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GENOTYPING OF HEPATITIS B VIRUS (HBV) BY RFLP AMONG CLINICAL GROUPS IN CHRONIC HEPATITIS B PATIENTS IN NORTH INDIA

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ABSTRACT

AIM: To study the distribution of Hepatitis B virus (HBV) genotypes among various clinical groups in chronic hepatitis B patients in a North Indian tertiary care centre.

MATERIALS AND METHODS: Consecutive samples were collected from 50 HBsAg positive cases which were divided into four clinical groups namely, a)Chronic viral hepatitis B(CVH), b)Acute exacerbation of chronic hepatitis B virus (CHB-AE), c)Incidentally Detected Asymptomatic HBsAg Positive Subjects(IDAHS) and d) Hepatocellular Carcinoma(HCC). Amplification of surface gene was performed by nested PCR using specific primers and results were confirmed on gel electrophoresis. Genotyping was done on amplified product by RFLP using NlaIV and specific bands were analysed.

RESULTS: Only genotypes A and D were observed and genotype D was predominated. Out of 50 patients genotype D was present in 49(98%) of chronic HBV patients. Among genotype D patients 36(73.5%) suffered from CVH, 4 (8%) from CHB-AE, 8(16.3%) were Incidentally Detected Asymptomatic HBsAg Positive Subjects (IDAHS) and 1(2%) from Hepatocellular Carcinoma. Genotype A was present in 1(2%) patient which was from CVH disease group.

CONCLUSION: Only HBV genotypes A and D were found in patients with chronic hepatitis B. No coinfections with genotype A and D were seen. Genotype D was the dominant genotype observed among the groups. During our study RFLP proved to be helpful tool owing to its high sensitivity and specificity. NlaIV can be used as a preliminary restriction endonucleases as it efficiently differentiates between genotype A and D on the basis band patterns, which are predominant genotypes in the region. Other restriction enzymes like AlwI, EarI, HphI, NciI may be used in consecutive step for determination of other genotypes.

KEYWORDS :

1.INTRODUCTION

Approximately 350 million [1] (Hepatitis B:Fact sheets WHO) persons worldwide are chronically infected with HBV. In India only, there are estimated 50 million [1] (Hepatitis B: Fact sheets WHO) hepatitis B carriers who are defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Till now Hepatitis B virus (HBV) has been classified into 10 genotypes (A-J) on the basis of intergroup divergence of 8% or more in the complete nucleotide sequence.[2](Sunbul et al 2014).There is a characteristic geographical distribution pattern of HBV genotypes, as genotype A is prevalent across Europe, India, Africa and America. Genotypes B and C are predominant in China Japan and Southern Asia whereas genotype D is distributed in Mediterranean and the Middle East region. Genotype E is reported from West Africa and genotype F in Central and South America. Genotype G was reported in France and the United States, and later from Mexico. [3](Schaefer S et al .2007) while Genotype H has been described in Mexico and Central America. In Indian scenario both genotypes A and D have been well documented from different parts of the subcontinent.

It is well established by previous studies [4] (Kao.H.J et al.,2000) that the severity of the disease, antiviral resistance, chronicity rate, length of HBeAg positivity and emergence of core promoter mutation are higher in genotype A and C than genotype D and B respectively. The age of development of hepatocellular carcinoma is higher in genotype B2 while lifelong risk is more in genotype C2 . Response to interferon therapy when compared is higher in genotypes A and B than genotype D and C respectively [5][6](Wai et al 2002 and Erhardt et al 2005). Also a study [7](Haddad .R et al .,2010) showed that the resistance to lamivudine which is associated with mutations in the conserved tyrosine (Y), methionine (M), aspartate (D), aspartate (D) motif of the hepatitis B virus DNA polymerase gene is more frequent in genotype D than in genotype A. Above variabilities in progression , management and outcome of the disease necessitates the genotyping of the virus using a simple, sensitive, rapid and cost effective tool.

