ORIGINAL RESEARCH PAPER

Volume-7 | Issue-3 | March-2018 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

HEPATITIS B VIRUS GENOTYPE AND DISEASE SEVERITY IN CHRONIC HEPATITIS B PATIENTS FROM NEW DELHI

Microbiology

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ABSTRACT

Introduction: Chronic infection with different genotypes of the hepatitis B virus (HBV) is known to affect disease progression and the clinical outcome. Information from this study will aid in gaining a better understanding of the pathogenesis of chronic hepatitis B (CHB) as well as to gauge the extent of liver damage caused by the different HBV genotypes prevalent in New Delhi.

Material and methods: In a study group of 50 HBsAg positive CHB patients, the HBV genotype was identified by using PCR-RFLP analysis of the S gene. Patients were evaluated clinically, and were subjected to various serological and histopathological investigations.

Results: The most common HBV genotype was D (31/50, 62%), followed by A (15/50, 30%) and mixed A/D genotype (4/50, 8%). Patients infected with HBV genotype D had significantly higher levels of serum viral load and a higher median histological activity index (HAI) score when compared to genotypes A and A/D ($p<0.05$).

Conclusions: These findings suggest that Genotypes A, D and A/D exist in CHB patients in New Delhi, and among these Genotype D is the commonest and is associated with a greater derangement of liver function.

KEYWORDS

HBV, Genotype, PCR-RFLP, Liver function, Hepatitis

Introduction

The Hepatitis B virus (HBV) is a member of the Hepadnaviridae family. HBV is a partially double stranded and enveloped DNA virus. HBV infection is one of the major global public health problems and is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[1] The virus is transmitted by perinatal, percutaneous, and sexual exposure. Since 1991, the World Health Organization has recommended the implementation of mass immunization programs. This has drastically reduced the incidence of HBV infection in many countries.[2,3]

Hepatitis B virus infection is characterized by an extremely variable course of disease comprising of a plethora of clinical manifestations. The clinical picture depends on the patient's age at infection, the patient's immune status, and the stage at which the disease is recognized. In majority of cases, hepatitis B infection is asymptomatic. Asymptomatic infection can be identified by virus-specific serologic tests and by detecting biochemical abnormalities in their blood. In others, early symptoms may be non-specific. Most adult patients recover completely. Rarely, some patients may develop fulminant hepatitis, but others may progress to become asymptomatic carriers or develop chronic hepatitis and progress to development of associated complications of chronic liver disease.[4,5] Chronic liver disease represents a series of liver disorders of varying causes and severity in which hepatic inflammation and necrosis continue for at least 6 months. HBV infection is a major cause of chronic liver disease, along with alcohol abuse and autoimmune disorders.

Several characteristics of hepatitis B virus appear to be associated with variations in the severity of liver disease. HBV genotype may determine the clinical outcome and the genotypic correlation has been associated with HBeAg seroconversion, activity of liver disease and treatment response.[6] Currently, at least 10 HBV genotypes and several subtypes have been identified; they have distinct geographic distribution. Genotype A and D occur frequently in India, Europe and Africa, while genotype B and C are prevalent in Asia.[7]

The measurement of serum HBV DNA is a marker of viral persistence and replication and is also used to monitor treatment response in HBV infection. A high response rate to antiviral chemotherapy in patients having a low baseline level of HBV DNA has been demonstrated. Therefore, the interplay of replication ability of the virus and the host immune response has been considered as the prime determinant of the likelihood of liver injury and its intensification to cirrhosis.[8,9]

In view of the above, this study was undertaken as among the few published reports comparing HBV genotypes with liver disease severity from the Indian subcontinent, there have been inconstant findings. [10-12] These conflicting findings emphasize the need for a thorough investigation between the HBV genotype, the Hepatitis B viral load and their relevance to Chronic Hepatitis B. The present study has been undertaken to investigate the association between the viral genotype and viral load among subjects with chronic hepatitis B in a tertiary care hospital.

Materials and methods

Study population. The study population comprised of adults who attended the inpatient and outpatient services in our hospital from October 2011 to March 2013. Patients who were seropositive for HBsAg for at least 6 months and who had never received antiviral therapy were selected for this study while those with other concomitant causes of liver disease were excluded. Informed consent was taken from all the subjects and their confidentiality was maintained throughout the study. The study group was subjected to a structured questionnaire, and were further evaluated on the basis of history, clinical examination, liver function tests, and serological tests for hepatitis B using commercially available ELISA kits. Other investigations included an ultrasound of the abdomen and a liver biopsy where indicated and feasible. Based on this criteria a study group of 50 subjects was formed and selected for this study. The present study was approved by the Institutional Ethics Committee of Maulana Azad Medical College, New Delhi.

