

Novel Assay Using Total Hepatitis C Virus (HCV) Core Antigen quantification for Diagnosis of HCV Infection in Dialysis Patients

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Dialysis patients remain a high-risk group for hepatitis C virus (HCV) infection. The current diagnosis of HCV infection among dialysis patients includes serological assays and nucleic acid amplification technology (NAT) for assessing serum anti-HCV antibody and HCV viremia, respectively. However, current NAT techniques are expensive and labor-intensive and often lack standardization. An assay prototype designed to detect and quantify total HCV core antigen (total HCV core Ag) protein in serum and plasma in the presence or absence of anti-HCV antibodies has been recently developed. A comparison between a total anti-HCV core Ag enzyme-linked immunosorbent assay (ELISA) and a quantitative HCV RNA assay based on reverse transcription-PCR (RT-PCR) (Amplicor HCV Monitor test) was performed using a large ($n = 305$) cohort of ELISA HCV 3.0 HCV-negative and -positive patients on maintenance dialysis. The concentrations of HCV core Ag and HCV RNA levels (measured by RT-PCR) were significantly correlated ($r = 0.471$, $P = 0.0001$) over a wide range of HCV RNA levels and were maintained among different HCV genotypes (HCV genotype 1, $r = 0.862$, $P = 0.0001$; HCV genotype 2, $r = 0.691$, $P = 0.0001$). We estimated that 1 pg of total HCV core Ag per ml is equivalent to approximately 19,952 IU of HCV RNA per ml, even if the wide range in the ratio of core Ag to HCV RNA (95% confidence intervals, 2.8×10^3 to 1.6×10^5 IU/ml) precluded definitive conclusions. In summary, total HCV core Ag proved to be useful for performing HCV RNA measurement among dialysis patients in routine laboratories without the need for special equipment or training. The present study supports the use of the total anti-HCV core Ag ELISA for assessing viral load among dialysis patients with HCV infection.

Dialysis patients remain a high-risk group for hepatitis C virus (HCV) infection (11). The Centers for Disease Control and Prevention, Atlanta, Ga., have recently included semianual anti-HCV testing for anti-HCV-negative patients in their infection control practices for hemodialysis units (6, 7).

Routine diagnosis of HCV is based on detection of anti-HCV antibody. However, due to the absence of an efficient *in vitro* culture system for HCV or assays capable of detecting viral antigens, direct detection of HCV has depended on nucleic acid amplification technology (NAT) techniques. Indeed, serological assays for detecting anti-HCV antibody cannot distinguish between patients with active infection and those who have cleared the virus (29). NAT tests are based on nucleic acid amplification (PCR and transcription-mediated amplification) (16, 37) or signal amplification (branched-chain DNA assay) and are currently used to detect viremia (2, 36). Recently both assay systems have become semiautomated. Several problems still persist with these methods; PCR requires considerable skill and has limited reproducibility, while the

branched-chain DNA assay requires a long incubation period. Both assays are also expensive.

An assay prototype designed to detect and quantify total HCV nucleocapsid core antigen (HCV core Ag) in serum and plasma in the presence or absence of anti-HCV antibodies has been recently developed (1, 3, 24). The HCV core Ag test is an enzyme-linked immunosorbent assay (ELISA) that can be performed in routine diagnostic laboratories. Confirmation of HCV infection in dialysis population requires the detection of HCV viremia in anti-HCV-positive patients; also, assessment of HCV viral load is needed for monitoring the response to antiviral therapy (12). The purpose of the present study was to compare HCV core Ag quantification and PCR-based technology for measuring HCV viral load in the dialysis population.

MATERIALS AND METHODS

Study group. The study group included 125 anti-HCV negative (42.8%) and 167 anti-HCV positive (57%) patients based on the anti-HCV ELISA HCV 3.0. There were 152 females (52%); the mean age of the study group was 65.3 ± 13.7 years. The median time on dialysis was 35 months (range, 1 to 247 months). Seven patients (2%) were Asians, and 278 (98%) were Caucasians. The etiology of end-stage renal disease (ESRD) was as follows: chronic glomerulonephritis ($n = 44$), diabetic nephropathy ($n = 65$), nephroangiosclerosis ($n = 105$), polycystic kidneys ($n = 18$), and ESRD of unknown etiology and others ($n = 60$). Routine hemodialysis techniques were performed by giving a 3- or 4-h treatment three times a week. Bicarbonate dialysis was used for all patients.

