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RESEARCH ARTICLE

Comparison study between biochemical and molecular assays in chronic hepatitis C virus patients in Egypt.

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Abstract

Biochemical parameters and Molecular assays are used in monitoring patients with chronic hepatitis C (CHC). The aim of the present study was to investigate the relationship between biochemical markers and HCV RNA titers in patients with CHC. The study was conducted on 50 known HCV-infected patients, recruitment of patients was random. All samples were collected from the medical department of Al-Azhar University, Cairo, Egypt during the period from January 2013 to June 2014. For the HCV-RNA positive patients, blood samples were collected for different biochemical analysis at the time of routine clinic attendance. All serum samples were assayed for anti-HCV by ELISA. Regarding to molecular assay HCV RNA was detected by RT-PCR. Our results indicated that ALT and AST biochemical markers were increased in CHC in relation with HCV RNA titre on the other hand WBCs and Platelets (PLT) were decreased. In Conclusion the results introduced in this work confirmed that biochemical analysis was correlated to HCV RNA titre and this indicated that Biochemical and Molecular assays are essential for monitoring patients with CHC.

Introduction:-

Hepatitis C virus (HCV), a family member of Flaviviridia, is a single-stranded 9.600 kb RNA virus (**Martro *et al.*, 2008; Pozzetto *et al.*, 2014; Webster *et al.*, 2015**). HCV RNA genome has genetic heterogeneity with its 6 major genotypes which are divided into more than 80 subtypes. HCV genotype distribution varies according to geographical location or route of transmission (**Rota *et al.*, 2013**). HCV is mainly transmitted via the parenteral route, by blood transfusion, substance abuse, and accidental needle pricks. Dental surgery, acupuncture, haemodialysis and procedures such as tattooing also pose a risk of transmission of HCV (**Chakravarti *et al.*, 2011; Lee *et al.*, 2014; Webster *et al.*, 2015**).

HCV infection is a significant public health issue. Currently, it is estimated that worldwide there are 175 million chronic hepatitis infection cases and 350.000 patients die every year due to complications of HCV such as cirrhosis and hepatic- cellular carcinoma (HCC) (**Zaltron *et al.*, 2012**). HCV infection is an insidious disease with slow progression. HCV infection can be manifested as an acute infection and in around 20% of the patients, the disease spontaneously resolves but becomes chronic in 80% of cases (**Saadeh *et al.*, 2001; Chan *et al.*, 2014; Pozzetto *et al.*, 2014**). HCV infection can lead to CHC, liver cirrhosis and hepatocellular carcinoma (HCC) (**Kuo *et al.*,2014; Shahid *et al.*, 2014**).The high rate of chronicity in HCV infections is explained by the escape of virus from immune control as a result of genetic heterogeneity due to the tendency to rapid mutation (**Farci *et al.*, 2000**). The natural history of HCV infection is affected by a number of host and virus variables (**Zechini *et al.*, 2004; Alberti *et al.*, 2005**). The duration and route of transmission of the disease, viral genotype, viral load, alcohol abuse and co-

infection with human immunodeficiency virus (HIV) and hepatitis B virus (HBV) are among the factors affecting the progression of the disease (**Chakravarti *et al.*, 2011; Chan *et al.*, 2014**). The diagnosis of HCV infection is established by detecting antibody formed against the virus (anti-HCV) and by measuring HCV RNA by nucleic acid amplification method (**Webster *et al.*, 2015**).

In HCV-positive patients, complete blood count, routine biochemical blood tests, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate (ALP) and measurement of serum HCV RNA levels are carried out (**Olga *et al.*, 2003; Ghany *et al.*, 2009**). There are several studies that have investigated the association between liver injury and serum ALT levels, HCV viral load, and HCV genotypes but the results were inconsistent (**Liu *et al.*, 2009; Al Swaff *et al.*, 2012; Shahid *et al.*, 2014**).

The main objective of this study was to evaluate and determine the potential correlation between HCV viral load and different biochemical parameters in chronic hepatitis C.

Material and methods:-

Blood Sampling:-

The study was conducted on 50 known HCV-infected patients, recruitment of patients was random. The median age of CHC patients was around 22. All samples were collected from the medical department of Al-Azhar University, Cairo, Egypt during the period from January 2013 to June 2014. For the HCV-RNA positive patients, blood samples were collected for different analysis at the time of routine clinic attendance.

