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Quantitative Analysis and Study of Correlation among Various Biological and Immunological Markers in Acute and Chronic HBV Infection

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Abstract: Hepatitis B is one of the most common infectious diseases globally that can progress to advanced liver diseases such as liver fibrosis, cirrhosis, cancer and ultimate death by liver failure. However, a systematic epidemiological study to find out disease risk factors as well as establishment of clinical correlation of hepatitis viral genotype with serological markers has not yet been fully understood. The present study was aimed to study the demographic data analysis according to age and sex and determine the avidity of different serological and immunological markers including HBsAg, HBV e antigen (HBeAg), IgM anti-HBc, anti-HBc and anti-HCV among hepatitis patients. In order to evaluate their molecular pattern, we also tried to establish a correlation of HBV load with the clinical parameters such as ALT, AST, ALP, bilirubin and Alpha-fetoprotein (AFP). This study revealed that, HBV patients were commonly found in ages ranged between 19-72 years. Among the 30 patients infected from HBV, 15 (50%) are male and 15 (50%) are female. It is also seen that the highest number of HBV positive male lies in 30-39 age group and HBV positive female lies in the age groups 20-29 and 50-59. There was a significant increase in ALT, AST, and ALP activities in patients with HBV. The levels of AFP among patients with chronic HBV was significantly higher. In addition, Anti-HBs, Anti-HbcIgM and HBeAg were detected by 93.33% (28/30), 50 % (15/30) and 40% (12/30) in HBV patients respectively. The levels of HBV DNA were found by 26.67% (8/30) in HBV patients which was above the linear detection range: 50-5×109 Copies/ ml (10-1×109IU/ml). This detectable HBV DNA level was significantly more common in HBeAg and Anti HBc positive patients with elevated ALT levels than patients with normal ALT level. Considering correlation of viral load with biochemical markers, the author observed a positive but very weak correlation of ALT with viral load.

Keywords: HBsAg, AntiHBs, AntiHBc IgM, HBeAg, AFP, ALT

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Introduction

Hepatitis means inflammation of the liver. There are many reasons for the liver to be inflamed by viral, toxic, metabolic, pharmacologic, or immune mediated attack on the liver. Viral hepatitis is a major global health problem all over the world and the spectrum of

hepatitis range from sub-clinical to milder and life-threatening illness including acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Zuckerman, 1997; Khan *et al*, 2000). The burden of infection by hepatitis B virus (HBV), a major cause of chronic hepatic damage and of hepatocellular carcionoma worldwide (Fattovich, 2003; Lai *et al.*, 2003). Quantitative methods for evaluating viral replication and disease activity need to be employed on this purpose, and such methods must also have a detection range wide enough to be employed also in the follow-up of chronic cases, either untreated or during antiviral treatment. As for HBV-DNA, current assays are technically complex and expensive and, although their value has been clearly established in controlled trials, they cannot be employed for routine monitoring outside of specialized clinical settings, and their cost allows for testing only at intervals of several months. The surface antigen of HBV (HBsAg) is used typically as a qualitative serological marker for diagnosing an ongoing HBV infection. Serum HBeAg and HBV-DNA are currently the most important markers for both purposes (Bonino and Brunetto, 2003; Lai *et al.*, 2003).

Another serological marker that gained importance as an indicator of the host's active immune response, and also for monitoring disease activity, is represented by the IgM antibodies towards the HBc antigen (anti-HBc/IgM) (Brunetto *et al.*, 1993; Colloredo *et al.*, 1996, 1999; Marinos *et al.*, 1994). The measurement of the avidity index of anti-HBc antibodies, that are detectable in all phases of HBV infection, may also represent an interesting tool. Alpha-fetoprotein (AFP) is a glycoprotein with a molecular Weight of approximately 70,000 Daltons, represents the major serum protein during fetal life. AFP is normally produced during fetal and neonatal development by the liver, yolk sac, and in small concentrations by the gastrointestinal tract. After birth, serum AFP concentrations decrease rapidly, and by the second year of life and thereafter only trace amounts are normally detected in serum (typically <10 ng/ml). High levels of AFP are observed during adulthood only under certain conditions, such as hepatocellular carcinoma-HCC, germ-cell tumors, and liver diseases.

We aimed to determine quantitatively both HBsAg and IgM anti-HBc, as well as to study the anti-HBc avidity, in a selected population of acute and chronic hepatitis B in order to evaluate their patterns and prospect their possible use in the serological diagnosis and monitoring of hepatitis B, and especially on the purpose of differentiating acute from chronic infection.

