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Consistency between biochemical and molecular assessments for chronic Hepatitis C virus in Egypt

Mohamed M.S. Farag[1*], Adel A. Mousa[1], Mohamed A. Alhemaly[1], Ahmed R. Sofy[1]

[1] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt
[*]Corresponding author: **Mohamed M.S. Farag**
E-mail: *moh_man_s1977@yahoo.com/Mohamed.farag@azhar.edu.eg*

Abstract

Hepatitis C virus (HCV) is a global health problem that may cause cirrhosis and progression to hepatocellular carcinoma. HCV infects approximately 170 million individuals worldwide. The use of biochemical, serological and virological tests has become essential in the management of HCV infection in order to diagnose infection, guide treatment decisions and assess the virological response to antiviral therapy. The main goal of this study was to estimate the prevalence of HCV infection and to compare between biochemical and molecular assessments for chronic Egyptian patients. To achieve this aim, a total of 75 anti-HCV-positive patients with chronic hepatitis C were examined for virological tools including all biochemical, serological and molecular assays. HCV RNA monitoring during interferon-ribavirin (INF-Rib) combination therapy is used to tailor treatment duration in HCV infection. Our results showed consistency between biochemical and molecular assessments for chronic infected patients. The infected patients were classified into different virus titer categories; 17/75 (22.7%) very low, 15/75 (20%) low, 15/75 (20%) intermediate, and 28/75 (37.3%) high virus titers. Of the 75 patients included in the study, 21 (28%) were not appropriate for treatment, while 54 (72%) patients were subject to INF-Rib combination therapy. Of 54 patient, 47 (87%) were responded to INF-Rib combination therapy, while 7 (13%) were non-responded to INF-Rib combination therapy. In conclusion, molecular assays are used to assess the end-of-treatment and, most importantly the sustained virological responses, i.e. the endpoint of therapy. Biochemical and molecular diagnostic tests for HCV shed light on the natural history of HCV.

Keywords: HCV, Biochemical, Molecular assays.

1. Introduction

Hepatitis C virus (HCV) infection is a global health problem [1]. HCV is recognized as one of the main causes of chronic liver disease worldwide [2]. HCV infection is usually asymptomatic during the acute phase, but more than 80% of patient's progress to chronic hepatitis C (CHC). Approximately 130-170 million people worldwide are chronically infected with HCV, and these people are at risk of developing hepatic complications, such as cirrhosis and/or hepatocarcinoma [3]. Hepatitis C virus is an enveloped, single-stranded, positive sense ribonucleic acid (RNA) virus with a 50nm diameter viral particle and is a member of the *Hepacivirus* genus of the *Flaviviridae* family [4]. Its RNA genome encodes a unique polyprotein of approximately 3,000 amino acids [5,6].

An estimated 2%-3% of the world's population, corresponding to approximately 130-170 million persons, has been infected with HCV [4]. Although recent advances in the treatment of HCV genotype 1 infection using directly acting antiviral agents are encouraging, there is still a need to develop vaccine strategies capable of preventing infection [6].

HCV is a small (36-65 nm in size) and positive sense single-stranded RNA virus [2]. It can be transmitted through contact with the blood of an infected person, organ transplantations, blood transfusions, renal dialysis, and intravenous drug abuse [3].

A recently acquired HCV infection is often asymptomatic [7]. Traditionally a recent HCV infection is determined by monitoring antibody IgG seroconversion, and/or the detection of viral RNA in the absence of anti-HCV. It can take between 1–4 weeks for RNA and 8–12 months for antibody to become detected after infection with HCV [8]. The presence of anti-HCV and HCV PCR can indicate either an acute or a chronic infection, even in the presence of elevated liver function tests. [9] IgM antibody in HCV can be detected during exacerbation of chronic HCV. [9] An anti-HCV positive and PCR negative result usually indicates a resolved HCV infection, but can also indicate an acute infection with low viraemia [8].