Laboratory Investigations. Serological tests were performed using commercially available ELISA kits according to the manufacturer's protocol. A battery of serological tests were performed in all the study samples and which included tests for HBsAg, Anti-HCV, HBeAg, IgG Anti-HBe, Anti-HBsAg, IgM Anti-HBc and IgG Anti-HBc. Those cases that had evidence of HBV infection were further subjected to HBV DNA viral load determination by RT PCR, and viral genotype was determined using PCR-RFLP analysis of the S gene.

Hepatitis B viral load by Real-Time PCR. Quantification of hepatitis B virus in serum was carried out using the COBAS® TaqMan® HBV Test according to the manufacturer's instructions. This is a highly sensitive test that offers a broader dynamic range than previous generation tests.

PCR-RFLP analysis of the S gene. The viral genotypes were determined through RFLP analysis of the S gene as described by

Mizokami et al.[13] Serum DNA was extracted using a commercial viral DNA extraction kit (Qiagen) following the manufacturer's protocol. In the next step, 1µL of extracted DNA was amplified by nested PCR. HBV S-gene sequence from nt120 to nt604 (485 bp) was amplified and subjected to restriction digestion by the enzymes: AlwI, Hphl, NciI, NlaIV and EarI at 37 ^{0}C. The digested PCR products were then electrophoresed on agarose gel and the RFLP pattern was visualized under U.V transilluminator. (Figure 1,2)

Based on the results of abdominal ultrasonography and liver biopsy, Histological Activity Index (HAI) scores were determined using the Ishak scoring system.[14] Patients with a HAI score of 4-8, 9-13 and 14-18 were classified as having mild, moderate and severe chronic hepatitis respectively.

Statistical analysis. Statistical analysis was performed using SPSS ver. 16 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism ver 7.0 (GraphPad Software Inc., La Jolla, CA, USA). The data was tabulated and presented in simple proportions and the difference between them was assessed by using either Pearson's chi-squared test or Fisher's exact test, for its statistical significance. ANOVA was used to analyze continuous numerical parametric data and for the analysis of non-parametric data, the difference between medians of three or more groups was assessed by Kruskal-Wallis test. This was followed by post-hoc tests if indicated. Pearson's correlation coefficient was used to determine the correlation between the HBV genotype, viral load and various host factors. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The demographic details of the study group are presented. (Table 1) The majority of study subjects were males between 36-45 years of age and male gender was significantly associated with severe chronic hepatitis. (Table 2)

Correlation of host and viral factors with severity of liver disease. Out of the 50 chronic hepatitis B patients, based on the patients' histological activity index (HAI) scores, 24 had severe, 15 had moderate and 11 had mild chronic hepatitis. The predominant genotype was genotype D (31/50, 62%), followed by genotype A (15/50, 30%) and a mixed genotype A/D (4/50, 8%). The median serum viral load was higher in patients with HBV genotype D infection. (Table 3,4) Patients with genotype D infection had greater disease severity as compared to patients infected with genotypes A and genotype A/D. (Table 5)

Discussion

The present study aimed at determining the HBV genotype and to find a correlation between the viral characters and the level of disease severity in patients with chronic hepatitis B infection. Chronic viral hepatitis infections likely account for the majority of both cirrhosis and HCC globally and in nearly all regions of the world. In 1990, 60% and 24% of global HCC could be attributed to HBV and HCV respectively.[15] An update of this analysis for 2002 yielded HBV- and HCV-attributable fractions of 54% and 31%.[16] Perz et al reported in 2006 that worldwide, 57% of cirrhosis was attributable to either HBV or HCV and 78% of HCC was attributable to HBV or HCV.[17] The lifetime risk of HBV carriers to develop cirrhosis, liver failure, or HCC may be as high as 15–40%.[18]

Recently hepatitis B viral factors have been identified to have a correlation with clinical outcomes. These viral factors include basal core promoter mutants, viral genotype, pre-S deletion mutants and the serum viral load among others. The patients with high HBV DNA level and infected with HBV genotype C or D have a poorer response to antiviral therapy with interferon and have a high incidence of adverse clinical outcomes. [19-22]

In the present study, male patients had more severe disease. This might be explained by findings provided by Chu et al and London et al, who in separate studies have postulated that, HBV is retained for a longer period of time and the chances of HBV DNA integration is higher in males than females. Therefore, males are more likely to become chronic carriers of HBV and as a consequence, are more likely to develop chronic hepatitis. However, further research is required to discover the ultimate mechanism which regulates this difference.[23,24]

Patients were classified as having Mild, Moderate or Severe chronic hepatitis based on the modified HAI scoring system proposed by Ishak et al. The Ishak system is a recent modification of the Knodell HAI, and is one of the most widely used quantitative histologic scoring systems. It provides consecutive scores for well-defined lesions within 4 separate categories (periportal hepatitis, confluent necrosis, focal inflammation and portal inflammation) that are added together for the activity grade. The maximum possible score for grading is 18. Pathologic examination of percutaneous biopsy specimens is considered to be the criterion for grading the severity of necroinflammation and for assessing the extent of fibrosis and the progression of liver cirrhosis.[25,26]

