ORIGINAL ARTICLE

Hepatitis B viral load in diagnosing different clinical stages of chronic hepatitis B in a tertiary care hospital in North Kerala

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ABSTRACT

Background: The diagnosis of chronic hepatitis B (CHB) infection has progressed from serological to molecular diagnostic methods. The newer sensitive technique of quantitation of hepatitis B virus (HBV) DNA by real-time polymerase chain reaction (RT-PCR) has helped in understanding the clinical stages of CHB, deciding on treatment and monitoring treatment response. **Aim:** This study aimed to determine the HBV DNA load by quantitative RT-PCR in the various clinical stages of CHB. **Materials and Methods:** Blood samples of CHB patients from the Gastroenterology Department received from June 2014 to December 2014 in the Microbiology Department were subjected to quantitative PCR analysis for HBV DNA. However, to facilitate analysis, only those patients' samples where hepatitis B e antigen (HBeAg) and alanine aminotransferase (ALT) status were known were selected for the study. Statistical analysis was done using SPSS (PASW statistics 18) for windows software. Chi-square test was used to analyse the differences in DNA level between the study groups. **Results:** A total of 71 CHB patients were included in the study. Of these, 29 (40.8%) were inactive carriers (HBeAg-negative ALT normal) and 42 (59.2%) were chronic active hepatitis B patients (ALT elevated with HBeAg-positive and HBeAg-negative cases). HBeAg was positive in 26 (36.6%) and negative in 45 (63.3%) patients. Among the 45 HBeAg-negative patients, 16 (22.5%) had CHB. Of the 71 CHB patients, 61 (85.9%) had detectable viral load. Serum HBV DNA load of 16 patients who were HBeAg-negative was significantly lower (median 5.5×10^5) than that of 26 patients who were HBeAg-positive (median 2.4×10^8) and higher than the 29 inactive carriers (median 1.6×10^3). Based on HBV load, 14 CHB patients who were HBeAg-positive and seven who were HBeAg-negative were started on antiviral therapy. **Conclusion:** Quantitation of HBV DNA based on HBeAg and ALT status helps to determine the stages of CHB. It could play an important role in assessing the status of those patients who are HBeAg-negative and inactive carriers with respect to viral load, as the former require treatment. The major role of HBV DNA determination is to evaluate patients with CHB (HBeAg-positive or HBeAg-negative) and to decide on antiviral therapy.

Key words: Alanine aminotransferase, chronic hepatitis B stages, hepatitis B e antigen, quantitative hepatitis B virus DNA

INTRODUCTION

Hepatitis B virus (HBV) is the leading cause of chronic viral hepatitis in humans and approximately, one million people die annually due to both acute and chronic infections.[1] Clinically, chronic infection ranges from the inactive carrier state to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[2] Less than 5% of adults, 10–25% of young children and 80–90% of infants progress to chronic hepatitis B (CHB) infection.[3] It has different clinical stages with each having varying degrees of liver damage. Assessing these clinical stages is essential to manage CHB infection.

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The diagnosis of CHB is based on the persistence of HBsAg for at least six months.[4] Molecular assays are now used to quantify HBV DNA in serum which is a reliable marker of active HBV replication. It helps in defining the phase of chronic infection, the treatment indication and in assessing the efficacy of antivirals. Studies have indicated

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that quantitative HBV DNA is also a strong predictor of progression to cirrhosis.[5]

The stages of CHB infection include the immune-tolerant phase, the immune-active phase (HbeAg-positive or HbeAg-negative CHB) and the inactive phase (inactive carrier). Not all patients go through all these stages.

There are definitions of clinical stages of CHB according to the American Association for the Study of Liver Diseases (AASLD) [Table 1].[6]

The immune-tolerant phase occurs most frequently in persons who are infected via perinatal transmission from HBeAg-positive mothers. It can last for a few years to more than 30 years. After some years in this phase, most persons eventually develop HBeAg-positive chronic hepatitis. This phase may last for several weeks to several years. After this phase, the infection may become inactive or may evolve into HBeAg-negative CHB after a quiescent phase. In HBeAg-negative CHB infection, patients are usually symptomatic. This stage is due to infection with a mutant HBV variant arising due to viral mutations in the pre-core or core promoter regions preventing HBeAg production.[7] Accurate diagnosis of HBeAg-negative CHB is imperative because the clinical management is markedly different from that of inactive carrier state. This is possible only with quantitative estimation of DNA.