The World Health Organization (WHO) has declared Hepatitis C a global health problem, with approximately 3% of the world's population infected with HCV [10]. In the US, approximately 3 million people are chronically infected, many of whom are still undiagnosed. In Egypt the situation is quite worse. Egypt contains the highest prevalence of hepatitis C in the world. The national prevalence rate of HCV antibody positivity has been estimated to be between 10-13% [11], since 30-40% of individuals clear the infection shortly after exposure based on national studies and village studies in Egypt, the estimated adjusted national prevalence rate of chronic HCV infection is 7.8% or 5.3 million people in 2004. Only one third of these individuals (1.75 million) are estimated to have chronic liver disease (elevated ALT) and, furthermore, among these one third (577,000 people) are suffering from advanced liver disease. Interestingly, genotype 4 represents over 90% of cases in Egypt. Chronic HCV is the main cause of liver cirrhosis and liver cancer in Egypt and, indeed, one of the top five leading causes of death. In Egypt, the major route of exposure appears to be due to injection therapy and inadequate infection control practices [12].

In most cases, diagnosing infection may occur accidentally or at late stage [13]. HCV-RNA is detectable in serum 1–2 weeks after infection, while anti-HCV antibodies are not detectable until 8–9 weeks [14]. In clinical practice, detecting anti-HCV antibodies is the first step towards identifying infected individuals that must be accompanied by RNA detection to declare active infection [15].

2. Materials and Methods

The study was included 75 patients with HCV infection during the period from April 2013 to April 2014.

2.1 Sample collection

Blood samples were collected for different analysis at the time of routine clinic attendance as following. Five ml whole blood was withdrawn into an anticoagulant (EDTA) tube from each patient included in our study. The 5 ml blood sample was covered on the side wall of a centrifuge tube containing 2.5 ml of Ficoll-Hypaque density gradient separating solution under aseptic conditions. The blood on the Ficoll-Hypaque was centrifuged at 2000 rpm for 20 min at room temperature. After centrifugation, the peripheral blood cellular components separated into 4 separate layers. The first layer was the plasma and platelets. The plasma was extracted carefully and kept frozen in asterile vial at -80 C until tested for antibodies and other biochemical tests. The layer between the plasma and Ficoll-Hypaque at a density of 1.77g/ml is the peripheral blood mononuclear cells (PBMC). The final layer was the erythrocytes.

2.2 Molecular Assay

2.2.1. Extraction

RNA was extracted using RTP® DNA/ RNA Virus Mini Kit. Briefly 200 µl of sample was transferred into the provided Extraction Tubes, 200 µl dd H2O was added. For samples which have a smaller volume than 200 µl were filled up to a total volume of 400 µl with ddH2O and incubated for 15 minutes at 65°C in a thermo-mixer after that incubated for 10 minutes at 95°C in a thermomixer (optional).For optimal binding conditions 400 µl **Binding Solution** was added and mixed completely by pipetting up and down. The sample was transferred on the RTA Spin Filter, incubated for 1 min then centrifuged for 2 mins at 11.000 x g (11.000 rpm), the flow-through with the RTA Receiver Tube was discarded and the RTA Spin Filter was put in a new RTA Receiver Tube. 500 µl **Wash Buffer R1** was pipetted onto the RTA Spin Filter, centrifuged for 1 min at 11.000 x g (11.000 rpm) then the flow-through and the RTA Receiver Tube were discarded. the RTA Spin Filter was transferred into a new RTA Receiver Tube. 700 µl

Wash Buffer R2 was pipette onto the RTA Spin Filter, centrifuged for 1 min at 11.000 x g (11.000 rpm), then the flow-through and the RTA Receiver Tube were discarded, after that, the RTA Spin Filter was transferred into a new RTA Receiver Tube**.** To eliminate any traces of ethanol, we centrifuged again for 4 min at maximum speed, discarded the RTA Receiver Tube. The RTA Spin Filter was transferred into an RNase-free 1.5 ml Elution Tube pipetted 60 µl of **Elution Buffer R** (preheated to 65°C) directly onto the membrane of the RTA Spin Filter, incubated for 3 min, centrifuged for 1 min at 11.000 x g (11.000 rpm) finally the RTA Spin Filter was discarded and the eluted viral DNA/ RNA was placed on ice.