Methodology

Patients

Patients of this study were the people referred to the Biochemistry and Immunology laboratory in Enam Medical College and Hospital for biochemical and serological tests over a period of two years (from July 2013 to June 2014) after ethical clearance. The samples were collected from both genders having ages ranging from <20 to ≥60 years. Informed consent was taken from every individual and history of individuals was recorded in the form of questionnaires. The processes of collecting specimens by withdraw about 3 ml blood sample per patient and centrifuged within 30 minutes at 3000 rpm for 5 minutes. Hemolysed samples were discarded and the sera were stored and frozen about -20C°until analysis.

Criteria for exclusion

Pregnant women, patients with leukemia, hemophilia, or autoimmune illnesses, patients who had been vaccinated against HBV, patients with hepatitis A, C, and D virus infections, patients with human immunodeficiency virus infection, or patients under dialysis were excluded from the study.

Ethics statement

Informed consent was taken from every individual being tested and approval will be obtained from institutional ethical review committee. History of individuals was recorded in the form of questionnaires. The laboratory test results were kept confidential and we shared the results with the participants. Infected individuals were provided with appropriate information on the prevention of spread of these infections to others and referred them to the nearest public health care facilities.

Clinical Data Analysis

Two groups of subjects were studied: 30 healthy individuals without hepatitis antecedents and 30 patients with HBV infection. The positive cases were tested with the detection of HBV e antigen (HBeAg), IgM anti-HBc antibodies and Anti-HBs antibodies by the enzyme-linked immunosorbent assay technique (Autobio-England). All patients and healthy group were screened for serum levels of Alanine aminotransferase (ALT), asparagine aminotransferase (AST), alkaline phophatase (ALP) and Bilirubin with respective Kits (Human-Germany). All sera samples were tested for the presence of the cancer marker alpha fetoprotein (AFP) by the enzyme-linked immunosorbent assay technique (Manufacturer: Autobio, England). Finally, correlations were analysed among biochemical variables of the study subjects and patients with HBV.

HBV Viral Assays

Samples

Thirteen hepatitis B surface antigen (HBsAg)-Reactive samples were collected from Enam Medical College and Hospital and will be stored at -70°C until DNA extraction.

Serum hepatitis B virus DNA isolation

Because the efficacy of PCR amplification may be affected by the presence of a number of inhibitors of Taq polymerase enzyme in the serum, such as EDTA (Greenfield And White, 1993), phenol (Katcher And Schwartz, 1994), polyamines (Katcher And Schwartz, 1994), polysaccharides (Shioda And Murakami-Murofushi, 1987) and calcium alginate (Wadowsky *et al*., 1994), DNA extraction is required before amplification (Kramvis *et al*., 1996, Khaja, *et al.*, 2003). DNA extraction can be done by many methods that some of them are standard methods. An efficient method of DNA extraction that yields pure and high-quality DNA is important for conducting PCR and sequencing reactions (Mcorist *et al*., 2002). Therefore, the aim of this study was comparison of three different methods, including QIAamp MinElute Virus Spin Kit, DNPTM Kit and phenol-chloroform extraction procedure.

(A) Phenol-chloroform extraction procedure: a total of 200µl of each sample was added to 200µl of lysis buffer (25mM EDTA, 200mM Tris-HCl pH 7.5, 250mM NaCl, 1%SDS) and was included at room temperature for 60 min. 400µl equilibrated phenol pH 7.8 was added, decanted and incubated for 10 min at room temperature. Tubes were centrifuged at 12000g for 5 min. Then mixed with an adequate volume of chloroform: isoamylalcohol (24:1). Then was precipitated with 3M sodium acetate pH 5.2 and 96% ethanol and incubated at -20°C for overnight. After centrifugation for 15 min at 4°C at 16000g, the pellet was washed with 70% cold ethanol and centrifuged at 16000g at 4°C. The pellet was air-dried and resuspended in 20µl double-distilled H2O (dd H2O) and then stored at -20°C until using.

(B) QIAamp MinElute Virus Spin Kit (QIAGEN, Germany): the extraction procedure was performed according to the manufacturer's instructions. 200µl buffer AL was added into the tube including 200µl serum and 25µl QIAGEN protease and incubated at 56°C for 15 min

in a heating block. 250µl of absolute ethanol was added and incubated at room temperature for 5 min.

All of the lysate from the previous step briefly was applied onto a specific column and centrifuged at 6000g for 1 min. 500µl of buffer AW1 was added. After centrifuging at 6000g for 1 min, 500µl buffer AW2 was added and centrifuged at 6000g for 1 min. 500µl of absolute ethanol was then added and centrifuged at 6000g for 1 min. The column was dried at 56°C for 3 min. In later step, 100µl buffer AVE was added and centrifuged at 20000g for 1 min and then stored at-20°C until using.

(C) DNPTM Kit (CinnaGen Inc.): DNPTM Kit was another method for nucleic acid extraction, purification and removal of amplification inhibitors that was used according to the manufacturer's instructions.