Serological Assay (ELISA):-

All serum samples were assayed for anti-HCV positive by ELISA (Third-generation enzyme-linked immunosorbent assay, murex anti- HCV version), following the manufacturer's instructions. All the reagents were allowed to reach room temperature before running the assay. Liquid reagents were mixed before use. Concentrate washing solution was diluted 1/10 with distilled water. The concentrated conjugate was diluted 1/51 with the conjugate diluents. Diluted samples or controls were loaded into a 96-well plate pre-coated with a recombinant HCV-specific antigen. The plate was then incubated for one hour at 37°C to allow for the formation of the Ag-B complex. The plate was washed, the conjugate was added, and the plate was incubated for 30 minutes at 37°C. After incubation, the washing step was carried out and a substrate solution (TMB) was added for detection. Finally, the reaction was stopped using H2SO4 and the colorimetric signal was measured by absorbance at 450 nm using a spectrophotometer.

Molecular Assay:-

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.

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.

The biochemical assessment included:-
AST, ALT, and Albumin (Alb.) which were measured using Spin React Kit respectively and Bilirubin (BIL.) which was measured using Diamond kit.

Aspartate aminotransferase (AST):-
Briefly, 1ml of AST working reagent(4 vol. R1(TRIS pH 7.8 + Lactate dehydrogenase + Malate dehydrogenase + 200 L-Aspartate) + 1 vol. R2(NADH + α-Keto glutarate))were pipetted and mixed with 100 µl sample into a cuvette, then incubated for 1 min at 37°C. After that initial absorbance (A) of the sample was read, also absorbances at 1-minute intervals thereafter for 3 minutes were read at 340 nm. Finally, the difference between absorbances and the average absorbance differences per minute (A/min) were calculated.

Aspartate aminotransferase (ALT):-
Briefly 1ml of AST working reagent(one tablet of R2 (NADH+1200 U/L Lactate Dehydrogenase+α-ketoglutarate) dissolved in 15 mL of R1(TRIS pH 7.8 + L-Alanine))were pipetted and mixed with 100 µl sample into a cuvette, then incubated for 1 min at 37°C. After that initial absorbance (A) of the sample was read, also absorbances at 1-minute intervals thereafter for 3 minutes were read at 340 nm. Finally, the difference between absorbances and the average absorbance differences per minute (A/min) were calculated.

Albumin (ALB):-
One ml of R (Bromocresol green PH 4.2) was pipetted and mixed with 5 µl of sample. Then incubated for 5 min at 37ºC. The absorbance (A) of the Blank (1 ml R). The colour is stable for 1 hour at room temperature.

Total Bilirubin (BIL.):-
200 µl of **R1 (**Sulfanilic acid+HCL) were pipetted and mixed with 1 drop of **R2** (Sodium Nitrite), 1 ml of **R3** (Caffeine+Sodium benzoate) and 100 µl of the sample. **Then** incubated for 1 minute at 20-25ºC / 37ºC.**After that**1 ml of R4(Tartarate+sodium hydroxide) was added and incubated for 5 mins at 20-25ºC / 37ºC.**Final** absorbance of sample (A_{sample}) was measured against sample blank at 578 nm (560-600 nm).The colour is stable for 30 mins at room.

Blood count:-
A complete blood count of haemoglobin (HB), white blood cells (WBC), and platelets (PLT), were counted using Beckman Coulter Machine. All obtained from the same automated blood sample at the time of admission to the study.

Results:-
Virological findings:-

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

Table 1:- Serological detection of Anti-HCV for our patients

No. of patients	**ELISA Results**	
50	HCV-Ab	HBsAg
	Positive	Negative

Molecular assay:-

The presence of the viral genome in serum was detected by qualitative polymerase chain reaction (PCR). All patients included in our study were positive for HCV RNA (**Table 2**).

Table 2:- Molecular detection of HCV RNA by PCR for our patients

No. of patients	**RT-PCR results (HCV RNA)**
50	**Positive**

Biochemical assays:-

Our results showed increasing in ALT, AST, and Bilirubin and decreased in WBCS and Platelets with a relation to HCV RNA titres (Table 3).

