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Research Article

Genotypic Distribution of Hepatitis B Virus in different Spectrum of its Viral Load in a Tertiary care Hospital in Northern India

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Abstract: Hepatitis B virus (HBV) genotyping is of clinical relevance to clarify the route, virulence, serologic reactivity pathogenesis and response to antiviral therapy. Current study characterizes the HBV genotypes and its distribution with respect to different spectrum of HBV DNA viral load. The distribution of different HBV genotypes among patients with relevance to DNA quantification was evaluated. 108 HBsAg positive serum specimens were considered, where 05 cases for HBV DNA viral load came negative and 08 samples fails to amplify. Further 95 HBV positive specimens were subjected for genotyping and HBV viral load was estimated. All the cases were categorized in the increasing order of their HBV DNA load into three groups as; $1.00x10^1$-$1.00x10^3$, $1.01x10^4$-$1.00x10^6$ and above $1.00\ x10^6$ IU/ml of HBV DNA with 58, 22 and 15 HBV positive cases respectively in these groups. HBV genotype D was the most prevalent in cases with higher viral load followed by Genotype C and B. The percentage of HBV genotypes D, C, B, A, E and F was 45.2%, 24.2%, 17.8%, 12.6%, 8.4% and 5.2% respectively. High HBV DNA viral load was seen in the age group 21-40 and 41-60 years. In conclusion HBV viral DNA titer was elevated in HBV genotype D, followed by genotype C and B assuming that HBV genotype D is the most replicated virus & need to be monitored thoroughly during the diagnosis that could potentially influence the outcome of chronic hepatitis B and the success of antiviral therapy.
Keywords: Hepatitis B virus, Real Time PCR, Chronic Hepatitis, Viral load, Hepatocellular carcinoma.

INTRODUCTION

Hepatitis B virus (HBV) is a well-known agent of acute and chronic hepatitis. Epidemiological studies have shown a strong association between chronic hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC), which is a major cause of morbidity and mortality in areas where chronic hepatitis B is endemic. Approximately 2 billion people in the world have been infected by HBV, 350 million of who are chronic carriers of this virus [1]. HBV strain isolated worldwide have been classified into eight genomic groups deduced from genome comparison and designated genotypes A to H [2]. Some genotypes have been associated with distinct clinical patterns, and their detection and identification are important for virus and disease surveillance [3]. Currently, seven therapeutic agents are approved by the Food and Drug Administration for the treatment of chronic hepatitis B, including two formulations of interferon (interferon alpha and pegylated interferon) and five nucleoside or nucleotide analogs (lamuvidine, telbivudine, abacavir, entecavir, tenofovir). Goal of chronic HBV therapy is to prevent cirrhosis, liver failure and liver cancer by reducing HBV Viral load and the loss of HBeAg (either with or without detection of anti-HBe) while improving liver enzyme levels. Study of HBV Genotypes and HBV DNA viral load is important for transmission of disease, viral pathogenesis and outcome of infection [4]. HBV genotypes characterization helps in early identification of potential sequel after chronic HBV infection and the same can help in implementation of appropriate therapeutic regimens. Thus, the present study was done to characterize the hepatitis B virus genotypes and its distribution with respect to different spectrum of HBV DNA viral load. The distribution of different HBV genotypes among patients with clinical manifestations of HBV infection with relevance to DNA quantification was evaluated.

MATERIAL AND METHODOLOGY

Specimens collection:

The study included 108 randomly selected patients with chronic hepatitis. All the serum specimens were collected from different departments of ShriMahantIndresh Hospital, Dehradun which includes the wards and outdoor patients departments of