Alanine aminotransferase (ALT) is most commonly used to evaluate liver disease.[8] In patients with HBeAg-positive CHB infection, ALT may show mild-to-moderate elevations. Patients in the immune-tolerant phase and inactive carriers have persistently normal ALT levels, while a proportion of patients with HBeAg-negative CHB infection may have intermittently normal ALT. According to the AASLD guidelines, ALT is raised when it is two times the upper limit normal for an ALT level of 30 U/L for men and 19 U/L for women.

Table 1: Phases of chronic hepatitis B

Phase	HBeAg	HBV DNA	ALT	Liver histology
Immune-tolerant	Positive	Elevated, typically >1 million IU/ml	Normal or minimally elevated	Minimal inflammation and fibrosis
Immune-active phase (HBeAg-positive)	Positive	Elevated >20000 IU/ml	Elevated	Moderate-to-severe inflammation or fibrosis
Immune-active phase (HBeAg-negative)	Negative	2000 IU/ml to 2 million IU/ml	Elevated	Moderate-to-severe inflammation or fibrosis
Inactive CHB phase	Negative	<2000 IU/ml or undetectable HBV DNA	Normal	Minimal necroinflammation but variable fibrosis

HBV: Hepatitis B virus deoxyribonucleic acid; ALT: Alanine aminotransferase; CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen

HBeAg is considered to be the principal marker of HBV replication and high infectivity and indicator of liver injury. Before the availability of molecular diagnostic methods, clinicians used to rely on HBeAg status and ALT level to assess infectivity of HBV.[9] However, in many populations, especially in the Mediterranean region, HBV DNA has been found in the serum in the absence of HBeAg and presence of anti-HBeAg.[10] In these patients who are HBeAg-negative, detection of HBV DNA is the only reliable marker of active HBV replication.

Treatment of CHB relies on the clinical status of the patient, serum HBV DNA and ALT levels, HBeAg status and liver histology if it has been done.[11] The European Association for the Study of the Liver (EASL) guidelines recommend a cutoff value of 2000 IU/ml (approximately 10^4 copies/ml) irrespective of HBeAg status for starting antiviral therapy. Drugs for the treatment of CHB include interferon-α, pegylated interferon-α, lamivudine, adefovir, entecavir, telbivudine and tenofovir.

MATERIALS AND METHODS

The study aimed to determine HBV DNA in different stages of CHB in patients with known HBeAg and ALT status.

A seven-month retrospective study on CHB patients visiting Gastroenterology Department from June 2014 to December 2014 was conducted in the Department of Microbiology, Government Medical College, Kozhikode. HBV DNA was quantitated in these patients with known HBeAg and ALT status. ALT had been done twice in six months.

Inclusion criteria

CHB patients (HBsAg-positive longer than six months) comprising HBeAg-positive with elevated ALT, HBeAg-negative with elevated ALT and HBeAg-negative with normal ALT (the three studied groups) were included in the study.

Exclusion criteria

Pregnant women, patients on treatment and patients co-infected with hepatitis C and HIV were excluded from the study.

The stage of the liver disease was determined with respect to the AASLD practice guidelines for CHB infection.

Blood samples (3–5 ml) were collected for HBV DNA quantitation.

Hepatitis B virus DNA extraction and amplification

Blood samples were centrifuged within four hours of receipt to obtain the serum fractions, which were then divided into aliquots and kept at −80°C till subjected to nucleic acid extraction. This was done using Qiagen DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. DNA was extracted by lysis of virus particles using proteinase and lysis buffer followed by alcohol precipitation.

