

STUDY OF HEPATITIS B VIRUS INFECTION AND ITS GENOTYPES IN TRIBAL PEOPLE

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ABSTRACT

Objective: This study was undertaken to screen the epidemiology of hepatitis B virus (HBV) in ethnically distinct, tribal dominated and of lower socioeconomic status area.

Methods: Briefly, 3 ml blood was collected from 50 random liver disease cases with jaundice, receiving care at Central Hospital, N.F. Railway, Guwahati, and Guwahati Medical College, with informed consent. The patients detected with hepatitis A virus (HAV)-immunoglobulin M positive status were included and were stratified as acute viral hepatitis and fulminant hepatitis failure based on the clinical profile. HAV genotyping was studied by polymerase chain reaction-direct sequencing-phylogenetic analysis approach. Statistical analysis was performed using SPSS 13.0 software.

Result: A total of 50 cases were HBV infected. HBV infection was predominant in the young and adult age group. HBV-RNA was detected in 19 cases.

Conclusion: This study shows that HBV genotype D is most commonly found hepatitis in all tribes of Assam and poor sanitation and alcohol consumption are a common reason for its widespread.

Keywords: Genotype, Hepatitis B virus, Liver disease, Molecular epidemiology, Phylogenetic study.

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INTRODUCTION

Hepatitis B is a worldwide health-care problem, especially in developing areas. An estimated one-third of the global population has been infected with this virus approximately 350 million people are lifelong carriers and only 2% spontaneously seroconvert annually [12]. Ongoing vaccination programmes appear to be promising in the attempt to decrease the prevalence of this disease [13]. Hepatitis B virus (HBV) is transmitted hematogenously and sexually. The outcome of this infection is a complicated viral-host interaction resulting in either an acute symptomatic disease or an asymptomatic disease. Patients may become immune to HBV or may develop a chronic carrier state. Later consequences are cirrhosis and the development of hepatocellular carcinoma (HCC) [14]. HBV belongs to the hepadnavirus family of enveloped DNA viruses containing a partially double stranded genome of 3182±3221 bp depending on genotype that encodes four overlapping open reading frames as follows:

- S for the surface or envelope gene encoding the pre-S1, pre-S2, and the S protein
- C for the core gene, encoding for the core nucleocapsid protein and the e antigen
- X for the X gene encoding the X protein
- P for the polymerase gene encoding a large protein promoting priming, RNA-dependent and DNA-dependent DNA polymerase and RNase H activities.

The genome is read in all three reading frames, and viral regulatory elements are all within coding regions which introduce constraints on the ability of the virus to accept mutations and remain viable [15]. Nevertheless, heterogeneity among the strains of HBV circulating globally is 10%-fold greater than that in the majority of DNA viral genomes. This is explained, at least partially, by the fact that hepadnavirus replication takes place via an RNA intermediate and reverse transcriptase is known to have a high error rate [16]. A nucleotide exchange rate of between 0-1 and 0-7 per year [17] has been estimated for the HBV [18] and woodchuck hepatitis virus [19]; genomes, respectively, which is similar to the most slowly evolving gene of retroviruses, the *gag* gene, and one

to two orders of magnitude lower than the mutation rates previously calculated for the positive- and negative-strand RNA viruses [19]. The virus persists in 2-10% of adult patients and approximately 90% of infected infants leading to chronic liver disease. In highly endemic areas, infection is predominantly acquired during the perinatal-neonatal period or by horizontal transmission in the first few years of life [20,21] which results in a high prevalence of long-term HBV carriers with a low average age of infection [22]; the virus has a long time span in which to evolve within its host.

Hepatitis B is the major etiological cause of advanced liver disease leading to HCC in India [1]. The seroprevalence of HBV, its different genotypes, and association with disease severity has been studied from different parts of India [2-9] excluding the Northeast; which may be a great underestimation of the HBV endemicity in India. Given that the population and indigenous tribes residing in Northeast are of different ethnicities than the rest of India and has a higher incidence of drug abusers as well as HIV infection, it is possible that the seroprevalence of HBV infection is very high in the North Eastern states.

