferon alfa response by the pretreatment viral load, the most appropriate cut off in pretreatment HCV RNA was calculated by using the ROC analysis. In this analysis, patients with a pretreatment viral load lower than the cut off

Table 4 Correlation between pretreatment levels of serum HCV RNA and response to interferon alfa treatment

Pretreatment serum HCV RNA level	CR (n=27)	NR (n=43)	p Value
Amplicor (log copies/ml)			
Low (≤ 3)	13 (68.4%)	6 (31.6%)	<0.05*
Median (3–4.5)	9 (39.1%)	14 (60.9%)	
High (> 4.5)	5 (17.9%)	23 (82.1%)	
bDNA-2 (log eq/ml)			
Low (≤ 5.3)	8 (66.7%)	4 (33.3%)	<0.05*
Median (5.3–6.5)	15 (38.5%)	24 (61.5%)	
High (> 6.5)	4 (21.1%)	15 (78.9%)	

CR, complete responder; eq, equivalent; NR, non-responder.

* χ^2 test with linear correlation.

Table 5 Prediction of complete response to interferon alfa treatment based on the calculated clinical cut off values for serum HCV RNA levels

Assays	Cut off values	Sensitivity (n/N)	Specificity (n/N)	Accuracy (n/N)
Amplicor (copies/ml)	8×10^3 (3.9 logs)	70.4% (19/27)	72.1% (31/43)	71.4% (50/70)
bDNA-2 (eq/ml)	0.65×10^6 (5.8 logs)	74.1% (20/27)	65.1% (28/43)	68.6% (48/70)

Seventy patients with chronic hepatitis C treated with interferon alfa, 6 MU thrice weekly for 24 weeks, were studied. The complete response rate was 38.6% (27/70).

value would be considered as probable complete responders, while those with a viral load higher than the cut off value would be regarded as probable non-responders. Using a cut off value of 3.9 logs for Amplicor and 5.8 logs for bDNA-2, both the Amplicor and bDNA-2 assays had acceptable sensitivity (70.5% and 74.1%), specificity (72.1% and 65.1%), and accuracy (71.4% and 68.6%) in predicting complete response to interferon alfa given at 6 MU thrice weekly for 24 weeks (table 5).

Discussion

Serum HCV RNA is an important predictive factor of response to interferon treatment in patients with chronic hepatitis C.⁶⁻⁸ Easy, reliable, standardised quantitative HCV RNA tests with good accuracy and reproducibility are therefore needed to popularise the routine use of viral load in the clinical management of HCV patients. We undertook this study to compare the commercially available Amplicor and bDNA-2 assays for clinical sensitivity, reproducibility, linearity of quantitation, and ability to quantify all HCV genotypes with equal sensitivity. Both assays showed good reproducibility, based on repetitive testing of a panel of 16 clinical samples in our study. The between run coefficients of variation were 5.3% and 2.6% in the Amplicor and bDNA-2 assays, respectively. This contrasts with the poor reproducibility in the Amplicor assay reported by Hawkins *et al.*¹⁶ Possible explanations for this discrepancy might be the small number of samples tested in the present study and in that by Hawkins, or differences in technical expertise in performing the test at various laboratories.

We observed that the Amplicor and bDNA-2 assays had similar clinical sensitivity, despite the vastly different quantification limits assigned by the manufacturers. Although quantification results by these two tests were positively correlated, the Amplicor values were 1.76 log lower than the values obtained with the bDNA-2 assay. This difference could result from factors such as quantification standards of different lengths, sequences, and chemical properties.^{10 17}

An important observation in our study was that there were discrepancies in the relation between genotypes and HCV viral load depending on the quantification method used. HCV genotype 1b samples were shown to have significantly higher viral load than other genotypes in the Amplicor assay. No genotype dependent differences in viral load was observed for the bDNA-2 assay, consistent with previous reports,¹⁶ as well as with larger surveys of blood donors and patients when values obtained with the first generation bDNA assay were corrected for lower sensitivities for genotypes 2 and 3.^{18 19} Furthermore, the mean difference in quantitation values between these two assays was 1.1 log for genotype 1 and 2.1 logs for non-genotype 1, even though the Amplicor and bDNA-2 results correlated well with each other for each of the HCV genotypes. Differences in viral load among HCV genotypes may have been caused by the absolute

efficiency of HCV RNA detection rather than being a real biological phenomenon. HCV exists as a heterogeneous group of viruses sharing approximately 70% homology, and shows significant variations even in the most "conserved" regions of the HCV genome.^{20 21} The Amplicor assay uses the most conserved regions of genotype 1 HCV as target sequences for amplification and probe hybridisation. However, minor sequence variations between the genotype 1 sequences used in PCR primers and target sequences of non-genotype 1 viruses,²⁰ and genotype specific differences in the strength of base pairings and in the secondary structure of the 5'NCR, may interfere with primer annealing and thus lead to underestimation of viral load for non-genotype 1 samples.²¹

An unexpected finding in our study was that male sex was associated with significantly higher levels of HCV RNA by the Amplicor results. Similar results had been observed in dialysis patients by using competitive PCR²² and in blood donors using the corrected bDNA-1 results,¹⁸ but not in other studies.^{4 9} The lower viral load in female subjects might be caused by sex specific differences in viral replication that could not be detected by the bDNA-2 assay in the present study. On the other hand, the observation might be an opportunistically statistical bias: confounding factors could not be controlled for with multivariate analysis owing to the fact that the Amplicor assay might have genotype specific differences in detection efficiency, and that a difference in genotype distribution among male and female subjects (42.4% male, 31.3% female for genotype 1 patients) contributed to sex specific differences in viral load.

Our study, as well as most other,⁶⁻⁸ documented that pretreatment HCV RNA level is one of the factors influencing the outcome of interferon alfa treatment, and may in fact be the single most important determinant.⁶ As it would be very useful for clinicians to be able to use a certain pretreatment viral load for gauging responsiveness to interferon therapy, we made an attempt to derive a suitable clinical cut off for each assay that would best predict the response to interferon. By using the ROC analysis, patients with an HCV viral load of less than 3.9 logs in the Amplicor assay or 5.8 logs in the bDNA-2 assay were considered likely to be complete responders to interferon given a dose of 6 MU thrice weekly for 24 weeks. When these cut off points were used to analyse the interferon response in our treated patients retrospectively, the predictive rate was 70% with either of the two assays, suggesting that either assay could be used for routine viral load quantification to tailor treatment regimens. In patients with a high viral load who are expected to respond poorly to conventional interferon treatment, a higher dose, a longer duration of interferon treatment, or a combination of ribavirin plus interferon may be appropriate.²³

CONCLUSIONS

Both the Amplicor and bDNA-2 assays are clinically useful methods for HCV RNA quan-

tification, despite the existence of method related discrepancies in quantification values and in the correlation between HCV genotypes and viral load. Both assays are reliable for monitoring virological changes throughout the course of HCV infection and in selecting patients for treatment, and also may provide decision making information for future therapeutic strategies. However, the two assays cannot be used interchangeably owing to inherent differences in technology and performance characteristics. Clinicians should weigh the advantages and weaknesses of each assay in selecting one particular assay for clinical studies and patient management.

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