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RESEARCH ARTICLE

Hepatitis C virus infection and its impact on Liver function in chronic HCV patients.

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Abstract

Hepatitis C virus (HCV) is a blood-borne virus whose target organ is the liver. It causes both acute and chronic hepatitis, acute infection becomes chronic in approximately 80% of cases, and patients with chronic hepatitis C areat high risk of life-threatening complications, including cirrhosis in 20% of cases and hepatocellular carcinoma at an incidenceof 4–5% per year in cirrhotic patients**.** Virological testing has become essential in the management of HCV infection in order to diagnose infection, and most importantly guide treatment decisions and assess the virological response to antiviral therapy**.** Thus, the aim of presenting work was to investigate the relationship between the viral load and Liver biochemical/function testing according to the results of the laboratory tests.To achieve that aim, 20 chronic hepatitis C patients, enrolled from National Institute of Liver Disease Shebeen EL Koom Menofyia Governorate, Egypt. Blood samples were collected for different analysis including serological test by ELISA and molecular assays by qualitative polymerase chain reaction (PCR), also a routine lab test for measuring liver function test has been done. 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 PCR. In conclusion, there is no significant variance between liver enzymes function and viral load. Data analysis for detecting HCV was an important factor for understanding the epidemiology and treatment strategies of HCV among Egyptian patients.

Introduction:-

HCV is a small single stranded ribonucleic acid (RNA) of positive polarity and is an enveloped virus belonging to the *Hepacivirus* genus within the *Flaviviridae* family **(Preciado *et al*., 2014**). It consists of approximately 9600 nucleotides in length, which encode three structural proteins (core, E1, and E2), the ion channelproteinp7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) **(Tang *et al*., 2009)**. Because each protein is involved in HCV entry, infection, replication, or maturation, they are potential antiviral targets. HCV replication takes place entirely within the cytoplasm, therefore, it does not establish latency, making it easier to cure **(Lambers *et al*., 2011).**

Hepatitis C is a complex disease of theliver.Due to its widespread nature and global burden, this disease has always drawn attention for insight into its causative agent (HCV), and for the development of new therapeutic approaches.Even after twenty-sevenyears of its discovery, HCV continues to be a major cause of concern and a huge burden on public health systems worldwide. WorldHealth Organization (WHO) estimates that a minimum of 3% (170 million people) of the world's population is chronically infected with HCV. Estimates over the last 15 years show HCV affection increased to 2.8% with an estimated 3 to 4 million new infections globally per year, which

means > 185 million infections worldwide **(Mohd et al., 2013).**
The prevalence of HCV infection in Egypt approximately 10 fold higher than in other countries **(El-Zanaty *et al*., 2009)**, including industrialized countries (ranging from 0.5% to 2.3%), as well as in limited resource countries, even Pakistan (high prevalence rate of 6.5) **(Baatarkhuu *et al*., 2008).**

HCV-infected people serve as a reservoir for transmission to others and are at risk for developing chronic liver disease, cirrhosis, and primary hepatocellular carcinoma (HCC). It has been estimated that HCV accounts for 27% of cirrhosis and 25% of HCC worldwide **(Alter, 2007; WHO, 1999**).

HCV causes infection in two phases, first involve acute attack that lasts for a few weeks and if untreated then it may persist for along time that is termed as chronic hepatitis C. This chronic infection may often lead to chronic liver disease (CLD) that may ultimately lead to hepatic failure. Several clinical trials showed that treatment of hepatitis C infection during the acute phase is associated with higher sustained virological response (SVR) rates ranging between 75% and 100 %**(Kamal, 2008).**

Virological diagnosis and monitoring of (HCV) are based on two categories of laboratory tests: indirect tests, namely serologic assays which detecting specific antibody to HCV (anti-HCV) and direct tests, namely molecular assays which detect, quantify, or characterize components of the viral particle, such as HCV RNA testing and HCV core antigen detection testing.Direct and indirect virological tests play a key role in the diagnosis of infection, therapeutic decision-making, and assessment of the virological response to therapy**(Pawlotsky, 2002).**

Anti-HCV antibodies are usually detected using third generationenzyme-linked immunosorbent assay (ELISA)**(Gonçales and Gonçales Junior, 2007)**– which serum or plasma antibodies or viral antigens are captured on the wells of microtiter plates by using the corresponding antigen or specific (generally monoclonal) antibodies, respectively. Antigen-antibody complexes are then specifically revealed in a colorimetric enzymatic reaction. After reading in a spectrophotometer, the result is expressed as the ratio of the optical density of the test sample to that of a kit control**(Pawlotsky, 2002)**.