Natural history

Acute infection with HBV in adulthood is rarely associated with the development of potentially fatal fulminant liver failure. Chronic infection, whether acquired in childhood or in adulthood, is associated with progressive liver disease; risk of cirrhosis, liver failure, and HCC; rarely with extrahepatic manifestations. Chronic HBV infection is characterized by four distinct phases: Immune tolerance, immune clearance, inactive carrier state, and reactivation [23]. The immune tolerance phase is characterized by detectable hepatitis B e antigen (HBeAg), high levels of HBV DNA (>105 copies/mL), and normal alanine aminotransferase (ALT) levels. The immune clearance phase, also called chronic hepatitis B, is characterized by detectable or undetectable HBeAg, undetectable or detectable anti-HBe antibodies, lower or fluctuating levels of HBV DNA, high or fluctuating ALT levels, and active inflammation as seen on liver biopsy. The inactive HBsAg carrier state is characterized by detectable HBsAg, undetectable HBeAg, detectable

anti-HBe antibodies, low levels of HBV DNA (<104 copies/mL), and normal ALT levels [10,11]. Later in the carrier phase, HBsAg may become undetectable and anti-HBs antibodies may appear [23] although reactivation can occur in inactive HBV carriers.

Epidemiology of HBV

HBV is a major public health problem worldwide. Approximately, 2 billion people worldwide have serological evidence of past or present HBV infection and 350 million people are chronically infected [1]. The prevalence of hepatitis B infection varies greatly throughout the world and is determined primarily by the predominant age at which the infection occurs. In areas where the prevalence is high, such as Southeast Asia, China, and Sub-Saharan Africa, more than half the population is infected at some time in their lives, and 10-20% are chronic carriers of the virus, the result of either neonatal transmission (vertical) or transmission from one child to another (horizontal). Risk of HBV infection continues after the first 5 years of life but its eventual contribution to the high rate of chronic infection is less significant. The risk of HCC is high in these areas. Areas with low levels of endemicity include North America, Western Europe, and Australia, where only a minority of people comes into contact with the virus, as a result of horizontal transmission among young adults. In intermediate-prevalence areas like the Mediterranean countries, Japan and India, the carrier rate is approximately 3-5%. In these areas, there are mixed patterns of infant, early childhood, and adult transmission.

In India, there are an estimated 42.5 million HBV carriers. In this group, transmission occurs in all the age groups, but early childhood infection accounts for most cases of chronic infection. The national average for chronic HBsAg positivity is 4.7% and around 24.4% of these patients are HBeAg positive and consequently highly infectious [24]. The HBV exposure rate has been estimated to range between 17% and 52% in the adult population. The HBsAg positivity below 1 year of age has been reported to range from 2.5% to 12.5% [25]. It is speculated that in 60% of subjects the HBV transmission occurs horizontally. HBV has been shown to be responsible for 26-62% of all sporadic acute viral hepatitis (AVH) illness and 22% of fulminant hepatic failure (FHF) in India. Nearly 60% of chronic liver disease in India is associated with HBV infection [2], as high as 80% of cases of cirrhosis of the liver are due to chronic HBV infection [26]. On the other hand, about 61.3% of patients with HCC seen at Chennai were positive for markers of HBV infection [27].

The exact epidemiology of HBV is important as the age at infection has significant impact on the clinical outcome because chronic infection occurs in approximately 90% of infected infants at birth, in 25-50% of children infected between the ages of 1 and 5 years, and in less than 5% of those infected during the adult life [24]. The World Health Organization estimates that the numbers of HBV carriers in the year 2000 are around 400 million.

Modes of transmission

HBV is a hardy virus with no animal reservoir. Man is the only known reservoir. Although HBsAg has been detected in urine, bile, tears, sweat, vaginal secretions, cerebrospinal fluid and synovial fluid, only serum, saliva, and semen are reported to be infectious. Respiratory, water-borne, or insect-related infections have not been documented. Before the screening of transfused blood was accepted universally, the risk of hepatitis B infection from transfusion of commercial blood was as high as 50%. The exclusion of commercial donors and application of hepatitis B serological screening in the 1970s dramatically reduced the incidence of post-transfusion hepatitis B [28]. At present, the risk of transfusion-related hepatitis B from blood donors who test negative for HBsAg and anti-HBc is estimated to be in 1 in 63,000. This risk can be further reduced by 42% by nucleic acid testing [29]. Now the common modes of transmission of HBV include sexual activity, injection-drug use, or occupational exposure. Other, less frequent causes of infection

include household contact, hemodialysis, and receipt of organs or screened blood products. No clear risk factors are found in 20-30% of patients, perhaps because of a reluctance to report high-risk behavior or possibly mucosal or other unrecognized routes of infection [30]. Transmission of HBV parentally has also been described. The risk is greatest for infants born to women who are HBeAg positive and ranges from 70% to 90% at 6 months of age and 90% of these children remain chronically infected. The risk of perinatal infection among infants born to HBeAg-negative mothers ranges from 10% to 40%, with 40-70% of these infected infants remaining chronically infected. Children born to HBsAg-positive mothers who do not become infected during the perinatal period remain at high risk of infection during early childhood. About 40% of infants born to HBeAg-negative mothers become infected by 5 years of age [30].

