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Original Article

Evaluation of the HCV Core Antigen Assay as a Second-Line Supplemental Test for Diagnosis of Active Hepatitis C Infection

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ABSTRACT

Background/Aim: Hepatitis C virus core (HCV) antigen assays have been produced to exclude infectious donations collected the preseroconversion window phase. For the same purpose, we evaluated the specificity and sensitivity of a novel hepatitis C virus core antigen detection immunoassay and the applications of this assay in clinical diagnosis.

Patients & Methods: Samples were collected from 30 anti-HCV antibody negative healthy subjects (G1), and 46 anti-HCV antibody positive patients (G2). All included samples were subjected to HCV core antigen and HCV-RNA PCR.

Results: Among the 46 anti-HCV Ab positive samples, HCV core antigen was positive in 38 samples from 40 samples positive for HCV-RNA with sensitivity of 95% (38/40). All the 30 anti-HCV Ab negative samples (n=30) were negative for both HCV core antigen and HCV-RNA with specificity of 100%.There was no significant variation in the sensitivity of HCV core antigen between genotype 1 (100%) and genotype 4 (94.5%). Viral load in HCV core antigen positive samples (906653±133803) was significantly increased than that of HCV core antigen negative samples (16342±5245) with P value < 0.05.

Conclusion: HCV core antigen assay is a useful method in screening strategy of HCV infection and provides a reliable means of distinguishing between current and cleared HCV infections that is well correlated with HCV-RNA testing.

Key words: HCV Core Antigen Assay, Second-Line Supplemental Test for Active HCV Infection**.**

INTRODUCTION

The hepatitis C virus (HCV) is a prevalent infectious disease generally contracted via blood and blood products infected with HCV. Currently about 170 million people worldwide are infected with HCV. Egypt has the highest country wide prevalence of HCV in the world, with an estimated 8 – 10 million among a population of 68 million have been exposed to the virus [(1)].

Due to the lack of efficient prevention such as therapy and vaccines, an accurate early diagnosis

is essential for preventing transmission of the disease. The method currently recommended for identifying subjects with HCV infection is an enzyme immune-assay for diction of anti-HCV antibodies. However, this assay sometimes generates false-positive or false-negative results. Furthermore, it is not always possible to distinguish between current and post cleared infections [(2)].

Although, the increased sensitivity of last generation assays has dramatically reduced the risk of HCV transmission by blood components by reducing the window period from 82 days to 66 days, the anti-HCV antibody detection is not applicable to confirm viral infection in the early phase of HCV infection before anti-HCV antibody has been produced [(2)]. Thus, supplementary tests are required. The strip immunoblot assay, a more specific anti-HCV serological test, is useful to distinguish true positive from false positive EIA results. HCV-RNA assay can indicate active HCV infection, but it is difficult and lacks reproducibility [(3)].

Assays that detect HCV-core antigen have been developed to diagnose current HCV infection. HCV core antigen can be used as a direct marker of viral replication in the chronic phase of infection and as a relevant marker for predicting and monitoring the response to therapy. Indeed, HCV core antigen assays have sensitivities close to that of HCV-RNA with mean detection difference of 1-2 days in the window period with qualitative assays type and 0.29 day with immuno assay for detection and quantitative assays of HCV core antigen. So, we investigated the usefulness of this HCV core antigen assay to confirm HCV infection in comparison to HCV-RNA as a golden marker [(4)].

HCV-RNA assay is the method of choice for confirmation of active infection in both immunocompetent patients who are anti-HCV antibody positive and immunosupressed patients who may not mount an antibody response, as well as for assessment of both spontaneous viral clearance and viral clearance as a result of therapy[(5)]. Qualitative HCV-RNA tests can also detect viremia within 1 to 3 weeks of infection, which is earlier than the time of detection of seroconvertion, which typically occurs within 4 to 10 weeks of the time of infection [(6)]. Nevertheless, HCV-RNA testing is expensive, difficult and lacks reproducibility. HCV-RNA testing is also, time consuming and requires a sophisticated molecular laboratory and may not always be readily available in underdeveloped parts of the world, where the greatest numbers of HCV infected patients are found [(7)].

SUBJECTS AND METHODS

The present study was conducted at the National Liver Institute, Menofia University wherein we recruited 46 anti-HCV positive subjects attending the outpatient clinics and the hepatology in-patient department. The control group comprised 30 apparently healthy non-obese individuals who were age and sex matched to the study group.

All subjects were subjected to:

-Thorough history taking, Clinical and ultrasonographic examination, and liver biopsy for HCV-RNA positive subjects only.

