Isolation of Hepatitis C Virus Antigen El and Establishment of a Comparison of its Level in Different Fibrosis Stages in Patients Infected with Chronic Hepatitis C

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ABSTRACT

Hepatitis C virus (HCV) is the main causative agent of chronic liver diseases that accounting for significant morbidity and mortality worldwide. Screening for HCV antigens presents an alternative marker to viral antibodies and RNA. Thus, this work aimed to detect HCV-E1 antigen level in different liver fibrosis stages and to evaluate the possible relationship between this level and the disease severity. The study was performed among one-hundred and forty one participants categorized into two groups (35 healthy individual and 106 chronic hepatitis C patients). All involved patients were recruited from Tropical Medicine Department, Mansoura University Hospitals, Mansoura, Egypt. HCV-E1 antigen was identified using western blotting and its level was quantified by ELISA. Fibrosis was staged according to METAVIR scoring system as the following: patients with fibrosis stage F1: 32.1% (34/106) of cases, F2: 29.2% (31/106) of cases, F3: 20.8% (22/106) of cases and F4: 19.7% (19/106) of cases. HCV-EI was identified at 38 kDa using their respective specific monoclonal antibody. HCV-EI antigen level (OD) significantly increased ($P< 0.0001$) with liver fibrosis progression, it was 0.77±0.27, 0.55±0.17,0.43 ±0.16, and 0.30 ±0.09, in F4, F3,F2, and F1;respectively. HCV-EI antigen level seems to be associated with progression of HCV infection. It could be served as a good supplemental assay for HCV-RNA and could be used to diagnose active HCV infection.

Keywords: Fibrosis, Hepatitis C virus, Diagnosis, HCV-EI, Antigen.

INTRODUCTION

HCV is an enveloped, single-strand RNA virus that belongs to the *Flaviviridae* family. Its genome of approximately 9.6 kb contains a single open reading frame that encodes for three structural (core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) and is flanked by untranslated regions (Saludes *et al.*, 2014). Hepatitis C infections are a major cause of chronic liver injury and other subsequent complications including hepatocellular carcinoma (HCC) (Westbrook and Dusheiko, 2014; Salas-Villalobos *et al.*, 2017). Internationally, it was estimated that approximately seventy one million individuals have chronic hepatitis C (CHC) infection and there are about 399,000 individuals die annually from hepatitis C, in particular, from liver cirrhosis and HCC (Niebel, 2017). In Egypt, HCV infection is endemic with the highest globally prevalence rate (Elgharably *et al.*, 2017).

Early detection of HCV infection is vital to put stop to further transmission and to permit clinicians to make a quick decision concerning treatment, which has been confirmed to have a high degree of efficacy for acute hepatitis C (Schnuriger *et al.*, 2006). HCV infection diagnosis depend on detection of anti-HCV antibodies by recombinant immunoblot assays and viral RNA detection by PCR (Salas-Villalobos *et al.*, 2017). However, anti-HCV antibody assay cannot distinguish between present and past infection and need additional HCV-RNA testing to assure active infection (Wasitthankasem *et al.*, 2017). In addition, the laboratory setup for detection HCV-RNA requires expensive reagents and equipment, technical expert, and dedicated procedure areas (Kamili *et al.*, 2012).

On the other hand, serologic techniques for HCV antigens have been established and show significant and potential for active HCV infection diagnosis (Kamili *et al.*, 2012). These serologic assays may be quick, simple, and lower in costs compared to nucleic acid tests for HCV infection diagnosing (Veillon *et al.*, 2003; Cresswell *et al.*, 2015). The core antigen of HCV has been reported to be an indirect marker for HCV replication comparable to the detection of HCV RNA. It can serve as a trustworthy marker to diagnose active infection of HCV as well as to evaluate the treatment response (Wasitthankasem *et al.*, 2017). NS4 antigen detection rate was superior to that have been found by using similar polyclonal or monoclonal anti-NS4 antibodies (Gerlach *et al.*, 2005).

HCV envelope glycoproteins, E1 and E2, are type I transmembrane proteins with a highly glycosylated N-terminal ectodomain and a short C-terminal transmembrane domain (TMD). TMDs involved in important protein functions, like endoplasmic reticulum retention, membrane anchoring, and formation of E1-E2 heterodimer that may be the prebudding E1-E2 form of the virus. HCV envelope proteins are thought to have a significant and vital roles in host-cell entry, viral particle assembly and stimulate fusion with a host-cell membrane (Penin *et al.*, 2004; Pène *et al.*, 2017).

