Original Article

Correlation of Serological, Biochemical, and Molecular Markers in Patients with Chronic Hepatitis-B Infection at Jaipur, Rajasthan

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ABSTRACT

Introduction: Monitoring of alanine transaminase (ALT), *Hepatitis B virus (HBV)* viral load, and Hepatitis B e-antigen (HBeAg) is important for the assessment of state of infection and prognosis following treatment of chronic hepatitis B (CHB) infection. The present work was planned to study the correlation of serological, biochemical, and molecular markers in patients with CHB infection.

Methodology: Out of 441 CHB cases, 411 were enrolled in the study based on IgM-anti HBc antibody results and were tested for biochemical (ALT), serological, and molecular (*HBV* DNA) parameters.

Results: Among 411 cases, males and females were 287 and 124, respectively and the median age was 39 years. The number of patients under immune tolerant, HBeAg positive immune active, inactive CHB, and HBeAg negative immune reactivation phase were 42, 48, 132, and 189, respectively as per the natural history of CHB. Significant association was seen in ALT level and *HBV* viral load (p value <0.0001) in all phases. *HBV* viral load was found to be significantly higher in HBeAg positive patients (median $5.1x10^7$) and those with elevated ALT (median 14800) than HBeAg negative patients (median 4700) and normal ALT (median 25750). ALT levels were also higher (94.6±161.71 U/L) in HBeAg positive individuals than HBeAg negative ones (50.78±22.93 U/L). In all three categories of viral load, a significant association was seen between ALT and *HB*V viral load (p value 0.00) and the same in case of HBeAg and *HBV* viral load (*p* value 0.00).

Conclusion: Majority of the patients belonged to the HBeAg negative immune reactivation (46%) phase followed by inactive CHB (33%). Estimation of serum *HBV* viral load along with ALT levels in patients with CHB infection is essential for appropriate management of the disease.

Keywords: ALT, *HBV* DNA, Immune active, Immune reactivation, Immune tolerant, Inactive CHB.

INTRODUCTION

Hepatitis B virus is a small DNA virus causing a broad spectrum of liver diseases which ranges from acute self-limiting disease to fulminant hepatitis, chronic hepatitis, symptomatic infection, cirrhosis, liver failure, and hepatocellular carcinoma (HCC). The mode of transmission of the virus is mainly horizontal by infected vaginal secretions, semen, blood, saliva, menstrual blood, and reuse of infected syringes and needles.[1] It can also get transmitted vertically from infected mother to fetus.[2] 5-10% of infected adults are unable to clear the virus and become chronic carrier.[3] Persistence of hepatitis B surface antigen (HBsAg) for or more than at least 6 months is defined as chronic hepatitis B (CHB). It is one of the emerging public health concerns. Over 257 million people are estimated to be suffering with CHB worldwide with development to HCC in 15-40% and deaths reported annually due to the same is about 887000.[4] India, with the prevalence of CHB reported to be 1.46% and an estimated 17 million chronic carriers, is considered to be a country with intermediate endemicity of CHB.[5] The natural history of CHB virus infection involves four phases that differ from each other in various parameters like serum alanine transaminase (ALT), HBeAg, status, and *HBV* DNA (*HBV* viral load). Phase1 (immune tolerant) is characterized by the presence of HBeAg, high levels of serum *HBV* DNA, and normal serum ALT. Phase 2 (HBeAg positive immune-active) is characterized by the presence of HBeAg, high or

fluctuating serum *HBV* DNA levels, persistent or intermittent elevation in serum ALT. Phase 3 (inactive CHB) is characterized by absence of HBeAg, presence of anti-HBe, relatively normal ALT levels, and relatively low serum HBV DNA. This phase is often overlapped or named as immune clearance. A hallmark of this phase is flares of ALT. An important outcome of this phase is HBeAg to anti-HBe seroconversion. Phase 4 (HBeAg negative immune reactivation) is characterized by negative HBeAg, positive anti-HBe, detectable *HBV* DNA, and elevated ALT. Serum *HBV* DNA levels are lower than in HBeAg positive patients but may be very high.[6] The aim of the present study was to observe correlation of serological, biochemical, and molecular markers in serum samples of patients with CHB infection from Rajasthan lying under all the above mentioned stages.