Gastroenterology and Medicine. The study was done in between the period of year 2012 to 2014 and was approved by institutional ethical clearance body and written consent was taken from the individual patients where ever required. The patients were selected on the diagnosis infection and include different parameters like liver function tests, clinical symptoms, endoscopy etc. Inclusion criteria: patients confirming anti HBV antibodies utilizing third generation ELISA kit from Erba diagnostics Mannheium, Germany and HBV DNA quantification was done by Roche Taqman 48 Real-time Polymerase Chain Reaction (PCR). HBV viral load: 5ml blood was collected from patients and serum was collected and further RNA was extracted from the separated serum using high pure viral DNA extraction kit as per the manufacturer's protocol (Roche Diagnostic,). CobasTaqMan HBV test uses PCR amplification primers that define a sequence within the highly conserved precore/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. The region from nucleotide position 2410 to 1314 was amplified using a nested PCR with the utilization of first and second round primers (Table1) [5]. The first reaction of the Nested PCR mix consisted of 5µl of 10x PCR Buffer, 4µl of 2mM dNTPs, 6µl of 25mM $MgCl_2$, 0.5µl of each primer 1 and primer 2, 1µl of 1mM AmpliTaq Gold DNA Polymerase and 8µl of Nuclease free water. To this reaction mix, 25µl of HBV DNA was added to make up the volume upto 50µl. The ABI veritti , Germany thermocycler was used and programmed to first incubate the samples for 10 min at 95°C, followed by 40 cycles consisting of 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min. A final extension step at 72°C for 5min was also included. For every specimen two second round PCRs were performed for each sample, with the common universal sense primer (B2) and mix A for types A through C and the common universal antisense primer (B2R) and mix B for types D through F (Table 1). A 5µl aliquot of the first PCR product was added to two tubes containing the second sets of 0.5µl of each of the inner primer pairs, 5µl of 10x PCR Buffer, 5µl of 2mM dNTPs, 5µl of 25mM $MgCl_2$, 1µl of AmpliTaq Gold DNA Polymerase and 7µl of Nucleus free water for a total volume of 30µl. These were amplified for 40 cycles with the following parameters: preheating at 95°C for 10 min, 20 cycles of amplification at 94°C for 20 s, 58°C for 20 s, and 72°C for 30 s, and an additional 20 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s. A final extension step was given at 72°C for 5min was also included. Genotypes of HBV for each sample were determined by identifying the genotype-specific DNA bands. Mix A allows for the specific detection of PCR products for types A, B and C, and Mix B allows for detection of types D, E, and F, type specific PCR products were recognized clearly by their distinct sizes in gel electrophoresis as described earlier [5]. Gel Electrophoresis of the PCR Amplicon. The two different second-round PCR products 20µl from one sample with 4µl of gel loading dye were separately electrophoresed on a 1.6% Agarose gel prepared in 1X TAE Buffer, stained with ethidium bromide, and evaluated under UV light. The sizes of PCR products were estimated according to the migration pattern of a 100bp DNA ladder. The typical electrophoresis patterns of PCR products from different HBV genotypes as determined by the PCR genotyping system can be observed. Amplicon products of 68 bp, 97bp, 119bp, 122bp and 281 bp for HBV genotypes A,F,D,C and B respectively were observed.

RESULTS

Out of 108 serum specimens collected for the proposed study, 05 showed target not detected when quantitated for HBV DNA by Real Time PCR (RT-PCR). Therefore 103 HBsAg positive specimens were quantitated for HBV DNA viral load by the COBAS TaqMan48 RT-PCR. The COBAS TaqMan48 Analyzer automatically determines the HBV DNA titer for the specimen as well as control. The HBV DNA titer is expressed in International Units (IU)/ml and can be converted in copies/ml. The conversion factor between HBV International Units/ml and HBV copies/ml is 1 IU is equal to 5.82 copies/ml using the WHO HBV International Standard for NAT testing, 97/746. In the present study, the lowest quantitation titre in the studied specimens was 2.33 X 10^1 IU/mland the highest was greater than 1.1 X 10^8 IU/ml. Further the quantitated specimens were genotyped through A to F by nested PCR. The first round PCR primers and second round PCR primers were designed on the basis of the conserved nature of nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the six HBV genotypes. Out of the 103 quantified specimens only 95 were genotyped because of the DNA degradation of 08 quantified specimens which were rejected for the further genotyping. 95 confirmed HBV positive specimens were subjected for genotyping and HBV viral load was estimated in all the cases which were categorized in the increasing order of their HBV DNA load into three groups as; $1.00x10^1$-$1.00x10^3$, $1.01x10^4$-$1.00x10^6$ and above $1.00x10^6$ IU/ml of HBV DNA with 58, 22 and 15 HBV positive cases respectively in these groups. HBV genotype D was widespread in cases with higher viral load, assuming to be the most replicating form of HBV genotype followed by Genotype C and B. The percentage of HBV genotypes D, C, B, A, E and F was 45.2%, 24.2%, 17.8%, 12.6%, 8.4% and 5.2% respectively, with genotype D as the most prevalent. The specimens were collected from the different age groups, which includes, 0-20, 21–40, 41–60 and above 60 years, with number of cases as 09, 45, 30 & 11 respectively. Different HBV genotypes were detected in

the different age groups was with high HBV DNA viral load (Table 3).

Fig. 1: Gel Electrophoresis of PCR products for different HBV genotypes

Table 1. Primer sequences used for HBV genotyping by nested PCR

Sr. No	Primer[b]	Sequence a (position, specificity, and polarity)
First PCR round	P1	5'-TCA CCA TAT TCT TGG GAA CAA GA-39 (nt 2823–2845, universal, sense)
	S1-2	5'-CGA ACC ACT GAA CAA ATG GC-39 (nt 685–704, universal, antisense)
Second PCR round	Mix A	B2-5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67–86, types A to E specific, sense)
		BA1R-5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113–134, type A specific, antisense)
		BB1R-5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324–345, type B specific, antisense)
		BC1R-5'-GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165–186, type C specific, antisense)
	Mix B	BD1-5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979–2996, type D specific, sense)
		BE1-5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955–2978, type E specific, sense)
		BF1 -5'-GYT ACG GTC CAG GGT TAC CA-3' (nt 3032–3051, type F specific, sense)

[a] An "M" represents a nucleotide that could be either an A or a C; a "Y" represents a nucleotide that could be either a C or a T. nt, nucleotide.

