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Assessment of Viral load for Human Cytomegalovirus in Patients with Hepatitis B Virus

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Abstract

This study was carried out on Seventy-Six samples, formed (55 males and 21 females) with 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. All patients were divided in six age groups. Blood samples were collected from patients and control for immunological (IgM, IgG & TFN-α) by using ELISA and molecular study by RT-PCR, respectively. The results showed that 76 HBsAg seropositive in all age groups but the age group (44-54) year revealed high significant($p<0.05$) than other age groups and male more infection than female. The ELISA test results showed that 68 (89.4%) out of the 76 samples were positive for anti-HCMV IgG antibodies, and 4(5.26%) samples were positive for anti-HCMV IgM. The results of the Real-Time PCR revealed that HCMV DNA were detected in 23(30.2%) out of 76 patients were found in all age groups with viral loads ranging from (0.24-1730000) Copies/ml, and the results of controls group in Real-Time PCR were HCMV negative. The results of cytokines profile (TNF-α) showed a highly significant ($P<0.05$) elevation in the serum of all patients than control groups.

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).

In Iraq, the CMV Seroepidemiology ranged from 8.02 %(un acceptable low rate) to 90.4%, a wide range of seroprevalence (Hassan *et al*., 2014).the fact that CMV can infect a wide range of tissues and organ systems. Therefore, the rapid and qualitative detection of CMV from clinical samples is a significant clinical importance (Binnicker and Epsy, 2013). A range of HCMV features like its lytic replication in multiple tissues, the lifelong persistence through periods of latency and intermitting reactivation, the extraordinary large proteome, and extensive manipulation of adaptive and innate immunity make HCMV a high profile candidate for involvement in autoimmune disease. (Hsu, 2014).

The development of RT- PCR technology has simplified nucleic acid quantification and is coming into more widespread use. Real-time, quantitative PCR has several advantages over older forms of quantitative PCR assays. Experience to date has reported comparable sensitivity but superior reproducibility and precision compared to previous methods, with a wide dynamic range (Ali, 2012).This study, designed to evaluate and compare the performance of real-time PCR assays for the

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qualitative detection of CMV from hepatitis patients and detection of TNF-α level among CMV patients.

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). Total of seventy six patients (55 males and 21 females) none of the studied patients had history of alcohol or had received antiviral treatment for at least 1 year ago, and Fifteen apparently healthy individuals were selected as normal control groups in this study which consists of (7 males) and (8 females) in age ranging(21-50) years. 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. Each sample was labeled and given a serial number together with the patient name, the serum samples were frozen at (-20°C) until used (Lewis, *et al*., 2006).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 was 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 of 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.

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Detection HCMV antibodies by ELISA technique:

All samples were tested for HCMV antibodies using Enzyme Linked ImmunoSorbent Assay (ELISA) techniques. Anti-virus IgM and IgG antibodies Human Serum estimated by ELISA. According to (Goura Kudesia and Wright, 2009).

Detection of IgM Antibodies to Cytomegalo Virus in human serum by ELISA kites (Human, Germany).

A- **Assay Procedure:**

1-The washing solution was diluted with deionised water Relation (1+20).
2-The samples and reagents were brought to room temperature.
3-Samples to be diluted with dilution buffer IgM (DIL-M) relation (1+100) e.g. 10µl serum +1ml (DIL-M)and mixed thoroughly.
4- For each test, one blank, two negative and two positive controls were set in the microtiter plate.
5-Dispense 100 µL of each Standard and diluted sample with new disposable tips into the respective wells of the Microtiter Plate.
6-. Covered plate with adhesive strip. Incubate for 30 min at 17-25°C.
7- Then the adhesive strip was removed and discards then the contents of the wells were aspirated completely filled with 350 µl 0f diluted washing solution. The process repeated of aspiration and washing 4 x, each column of wells soak for at least 15 seconds before the next aspiration cycle. After the last washing blot the microplate on paper towel and removed any excess liquid from the wells.
8-Hundred microliters of conjugate was transferred into each well of the microplate, except the blank well.
9-The plate covered with new adhesive strip and incubated for 30 minutes at 17-25°C.
10- Adhesive strip was removed. Discard incubation solution. Wash plate 5 x with 350 µL of diluted Wash solution. Removed excess solution by tapping the inverted plate on a paper towel.
11-Added 100 µL of TMB Substrate Solution into each well.
12-**.**Incubate 15 min at 17-25°C in the dark (without adhesive strip).
13-Stopped the reaction by added 100 µL of Stop Solution (Sulphuric acid) into each well. Briefly mix contents by gently shaking the plate.
14- Measured the absorbance at 450 nm of each well, within 30 minutes.
15-Calculated the cut-off value according to: COV= MNC + (0.2 × MPC

Detection of IgG Antibodies to Cytomegalo Virus in human serum by ELISA kit (Human, Germany).