After adding 5µl protease to 100µl serum, it was mixed with 400µl lysis solution and vortexed 20 sec. 300µl of precipitation solution was added, placed in -20°C for 20 min and centrifuged at 12000g for 10 min. 1ml washing buffer was then added to pellet and mixed gently and then centrifuged at 12000g for 5 min. The pellet was dried at 65°C for 5 min, suspended in 50µl of solvent buffer, shacked and placed at 65°C for 5 min. The mixture was centrifuged. Supernatant contained purified DNA. In this present study, Viral DNA was isolated with QIAamp Blood Mini Kit (Qiagen, Germany) following the manufacturer's instructions since this is the most efficient method for viral DNA extraction of serum samples.

Detection of hepatitis B virus DNA by PCR

Conventional nested PCR was used to detect hepatitis B virus DNA in sera of patients. Nested PCR, in first- and second-round PCR, was performed for 3 min at 94°C, following of denaturation at 94°C for 45 sec, annealing for 60 sec at 55°C and extension at 72°C for 90 sec. Final extension was done at 72°C for 6 min. PCR solution containing 2.5µl of extracted DNA, 0.5µl dNTP mix, 2.5µl 10x Taq polymerase buffer, 0.75µl MgCl2, 0.2µl Taq polymerase and 1µl of each primer (10 pmol), PrsS3 and S1R in first-round PCR and YS1 and YS3 in second-round PCR. PCR products were detected in 1% ethidium bromide-stained agarose gel.

Quantification of HBV viral DNA in serum by real time PCR

Real-Time PCR is designed based on nucleic acid profile.

Test Method

Analytical Specificity	**:**	All HBV genotypes (A to H) with equal specificity
Instrument Platform	**:**	Smart Cycler II Real Time PCR System, Cepheid, Sunnyvale, USA.
Real Time PCR Kit	**:**	RoboGene®HBV DNA Quantification Kit, Roboscreen GmbH
DNA extraction	**:**	RoboGene® DNA Extraction Kit, Roboscreen GmbH
Probe/Primer	**:**	TripleHyb (Taqman, Molecular Beacon, Hybridization Probe)

Linear Detection Range: 50-5×109 Copies/ ml (10-1×109IU/ml)

Statistical analysis

For a statistical analysis of quantitative variables, the mean and standard deviation were calculated. One sample t-tests were performed: comparisons were done with the independent samples T test and paired samples T test. All values are presented as mean SD. Data regarding age, sex, liver function test (ALT, AST, ALP), Bilirubin, AFP test, HBV and control. Statistical analysis and correlation of lab parameters with viral factors was determined by using SPSS version 16. It would be significant if $p \leq 0.05$.

Results

Demographic and laboratory data analysis of 30 HBV studied patients

Table 1. Demographic and laboratory data of 30 HBV studied patients

Gender	
Male	15 (50%)
Female	15 (50%)
Age (years)	
Range	19-72
Mean ± SD	42.10 ± 15.88
ALT	
< 40	15 (50%)
≥ 40	15 (50%)
Mean ± SD	256.0167 ± 461.2412
AST	
< 40	17 (56.66667%)
≥ 40	13 (43.33333%)
Mean ± SD	119.4627 ± 237.1219
ALP	
< 40	9 (18%)
≥ 40	41 (82%)
Mean ± SD	65.1353 ± 57.2438
Bilirubin	
< 0.9	20 (40%)
≥ 0.9	30 (60%)
Mean ± SD	2.7187 ± 5.83031

From the above demographic and laboratory data in table 1, we can say that among the 30 studied patients 15(50%) are male and 15(50%) are female. The lower limit of the respondents' age is 19 and its upper limit is 72. 15, 17, 9 of the respondents have less than 40 ALT, AST and ALP level respectively and remaining are greater than or equal to 40.20 (40%) of the respondents have Bilirubin level that is less than 0.9 and remaining 30 (60%) have Bilirubin value greater than or equal 0.9.

Study of distribution of the Hepatitis B patients and control groups according to age and sex

Table 2. Distribution of the patients and control groups according to age and sex

Groups (year)	HBV		Control	
	Female	Male	Female	Male
< 20	0	1	4	0

20-29	**4**	2	11	1
30-39	3	**6**	4	3
40-49	3	0	3	2
50-59	**4**	2	0	1
≥ 60	1	4	0	1
Net Total	15	15	22	8
Total=60				

The distribution of the studied groups according to age and sex are summarized in table 2. Our data include 30 HBV infected patients and 30 are healthy (control). Among the 30 patients infected from HBV, 15 (50%) are male and 15 (50%) are female. Out of 30 healthy 8 are male and 22 are female. It is also seen that the highest number of HBV positive male lies in 30-39 age group and HBV positive female lies in the age groups 20-29 and 50-59.