[b]The sequence for primer P1 was determined by Lindh*et al.* [5].

Table 2: HBV DNA titer and genotypes detected

HBV DNA titer (IU/ml)	No. of cases	HBV genotypes	HBV genotypes detected					
			A	B	C	D	E	F
$1.00x10^1$-$1.00x10^3$	58	Type A, B, C, D,E & F	06	10	20	28	04	02
$1.01x10^4$-$1.00x10^6$	22	Type A, B, C, D, E & F	04	06	02	12	02	03
Above $1.00x10^6$	15	Type A,B,C,D & E	02	01	01	03	02	00
Total = 95			12(12.6%)	17(17.8%)	23(24.2%)	43(45.2%)	08(8.4%)	05(5.2%)

Table- 3: Age wise distribution of HBVgenotypes

Age Groups (in Years)	No. of cases	HBV genotypes detected	HBV genotypes detected					
			A	B	C	D	E	F
0-20	09	Type C & E	01	00	02	01	02	00
21-40	45	Type A, B, C, D & E	05	06	04	11	03	03
41-60	30	Type B,C, D, E & F	02	08	06	09	04	00
Above 60	11	Type A, B, C, D & E	02	02	02	02	00	02
Total=95			10(13%)	16(21%)	14 (18%)	23 (30%)	09(12%)	05(6%)

DISCUSSION & CONCLUSION

In this study we found that HBV genotype D followed by genotype C is the most prevalent genotype associated in HBV infected individuals and in patients with high HBV DNA viral load. HBV genotypes have distinct geographical distribution. Genotypes B and C

are prevalent in the Far East and in South-East Asia, where HBV infection is highly endemic and perinatal or vertical transmission is the predominant mode of transmission [6-8].The HBV genotype is a variable that could potentially influence the outcome of chronic hepatitis B and the success of antiviral therapy. The influence of the HBV genotype on chronic infection has been most intensively studied in high prevalence populations in Asia, where genotypes B and C predominate. The geographical variability in the distribution of HBV genotypes has resulted in most studies comparing either genotype B with genotype C or genotype A with genotype D. Studies from southeast Asia comparing genotypes B and C show that genotype C is associated with higher virus loads [9] and more aggressive liver disease [10, 11]. In India, genotype D is reported to be associated with more-severe liver disease than that associated with genotype A [12]. In a study from western India, genotype D was detected in 92% of subjects, and genotype A was detected in a very small proportion of the population [13]. In another study from northern India, genotypes A and D were found in 46% and 48% of patients with chronic HBV infection, respectively [12]. An HBV genotype-dependent response to antiviral therapy has been observed for some drugs but not for others. For IFN-α treatment of $HBeAg^+$ Chronic Hepatitis (CHB), greater rates of HBeAgseroconversion have been observed for genotype A than for genotype D (49% versus 26%) [14] And for B than for C (39% versus 17%) [15]. Similar results have been found with pegylated IFN-α 2b [16, 17]. A trend of higher HBeAgseroconversion rates after pegylated IFN- α 2a treatment of $HBeAg^+$ CHB has been observed for genotype A compared to genotypes B, C, and D [18].

In conclusion, 95 serum specimens were quantified for HBV DNA vial load and their titer values were observed in-between $2.33x10^1$- $5.12x10^9$ IU/ml. HBV genotype D (45.2%) followed by HBV genotype C (24.2%) & B (17.8%) were the most prevalent genotypes detected. The findings also included that HBV viral DNA titer was elevated in HBV genotype D, assuming that HBV genotype D is the most replicated virus & need to be monitored thoroughly during the diagnosis. Type D was most prevalent in age group between 21-40 years. Although type A, E & F were also present but in lower proportion. The utility of novel nested PCR is that it consists of multiple primer sets within a single PCR reaction to produce amplicons of varying sizes that are specific to different DNA sequences for HBV genotyping. This assay system is very useful for rapid and sensitive genotyping of the HBV genomes when either epidemiological and transmission studies of this agent are carried out in large scale. In particular, the assessment of sequence assortment among dissimilar isolates of the virus is important, because variants may differ in their patterns of serologic reactivity, pathogenicity, virulence, and response to therapy. Thus the arena of molecular testing for the diagnosis and management of HBV infection has shown steady improvement in technology and standardization for the proper therapeutics and clinical outcomes.

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