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TABLE 1. Results obtained with anti-HCV-positive and -negative sera by the total HCV core Ag assay and the Amplicor HCV Monitor test

Result of Amplicor test	Total HCV core Ag ELISA		
	No. positive	No. negative	Total no.
Positive	89	7	96
Negative	5	191	196
Total	94	198	292

Study design. The patients enrolled in the present study were from a population of 812 patients undergoing regular dialysis for ESRD in six dialysis units in Italy. The epidemiological features of HBV and HCV infections in this population had been previously reported (13). In our panel of specimens, we identified by ELISA HCV 3.0 the anti-HCV-positive patients ($n = 180$) present in a population of 812 patients; the anti-HCV-negative sera used ($n = 125$) were randomly included from the subset of 632 anti-HCV-negative patients.

The study group included 305 patients with ESRD who were on maintenance dialysis. Thirteen patients (4%) were excluded because their stored sera were inadequate. Thus, the comparison between the total HCV core Ag ELISA and Amplicor HCV Monitor test was performed with 292 patients on maintenance dialysis.

Samples (processing). All patient specimens were collected at the beginning of the HD session (midweek session). Aliquots of serum from each patient were immediately separated and stored at -70°C until tested by the total HCV core Ag ELISA and the Amplicor HCV Monitor test. An additional aliquot was used for prompt measurement of alanine aminotransferase ALT levels.

Laboratory tests (i) ELISA. Anti-HCV antibody was detected by a third-generation HCV ELISA (ELISA HCV 3.0 system [Ortho-Clinical Diagnostics, Raritan, N.J.]). The ELISA HCV 3.0 uses three recombinant antigens (c22-3, c200, and NS5) originating from four regions of the viral genome (core, NS3, NS4, and NS5).

(ii) Ortho trak-C assay. The Ortho trak-C assay is a quantitative immunoassay that measures the total HCV core Ag in human serum or plasma in the presence or absence of anti-HCV antibodies. It is a microwell plate format test and a manual immunoassay that uses several monoclonal antibodies with specificities to different regions of the HCV core antigen. Monoclonal antibodies coated onto the microwell surface capture the antigen, and monoclonal antibody Fab fragments conjugated to horseradish peroxidase bind to the captured antigen. The assay procedure is a four-step test. In step 1, the patient sample or control material ($100\ \mu\text{l}$) is incubated with a pretreatment reagent ($50\ \mu\text{l}$) in an uncoated microwell for 30 min at 56°C . The aim of this pretreatment step is to dissociate any immune complexes in order to liberate the HCV core Ag for detection. In step 2, the pretreated sample ($100\ \mu\text{l}$) is diluted and incubated for 60 min at 25°C in a microwell coated with monoclonal antibodies that capture the immunoreactive HCV core Ag. At the end of the second step, the wells are washed to remove any unbound material. In step 3, the conjugate ($200\ \mu\text{l}$), consisting of monoclonal antibody Fab fragments conjugated to horseradish peroxidase, is added to the microwell. The mixture is incubated for 30 min at 25°C . The conjugate binds to the HCV core Ag that is bound to the capture antibody coating the microwell surface. The HCV core Ag forms a bridge between the capture and conjugate antibody reagents. At the end of step 3, the microwells are washed to remove any unbound conjugate. In step 4, an enzyme detection system composed of *o*-phenylenediamine (OPD) and hydrogen peroxide is added to the microwell. In the presence of bound conjugate, the OPD is oxidized, resulting in a colored end product. In this reaction, peroxidase is divalently oxidized by hydrogen peroxide to form an intermediate compound, which is, in turn, reduced to its initial state by subsequent interaction with hydrogen-ion-donating OPD. The resulting oxidized form of OPD is orange. Sulfuric acid is added to stop the reaction. The intensity of the color is proportional to the amount of bound conjugate and therefore is a function of the concentration of HCV core Ag present in the sample. The color intensity is measured with a microwell reader (photometer) designed to measure light absorbance in a microwell (at 490 nm, using a 620 nm reference). The samples and controls were tested in duplicate, and the mean optical density (OD) of the duplicate tests was used. Samples that exhibited more than 25% variation between the two ODs were considered invalid and retested. As recommended by the manufacturer, the lower detection cutoff

