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Study of Genotypic Variants of Hepatitis B Virus, Their Viral Load and Correlation with Alanine Aminotransferase Levels in HBsAg Positive Patients

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Abstract: Hepatitis B virus (HBV) is the most common potentially life threatening chronic viral infection affecting millions worldwide. More than 240 million people have chronic HBV liver infections. (serum Alanine amino transferase (ALT) level is the most commonly aced variable for assessment of liver disease) High genetic variability is a characteristic feature of the HBV as the viral polymerase lacks proof reading.Genotyping of HBV is essential for characterization of patients groups and for epidemiological studies. Typical chronic hepatitis B is marked by the presence HBsAg and high levels of HBV DNA with variable elevation of ALT and histological activity.The study was done to study in patient attendance OPD of SMIH Dehradun to study prevalence of various genotypic variants, their association with ALT levels and estimation of HBV viral load in various genotypes. A total of 78 patients were taken 53 were HBsAg Positive (test) and 25 were HBsAg negative (control) all blood samples were subjected to estimation of ALT and quantification genotyping of HBV. The HBV genotypes were characterized through A-F. 43 specimens were subjected for HBV genotyping of which genotype D was most prevalent and was found in 23 patients (53.48%) followed by genotype A in O (23.33%) patients. Genotype B, C and E were found in 05 (11.62%), 02 (4.65%) patients respectively and a rare genotype F in 1 (2.33%) was also found correlation. There is no significant (p=0.121) between various HBV genotype and HBV DNA load. But significant correlation (p=0.012) was found between HBV genotype D and A and viral load. There was no association found between HBV genotype and serum ALT levels (p=0.575).
Keywords: Hepatitis B, Alanine amino transferase,HBsAg, HBV DNA.

INTRODUCTION

Hepatitis B virus (HBV) is the most common, potentially life-threatening chronic viral infection affecting millions worldwide. More than 240 million people have chronic liver infections. About 600 000 people die every year due to the acute or chronic consequences of hepatitis B [1]. HBV is 42nm DNA virus belonging to the hepadnavirus family. The DNA is partially double stranded and contains 3200 nucleotides with overlapping coding regions, leading to several major open reading frames [2]. Different genotypes of HBV have shown different characteristics [3]. Currently used diagnostic techniques for HBV includes HBsAg, PCR and anti-HBcIgM [2].HBs Ag is the first serologic marker to appear, although HBV DNA may be detected slightly earlier. HBs Ag usually appears 1 to 2 months after infection and before the onset of clinical illness, and is the last protein marker to disappear [2]. The ability to detect HBV DNA in serum has been reported to have prognostic value for the outcome of acute and chronic HBV infections. A quantitative test for the measurement of HBV DNA is a valuable tool that can be used in conjunction with other serological markers in the management of HBV infection. Quantitation of Hepatitis B virus DNA (genotypes A to G) can be done over the range 6-110,000,000 IU/mL (Conversion factor: 5.82 copies= 1 IU). This is intended for use in conjunction with clinical presentation and other laboratory markers as aid in assessing viral response to antiviral treatment as measured by change in HBV DNA levels [4, 5]. Serum alanine aminotransferase (ALT) level is the most commonly used variable for assessment of liver disease [6].The most common marker of HBV infection is the presence of HBV surface antigen (HBs Ag) [2]. HBV replication persists throughout the whole course of

chronic HBV infection [7]. Typical chronic hepatitis B is marked by the presence of HBe Ag and high levels of HBV DNA with variable elevations in ALT and histological activity [8].High genetic variability is a characteristic feature of the HBV as the viral polymerase lacks proof reading activity and uses an RNA intermediate during its replication.

According to the molecular evolutionary analysis of genomic DNA sequence, HBV strains isolated in various countries are classified into ten genotypes, designated A–J, and arbitrarily defined by an inter-group sequence divergence of more than 8% based on complete genomes [9]. HBV genotypes have distinct geographical distribution and correlate with severity of liver disease. Higher rates of HCC have been found in persons infected with genotypes C and F (compared with genotypes B or D), and in those infected with certain subtypes of genotype A found in southern Africa. Likewise, patients infected with genotype D appear to have a higher incidence of HCC [10], a higher risk for HBV recurrence and mortality after liver transplantation than patients with genotype A [11].Genotyping of HBV is essential for characterization of patient groups and for epidemiological studies. The course of HBV infection depends on several factors such as host genetic factors, age and genetic variability of the virus. In addition to the epidemiological importance, HBV genotypes may influence the disease pattern and response to treatment [9].HBV genotype A and D are the commonest in Indian population. Hepatitis B virus genotype A is more often associated with severe liver disease in northern India than genotype D. Genotype A is more often associated with ALT elevation, HBeAg positivity, absence of anti-HBe and, among those aged 25 years and above, cirrhosis of liver, than was genotype D [12].HBsAg is the first serologic marker to appear, although HBV DNA may be detected slightly earlier. HBsAg usually appears 1 to 2 months after infection and before the onset of clinical illness, and is the last protein marker to disappear.HBV replication persists throughout the whole course of chronic HBV infection [7].Persistence of HBsAg for longer than 6 months beyond the onset of acute hepatitis indicates chronic infection.The main objective of the present study was to study the prevalence of typical genotypic variants in the population and their association with the serum ALT levels and their viral loads.

