Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 55 (1): 91-98. Oct. 2017
DOI: 10.5455/ajvs.271779

Evaluation Of An Enzyme Immunoassay For Hepatitis C Virus Core Antigen As A Diagnostic Test For Detection Of Hepatitis C Virus Infection In Comparison To Real-Time RT-PCR

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Key words:
HCV, ELISA, real-time RT-PCR, HCV core antigen

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ABSTRACT

Hepatitis C virus (HCV) is a global public health care problem. Diagnosis of HCV infection was mainly based on the detection of anti-HCV antibodies as a screening test with serum samples and detection of HCV RNA by using real-time RT-PCR, which considered as a golden standard test. In this study, we compared the HCV core antigen (HCV cAg) test with the HCV RNA assay for confirming anti-HCV results to determine whether the HCV cAg test may be used as an alternative confirmatory test to the HCV RNA test and to assess its diagnostic values by determining the specificity and sensitivity rates compared to the real-time RT-PCR test. Sera samples from 100 persons were analyzed by ELISA for anti-HCV and HCV core antigen and with the molecular HCV RNA assay as a confirmatory test. The diagnostic sensitivity, specificity, positive and negative predictive values of the HCV cAg assay compared to the HCV RNA test were 76.1%, 86.2%, 80%, and 82.5%, respectively. The HCV cAg levels showed a poor correlation with those from the HCV RNA quantification (r = 0.321, p= 0.079). In conclusion, the core antigen detection test can be more valuable in HCV diagnosis than anti-HCV antibodies but it cannot replace HCV RNA in confirmation of the diagnosis of HCV because the sensitivity of the HCV cAg detection assay is significantly lower than that of real-time RT-PCR based methods

1. INTRODUCTION

Hepatitis C Virus (HCV) is a major threat to global public health. More than 170 million people worldwide are estimated to be infected, and about 20 % are chronically ill and at risk for liver cirrhosis and liver cancer (Li and Lo, 2015). Furthermore, the frequency of patients with HCV complications is expected within the next 20 years resulting in increased liver transplants (Rosen, 2011).

HCV is a small enveloped virus with a positive-sense, single-stranded RNA genome that belongs to genus hepacivirus, family flaviviridae. In 1989 HCV was identified as major causative agent of hepatitis C (Houghton, 2009). HCV encodes a large polyprotein of 3010 amino acids and contains structural and non-structural proteins (Shimoike et al., 1999; Wakita et al., 2005). This polyprotein is processed by cellular peptidases (proteases) and virally encoded proteases to produce the mature structural (E1, E2, core, p7) and non-structural (NS) proteins (NS2, NS3, NS4a, NS4b, N4a and NS5B) (Akeno et al., 2001; Luis et al., 2009). Replication occurs by means of a negative-strand RNA intermediate and is catalyzed by the NS proteins, which form a cytoplasmic membrane-associated replicase complex (Blanchard et al., 2002).

HCV core is a highly conserved basic protein that forms viral nucleocapsid. The core of HCV is composed of the first 191 amino acids and can be divided into three domains based on hydrophobicity (Bukh et al., 1994). The core protein can bind viral RNA across domain 1 (amino acids 1 - 74) (Santolini et al., 1994). Also core protein directly or indirectly involved in hepatocarcinogenesis (Hope et al., 2002; Lerat et al., 2002).

HCV infection is characterized by a high tendency to develop lifelong viability of the virus. Only one out of every five acute infections is removed automatically, usually within the first six months after infection (Verstrepen et al., 2015).

Egypt has possibly the highest prevalence of hepatitis virus in the world; 10%-20% of the general populations are infected. In addition, HCV is important cause of liver cancer in the country (Hassan et al., 2001). A recent published Egyptian Demographic Health Survey (EDHS) of samples of the Egyptian resident population was conducted. This report recoded the prevalence of anti-HCV antibody at 14.7% and the estimated number of patients with hepatitis was 9.8% (El-Zanaty and Way, 2009; Chemaitelly et al., 2013).

The HCV diagnosis is based on the detection of any anti-HCV antibody by enzyme linked immunosorbent assay (ELISA) or HCV RNA by real time polymerase chain reaction (real-time RT- PCR) and both tests have limitations and defects. Low sensitivity was recorded with anti-HCV antibody test, in detecting the virus in early intervals because it gave high false positive rates and no distinction could be made between patients who were recovered and actively injured. In addition, it can be negative during hemodialysis and patients with immunodeficient despite ongoing HCV replication (Medhi et al., 2008).

HCV RNA detection by real-time RT- PCR is highly sensitive and is a reliable test in the early diagnosis of HCV Infection (Tanaka et al., 2003). However, since testing is costly and requires certain amount of experience, it is not used routinely in the diagnostic laboratories, especially in developing countries as Egypt. in addition, researchers sought to replace detection HCV RNA or quantification through other techniques.