- Laboratory investigations: Liver function tests including serum bilirubin, ALT, AST, alkaline phosphatase, gamma glutamyl transpeptidase (GGT), serum albumin (using Integra 800, Roche) and prothrombin activity (by Quick one stage method, Thromboreal S human thromboplastin containing calcium, Behring, 1991, Germany). Hepatitis markers for Anti-HCV antibodies (ELISA by Murex VK47148, England IV version), HBsAg, HBcAb (IgG & IgM), HBs Ab, HBe Ag, and HBe Ab all detected by ELISA (Dia Sorin Diagnostics, Italy). This kit implement qualitative methods based on enzyme linked immunosorbant assays (ELISA). The procedures were done according to the manufacture's instructions.

A venous sample of blood was withdrawn from each subject under strict aseptic conditions in sterile vacutainer tubes. One tube was left to clot in water bath (37°) for 20 minutes and centrifuged. The serum was then separated and used for assay

of liver function tests, hepatitis markers. Another tube was left to clot and centrifuged in the PCR unit and the serum was divided into 3 aliquots under strict sterile conditions and stored at -80°C to be used for PCR, HCV core antigen and for HCV genotyping.

Quantitative HCV-RNA PCR

By COBAS AMPLICOR Roche Diagnostic System, Inc. Hacienda Drive, Pleasanton, CA 94588, USA. HCV monitor test, version 2.0, from Roche. It is an in vitro nucleic acid amplification test for the quantification of hepatitis C virus RNA in human serum or plasma on the COBAS AMPLICOR analyzer. It is based on five major processes: specimen preparation; reverse transcription of the target RNA to generate complementary DNA (cDNA); PCR amplification of target cDNA using HCV specific complementary primers; hybridization of the amplified DNA to oligonucleotide probes specific for the target(s); and detection of the rpobe-bound amplified DNA by colorimetric determination [8].

HCV genotyping: by REFLP technique:

Principle:

Owing to conserved sequence in 5' UTR, consistent patterns of fragment lengths with each isolate could be produced. Variable sequences in this region result in diverging restriction sites, and hence, produce different length products. Signature sequencing, which differs among all types, provides a defined cut site with specific patterns with each viral type. A pattern of restriction enzymes to differentiate between HCV types 1 through 3 using Hae III/Rsa I, and Scr FI restriction enzymes was used. Hinf 1 was used to provide good recognition of type 4 since the Hinf 1 cut site is absent from types 1 and 2. Subtyping of type 1 was done using Bst UI, while types 2 and 3 was subtyped using Scr FI. Kir produced by Invitrogen Tech-line, 8009556288, USA

Procedure*:*

From nested PCR product we put into two different tubes:

Mixture 1	Mixture 2
10 µl of nested PCR product	10 µl of nested PCR product
2 µl of buffer L	2 µl of buffer H
0.75 RSA I	0.75 µl of MVa I
0.75 of Hac III	0.75 µl of Hinl I
6.5 of H_2O	6.5 µl of H_2O
Total 20 µl	Total 20 µl

They were digested for 2 hours at 37°C simultaneously then electrophoresis was done in a 4% metaphore gel (FMC Bioproducts). Interpretations of the results of these two combinations of restriction endonucleases are shown below. The electrophoretic pattern is shown in figure (X) and figure (XI). The type 4 bands are seen at 205 bp and type 1 at 144 bp.

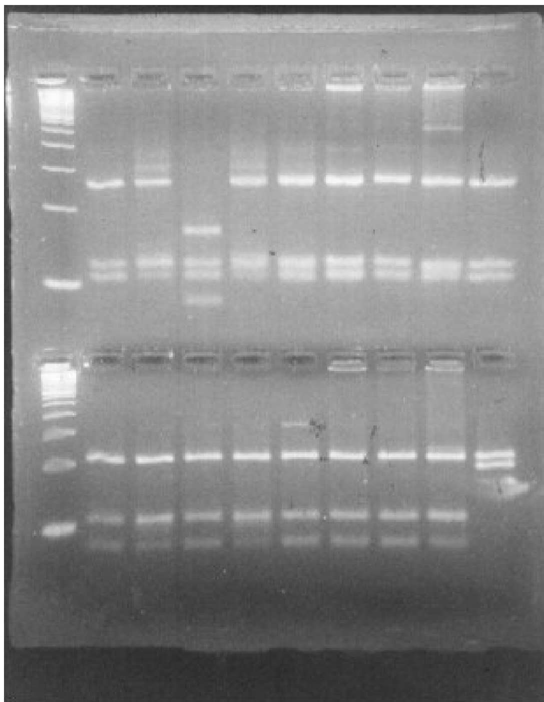

Fig. (1): HCV genotype 1
Φ x 174 DNA/Hae III fragments.
Genotype 1 equals 144 bp.