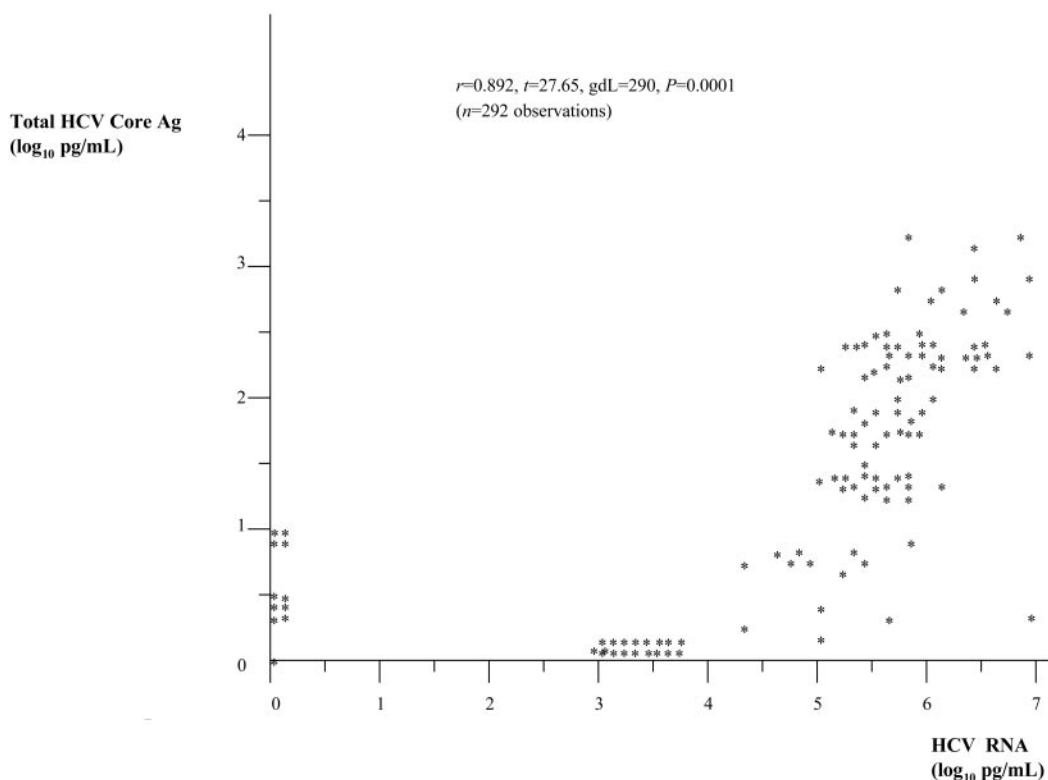


FIG. 1. Relationship between total HCV core Ag (y axis) and HCV RNA (x axis) in the study group ($n = 292$).

