Al-Kufa University Journal for Biology / VOL.8 / NO.2 / Year: 2016
Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Detection of Autoimmune Hepatitis among Chronic Cases of Hepatitis B virus in AL-Najaf province

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Abstract

This study was amid to investigate the existence of HBV and the diagnosis of autoimmune hepatitis (AIH) in addition study (C3, C4) Levels among chronic hepatitis B patients. The current study was conducted on 360 patients suspected with hepatitis B virus infection, which have age ranging (11-72) year were collected from center health laboratory, AL-Hakeem hospital, and AL-Sadder medical city in AL-Najaf city, during the period from January (2013) to August (2013). Fifteen healthy individuals without any evidence of chronic inflammatory disease involved as control, age ranging (21-50) years. The results showed that only 76 were seropositive hepatitis B in ELISA technique , the age group (44-54) year revealed high significance($p<0.05$) than other age groups. While 35 (46%) out of 76 seropositive with HBc Ab, the age group (55-65) year showed high significant ($p<0.05$) than other age groups, and male more infection than female. The result also revealed that the autoimmune hepatitis disease was 5 (6.5%) out of 76 patient infected with Type 1autoimmune hepatitis. Complement fractions C3, decreased in all patients compared to those of a healthy control, while the autoimmune hepatitis patient recorded high level was 142.2±8 mg/dI. In regard to C4 was revealed normal concentration in all patients compared with control groups while the autoimmune hepatitis patient recorded high level was 41.7±5.1 mg/dI and non-significant ($P<0.0$) in males than female in concentration of (C3 and C4).

Introduction

Hepatitis B virus is one of the most common infectious diseases globally. It is a major public health problem accounting to 400 million chronic infections worldwide. About 2 billion people (or 30% of world population) worldwide have serological evidence of current or past HBV infection, of whom about one million die annually (Lindenberg *et al*., 2013; Daw *et al*., 2014; Mehta *et al*., 2014 ; Salih, 2014).

Iraq is a developing country, where HBV virus infections are still prevalent, with an HBV carrier rate of 2%–5%. Although Iraq depended the HBV vaccination in its expanded programmer on Immunization, the coverage rate is less than 80%. Hospital-acquired HBV infections continue to occur despite increased awareness of the problem among the medical community (Al-Jadiry *et al*., 2013).

Autoimmune hepatitis (AIH) is a progressive, chronic inflammatory liver disease of unknown etiology that occurs in children and adults with a prevalence of female. This clinical syndrome is caused by an immune response that is misdirected against self or foreign antigens that resemble self-antigens, leading to a progressive inflammatory and fibrotic process of the liver (Manns and Vogel, 2006; Czaja, 2007a; Czaja 2007b;Vergani and Mieli-Vergani, 2008). The complications of AIH are the same as any other progressive liver disease. Primary hepatocellular carcinoma is a known consequence; in some patients, chronic hepatitis progresses to cirrhosis and, ultimately, to carcinoma. Liver transplantation is required when end stage liver disease develops (Krawitt, 2006; Pierpaolo *et al*., 2012).The humeral immune responses were mediated by specific antibodies that recognize and react to any challenges, as well as by complement components, so estimation of serum immunoglobulin and complement

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may provide a useful marker for disease progression and therapeutic monitoring (Ahmad *et al*., 2014).

Materials and Methods

All samples were collected from AL-Hakeem hospital (center laboratory) and AL-Sadder medical city in AL-Najaf city, during the period from January (2013) to August (2013).Fifteen apparently healthy individuals were selected as normal control groups in this study. Approximately (5 ml) of fresh blood was drawn from each patient by vein puncture, collected in tubes were without anti-coagulant as plain tubes thereafter they were centrifuged for 5 minutes at 3000 (rpm) to separate serum, The serum samples were liquated in sterile test tubes using micropipette with sterile disposable tips to use for subsequent serological tests. Each sample was labeled and given a serial number together with the patient name, the serum samples were frozen at (-20°C) (Lewis, *et al*., 2006) . Until used investigation included: Virological diagnostic and immunological investigations.