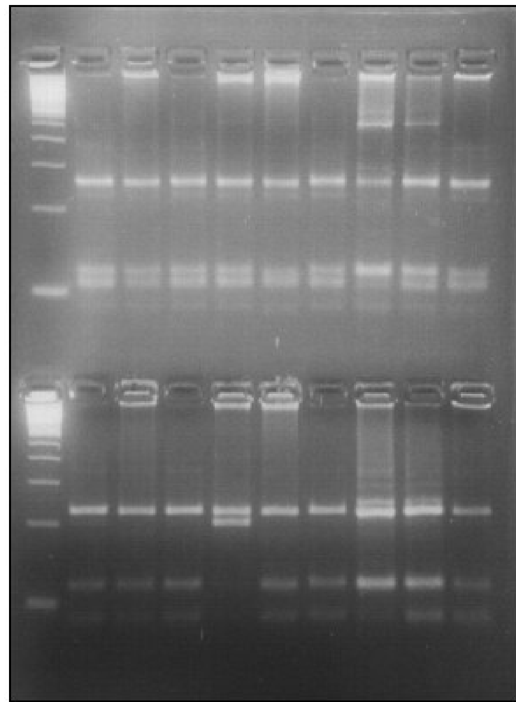

Fig. (2): HCV genotype 4
174 DNA/Hae III fragments
Genotype 4 equals 205 bp.

HCV core antigen

By using an Enzyme- Linked Immunosorbent assay (ELISA) kit (Code 933260- 480 test) manufactured by Ortho-Clinical Diagnostics, Inc., Rarita.ii, New Jersey, USA. Ortho antibody to HCV core antigen ELISA test system is a qualitative, enzyme linked immunosorbeiit assay for the detection of hepatitis C nucleocapsid core antigen (HCV core antigen) in human serum or plasma. It is intended for use as a screening test for blood donors and as an aid in the early diagnosis of hepatitis C infection.

Principle of the procedure:

The assay procedure is a three- stage test carried out in micro-wells coated with monoclonal antibodies that recognize HCV core antigen. In the first stage, a diluted test specimen is incubated in the test well for a specified length of time. The addition of the specimen to the diluent results in a distinctive color change from green to blue. If antigen reactive to any of the coated antibodies is present in the specimen, antigen-antibody complexes will be formed on the micro-well surface. If HCV core antigen is not present, these complexes will not be formed. In the subsequent washing step, unbound serum or plasma proteins will be removed [9].

In the second stage, murine monoclonal antibodies conjugated to horseradish peroxidase are added to the micro-well. The conjugate binds to the antigen- antibody complexes. If antigen-antibody complexes are not present, the unbound conjugate will be removed by subsequent washing. In the third stage, an enzyme detection system composed of o- phenylenediamine (OPD) and hydrogen peroxide is added to the test well. If bound conjugate is present, the OPD will be

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 has an orange color. Sulfuric acid is then added to stop the reaction the color intensity of the microwell strip plate was read by a photometer at a wave length of 490 nm with a reference wave length at 620 nm. The intensity of the color is dependent upon the amount of bound conjugate and, therefore, is a function of the concentration of HCV core antigen present in the specimen. The color intensity is measured with a micro-well reader (photometer) designed to measure light absorbance in a micro-well [9].

STATISTICAL ANALYSIS:

Data collected, tabulated and analyzed by SPSS 11.0 statistical package (Chicago, Illinois, USA).Quantitative data expressed as mean and standard deviation (X±SD). Student t-test was used for comparison of the means of two groups of normally distributed data. Qualitative data expressed as number and percentage and analyzed by Chi-square (X^2) test.

$$X^2 = (O-E)^2/E$$

Where O is the observed frequency and E the expected frequency.

Level of significance was set as $P<0.05$.

RESULTS

Table (1) shows that there was no statistically significant difference between positive rates of HCV core antigen and HCV-RNA by PCR in anti-HCV antibody positive individuals with $P>0.05$.

Table (2) shows that there was no statistically significant difference between HCV core Ag and HCV-RNA by PCR in detecting active hepatitis C infection $P>0.05$.

Table (3) shows no significant variations as regards HCV core antigen detection among different HCV genotypes $P>0.05$.

Table (4) shows no significant variation between two markers HCV core antigen and HCV-RNA in detecting hepatitis C virus infection , it shows that the viral load in individuals positive for HCV core antigen was significantly elevated than that of that in individual negative for HCV core antigen.