The target of this work was to determine the level of HCV-E1 antigen in different liver fibrosis stages as well as evaluation the possible relationship between this level and the disease severity.

MATERIALS AND METHODS

Patients

One hundred and sex CHC patients (28 females and 78 males) at the Tropical Medicine Unit, Mansoura University Hospitals, Mansoura, were enrolled in this work. They were positive for both HCV RNA and anti-HCV antibodies, and aged between 23-58 years with mean age ± standard deviation (SD)=42.2± 8.2 years. Moreover, 35 normal individuals (26 males and 9 females) aged between 21-56 years with mean age ± SD=41.2± 9.8 years as a negative control group were included. All negative controls were negative for anti-HCV antibodies

Individuals with the subsequent conditions were excluded from this work: hepatitis B virus co-infection, prior antiviral bleeding or immunosuppressive treatment and decompensated liver disorders (jaundice, ascites, bleeding, variceal or encephalopathy. Also, individuals with low platelet production other than hepatic disease

with HCV like typhoid, leukemia and vitamin B12 insufficiency were expelled from the study.

Samples and liver biopsies

Blood samples were collected after written consent from all populations. Portion of the blood was treated with EDTA-K3 to measure complete blood count by using KX-21 Sysmex hematology analyzer (Sysmex Corporation, Kobe, Japan). The last portion was left to clot and serum was separated by centrifugation. Liver function tests were detected on biochemistry analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany) on fresh serum.

Needle liver biopsy specimens were obtained with an 18-gauge or larger needle. Biopsies had to measure fifteen mm and/or contain 5 portal tracts at least, to be adequate for scoring, except for cirrhosis there was no required limitation. Interpretation of biopsies was according to METAVIR scoring system. (Poynard *et al.*, 1997). Fibrosis was scored on a 5-point scale: no fibrosis, F0; moderate fibrosis, F1; intermediate fibrosis, F2; extensive fibrosis, F3; cirrhosis, F4.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS - PAGE)

SDS-PAGE , through 16% resolving gels and 4% stacking was carried out according to the method of Laemmli (1970) in 0.75 mm-thick vertical slab gels. 50 µg/lane of selected serum samples from healthy individuals and patients with CHC were mixed with sample buffer (0.25 M Tris base, 10% mercaptoethanol, 0.1% bromophenol blue as a tracking dye, 20% glycerol, and 4%sodium dodecyl sulfate) and immediately boiled for 3 minutes. Reference proteins (BiorRad Laboratories, CA) were run in parallel. Then the gels were stained with coomassie blue.

Western immunoblotting

According to Towbin *et al.* (1979) method, samples were separated on SDS-PAGE and electrotransferred onto nitrocellulose (NC) membrane (0.45 μm pore size, sigma). Prestained molecular weight standards (Sigma) were run in parallel. The NC membrane was blocked using non-fat dry milk 2% (w/v) dissolved in Tris-buffered saline (TBS) (0.05 M) containing 200 mM NaCl (pH 7.4), rinsed in TBS and incubated with mono specific antibody (ABC Diagnostics. New Damietta, Egypt) directed against HCV-E1 antigen diluted in blocking buffer with constant shaking. Then it was washed three times in TBS (thirty min each) then incubated for two hours with anti-rabbit lgG alkaline phosphatase conjugate (Sigma) diluted in TBS (1:500) followed by washing 3 times with TPS. The blots were then visualized by soaked the membranes in premixed substrate, 5-bromo-4-chloro-3-indolylphosphate [BCIP]/nitro-blue tetrazolium [NBT] in 0.1 M Tris buffer, pH 9.6, (Sigma). Within ten min, the color was developed and the NC membrane dipped in distilled H_2O in order to stop the reaction.

Detection of HCV-E1 antigen using indirect ELISA

HCV-E1 was detected according to Attallah *et al.* (2008) with some modifications. Polystyrene 96-well microtiter plates were coated with diluted sample (fifty μL) in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Then, plates were washed with PBS-T20

(phosphate buffered saline containing 0.05% Tween 20). Free binding sites were blocked with bovine serum albumin (0.5%) for one hour at room temperature. After washing, 50 µl/well of monoclonal anti-E1 antibody (ABC Diagnostics. New Damietta, Egypt) diluted in PBS-T20, was added and incubated for two hours at 37 ºC. After washing, fifty µl/well of anti-mouse IgG (Sigma Chemical company, USA) alkaline phosphatase conjugate diluted in 0.2% (w/v) BSA in PBS-T20, was added and incubated for one hour at 37 °C. Then the plate was incubated with *p*-nitrophenyl phosphate substrate (Sigma Chemical Company, USA) at 37 °C for thirty min to determine the amount of coupled conjugate. then stop the reaction and read the absorbance using ELISA reader (Metreiteck, Axiom, Burstadt, Germany) at 405 nm.