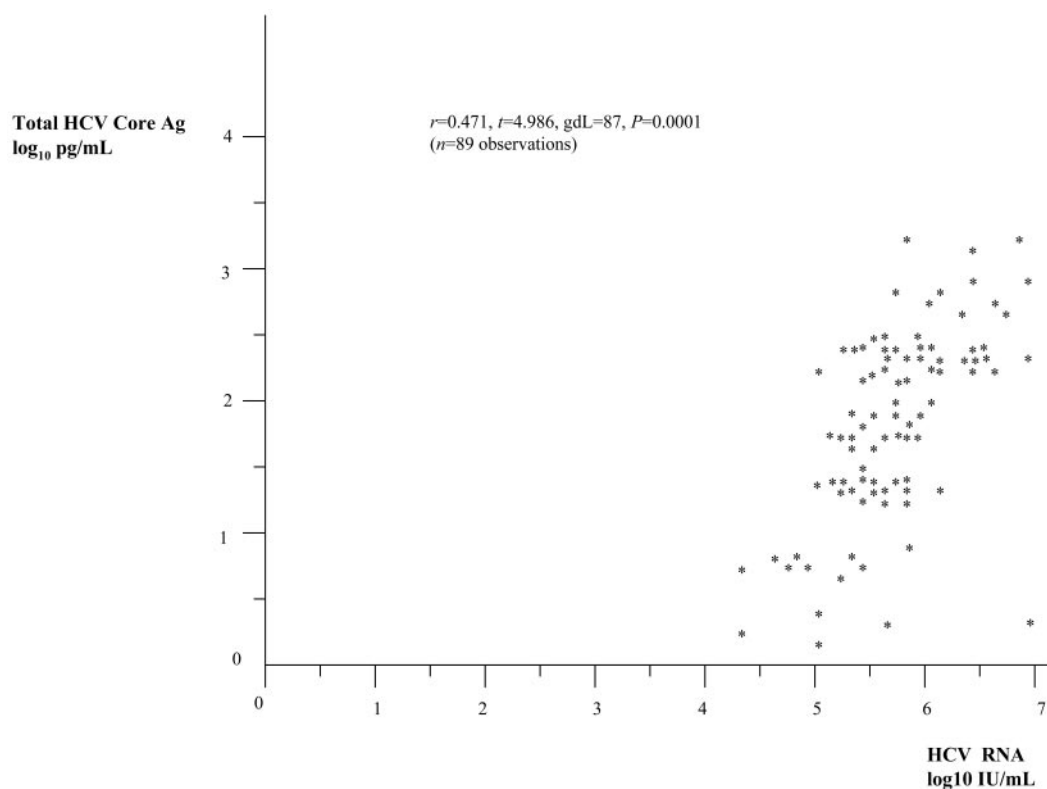


FIG. 2. Relationship between total HCV core Ag (y axis) and HCV RNA (x axis) in the group of patients who were positive for both markers ($n = 89$).

was established for each run and corresponded to the mean OD of the two negative controls plus 0.041. A sample was considered positive only when the mean OD was higher than the cut off of the corresponding run. The amount of total HCV core Ag (in picograms per milliliter) was calculated by means of a standard curve established in each run by testing serial dilutions of a standard containing 4×10^2 pg of HCV core Ag per ml.

(iii) **HCV RNA detection and quantification.** The Amplicor HCV Monitor test (Roche Molecular Systems, Branchburg, N.J.) is an in vitro assay that uses PCR-based nucleic acid amplification for the quantitative assessment of HCV RNA in human serum or plasma. The Amplicor HCV Monitor quantitates virus titers from 6×10^2 to 6×10^7 viral particles (IU) per ml of serum or plasma.

(iv) **HCV genotyping.** A second-generation line probe assay (INNO-LIPA II test; Innogenetics, Zwijndrecht, Belgium) was performed, as specified by the manufacturer, instructions to determine HCV genotypes (33). Genotypes 1 and 1b were classified as genotype 1 only, due to the limited subtype discriminatory capacity of this assay.

(v) **Liver tests.** Serum alanine aminotransferase (ALT) levels in dialysis patients were measured with a Hitachi 7450 analyzer as specified by the manufacturer.

Statistical analysis. The correlations between the total HCV core Ag ELISA and Amplicor HCV Monitor test were analyzed by linear regression. The statistical package JMP In, version 3.1.7 (SAS Institute, Cary, N.C.) was used. Results are presented as \log_{10} mean and standard deviation. Statistical significance was assessed at the two-sided $P \leq 0.05$ level.

For results obtained by the Amplicor HCV Monitor test, the sensitivity, specificity, efficiency, false-negative and false-positive rates, and positive and negative predictive values of total HCV core Ag quantification were calculated by standard formulae (17).

RESULTS

The results obtained by the total HCV core Ag ELISA and Amplicor HCV Monitor test are shown in Table 1.

The relationship between HCV RNA (in international units

per milliliter) and total HCV core Ag (in picograms per milliliter) was given by the following equation: HCV core Ag (\log_{10} picograms per milliliter) = $0.9716 \times \text{HCV RNA} (\log_{10}$ international units per milliliter) - 1.4996. Based on this equation, we calculated that 1 pg of total HCV core Ag per ml is equivalent to approximately 19,952 IU/ml (95% confidence interval [CI], 2.8×10^3 to 1.6×10^5 IU/ml).

Of 292 clinical samples, 12 (4%) showed a discrepancy between the results obtained by the total HCV core Ag ELISA test system and the HCV RNA by Amplicor HCV Monitor test. Five patients positive by the total HCV core Ag ELISA system were negative by the Amplicor HCV Monitor assay; in these samples, weakly positive results were found by the anti-HCV core Ag ELISA (ranging between 0.25 and $0.87 \log_{10}$ pg/ml). These samples were negative on repeat HCV core Ag testing and were therefore interpreted as negative.

Seven other samples were positive by the Amplicor HCV Monitor test but negative by the anti-HCV core Ag ELISA. For these samples, very low titers of HCV RNA were observed (ranging between 3.2 and $4.13 \log_{10}$ IU/ml). These discordant samples were retested by the anti-HCV core Ag ELISA, and the same results were obtained. Detectable HCV viremia in these patients was confirmed by in-house PCR (data not shown).

