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RESEARCH ARTICLE

DISTRIBUTION OF HEPATITIS B VIRUS GENOTYPES AMONG CHRONIC HEPATITIS B PATIENTS IN A TERTIARY CARE HOSPITAL IN NEW DELHI

[1,*]Bibhash Nandi, [1]Anita Chakravarti, [1]Preena Bhalla and [2]Premashis Kar

[1]Department of Microbiology, Maulana Azad Medical College, New Delhi, India
[2]Department of Medicine, Maulana Azad Medical College, New Delhi, India

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ABSTRACT

Introduction: Hepatitis B Virus (HBV) genotypes A and D are well documented from different parts of India, while genotype C in addition to mixed genotype A and D (A/D) has been reported and characterized from Eastern India. HBV genotyping is a useful tool for understanding the epidemiology of HBV infection. HBV genotype is not only predictive of clinical outcomes but has also been associated with response to interferon treatment.

Material and methods: The HBV genotype was identified by using PCR-RFLP analysis of the S gene in 50 HBsAg positive adult patients. Restriction enzyme digestion was carried out by using AlwI, HphI, NciI, NlaIV and EarI nuclease enzymes to see the different patterns of cleavage that would occur at the specific sites.

Results: The most common HBV genotype was D (31/50, 62%), followed by genotype A (15/50, 30%) and mixed A/D genotype (4/50, 8%).

Conclusions: These findings suggest that Genotypes A, D and A/D exist in chronic hepatitis B patients in New Delhi, and among these Genotype D is the commonest.

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INTRODUCTION

With an estimated 350 million people worldwide chronically infected with hepatitis B, it has been recognized as a major global health problem. India is placed in the intermediate HBV endemicity zone. The prevalence of HBsAg ranges from 2% to 8%, and the number of HBV carriers is estimated to be about 50 million (Datta, 2008; Lavanchy and Hepatitis, 2004). Chronic HBV infection is one of the most important risk factors of liver cirrhosis and hepatocellular carcinoma (HCC), and it is associated with substantial morbidity and mortality, forming an immense economic burden and putting the state health services through considerable strain. An effective vaccine against hepatitis B has been available for the last three decades and it is highly effective in protecting against HBV infection and its consequences. It is the first major vaccine that protects against cancer and it represents the most effective way of preventing chronic HBV infection and related end stage liver disease (Longo *et al*., 2007). In India, HBV is mostly transmitted horizontally and perinatal transmission during early life plays a less important role (Nayak *et al*., 1987 and Chakravarty et al., 2005). Infection with HBV is recognized as one of the most important reasons for viral hepatitis worldwide, and it is associated with a broad spectrum of clinical manifestations, ranging from acute or fulminant hepatitis, to diverse forms of chronic infection, including asymptomatic carrier (ASC) state, chronic hepatitis B (CHB), liver cirrhosis and carcinoma (Kann *et al*., 2007). The clinical course of hepatitis B may be extremely variablewith asymptomatic infection constituting the majority of cases. The patient's age at infection and the immune status largely determine the stage at which the disease is diagnosed.The course of the disease and prognosis is known to be influenced by several viral factors viz., serum HBV DNA levels, specific viral mutations and the virus genotype.Among these, the genotype is known to be a useful prognosis marker as it is associated with the response to antiviral therapy, specifically interferon treatment and therefore, viral genotype is predictive of clinical outcomes. Based on comparison of the complete nucleotide sequences, worldwide HBV isolates have been classified into ten genotypes: A-J, and several subtypes have been identified by divergence in the entire HBV genomic sequences, >8% for genotypes and 4-8% for subtypes respectively. The geographical distribution of HBV genotypes is singularly distinct.Genotype A and D occur frequently in India, Europe and Africa, while genotype B and C are prevalent in Asia, genotype E is almost exclusively found in Africa and genotype F in Central and South America. Genotype G has

Corresponding author: **Bibhash Nandi,**
Department of Microbiology, Maulana Azad Medical College, New Delhi, India.

been reported from the United States of America and France while genotype H has been described in Central America (Kumar *et al*., 2011). The present study has been undertaken to investigate the distribution of HBV genotypes among chronic hepatitis B patients in a tertiary care hospital in NewDelhi, India.

MATERIALS AND METHODS

Study population

We carried out a cross-sectional study of cases that included adult patients of either sex who were seropositive for HBsAg for at least 6 months and who had never received antiviral therapy. All the patients were recruited from the wards and OPD of the Department of Medicine, Lok Nayak hospital, New Delhi between October 2011 to March 2013. After obtaining a written informed consent, the patients were assessed through a structured questionnaire, a clinical examination and subjected to an extensive panel of tests forserum viral markers. The present study was approved by the Institutional Ethics Committee of Maulana Azad Medical College, New Delhi. Patients with other (infectious and non-infectious) concomitant causes of liver diseasewere excluded.