Table 3:- Biochemical analysis for chronic HCV patients

No.	ALT	AST	BILL.T	BILL.D	S.Alb	Hb	WBC	PLT
1	11	10	0.8	0.2	3.6	13.9	4,700	205,000
2	27	29	0.7	0.2	5.1	11.2	4,700	354,000
3	42	34	0.02	0.6	4.7	10.3	4,900	339,000
4	30	24	1.1	0.8	4.9	12	2,000	150,000
5	31	30	0.7	0.4	4.6	14.4	7,000	292,000
6	40	40	1	0.2	3.9	11.8	4,500	160,000
8	33	38	1.2	0.5	4	13	5,100	190,000
9	34	44	1.1	0.7	3.8	12.5	4,300	150,000
11	30	22	1	0.6	3.7	13.7	4,400	138,000
12	32	36	0.8	0.4	4	12.8	6,300	177,000
13	40	35	1	0.3	4.7	13	4,000	165,000
14	44	23	1.1	0.8	4	12.3	4,500	170,000
15	28	25	2.1	0.6	4.5	10.6	3,800	188,000
16	44	36	1.1	0.5	3.9	13.2	4,400	159,000
17	21	25	1	0.7	4.1	11.2	3,200	243,000
18	24	33	1.4	0.7	3.1	13.6	4,600	200,000
19	43	36	1.2	0.5	3.4	12.8	5,000	196,000
20	30	28	2.2	0.7	3.7	11.8	4,600	170,000
21	41	38	1.3	0.4	4	14,0	3,700	210,000
23	35	32	1.5	0.6	4.8	13.3	4,700	187,000
24	18	25	2.1	1	3.8	14	4,600	212,000
25	60	70	1.8	0.8	4.5	11.8	2,900	120
26	90	81	0.8	0.6	4.8	13.5	6,300	241,000
27	21	25	0.6	0.2	4.4	11	3,200	243,000
28	30	24	1.1	0.4	4	15.8	5,100	202,000
36	38	39	0.9	0.7	4.9	13.2	2,300	238,000
38	47	50	0.7	0.13	4	13.6	7,000	225,00
39	30	28	1.1	0.7	4.2	15.8	9,500	323,000
40	25	28	2	0.4	4	12.5	4,300	177,000
41	28	33	1.1	0.8	4.6	12.2	4,000	290,00
42	39	33	1	0.6	4.6	11.1	3,500	162,000
43	34	30	1.1	0.7	4	15.1	3,200	190,000
44	34	39	0.71	0.25	4.1	11.3	1,900	256,000
45	29	12	1	0.8	4	11.3	2,900	166,000
46	32	29	1.1	0.4	4.5	13.5	7,900	196,000
47	32	45	0.9	0.8	4.5	12	2,200	158,000
49	22	19	1	0.7	4.7	13.4	3,900	132,00
50	28	25	2.3	1.2	3.8	12.5	4.4	178,000

Correlation between biochemical and molecular assays:-

The present study was aimed to investigate the relation between biochemical and molecular assays in CHC patients. Our results indicated that some biochemical analysis including ALT and AST were increased in relation with HCV RNA titre (Viral load) on the other hand WBCs and Platelets (PLT) were decreased. These results have implied that biochemical parameters may contribute to monitoring patients with CHC (Table 4).

Table 4:- Relation between PCR and biochemical tests in chronic HCV patients

No.	Age	weight	PCR	ALT	AST	BILL.T	BILL.D	S.Alb	Hb	WBC	PLT
1	24	90	250,000	11	10	0.8	0.2	3.6	13.9	4,700	205,000
2	20	67	171,000	27	29	0.7	0.2	5.1	11.2	4,700	354,000
3	21	62	312,000	42	34	0.02	0.6	4.7	10.3	4,900	339,000
4	23	80	107,000	30	24	1.1	0.8	4.9	12	2,000	150,000
5	21	61	105,600	31	30	0.7	0.4	4.6	14.4	7,000	292,000
6	26	75	350,000	40	40	1	0.2	3.9	11.8	4,500	160,000
8	22	100	70,000	33	38	1.2	0.5	4	13	5,100	190,000
9	23	75	16,000	34	44	1.1	0.7	3.8	12.5	4,300	150,000
11	19	60	689,000	30	22	1	0.6	3.7	13.7	4,400	138,000
12	32	92	150,000	32	36	0.8	0.4	4	12.8	6,300	177,000
13	20	64	85,566	40	35	1	0.3	4.7	13	4,000	165,000
14	20	66	130,000	44	23	1.1	0.8	4	12.3	4,500	170,000
15	25	65	66,670	28	25	2.1	0.6	4.5	10.6	3,800	188,000
16	24	70	320,000	44	36	1.1	0.5	3.9	13.2	4,400	159,000
17	23	78	660,000	21	25	1	0.7	4.1	11.2	3,200	243,000
18	22	70	370,000	24	33	1.4	0.7	3.1	13.6	4,600	200,000
19	22	68	6,261,000	43	36	1.2	0.5	3.4	12.8	5,000	196,000
20	25	65	900,405	30	28	2.2	0.7	3.7	11.8	4,600	170,000
21	26	73	850,000	41	38	1.3	0.4	4	14,0	3,700	210,000
23	23	63	1,169,000	35	32	1.5	0.6	4.8	13.3	4,700	187,000
24	22	73	2,300	18	25	2.1	1	3.8	14	4,600	212,000
25	23	64	2,21,000	60	70	1.8	0.8	4.5	11.8	2,900	120
26	24	77	1,84,000	90	81	0.8	0.6	4.8	13.5	6,300	241,000
27	23	74	340,387	21	25	0.6	0.2	4.4	11	3,200	243,000
28	24	70	5,600,000	30	24	1.1	0.4	4	15.8	5,100	202,000
36	21	70	210,000	38	39	0.9	0.7	4.9	13.2	2,300	238,000
38	22	74	842,000	47	50	0.7	0.13	4	13.6	7,000	225,00
39	22	84	4,392,966	30	28	1.1	0.7	4.2	15.8	9,500	323,000
40	21	70	2,700	25	28	2	0.4	4	12.5	4,300	177,000
41	23	61	1,500	28	33	1.1	0.8	4.6	12.2	4,000	290,00
42	22	64	34,453	39	33	1	0.6	4.6	11.1	3,500	162,000
43	23	75	7,920,00	34	30	1.1	0.7	4	15.1	3,200	190,000
44	21	65	162,000	34	39	0.71	0.25	4.1	11.3	1,900	256,000
45	24	72	220,000	29	12	1	0.8	4	11.3	2,900	166,000
46	22	68	998,399	32	29	1.1	0.4	4.5	13.5	7,900	196,000
47	24	70	45,000	32	45	0.9	0.8	4.5	12	2,200	158,000
49	21	95	2,200,000	22	19	1	0.7	4.7	13.4	3,900	132,00
50	25	80	110,000	28	25	2.3	1.2	3.8	12.5	4.4	178,000