Table (1): Comparison between the positive rate of HCV core antigen and HCV-RNA by PCR in the anti- HCV positive individuals

Variable	Anti-HCV antibody positive individuals (G2) n=46			
	No	%	Chi^2	P
HCV-RNA	40	86.95	1.95	> 0.05
HCV core Ag	38	82.6		

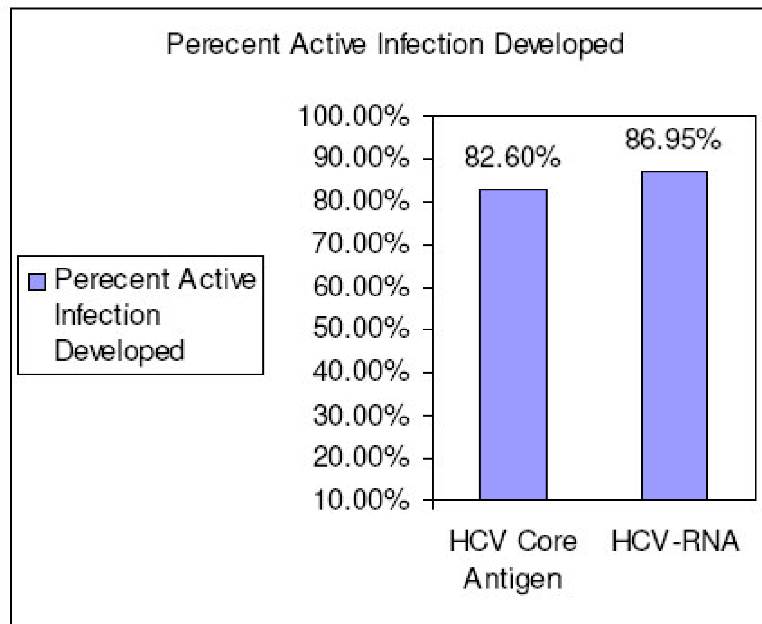

Fig. 3: Percentage of active infection demonstrated by detection of HCV core antigen versus detection of HCVRNA by quantitative HCV-RNA PCR

Table (2): Comparison between HCV core antigen and HCV-RNA by PCR as a marker for active hepatitis C infection

	Cirrhotic patients (n=40)				Chi²	P
	HCV-RNA		HCV-core Ag			
	No.	%	No.	%		
Positive	40	100%	38	95%	1.85	>0.05
Negative	0.0	0.0	2	5%		

Table (3): Shows HCV core antigen estimation among different genotypes

Variable	Genotype I N=3		Genotype 4 N=37		Chi²	P
	No	%	NO	%		
HCV core Ag -ve	0.0%	0.0%	2	5.4%	0.34	>0.05
HCV core Ag +ve	3	100%	35	94.5%	0.36	>0.05

Table (4): Comparison between HCV core antigen in the studied patients and HCV viral load

HCV Core Ag	HCV-RNA viral Load		
	M±SD	t	P
Negative	16342±5245	2.5	< 0.05
Positive	906653±133803		

DISSCUSSION

Several different serological tools are available to identify HCV infection. Positivity for anti-HCV antibodies indicates past or current HCV infection but gives no indication about viral replication. In contrast, molecular detection of HCV-RNA indicates active viral replication and calls for thorough clinical evaluation. Qualitative RNA assays are currently used to diagnose ongoing HCV infection among anti-HCV antibody-positive individuals. They are also used to detect viremia in subjects during early phase of infection prior to anti-HCV seroconversion (window period), for instance, in blood banks [10].

Detection of HCV core antigen, a new serological assay, was originally designed to monitor the efficacy of HCV treatment as an alternative to measurement of HCV-RNA viral load. This test has also been shown to be appropriate for screening for HCV infection in blood donors. HCV core antigen is now routinely used by French blood banks to detect HCV viremia in donors during the window period. Retrospective studies carried out with HCV core assay showed that it can detect early HCV viremia during this window period and might represent an alternative to HCV-RNA assays. However, further studies are necessary to evaluate the HCV core antigen assay prospectively before it can be used routinely in blood banks [11].

The present study planed to evaluate HCV core Ag as a marker for viral replication and for differentiation between past HCV infection and ongoing one. Also we aimed to evaluate its sensitivity, specificity and efficacy as simple assay, not in need to sophisticated precaution in comparison to a golden marker as HCV-RNA. So, the present study was conducted at the National Liver Institute, Menofia University wherein we recruited 46 anti-HCV positive subjects attending the outpatient clinics and the hepatology in-patient department. The control group comprised 20 apparently healthy non-obese individuals who were age and sex matched to the study group.