Figure 1 shows the relationship between the total HCV core Ag ELISA and HCV RNA by PCR (Amplicor HCV Monitor test) for the entire cohort of 292 samples; a strong association was observed ($P = 0.0001$). This association was obtained over a wide range of HCV RNA levels: $4.26 \log_{10}$ to $6.9 \log_{10}$ IU/ml.

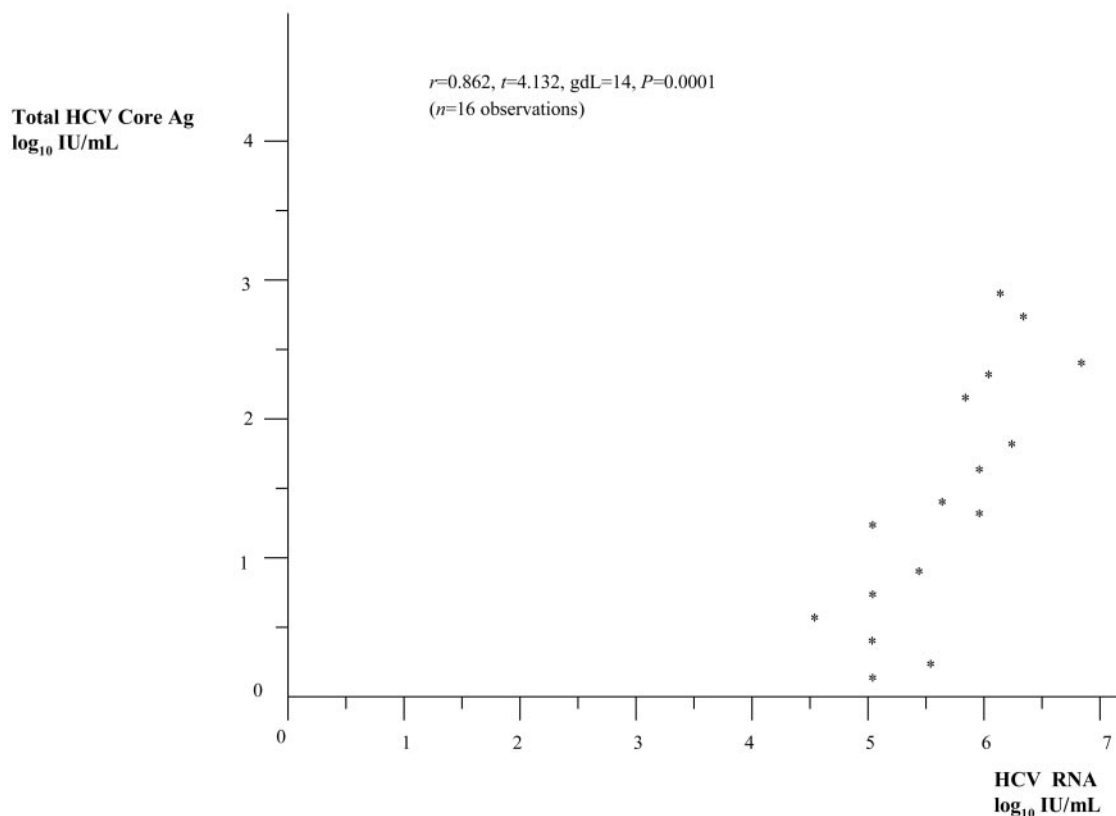


FIG. 3. Relationship between total HCV core Ag (y axis) and HCV RNA (x axis) in the group of patients infected with HCV genotype 1 ($n = 16$).

Figure 2 shows the relationship between total HCV core Ag and HCV RNA for the 89 clinical samples that were positive in both markers. Total HCV core Ag and HCV RNA were significantly related ($P = 0.0001$). The relationship between total HCV core Ag and HCV RNA levels was genotype independent: they were significantly related in 16 clinical samples having the HCV genotype 1 ($P = 0.0001$) (Fig. 3), and the correlation in 21 clinical samples having the HCV genotype 2 was also significant ($P = 0.0001$) (Fig. 4).

In relation to the results obtained with the Amplicor HCV Monitor test, the sensitivity of the total HCV core Ag assay was 92.7% and the specificity was 97.4%. The positive and negative predictive values were 94.7 and 96.5%, respectively. The efficiency of the HCV core Ag ELISA was 95.9%.