Detection of HBsAg in human serum by ELISA technique (Dialab-Austria Kit)

A- Assay Procedure

The test was performed according to restrictions manual of manufacturing company as following:

1- The washing buffer was diluted with distilled water Relation (1:20).
2-The samples and reagents were brought to room temperature.
3- Before commencing the assay.
4- For each test, one blank, two positive and three negative controls were set, then 50µl of positive and negative control serum were added into positive and negative control wells respectively.
5- A50µl of sample were added into each test well.
6-The wells covered with the seal paper and incubated for 60 minutes at 37˚C.
7- By using of ELISA washer, ELISA System EL 800, the liquid in all wells discarded and filled with 300 µl per well of the washing solution, laid aside For 30-60 seconds and discarded of the liquid in all wells which than filled with wash solution. Repeat 5 times and dry wells after last wash
8- Dispensed 50µl of substrate solution B and substrate solution A into each well respectively, mixed gently, protected from light and incubated for 15 minutes at 37°C.
9- Stopping solution in amount of 50 µl were added into each well and mixed gently to stop the reaction including blank well.

10- The Optical Density (O.D.) was read at wave length 450 nm using a Micro well system reader ELISA reader within 5 minutes after stopped the reaction

B. Interpretation of the Results:

Cut off Value (**COV**) was calculated as following:
COV = 2.1× the average OD of negative controls.
Positive sample at whichOD450 of the sample ≥ **COV.**
Negative sample at whichtheOD450 of the sample ＜**COV.**

Detection of HBc Ab in Human Serum by ELISA technique (Foresight-USA Kit).

A-Assay Procedure

1- Prepared working wash buffer by diluting the concentrated wash buffer 1:25.
2- Leaved A1 as blank well.

3- A50 µl positive controls were added to B1 and C1.
4- A50 µl negative controls were added to D1 and E.
5- Starting F1: added 50 µl specimen.
6- Added 50 µl of conjugate to each well except for the blank well.
7- Mixed gently.
8- Covered the microwell plate with plate sealer and incubated at 37 ºC for 30 minutes.
9- Removed the plate sealer.
10- Washed each well 5 times with 350 µl of working wash buffer.
11- Turned the microwell plate upside down on absorbent tissue.
12- Added 50 µl of substrate A to each well. (Clear reagent)
13- Added 50 µl of substrate B to each well. (Clear reagent)
14- Mixed gently then covered microwell pate with plate sealer and incubated in a water bath or incubator at 37 ºC ± 2 ºC for 15 minutes ± 1minute.
15- Removed the plate sealer.
16- Added 50 µl of stop solution to each well.
17- Read at 450/630-700 nm within 30 minutes.

B. Interpretation of the Results
Cut off Value (**COV**) was calculated as following:
COV = 0.2× **NCx**
NCx=Mean Absorbance of Negative Control-Blank Absorbance
Negative Control= Mean Absorbance after subtraction of Blank Absorbance should b≥ 1.000
Positive Control= Mean Absorbance after subtraction of Blank Absorbance should be< 0.080

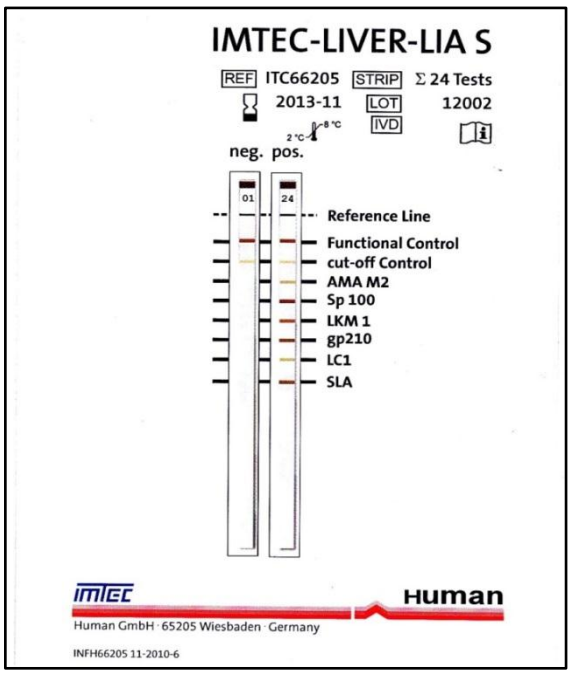
Detection of Autoimmune Liver Diseases
A-Assay Procedure:
1- The samples and reagents were brought to room temperature before commencing the assay.
2- Wash Buffer to be diluted 1part (wash 20x) with 19 parts distilled water.
3-About 30 ml of wash added to buffer (RCNS 30 ml) and mixed well. This solution was called Buffer Solution (DIL).
4-The sample diluted by added 10µl serum to 1ml DIL.
5- The strips put into the incubation tray and moist in 1ml (DIL) to wet the membrane. And then removed (DIL) after 1 min.
6- 1ml of diluted patient sample was transferred into each strip of the incubation tray, and incubated for 30 min at room temperature with gentle agitation.
7- Removed diluted sample completely.
8- Washed strip 3 times used 1.5 ml wash for 5 min.with gentle agitation.
9- Removed wash after ever washing step.
10-1ml of conjugate Solution was transferred into each strip of the incubation tray, and incubated for 30 min at room temperature with gentle agitation.
11- Removed conjugate solution.
12- Washed strip 3 times used 1.5 ml wash for 5 min.with gentle agitation, and removed wash after ever washing step.
13- 1 ml of TMB LIA solution (SUB) were added to each strip, and incubated for 10 min at room temperature with gentle agitation.
14- Removed TMB LIA solution (SUB).
15- Washed with 1.5 ml distilled water (with gentle agitation).
16- Removed distilled water after 1min.