Serum HBV DNA was quantified by real-time polymerase chain reaction (RT-PCR) in 48-well MiniOpticon (Bio-Rad, USA) based on TaqMan principle and using Geno-sense HBV quantitative PCR kit (Genome Diagnostics, New Delhi, India). HBV-specific primers were derived from the conserved core/pre-core region of the HBV genome. The direct detection of the specific amplicon in fluorescence channel in the MiniOpticon thermocycler was by measuring the fluorescence emitted during amplicon production at each PCR cycle. Amplification and quantification of PCR product occurred simultaneously in the same closed reaction vessel and viral load was determined based on the quantity of standards. Known amounts of standards 2.5 × (10^3, 10^4, 10^5, 10^6 and 10^7 IU/ml) of HBV DNA were used as positive controls. Molecular grade water was used as the negative control. The reaction mix included 15 µl of the master mix and 10 µl of extracted DNA which was added to labelled 0.2 ml PCR tubes and loaded into 48-well PCR machine. The PCR cycle (45 cycles) was programed in the computer and the reaction was set up. Quantitation was done by the machine using the external positive controls and viral load was expressed in IU/ml (1 IU/ml = 5.6 copies/ml).

Data analysis

Statistical analysis was done using SPSS (PASW statistics 18) for windows software. Age was expressed as mean ± standard deviation (SD). HBV DNA level of each group was expressed as the median value and range. The differences in DNA between groups were analysed using Chi-square test. $P < 0.05$ was considered as statistically significant.

RESULTS

The demographic and clinical data of the three studied groups were compared with respect to age, sex, ALT levels and HBV DNA levels [Table 2]. There was no significant difference in age and sex between the three groups. The mean age of the patients was 37.80 ± 13 years, SD ranging from 16 to 66 years. Males dominated the study groups with a total of 44 (61.9%) and 27 (38%) were females. Of the 71 CHB patients, 26 (36.6%) were HBeAg positive and 45 (63.3%) were HBeAg-negative. HBV DNA was detected in all the 26 HBeAg-positive patients who had elevated ALT. Of the 45 HBeAg-negative patients, 35 (77.7%) had detectable DNA. Of these, only 16 patients (22.5%) had raised ALT and were diagnosed as CHB patients with HBeAg-negative status [Figure 1]. The remaining 19 patients with normal ALT and detectable DNA comprised the inactive carriers. DNA was not detected in the remaining 10 HBeAg-negative patients with normal ALT. A total of 29 (40.8%) patients comprised inactive carriers [Figure 1].

Baseline serum HBV DNA levels among patients who were HBeAg-negative were significantly lower than the HBeAg-positive patients (median 5.5 × 10^5 vs. 2.4 × 10^8; $P < 0.05$) and significantly higher than the inactive carriers (median 5.5 × 10^5 vs. 1.6 × 10^3; $P < 0.05$) [Figure 2]. We

Table 2: Characteristics of CHB patients

Patient characteristics	HBeAg-positive *n*=26	HBeAg-negative *n*=16	Inactive carriers *n*=29
Age			
Mean+SD	35.15+14.6	36.8+10.7	40+12,9
Range	16-66	20-55	18-61
Sex			
Male	15 (57.7%)	12 (75%)	17 (58.6%)
Female	11 (42.3%)	4 (25%)	12 (41.4%)
HBsAg	Positive	Positive	Positive
HBeAg	Positive	Negative	Negative
ALT (IU/L)	Raised	Raised	Normal
DNA LOAD (copies/ml)			
No	26 (100%)	16 (100%)	19 (65.5%)
Median	2.4×10^8	5.5×10^5	1.6×103
Range	(2.1×10^5-1.5×10^{10})	(1.3×10^4-2.9×10^7)	(1.9×10^2-8.3×10^3)

SD: Standard deviation; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; DNA: Deoxyribonucleic acid