2.2.2. Real Time PCR (RT-PCR)

A RT-PCR test was done using RT-PCR reagents that constitute a ready-to-use system for the detection of HCV RNA by PCR in a Stratagene' Mx3000P quantitative RT-PCR system. The HCV RT-PCR kit included reagents and enzymes for the reverse transcription and specific amplification of a specific region of the HCV genome in a fluorescence detector FAM (reporter dye). The kit has a second heterologous amplification system to identify possible PCR inhibition. ***HCV*** PCR Master Mix (Applied Biosystems) was added including an optimized RT-PCR buffer, MgCl2, Taq DNA polymerase, and Reverse transcriptase, and stabilizers. HCV-RNA was amplified by RT-PCR using primers KY80 (5′GCAGAAAGCGTCTAGCCATGGCGT) and KY78 (5′CTCGCAAGCACCCTATCAGGCAGT) targeting the 244-base region located within the highly conserved 5′ noncoding region of the HCV genome. The reaction took place under stander thermal profile: incubation at 40°C for 60 minutes to transcribe viral RNA to cDNA by RT. This was followed by AmpliTaq gold activation at 95°C for 3 minutes. Denaturation was performed at 95°C for 15 seconds, followed by annealing at 94°C for 5 second and extension at 62°C for 10 second with end point fluorescence detection. 34 The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase has started to have an effect on the efficiency of amplification. Software provided in the computer system should connect to the apparatus allowing real-time amplification plots to be viewed and to be analyzed during the PCR run.

2.3. Biochemical analysis

2.3.1. Aspartate aminotransferase (AST/SGOT) colorimetric method

Five hundred micro-litter of AST reagent one was added to 100µl of serum/or blank in the test tube, the tube was mixed and incubated for 30 min at 37°C. Then, 500 µl of AST reagent two was added to the tested tube, mixed and the tube was incubated for 20 min at 37°C. After the incubation, 500 µl of sodium hydroxide was added to the tested tube. The reaction has measured the absorbance of each at 546 nm after 5 minutes.

2.3.2 Alanine aminotransferase (ALT/SGPT) colorimetric method

Five hundred micro-litter of ALT reagent one was added to 100µl of serum/or blank in the test tube, the tube was mixed and incubated for 30 min at 37°C. Then, 500 µl of ALT reagent two was added to the tested tube, mixed and the tube was incubated for 20 min at 37°C. After the incubation, 500 µl of sodium hydroxide was added to the tested tube. The reaction has measured the absorbance of each at 546 nm after 5 minutes.

2.3.3 Total bilirubin

Two hundred micro-litter of reagent 1, One drop reagent 2, 1000 µl of reagent 3, and 200 µl of serum samples/or blank was added in the tested tube, the tube was mixed and incubated for 10 min at 20- 25°C. After incubation, 1000 µl of Reagent 4 was added. The reaction was measured the absorbance of each sample at 578 nm (560- 600nm), the color intensity was stabled for 30 min.

2.4. Statistical Analysis

Patients' age represented by a box plot constructed in SigmaPlot® 12.5 software. Patients' gender represented by a column plot constructed in MS Excel®2013 software. It was necessary to determine the level of disease to classify patients according to the study hypotheses, therefore, a preliminary measurement of PCR values were performed. Data then analyzed using a one-way model of analysis of variance (ANOVA) to determine the significance between groups.

3. Results

The study was conducted on 75 patients who were positive for anti-HCV by single enzyme linked immunosorbent assay (ELISA). Recruitment of patients was random. Overall samples were used for the establishment of the procedure for detection of HCV RNA by RT PCR. For the HCV-RNA positive patients, blood samples were collected for different biochemical analysis, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, platelets (PLTS) and albumin.

3.1. Patients age

The age is roughly symmetric around the median of low and high level patients (Table 1 and Figure 1).

Table 1: Shows the descriptive statistics for Egyptian patients' age

Symbol	Denotation	Descriptive values			
		Very low	Low	Intermediate	High
---	Mean	26.235	29.467	44.800	59.429
—	Median	22.0	30.0	49.0	57.5
□	25th% - 75th% quartile	16.50 – 33.50	25.00– 33.00	33.00– 52.00	54.00 – 65.00
I	10th% - 90th% percentiles	15.80– 46.00	20.60 – 36.40	27.80– 56.40	53.00– 66.10
	Non-outliers (min. to max.)	15.00– 50.00	20.00– 40.00	23.00– 60.00	51.00– 69.00
○	Standard Deviation (SD)	± 10.975	± 5.566	± 10.557	± 5.527
	Standard Error (SE)	± 2.662	± 1.437	± 2.726	± 1.045

