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Total HCV core antigen assay: a new marker of HCV viremia and its application during treatment of chronic hepatitis C

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Abstract
The present study assesses the clinical usefulness of the hepatitis C core antigen assay for monitoring of patients being treated for chronic hepatitis C virus (HCV) infection. Eighty-six serum samples were selected at random from 16 patients and levels of HCV RNA and HCV core antigen were determined simultaneously and in parallel to compare both techniques. The data obtained were compared by Pearson's correlation and the coefficients calculated by Fisher transformation and by calculating the difference and standard error. A good linear correlation was observed between both techniques. Maximum correlation, with significant difference, was found between patients infected with the 1a genotype and other genotypes. In conclusion, the HCV core antigen assay is useful for the diagnosis of early infection; however, its use for determining the exact timing of viral elimination during treatment is clearly unsuitable.

Keywords
Hepatitis C; HCV core antigen assay; Treatment; Diagnosis

1. Introduction

The presence of hepatitis C virus (HCV) can be demonstrated by direct detection techniques using monoclonal antibodies that recognize specific viral antigens. Chemiluminescent substrates and photometry are used frequently, given the high sensitivities achieved. Although HCV infection does not induce high viremia, attempts to develop procedures for antigen detection in serum date back to 1992 (Katayama et al., 1992; Tanaka et al., 1995; Kashiwakuma et al., 1996; Orito et al., 1996; Tanaka et al., 1996; Jolivet-Reynaud et al., 1998; Masalova et al., 1998; Kobayashi et al., 1998). The target of these assays was the core protein of HCV, since it is the most abundant protein in the virion and its immunodominant epitopes are well-conserved in different strains. Although the performance of these methods seemed promising, it was not until 1999 that the first useful report was published. In the original description (Aoyagi et al., 1999), this method was able to detect antigen concentrations of up to 0.06 pg/ml, roughly 500 copies per ml. Moreover, it was able to detect infections in any serotype. The correlations with the PCR techniques ranged from 94% to 96%. The presence in the sample of substances, such as anticoagulants, hemoglobin, rheumatoid factor, bilirubin or lipids, that could possibly interfere with these assays had no effect on the method. The linear response range was broad and the coefficient of variation between 4% and 7.9%. After the optimization and standardization of the use of detergents (Peterson et al., 2000), a new method for the detection of core antigen in serum was developed. This method was able to detect viremia in all stages of infection and not only in the antibody negative window phase. In the first studies, the cutoff value was 2.3 pg/ml, which was equivalent to 50,000 IU/ml, measured by PCR (Icardi et al., 2001). However, more recent studies have demonstrated that it is possible to reduce the cutoff value of the assay to 1.5 pg/ml without loss of specificity (Zanetti et al., 2002; Picchio, 2002). The present study aims to assess the clinical usefulness of this new technique for monitoring patients under treatment for chronic HCV infection.

2. Materials and methods

This study was conducted at the Department of Internal Medicine B of Juan Canalejo Hospital (La Coruña, Spain) during 2002. Prior informed consent was obtained from all patients. Serum samples were obtained from the serum bank of the hospital and stored at −85 °C until use. Eighty-six serum samples were selected at random from 16 patients with chronic HCV hepatitis on different antiviral treatment schedules (interferon alpha plus ribavirin) and then tested simultaneously and in parallel to determine:

1. HCV RNA using the Cobas Amplicor Monitor kit (Roche version 2.0), following the manufacturer's instructions. The results, expressed in IU/ml, ranged from 250 to 800,000. This method is a PCR-based quantitative and non-competitive assay. The process included RNA extraction and reverse transcription and amplification from the 5′-untranslated region using primers. The samples and the quantitation standard were detected by hybridization using a biotinylated-labelled probe. All procedures, except for the extraction step, were automated.
2. HCV core antigen tests using a Trak-C ELISA kit (Chiron-Ortho, Ortho-Clinical Diagnostics, Johnson & Johnson Company), following the procedure recommended by the manufacturer. This is a microplate test, consisting of a two-step manual immunoassay that uses several monoclonal antibodies with specificity for different regions of the HCV core antigen. The antibodies covering the surface of the microplate bind the antigen and Fab fragments of the horseradish peroxidase-conjugated monoclonal antibodies unite with the bound antigen. HCV core antigen levels ranged between a maximum value of 160 pg/ml and a minimum of 0 pg/ml.