Serology

Serological tests were performed using commercially available ELISA kits according to the instructions given in the manufacturer's manual. The various serological tests performed in all the study samples included, HBsAg and Anti-HCV (Transasia Biomedicals Ltd, India), HBeAg (Genedia SRL, Italy), IgG Anti-HBe (DSI SRL, Italy), Anti-HBsAg (DSI SRL, Italy), IgM Anti-HBc and IgG Anti-HBc (DSI SRL, Italy). Viral genotype was determined using PCR-RFLP analysis of the S gene in those cases that had evidence of HBV infection. The viral genotypes were determined through RFLP analysis of the S gene as described by Mizokami *et al*. (Mizokami *et al*. 1999). Serum DNA was extracted using a commercial viral DNA extraction kit (Qiagen) following the manufacturer's protocol.

Amplification of the S gene by Nested PCR

One µL of extracted DNA was used to amplify HBV S-gene sequence from nt120 to nt604 (485 bp) in two rounds of PCR, following which 12 µL of the second round PCR product was electrophorosed in 2% agarose gel and then visualized after staining with ethidium bromide. Band size of 485 bp was confirmed by comparing with known molecular weight marker. (Figure 1)

Restriction Digestion and RFLP analysis

HBV S-gene sequence from nt120 to nt604 (485 bp) was subjected to restriction digestion, with 15 µL of the second round PCR product, using 10 units of each of these nuclease enzymes: AlwI, Hphl, NciI, NlaIV and EarI (MBI Fermentas) at 37^0C following manufacturer's protocol. The digested PCR products were then electrophoresed on agarose gel and the RFLP pattern was visualized under U.V transilluminator after staining with ethidium bromide. HBV genotype B was distinguished by the fact that S-gene fragment remains uncut by EarI, while no AlwI site exists in S-gene sequences of genotype C.

Only genotype E has a restriction site for NciI at position 461 and will yield two bands of 379 bp and 106 bp size. Similarly, only genotype F has a restriction site for HphI at position 82 and yields two bands of 438 bp and 47 bp size. For genotype A, the specific restriction site for NlaIV is found at position 299, yielding two bands of size 265 bp and 220 bp. Genotype D is digested at positions 265 and 299 by NlaIV and therefore, it produces three bands of 265 bp, 186 bp and 34 bp size. (Figure 2)

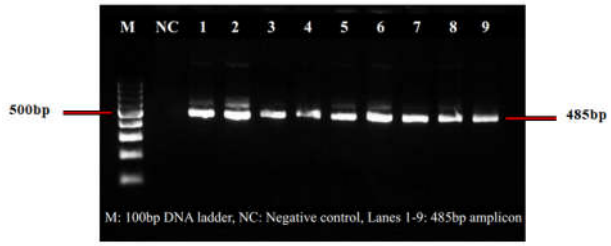

Figure 1 Gel electrophoresis confirming PCR products of 485 bp

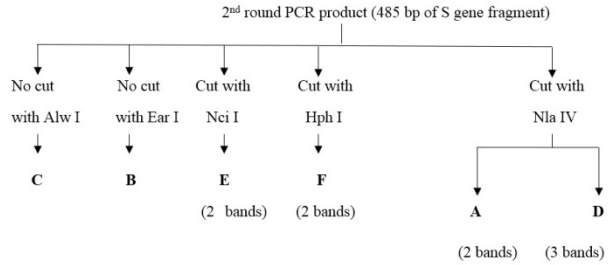

Figure 2. Brief outline of the strategy for HBV genotyping by PCR-RFLP method

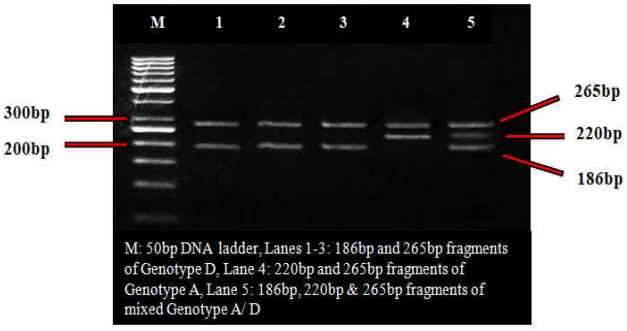

Figure 3. Restriction digestion of 485 bp PCR products into fragments by Nla IV

RESULTS

Demographic characteristics

When the age and sex distribution of the study group were analysed (Table 1), highest HBsAgseropositivitywas notedin male subjects aged between 36-45 years.

Table 1. Demographic data of the study group

Age (Years)	Sex		Total (n=50)
	Male	Female	
18-25	0	3	3 (6%)
26-35	8	4	12 (24%)
36-45	16	4	20 (40%)
46-55	9	1	10 (20%)
56-65	5	0	5 (10%)
TOTAL	38 (76%)	12 (24%)	50 (100%)

Table 2. HBV Genotypes

HBV Genotype	Male (n=38)	Female (n=12)	Total (n=50)
A	11 (29%)	4(33%)	15 (30%)
D	24(63%)	7(58%)	31 (62%)
A + D	4 (10%)	0 (0%)	4 (8%)

Overall, the male: female ratio was 3.17: 1 and the mean age of presentation was 41.86 years. HBV genotype distribution. Treatment of the 485 bp fragment of S gene with AlwI, Hphl, NciI and EarI restriction enzymes yielded no DNA fragments, while treatment with NlaIV restriction enzyme yielded different fragments of characteristic length. Characteristic fragments of genotype A (220 bp and 265 bp) were observed in 15 (30%) patients. Fragments characteristic of genotype D (186 bp and 265 bp) were found in 31 (62%) patients. Interestingly, mixed genotype A/D was found in 4 (8%) patients (Figure 4). Overall, HBV genotype D was dominant in the study group (Table 2).