A-Assay Procedure:

1- The washing solution was diluted with deionised water Relation (1+20).
2-The samples and reagents were brought to room temperature (15-25 C°).
3-Samples to be diluted with dilution buffer IgG (DIL-G) relation (1+100) e.g. 10 µl serum + 1ml (DIL-G) and mixed thoroughly.
4-For each test, one blank, two negative and two positive controls were set in the microtiter plate.
5-Transefered 100 µL of each Standard and diluted sample with new disposable tips into the respective wells of the Microtiter Plate. And covered plate with adhesive strip. Incubated for 30 min at 17-25°C.
6-Then the adhesive strip was removed and discards then the contents of the wells were aspirated completely filled with 350 µL 0f diluted washing solution. The process repeated of aspiration and washing 4 x.

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7- One-hundred microliters of conjugate was transferred into each well of the microplate, except the blank well. And covered with new adhesive strip and incubated for 30 minutes at 17-25°C.
8-Adhesive strip was removed. Discard incubation solution. Washed plate 5 x with 350 µL of diluted Wash solution. And removed excess solution by tapping the inverted plate on a paper towel.
9-A100 µL of TMB Substrate Solution were added into each well and **i**ncubated 15 min at 17-25°C in the dark (without adhesive strip).
10-Stopped the reaction by added A hundred microliters was transferred one of stopping solution and mixed contents by gently shaking the plate.

11-Measured the absorbance at 450 nm of each well, within 30 minutes and
Calculated the cut-off value according to:

COV= MNC + (0.1 × MPC).

Molecular Tests:

DNA extraction of HCMV:

A total of 76 HBV positive serums of samples were used for the relative detection of HCMV genotyping in this patient. The HCMV DNA was extracted from 200 µl serum samples using Viral Nucleic Acid Extraction Kit II (Geneaid,U.S.A), then, resulting DNA solution was subjected to Real Time polymerase chain reaction (RT-PCR).

A-Content of the kit:

Table (1): Viral DNA extraction kit reagents and contents

Components	Quantity
VB Lysis Buffer	60 ml
AD Buffer[1] (Add Ethanol)	8 ml (6oml)
W1 Buffer	50 ml
Wash Buffer[2] (Add Ethanol)	25 ml (100 ml)
RNase-free Water	6 ml
VB Column	100 pcs
2ml Collection Tube	200 pcs

B-Materials Preparation:

- About 60 ml of absolute ethanol was added to 8 ml of AD buffer and shake well prior to initial used.
- A100 ml of absolute ethanol was added to 25 ml of wash buffer.
- Prepared Additional requirement: microcentrifuge tubes (RNase free), phosphate-Buffered Saline (PBS).

A- Procedure:

1- Labeled the microcentrifuge tubes according to the numbering of samples.
2- Transferred a 200 µl from each sample (serum) into a 1.5 propermicrocentrifuge tubes.
3- Four-hundred µl of VB Lysis Buffer was added to each sample and mixed by vortex, then incubated for 10 minutes at room temperature.
4- About 450 µl of AD buffer (prepared) was added to the sample lysate and shake vigorously.

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5-VB Columns placed in a 2 ml Collection tubes with labeled.
6-Transferred 600µl of the lysate mixture to the VB Column.
7-All tubes were centrifuged at 14-16,000 x g for 1 minute. Carefully discard supernatant from each tube without disturbing the pellet and placed the VB columns back in the 2 ml Collection tubes.
8-The remaining lysate mixture was Transferred to the VB column and centrifuged at14-16,000 x g for 1 minute. Remove and discard supernatant from each tube.
9-The VB column transferred to a new 2 ml collection tubes.
10-Added 400µl of W1 Buffer to the VB column and centrifuged at14-16,000 x g for 30 second, discard supernatant from each tube, and placed the VB columns back in the 2 ml collection tubes.
11-Six-hundred µl of Washing Buffer was added to each VB column. centrifuged centrifuge for 30 sec at14-16,000 x g. for 30 sec at14-16,000 x g. Remove and discard supernatant from each tube and placed the VB Columns back in the 2 ml Collection tubes.
12- All tubes were centrifuged for 3 minutesat14-16,000 x g to dried the column matrix.
13- The dried VB column placed in a clean 1.5 proper microcentrifuge tubes.
14- Added 50 µl of RNase-free Water to the center of the VB column matrix.
15- The VB Column matrix let for 3 minutes to ensure the water was absorbed by the matrix.
16- The end step Centrifuged for1 minuteat14-16,000 x g to eluted the purified nucleic acid.

Quantitative Real Time Polymerase Chain Reaction (RT-qPCR) (BIONEER, Korea).