Briefly, the protocol involved four stages:

(a) Prior to starting the assay, samples were incubated with pretreatment reagent in uncoated microplates, as indicated in the protocol (Ortho-Clinical Diagnostics Chiron-Ortho Trak-C Protocol of Use), to break any immunocomplexes formed.
(b) The pretreated sample was diluted and incubated in a microplate coated with monoclonal antibodies that bind the immunoreactive HCV core antigen. At the end of the incubation, the microplate was washed to remove unbound material.
(c) The conjugate (Fab fragments of monoclonal antibodies conjugated to peroxidase) was added to the microplate and the mixture incubated. The conjugate binds the antigen attached to the antibodies on the surface of the microplate. The plate was washed again to eliminate unbound material.
(d) An enzymatic detection system composed of *o*-phenylendiamine (OPD) and hydrogen peroxide was added to the microplate. In the presence of bound conjugate, OPD is oxidized, yielding a reddish product. In this reaction, peroxidase is oxidized divalently by hydrogen peroxide to form an intermediate compound that is also reduced to the initial state by a subsequent interaction with the hydrogen ion donated by OPD. The resulting form of OPD is orange and the color intensity is proportional to the amount of core antigen present in the plate. This is measured in a photometer at 490 or 492 nm with a reference filter at 620 or 630 nm.

Genotyping was carried out prior and during testing of one sample from each patient by INNO-LiPA (Boehringer-Ingelheim), according to the standard method described by the manufacturer. Data comparisons were performed by Pearson's correlation coefficient (confidence interval $P < 0.05$, unless otherwise indicated). Comparison of the coefficients calculated was carried out by Fisher transformation and by calculating the difference and standard error.

3. Results

Eighty-six samples from 16 patients (mean of samples per patient was 5.3 with a maximum of 12 and a minimum of 4) were studied. The distribution of sample genotypes was 51% for 1b and the rest were grouped as shown in Fig. 1. Using the sample analysis by PCR as a reference, the distribution of viral RNA expressed in IU/ml showed that over 50% of the samples appeared at end values (>700,000 and <100,000 IU/ml), as depicted in Table 1.

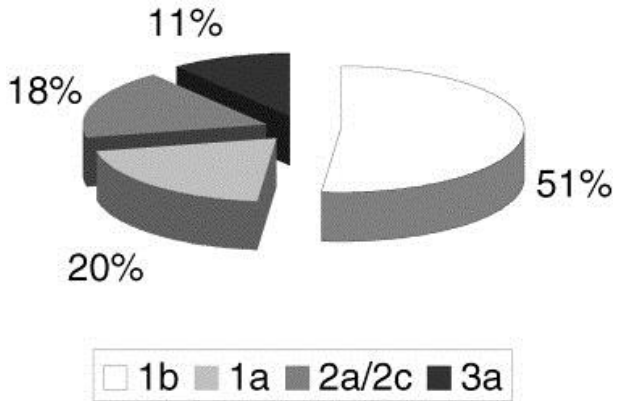

Fig. 1. Genotype distribution in the sample group.

Table 1. Sample distribution as a function of viral load

Viremia RNA (IU/ml)	%	Number of cases
>800,000–700,000	27.9	24
700,000–500,000	10.4	9
500,000–300,000	12.7	11
300,000–100,000	13.9	12
100,000–<250	34.8	30

To compare both techniques, a correlation study was undertaken between the continuous quantitative values of viral load, expressed in pg/ml of core antigen in serum, and viral RNA in IU/ml. Good linear correlation was observed, giving a Pearson's correlation coefficient value of 0.887 that was significant with $P < 0.001$.