Intra-assay precision was assessed on the basis of eight measurements of eight specimens of human sera (samples 1, 5, 6, 7, and 8 [HCV RNA-positive control sera] and samples 2, 3, and 4 [HCV RNA-negative control sera]). The control sera were obtained from our study group of dialysis patients. The mean amount of viral RNA ranged from 1.52 pg/ml (95% CI, 1.846 to 1.195 pg/ml) to 290.25 pg/ml (95% CI, 304.95 to 275.5 pg/ml) for the positive control sera, and the coefficients of variation (CVs) ranged from 4.1 to 26.3% (mean, 10.4%) (Table 2).

The reproducibility between runs (interassay reproducibility) was assessed by using the same specimens. The mean quantity of viral RNA ranged from 0.98 pg/ml (95% CI, 1.02 to

0.94 pg/ml) to 313.7 pg/ml (95% CI, 316.6 to 310.76 pg/ml) for the positive control sera during 3 days of measurements, and the CVs ranged from 0.25 to 8.4% (mean, 3.9%) (Table 3).

We did not find any relationship between HCV core Ag and ALT levels ($P = 0.9$).

Figure 5 shows the distribution of the HCV core Ag level in the 125 anti-HCV negative dialysis patients; the mean value was 0.08 pg/ml (95% CI, 0.081 to 0.078 pg/ml).

DISCUSSION

HCV belongs to the *Flaviviridae* family. Its genome is contained in an icosahedral capsid (or core), itself contained within the viral envelope (31). The capsid is formed by polymerization of the HCV core protein, a structural viral protein encoded by the 5' end of the HCV open reading frame. The mature HCV protein is located in the cytoplasm of infected cells, in close vicinity to the perinuclear membranes and the endoplasmic reticulum, where it polymerizes in the presence of genomic RNA to form viral capsids (31). The HCV core protein is highly antigenic, induces specific cellular and humoral responses, and probably plays a pivotal role in the pathogenesis of HCV infection (25, 27). The availability of an anticore monoclonal antibody allowed the development of an ELISA to detect HCV core Ag in peripheral blood of patients with HCV (3, 28). A standardized commercial assay using the same monoclonal antibody has been developed for the quali-

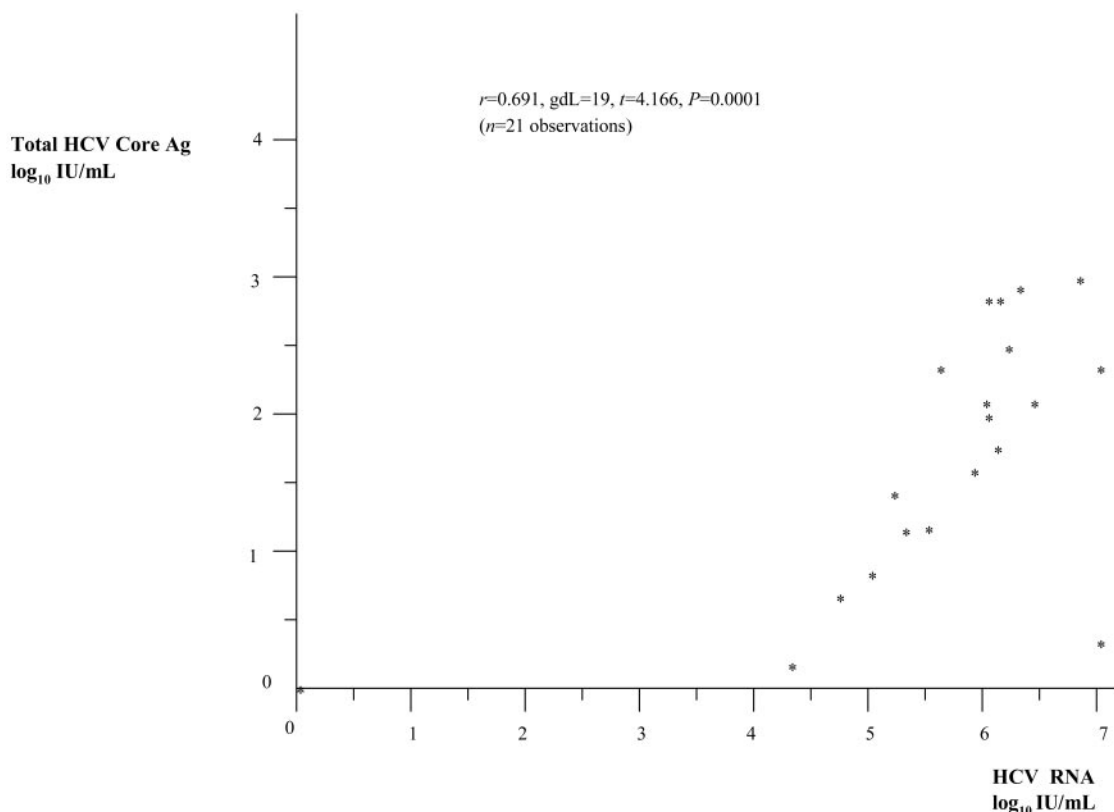


FIG. 4. Relationship between total HCV core Ag (y axis) and HCV RNA (x axis) in the group of patients infected with HCV genotype 2 (n = 21).