HBV genotyping methods currently available are direct sequencing [8](Stuyver et al., 2000), Restriction fragment length polymorphism analysis (RFLP) [9](Mizokami et al., 1999), Line probe assay [10](Grandjacques et al., 2000), PCR using type specific primers [11](Naito et al., 2001), Colourimetric point mutation assay [12](Ballard et al.,1997), Ligase chain reaction assay [13](Minamitani et al., 1997) and Enzyme linked immunosorbent assay for genotype specific epitopes [14](Usuda et al.,1999). This centre has standardized HBV genotyping by PCR, which was done by Sami.H et al 2012[15], using Kirschberg's type specific primers (TSP-PCR) and Naito's monoplex PCR. In the study feasibility of RFLP for genotyping HBV was explored, to assess its sensitivity as a tool. A total of 53 samples were genotyped using a modified protocol of Kirschberg et al[16]. However 26 samples could not be genotyped despite amplification by heminested PCR.

2.MATERIALS AND METHODS

2.1.SELECTION OF CASES:

SUBJECTS:

Over two hundred patients presenting at Department of Medicine, J.N. Medical College and Hospital, AMU Aligarh, between January 2014 and March 2015 ,with the sign and symptoms of liver disease were evaluated clinically as well as on the basis of various investigations including liver function test (Serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), Total bilirubin and Alkaline phosphatase, MELD, PT-INR HBsAg, AntiHBs, HBeAg, Anti HBeAg, abdominal ultrasonographic and computed-tomographic scans). Ethical clearance was obtained from institutional ethics committee of J. N. Medical College. Fifty chronic HBsAg positive subjects (positivity more than six months) along with thirty healthy control were selected for the study and were further divided in four study groups:

a) Chronic hepatitis B (CHB) is defined as a condition associated with fatigue, anorexia, jaundice, hepatomegaly, density of the liver harder than normal, splenomegaly, hyperbilirubinemia more than twofold higher than in healthy individuals, serum AST, and ALT twofold higher than in the healthy control group and HBsAg (+/) for longer than 6 months.[17] (Song et al,2003).
b) Hepatocellular carcinoma (HCC) is associated with clinical

features like hyperbilirubinemia, increased AST and ALT, serum α-fetoprotein (AFP) levels greater than 500 mg/l, ultrasound showing tumour, liver biopsy and histopathology showing tumour cells; and HBsAg (+) [17](Song et al.,2003).

c) Acute exacerbation of chronic hepatitis B virus (CHB-AE) was diagnosed if the patients developed derangement of liver functions seen in HBV immune clearence phase or as reactivation in patients with inactive or resolved HBV infections .[18](Puri .P. jcehdec 2013).

d) Incidentally Detected Asymptomatic HBsAg Positive Subjects (IDAHS): These subjects have no present / past symptoms or signs of liver disease and have HBsAg positivity on two occasions more than six months apart.

Socioeconomic status: High middle or low status was determined by Modified Kuppuswamy scale on the basis of education, occupation and per capita income and classified as lower (score below 10), middle (score11-25),upper (score >25) [19](Mishra et al., 2003).

Clinical grading of severity of liver disease

Patients with no sign and symptoms were classified as having mild, while those with icterus, pallor, anorexia, jaundice, nausea, vomiting as moderate and those with splenomegaly, ascites, variceal bleeding, weight loss and abdominal discomfort as having severe liver disease.

ii) Exclusion criteria:

Clinical or serological or radiological examination suggestive of alcoholic hepatitis or any other diseases like; Obstructive jaundice, haemolytic jaundice, non-alcoholic fatty liver disease, autoimmune hepatitis, and patients with hepatitis resulting from hepatotoxins and drugs like anti tubercular therapy were excluded from the study.

2.6.Biochemical investigations

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (T. Bil.), MELD scores and PT-INR tests were done weekly when the patients were admitted to the wards, while routine biochemical tests were done at monthly follow-up after the patients were discharged from the hospital.