While direct tests that detect HCV RNA in the blood are so important in thedetermination of the viralload since the presence of viral RNA indicates that the virus is actively replicating (reproducing and infecting new cells). A viral load test is usually first done after a person has tested positive for exposure to HCV based on an antibody test. A blood sample is taken and the amount of HCV RNA in a milliliter of blood is measured.**(Franciscus and Highleyman, 2014).**

In addition to the previous categories, there are routine lab tests that must be done to monitor liver function of HCV infected patients including the following: Liver biochemical/function testing, Complete blood counts (CBC) and Chemistry panels. The aim of the presented work was an attempt to evaluate the relationship between the viral load and Liver biochemical/function tests.

Materials and Methods:-

Study Design and Patient EnrollmentPlans:-
The study was conducted atthe National Institute of Liver Disease Shebeen EL Koom Menofyia Governorate, Egypt and included 20 patients with HCV infection during the period from January 2013 to January 2014.

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.

Virological diagnosis of hepatitis C virus (HCV:-

Serologic assay:-

All patients included in our study were confirmed as anti-HCV positive by ELISA (third-generation enzyme-linked immunosorbent assays murex anti- HCV version). The wells of microplate were coated with recombinant antigens representing epitopes of HCV: core NS3, NS4 and NS5. The intensity of thecolor is proportional to the HCV antibody concentration in the sample. Serum samples were added to ELISA plate wells. Excess sample is removed by a wash step and a rabbit anti-human IgG conjugated with peroxidase was then added and incubated. The conjugate was bound to form antigen-antibody complexes. After the second wash, a solution of enzyme substrate and chromogen was added. This solution was developed to blue color if the sample was positive. The blue color changes to yellow afterblocking the reaction with sulfuric acid. The intensity of color was proportional to anti-HCV antibody concentration in the sample; wells containing negative samples remains colorless.

Molecular assays for detection and quantity of the viral HCV RNA:-

The presence of the viral genome in serum was tested by qualitative polymerase chain reaction (PCR) using the Amplicor- method. The commercial GoTaq Green master mix "Promega Co". Gotaq DNA polymerase was supplied in 2X Green Taq reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2. The Green GoTaq reaction buffer was a branded buffer containing a compound that increases sample density, and yellow and blue dyes, which function as loading dyes when reaction products are analyzed by agarose electrophoresis. The blue dye migrates at the same rate as 3-5 kb DNA fragments, and the yellow dye migrates at a rate faster than primers (<50bp), in a 1% agarose gel. All patients were infected with HCV had a minimum follow-up of 6 months from the start of antiviral therapy. Diagnostic liver enzymes, bilirubin, total leukocyte count, platelets, RBCs, and hemoglobin were available

RNA Extraction:-

Viral RNA was extracted using the viral RNA min kit according to the manufacturers' instructions by using spin column protocol. Briefly, 560 µl of prepared AVL buffer containing carrier RNA and 140 µl of serum were pipette together in 1.5 ml microcentrifuge tube and incubated at room temperature for 10 min. Five hundred and sixty µl of ethanol (97%) were added to each sample and mixed by pulse-vortexing for 15 sec. Six hundred and thirty µl of the previous solution were carefully applied to the QIAamp spin column (in a 2- ml collection tube) and centrifuged at 8000 rpm for one min. the QIAamp spin column was placed into a clean 2-ml collection tube and 500µl of AW1 buffer was added and centrifuged at 8000 rpm for 1 min. The QIAamp spin column was placed again in a clean 2-ml collection tube and 500 µl of buffer AW2 was added and centrifuged at full speed 14000 rpm for 3 min. Finally, 60 µl of AVE was added equilibrated to room temperature for one min, then centrifuged at 8000 rpm for one min. A total HCV RNA was extracted and collected in sterile vials for amplification.

Qualitative polymerase chain reaction (PCR):-

After HCVRNA was extracted, the first strand complementary DNA (cDNA) was synthesized. Initial denaturation was performed at 95°C for 5 min. Polymerase chain reaction amplification was carried out at 94°C for 1 min, 57°C (annealing temperature) for 1 min, and 72°C for 1 min for a total of 40 cycles and afinal extension at 72°C for 7 min. The primer sequences were used as follows: forward primer was 5′CGCGCGACTAGGAAGACTTC3′ and thereverse primer was 5′ACCCTCGTTTCCGTACAGAG 3′.