Quantitative analysis of various biological and immunological markers with HBV infection

Table 3. Clinical and laboratory data of patients with HBV and control

	HBV patients		**P HBV**	**P Control**
N	30	30		
Gender (M/F)	15/15	8/22		
Age	42.1000±15.88173	30.6000±12.21531	.000	.000
ALT	256.0167±461.24126	22.4543±7.66408	.005	.000
AST	119.4627±237.12192	34.1427±6.84720	.010	.000
ALP	65.1353±57.24382	1140840±40813054	.000	.137
Bilirubin	2.7187±5.83031	.4980±.19887	.016	.000
HBsAg	2.3227±.55557	.0270±.01821	.000	.000
AntiHBs	3.6518±6.03082	653.2667±41417537	.002	.000
AntiHBc IgM	1.0765±2.7174	.0467±.05741	.042	.000
HBeAg	.5336±.88694	.0470±.02662	.003	.000
AFP	25.8647±46.62111	3.6370±1.51024	.005	.000

Clinical and laboratory data for the studied groups are given in table 3. Serum AFP levels are significantly higher in HBV (25.8647 ± 46.62111) in comparison with control group (3.6370 ± 1.51024). Patients with HBV had also a significant higher AST, ALT, ALP and TSB ($p < 0.00$) compared to control as clear in table 3.3.

Table 4. Spearman's Correlation Analysis between Serum AFP and patients with HBV and Biochemical Variables of the study subjects

Parameters	**HBV Patients**	**P - value**
N	30	
Age Years	-0.010	0.958
ALT	**0.099**	**0.604**
AST	-0.036	0.852
ALP	**0.023**	**0.905**
Bilirubin	-0.120	0.526
HBsAg	-0.137	0.469
AntiHBs	-0.112	0.554

AntiHBc IgM	-0.106	0.577
HBeAg	**0.266**	**0.156**

. Correlation is significant at the 0.05 level (2-tailed)

****. Correlation is significant at the 0.01 level (2-tailed)**

The relationship between serum AFP and other biochemical parameters for HBV are shown in **table 4**. The above results also indicate that there is a positive correlation between serum AFP levels & ALT (r=0.099, p=0.604), ALP (r=0.023, p=0.905) & with HBeAg (r=0.266, p=0.156) in HBV patients.

Study of correlation among various biological and immunological markers of patients with HBV with respect to gender and age: The Spearman's correlation analysis between the different Biochemical and serological Variables of the Control study subjects and patients with HBV.

Table 5. Spearman's correlation Analysis between Age and Biochemical Variables of the study subjects

	Anti-HBs (N=30)	p-value
Age	0.161	0.394
ALT	-0.429	0.018*
AST	-0.466	0.009**
ALP	0.044	0.817
Bilirubin	0.119	0.533
HBsAg	-0.202	0.286
antiHBcIgM	-0.063	0.741
HBeAg	-0.158	0.405
AFP	-0.09	0.638
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

Table 6. Spearman's correlation Analysis between ALT and Biochemical Variables of the study subjects

	Anti- HBcIgM (N=30)	p-value
Age	0.097	0.61
ALT	-0.276	0.14
AST	-0.138	0.466
ALP	-0.198	0.295
Bilirubin	0.009	0.961
HBsAg	-0.162	0.394
antiHBs	-0.063	0.741
HBeAg	-0.078	0.681
AFP	0.177	0.35
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-5** we have seen that, the correlation coefficient between the Anti-HBs and different Biochemical Variables of the control study subjects are very low. Some of the cases there is a negative correlation. Since P-value is greater than 0.05 all the cases so we may not reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0, i.e., there is no linear association between the Anti-HBs and different Biochemical Variables of the control study subjects. But P value of correlation between Anti-HBs and ALT, Anti-HBs and AST is 0.018 and 0.009 respectively which is less than 0.05 and 0.01, so we may reject the null hypothesis at 5% and 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between the Anti-HBs and ALT, Anti-HBs and AST.

From the above table-6 we have seen that, the correlation coefficient between the Anti-HBcIgM and different Biochemical Variables of the control study subjects are very low. Some of the cases there is a negative correlation. Since P-value is greater than 0.05 all the

cases so we may not reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0, i.e., there is no linear association between the Anti- HBcIgM and different Biochemical Variables of the control study subjects.

Table 7. Spearman's correlation Analysis between HBeAg and Biochemical Variables of the study subjects

	HBeAg (N=30)	p-value
Age	-0.147	0.438
ALT	0.126	0.507
AST	0.054	0.777
ALP	0.021	0.914
Bilirubin	-0.008	0.966
HBsAg	0.222	0.238
antiHBs	-0.158	0.405
antiHBcIgM	-0.078	0.681
AFP	0.346	0.061
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

Table 8. Spearman's correlation Analysis between AFP and Biochemical Variables of the study subjects

	AFP (N=30)	p-value
Age	0.184	0.329
ALT	0.191	0.312
AST	0.013	0.946
ALP	0.061	0.748
Bilirubin	0.047	0.805
HBsAg	-0.219	0.245
antiHBs	-0.09	0.638
antiHBcIgM	0.177	0.35
HBeAg	0.346	0.061
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-7** and **table-8** we have seen that, the correlation coefficient between the HBeAg, AFP and different Biochemical Variables of the control study subjects are very low. some of the cases there is a negative correlation. Since P-value is greater than 0.05 all the cases so we may not reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0, i.e., there is no linear association between the HBeAg, AFP and different Biochemical Variables of the control study subjects.