tative assessment of HCV core Ag (8, 20, 22, 26, 30, 34), this assay has significantly reduced the serological window occurring before seroconversion during acute infection. Recently, first- and second-generation quantitative assays have been developed and have confirmed that HCV core Ag quantification allows an indirect but accurate measurement of HCV replication in immunocompetent patients (1, 3, 24, 35).

The results of the present study indicate that HCV core Ag quantification is significantly related to the HCV viral load in dialysis patients. This association was observed over a large range of HCV RNA levels and was maintained across HCV genotypes in our cohort of dialysis patients.

The availability of a serological assay for assessing the viral load in HCV-infected patients on dialysis will be valuable for several reasons. The prevalence of HCV infection remains high in the dialysis population (11), assessment of viral load is crucial for monitoring the antiviral therapy (9–11), and low HCV viremia is a predictive factor of a successful response to interferon therapy (5, 14, 21).

An apparent drawback of the total HCV core Ag ELISA is its lower limit of detection, and HCV-positive patients on dialysis often have low HCV RNA levels (11). In our hands, the total HCV core Ag assay could not assess HCV viral load below 4.1 log₁₀ IU/ml: seven samples testing positive for HCV RNA by the Amplicor HCV Monitor test (ranging between 3.2

TABLE 2. Intra-assay precision of the total HCV core Ag ELISA

Sample no.	Genotype	Amt of viral RNA (pg/ml)		CV (%)
		Mean	SD ^b	
1	2	19.86	1.91	9.6
2	NT ^a	1.30	0.12	9.23
3	NT	0.96	0.10	10.4
4	NT	1.01	0.12	11.8
5	1	2.77	0.16	5.8
6	4	313.4	12.89	4.1
7	4	290.25	18.03	6.2
8	2	1.52	0.40	26.3

^a NT, not typeable (HCV RNA-negative controls).

^b SD, standard deviation.

TABLE 3. Reproducibility of the total HCV core Ag ELISA between runs

Sample no.	Amt of viral DNA (pg/ml) on:			Mean amt (pg/ml) ± SD ^a	CV (%)
	Day 1	Day 2	Day 3		
1	21.9	18.6	19.57	20.03 ± 1.7	8.4
2	1.33	1.3	1.27	1.3 ± 0.03	2.3
3	0.93	0.97	1.03	0.98 ± 0.05	5.1
4	0.93	1.07	1.03	1.01 ± 0.07	6.9
5	2.87	2.77	2.63	2.76 ± 0.12	4.34
6	317.8	310.9	312.4	313.7 ± 3.6	1.1
7	285.8	301.57	290.37	292.6 ± 8.1	2.76
8	1.33	2.13	1.5	1.65 ± 0.42	0.25

^a SD, standard deviation.