To overcome limitations of the previous methods of diagnosis of hepatitis infection, a new enzyme immunoassay (EIA) test was recently developed to detect HCV core antigen (HCV cAg). This protein is the ideal target for detecting the HCV antigen. This test has the advantages of easy testing and rapid reporting (Park et al., 2010).

The detection of the Core antigen detection by ELISA method has a comparable sensitivity to that shown by the PCR-based assay (Buket, 2014). Therefore, HCV cAg tests have been introduced to complete anti HCV tests or HCV real-time RT-PCR analysis (Aoyagi et al. 1999; Tanaka et al., 2000). This assay will be useful to monitor HCV infection and to distinguish active virus replication states from stable infection by detecting the HCV cAg with comparative evaluation of ELISA and real-time RT-PCR.

The aim of this study is to evaluate a new test for the environmental impact assessment of HCV cAg as a diagnostic test for HCV infection compared to real-time RT- PCR.

2. MATERIAL AND METHODS

2.1. Clinical history and blood sampling

This study was carried out over 10 months period from June 20I3 to April 2014 on total number of 100 persons (eighty persons were selected from inpatients and outpatients of Kafrelsheikh- Liver Research Centre and twenty healthy volunteers were also included in this study after obtaining an informed consent).

Whole blood samples were collected from the patients by vacuum venipuncture, using a dry 10-mL tube. The serum was separated. Aliquots of serum from each patient were immediately separated and stored at -70°C until tested. Grossly hemolytic or turbid samples were excluded. All samples were handled as potentially infective.

All serum samples are checked for HCV antibody with abcam ELISA Kit (Abcam protocol book, 2010) whereas eighty samples were positive for HCV antibodies and the remaining twenty were negative.

2.2. Extraction of RNA from serum and Real Time –PCR

RNA was extracted from 200 µl of serum with the QIAamp Viral RNA kit (Qiagen), eluted in 60 µl and stored at −80°C. RNA (4µl) was reverse transcribed to

cDNA. QuantiTechTMProbe RT-PCR Kit (QIAGEN) was used for quantitative one- step real-time RT-PCR using fluorogenic Probe FT-275: 5´-(FAM) CAC CCT ATC AGG CAG TAC CAC AAG GCC (TAMRA)-3´ and Primers: C-149 (sense primer), 5´- TGC GGA ACC GGT GAG TAC A-3´ and C-342 (antisense primer), 5´- CTT AAG GTT TAG GAT TCG TGC TCA T-3´ (Brass et al., 2002).
The viral RNA was quantitated by using a set of five external HCV RNA standers, which have been calibrated against HCV WHO Standers, 96/790 and assigned concentration values in IU/ml. The five standards cover a range of 4 logs to enable generation of a standard curve over approximately $1x10^3$ to $1x10^7$ IU/ml. The standard curve is generated automatically by plotting the threshold cycle (CT, first cycle with a reporter fluorescence above the baseline) versus log10 (N), where N is the initial concentration of the standard in IU/ml, and calculating the best–fit line through linear regression analysis. The level of HCV RNA in each specimen is determined by detemining its CT on the standard curve.

2.3. Detection HCV cAg by ELISA

HCV cAg was detected in serum samples by ELISA according to abcam Protocol book (**2010**) by visual density measurement (O.D) which is proportional to the amount of the core antigen in the serum. The signals from the microwell plate coated with serum samples after incubation with the primary antibody (Mouse monoclonal antibody against core antigen of HCV) and the secondary antibody (Anti mouse antibody).
The cutoff level of HCV core antigen was calculated by ELISA above or below where the tested samples is considered positive or negative as the mean optical densities of 20 serum samples from healthy volunteers (MNC) Mean Negative Control + 3 standard deviation (SD) i.e 0.422 ± 3x (0.065). Cutoff value= MNC+ 3 SD. Cutoff value = 0.617.

3. Results

3.1. Real-time PCR assay

From one hundred samples, 42 samples were positive HCV RNA and 38 samples were negative HCV RNA. Samples were divided into 3 groups **(group I, II and III)** according to results of real-time RT-PCR assay (Table 1).

3.2. ELISA assay for HCV cAg

ELISA assay for detecting HCV cAg showed that 32 samples were positive in group1 and 8 samples were positive in group II, while all samples in group 3 were negative (Table 2).

The percentage values of the results in the three different groups of the study were calculated. Group I showed 76.2% positivity and 23.8% negativity, while the positivity was 21.1% and negativity 78.9 % in Group II and Group III showed l00% negative (Fig 2).

The mean value and range of HCV cAg test by ELISA was calculated in the three groups, Cutoff point was (0.617). P. value was significant in groups, (I& II) (1& III) but P. value of group (II& III) was insignificant (Table 3).