17- Pipette 1 ml of Stop LIA Solution into all strips, and incubated developed strip for 5 min. with gentle agitation to terminate the enzymatic reaction.
18-Dried strip between filter paper.

D- Interpretation of the Results:

Fixed strip onto scored sheet and aligned the reference line of the strip with the reference line on the score sheet.
Aligned the dotted reference line of the evaluated template with the reference line of the strip.
The Interpreted of the test Results taken place exclusively on basis of the respective cut-off control regarded for each strip:
The test result was negative, if no band was recognized or if the band exhibited a smaller intensity in compared to the cut-off control.
The test was equivocal, if the intensity of the band and the intensity of the cut-off control did not significantly differ.
The test result was positive, if a band exhibit a strong stain in compared to the cut-off control

- A normal test indicated by a visible function control.
- The cut-off control was visible.
- Intensity function control was > Intensity cut-off control
- The evaluated template with the reference line of the strip was showing in
- following Figer(1)



Figer (1): The evaluated template with the reference line of the strip (Human, Germany).

Complement (C3) and (C4):

The Determination of the (C3 and C4) protein by (RID) radial immunodiffusion plate was performed according to the manufacturing company (LTA s. r. l, Italy) as follows:

A- Reagents for C3 Complement:

Plate of Agarose gel containing the goat antiserum C3**.**

B- Reagents for C4 Complement:
Plate of Agarose gel containing the goat antiserum C4**.**
C-Procedure According to manufacturer Company:
1- The plate was removed from its envelope and leaved at room temperature for few minutes, for the any condensed water in the well evaporated.
2-5µl of sample and controls was filled the wells and waited to completely adsorbed before handed the plate.
3- The plate was closed and placed it in a moist chamber. Then incubated for (72 hours).
D-Results Interpretation:
The concentration value corresponding to the precipitating ring diameter was read on enclosed reference table, for each complement (C3 and C4).
Statistical Analysis
Data were translated into a computerized database structure. An expert statistical advice was sought for. Statistical analyses were computer assisted using SPSS version 17 (Statistical Package for Social Sciences). Frequency distribution for selected variables was done first. Standard error (SE) and the parametric statistical tests of significance was applied T test. One way ANOVA test model and LSD test at level of significance $p< 0.05$ were applied to estimate the significant differences in viral loads between study groups (Al-Rawi and Khalaf Allah, 2000).

Results
Detection of HBsAg by ELISA technique
The results revealed high significance ($p < 0.05$) in the specific anti-HBsAg in all patients compared with negative control while the group with aged (44-54) year recorded the highest mean level (2.334 IU/ml) compared with other patients shown in the (Table 1).

Table (1): The Levels of HBsAg IU/ml among patients

Age group (year)	Number	anti - HBsAg positive	mean level of negative control	mean level of positive control	mean level of positive sample
11-21	9	9	0.070	2.237	2.076
22-32	19	19	0.070	2.237	2.210
33-43	19	19	0.070	2.237	2.304
44-54	14	14	0.070	2.237	2.334
55-65	12	12	0.070	2.237	2.185
> 65	3	3	0.070	2.237	2.165
Total	76	76			

LSD=0.223
Detection of chronic hepatitis patients HBc Ab

The patients with HBsAg showed percent of infectivity with HBc Ab included 35(46 %) patients With age ranging (11-72) years old , all patients with HBc Ab revealed high mean level ($p<0.05$) compared with negative control while patients with aged (55-65) recorded high mean level (0.206) compared with other patients as in (Table 6). In the samples taken from all patients with HBc Ab infection, it had been shown that consisted of 25(32.8%) males and 10(13.1%) female Table (2).