2.2.SEROLOGICAL TESTS

1. For HBsAg ELISA 3.0 :

All patients were tested for serum HBsAg by double sandwich ELISA kit (SD bioline). Patients positive with HBsAg were screened for serum HBeAg and anti-HBe by HBeAg and anti HBe combined ELISA kit, (DIA-PRO Sesto San Giovanni, Italy), HCV (hepatitis C virus) by third generation anti-HCV (J. Mitra & Co, India), HIV by fourth generation anti-HIV ELISA kits (J. Mitra & Co, India). The tests were performed according to the manufacturer's instructions.

2.3.DNA EXTRACTION FOR HEPATITIS B VIRUS:

DNA extraction was performed by using phenol chloroform iso-amylalcohol method.[20] (Saiki et al., 1988)

2.4.METHODOLOGY FOR DETECTION OF HBV GENOTYPES:

2.4.1.Amplification of Hepatitis B Virus:

Polymerase Chain Reaction:

After extraction, amplification of HBV surface gene was done by Nested Polymerase Chain Reaction. The Master Mix used was obtained commercially from FERMENTAS, Life sciences, USA**.**

1)Primers used for outer PCR were:

Forward primer (HBMF1; 5'-YCCTGCTGGTGGCTCCAGTTC-3')

Reverse primer (HBMR2; 5'-AAGCCANACARTGGGGGAAAGC-3')

2)Primers used for inner/nested PCR were:

Forward primer (HBMF2; 5'-GTCTAGACTCGTGGTGGACTTCTCTC-3')

Reverse primer (HBMR2; 5'-AAGCCANACARTGGGGGAAAGC-3').

Cycling condition:

HBV DNA was amplified by nested PCR with the first-round sense (HBMF1; 5'-YCCTGCTGGTGGCTCCAGTTC-3') and antisense primers (HBMR2; 5'-AAGCCANACARTGGGGGAAAGC- 3') and then the second-round inner sense (HBMF2; 5'-GTCTAGACTCGTGGTGGACTTCTCTC-3') and antisense primers (HBMR2; 5'-AAGCCANACARTGGGGGAAAGC-3').The amplification mixture contained 2-µl DNA sample mixed with the reaction mixture. [1x PCR buffer, MgCl2 (1.5 mM), 200mM dNTP's, a 20 pmol concentration of each primer; and 2.5 units of Dream Taq DNA polymerase; MBI Fermentas, USA]. The amplification profile was 2 min at 96°C, followed by 25 cycles at 94°C for 15 s (denaturation), 45 s at 60°C (annealing) and 45 s at 72°C (extension) and was performed in 96 welled thermocycler (Labanics, USA). 1 µl of the first-round PCR product was then added to a second-round PCR mixture with the same composition and same profile but with a set of inner sense. 5 µl of the second-round PCR product was analyzed by electrophoresis in 3% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

2.4.2.HBV genotyping by RFLP analysis

Restriction digestions was carried out by incubating the reaction mixture consisting ; 5 µl of the second-round PCR product, 5 µl of 10×FastDigest Green buffer, 10 units of NlaIV (New England BioLabs) and 10 µl of ddH2O for three hours at 37°C. It was followed by inactivation of the restriction endonuclease by incubation at 65 °C on heat block for 10 minutes. The digested PCR products were then electrophoresed on 3.0% agarose gel and the restriction patterns were read under gel doc (Biorad,Aust.). The digest products were found at 220 bp for genotype A and 186 bp for genotype D. We used NlaIV as the preliminary enzyme keeping the predominance of genotype D and A in this region. Other enzymes like AlwI, HphI, NciI or EarI were not used as all 50 PCR products were well digested and genotyped into genotype D and A which are predominant genotypes in the region.