3.3. Evaluation of HCV cAg test sensitivity and specificity

Parallel testing of 100 serum samples, 80 of them with HCV-positive antibody patients and 20 are volunteers performed the comparative analysis of the ELISA HCV cAg assay. The analytical sensitivity of the core antigen methods versus that of the real-time RT-PCR showed 76.1% sensitivity and specificity 86.2%. The Positive predictive value was estimated as 80%, while the negative predictive value was 82.5%. Moreover, the likelihood ratio, which refers to the utility of the test was calculated and if more than 5 it can differentiate between patients and non-diseased was 5.4. The correlation between HCV cAg and HCV-RNA by (real-time RT-PCR) was calculated from group I and it was (r = 0.321), where it was insignificant.

Table 1. The results of the three groups according to anti-HCV antibodies and real-time RT-PCR.

	HCV Antibodies	Real-time PCR

Groups	Positive	Negative	Positive	Negative
Group I	42	0	42	0
Group II	38	0	0	38
Group III	0	20	0	20

Table 2. Results of HCV core antigen (HCV cAg) detection by ELISA in 3 different groups of the study (Number and Percentage).

HCV core Ag		Groups			
		Group I	Group 11	Group III	Total
Negative	No.	10	30	20	60
	Percent	23.80%	78.9%	100%	
Positive	No.	32	8	0	40
	Percent	76.2%	21.1%	0.00%	
Total	NO.	42	38	20	100
	Percent	100%	100%	100%	
Chi-Square	X^2	44.70			
	P-value	< 0.001 *			

P value of the test was significant< 0.001*.

Table 3. Statistical analysis of the HCV core antigen (HCV cAg) detection results by ELISA in the three different groups of the study.

HCV core Ag							ANOVA	
	Range			Mean	±	SD	f	P-value
Group I	0.316	-	1.269	0.725	±	0.257		
Group II	0.182	-	0.805	0.479	±	0.134	24.31	<0.001 *
Group III	0.309	-	0.522	0.422	±	0.065		
TUKEY'S Test								
II&I			III&I				III&II	
<0.001 *			<0.001 *				0.502	

P.value of the test was significant< 0.001*.

4. DISCUSSION

HCV infection is a global problem affecting more than 170 million people worldwide (**Li and Lo, 2015)**. The WHO estimates that 3% of the world's populations is infected (World Health Organization, 2009). HCV causes prolonged and persistent diseases in virus carriers. Often leading to chronic hepatitis C, cirrhosis, and hepatocellular carcinoma (Afdhal, 2004). In addition, the number of patients with HCV complications is expected to triple over the next 20 years leading to increased demand for liver transplantation (Banker, 2003).

Moreover, due to the lack of an efficient laboratory culture system for HCV or assays capable of detecting viral antigens. Direct detection of HCV has depended on nucleic acid amplification technology (NAT). In fact, serological assays to detect antibodies against HCV cannot distinguish between patients with active infection and those who cleanse the virus (Pawlotsky, 1999). ElAs are based on the diagnosis of HCV infection on the detection of antiHCV antibody, and it is confirmed by a positive result usually obtained by immunoblot assay or by the presence of HCV RNA by real-time RT-PCR (Pawlotsky, 2002). However, anti-HCV assays have limitations, including the

absence of sensitivity detection at window intervals as early as 45 to 68 days after infection (Glynn et al., 2005).

The breakthrough in diagnosing the early HCV infection is by detecting HCV cAg that occurs during the early stage or before seroconversion. HCV cAg tests have been introduced to supplement anti-HCV tests or HCV real-time RT-PCR analysis and these HCV Ag assays could be used for the monitoring of antiviral therapy as well as to diagnose HCV infection (Omura et al., 2005). Furthermore, the HCV Ag assay could also be useful in monitoring immunocornpromised patients and those undergoing hemodialysis (Medhi et al., 2008).

Some assays were designed to detect and quantify HCV cAg in serum and plasma in the absence of anti-HCV antibodies (Kesli et al., 2009). HCV detection using real-time RT-PCR is expensive, time-consuming and requires specialized equipment and skilled personnel. A less costly and less time-consuming test for acute HCV infection may be core-antigen testing (Cresswell et al., 2014). In previous studies, HCV cAg was proven to be more stable than HCV RNA and did not need particular precautions to prepare and store samples (Takahashi et al., 2005).

In this study, we had evaluated the HCV cAg assay detection in serum samples of different groups of patients and volunteers compared to the detection of HCV-RNA using real-time RT-PCR as a gold standard test. Furthermore, it is based on data from previous studies suggesting that HCV viremia appear and disappear in parallel (Tanaka et al., 2000). In addition, pervious reports with various HCV antigen assays indicated that HCV antigen kinetics were similar to those of HCV RNA in all stages of infection, and that the concentrations of HCV antigen and HCV RNA were well correlated (Seme et al., 2005; Fabrizi et al., 2005).