In order to detect the exact quantities of viral blood levels of CMV, the absolute standard curve method for viral quantification have been applied which considered as the most accurate method for viral quantification by determination the input copy number of transcript of interest by relating the PCR signal to a standard curve, The method characterized by high dynamic range of detection. There for this Viral load assay had shown that the amount of CMV in blood could be used to identify patients at risk of developing viral disease in those patients which infected with HBV and to monitor antiviral therapy.

A-Content of the kit:

Table (2): Quantitative Real-Time PCR kit reagents and contents.

NO.	Labeling and Contents	Volume
1	CMV Quantitative PCR Premix	8-well strip x 12ea(96 tests)
2	CMV Standard DNA	15 µl/ tub x 8 strips (Natural)
3	Internal Positive Control DNA	15 µl/ tub x 2strips **(**Yellow)
4	DEPC-DW (NTC)	15 µl/ tub x 2strips **(**Purple)
5	DEPC-DW	1800µl/ tub x4 ea.

B-Procedure:

The steps have been achieved inside biological cabinet according to manufacturer's instructions.

1-The tubes of the purified nucleic acid. Thawed and centrifuged at 50 x g for 20 second then all reaction tube was prepared for used in the follows steps.

2-Sterile 0.2 PCR tubes marked and put on ice.

3-From tube contents DEPC-DW, 44µl were transferred and added to each PCR tube.

4-One µl of tubes contentsInternal Positive Control DNA was added to each PCR tube with avoiding of touching the wall of the microtubes.

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5- Five µl of CMV Standard DNA were added to 5 PCR tube, the following putative concentrations were ensue in comparison to the standard concentration (10^6copies /ml) (2×10^2~2×10^6) respectively and the total volume in this tubs 50 µl.

6-Five µl of DNA clinical sample were added to 76 Perturbed, total volume in this tubs 50 µl.

7-Five µl of DEPC-DW (NTC) were added to one PCR tube, total volume 50.

8- All PCR tubes vortex thoroughly and centrifuges briefly and then inserted in The Real Time PCR Thermo cycler: Exicycler™ Quantitative Thermal Block, Version 3.0. And ran as following programme: Table (3)

Table (3): Real Time- PCR Thermocycler conditions.

STEP	Temperature	Running Time
Line 1:Pre-Denaturation	95C°	10 minutes
Line 2:Denaturation	95 C°	20 seconds
Line3: Annealing&Extension	55 C°	30 seconds
Scan	FAM:Target /TAMRA:Internal Positive Control	
Go to	Line 2, 45 cycles	

9- **Calculating of Results (viral Quantification):**

The HCMV copies per milliliter of blood (viral concentrations) were calculated by extrapolating the quantities from the standard curve. After obtaining the Ct values of the samples, the viral concentration in the samples were determined by comparing the Ct values of the samples to the standard curve. Samples with HCMV DNA levels falling above the upper limit of quantification of the assay (10^6) are underestimated and achieve accurate quantification.

Estimation of Tumor Necrosis Factor-Alpha (TNF-α) (Komabiotech, Korea)

1-The components of the kite were equilibrated at room temperature before use.

2-The standards and samples Diluted in Assay Diluent at 1:2 serial dilutions as follows: Table (4):

Table (4): TNF-α standards concentration

Step	Dilution Method	Standard conc.
Step A	15µl of standard+ 0.485 ml o Assay Diluent	1500 pg./ml
Step B	0.25 ml of stepA+0.25 ml of Assay Diluent	750 pg./ml
Step C	0.25 ml of step B +0.25 ml of Assay Diluent	375 pg./ml
Step D	0.25 ml of step C +0.25 ml of Assay Diluent	187.5pg/ml
Step E	0.25 ml of step D +0.25 ml of Assay Diluent	93.75 pg./ml
Step F	0.25 ml of step E +0.25 ml of Assay Diluent	46.87 pg./ml
Step G	0.25 ml of step F +0.25 ml of Assay Diluent	23.43 pg./ml

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3- **Procedure According to manufacturer Company:**

A- Two-hundred µl of washing solution was added to each well. Washed the plate 3 times using 300 µl of washing solution per well. Then inverted plate to removed excess solution and blotted on paper towel.

B- One-hundred µl of standard and sample was added to each well. And covered with the plate Sealer and then incubated for two hours at room temperature.

C- The plate was washed four times with washing Solution (300µl /well).

D- A volume of 100µl/well of diluted detection antibody was added. And covered with new plate Sealer and then incubated for two hours at room temperature.

E- The microplate was aspirated and washed four times as described above.

F- One-hundred µl/well of diluted color development (Streptavidin-HRP conjugate) were added and covered with the sealing tap**.** Then incubated for 30 minutes at room temperature**.**

G- The plate was washed four times with washing solution (300µl/well).