Hence, there is strong positive correlation between the two techniques, as seen in Fig. 2. To extrapolate these results to the population, the confidence interval of the correlation coefficient was calculated for a confidence interval of 99% ($P < 0.01$), obtaining maximum and minimum values of 0.943 and 0.781, respectively. Both were within the significance level for a sample of 86 cases. This strong correlation was maintained throughout the entire range, except at the lower and upper limits. In Table 2, the correlation coefficient is expressed for RNA values from 800,000 to 250 IU/ml, grouped according to levels of viremia. It also depicts the confidence intervals for $P < 0.05$. However, differences between the plots were not significant.

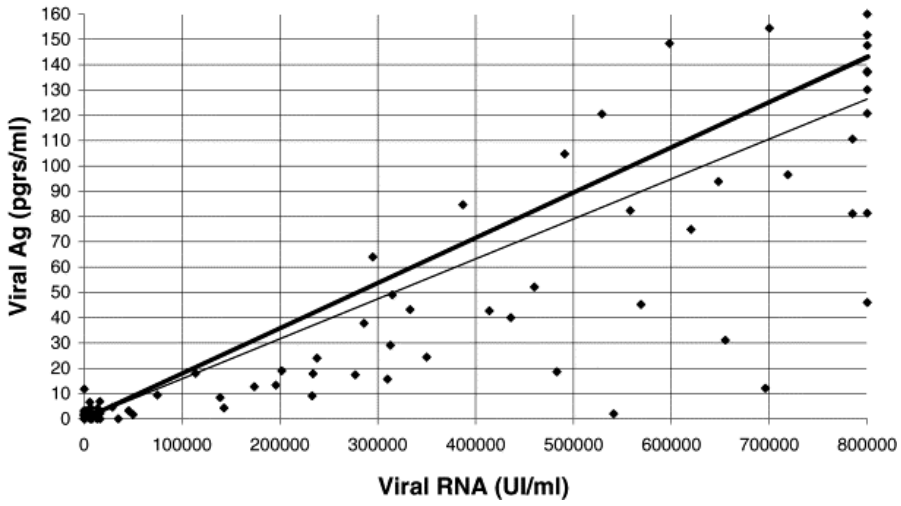

Fig. 2. Linear correlation calculated of viral RNA vs. core Ag.

Table 2. Correlation coefficient as a function of viral load

Viremia RNA (IU/ml)	Correlation coefficient[a]
>800,000–700,000	0.157 (−0.4 to 0.5)
700,000–500,000	−0.12 (−0.6 to 0.59)
500,000–300,000	0.41 (−0.6 to 0.81)
300,000–100,000	0.66 (−0.58 to 0.90)
100,000–<250	0.32 (−0.36 to 0.61)

[a] Correlation coefficient with confidence interval ($P < 0.05$).

Once the existence of correlation was established, formulaes of linear regression were calculated using the data points, where the amounts of antigen and RNA were obtained, respectively, as

$$\text{antigen}(\text{pg/ml}) = 0.000158\text{RNA (UI/ml)}$$

$$\text{RNA}(\text{UI/ml}) = 5590\text{Ag}(\text{pg/ml}),$$

assuming 0 pg/ml = 0 IU/ml. The correlation was equally good for each of the genotypes present in the sample group, varying between 0.75 and 0.99. Differences between correlation coefficients were analyzed and maximum correlation, with significant difference ($P < 0.05$), was found for patients having 1a genotype when compared with the other genotypes. Significant differences were not found for the other genotypes (Table 3).

Table 3. Correlation coefficient as a function of viral genotype

Genotype	Correlation coefficient[a]
3a	0.934 (0.72–0.97)
1a	0.991 (0.98–1)
1b	0.88 (0.79–0.94)
2a/2c	0.753 (0.5–0.95)

[a] Confidence interval ($P < 0.05$).

Finally, a qualitative comparison (whether positive or negative for viremia) of both techniques was performed to assess the sensitivity and specificity of this new technique (Table 4). The cutoff values of RNA in serum were set at 250 IU/ml for the PCR technique and 1 pg/ml for the HCV core antigen technique. In the sample group studied, specificity (the probability of a negative patient being evaluated accurately) was 0.4, that is, two out of a total of five patients were negative; however, sensitivity (the probability of the result being positive when the patient was positive) was 0.959. The predicative value for a negative result was 0.166, that is, the probability of a negative value being real is low in this series (in fact, the number of false negatives surpassed the real negative values). In contrast, the predicative value for a positive result was high, showing a value above 0.9. Three antigen positive, PCR negative samples were found and all showed low

levels of Ag (1.2–3 pg/ml). For other factors studied (age, sex, evolution of the infection or degree of inflammatory activity), no significant differences were found in the correlation of both methods.