Table 9. Spearman's correlation Analysis between ALT and Biochemical Variables of the study subjects

	ALT (N=30)	p-value
AST	0.58	0.00**
Age	0.60	0.00**
ALP	0.35	0.06
Bilirubin	0.58	0.00**
HBsAg	0.12	0.53
antiHBs	-0.15	0.44
antiHBcIgM	0.17	0.37
HBeAg	-0.06	0.73
AFP	0.46	0.01*
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

Table 10. Spearman's correlation Analysis between AST and Biochemical Variables of the study subjects

	AST (N=30)	p-value
ALT	0.58	0.00**
Age	0.38	0.04*
ALP	0.36	0.05
Bilirubin	0.57	0.00**
HBsAg	-0.07	0.72
antiHBs	-0.23	0.23
AntiHBcIgM	0.37	0.04*
HBeAg	0.15	0.44
AFP	0.50	0.00**
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-9** of Spearman's correlation Analysis between ALT and Biochemical Variables of the study subjects we have seen that, the correlation coefficient between the

ALT and AST is 0.58 which implies that there is a moderate positive association between these variables. Since p-value is less than 0.01, we may reject the null hypothesis at 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between ALT and AST. Similarly, for the ALT and Age, ALT and Bilirubin, ALT and AFP. Since the p-value of correlation between the ALT and ALP is greater than 0.05 so may not reject the null hypothesis that is we conclude that the population correlation is equal to 0 i.e. there is no linear association between the ALT and ALP. Some of cases the correlation coefficient is low and negative.

From the above **table-10** we have seen that, the correlation coefficient between the AST and ALT is 0.58 which implies that there is a moderate positive association between these variables. Since p-value is less than 0.01, we may reject the null hypothesis at 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between AST and ALT. Similarly, for the AST and Bilirubin, AST and AFP. Since the p-value of correlation between the AST and ALP is greater than 0.05 so may not reject the null hypothesis that is we conclude that the population correlation is equal to 0 i.e. there is no linear association between the AST and ALP. Some of cases the correlation coefficient is low and negative.

Table 11. Spearman's correlation Analysis between Age and Biochemical Variables of the study subjects

	Age (N=30)	p-value
ALT	0.60	0.00**
AST	0.38	0.04*
ALP	0.37	0.05
Bilirubin	0.52	0.00**
HBsAg	0.19	0.31
antiHBs	-0.31	0.09
antiHBcIgM	0.04	0.82
HBeAg	-0.19	0.32
AFP	0.28	0.14
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

Table 12. Spearman's correlation Analysis between ALP and Biochemical Variables of the study subjects

	ALP (N=30)	p-value
ALT	0.35	0.06
AST	0.36	0.05
Age	0.37	0.05
Bilirubin	0.39	0.03*
HBsAg	0.07	0.71
antiHBs	-0.42	0.02*
antiHBcIgM	-0.06	0.76
HBeAg	0.02	0.93
AFP	0.24	0.19
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-11** we have seen that, the correlation coefficient between the Age and ALT is 0.60 which implies that there is a moderate positive association between these variables. Since p-value is less than 0.01, we may reject the null hypothesis at 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between AST and ALT. Similarly, for the Age and Bilirubin. Some of cases the correlation coefficient is low and negative. In that case the P-value is greater than 0.05, So we may not reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0, i.e., there is no linear association between these variables. From the above **table-12** we also observe that, the correlation coefficient between the between ALP and different Biochemical Variables of the case study subjects are low and some of the cases are negative. From observing the p-value

we conclude that only there is linear association between ALP and Bilirubin, ALP and anti HBs.