2.5 Sequencing The amplified product of 10 genotype D samples was purified and sequenced by Macrogen, Inc. (Seoul, Korea), using same primers as were used for PCR. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

2.6 Phylogenetic Analysis. For sequence alignment as well as phylogenetic analysis, we selected the GenBank sequences with the best high scoring matching with HBV reference sequences for each genotype [17]. Sequences, were edited, aligned and analyzed using Clustal W Bioedit software. Genetic distances were calculated using the Kimura two parameter algorithms and phylogenetic trees were constructed by the neighbour joining (NJ) method. To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Phylogenetic analysis was done using MEGA version MEGA 4 package.

2.5.Statistical analysis Statistical analysis was performed with the IBM SPSS Statistics 19. Results were expressed as means ± standard deviation or as percentages. Means were compared between groups by using the t –test, ANOVA (one way analysis of variance) and frequency distributions were compared by using the chi- square test.

3. RESULTS

In the present study 50 chronic HBV cases were genotyped. Out of the 50 subjects 74% patients were in CVH group, while in groups IDHAS, CHB-AE and HCC, they were 16%, 8% and 2% respectively. As seen in table 1, 16% prevalence of HBsAg in IDAHS suggests that the routine screening of HBsAg in population may be beneficial. Out of 50 patients majority 15(30%) were in the age group 21-30 years.

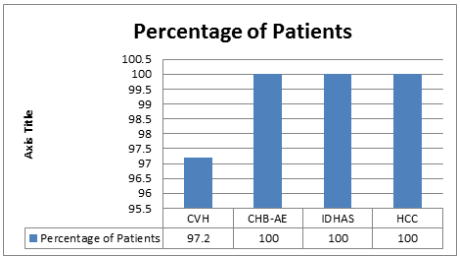
Table 1: Prevalence Of Hbv Genotypes In Different Liver Disease Groups,by-restriction Fragment Length Polymorphism (rflp) Analysis(n=50)

Disease groups	Genotype D n (%)	Genotype A n (%)
CVH(37)	36(97.3)	1(2.7)
CHB-AE(4)	4	0
IDAHS(8)	8	0
HCC(1)	1(100)	0
Total	49(98%)	1

Of the 50 patients in the study groups **49(98%)** were positive for HBV genotypeD by using RFLP. 1 (2%) patient of CVH disease group was

found to be positive for genotype A.No other genotype was detected in this study.

Figure 1:Distribution of HBV genotype D in different liver disease groups by RFLP



Male to female ratio was 1.2:1. Among the demographic factors, notable observation was association of low socioeconomic status in majority of patient with genotype D as seen in table 2. Abdominal discomfort was the most common presenting complaint found in 29(58%) of cases followed by fever in 16(32%) patients while icterus was the most commonly presenting sign appreciated in 22(44%) of the patients(Table 2). On analysing liver function test, 33(66%) had deranged alanine transferase level (ALT), while 34(68%) had deranged aspartate transferase level (AST), and 26(52%) patient had deranged alkaline phosphatase (ALP) level. International normalized ratio (INR) was found to be deranged in 35(70%) of the patients while 38(76%) of the patients had deranged Model for End Stage Liver Disease (MELD) Score. On the basis of clinical and biochemical grading, majority 25(51%) of patients with genotype D were graded clinically as moderate level of liver disease while 11(22.4%) patients had severe liver disease.

Table 2:Demographic profile, hepatitis Be antigen (HBeAg) status, and Biochemical profile of patients with HBV genotypes A and B.