Statistical Analysis

All data analyses were done by the SPSS software (SPSS Inc., Chicago, IL). Continuous variables differences were evaluated using Student's t-test or variance (ANOVA). Descriptive findings were expressed as number (percentage) or range and mean $\pm$ SD. All tests were considered significance at 0.05 levels.

RESULTS

Classification of CHC according to METAVIR scoring

CHC patients was evaluated and classified by METAVIR scoring as follows: 32.1% (34/106) of cases with F1fibrosis stage, F2: 29.2% (31/106) of cases, F3: 20.8 % (22/106) of cases and F4: 17.9 % (19/106) of cases; (Figure 1).

Figure 1. CHC patients classification according to METAVIR scoring system.

Laboratory parameters of patients with different liver pathology

As shown in Table 1, CHC patients showed higher values of AST, ALT, ALP, total bilirubin and platelet count compared to controls (P < 0.0001 for all comparisons except for serum ALP *(P* < 0.001)) while they had lower albumin concentrations (P < 0.01). Moreover, there was a significant difference among all CHC patients with different liver fibrosis stages in total bilirubin level and platelet count (P< 0.01 and P< 0.05; respectively).

Identification of HCV-E1:

As shown in Figure 2, in case of samples from CHC patients with different fibrosis stages a single immunoreactive band for HCV-E1 was observed at 38-kDa due to their binding with their respective specific monoclonal antibody, but in case of serum samples from normal controls there was no observed reaction.

Table 1.The laboratory characteristics of chronic hepatitis C patients with different fibrosis stages and normal individuals

Groups		ALT (U/ml)	AST (U/ml)	ALP (U/L)	Total bilirubin (mg/dl)	Albumin (g/L)	Platelet count × 10⁹/L
Normal	Mean ± SD	27.5±5.6	25.8±5.6	71.2±17.2	0.56±0.13	4.42±0.45	235.0±52.0
	Range	21.0-40.0	15.0-38.0	39.0-107.0	0.43-0.92	3.90-5.20	183.0-338.0
CHC	Mean ± SD	72.0±39.3	63.4±32.4	98.4±43.1	0.82±0.35	4.12±0.37	205.0±53.0
	Range	24.0-189.0	16.0-146.0	46.0-213.0	0.34-1.77	3.0-4.8	100.0-328.0
F1	Mean ± SD	64.0±32.5	54.2±23.0	90.0±38.2	0.79±0.27	4.2±0.39	214.0±63.0
	Range	24.0-160.0	17.0-142.0	46.0-158.0	0.34-1.34	3.60-4.80	115.0-338.0
F2	Mean ± SD	67.3±31.4	62.3±35.0	94.2±34.3	0.81±0.32	4.16±0.31	212.0±46.0
	Range	26.0-179.0	16.0-146.0	50.0-160.0	0.42-1.77	3.99-4.70	104.0-290.0
F3	Mean ± SD	74.0±49.6	73.2±38.1	107.7±49.2	0.88±0.46	4.08±0.37	206.0±46.0
	Range	24.0-188.0	28.0-144.0	50.0-195.0	0.50-1.90	3.47-4.80	103.0-268.0
F4	Mean ± SD	90.2±45.1	76.8±38.8	108.7±56.1	1.16±0.68	4.06±0.41	176.0±50.0
	Range	26.0-189.0	24.0-142.0	55.0-213.0	0.50-2.76	3.00-4.50	100.0-268.0
P value *(ANOVA)*		>0.05	>0.05	>0.05	< 0.01	>0.05	<0.05
P value (*t* test)		< 0.0001	< 0.0001	< 0.001	< 0.0001	< 0.01	< 0.0001

References values: Alanine aminotransferase (ALT) up to 45 U/ml; aspartate aminotransferase (AST) up to 40 U/ml; alkaline phosphatase (ALP) 22-92 IU/L; total bilirubin up to 1 mg/dl; albumin 3.8-5.4 g/L; platelet count 150-400 × 10⁹/L.