Table 13. Spearman's correlation Analysis between Bilirubin and Biochemical Variables of the study subjects

	Bilirubin (N=30)	p-value
ALT	0.58	0.00**
AST	0.57	0.00**
Age	0.52	0.00**
ALP	0.39	0.03
HBsAg	0.16	0.40
antiHBs	-0.05	0.78
antiHBcIgM	0.16	0.39
HBeAg	-0.04	0.83
AFP	0.54	0.00**
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

Table 14. Spearman's correlation Analysis between HBsAg and Biochemical Variables of the study subjects

	HBsAg (N=30)	p-value
ALT	0.12	0.53
AST	-0.07	0.72
Age	0.19	0.31
ALP	0.07	0.71
Bilirubin	0.16	0.40
antiHBs	-0.08	0.66
antiHBcIgM	0.04	0.84
HBeAg	0.02	0.92
AFP	0.05	0.81
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-13** we have seen that, the correlation coefficient between the Bilirubin and ALT is 0.58 which implies that there is a moderate positive association between these variables. Since p-value is less than 0.01, we may reject the null hypothesis at 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between Bilirubin and ALT. Similarly, for the Bilirubin and AST, Bilirubin and Age, Bilirubin and AFP. Some of cases the correlation coefficient is low and negative. In that case the P-value is greater than 0.05, So we may not reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0, i.e., there is no linear association between these variables. From the above **table-14** we also observe that, the correlation coefficient between the between HBsAg and different Biochemical Variables of the case study subjects are low and some of the cases are negative. From observing the p-value we conclude that there is no linear association between HBsAg and different Biochemical Variables of the case study subjects.

Table 15. Spearman's correlation Analysis between HBeAg and Biochemical Variables of the study subjects

	HBeAg (N=30)	p-value
ALT	-0.06	0.73
AST	0.15	0.44
Age	-0.19	0.32
ALP	0.02	0.93
Bilirubin	-0.04	0.83
HBsAg	0.02	0.92
antiHBs	0.13	0.50
antiHBcIgM	0.45	0.01*

Table 16. Spearman's correlation Analysis between AFP and Biochemical Variables of the study subjects

	AFP (N=30)	p-value
ALT	0.46	0.01*
AST	0.50	0.00**
Age	0.28	0.14
ALP	0.24	0.19
Bilirubin	0.54	0.00**
HBsAg	0.05	0.81
antiHBs	-0.14	0.45
antiHBcIgM	0.41	0.02*

AFP	0.17	0.37
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

HBeAg	0.17	0.37
*. Correlation is significant at the 0.05 level (2-tailed).		
**. Correlation is significant at the 0.01 level (2-tailed).		

From the above **table-15** we observe that , the correlation coefficient between the HBeAg and Biochemical Variables of the case study subjects are low but some of the cases negative and p value is greater than the 0.05 so we may reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0 i.e. there is no linear association between HBeAg and Biochemical Variables of the case study subjects except HBeAg and anti-HBcIgM.

From **table 16** we can say that the correlation coefficient between the AFP and AST is 0.50 which implies that there is a moderate positive association between these variables. Since p-value is less than 0.01, we may reject the null hypothesis at 1% level of significance and conclude that the population correlation coefficient ρ is not equal to 0, i.e., there is a linear association between AFP and AST. Similarly, for the AFP and Bilirubin.

The correlation coefficient between the AFP and ALT is 0.46 which implies that there is a positive association between these variables and p-value is less than the 0.05 so we may reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is not equal to 0 i.e. there is a linear association between AFP and ALT. The correlation coefficient between the AFP and anti-HBcIgM is 0.41which implies that there is a positive association between these variables and p-value is less than the 0.05 so we may reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is not equal to 0 i.e. there is a linear association between AFP and anti-HBcIgM. Observing the P-value we see that, p-value is greater than 0.05 so we may reject the null hypothesis at 5% level of significance and conclude that the population correlation coefficient ρ is equal to 0 i.e. there is no linear association between AFP and Age, ALP, HBsAg, antiHBs and HBeAg.

Detection of HBV DNA level in HBV carriers (n=13)

The detection of HBV DNA gives a measure of active viral replication in plasma and is important in detecting HBV infection. So, the value of HBV DNA is important for treatment and in monitoring response to treatment. Out of 30 HBV patients, 15 were acute and 15 were chronic in disease progression.

In the present study, HBV DNA level was detected in serum samples of 13 HBV patients (6 acute+13 chronic patients) **(Table 17)**. Among the 6 acute patients, HBV DNA was not detectable that is below the linear Detection Range: 50-5×109 Copies/ ml (10-1×109IU/ml) in 5 patients and only one patient carried detectable amount of HBV DNA which was 1.82×10^4 copies/ml, 3.64×10^3IU/ml.

On the other hand, among the 7 HBV chronic patients, every individual carried a detectable amount of HBV DNA level that is above the linear Detection Range: 50-5×109 Copies/ ml (10-1×109IU/ml).