HBV genotype

HBV is classified into eight genotypes (A-H) that are geographically dispersed - Genotype A is predominant in North America, Western Europe, and Africa; genotypes B and C in Asia; genotype D in Southern Europe, Africa, and India; genotype E in West Africa; genotype F in Central and South America and Alaska; genotype G has been found in the United States, France, and Germany; genotype H in Central America. Genotypes A, B, C, and D predominate in the United States, while the other genotypes are less common [31]. Genotype B is associated with less active disease, slower progression, and lower incidence of HCC compared with genotype C. Genotypes A and B generally respond better to treatment with interferon than do genotypes C and D. No relation between HBV genotypes and response to nucleos(t)ide analogue-based therapies has been demonstrated [31].

Originally, four genotypic groups of HBV (A-D) were defined based on an inter-genotypic divergence score of 8 ± 5 to $10 \pm 0\%$ between 18 complete genomes, as compared to a score of 1 ± 1 - $2 \pm 7\%$ between isolates within the same genotype [32]. This genotypic classification was extended to six genotypes (A-F) by phylogenetic analysis of 122 surface antigen (HBsAg) genes [33]. The genotypic groups are geographically arranged [34] with genotypes B and C confined to Asia, while genotype A predominates in Northern Europe giving way to genotype D as one moves toward the Mediterranean region. Genotype E is mainly found in parts of East, Central, and West Africa and genotype F is only found in the New World and the Pacific which is also home to the Cq- subgroup of genotype C [35]. Two subgroups of genotype A, subgroups A and A, were found in approximately equal amounts in an urban population from South Africa together with 10% of genotype D [36].

HBV genotype and its association with severity of liver diseases

Studies from Southeast Asia have shown that HBV genotype C is associated with severe liver disease [38] and with lower rates of response to interferon- α , compared with rates of response associated with genotype B [39]. In Taiwanese patients, genotype B has been shown to be associated with the development of HCC at a younger age [40]. Differences among HBV genotypes in the rate of seroconversion to HBe antibody (anti-HBe) have been demonstrated in patients from Hong Kong [41]. In studies from Europe, differences among HBV genotypes in the selection of basal core promoter and/or precore mutations [42] and disease progression have been demonstrated [43].

A study from Northern India showed that HBV genotype D has been shown to be associated with more severe liver disease and may predict the occurrence of HCC in younger patients [44]. Genotype A was 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 [45]. The frequency of mixed HBV genotypes has been reported to be 6% [39]. The clinical significance of HBV genotypes is becoming increasingly relevant. The identification of HBV genotypes may help in the early identification of potential sequelae after chronic HBV infection and may also help in implementation of appropriate therapeutic regimens in the North eastern states.

METHODS

Diagnostic criteria

Fulminant hepatitis failure (FHF)

The diagnosis of FHF was considered when after a typical acute onset; the patient became deeply jaundiced and went into hepatic encephalopathy within 4 weeks of the onset of the disease with no history of pre-existing liver disease.

AVH

The patients of AVH will be evaluated on the basis of clinical and biochemical features of acute hepatocellular disease untreated to any toxic or drug etiology. The liver function test (LFT) suggested hyperbilirubinemia, rise in transaminase level.

Chronic hepatitis

Chronic hepatitis will be diagnosed as per AASLD practice guideline (Lok *et al.*, 2001):

1. HBsAg positive >6 months
2. Serum HBV DNA $\geq 10^5$ copies/mL for HBeAg +ve Ch-B and $\geq 10^4$ copies/mL for HBeAg -ve Ch-B
3. Persistent or intermittent elevation in ALT/aspartate aminotransferase levels
4. Liver biopsy showing chronic hepatitis (necroinflammatory score ≥ 4).

Liver histology of HBV related disease severity will be assessed by modified version of the Knodell histological activity index (Ishak *et al.*, 1995) and the French METAVIR score (Bedossa *et al.*, 1996 and Brunt, 2000).

Cirrhosis

The diagnosis of cirrhosis will be considered on the basis criteria:

- i. Ascites, splenomegaly, shrunken liver
- ii. Endoscopy shows esophageal varices
- iii. Biochemical features of hepatocellular failure, characterized by low serum albumin level (<2.5 g%), increased BSP (Bilirubin serum Protein) retention of more than 16%
- iv. Liver biopsy will be done to substantiate the diagnosis whenever feasible
- v. The presence of activity will be decided on the basis of raised serum bilirubin (moderate elevation of bilirubin up to 5 mg) and raised transaminases (3-5 times than normal)
- vi. Liver biopsy is suggestive of activity, when it demonstrates evidence of focal cellular necrosis and mononuclear cell in filtration into the portal tracts as well as in to hepatic lobules.