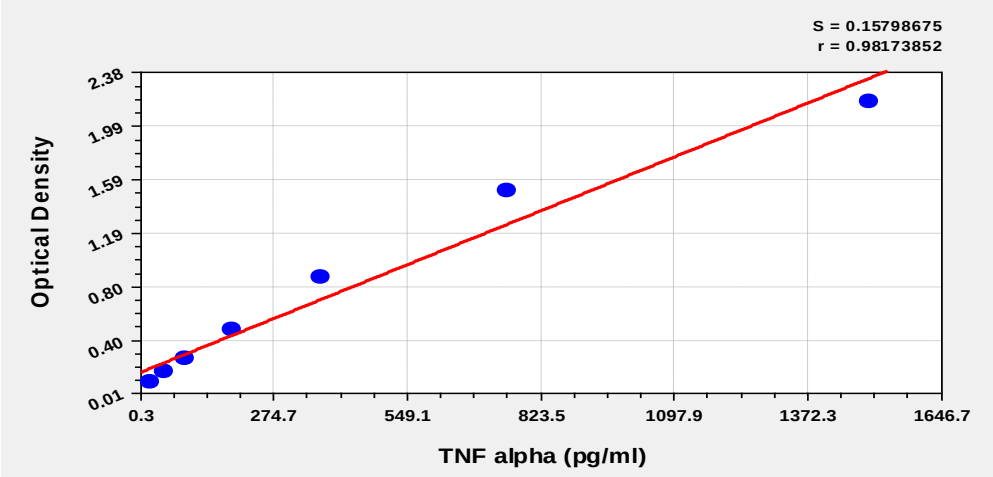
H- One-hundred µl/well of Color Development Solution were added and the plate was incubated for (9-19minutes) at room temperature.

I- One-hundred µl of Stop Solution were added to each well to stop the color reaction.

J- The absorbance on bioelisa readerEL_x800 was read at a wavelength of 450 nm immediately .Results was provided within 1 minute on the LCD display.

4- Calculation of results:

Results were calculated by using standard concentration curve. The standard curve measures the relationship between optical density (O.D) and standard (pg/ml) and the results were interpolated by using Graph pad prism. Figer (1)



Figer (1): standard curve of INF- α.

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).

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Results

Detection of HBsAg by ELISA technique

The results revealed significance increase ($p < 0.05$) in the specific anti-HBsAg in all patients compared to 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 5).

Table (5): Tests result of anti HBsAg in patients estimated by ELISA test.

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 HCMV infection sample among hepatitis B patients

After conducting several serological tests which confirmed by molecular test were detected 23(30.2%) cases of HCMV infection among the hepatitis B patients consisted of 17 males and 6 females, age ranged 11-72 years old.

Seropositivity of anti-HCMV IgG and IgM antibodies among hepatitis B patients by ELISA Technique

The ELISA Technique was aimed to detect Anti-HCMV IgM and IgG antibodies among hepatitis B patients the result was 68 (89.4%) out of the 76 samples were positive for anti-HCMV IgG antibodies. Whereas 4 samples out of 76 samples (5.26%) were positive for anti-HCMV IgM antibodies **(**Figure 2).

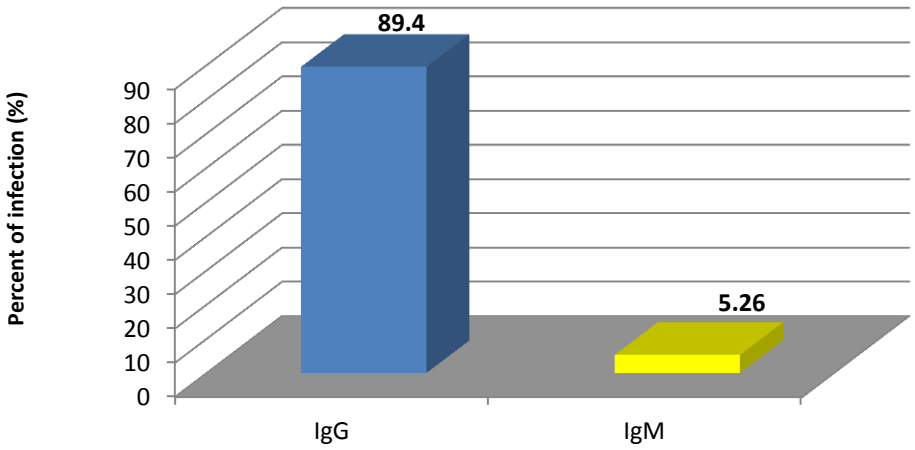

Figure (2): Seropositivity of anti-HCMV IgG and IgM among hepatitis B patients by ELISA Technique

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Distribution of anti-HCMV IgG among hepatitis B patients

From figure(3),it was shown that the highest percentage of anti-HCMV IgG antibodies positivity was detected in the age group (22-32) that represents 17(25 %) of the total anti-HCMV IgG antibodies positive samples for HCMV among hepatitis B patients and the lowest one was the age group (65-72) years which represents 3(3.9%).