Table 4. Qualitative comparison of both techniques

	RNA+	RNA−	Total
Positive antigen	71	3	74
Negative antigen	10	2	12
Total	81	5	86

4. Discussion

The results show excellent correlation between the HCV core antigen detection technique and the PCR-based method, especially at intermediate values, between 100,000 and 500,000 IU/ml of viral RNA. In comparison with PCR analyses, the clinical sensitivity of the HCV core antigen detection technique was close to 100% in at least three independent studies (Tanaka et al., 2000; Bouvier-Alias et al., 2002; Shah et al., 2003). In the chronic stage of infection, correlation with the PCR technique ranged between 88.5% and 100% (Peterson et al., 2000; Picchio, 2002; Lee et al., 2001; Bahl et al., 2001). With reference to monitoring treatment, thus far, a good correlation was shown between the method presented and quantitative PCR, fluctuating between 87% and 100% (Tokita et al., 2000; Busch et al., 2001). These sensitivities are accompanied by a specificity between 99.2% and 99.9% after studying more than 1350 negative samples (Busch et al., 2001; Bahl et al., 2001). However, this excellent relation at intermediate values deteriorates as limit values of distribution were reached. This phenomenon could be related at least in part to the fact that both techniques have a limit; hence, values outside of this range are no longer linear and extrapolation is not possible. The same occurred with values near the upper limit, linearity also being lost progressively. In this study, only 37% of samples were within the linear range of the assay.The minimum values obtained in the sample group also showed diminished correlation. As described in the literature, in general, this lack of correlation is produced in samples with viremia in the order of 1000 copies per ml. The clinical relevance of these errors is debatable, given in the early phases of infection, the correlations are high (the viral loads are within the range) and for the same reason, this technique also serves to detect early responders (Zanetti et al., 2002), for early prediction of the response (Bahl et al., 2001) and the absence of this response (Zanetti et al., 2002) in treated patients. The presence of low viremia in intermediate phases of treatment (where the discrepancies are found) could affect the ability of the method to identify patients who have a relapse after treatment. Nonetheless, the method offers linear quantitation between 2.3 and 100 pg/ml with a specificity of 99.2% and variation coefficient between 7% and 33% (Busch et al., 2001).

The core antigen technique examined in the present study showed low negative predicative value (due to the low number of RNA negative patients in the sample, 5 out of 86); however, this predicative value would be higher in the general population, where there are more non-infected patients and viremia below 5 logs is infrequent. This limitation in detecting the absence of viremia should be considered when using this technique to monitor patients during treatment. These findings have been reported in previous studies (Zanetti et al., 2002; Bahl et al., 2001; Busch et al., 2001) and undoubtedly are due to the presence of low viremia in this group of patients.

It is true that some studies noted the lack of correlation as a function of the different viral characteristics, for example, the presence of certain mutations (Tokita et al., 2000), yet the correlation difference in favor of a particular genotype is striking, specifically because it is clear when compared with each of the other genotypes. There is no explanation for the phenomenon, since when other factors that could possibly be involved, such as magnitude of viral load, are studied, no significant differences are found. To the best of our knowledge, this finding has not been described in the literature.

There is good correlation between the measurements of viral RNA and of core antigen in the serum of patients with chronic infection by HCV under treatment. This correlation was maintained throughout the entire range of RNA values and it was apparently better for intermediate viral loads (100,000–300,000 IU/ml), although it never reached significant differences. If only the ability of the test to distinguish between positive and negative patients for circulating virus is considered, this method is useful for the diagnosis of early infection. Although it is easier and cheaper than PCR–RNA, it is clearly inappropriate for determining the exact moment of viral elimination during treatment. In studying other factors that could influence the degree of correlation, it was found incidentally that this correlation was significantly greater between patients infected with the 1a genotype, independently of the level of viral load or the number of cases studied, which requires further study.

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