METHODS

Patient selection: The study was conducted at the Advance Research laboratory, Department of Microbiology, SMS Medical College, Jaipur from January 2021 to December 2021. In this prospective study, 441 HBsAg positive, treatment naïve cases were shortlisted. Patients with alcoholic liver disease, *HCV* and *HIV* infection were excluded from the study. All these cases were tested for IgM-anti HBc antibody and finally 411 chronic *HBV* cases were selected for further study. Sample size was determined by the patients for whom almost complete clinical data was available. Informed consent and Institutional ethical clearance (Ethical clearance no 2273/MC/EC/2016 dated 29.03.2016) was obtained. Blood samples from all these patients were collected in EDTA (plasma for PCR) and plain vial (serum for serology and biochemistry). All the samples were tested for the below mentioned parameters

Biochemical parameters: Measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels was done using AU680 Chemistry Analyzer system by Beckman Coulter, USA. Quality control measures were strictly followed. The results were expressed as U/L. The values above 35 U/L and 40 U/L for AST and ALT, respectively were considered elevated as per the manufacturer's protocol.

Serological parameters: HBsAg, HBeAg, IgM-anti HBc, and antiHBe were performed by Enzyme Linked Immunosorbent Assay (ELISA) as per the manufacturer's protocol with commercially procured kits by Dia. Pro Diagnostic Bioprobes Srl (Italy).

Molecular parameter: Extraction of DNA was done using High Pure viral nucleic acid kit which is a silica column-based assay and quantitative PCR assays were carried out with Cobas Taqman *HBV* test kit on Cobas Taqman48 Analyzer (Roche Molecular Systems, New Jersey, USA) according to the manufacturer's protocol with detection range from < 6.00E+00 IU/mL to > 1.10E+08 IU/mL.

Statistical analysis was performed by SPSS (IBM SPSS Statistics 24.0, USA). Analysis of continuous variables was done and presented as median (range) with 95% CI. Comparison between means was performed using the Mann-Whitney test according to the underlying distribution of the variables. The association between *HBV* DNA viral load (IU/mL), ALT level, and HBeAg were assessed by Chi-square test for *HBV* viral load as categorical variable. The level of statistical significance was set at 0.05 in all cases.

RESULTS

In this study, male predominance was seen with 287 (70%) out of 411 CHB cases while the number of females was 124 (30%). The median age of the patients was found to be 39 years (ranging 7-89). As per the evidence of HBeAg, *HBV* viral load, and ALT status, 42 (10.21%) patients were found to be in immune tolerant phase, 48 (11.67%) were in HBeAg positive immune active phase, 132 (32.11%) were in inactive CHB phase, and 189 (45.98%) were found to be in HBeAg negative immune reactivation phase. The median of ALT level represented in U/L was 53 ranging from 10 to 1440 while for AST it was 43 ranging from 10-1286 and median for *HBV* viral load was $2.64x10^4$ IU/mL ranging from 6 to $>1.7x10^8$ (Table 1).

Females were having comparatively lower levels of *HBV* viral load (median 16900) than men. These levels were significantly higher in HBeAg positive CHB patients (median $5.1x10^7$) and those with elevated ALT levels (median 14800) than HBeAg negative CHB patients (median 4700) and normal ALT levels (median 25750) (Table 2). ALT levels were also higher in HBeAg positive CHB patients (mean 94.6±161.71) as compared to HBeAg negative CHB patients (mean 50.78±22.93) (Table 3). Significant association was found between ALT levels and *HBV* viral load (p value 0.00) as well as HBeAg status and *HBV* viral load levels (p value 0.00) in all three categories (Table 4).

DISCUSSION

Identification of the phase of infection is important in

Table 1: Clinical characteristics of patients with *HBV* infection

	Total	Immune tolerant	HBeAg positive immune active	Inactive CHB	HBeAg negative immune reactivation	p value
	411	42 (10%)	48 (11.6%)	132 (32.1%)	189 (45.9%)	–
Male, n (%)	287 (70%)	24 (57%)	36 (75%)	92 (70%)	135 (71%)	–
Female, n (%)	124 (30%)	18 (43%)	12 (25%)	40 (30%)	54 (29%)	–
Age (years)	39 (7, 89)	30 (7,67)	33 (11, 86)	37.5(7, 74)	45 (9, 89)	<0.00001
HBeAg		Positive	Positive	Negative	Negative	–
ALT (U/L)*	53(10,1440)	29 (12,39)	98 (62,1440)	28(10,40)	64 (42,98)	<0.00001
AST (U/L)*	43 (10,1280)	25 (11,39)	83.5 (42,1280)	28 (10,35)	53 (37,93)	<0.00001
HBV* DNA	2.04 x 10^4 (<6 - $>1.7x10^8$)	1.1x10^8 (1.1x10^8 ->1.7x10^8)	7.81 x 10^5 (2.2x10^3 ->1.7x10^7)	7.41 x 10^1 (<6-2x10^3)	8.39 x 10^4 (2.2x10^3 -7.4x10^8)	<0.00001

* values represent median (minimal, maximal)
p value <0.05 considered to be statistically significant