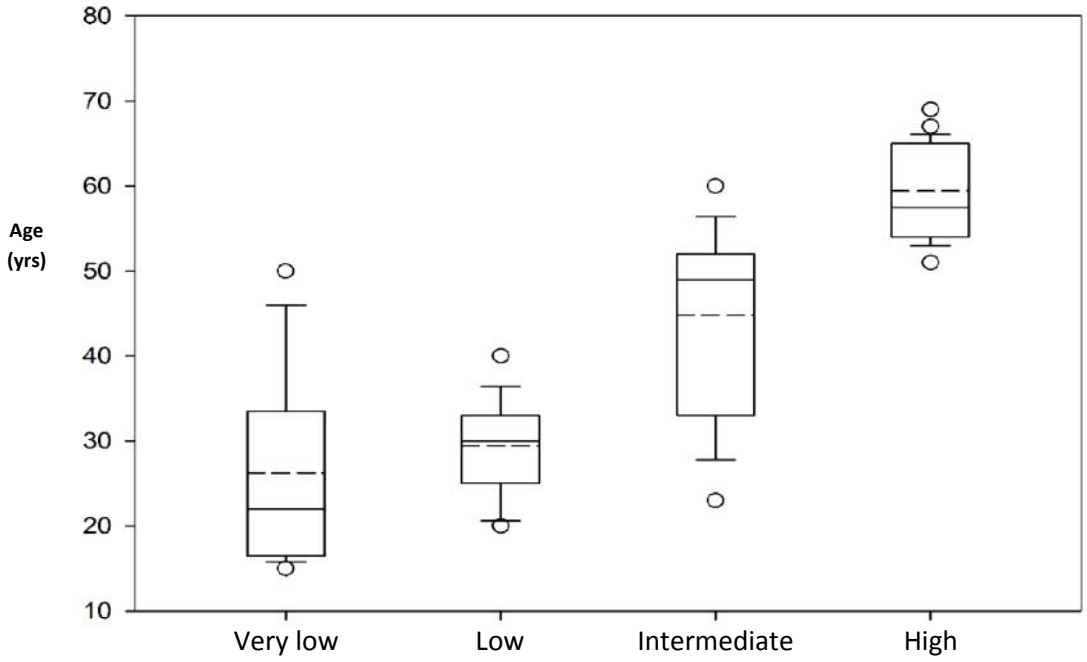

Figure 1: Shows the descriptive statistics for Egyptian patients' age

3.2. Virological findings

3.2.1. Serological assay

All patients included in our study were anti-HCV positive by third-generation enzyme-linked immunosorbent assays (ELISA) (Table 2).

Table 2: Detection of anti-HCV for our patients by ELIZA test

No. of patients	ELISA results HCV-Ab
75	Positive

3.2.2. Molecular assay

The presence of the viral genome (HCV RNA) in serum was detected by real time polymerase chain reaction (RT-PCR). All patients included in our study were positive for HCV RNA (Table 3).

Table 3: Molecular detection of HCV RNA by RT-PCR for patients included our study.

No. of patients	RT-PCR results HCV RNA
75	Positive

3.3. Patient classification

The present study showed that infected patients were classified into different virus titer categories; 17/75 (22.7%) (7 males and 10 females) very low, 15/75 (20%) (9 males and 6 females) low, 15/75 (20%) (15 males and NO females) intermediate, and 28/75 (37.3%) (6 males and 22 females) high virus titers (Figure 2 and table 4).

Table 4: distribution male and female in our patients groups

Gender	Number of patients			
	Very low	low	Intermediate	High
Male	7	9	15	6
Female	10	6	-	22
Total	**17**	**15**	**15**	**28**