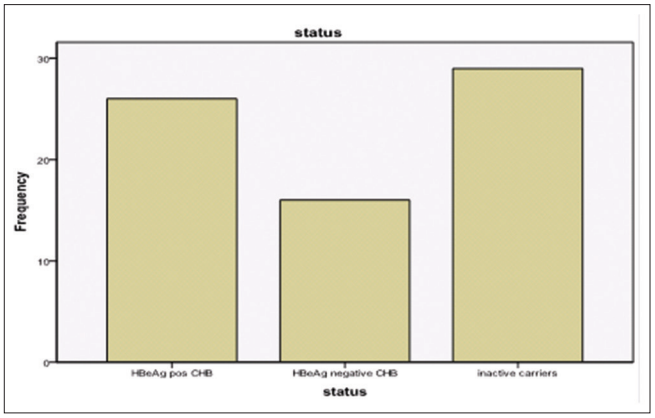

Figure 1: Distribution of patients in the three studied groups

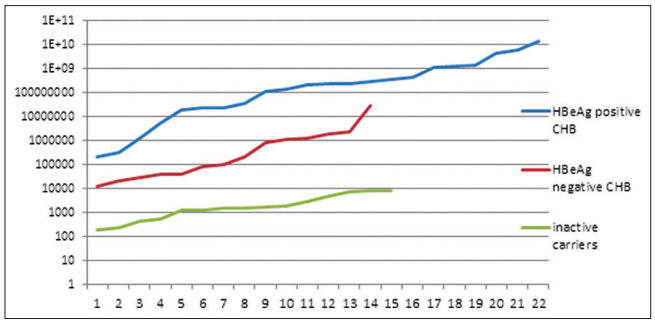

Figure 2: Hepatitis B virus DNA load in the three studied groups

had a follow-up of 21 patients (HBeAg-positive 14 and HBeAg-negative 7) who were started on antiviral therapy based on their viral load. According to the EASL practice guidelines for the treatment followed in Gastroenterology Department, antiviral therapy is started when DNA load is $>10^4$ copies both in HBeAg-positive and HBeAg-negative CHB patients.

DISCUSSION

The study aimed at determining the stages of CHB based on HBV DNA load, HBeAg and ALT status. The stages included CHB (HBeAg-positive and HBeAg-negative) and the inactive carriers. HBV DNA was detected in all the 26 HBeAg-positive patients. Studies have shown the presence of HBV DNA in 83–100% of HBeAg-positive patients.[12]

There was a significant difference in serum HBV DNA viral load between HBeAg-positive and HBeAg-negative chronic hepatitis patients and inactive carriers. The median HBV DNA viral load was higher in CHB patients who were HBeAg-positive (median 2.3×10^8 copies/ml) than in those who were HBeAg-negative CHB (5.5×10^5 copies/ml). Xie *et al*.[13] and Kessler *et al*.[14] have also reported that HBV DNA levels in HBeAg-positive group were significantly higher than those in HBeAg-negative group. The latter group had higher DNA load than inactive carriers (median 5.5×10^5 vs. 1.6×10^3). Similar findings were seen in studies by Heo *et al*.[15] and Changotra *et al*.[16] ALT levels were raised in both the groups. In many studies, raised ALT has been seen associated with HBV DNA load.[17] In the present study, the highest load in HbeAg-negative chronic hepatitis patients was 2.9×10^7 whereas in HBeAg-positive patients, the highest load was 1.5×10^{10}. HBeAg-negative strain emerges during immune clearance of a wild-type strain, when increased immune pressure on the wild-type strain leads to selection of the HBeAg-negative mutant. It may be spontaneous or may occur during treatment.[5]

Among the 29 inactive carriers who were also HBeAg-negative and on follow-up, 19 had viral load and ALT was normal. The highest load detected was 8.3×10^3. Inactive carriers form the largest group in chronic HBV-infected patients with a low risk of progressing to cirrhosis and HCC.[18] Periodical follow-up with HBV DNA level and liver enzymes is necessary in them to diagnose the development of active hepatitis. It is difficult sometimes to differentiate between HbeAg-negative chronic hepatitis and inactive carriers as they share their serological status.[19] This is where HBV DNA load determination plays an important role.

CONCLUSION

CHB infection is a serious viral disease; in the absence of careful monitoring, it can lead to the development of HCC and cirrhosis in more than one-third of the patients. HBV DNA load varies between the different clinical stages. Hence, patients should be followed regularly with ALT and HBV DNA level. HBV DNA viral load testing is a crucial tool to monitor and manage CHB patients and to differentiate clinically between HbeAg-negative CHB and inactive carriers.[13] RT quantitative PCR enables accurate determination of the viral DNA levels in these patients.

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Conflicts of interest

There are no conflicts of interest.

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