MATERIALS AND METHODS

Sample collection

A total of 78 patients were included in this study out of which 53 were infected cases of HBV (HBsAg positive) which were untreated and rest 25 were HBsAg negative (taken as control) and all the blood samples were subjected for biochemical estimations like serum ALT & AST, and quantification of HBV and genotyping of Hepatitis B virus. These were obtained after taking written informed consent from patients catering to SMI hospital OPD and admitted in different wards of ShriMahantIndiresh (SMI) Hospital, Patel Nagar, and Dehradun.

Test Group:

Inclusion Criteria

- Patients who are confirmed cases of Hepatitis B and positive for HBsAg.
- Showing Increased/ normal ALT levels in their blood.
- Patients from all age group and of both the sexes.

Exclusion criteria

- Patients positive for HBsAg but showing co-infections with other HBV or HIV.
- Patients undergoing treatment or has recently taken treatment with Anti-viral Therapy.
- Patient diagnosed with alcoholic hepatitis.

Control Group

Inclusion Criteria

- Normal healthy individuals who are HBsAg negative.

Exclusion Criteria

- Patients/Subjects who are HBsAg negative but positive for other HBV or HIV.
- Patients having history of alcohol intake

5ml blood was collected from patients and serum was separated and further DNA was extracted from the separated serum using high pure viral DNA extraction kit as per the manufacturer's protocol (Roche Diagnostic,). CobasTaq Man HBV test uses PCR amplification primers that define a sequence within the highly conserved pre-core/core region of the HBV genome. HBV genotypes characterization: Collect blood in a clean, dry, sterilized vial and allow it to clot. Separate the serum by centrifugation at 5000 rpm for 15minutes at room temperature.

Further HBV genotyping was done as demonstrated by Naito et al(13).ALT estimation was done by Auto-analyzer by VITROS ALT Slide method (multilayer system).

Primer detail

Table-1: Primer sequences used for HBV genotyping by nested PCR PrimerSequence[a](position, specificity, and polarity)

First PCR
P1[b].....59-TCA CCA TAT TCT TGG GAA CAA GA-39 (nt 2823–2845, universal, sense)
S1-2.....59-CGA ACC ACT GAA CAA ATG GC-39 (nt 685–704, universal, antisense)
Second PCR
Mix A
B2.....59-GGC TCM AGT TCM GGA ACA GT-39 (nt 67–86, types A to E specific, sense)
BA1R .....59-CTC GCG GAG ATT GAC GAG ATG T-39 (nt 113–134, type A specific, antisense)
BB1R.....59-CAG GTT GGT GAG TGA CTG GAG A-39 (nt 324–345, type B specific, antisense)
BC1R.....59-GGT CCT AGG AAT CCT GAT GTT G-39 (nt 165–186, type C specific, antisense)
Mix B
BD1 .....59-GCC AAC AAG GTA GGA GCT-39 (nt 2979–2996, type D specific, sense)
BE1.....59-CAC CAG AAA TCC AGA TTG GGA CCA-39 (nt 2955–2978, type E specific, sense)
BF1 .....59-GYT ACG GTC CAG GGT TAC CA-39 (nt 3032–3051, type F specific, sense)
B2R.....59-GGA GGC GGA TYT GCT GGC AA-39 (nt 3078–3097, types D to F specific, antisense)

OBSERVATION AND RESULTS

A total of 78 patients were enrolled in this study. Out of 78 patients, 53 were found to be positive for HBsAg, taken as "Tests" and remaining 25 subjects which were HBsAg negative were taken as "Controls". All blood samples were subjected for biochemical estimations like serum ALT & AST, and quantification of HBV and genotyping of Hepatitis B virus. Patients were selected randomly from different Departments of ShriMahantIndiresh Hospital, Dehradun (U.K.).