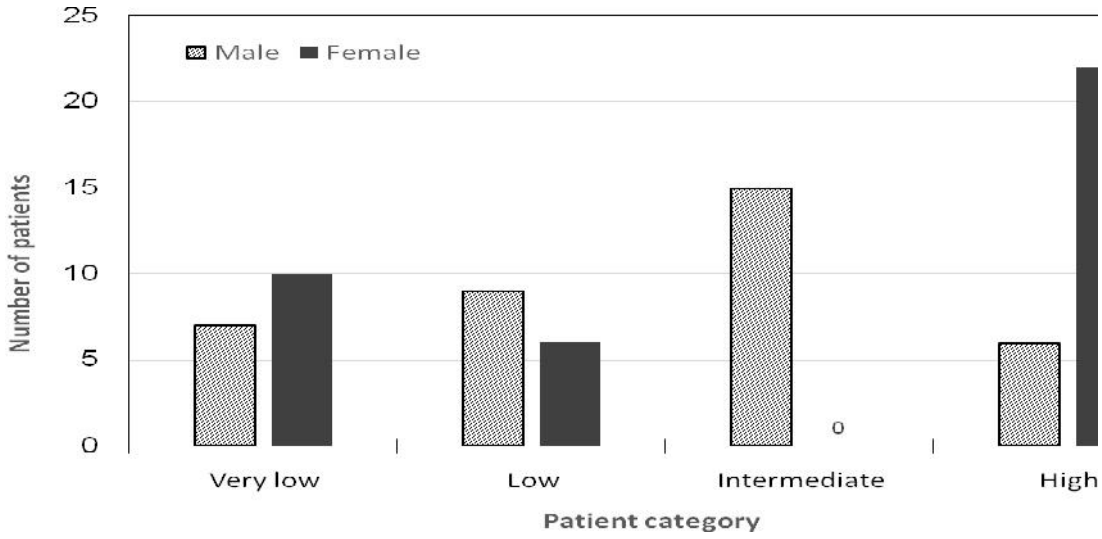

Figure 2: Showed patients classification

3.4. Virus titers among patients groups

Our results showed that virus titer was significantly high ($p < 0.001$) in fourth group (high patients category), greater than in other groups. However, No significant differences were detected between other patients' groups (Figure 3 and table 5).

Table 5: Showed virus titers among patients groups

Source of variation	PCR (IU ml^{-1})				
	df	SS	MS	*F*-ratio	*p*-value
Between Groups	3	6.680E+014	2.227E+014	96.618	< **0.001**
Residual	71	1.636E+014	2.305E+012		
Total	74	8.317E+014			

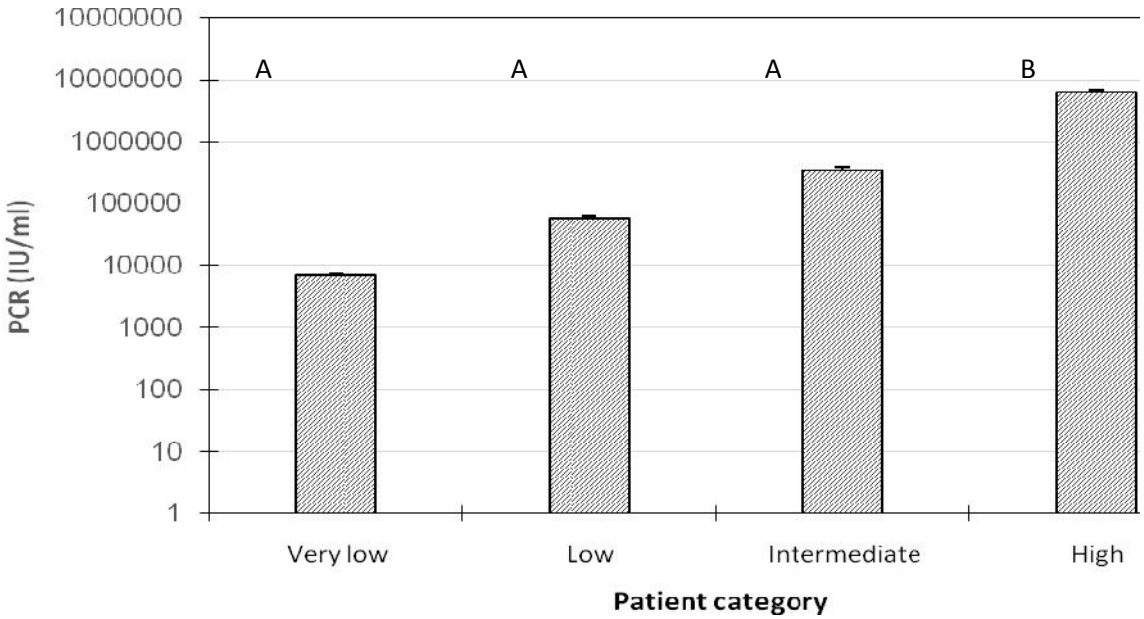

Figure 3: Showed virus titers among patients groups detected by RT-PCR. Capital letters represent the significance between groups. Error bars represent standard error (SE). The scale of *y*-axis was transformed to *log*-scale due to very small and very large values.