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Table (2): The level of anti-HBc Ab IU/ml in patients by ELISA technique

Age groups (year)	Number	anti-HBsAg positive	HBc Ab positive	mean of the titer of negative control	mean of the titer of positive control	mean of the titer of positive sample
11-21	9	9	4	0.575	0.016	0.168
22-32	19	19	6	0.575	0.016	0.067
33-43	19	19	7	0.575	0.016	0.077
44-54	14	14	8	0.575	0.016	0.099
55-65	12	12	8	0.575	0.016	0.206
>65	3	3	2	0.575	0.016	0.196
total	76	76	35			

LSD=0.092

Detection of Autoimmune hepatitis

The percentage of Type 1 autoimmune hepatitis diseases was 5(6.57%) out of 76 patients consisted of three male and two female with age ranged 35-72 years old. As in (Table 3) and Figure (2)

Table (3): characteristics of autoimmune hepatitis patients

Age of patients year	males	females	Anti HBsAg+	Anti HBc Ab+	autoimmune hepatitis	Type of autoimmune hepatitis
35	-	1	+	+	+	Sp100
50	1	-	+	+	+	Sp100
60	1	-	+	+	+	Sp100 and Am Am2
65	1	-	+	+	+	Sp100
72	-	1	+	+	+	Sp100

Al-Kufa University Journal for Biology / VOL.8 / NO.2 / Year: 2016
Print ISSN: 2073-8854 & Online ISSN: 2311-6544

-A- **-B-**

Figure (2): Immune Assay (LIA) for the Detection of Autoimmune hepatitis.
A- Positive Autoimmune hepatitis type1 (SP100). B- Negative

Complement detection (C_3 & C_4)

The results demonstrated low significance in (C3) level in all patients groups compared with their control (140.5 ±13.9 mg/dI).While significance increase in autoimmune hepatitis to (142.2±8.5) mg/dI .The results showed normal concentration of C4 level in patients with HBsAg and HBc Ab compared with control group while the level of C4 in Autoimmune revealed high significant were (41.7±5.1) compared with control group (30.5 ±2.4) as in(Table4).

Table (4): The Level of C3 & C4 mg/dI among patient groups in comparison to healthy control.

Complement	control M ± SE	HBsAg M ± SE	HBc Ab M ± SE	Autoimmune M ± SE	LSD
C3 mg/dI	141.5 ±13.9	140.0 ±5.4	136.8 ±5.7	142.2 ±8.5	LSD=1.6
C4 mg/dI	30.5 ±2.4	31.8 ±1.9	29.3 ±2.6	41.7 ±5.1	LSD=3.3

Complement detection with sex

The concentration of C3 was significant decrease at ($p < 0.05$) in both sexes compared with control group figure (3). The concentration of C4 showed no-significance difference between female and male, while male increased significant compared with their control figure (4).

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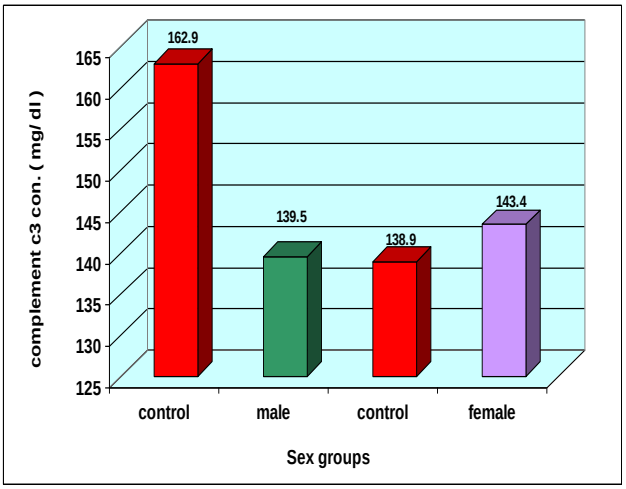

Figure (3): Level of C_3 in all patients groups according to sex compared with healthy control group.