In this prospective study, mean age of the patients was 46 years, and 37 patients (69.8%) were male while 16 (30.2%) were female. Patients were selected from SMI hospital, Dehradun where patients come from all the areas surrounding to Dehradun. These include patients mainly from Dehradun, Roorkee (Uttarakhand) and Saharanpur (Western Uttar Pradesh).A few patients were from Srinagar and Uttarkashi of Uttarakhand and few were from Poanta sahib (Himachal Pradesh).

Majority of the patients (41 out of 53, 77.4%) had acquired infection from unknown risk factors while 7 patients (13.2%) reported previous history of blood transfusion, 3 (5.6%) reported previous history of surgery (among 3, one included surgery with blood transfusion history) and only 2 (3.7%) had previous history of intravenous drug abuse.

Most of the patients (27 out of 53, 50.94%) were asymptomatic, 21 (39.62%) were with mild symptoms, 4 (7.54%) were presented with Cirrhosis and only 1 (1.88%) had HCC.

Out of 53HBsAg positive samples, HBV DNA was quantified only in43samples by the utilization of TaqMan probes in COBAS TaqMan 48 Real Time PCR and their titre values were observed and ranged in between from $2.33x\ 10^1 – 5.12\ x\ 10^9$ IU/ml (Table 9). In the remaining 10 samples which were HBs Ag positive target was not detected which means HBV DNA was below the detection limit. Further HBV genotypes were detected by the method as described earlier by Hideo Naito *et al*. [19].

The HBV genotypes were characterized through A-F. 43 specimens were subjected for HBV genotyping out of which genotype D was most prevalent one and was found in 23 (53.48%) patients followed by genotypes A which was present in 10 (23.33%) patients. Genotype B, C and E were found in 05 (11.62%), 02 (4.65%), 02 (4.65%) patients respectively. Electrophoretic pattern (Gel picture) of PCR products from different HBV genotypes was read with the help of ABI E-Gel Imager with UV light (work as UV Transilluminator). Of these two images are shown here (Figure 1 &2).

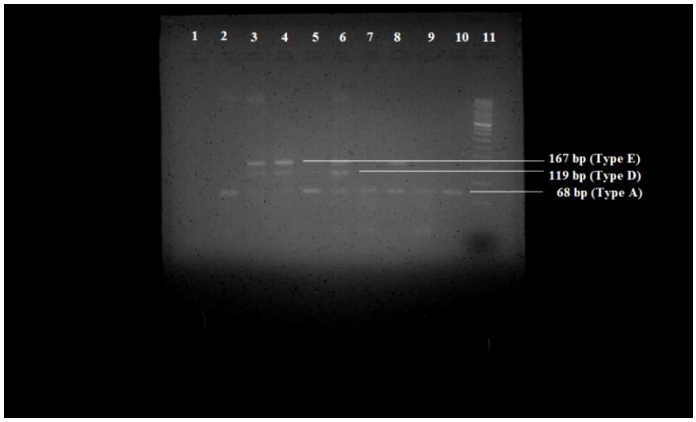

Fig-1: Electrophoresis patterns of PCR products from different HBV genotypes as determined by PCR genotyping system

Well 1:Target Not Detected, Well 2: Genotype B, Well 3: Genotype B, Well 4: Genotype B
Well 5:Genotype B, Well 6: Genotype B, Well 7: Genotype A, Well 8: Genotype A, Well 9:Target Not Detected, Well 10: Genotype A, Well 11- DNA ladder (50 bp).

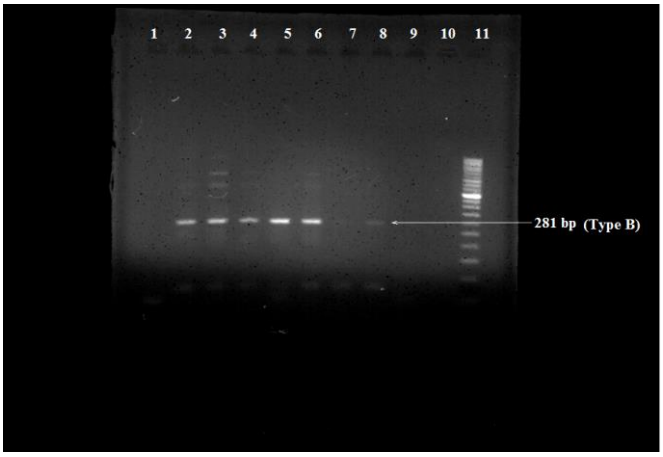

Fig-2: Electrophoresis patterns of PCR products from different HBV genotypes as determined by PCR genotyping system