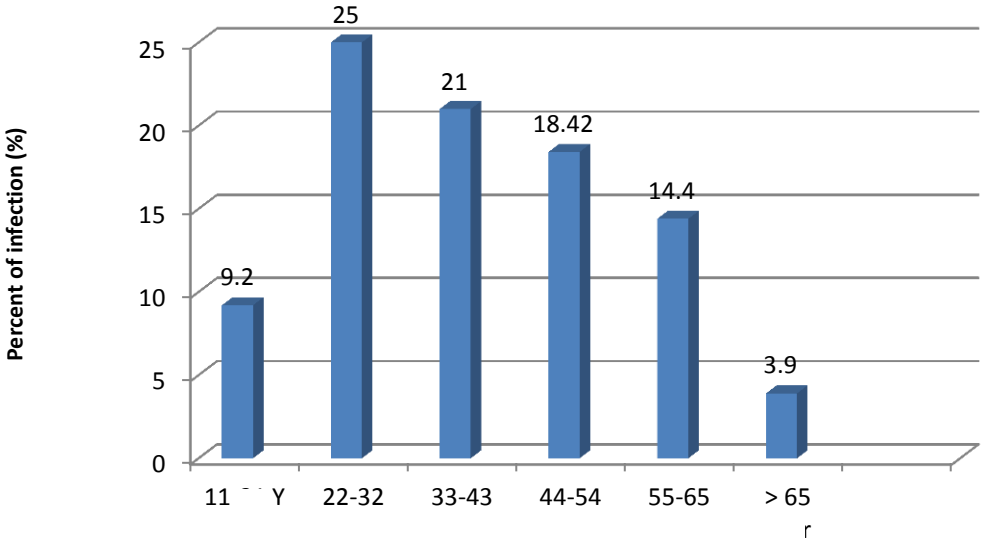

Figure (3): Distribution of anti-HCMV IgG among hepatitis B patients in Regards to their age groups

Regarding to the distribution of the sex of patients, the rate of anti-HCMV IgG antibodies Seropositivity among males 49(72%) out of 68.While the rate of anti-HCMV IgG seropositive among Female was 19(27.9%).

Distribution of anti-HCMV IgM among hepatitis B patients

For anti-HCMV IgM antibodies, it was shown from Figure (4) that the highest age group in regards to anti-HCMV IgM antibodies positive was the age group (33-43)year and it represent 2(2.63%) from the total anti-HCMV IgM antibodies cases while the age groups (22-32) and (55-65)year recorded equal percentage which was (1.31%) **.** In regarded to the sex the percentage of anti-HCMV-IgM antibodies Seropositivity among males 2 (2.63%) it is equal to the proportion of women Figure (4).

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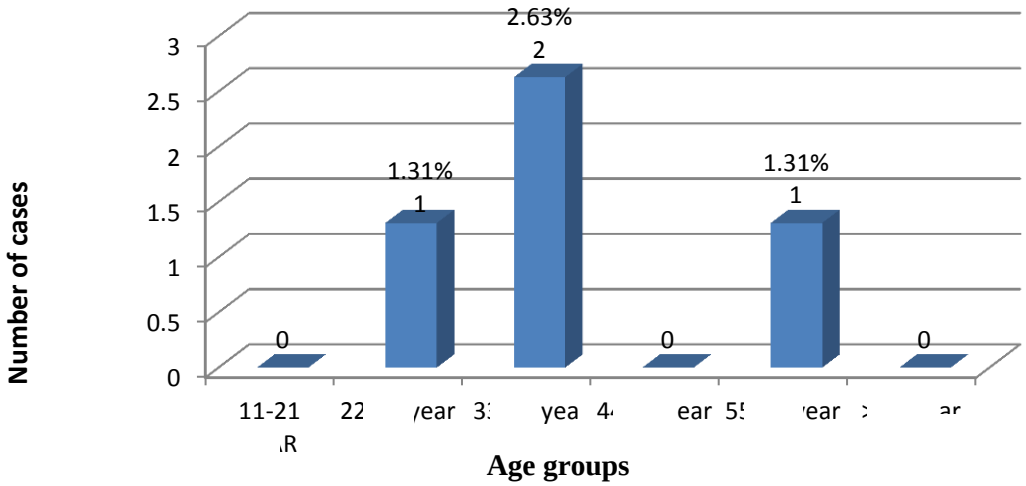

Figure (4): Distribution of anti-HCMV IgM among hepatitis B patients in Regards to their age groups

Seropositivity of anti-HCMV IgG and IgM antibodies among hepatitis B patients in regards to their age groups

The ELISA result showed that the mean titer of IgG antibodies were (2.61±0.28) IU/ml at age group (>65) increased significantly at (P value <0.05) compared with other groups while the IgM value were (0.123±0.01) IU/ml at the age groups (44-54) increased significantly at (P < 0.05) compared with other groups as in table (6).