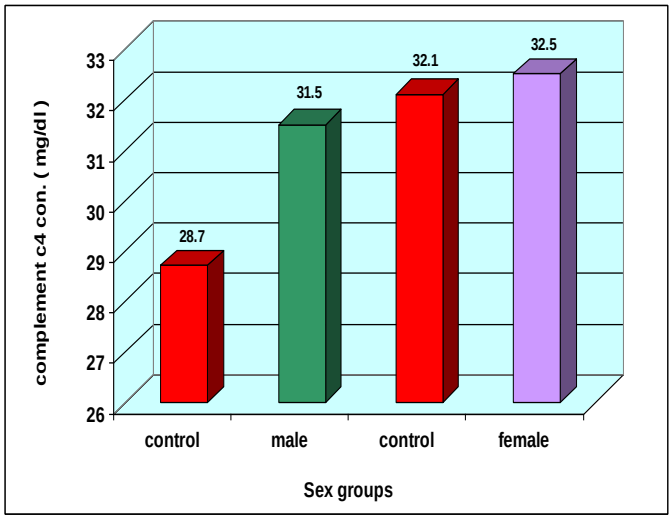

Figure (4): Level of C_4 in all patients groups according to sex compared with healthy control group

Discussion

The age distribution

The present study according to age distribution was adopted the anti-HBsAg is global marker of infection with HBV revealed that significant increase at ($p < 0.05$) in the specific anti-HBsAg in all patients while the age groups (44-54) year old were the highest significance in mean level(2.334 IU/ml) compared with other patients (Table 1) .These data were closer to study by Daw *et al*., (2000) in Libya that appeared the largest group between 30 to 45 years, and the results of present study is similar to results Khan *et a*l.,(2011) in Pakistan that showed the highest frequency of infection was found in the age of 21-30 was 34.93% followed by 23.83% in 31-40 only 13.39% were belonging to the age group 11-20 years and agree with recent study in Iraq that revealed the higher prevalence of hepatitis B among young aging groups (31-40) and (41-50) years compared to older age groups Abdul-Husin, (2013).

In table (2) shows the distribution of chronic HBV patients according to age .It has been found that age (55-65) year recorded high mean titer (0.206) compared with other patients .The results of the present study were similar to those done in California by Brown *et al*., (2013) who found that the chronic HBV infection were among persons aged 25-54 years, less

than 18 years age had the lowest rate of chronic HBV infection. The results of the present work agrees with other study Machado *etal*., (2013) that revealed the prevalence of positive anti-HBC was 23% among the elderly which is significantly higher than that found for younger individuals.

The Sex influence

The results showed that the prevalence of acute HBV infection in males 55(72%) higher than females, There is a high similarity between this results and previous work in Iraq by AL-Khozai, (2006); AL-Saidi, (2012) that the males are more affected with HBV than females, also several studies in the world agreed with this study results as Baig, (2009) who reported the frequency of hepatic infection in males was 79.5%and in females 20.5%.

These results are comparable with other results reported a high prevalence of HBV infection. Moosa *et al*, (2009); Awan *et al*, (2010) reported a high (59.1%, 58.3%) prevalence in males than females (40.9%, 41.7%) respectively. These results accordance with a Pakistan study in which male were found to be more frequently infected with HBV as compared to female with a positivity ratio of 2.14:1.0 respectively. The rate of infection in both male and female tends to increase with the passage of time. The highest rates of infection 44.54% were 0 followed by 30.13% 2009 and 25.32% in 2008 respectively (Khan *etal*, 2011). Also this fact is well documented by (Blumberg, 2006) that higher anti-HBsAg seroprevalence has been reported in male than in female for populations in some Asia country.

The study under discussion agrees with other study that has revealed the chronic HBV infection was 2.9 times more frequent in males than in females Kursad *et al*., (2005); Alexander and Kourtis, (2007) found that men predominate women in all populations of HBsAg carriers. The gender is well established but poorly understood, determinant of chronicity; however women are more likely than men to clear HBsAg (David and Daniel, 2003). Higher HBV Infection in males as compared to female may be due their being employed outsides their homes, visiting barber shops and also their involvement in blood transfusion practices. While women are mostly involved in house hold activates based on the social, cultural and religious preferences and influence. Qureshi *et al.*, (2009); Khan *et al*., (2011).

Detection of Autoimmune hepatitis

The current study Showed that the proportion of Type 1 Autoimmune hepatitis diseases 5(6.57%) out of 76 patients, consisted of three male and two female with age ranged 35-72 years old. as in (Table 3) The results of the presented study in agreement with Manns *et al*., (2010**)** who founds Type 1 can occur at any age; however, it most often starts in adolescence or young adulthood .About 70 percent of people with Type 1 autoimmune hepatitis are female .Type 2 autoimmune hepatitis is less common and occurs more often in children than adults. Krawitt, 2006; Manns and Vogel, 2006**)** demonstrated that the Autoimmune hepatitis (AIH) is a progressive, chronic inflammatory liver disease of unknown etiology that occurs in children and adults with a prevalence of female. This clinical syndrome is caused by an immune response that is misdirected against self or foreign antigens that resemble self-antigens, leading to a progressive inflammatory and fibrotic process of the liver the complications of AIH are the same as any other progressive liver disease. Primary hepatocellular carcinoma is a known consequence; in some patients, chronic hepatitis progresses to cirrhosis and, ultimately, to carcinoma. Liver transplantation is required when end stage liver disease develops.