CHC: Chronic hepatitis C; SD: standard deviation; F Fibrosis was scored according to METAVIR scoring system: F1, moderate fibrosis; F2, intermediate fibrosis; F3, extensive fibrosis; F4, cirrhosis; *P*<0.05 is considered significant.
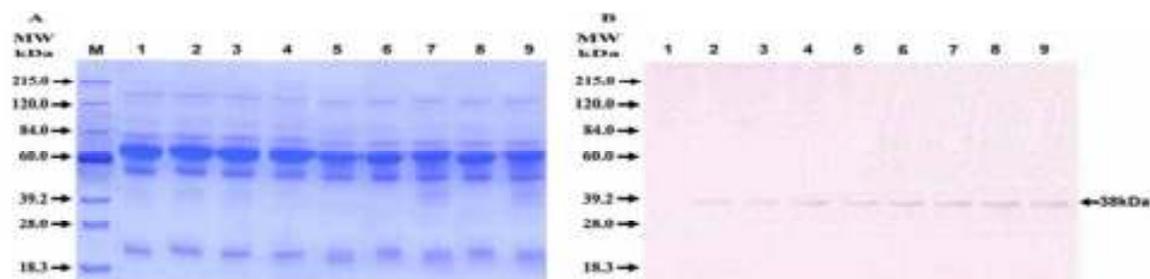

Figure 2. (A): Cooomassie blue stained SDS-PAGE showing the ploypeptide pattern of samples from CHC patients and controls. (B): Western blot analysis of samples from CHC patients and controls with monoclonal antibody against HCV-E1. Lane 1: sample from normal individual, lanes 2-3: samples from F1 patients, lanes 4-5: samples from F2 patients, lanes 6-7: samples from F3 patients and lanes 8-9: samples from F4 patients. Molecular weight marker (Mr.) including: lysozyme (18.3 kDa), trypsin inhibitor (28.0 kDa), carbonic anhydrase (39.2 kDa), ovalbumin (60.0 kDa), bovine serum albumin (84.0 kDa), phosphorylase B, (120.0 kDa) and myosin (215.0 kDa).

Standardization of ELISA for HCV-E1 antigen detection

The cut-off level of ELISA technique below or above of which the analyzed sample can be considered negative or positive respectively, was determined using sera of sixteen CHC patients and sixteen normal individuals as the mean optical densities (at 405nm) of serum samples from normal individuals + 3SD (i.e. OD+ 3SD). It was set at OD = 0.21 as shown in Figure 3.
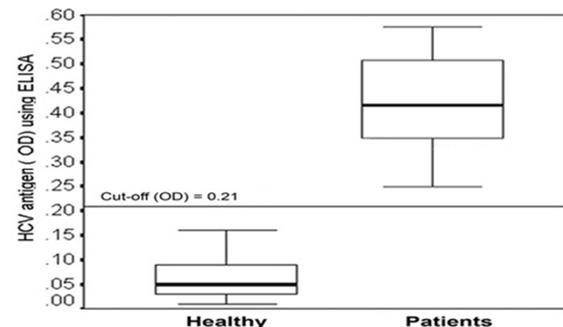

Figure 3. Determination of HCV-E1 antigen cutoff. The cut-off value o is 0.21.

Association between HCV-E1 antigen level and liver disease progression

The level (OD) of HCV-E1 antigen in total CHC patients was higher than in healthy individuals with extremely high significant difference (0.47±0.28 *vs* 0.17±0.06; *P* <0.0001). In addition, HCV-E1 antigen level significantly increase ($p < 0.0001$) with progression of liver fibrosis where the means OD± SD were 0.77±0.27, 0.55±0.17, 0.43 ±0.16, and 0.30 ±0.09, in F4, F3,F2, and F1;respectively, (Table 2 and Figure 4).