3.5. Relation between virus titers and biochemical assays

Our results showed consistency between biochemical and molecular assessments for chronic infected patients included in our study (Table 6).

Table 6: Showed relation between viral load and biochemical analysis

Bio-chemical test	Patient category			
	Very low	Low	Intermediate	High
ALT	25.41176471 ±1.277804941	23.89333333 ±1.088444354	35.66666667 ±1.55430789	76.78571429 ±3.9290524
AST	26.17647059 ±1.348297658	27.46666667 ±1.853867686	34.89333333 ±3.863128102	76.07142857 ±3.97210131
Albumin	4.376470588 ±0.090557672	4.4200 ±0.094213537	4.0200 ±0.171603086	2.664285714 ±0.102767818
Total Bilirubin	0.705882353 ±0.037837295	0.700666667 ±0.026121815	0.927333333 ±0.048348602	2.3875 ±0.114596861

PT	93.52941176 ±0.814020735	94.800 ±1.096314171	87.33333333 ±1.953425969	65.92857143 ±1.381978272
HB	11.87058824 ±0.334945639	12.49333333 ±0.402310786	12.90666667 ±0.617943337	12.00714286 ±0.253020304
RBCs	3.973529412 ±0.170740044	4.17600 ±0.132384001	4.4520 ±0.208582974	3.796785714 ±0.110037336
HCT	36.94117647 ±0.847316648	40.8800 ±1.319278302	40.06666667 ±1.890997889	38.53571429 ±0.789283619
Platelets	278.4705882 ±26.65894586	273.1333333 ±18.50762177	165.9333333 ±19.55078044	115.5357143 ±4.006437328
WBCs	6.952941176 ±0.701737217	6.7200 ±0.489236529	6.9800 ±0.71602075	6.557142857 ±0.394117747

Discussion

Hepatitis C virus (HCV) constitutes a significant health burden worldwide. Indeed, this virus possesses a high susceptibility for establishing a chronic infection and it is estimated that 130–170 million people suffer from chronic hepatitis C. In the long-term, this can lead to advanced liver fibrosis, cirrhosis, and hepatocellular carcinoma. As a consequence, HCV is the most common indication for liver transplantation in developed countries [16].

Globally, about 1 person in 50 is infected with HCV. In Egypt, a recent study reported that about one person in seven of Egypt's 83 million population tested positive for antibodies against HCV, indicating that these individuals have been infected with the virus at some point [17]. However, nearly one person in ten carries its viral RNA and is therefore chronically infected. Firm data for the infection rate are hard to come by. Another study in 2010 estimated that more than half a million people are newly infected each year [17]. The use of Serological and molecular markers in clinical practice has become essential in managing chronic hepatitis C, guide treatment decisions and monitor the antiviral efficacy of treatment.

Several virological assays are available to help diagnose patients infected with the (HCV). These include the anti-HCV antibody assays, measurement of HCV RNA viral load and HCV genotyping. In addition to the previous tools, there are routine lab tests are thus important for monitoring liver function of HCV infected patients as follows; Liver biochemical/functional testing (ALT/AST), Complete blood counts (CBC) and Chemistry panels.

This study highlights on one of the routine laboratory tools which can be employed as an indicator of the state of the activity of the liver in patients with HCV infection, the tool estimates the level of concentration of two liver enzymes (ALT/AST), and study the impact of these enzymes with the HCV load. In the present study, Blood samples were collected from all subjects under study for preliminary diagnosis of the infection with HCV, also routine lab tests for assessing liver function have been made out. All patients included in our study were positive for anti-HCV antibody by ELISA test and were positive for the presence of HCV-RNA which detected by RT- PCR. In our study, all biochemical analysis were increased with HCV RNA titres and decreased with low HCV RNA titres. Our results were in agreement with previous studies [18,19] On the other hand,our results were in contrast with previous studies [20,21] that have been showed there was no association between HCV RNA level and grade of liver injury in chronic HCV carriers but serum ALT level was associated with portal inflammation and necrosis. In some studies, no clinically feasible association was found between ALT level and liver injury or liver fibrosis [21]. In conclusion, molecular assays are used to assess the end-of-treatment and, most importantly the sustained virological responses, i.e. the endpoint of therapy. Biochemical and molecular diagnostic tests for HCV shed light on the natural history of HCV.

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