Distribution of autoimmune disease among hepatitis B and coinfection hepatitis B with cytomegalovirus HCMV Can be explained by five main mechanisms through which an

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infector can contribute to the pathogenesis of an autoimmune disease (Wucherpfennig, 2001; Barzilai *et al.*, 2007) first is molecular mimicry, where the infecting agent may incorporate an epitope that is structurally similar to a self-antigen. This structural resemblance confuses the immune system and induces the autoimmune response. Another mechanism is a phenomenon known as epitope spreading, where an exaggerated local activation of antigen presenting Cells due to an inflammatory state may cause over processing and over presentation of antigens. In chronic autoimmune disease, this exaggerated Activation may cause the priming of large numbers of T cells with broad specificities, thus encouraging the development of the autoimmune disease. Viral and bacterial super antigens possess the ability to bind to the variable Domain of the T cell receptor beta chain along with the ability to bind to a wide variety of MHC class 2 molecules. These capabilities allow them to bind to awide variety of T cells, irrespective of their specificity and to therefore induce an autoimmune reaction. Bystander activation is a mechanism describing a situation where enhanced cytokine production induces the expansion of autoreactive T cells whose prior number was insufficient to produce an overt disease. The last mechanism is known as polyclonal activation or activation of lymphocytes by lymphotrophic viruses. In this case, an infection of B cells results in B cell proliferation, enhanced antibody production, and the generation of circulating immune complexes that may cause damage to self-tissues.(Ferri and Zignego,2000 ;Varani and landini,2011).

In present study it has been found that autoimmune hepatitis was 3(60%) in male and 2(40%) in female, while studies from some researchers show that autoimmune hepatitis in female to male ratio ranges from 3:1,in type 1, to 9:1, in type 2 AIH. Sex differences in the immune response are also observed in other liver diseases, men are more likely to become chronic carriers of hepatitis B than women Alvarez *et al*., (2001). Another study conducted by Oo *et al*., (2010); Toyoda-Akui *et al*, (2011) stated that the rate of autoimmune hepatitis was more common in females (70%) and it affects all age.

Several studies have investigated the effect of donor: recipient sex matching in the outcome of orthotropic liver transplantation (OLT) and have found that male to female grafts have the most, and female to male the least favorable. This gender discrepancy could be the result of existing differences in basic immune responses between females and males, higher levels of antibodies and stronger T cell activation are observed in women after vaccination Women have higher absolute numbers of CD4+ T-cells and produce higher levels of Th1 cytokines than men. Interestingly, *in vitro* Oestrogen increases Th1 cytokine production by Tlymphocytes, while a decrease is observed in presence of androgen. (Béland *et al*., 2009).

Complement levels (C_3 and C_4)

In the present study, C3 level showed a significant decrease in all patients .the results recorded no significant difference ($p< 0.05$) between male and female in serum level (C3) in all patients Figure (3) While the results of C4 level was normal in all patients as compared to healthy controls group as in Table (4). The results also recorded no significant difference between male and female in serum level (C4) in all patients figure (4). These results in agreement with Castro and Gourley, (2010) in America who founds the Serum levels of complement components can serve as markers of disease activity. In immune complex deposition disease, serum complement proteins are consumed and serum levels decrease. Immune complex disease results from the deposition of antigen-antibody complexes in involved organ tissues. In immune complex glomerulonephritis in SLE, decreased C3 and C4 indicate increased consumption and indicate disease activity. In contrast, increases of C3and

C4 indicate inflammatory disorders as these proteins are also acute phase reactants**,** and demonstrated Comprehensive laboratory evaluation of a suspected autoimmune illness in conjunction with a thorough clinical evaluation provides a better understanding of a patient's immunologic disease. The results of the current study also coincided with other studies in Iran showed serum complement levels in 53 patients with various liver diseases. The total serum complement level, C4 and C3 was raised in 28, normal in 12 and low in 13 patients (Fatemeh *et al*., 2010)

These results are in agreement with what were found by Porta, (2000) who found Complement concentrations, particularly C4, may be low, with levels Much lower in type 2 when compared to type 1 autoimmune hepatitis; C3 may be low in both types.

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