Table 2. association between HCV-E1 antigen and liver disease progression

Groups	n	HCV-E1 antigen Mean OD ± SD[b]	*p* value[d]
Normal	35	0.17±0.06	*p* < 0.0001
CHC patients[a]	106	0.47±0.28	
F1[c]	*34*	*0.30±0.09*	
F2	*31*	*0.43±0.16*	*p* < 0.0001
F3	*22*	*0.55±0.17*	
F4	*19*	*0.77±0.27*	

[a]CHC: Chronic hepatitis C; [b]SD: standard deviation; [c]F: Fibrosis was scored according to METAVIR scoring system: F1, moderate fibrosis; F2, intermediate fibrosis; F3, extensive fibrosis; F4, cirrhosis; [d] *P*<0.05 is considered significant
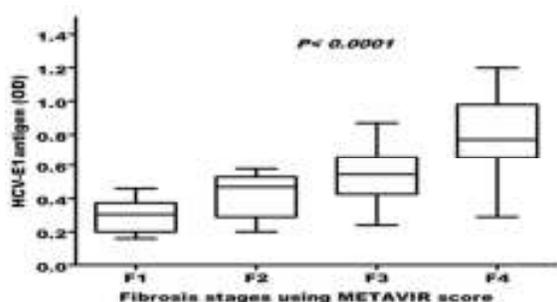
Figure 4. Association between HCV-E1 antigen level and liver disease progression.

DISCUSSION

There is an increasing need for a simple, cost-effective and supplemental assay besides the costly HCV RNA testing for evaluation HCV disease severity (Wasitthankasem *et al.,* 2017). Also, many studies documented the relation between the liver disease severity and HCV proteins in hepatic tissues (Kasprzak *et al.*, 2004; Galy *et al.*, 2007).

Therefore, this study evaluates the potential role of HCV-E1 antigen to assess and diagnose active HCV infection besides measuring it in different histological degrees to establish relationship between its level and the disease severity. The reason why E1 antigen is chosen for this purpose is because of its good neutralizing properties, Interestingly, E1-neutralizing antibodies combined with T-helper (Th)-recruited that seem to have facilitated HCV 1b clearance (Verstrepen *et al.*, 2011).

Laboratory liver tests are defined as a valuable tool for evaluation and treatment of patients with hepatic dysfunction (Gowda *et al.*, 2009). In this study, among the CHC patients, many laboratory parameters were examined to provide the clinical usefulness information. The examination of these parameters revealed higher ALT, AST and ALP activities, elevated total bilirubin levels, and lower albumin and platelet count levels in CHC patients in comparison to the normal individuals (control), ($P < 0.0001$).

In many cases of hepatocyte injury including hepatitis, AST and ALT can be extremely increased, exceeding 2,000 U per L. Moreover, the AST/ALT ratio varies in (Thapa and Walia, 2007; Lui, 2015). Albumin synthesis is a significant function of the liver; nearly 10g albumin is synthesized and secreted daily. With the progression of liver disease, circulating albumin levels reduce which reflect diminished synthesis (Limdi and Hyde, 2003). However, these laboratory liver tests may be of little value in liver disease screening and it can only aid in directing the subsequent diagnosis. Many serious hepatic disorders such as cirrhosis and congenital hepatic fibrosis may be related with normal levels (Gowda *et al.*, 2009). Moreover, these laboratory tests are not specific for any certain disease. Albumin can be reduced in chronic disease as well as in nephrotic syndrome. Furthermore, aminotransferases can be elevated in cardiac disorders as well in liver disorders. Liver function tests, except serum bilirubin, are not specific for hepatic disorders and can be elevated for extra-hepatic diseases (Thapa and Walia, 2007).

Also, in CHC, among the hematological disturbance, the platelet count decrease seems to be the most prevalent. The platelet number may be lower than 50,000/µL. Clinical variables such as age, gender, viremia degree and disease severity might influence platelet reduction severity (Olariu *et al.*, 2010).

In biological samples, western blot analysis provides an adequate method to identify and measure any changes in specific proteins concentrations. In western blot techniques, examiners take advantage of the antibodies sensitivity to identify interested proteins in complex samples (Eslami and Lujan, 2010; Palmisano and Meléndez, 2016). In this study, HCV-E1 antigen was detected at 38-kDa in CHC patients serum using specific monoclonal antibody and western blotting. Similar results were obtained by Fournillier-Jacob *et al.* (1996) and Lee *et al.* (1997), they reported tha structural proteins of HCV were composed of two glycosylated envelope proteins, E2 of 58 –74 kDa and E1 of 31–35 kD and the core protein. Also, Attallah *et al.* (2015) mentioned that HCV-E1 was detected in both cord blood and serum of HCV-infected pregnant women at 38 kDa.