Well 1:Target Not Detected,Well 2: Genotype B,Well 3: Genotype B,Well 4: Genotype B
Well 5:Genotype B,Well 6: Genotype B,Well 7:Genotype A,Well 8:Genotype A,Well 9:Target Not Detected,Well 10: Genotype A,Well 11- DNA ladder (50 bp)

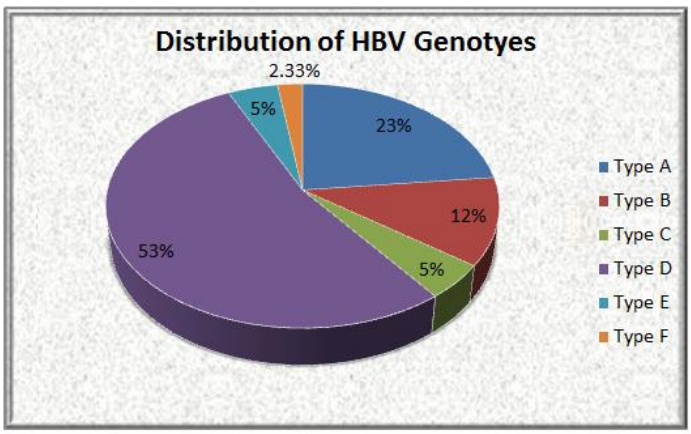

Fig-3: Pie chart for HBV genotypes in ratio

HBV genotype A =10 (23.33%), HBV genotype B = 05 (11.62%), HBV genotype C = 02 (4.65%)
HBV genotype D = 23 (53.48%), HBV genotype E = 02 (4.65%), HBV genotype F = 01 (2.33%)

Table 2 shows Hepatitis B viral titre and different genotype detected. It was observed that in cases having viral load more than 1.1 x 10^6 only genotype A and D were present with prevalence of genotype D, while other genotypes were absent. It was also seen that maximum HBV infections were between viral loads1.00 x10^3 -1.00 x10^6 and all genotypes were present except genotype C. Out of 43 patients 18 (41.86%) had viral load in range between 1.01 x10^3 to1.00 x10^6, next major group was of 15 (34.88%) patients and their Viral load was between 1.0x10^1to1.00 x10^3 and rest 10 (23.26%) patients had very high viral load (more than 10^6). Table 3 shows age wise prevalence of HBV Genotype. The patients of different age were included in this study, with minimum of 12years and maximum age of 80 years with mean age of 46. Patients were categorized in four age groups viz. 0-20, 21-40, 41-60 and above 60 years. Maximum cases were in the age group 21-40 years.

Table-2: Titre wise HBV Viral Load and Genotypes detected

S. No.	HBV DNAtiter(IU/ml)	No. of cases	HBV genotypes	HBV genotypes detected						Total
				A	B	C	D	E	F	
1.	1.00 x10^1 to 1.00 x10^3	15	Type A, B, C, D & E	05	04	02	03	01	00	15 (34.88%)
2.	1.01 x10^3 to 1.00 x10^6	18	Type A, B, D, E & F	02	01	00	13	01	01	18 (41.86%)
3.	More than 1.00 x10^6	10	Type A & D	03	00	00	07	00	00	10 (23.26%)
TOTAL = 43				10	05	02	23	02	01	43
				23.33%	11.62%	4.65%	53.48%	4.65%	2.33%	

Table-3: Age wise Prevalence of HBV Genotype

S. No.	Age group (in years)	No. of cases	HBV genotypes	HBV genotypes detected						Total
				A	B	C	D	E	F	
1.	0-20	03	Type D & E	00	00	00	02	01	00	03
2.	21-40	16	Type A, B, D, E & F	05	01	00	08	01	01	16
3.	41-60	15	Type A, B, C & D	02	03	02	08	00	00	15
4.	60 & Above	09	Type A, B & D,	03	01	00	05	00	00	09
TOTAL = 43				10	05	02	23	02	01	43
				23.33%	11.62%	4.65%	53.48%	4.65%	2.33%	

STATISTICAL ANALYSIS OF RESULT

Data are analyzed using the Statistical Package for the Social Sciences (SPSS), version 23.0 (SPSS Inc., Chicago, IL, USA). Statistical significance is defined as a *p*-value less than 0.05. Table 4 shows mean and SD of ALT levels of 'tests' and 'control'. With the help of t-test p-value is calculated which shows significant relationship between increased ALT levels and HBsAg positive patients.In table 5 shows mean and SD of ALT levels and its association with different genotypes detected. The SPSS version 23 was used and oneway ANOVA was applied to find out the statistical significance. It was observed that association between HBV Genotypes detected and raised ALT level is not significant. Table 6 shows mean and SD of ALT levels and its association with more prevalent HBV genotype (A and D). The SPSS version 23 was used and Oneway ANOVA was applied to find out the statistical significance. It was observed that association between HBV Genotype A and D, and raised ALT level were not significant.