No clear association between the disease severity and other clinical features could be established. The reason for this could be that chronic hepatitis is characterized by an inefficient T cell response, that is unable to completely clear HBV from the liver. Consequently, the virus sustains continuous cycles of low-level cell destruction and over long periods of time, recurrent immune-mediated liver damage contributes to the development of cirrhosis and hepatocellular carcinoma. Therefore, even while the expression and retention of viral proteins in hepatocytes may influence the severity and progression of liver disease, the mechanisms of liver injury in viral hepatitis are believed to be due to the host immune response to viral proteins expressed by infected hepatocytes and not due to the direct cytopathic effects of viruses.[27] Several authors have reported that the major feature of the symptomatology of early or slowly progressive liver disease in chronic viral hepatitis is its highly variable nature. A long asymptomatic phase followed by signs associated with cirrhosis or decompensation is not uncommon.[28-30]

In our study, through RFLP analysis of the S gene, it was discovered that Genotype D was predominant (62%), followed by Genotype A (30%), and a mixed genotype (A/D) (8%). HBV genotypes have distinct geographical distributions. Genotype A is most frequently found in Europe, North America, and Sub-Saharan Africa. Genotypes B and C are confined to Asia and Oceania. Genotype D is the most widely distributed genotype and has been found universally in southern Europe, North Africa, India, China, and West and South Africa, and intravenous drug users on all continents.[31] The present study demonstrates that HBV genotype D is highly prevalent. The predominance of genotype D in India has also been reported by various authors.[32-35]

HBV genotypes have been correlated with various epidemiological, virological and clinical variables. In our study, we found a significant positive correlation between genotype D cases and severe histopathological findings and a higher viral load, as compared to genotype A and A/D. These results are in accordance with a previous study by Thakur et al[10] who studied the association of HBV genotypes and disease severity in 130 patients with histologically proven CHB and concluded that CHB patients with viral genotype D infection had more severe disease as compared to genotype A. In a western study, Sanchez et al[36] have reported that genotype A patients are more likely to enter remission, have a better prognosis and experience clearance of HBV DNA as opposed to those infected with genotype D. While studying patients from Romania, Constantinescu et al[37] concluded that genotype D is associated with active viral infection and a greater risk of hepatocellular carcinoma as compared to genotype A. However, our result differs from the findings of Kumar et al[11] who studied 70 patients attending the outpatient and inpatient services of a tertiary care hospital in Lucknow, India. They reported that genotype A was associated with more severe disease as compared to genotype D. In a study carried out in 2012 to investigate genotype impact on long-term virological outcome of chronic HBV infection, Malmström et al[19] reported that genotype C or D infection often remained highly active, implying a risk for progressive liver damage. This suggests that HBV genotypes may have clinical significance; however, pathogenetic mechanisms contributing to the differences in disease between HBV genotypes remain to be clarified.

HBV genotype has been reported to correlate with response to interferon. Kao JH et al[38] and Wai CT et al[39] have reported that patients with genotype B had a higher rate of HBeAg seroconversion compared to genotype C. Another study, from Spain, suggested that genotype A had a higher rate of seroconversion than genotype D.[36] Recent studies with pegylated interferon (IFN) confirmed that HBeAg seroconversion occurred more often with genotypes A (47%) and B

(44%) as compared to genotypes C (28%) and D (25%).[40] The impact of HBV genotypes on response to lamivudine therapy has been studied in various countries. Sanchez-Tapias et al compared the rate of resistance to lamivudine in patients with HBV genotype A and those with genotype D infection and came to the conclusion that the rate of lamivudine resistance was higher in the former group. They reported no significant difference in the risk of lamivudine resistance when comparing patients with genotypes B and C. However, the virological response was worse in patients with genotype C during lamivudine therapy.[36] The response to lamivudine is poorer in patients infected with subtype Ba, which contains a recombination with genotype C, than in those with subtype Bj without such a recombination.[41] Influence of genotypes A and D on therapeutic response was examined by Chattopadhyay et al[34] , who observed sustained virological response on treatment with lamivudine in 28.5% of patients of genotype A while it was seen in 37.5% of patients of genotype D, but their result was not statistically significant. There is also paucity of information on the correlation between HBV genotypes and outcome of acute HBV infection. A report from Switzerland showed that 80 patients with acute HBV had genotype D and 80 patients with chronic hepatitis B had genotype A.[42] This implies that rates of progression from acute to chronic infection may differ for different HBV genotypes.