All the test properties of HCV cAg detection test were high. The sensitivity and specificity of the test were calculated as 76.1% and 86.2%, and positive and negative predictive values were calculated 80% and 82.5% respectively. As expected, the real-time RT-PCR was more sensitive than HCV cAg test. The core antigen was 32 positive samples of 42 real-time RT-PCR positive samples (76.1%) and 10 negative samples (23.8%), and real-time RT-PCR was more specific than HCV cAg assay as 30 samples were negative of 38 PCR negative samples 78.9% and 8 negative samples 21%. Chakravarti et al. (2013) reported the sensitivity of HCVcAg assay was 92.86% when compared to HCV RNA in anti-HCV Ab negative cases, and the specificity was 100%. The positive and negative predictive values were 100% and 99.22% respectively. The sensitivity, specificity of the HCVcAg assay for detection HCV infection, compared to RT-PCR in cases of acute liver disease (ALD) was 86.36%, 100%, respectively. This difference between our studies may be due to acute liver disease patients.

The effectiveness of HCV cAg was assessed in the diagnosis of acute HCV in HIV-infected parity and compared with HCV real-time RT-PCR. Core antigen has demonstrated sensitivity (100%) and specificity (97.9%). As a quick, simple, and cost-effective test, it is of great benefit in testing HCV (Cresswell et al., 2014). In current study, the test sensitivity and specificity were 76.1% and 86.2%. This difference in results may be due to the co-infection with immunodeficiency virus (HIV).

The benefit of HCV cAg ELISA has been studied in the examination of HCV infection of patients with hemodialysis. The sensitivity and specificity were 60% and 83% respectively, while the positive predictive value was 14.3%, negative predictive value was 97.7% and the efficiency was 81.9%. (Reddy et al., 2006). In this study, positive and negative predictive values were 80% and 82.5% respectively, and this difference in results may be due to the difference in patients groups between two studies. In a previous study that assessed the clinical usefulness of the HCV cAg assay for monitoring of patients being treated from chronic HCV infection on a total number of 86 samples, the core antigen test showed low negative predictive value of 16.6% due to the low number of RNA negative patients in the sample, (5 negative samples out of 86). However, the positive predictive value was above 90% (Lorenzo et al., 2004).

In this study, Thirty-two patients of the forty-two real-time RT-PCR positive patients were positive by HCV cAg, while 10 patients were negative. These negative samples can be explained by a low viral load « is 04)

in the sera. This interpretation was agreed with previous studies which also found that some individuals during pre-seroconversion period may have low viral load « is 04) and their HCV cAg cannot be detected through this examination (Lunel et al., 2003). Kesli et al., (2011) recorded that the diagnostic specificity and positive predictive values of the HCV cAg test compared with the HCV RNA test were 100%, 100%, respectively. The levels of HCV core antigen showed a good correlation with those from the HCV RNA quantification (r = 0.907). In our study specificity was 86.2%, positive predictive values was 80% respectively with a statistically insignificance correlation with HCV RNA and this may due to lack of false-positive results in previous study.

The correlation between HCV cAg and HCV RNA titer (r=0.321, p= 0.079) was statistically significant. We found (2) patients with high viral load and low optical densities (OD) obtained by detecting the core antigen with ELISA. Daniel et al. (2007) and Zhang et al., (2007) explained the high virus load (10^6) and low core antigen level in five patients may be attributed to ongoing anti-viral therapy. While the statistical analysis of relative results of HCV RNA and HCV cAg assays did not have statistically significant differences (p= 0.07) (). On other hand Buket et al. (2014) recorded that HCV core Ag assay displayed good correlation with HCV RNA assay (r= 0.935, P < 0.001) and this may due to the sensitivity, specificity were high in this study (86.5%, 100%) respectively than our study (76.1%, 86.2%).

The present study focused on the evaluation of the HCV cAg in relation to HCV RNA by real-time RT-PCR, thus we could not assess the effect of antiviral therapies on levels of HCV cAg and this agreed with (Ross et al., 2010), while Veillon et al. (2003), concluded that the total HCV cAg assay is a useful test for the detection of HCV viremia and the monitoring of patients treated with IFN alone or in combination with ribavirin.

5. CONCLUSION

Core antigen detection testing can be more valuable in HCV diagnosis than anti-HCV antibodies. It can be used to diagnose HCV infection in early infection before the appearance of anti-HCV antibodies in patient serum, but we must confirm that this examination cannot replace HCV RNA in confirming HCV diagnosis because the sensitivity of the HCV cAg checks detection is much less than real-time RT-PCR-based methods.

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