Table 2: *HBV* DNA level according to gender, ALT level, and HBeAg status

Characteristics	N	*HBV* DNA (IU/mL) Mean ± SD	Median	p value
HBeAg				
Negative	321	7.8x106 ± 2.7x10^7	4.7x10^3	0.000
Positive	90	6.3x107 ± 5.8x10^7	5.11x10^7	
ALT				
Normal	172	2.7x107 ± 5.1x10^7	2.5x10^2	0.000
High	239	1.4x107 ± 3.5x10^7	1.48x10^5	
Gender				
Female	124	2.3x107 ± 4.7x10^7	1.6x10^4	0.759
Male	287	1.8x107 ± 4.1x10^7	2.48x10^4	

p value < 0.05 considered to be statistically significant

Table 3: ALT level according to HBeAg status

Characteristics	N	ALT (U/L) Mean ± SD	Median	p value
HBeAg				
Negative	321	50.78 ± 22.93	53	0.002
Positive	90	94.6 ± 161.71	73.5	

p value < 0.05 considered to be statistically significant

Table 4: Association of *HBV* DNA with ALT levels and HBeAg status

Viral load	ALT		HBeAg	
	Normal	High	Negative	Positive
<2000	130 (100%)	0 (0%)	130 (100%)	0 (0%)
2000- 20000	2 (2.7%)	7 1(97.3%)	68 (93.2%)	5 (6.8%)
>20000	40 (19.20%)	168 (80.8%)	123 (59.1%)	85 (40.9%)
Total	172 (41.8%)	239 (58.2%)	321 (78.1%)	90 (21.9%)

patients with CHB infection presenting to the clinicians to plan appropriate antiviral therapy. As CHB infection is dynamic in nature, continuous monitoring of *HBV* viral load and ALT levels is important to characterize the phase of infection and for starting, continuing, and stopping the antiviral treatment.[6] Appropriate management of the disease is important for the prevention of mortality and morbidity due to the disease.

In the present study, females showed comparatively lower levels of *HBV* DNA than that of men which is similar to the

findings of other studies like Nita et al.[7] Along with *HBV* viral load, status of HBeAg and ALT levels are also important to be assessed. *HBV* viral load levels were found to be higher in HBeAg positive patients and those with elevated ALT levels which are in agreement with other studies.[8,9]

In the present study, the majority of the patients (189, 45.9%) belonged to HBeAg negative immune reactivation category which is characterized by negative HBeAg, positive anti-HBe antibody, detectable *HBV* viral load, and elevated ALT levels. It is reported that 10-30% patients showing conversion from HBeAg to anti HBe antibody, continue to possess high levels of ALT and *HBV* viral load and 10-20% patients may show reactivation even after many years of inactive carrier state. Most of such patients may harbor mutations in precore and core promoter region.[10] Serum *HBV* viral load levels are lower than in HBeAg-positive patients but it may fluctuate.[10,11]

Next common was the inactive CHB phase with 132 (32.1%) patients in it. This stage is characterized by negative HBeAg, positive anti-HBe antibody, relatively normal ALT levels, and relatively low serum *HBV* viral load. As per the data available, it is estimated that 0.5% of inactive carriers clear HBsAg every year and develop HBs antibody, this increases survival and decreases the risk of liver decompensation.[10]

Whereas only 42 (10%) patients were found to be in immunetolerant phase which is characterized by positive HBeAg, high serum *HBV* viral load, and normal serum ALT levels. It is recommended to test ALT levels every 6 months in such patients to check for transition to immune active or inactive CHB. Antiviral therapy is recommended for adults of > 40 years of age with high *HBV* viral load or with moderate to severe liver necroinflammation. [10]

Among all, only 48 (11.6%) patients were in the immune active phase which is characterized by positive HBeAg, high or fluctuating serum *HBV* viral load, and persistent or intermittent elevation in serum ALT levels. A hallmark of this phase is considered to be flares of ALT levels. An important outcome of the 'Immune active' phase is HBeAg to anti-HBe seroconversion. In children seroconversion occurs in about 2% per year and about 12% in adults per year.[10] It is recommended to give antiviral therapy like Peg-IFN, Tenofovir, or Entecavir in this phase as per guidelines of AASLD to reduce the complications. The reason for choosing these drugs is low chance of drug resistance on long duration of therapy. However, a number of factors need to be considered when giving antiviral therapy like age of patient, family history for HCC, previous treatment etc. As per the reports *HBV* genotype A and B are considered to get better response with Peg-IFN than non A/B genotypes. In untreated individuals the risk of cirrhosis is reported to be 8%-20% while in person with cirrhosis, risk of hepatic decompensation is 20% and for hepatocellular carcinoma it is 2%-5%.[10]