Results showed that HCV-E1 antigen levels were elevated in CHC patients ($p < 0.0001$) than normal individuals. In addition, HCV-E1 antigen levels increase with progression of liver disease with highly significant difference among difference fibrosis stages ($p < 0.0001$). Our findings were in accordance to El Awady *et al.* (2006) who found that the mean OD reading E1 antigen was significantly ($P < 0.05$) elevate in viremic individuals in comparison to negative controls.

HCV envelope glycoproteins E1 and E2 have pivotal roles at various steps of the HCV life cycle, such as virus entry, fusion with the endosomal membrane and infectious particle assembly (Moradpour and Penin, 2013). Chronic HCV infection developed in patients where the immune system is not fully able of controlling the infection due to the emergence of various escape mutants (Farci *et al.*, 1997; Thimme *et al.*, 2012). Such mutations within either core or envelop proteins can increase viral aggressiveness or block the viral infectivity (Hong *et al.*, 1999). It has been reported that viral mutations not only differ in infection but also in their intracellular pathogenesis. Many studies have suggested that HCV core protein has a main significant role in liver fibrosis development (Shin *et al.*, 2005), hepatic steatosis (Koike and Moriya, 2005), and HCC (Xue, 2005). Moreover, patients may have absent or mild hepatic changes though high viral load (Moatter *et al.*, 2002). These results indicated that viral proteins expression in each case is the most effective agents on the disease morbidity than viral loads (El Awady *et al.*, 2006).

In conclusion, a cost-effective quick and easy assay for screening and identifying HCV-E1 antigen would be a useful clinical marker for identifying active HCV infection. More studies are necessary to evaluate the diagnostic efficacy of this antigen in larger multicentric studies.

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فصل أنتيجين الغلاف-1 لفيروس الالتهاب الكبدي ج ومقارنه مستواه فى مراحل التليف الكبدى المختلفه فى مرضى مصابين بالتليف الكبدى المزمن ج

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الإصابة بفيروس الالتهاب الكبدي الفيروسي ج تمثل اهم المشاكل الصحية التي تواجه العالم نظرا لإنتشارها وأرتفاع معدلات الوفيات الناتجة عنها. تشخيص العدوى بفيروس الالتهاب الكبدي الفيروسي ج وتتبع تطور المرض يفيد كثيرا في قرار العلاج. وقد وضعت أساليب للكشف عن المستضدات الفيروسية (antigens) في الأمصال باستخدام الأجسام المضادة وحيدة النسيلة . و تهدف هذه الدراسة إلى تحديد مستوى مستضد فيروس ج E1 (HCV-E1 antigen) في مراحل مختلفة من تليف الكبد. تضمنت هذه الدراسه 106 من المرضى المصابين بالتهاب الكبد المزمن بالاضافه الى 35 من الأشخاص الأصحاء لإستخدامهم كمجموعة قياسية. تم تقسيم مرضى التهاب الكبدي ج المزمن على حسب التحليل الباثولوجى لعينات الكبد الى 34 من درجة التليف الأولى (F1) و 31 من درجة التليف الثانية (F2) و 22 من درجة التليف الثالثة (F3) و 19 أخرون يعانون من تشمع الكبد (F4). تم التعرف على هذا المستضد في أمصال دم المرضى باستخدام طريقة النقل المناعي وقد أظهرت النتائج أن الوزن الجزيئي له 38 كيلو دالتون. كما تم تقديرمستويات هذا الأنتيجين في أمصال الدم باستخدام تقنيه الادمصاص المناعى الإنزيمي فوجد انها أعلى بكثير في المرضى المصابين بالتهاب الكبد المزمن مقارنة بالأفراد الأصحاء مع وجود فرق إحصائي كبير ($P<0.0001$) وعند تقدير نسبته في مراحل تطور المرض المختلفة من التليف والتشمع وجد ان نسبته تختلف بفرق إحصائي واضح($p < 0.0001$) مع تطور هذا المرض حيث أن مستواه في مراحل التليف المختلفة كان كالأتى: المرحلة الأولى للتليف (0.30±0.09), المرحلة الثانية للتليف (0.43±0.16), المرحلة الثالثة للتليف (0.55±0.17), المرحلة الرابعة للتليف (التشمع) (0.77±0.27). مما سبق يمكن إستنتاج انه يمكن إستخدام المستضد (HCV-E1) كاحدى الطرق البديله لتشخيص الاصابه بفيروس الالتهاب الكبدي ج.

كلمات مرشده: فيروس الالتهاب الكبدي الفيروسي ج، مستضد، التليف، التشخيص.