Table (6): Anti-CMV IgG & IgM IU/ml in hepatitis B patients according to age groups.

Parameters	Control (n=15)	11-21 (n= 9)	22-32 (n=19)	33-43 (n= 19)	44-54 (n= 14)	55-65 (n=12)	>65 (n=3)	LSD
HCMV IgG IU/ml	0.976 ± 0.22	1.659 ±0.3	1.949 ±0.21	2.193 ±0.26	2.003 ±0.31	2.41 ±0.31	2.61 ±0.28	LSD=0.7
HCMV IgM IU/ml	0.206 ±0.02	0.214 ±0.03	0.166 ±0.02	0.263 ± 0.08	0.123 ±0.01	0.187 ±0.0 3	0.152 ±0.04	LSD=0.1 4

Molecular study

Detection of viral HCMV by RT-PCR techniques

The results of RT-PCR amplification revealed that the presence of HCMV DNA in 23 (30.2%) out of the 76 patients suffering hepatitis B with viral loads ranging from (0.24- 1730000) Copies/ml, including all age groups. The result showed that the age group (44-54) years increased significant at p< 0.001 with mean viral load (441502.6) Copies/ml compared to other groups as in table (7).

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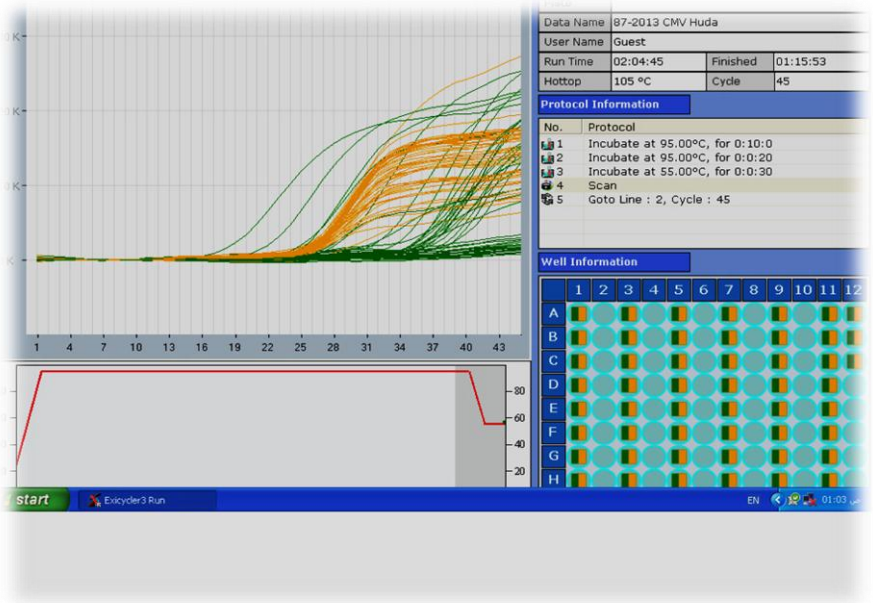
Table (7): HCMV DNA detection from hepatitis B patients by RT-PCR amplification in different age groups.

Age group (year)	Number test	Anti HBsAg Positive	HCMV DNA Positive	mean of Viral load Copy/ml
11-21	9	9	5	33203.6
22-32	19	19	4	2528.3
33-43	19	19	4	1978.9
44-54	14	14	6	441502.6*
55-65	12	12	2	1000.3
>65	3	3	2	400.1
Total		76 (100%)	23 (30.3%)	

LSD=1323 *P< 0.001



(A)

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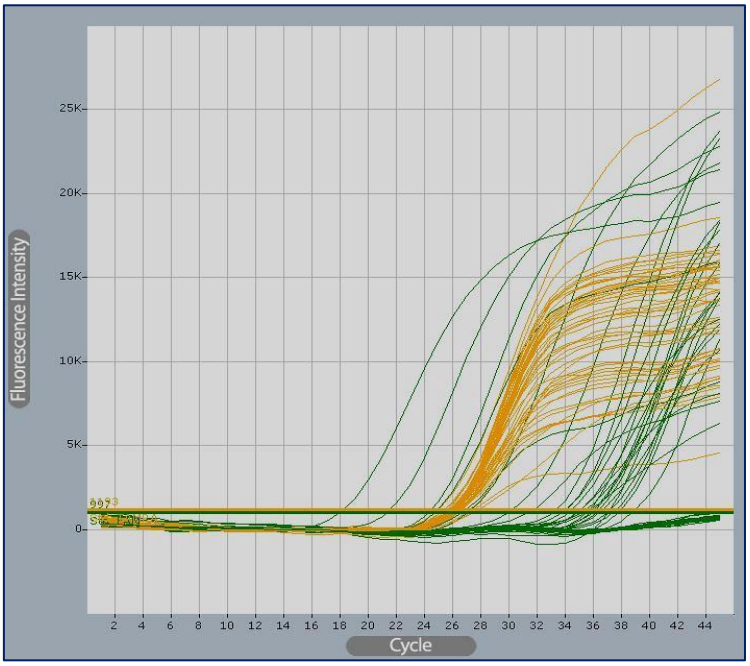

(B)

Figure (5 A and B): Real-time PCR amplification of commercial standards (blue) and specimens (orange), internal control positive (violate) for the quantitative detection of human *Cytomegalovirus* DNA.