In the present study among 46 anti-HCV antibody positive subjects, we found that 40 subjects of them were positive for HCV-RNA., 38 of them were positive for HCV core Ag (95%). At the same time, both HCV-RNA and HCV core antigen were negative in 20 anti-HCV antibody negative subjects, included as control group. HCV core Ag shows no significant variations between different HCV genotype 1 and 4. Of 37 genotype 4 subjects, 35 were positive for HCV core Ag (94.5%) while 3 of 3 genotype 1 subjects were positive fore HCV core Ag (100%).In our study, the viral load of subjects with HCV core Ag positive (n=38) was significantly higher than HCV core Ag negative subjects (n=2) with mean value (906653±1338037) and (16342±5245) respectively. We further explored the specificity of the HCV core antigen assay in 10 HCV-RNA negative subjects. This revealed that all subjects negative for HCV-RNA

(n=20) were negative for HCV core antigen, leading to specificity of 100%.

Our results show that the HCV core antigen assay is an efficient alternative to HCV-RNA assays to distinguish between past and ongoing infection. Among anti-HCV antibody-positive subjects; we estimated that the sensitivity of HCV core antigen assay to detect HCV viremia compared to HCV-RNA PCR was 95% (38 of 40). The lack was not related to genotype but was related to low viral load as the lack was in cases with viral load below 10,000 IU/ml as the level of viremia was significantly higher in patients showing positive HCV-Ag than in those with negative HCV Ag as reported by Daniel, et al [12].

The same results were obtained by Gaudy, et al who reported that HCV core antigen can be used with efficacy as a marker for HCV infection and viral replication. Among anti-HCV antibody positive subjects, they estimated that the sensitivity of HCV core Ag assay to detect viremia compared to RT-PCR was 96.7% (117/121). They also found a good correlation between HCV core Ag levels and viral load. HCV core Ag in 2395 anti-HCV antibody subjects was negative leading to specificity of 100% [13]. The same specificity for HCV core Ag was previously reported by Schttler, et al [14] .

Li, et al reported that the HCV core antigen was highly specific and easy reliable test for the early detection of HCV infection. They also found a good correlation between the levels of HCV core antigen and HCV-RNA [2]. The results of Fabrizi et al indicate that HCV core antigen quantification is significantly related to HCV viral load in dialysis patients. This association was observed over a large range of HCV-RNA levels and was maintained across different HCV genotypes. They estimated that 1 pg of total HCV core antigen per ml is equivalent to 19.952 IU HCV-RNA per ml. They referred the fluctuations in the ratio of HCV core antigen to HCV-RNA among HCV positive patients to the differences in the amount of HCV core protein per HCV-RNA molecule in the peripheral blood [15].

Krajdem and his co-workers estimated the percentage of individuals actively infected with HCV, as demonstrated by detection of HCV core antigen and HCV-RNA in the anti- HCV antibody positive specimens. HCV core antigen was detected in 331 of 478 (69%) specimen, and HCV-RNA was detected by quantitative HCV bDNA was 348of 478 (73%) specimens. HCV core antigen was positive in 331 of 348 specimens positive for HCV-RNA (95%). Results similar to that obtained by the present study. They found good correlations between the concentrations of HCV core antigen (pg/ml) and concentrations of HCV-RNA (IU/ml) [16].

The same results was reported by earlier studies, Bouvier-Alias et al, reported correlation of 1 pg/ml HCV core antigen to 7,688 IU/ml of HCV-RNA for genotype 3 [17]. Similarly, the correlations estimated from differences between the mean concentrations of HCV core antigen and HCV-RNA for each genotype reported by Veillon et al, suggests that 1 pg/ml of HCV core antigen corresponds to HCV-RNA concentrations of 8,128 IU/ml of genotype 1, 8,511 IU/ml for genotype 2 and 9,550 IU/ml for genotype 3 with no significant variations between different genotypes. So they reported that HCV core antigen sensitivity nearly was the same among different genotypes [18]. A recent study suggests that core antigen levels are not influenced by mutations in the core region and the HCV core antigen assay seams to identify genotype 1-4 with equal sensitivity [12]. In conclusion, The HCV core antigen assay, which is easier to perform than HCV-RNA assays and less expensive, gives reliable information about viremia and might help to ensure an accurate medical follow-up. The HCV core antigen is an appropriate serological tool to indicate HCV status. It can be efficiently distinguish recovery from current infection.

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