In summary, the higher viral load and severe histopathological findings in our patients with genotype D infection, indicates a possible association of genotype D with occurrence of a more severe liver disease. Our observations support the view that HBV genotypes influence the severity of liver disease and have an important impact on the selection of patients for antiviral therapy and response to therapy. This may also help design individualized medicine for the effective treatment of patients with chronic hepatitis B. It might be prudent to consider undertaking HBV genotyping before the onset of treatment and all genotyping data must be correlated with liver biopsy assessments. Since the distribution of HBV genotypes within the Indian subcontinent appears to be markedly different, large scale studies are required to understand the clinical, therapeutic, and epidemiological differences among all HBV genotypes prevalent in our country.

Acknowledgements
The authors sincerely thank all the study subjects and the Department of Medicine, Lok Nayak Hospital, New Delhi, for assistance in recruiting the subjects.

Conflict of Interest
The authors declare no conflict of interest.

TABLE 1: ASSOCIATION OF HOST DEMOGRAPHIC FACTORS WITH DISEASE SEVERITY

		Mild (n=11)	Moderate (n=15)	Severe (n=24)	p value
Age (mean ± SD)		32.16 ± 8.90	34.87 ± 6.08	41.36 ± 9.91	0.100
Sex	Male	7 (64%)	9 (60%)	22 (92%)	**0.036***
	Female	4 (36%)	6 (40%)	2 (8%)	

*statistically significant value

TABLE 2: UNIVARIATE ANALYSIS OF SIGNIFICANT HOST DEMOGRAPHIC FACTORS

	Severe (n=24)	Non-severea (n=26)	p value	Odds ratio at 95% CI
Age > 40 year	14	9	0.092	2.64
Male gender	22	16	**0.012***	**6.87**

a. Mild and Moderate chronic hepatitis. **statistically significant value*

TABLE 3: ASSOCIATION OF VIRAL GENOTYPE WITH VIRAL LOAD

Genotype	**Viral load**			
	Median	Range	IQR	p value
A	110000	16800 - 223008	72500 - 145500	< 0.001*
D	412650	89000 - 4171000	138500 - 1910000	< 0.001*
A/D	52900	11000 - 114800	25800 - 87915	< 0.001*

*statistically significant value

Table 4: DUNN'S MULTIPLE COMPARISONS TEST

Comparisons (Viral load)	**Mean rank difference**	**p value**
Genotype A/D vs Genotype A	-10.08	p > 0.05
Genotype A/D vs Genotype D	-26.09	**p < 0.05***
Genotype A vs Genotype D	-16.01	**p < 0.05***

*statistically significant value

TABLE 5: ASSOCIATION OF VIRAL GENOTYPE WITH DISEASE SEVERITY

Genotype	Mild (n=11)	Moderate (n=15)	Severe (n=24)	p value
A	3 (27%)	1 (7%)	4 (17%)	0.413
D	4 (36%)	6 (40%)	18 (75%)	0.040*
A/D	1 (9%)	3 (20%)	0 (0%)	0.057

*statistically significant value

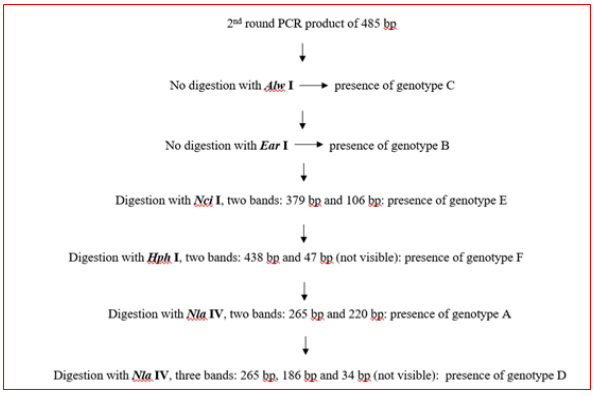

Figure 1: Strategy for HBV genotyping by PCR-RFLP method

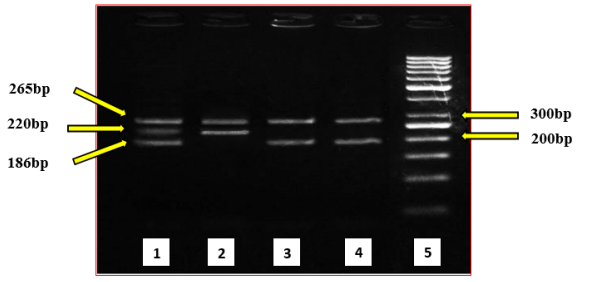

Figure 2: Restriction digestion by Nla IV

Lane 1: 186bp, 220bp & 265bp fragments of mixed Genotype A/ D, Lane 2: 220bp and 265bp fragments of Genotype A, Lanes 3-4: 186bp and 265bp fragments of Genotype D, Lane 5: 50bp DNA ladder

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