Detection TNF-α

The level of TNF-α was raised significantly at ($p < 0.05$) in all groups of patients suffering from HBs Ag, HCMV, in comparison to the healthy control group (419.3±27.8)Pg/ml, as in Figure (6).

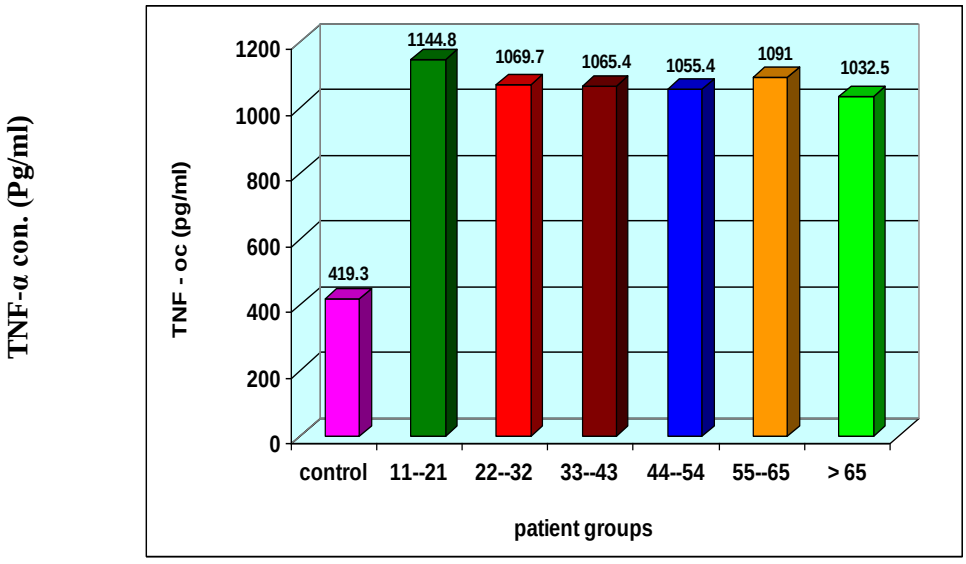

Figure (6): The level Cytokines (TNF-α) in all groups of patients compared with healthy control.

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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 were the highest significant increase in mean titer(2.334)(IU/ml) compared with other patients (Table 5) .These data were closer to study by Daw *et al*., (2000) in Libya that appeared the largest group being 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 (6) 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).

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Detection of anti-HCMV IgG and IgM among hepatitis B patients

Accuracy, speed and efficiency are important considerations in assaying for CMV antibody therefor two main reasons for the clinical use of serologic tests for CMV are for determining susceptibility to primary infection and for screening blood and therefore the potential for transmitting latent CMV. Seroconversion (seronegative to seropositive) remains a reliable means of diagnosing primary CMV infection. ELISA for detection of CMV antibody is available from several manufacturers. The ELISAs give higher antibody titers and is as accurate as CF assays in determining serologic status, are much easier to perform and eliminate the problem of anticomplimentary sera. Results are typically available in few hours (Jahan, 2010).

The high frequency of positive samples increased to 68(89.4%) out of the 76 samples were positive of HBV for anti-HCMV IgG antibodies, IgG-positivity was found in all age groups, whereas only 4(5.26%) were positive sample for anti-HCMV IgM antibodies by ELISA test**.** The high prevalence of IgG seropositive was probably due to cumulative effect of previous infection, reactivation or new infection lead to high percentage of Seropositivity. Because hepatitis B patients conceder immunosuppressed individuals. Apositive test for CMV IgG indicates that a person may be was infected with CMV at some time during their life but the IgG test cannot determine when a person was infected (Drew, 2011**).**These results were in agreed with Barzilai *etal*, (2007) who showed that the CMV IgG antibodies a significant titer increase in SLE patients when compared with matching controls. With regards to other autoimmune disease.