High replication of *HBV* DNA represents disease conditions in both HBeAg positive and negative CHB infection. It is a main independent risk factor in comparison to HBeAg and ALT for cirrhosis and HCC.[12,13] It may not always be associated with the progression of liver disease.[14,15] Along with *HBV* viral load, ALT and HBeAg status are also equally important markers. HBeAg negative CHB infection is considered to be associated with low levels of *HBV* viral load, more significant intrahepatic necro-inflammatory damage, more progressive disease and high number of cirrhosis and HCC as compared to HBeAg positive CHB cases.[16-18] Positivity for HBsAg, absence of HBeAg, elevated ALT levels, and *HBV* viral load are diagnostic for HBeAg negative CHB infection. Usually in HBeAg negative CHB patients, ALT levels reflect the degree of liver damage but that's not in every case. Fluctuation of *HBV* viral load and ALT levels can be seen in different cases of HBeAg negative CHB infection.[18]

Measurement of only single parameters such as either ALT or *HBV* viral load is not sufficient for diagnosis. At times HBeAg negative CHB patients with normal ALT may be misdiagnosed as 'inactive carriers' and mistakenly receive no appropriate treatment. Also in recent studies CHB patients with normal ALT levels were found to be at high risk of cirrhosis and HCC.[19,20]

In the present study, ALT levels showed a significant association with *HBV* viral load in patients categorized in all the four phases. Similar findings were found in a study from Hong Kong on 57 patients.[21]

CHB infection is considered to be a serious condition because of its worldwide distribution and adverse sequelae. The importance, particularly in Asian Pacific region becomes high due to the high prevalence rate. Also in many parts of the world *HBV* infection is also acquired perinataly or in early childhood. The prevalence of CHB in Indians is at an intermediate level with an estimated 40 million subjects infected.[22] Monitoring of CHB patients during antiviral treatment is also very important. Methods for monitoring response to the treatment include

investigations such as serum ALT, AST, HBV viral load, HBeAg, anti HBe antibody, HBsAg, anti HBs antibody, and liver histology.[23] Similar to our findings; recent studies have provided evidences that relationship exists between HBeAg, *HBV* DNA viral load, ALT levels, and patient response to antiviral therapy. Negative or poor correlation has also been reported by few studies.[24,25]

The most important factors influencing progression towards cirrhosis are ALT levels, *HBV* viral load levels and HBeAg status, moreover, *HBV* viral load level >2000 IU/ml and HBeAg affect progress to HCC. Basically host, environment, and viral factors all have a combined affect in the development of cirrhosis and HCC. Factors like use of alcohol, family history of HCC, diabetes, infection with genotype C, presence of precore and core promotor mutations, and *HIV* infection can affect morbidity and mortality related to liver condition.[26,27]

Positive association between *HBV* viral load and ALT levels was found in HBeAg negative CHB patients. Similar findings were reported by Gupta et al[28] and Thompson et al[29] where *HBV* viral load is associated with ALT levels in HBeAg negative CHB patients. Kim et al[19] also reported association between ALT levels and *HBV* viral load in 82 HBeAg negative CHB patients. Thus, levels of ALT may reflect the replicative status of the virus and are associated with the activity of hepatitis in *HBV* carriers.

Limitation of our study was that liver biopsy was not done which would have given the status of fibrosis and severity of inflammation and helped in ruling out other liver related disorders. Other noninvasive methods which indicate fibrosis like FIB-4, vibration controlled transient elastography, and AST to platelet ratio index (APRI) could also be tried but are more useful in excluding advanced fibrosis. Moreover detection of precore and core promoter regions was also not done which would also have helped in guiding clinical management better.

Many point of care methods are now available to test viral load which has made the monitoring of *HBV* easy. Moreover the National Viral Hepatitis Control Program (NVHCP) is providing free diagnostic, treatment, and monitoring facility to all the hepatitis patients and has a mandate to reduce numbers of *HBV* cases and elimination of *HCV* by 2030.[30] With easy and free access to *HBV* viral load testing under NVHCP the patients who were tested negative for HBeAg and having normal ALT levels but high *HBV* viral load are being treated timely which would have been mislabeled as inactive carrier earlier in absence of *HBV* viral load testing. The present information will help them to plan the logistics for management of CHB patients.

CONCLUSION

Our findings have practical advantages of correlating serological, biochemical, and molecular viral markers in CHB patients which will help the NVHCP program managers to plan for monitoring and treatment of the patients. Majority of the patients belonged to the HBeAg negative immune reactivation (46%) phase and were more than 45 years old followed by inactive CHB (32%) phase. Higher prevalence of CHB was seen in males than females.

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Conflict of interest: The authors declare no conflict of interest.

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