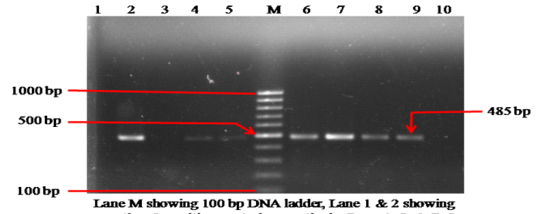
Parameters	Genotype D (n=49)	Genotype A(n=1)	Total study group(n=50)
a)Demographical Age, mean years ± SD	33.10 ±15.01	46	33.45±15.58 years
Ratio of male to female subjects	1.1:1	1:0	1.2:1
Socioeconomic status	Mostly low	Middle class	Mostly low
Predominant patient complaint	Abdominal discomfort	-	Abdominal discomfort
Predominant clinical sign	Icterus	-	Icterus
b)Biochemical ALT (IU/L) (Normal 2-15)	38.44±36.12	34	38.34±36.35
AST (IU/L) (Normal 2-20)	36.11±34.43	39	36.23 ± 34.72
PT-INR	2.01 ±1.94	2.1	2.04 ± 1.96
MELD	18.28 ±9.46	16	18.54 ± 9.51
c)Serological HBeAg positive,no (%)	21(42.8%)	1(100%)	22(44%)
Anti HBe positive,no (%)	17(34.7%)	0	17(34.7%)
Both HBeAg and anti HBe negative (%)	11(22.4%)	0	11(22.4%)

No significant findings were observed exclusively in any particular genotype. Genotype A was associated with mildly elevated levels of ALT,AST,PT-INR and MELD score.

3.1.Prevalence of HBV genotypes

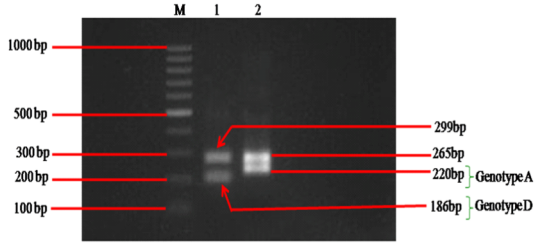
In this study, samples from 50 chronic hepatitis B patients were collected followed by DNA extraction, amplification (Fig 2) and finally were genotyped using RFLP [9] (Mizokami M, Nakano T et al 1999). It was observed that 49 (98%) patients with chronic hepatitis B had genotype D which is the most prevalent genotype in this region while only one patient had genotype A (Fig 3). Similar results were also observed in previous studies from the same region which also reported genotype D to be predominant (84%) with a low frequency of genotype A (16%) in northern Indian patients [21](Chattopadhyay et al.,2006). These results were also comparable to the HBV genotype distribution documented from western and southern parts of India [22] [23].(Vivekanandan et al.,2004, Gandhe et al.,2003) All the cases were genotyped and no other genotypes were found.

FIG 2



Lane M showing 100 bp DNA ladder, Lane 1 & 2 showing negative & positive control respectively, Lane 4, 5, 6, 7, 8 & 9 showing 485 bp amplified PCR product of HBV surface gene, while Lane 3 & 10 showing no amplification.

FIG 3



RFLP pattern of the PCR products of S gene: Lane M showing 100 bp DNA ladder, Lane 1 showing 186 bp & 299 bp digested PCR products & Lane 2 showing 220 bp & 265 bp digested PCR products of HBV surface gene.

4. DISCUSSION

The study emphasises the role of RFLP as an important tool for HBV genotyping and the utility of using of NlaIV as initial restriction endonucleases, considering its efficiency in digesting genotype A and D, which are predominant genotypes in the region. RFLP was found to be an effective genotyping tool with good discriminating ability. Genotyping of HBV is essential as the the progression, management and outcome of the disease often is genotype specific. Previous studies [4](Kao.H.J et al.,2000) have reported that the chronicity rate, duration of HBeAg positivity and emergence of core promoter mutation are higher in genotype A than genotype D while response to interferon therapy when compared is higher in genotypes A than genotype D. Also a study [7](Haddad .R et al .,2010) showed that the resistance to lamivudine which is associated with mutations in hepatitis B virus DNA polymerase gene is more frequent in genotype D than in genotype A.