Biochemical analysis:-

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 thetested tube, mixed and the tube was incubated for 20 min at 37°C. After the incubation, 500 µl of sodium hydroxide was added to thetested tube. The reaction has measured theabsorbance of each at 546 nm after 5 minutes.

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 thetested tube, mixed and the tube was incubated for 20 min at 37°C. After the incubation, 500 µl of sodium hydroxide was added to thetested tube. The reaction has measured theabsorbance of each at 546 nm after 5 minutes.

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 theabsorbance of each sample at 578 nm (560- 600nm), the color intensity was stabled for 30 min.

Results:-

Serological assay:-

All patients included in our study were anti-HCV positive by third-generation enzyme-linked immunosorbent assays (ELISA) (Table 1).

Table 1:- Detection of anti-HCV for our patients by ELIZA test

No. of patients	HCV-Ab By ELISA
20	Positive

Molecular assay:-

Qualitative polymerase chain reaction (PCR) confirmed that all the patients enrolled in the study were HCV RNA positive (Table 2).

Table 2:- Detection of HCVRNA by PCR

No. of patients	HCV-RNA by PCR
20	Positive

Biochemical analysis:-

(AST/SGOT), (ALT/SGPT) & Total bilirubin:-

The biochemical analysis resultsas in table 3, presentedthat there is variance in the level of both of ALT, AST and total bilirubin for all study enrolled patients in relation to viral load as follows;

It was foundthat an ALT level was increased than normal in 13 HCV infected patients (represented 65%) also AST level was elevated in 13 HCV infected patients.While the total bilirubin level increased in 5 HCV infected patients (represented 25% of all subjects) (Table 3).

Table 3:- Relation between viral load and liver enzymes

Viral Load	ALT: U/I Refre.range up to 41	AST: U/I Refre.range up to 40	Bilirubin: mg/dl Refre.range (0.20-1.20)
2000000	25	48	0.8
400000	32	37	0.7
16500	24	17	1.2
66000	52	64	2.6
360000	42	28	1.3
150000	43	41	0.37
163043	45	25	1.2
485000	37	32	1.8
420000	49	41	2.1
551000	27	25	0.5
25700	99	68	0.9
90000	92	71	1.3
320000	28	70	0.45
256000	30	34	1.7
190000	89	35	0.5
63000	28	74	1
57000	14	10	0.7
88000	39	55	2.7
738.079	43	47	0.8
94000	68	32	0.7

Discussion:-

Hepatitis C virus (HCV) constitutes a significant health burden worldwide. Indeed, this virus possesses a high susceptibility for establishing a chronic infectionand 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, andhepatocellular carcinoma. As a consequence, HCV is the most common indication for liver transplantation in developed countries **(Thomas, 2013)**

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**(Brody et al., 2011)**. 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**(Brody et al., 2011).** 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 presentstudy, 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 thepresence of HCV-RNA which detected by PCR.

Our results showed that the amount of HCV-RNA in milliliter of blood (viral load) were accompanied by variability in the level of both of ALT, AST and total bilirubin in our study. It was reported an elevation of both ALT and AST levels during the infection with HCV in just 10 cases which represent 50% of the study enrolled patients (n20), on the other hand, ALT and AST levels remain normal on the rest of the samples although HCV-RNA was confirmed in the serum, while the total bilirubin exhibit an elevation by 30% (6 cases only). In agreement with previous **studies (Alberti et al.,1992; Healey et al.,1993; Stanley et al.,1996; Shindo et al.,1995)**, our data indicate that the presence of serum HCV RNA is almost always associated with significant liver disease which may occur regardless of viral load, ALT levels, AST level, total bilirubin and HCV genotypes. In contrast to reports**(McLindon et al.,1995; Ishak et al.,1995)**,suggesting a relationship between advancing liver damage and elevated concentrations of serum AST and ALT, we didn't see any correlation between ALT levels and the severityof liver damage. In conclusion, the main finding of our study proves that chronic hepatitis and cirrhosis may take placeregardless of clinical symptoms, ALT and AST levels, viral load, and HCV type.

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