In Table (6).was recorded the highest percentage distribution of anti-HCMV IgG among hepatitis B patients in the age group (22-32) year were 17(25 %) and the lowest one was the age group (65-72) years were 3(3.9%).While the ELISA result showed that the mean titer of IgG antibodies were (2.61±0.28) IU/ml at age group (>65) increased significantly at (P value <0.05) compared with other groups. These results agreed with Masoodi *et al*, (2013) in India whom conclude that Out of the 50 patients 32 (64%) were found to have CMV disease. 9 patients had low CMV IgG Avidity, those having high CMV IgG Avidity were 11 and those with borderline CMV IgG Avidity were 12. Anti-CMV IgG antibody avidity was found to have a marginal role in diagnosis of this disease. Out of the 32 diseased patients, 12 had CMV virus syndrome, 8 had gastrointestinal involvement, 5 had allograft dysfunction, 3 had hepatitis, 3 had nephropathy and 1 had pneumonitis.

Distribution of anti-HBsAg and anti-HCMV positive Samples according to sex were revealed a high HCMV Seropositivity in male (73.9%) than in female (26.1%). Is similar to results of Wreghitt *et al*, (2003) who found the ratio of male to female patients was 1.2:1. With CMV infection, was most frequently found in patients aged 20–59 years (90% of all patients) and most studies have involved patients with hepatitis, but there are reports of patients with neurological and intestinal symptoms. While the other recent studies by (Paixão *et al*, 2012; Liu *et al*, 2014) showed that the odds ratio of HCMV infection in males was slightly higher than that in females, there was no significant difference. Similar results of no significant differences in age and duration of illness were found among the different HCMV infection groups.

Detection of viral HCMV by RT-PCR techniques

Traditionally, the laboratory diagnosis of CMV infection has been made through a combination of culture-based methods, histopathology, serological assays and more recently, molecular detection by real-time PCR. Routine viral culture demonstrates adequate sensitivity for identification of CMV;

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however, viral culture is labor-intensive and subjective and may require up to 14 days for the virus to be propagated and identified. Due to the limitations of culture based methods, real-time PCR has become an important laboratory tool for the diagnosis and management of CMV disease**.** (Vries *et al*, 2012; Binnicker and Espy, 2013)

Real-time PCR has been developed to detect HCMV because of its time-saving feature, and high sensitivity and specificity. (Liu *et al*, 2014)

The results in this study reported that there is about 23 (30.2%) out of the 76 patients suffering from hepatitis B with viral loads ranging from (0.24- 1730000) Copies/ml, including all age groups. gave positive viral DNA existence. The result also showed that the age group (44-54) years increased significant at p< 0.001 with mean viral load (441502.6) Copies/ml compared to other groups .In regarded to sex distribution of patients the results showed the high percentage in males as 17(73.9%) with mean viral loads 156292.84 copies/ml while in females 6(26.1%), with mean viral loads 29351.94 copies/ml .Table (7) .The thresholds of PCR copy numbers was taken from 200 to 2000,000 of the 76 patients studied, In our study we found that CMV DNA copy numbers of >200 detected by the Real-time PCR correlated 100% with the presence of CMV disease Figure (5 A and B).These results in agreement with Masoodi *et al*, (2013) in India who found in his study population of 50 patients, 34(68%) tested positive for CMV DNA with CMV Real Time PCR. The thresholds of PCR copy numbers was taken from 500 to 500,000. Found that CMV DNA copy numbers of >500 detected by the Real-time PCR correlated 100% with the presence of CMV disease.

When comparing serology with the polymerase chain reaction for cytomegalovirus diagnosis, it is important to remember that serology is a diagnostic test that detects circulating antibodies and identifies the history of previous infections through IgG and acute infections using IgM. On the other hand, the most significant advance test that the real-time systems come from is its rapid thermocycling and simultaneous detection characteristics. Furthermore, increases or decreases in antibody levels, in general, do not necessarily support an actual diagnosis of HCMV infection in the immunosuppressed patient populations, due to frequent reactivation of the Virus. Therefore, serology has limited diagnostic value in the transplant patient group (Andrew *et al.*, 2008)**.**

Immunological study

Tumor Necrosis Factor alpha (TNF-α)

In this study the results showed increased the concentration of TNF-α in all patients hepatitis B in compared with healthy control groups Figure (6) The results also revealed no significant difference (p>0.05) between male and female .These results in agreements with other studies Park *et al*,(2012) who founds TNF-α levels were higher in CHB patients than in controls.

evaluated of level of the concentration serum TNF-α indicate to found viral infection in patients these results agree with Rehermann, (2000) who showed that in HBV infection the cellular immune response is thought to contribute to both viral clearance and liver cell injury .These two opposing function have peptide on MHC class I molecules of HBV-infected cells,CD8+ Tcells acquire the capacity to either cure HBV infected cell via noncytpathic, cytokine-mediated inhibition of HBV replication or to destroy them via perforin-Fas ligand(FasL),and TNF-α mediated death pathways Both effector functions have been observed during resolution of acute hepatitis B.

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Plasma TNF- a level is increased in the patients with chronic HBV and HCV infections, However elevated plasma as well as soluble TNF receptor levels were mainly observed with acute hepatitis and correlate with serum transaminase levels .(Tokushige *et al.*, 2000).

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