The present study was performed to evaluate the prevalence of HBV genotypes in patients of chronic hepatitis and to assess the clinical and demographic characteristics in relation to their genotypes. In our study HBV genotype D was detected in 49 (98%) out of fifty subjects, and was by far the most prevalent genotype in this region while genotype A was detected in only one patient. Similar distribution among genotypes was reported in a study at the same centre by [15]Sami et al .,2013. Chattopadhyay et al.,2006[21] also reported genotype D to be predominant genotype (84%) with a low frequency of genotype A (16%) in northern Indian HBV infected patients 12]. The distribution of genotypes is interestingly similar to other studies from South East Asia, more closely in countries sharing borders with India like Pakistan where also genotype D was predominant genotype followed by genotype A [24](Ali et al.,2011). This study confirms the high prevalance of genotype D in south-eastern Asia and also highlights the genotypic link between various ethnic groups within the country and people of the neighbouring countries.

It was observed that the number of male cases were higher, as male to female ratio was 1.2:1. This may be due to higher transmission rate of hepatitis B virus among young males [25](Singh et al., 2003). Majority of the patients had CVH followed by individuals with IDAHS. Majority (62%) of our cases were having low socioeconomic status which was also reported in previous studies [26](DH Lee et al 2002). Abdominal discomfort (58%) was the most common presenting complaint while predominant sign reported was icterus (44%). On genotyping it was found that majority of genotype D positive patients

belonged to low (62%) followed by middle (26%) socioeconomic status which was again in accordance with Lee et al 2002. Genotype D was serologically associated with moderate liver disease based on clinical grading with maximum distribution in the age group 20-40 years thus demonstrating the relation genotype D to chronicity of disease which was also mentioned by Zekri et al 2007[27]. There was also an association between genotype D and Cirrhosis as the latter was observed in 6(16.7%) of the genotype D patients probably because it is predominant genotype in the region. Similar findings were also reported by Nastaran Ansari et al 2015[28] where mostly patients with cirrhosis were found to be genotype D. However the relationship between HBV genotypes and liver cirrhosis remains debatable as some studies suggested that genotype C had a higher risk of cirrhosis, whereas others reported no significant relation with cirrhosis. [29][30](Chen CH et al 2007, Sumi H et al 2003). Biochemically, it was found that the most of the genotype D patients showed higher levels of ALT (38.44±36.12) and AST(36.11±34.43) levels than genotype A patient where the values were 34 and 39 respectively which was in accordance with the previous studies in the same region.[31] (thakur, kazmi et al 2002). However the level of derangement in various parameters in genotype D patients were mild as it was found that, ALT levels were deranged mildly in 16 (32.6%), moderately in 6(12.2%) and severely in 10(20.4%) patients, on the otherhand AST levels were deranged mildly in 16(32.7%), moderately in 7(14.2%) and severely in 10(20.4%) cases. Similarly INR was mildly deranged 23(46.9%), moderately in 8(16.3%) and severely in 3(6.1%) patients while MELD Scores were elevated mildly in 16(32.6%), moderately in 10(20.4%) and severely in 11(22.4%). Thus it was observed that most of the genotype D patients had mildly or moderately elevated levels of biochemical parameters. Similar findings were also reported by Ajay Kumar et al 2011[32] where genotype A was associated with higher levels of ALT/AST than genotype D. However no conclusion can be drawn for genotype A as it was found in only one patient who was from CVH disease group, although he had mild elevation in liver enzymes, PT-INR and MELD score. The patient was positive for HBeAg while among genotype D patients mostly were HBeAg positive (42.8%).

To conclude, in our study only genotypes A and D were found in patients with chronic hepatitis B. While Genotype D was the dominant genotype observed among the groups, Genotype A was found in only one patient. No coinfections with genotype A and D were seen. We found RFLP as a very helpful tool for detection of genotypes owing to its high sensitivity and specificity. NlaIV can be used as a preliminary restriction endonucleases as it efficiently differentiates between genotype A and D on the basis band patterns, which are predominant genotypes in the region. Other restriction enzymes like AlwI, EarI, HphI, NciI may be used in consecutive step for determination of other genotypes.

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