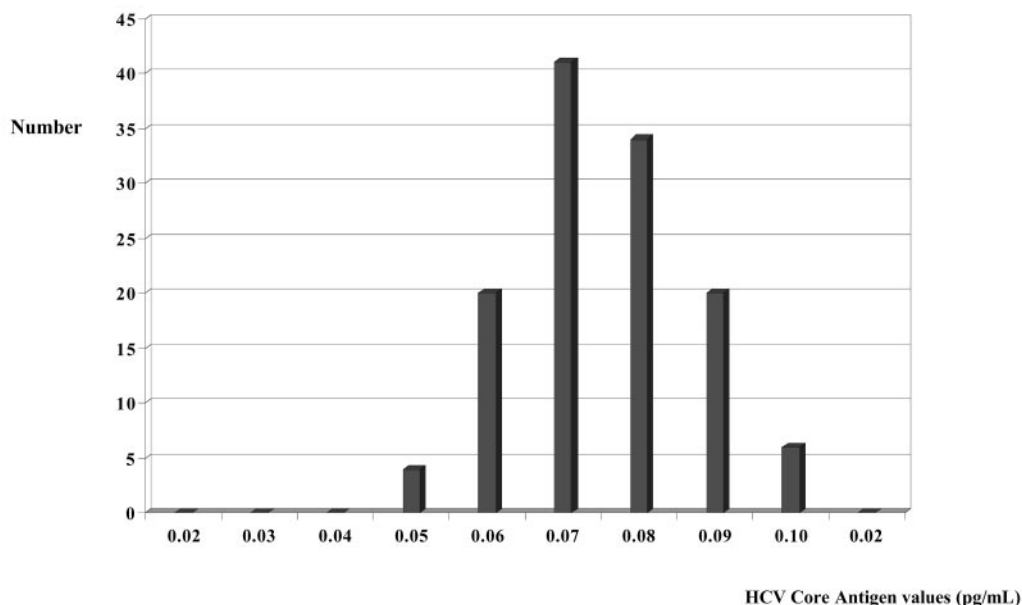


FIG. 5. Distribution of HCV core Ag levels in the group of anti-HCV-negative dialysis patients ($n = 125$).

and $4.13 \log_{10}$ IU/ml) and an in-house PCR were negative by the total HCV core Ag ELISA. This result is in keeping with those obtained with samples from patients with HCV infection and normal renal function; i.e., the total HCV core Ag assay cannot be used as a marker of viral replication for HCV RNA levels below approximately $4.2 \log_{10}$ IU/ml (1). Nevertheless, our results suggest that the total HCV core Ag assay meets the clinical sensitivity needs necessary to identify most (92.7% of) dialysis patients with HCV viremia. Other authors have calculated that the lower detection cutoff of the current version of the total HCV core Ag assay is $4.4 \log_{10}$ IU/ml (95% CI, 4.10 to $4.69 \log_{10}$ IU/ml) (23), and the majority of our dialysis patients with HCV had a viral load larger than this cutoff.

HCV viremia can be demonstrated in a small but significant minority of dialysis patients who are negative by anti-HCV serological testing (4, 15, 18, 19, 32). Immunocompromise due to chronic uremia may limit the ability of dialysis patients to mount an antibody response to viral antigens. This could affect the role of HCV core Ag quantification as a marker of HCV replication among dialysis patients. However, we have observed a strong relationship between HCV core Ag quantification and HCV viremia, as measured by PCR-based technology, in our cohort of anti-HCV-positive and -negative patients on maintenance dialysis.

We estimated that 1 pg of total HCV core Ag per ml is equivalent to approximately approximately 19.952 IU of HCV RNA per ml even if the large 95% CI precluded definitive conclusions. The fluctuations in the ratio of HCV core Ag to HCV RNA among HCV-positive dialysis patients suggests that there are differences in the amount of core protein per HCV RNA molecule in the peripheral blood of dialysis patients. It has been reported that the number of capsomers per virion is constant and that each virion contains only one genome molecule (25, 27, 31). Thus, our results strongly support the pos-

sibility that HCV core proteins are not associated with the HCV genome in peripheral blood of dialysis patients; alternatively, repeated freeze-thaw cycles or other techniques of RNA preservation could affect the ratio of core protein to HCV genome molecules, although this was not our experience.

It has already been reported that 1 pg of HCV core protein per ml is equivalent to approximately 8,000 IU of HCV RNA per ml in clinical samples from HCV-infected patients with normal renal function (1). The clinical significance of these fluctuations remains to be established. Despite these variations, the performance of the HCV core Ag quantification assay was very good; the specificity of the total HCV core Ag assay among dialysis patients was high (97.4%), and the reproducibility was satisfactory irrespective of the genotype and viral HCV burden.

Serological detection of HCV core Ag may be an alternative to PCR-based technology; the HCV core Ag ELISA can be easily performed in clinical laboratories; it is reliable, rapid, and not expensive. At present, a single HCV core Ag ELISA is roughly three times less costly than the quantitative RT-PCR assay (Amplacor HCV Monitor test); these prices reflect the current cost of each assay to the hospital (labor costs are not included). However, the possibility of developing additional automation for ELISA and/or NAT-based technology hampers definitive comments on cost efficacy.

In conclusion, our study shows that total HCV core Ag quantification is an accurate marker of viral replication in dialysis patients with HCV. In addition, the total HCV core Ag test results could be obtained in routine laboratories without the need for special equipment or training. The management of HCV infection in dialysis population requires assessment of the HCV viral load under various circumstances. The total HCV core Ag ELISA may be a useful tool for assessing the viral HCV load in clinical practice within dialysis units.

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