DISCUSSION

The present study attempted to uncover the distribution of HBV genotypes in a tertiary care hospital in New Delhi. In the past, several viral factors such as the serum viral load, viral mutations and the genotype, have been associated with clinical outcomes. A significantly high risk of severe liver disease characterized by cirrhosis and hepatocellular carcinoma has been linked to viral factors such as infection with genotype C, basal core promoter mutation, pre-S deletion and high serum viral load. A high viral load and infection with genotypes C or D have been linked to a worse response to interferon therapy (Coursaget *et al*., 1987; McMahon *et al*., 2009; Fung and Lok, 2004; Baker *et al*., 1991 and van Bömmel *et al*., 2006). Our study subjects were aged between 18 to 65 years and a majority of them were males between 36-45 years of age. The mean age of presentation was around 42 years and the male to female ratio was 3.17:1.

This observation is in accordance to the results of a study conducted in New Delhi (Chakravarti and Verma, 2005). and to a WHO collaborative study conducted by Sobeslavsky (Sobeslavsky, 1980). in which there is a clear tendency for HBsAg prevalence to be higher in males belonging to the age group of 40-49 years. A comparable high age distribution (42%) of CLD cases in age < 50 years was observed by Bukhtiari *et al* in Pakistan (Bukhtiari *et al*. 2003). It is postulated that male patients with chronic hepatitis B virus infection might have a greater ability to clearHBeAg that results in more frequent hepatitis B virus DNA integration and longer retention of the virus with subsequent development of chronic liver disease associated with hepatitis B. That is, males are more likely to become chronic carriers of HBV and as a consequence, are more likely to develop chronic hepatitis, post-necrotic cirrhosis and primary hepatocellular carcinoma. However, the exact mechanism that causes this difference awaits further study. (Chu *et al*. 1983; London, and Drew, 1977) it is widely recognized that in many of the Asian communities, the lesser access and adherence of the female patients to the available health care facility may also be a probable reason for this lower incidence in women due to lower reporting and care-seeking behavior of women. Determination of the HBV genotypes by RFLP analysis of the S gene using nested PCR has been known to be a simple, cost effective and accurate method that is considered to be useful for research on HBV (Mizokami *et al*. 1999). Therefore, in our study this method was used to determine the viral genotype.The present study demonstrates that HBV genotype D is highly prevalent. The predominance of genotype D in India has been reported by Banerjee *et al* (Banerjee *et al*. 2005). Gandhe *et al* (Gandhe *et al*. 2003) and in the Andaman Nicobar islands by Arankalle *et al*. (Arankalle *et al*. 2003). More recently, Chattopadhyay *et al* (Chattopadhyay *et al*. 2006 and Chattopadhyay *et al*. 2006). Have reported predominance of Genotype D in New Delhi, India. However, Thakur *et al* (Thakur *et al*. 2002) and Kumar *et al* (Kumar *et al*. 2005) reported that genotype A and D are prevalent in equal proportions in patients of chronic hepatitis B.Genotypes A and D, along with genotype C have been detected exclusively from eastern Indian HBV carriersby Vivekanandan *et al* (Vivekanandan *et al*. 2004). This genotype C virus infecting carriers in eastern India is reported to have a high nucleotide similarity with south East Asian subgenotype Cs/C1 strain, suggesting a recent introduction (Vivekanandan *et al*. 2004). Novel recombinants between HBV genotypes A, G and C referred as genotype I has been recently reported from Eastern India (Ashrafali *et al*. 2013). The major reason of the different results might be due to the differential demographic distribution of the HBV genotypes. The previous study (Kumar *et al*. 2005) was carried out in Lucknow, which is a city situated 600 km away from New Delhi, India. Also, the majority of the cases in Lucknow come from semi-urban and rural areas.

The patients in New Delhi are more or less from urban or semi-urban areas. Another reason could be the possibility that the Indian population originally had HBV genotype D, which has been partially replaced by genotype A, particularly in northern India, due to human migration from Europe and North America, which has been extremely common over the last few decades. In this study, mixed infection of genotypes A and D of HBV were found in a small percentage (8%) of CHB cases. This is in accordance with what has been reported earlier (Kumar *et al*. 2005). In hepatitis C virus infection, viral genotypes have been related to clinical illness and response to therapy. However, in the case of HBV infection, data on relationship of clinical illness with viral genotype are limited. Most of these have compared genotypes B and C in South-East Asian countries where genotypes A and D are rare. In conclusion, since the distribution of HBV genotypes within the Indian subcontinent appears to be markedly different, our observations support the view that larger prospective studies are required to study the natural history of different HBV genotypes that are prevalent in India, and their association with disease severity and prognosis.

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Conflict of Interest: The authors declare no conflict of interest.

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