Table 17. Detectable HBV DNA level in HBV patients (n=13)

HBV Patients	Number of Patients	Sex	Age	HBV DNA level linear Detection Range: 50-5×109 Copies/ ml (10-1×109IU/ml
Acute	1	M	42	Not detectable
	2	F	42	Not detectable
	3	M	40	Not detectable
	4	F	45	Not detectable
	5	F	42	1.82×10^{4} copies/ml, 3.64×10^{3}IU/ml
	6	M	42	Not detectable
Chronic	1	M	28	2.59×10^{8} copies/ml, 5.19×10^{7} IU/ml
	2	M	36	8501 copies/ml, 1700 IU/ml
	3	M	58	1.58×106 copies/ml ,7.92×10^{5} IU/ml
	4	F	40	1. 172 copies/ml,34 IU/ml
	5	M	36	8501 copies/ml 1700 IU/ml
	6	M	26	1. 832 copies/ml ,166 IU/ml
	7	M	18	1. 5.35×10 copies/ml ^{8,} 1.07×10^{8} IU/ml

Comparative study among HBV DNA level with HBeAg, Anti- HBC status and ALT

The HBV DNA level was compared with HBeAg, Anti- HBC status and the level of liver enzyme ALT in **table 18.**

Table 18. Serum HBV DNA and ALT level in different HBeAg, Anti- HBC status

HBV Patients	Number of Patients	HBV DNA level linear Detection Range: 50-5×109 Copies/ ml (10-1×109IU/ml	HBeAg	Anti-HBc	ALT
Acute	1	Not detectable	-	+	21.68
	2	Not detectable	-	-	65
	3	Not detectable	+	+	63
	4	Not detectable	+	-	397
	5	detectable	+	+	70.45
	6	Not detectable	-	-	42
Chronic	1	Detectable	+	+	535.23
	2	Detectable	+	+	300
	3	Detectable	+	+	512
	4	Detectable	+	-	659
	5	Detectable	+	-	1121
	6	Detectable	+	+	1832
	7	Detectable	+	+	1500

Out of 6 acute HBV patients, 5(83.33%) were found without detectable HBV DNA level with normal ALT level and HBeAg negative condition. The rest one (16.66%) HBeag-positive and anti-HBc positive patient had elevated ALT level who had also detectable amount of DNA. Out of 7 chronic HBV patients, all of the 7(100%) HBeAg-positive and 5(71.42%) Anti-HBc positive patients had elevated ALT level and all of them had detectable HBV DNA. Only two

(28.57%) Anti-HBc-negative ptients were found who had detectable level of DNA with elevated ALT level.

So, it is seen in the present study, detectable HBV DNA level was significantly more common in HBeAg and Anti HBc positive patients with elevated ALT levels than patients with normal ALT level.

Discussion

In the present study Hepatitis B patients were compared with control subjects according to age and sex**.** Interestingly, it is also seen that the highest number of HBV positive male lies in 30-39 age group and HBV positive female lies in the age groups 20-29 and 50-59. Among the 30 patients infected from HBV, 15 (50%) are male and 15 (50%) are female. These findings are related to the author observed that infection with HBV were most prevalent among people age between 30 to 49 years (Koulentaki *et al*., 2001).

The ALT, AST ALP and bilirubin activities were important as a biological marker that is widely used for liver diseases. This study revealed that there was a significant increase in ALT, AST ALP and bilirubin activities in patients with HBV, damaged liver cells may be the result of increase these Research enzymes activities (Chopra and Griffin, 1985).

High bilirubin level is usually associated with liver metastases and liver tumor involvement leading to hepatocellular carcinoma and liver cirrhosis by active or non-active HBV (Raymond *et al*, 2003). Bilirubin has been reported as marker of liver injury and to determine the proper dose of interferon in patients with different genotypes (Imbert-Bismut *et al*, 2001). Patients with HBV had a significant higher AST, ALT, ALP & TSB ($p < 0.00$) compared to control as clear in the study. Evaluating the correlation between different clinical markers, we observed that there is a moderate positive association between these variables. This may lead to conclude that these elevated biochemical markers act as indicators of liver cell injury (Murakami *et al*, 2004; Ong *et al*, 1999) and are usually predominant in liver cirrhosis with increased ALT levels (Furukawa *et al*, 1984). However, we could not observe any correlation between viral load and AST that may be due to poor immune response resulting in uneven AST level and viral load and lead to liver damage (Zechini, Pasquazzi and Aceti, 2004).

The levels of AFP among patients with HBV patients were determined and compared to control subjects, the results in the current study indicated that the serum AFP levels were significantly higher in HBV patients ($p <0.05$) in comparison with control subjects, Our results were consistent with the previous studies (Abd El Samiee *et al*, 2011; Ong *et al*., 1999; Furukawa *et al*, 1984; Di Bisceglie *et al*., 1989) which revealed that there were an increase in AFP level in viral hepatitis (HBV and HCV) and liver cirrhosis without evidence of HCC.

The study was aimed to analyze and find out the association of age, gender and viral load with host serum markers in HBV patients.