Table- 4: Significance of ALT in Tests and Controls group

Parameter	Tests	Controls	*t*-Value	*p*-Value	Significance
ALT (Mean ± SD)	270.51 ± 426.78	30.80 ± 11.47	1.99254	0.00878	Significant

Table-5: Association between HBV Genotypes detected and ALT

ALT	Sum of Squares	Df	Mean Square	F	p-value	Significance
Between Groups	835467.166	5	167093.433	0.773	0.575	
Within Groups	7992945.439	37	216025.552			Not significant
Total	8828412.605	42				

Table-6: Association between HBV Genotype A and D, and ALT

ALT	Sum of Squares	Df	Mean Square	F	p-value	Significance
Between Groups	220633.740	1	220633.740	0.859	0.361	
Within Groups	7966722.139	31	256991.037			Not Significant
Total	8187355.879	32				

DISCUSSION

Several studies from around the Globe over Hepatitis B infectivity, its Genotyping, effect of drugs and resistance shown by HBV and progression and clinical outcome of Hepatitis B is already done. Different studies have shown variable results depending upon several factors like geographic distribution of HBV genotype, HBeAgseroconversion and ALT levels.

Few studies from different corners of India were also conducted showed certain similarities and some differences. HBV genotype A and Dare the commonest in Indian population [12, 14].

Most of the studies from western side of the world and from China and Japan have shown the prevalence of genotypes B and C and their correlation with clinical outcome. However there are very few studies done in India to show association between HBV genotype A and D and their clinical outcome.

In this study we found that majority (53.48%) of patients were infected with HBV Genotype D followed by genotype A (23.33%).This result is consistent with previous finding from a study done in northern India [15], one study from western India [16] and two studies from Saudi Arabia [11, 17]. This result differs from two other studies done earlier in northern India in which HBV genotype A was most prevalent followed by genotype D [12,14]. In our study we also found HBV genotype B (11.62%), C (4.65%) and E (4.65%), but in low prevalence. This result is consistent with one study done earlier in northern India [93].A rare genotype F was also found in our study which was present only in one case accounting 2.33% of total genotypes detected and this result is similar to one study done earlier in northern India [15].

In our study there was no significant association between HBV genotype and serum ALT level. This finding is consistent with a previous study done in Saudi Arabia where they did not find any significant association between HBV genotype and age, gender, liver function tests, or HBV viral load [11]. In contrast to this, a study done in northern India showed that genotype A was most often associated with raised ALT levels as compared to genotype D [12].

In our study when association between HBV genotype and HBV DNA load was observed as a whole, no significant correlation was observed ($p = 0.121$) but when only two genotypes having greater prevalence i.e. genotype D and genotype A were considered, significant relationship was found ($p = 0.012$). This was contrary to one study done earlier in northern India [12] and two previous studies done in Saudi Arabia [9, 17].

A rare genotypes B, C and E were also found in our study. Finding of these genotypes are consistent with one study done in northern India [14] and genotype C was found in one study in northern India [12]. One reason for these findings may be because of Dehradun and adjoining areas like Mussoorie being tourist destinations and people from all over the world visit these places. Other possible reason may be the migration of people from Bihar and Uttar Pradesh to Dehradun and adjoining areas like Haridwar and Mussoorie in search of job. In an era of frequent international travel and human migration, introduction of new HBV genotype to a community might have far reaching effects, including recombination between genotypes or replacement of one genotype by another.

However, there is an urgent need to explore other possible reasons for this unusual prevalence of genotype F. This suggests that genotype F may be indigenous to certain pockets of North India, clustering in and around western UP and Haryana. This unusual finding apparently contradicts the conventional knowledge that HBV genotype closely mirrors ethnic and geographical migration.

CONCLUSION

It is found that HBV genotype D is the most prevalent one followed by genotype A in northern India and a very rare genotype F is also found.There is no significant association between various genotypes of HBV and the increased levels of ALT. Although there is no significant association between the various HBV genotypes and HBV DNA load, but a significant association is found between HBV genotype A and D and HBV DNA load. Emergence of genotype F in India needs further study regarding its severity, clinical implications and treatment modalities.

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