Discussion:-

HCV currently infects nearly 2% of the world's population [**Fatma *et al.*, 2015**]. In Egypt, the situation is very critical. Hepatitis C virus constitutes an epidemic in Egypt which is having the highest prevalence in the world. Nowhere else is there an HCV epidemic that affects a whole country. In all other countries, the prevalence of HCV is between ''1 % to 2 %''(**Alter et al., 2007)**. There are a few exceptions where the prevalence of HCV is 3%. In Egypt however, the prevalence of HCV is 14.7%. Where every family in Egypt is touched by hepatitis C. The blood-borne virus, which is highly infectious, infects at least 1 in 10 of the population aged 15 to 59 [**Fatma *et al.*, 2015**]. There are several studies that have investigated the association between liver injury and serum ALT levels, HCV viral load, and HCV genotypes but the results were inconsistent (**Liu *et al.*, 2009; Al Swaff *et al.*, 2012; Shahid *et al.*, 2014**).

In recent years, various studies investigated the association between the grade of liver injury and serum ALT levels, HCV RNA titers in CHC patients and HCV genotype were performed, but the results were inconsistent.

Fanning et al have found that serum HCV RNA viral load and ALT level were significantly correlated with the grade of liver inflammation but no such correlation was found between these parameters and liver fibrosis (**Fanning *et al*., 1999**). Al Swaff have found an association between grade 1 and grade 4 liver fibrosis and higher ALT levels in patients with CHC (genotype 4) infection and have detected higher HCV RNA levels in grade 3 liver fibrosis(**Al Swaff*et al*., 2012**).

Zechini et al have found a significant correlation between HCV RNA and ALT. in CHC patients and have also found a correlation between histological activity index (HAI) and HCV RNA levels aswell as between HAI and AST and ALT levels. They have reported in their study that particularly AST might be associated with liver injury (**Zechini*etal*., 2004**). Shahid et al have found that HCV RNA titers, AST, ALPand total bilirubin were correlated with grade of fibrosis in patients with CHC (genotype 3a) infection (**Shahid *et al*., 2014**). Other studies have shown that ALT, AST, ALB and Bilirubin values are increased in CHC patients, but LYM and PLT were decreased. Also, levels of ALT and WBC have a significant correlation with HCV RNA titers in CHC patients **(Rukiye and Fikriye., 2016)**.

In our study ALT, AST and BILI values were increased with HCV RNA titres while WBCs and Platelets values were decreased with HCV RNA titres. Our results were in agreement with previous studies (**Fanning *et al*., 1999; Zechini *et al*., 2004; Shahid *et al*., 2014; Rukiye and Fikriye., 2016).** On the other hand,our results were in contrast with previous studies(**Liu *et al*., 2009; Lee *et al*., 2014**) 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 periportal necrosis. (**Lee *et al* .,2014**)In some studies, no clinically feasible association was found between ALT level and liver injury or liver fibrosis (**Liu *et al*., 2009**).

In Conclusion The present study have implied that non-invasive biochemical parameters may contribute to the monitoring of CHC disease and evaluation of its grade. However, further studies including larger patient population and measuring biochemical parameters and HCV RNA titers simultaneously with histopathological evaluation are needed.

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