The serological diagnosis of hepatitis B virus infection has relied for more than three decades mainly on a combination of qualitative assay results, whose different patterns were considered representative of acute and chronic disease or of a chronic “healthy” state. In chronic hepatitis B quantitative testing of serological markers may represent an attractive alternative to the evaluation of ALT and HBV-DNA. In fact, while such determinations are both quantitative, and thus appear more suited for following up a chronic disease, the former

lacks specificity, being just an index of hepatic cytonecrosis (Brunetto *et al*., 1993; Lai *et al*., 2003) and the latter is still fairly imprecise, difficult to standardize and, for the time being, too expensive and technically demanding to be adopted on a routine basis in general hospital settings. Concerning correlation of viral load with biochemical markers, we found a positive but very weak correlation of ALT with viral load.

For many years anti-HBc IgM have been considered a specific marker of acute hepatitis B. Indeed, this was substantially true with the old enzyme immunoassays, that were standardized at a threshold value corresponding to 600–700 Paul-Ehrlich (PEI) units and were then able to give a positive result only in the presence of the high IgM levels that are usually present in acute HBV infections (Gerlich *et al*., 1986; Brunetto *et al*., 1993; Marinos *et al*., 1994). However, it has been demonstrated by several authors that lower levels of IgM anti-HBc are often detectable also in chronic hepatitis B (Brunetto *et al*., 1993; Colloredo *et al*., 1996; Gerlich *et al.*, 1986; Marinos *et al.*, 1994) by more sensitive methods that may attain an analytical sensitivity as low as 7 PEI units (Brunetto *et al*., 1993). This level seems adequate to differentiate chronic, inactive carriers (Brunetto *et al*., 1993; Cacciola *et al*., 2005) from asymptomatic patients suffering from chronic hepatitis B (Brunetto *et al*., 1993; Colloredo *et al.*, 1996; Gerlich *et al*., 1986; Marinos *et al*., 1994), an important clue in countries where the negativity for HBeAg is a common trait for both groups (Bonino and Brunetto, 2003; Fattovich, 2003). The quantitation of IgM values allows also establishing their relationship with the HBV replicative flares and with disease activity (Colloredo *et al*., 1996, 1999).

The possible role of the quantitative determination of HBsAg has been initially prospected by Froesner *et al*., (1982), who studied the concentration of the surface antigen over time in patients with acute and chronic hepatitis B and concluded that in acute hepatitis B cases a reduction of at least 50% of values was attained on average in 16.6 days in patients who recovered spontaneously. The diagnostic assays available at that time did not allowroutine quantitative testing for HBsAg, and a decade passed before other authors (Zoulim *et al*., 1992), employing an automated assay, suggested the possible role of this marker also in the monitoring of chronic HBV infection, and in particular its predictive value on the outcome of interferon therapy (Burczynska *et al*., 1994).

Two weak points of those studies were the limited dynamic range of the assays, with the ensuing need of performing multiple dilutions, and the need to employ an external standard, also at serial dilutions, since all HBsAg assays were qualitative. The dynamics of HBsAg levels in acute infections and their relationship with HBV-DNA and other HBV markers have been recently reviewed (Chulanov *et al*., 2003; Erhardt *et al*., 2000). While Laurell's electrophoresis has been demonstrated to be accurate and clinically useful for the quantitation of HBsAg (Gerlich *et al*., 2004), the recent availability of a quantitative, fully automated assay has started a new wave of studies: a correlation between HBsAg and HBV-DNA was found by Chen *et al*. (2004) in asymptomatic carriers stratified by HBV-DNA levels and by Kohmoto *et al*. (2005) in lamivudine-treated patients.

In contrast, a retrospective study (Kuhns *et al*., 2004) failed to find a correlation between HBsAg and HBV-DNA in untreated blood donors; a recent observation (Werle-Lapostolle *et al*., 2004) linking HBsAg load to the amount of covalently closed circular HBV-DNA (ccc-DNA), whose dynamics are different from serum HBVDNA, may help to explain the contrasting finding among studies carried out only on serum specimens. Our findings supported the above hypothesis. The presence of HBeAg in serum correlates with the presence of viral replication in the liver (Badur and Akgun, 2001). Monitoring of ALT level

is of value in accessing hepatocellular damage in patients with chronic hepatitis B virus infection (Hussain *et al*., 2004). In the present study, 13 HBV DNA PCR positive hepatitis B virus carriers were considered. Out of 6 acute HBV patients, 5(83.33%) were found without detectable HBV DNA level with normal ALT level and HBeAg negative condition.

The rest one (16.66%) HBeag-positive and anti-HBc positive patient had elevated ALT level who had also detectable amount of DNA (Alagiozian *et al*., 1998). Out of 7 chronic HBV patients, all of the 7(100%) HBeAg-positive and 5(71.42%) Anti-HBc positive patients had elevated ALT level and all of them had detectable HBV DNA. Only two (28.57%) Anti-HBc-negative ptients were found who had detectable level of DNA with elevated ALT level.

Taken together all of the findings and further studies of genotype distribution might guide eventually to the development, adaptation and evaluation of preventive strategies of HBV infections.

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