HCC

The EASL diagnostic criteria for the diagnosis of HCC will be followed (Bruix *et al.* 2001). Diagnostic criteria for HCC will be based on either fine-needle aspiration cytology or any two of the following inclusion criteria:

- Alpha fetoprotein (AFP) more than 400 ng/ml
- Arterialization on color Doppler
- Arterialization of triple-phase computed tomography (TPCT)
- Arterialization on contrast enhanced magnetic resonance imaging (MRI) (Contrast enhanced MRI will be used in the rare occasions [ESAL criteria: Bruix *et al.*, 2001] when diagnosis could not be founded by US, TPCT and AFP).

Inclusion criteria

All those patients who will be clinically diagnosed as AVH, FHF, chronic active hepatitis, cirrhosis, HCC will be included in the study. Voluntary blood donors and health-care workers will be included in this study.

Exclusion criteria

Professional blood donors, high risk group like intravenous drug abusers will be excluded from the study.

MATERIAL AND METHOD

The patients of AVH were evaluated based on history and clinical examination. The LFTs will be done at first visit. The patients of AVH, FHF will be evaluated based on history, physical examination, LFT, and serological test for HBsAg. Samples, which are positive for HBsAg, will also be screened for immunoglobulin M (IgM) anti-HBc and HBV-DNA. To rule out any coinfection with other hepatotropic viruses IgM anti-hepatitis A virus, anti-hepatitis C virus (HCV), IgM, anti-HEV infection will also be carried out. The patients of chronic liver disease, viz., chronic hepatitis, cirrhosis of liver, and HCC will be also evaluated based on history, physical examination, liver function profile, prothrombin time, serological markers for HBsAg. Samples which are positive for HBsAg will be further tested for IgG anti-HBc and HBeAg followed by HBV DNA. Anti-HCV and HCV RNA will be done in the cases which are positive for HBV DNA to rule out cases of coinfection.

The serum of voluntary blood donor was collected from blood banks Medical Institution of NE India. These blood donors were evaluated based on a preform and their LFTs was done. They will be screened for HBsAg using commercially available enzyme-linked immunosorbent assay (ELISA) kits (3rd generation) at each center. Those samples of blood donors, who are found positive for HBsAg, shall be reported and despatch to the respective reference laboratory, viz., Gauhati University, Department of Applied Sciences, with regard to molecular biology works.

Extraction of HBV DNA

Isolation of DNA from serum

From each serum sample, 300 μ l will be incubated at 65°C for 4 hrs in 20 mM Tris-HCl (pH 8.0) - 10 mM EDTA - 0.1% sodium dodecyl sulfate - 0.8 mg of proteinase K per ml. The DNA was then extracted with phenol, subsequently extracted with chloroform, and precipitated with ethanol. The pellet will be ultimately dissolved in 20 ml of H₂O.

Polymerase chain reaction (PCR) amplification of HBV DNA

Surface gene amplification

Part of the surface gene (nucleotide position 425 to position 840) will be amplified by nested PCR. 3 μ l of extracted DNA from sera samples will be used as template. The amplification step will be performed for 30 cycles with the outer primers 5'-ATCGCTGGATGTGTCTGCGG-3' and 5'-GGCAACGGGGTAAAGTTCA-3' 10 μ l of amplified product will be analyzed on 1.2% agarose gel in $\times 1$ tris/borate/EDTA (TBE). Samples, which will not show amplification, would be directly used for nested amplification. Where 787 bp fragment will be generated, nested PCR will be done after appropriate dilution. 3 μ l of the first round PCR product (directly or after dilution depending on the band intensity visualized on the gel) will be used as a template in the second amplification step performed for 30 cycles with inner primers 5'-TTAGGTTTAAATGTATACCC-3' and 5'-CATCTTCTGTGGTCTTCTG-3'. 10 μ l of nested PCR product (416 bp) will be analyzed by the agarose gel electrophoresis on 1.2% gel in $\times 1$ TBE or tris base, acetic acid and EDTA buffer.

Pre-core and core-gene amplification

The amplification of core promoter, precore, and core genes will be performed using 5'CATAAGAGACTCTTGACT3' and 5'GGCGAGGGAGTTCTTCTC3'. The amplification of 742 bp will be checked for on 1.2% agarose gel.

Type specific primer for HBV genotyping

Genotyping of HBV

HBV genotypes were determined by multiplex PCR, i.e., surface region designed by Oliver *et al.* Briefly, 5 μ l of extracted HBV DNA was subjected to PCR using primers used by Oliver *et al.* The size of the expected genotype specific amplification is: Genotype A 370 bp, genotype B 190 bp, genotype C 701 bp, genotype D 147 bp, genotype E 787 bp, and genotype F 481 bp.

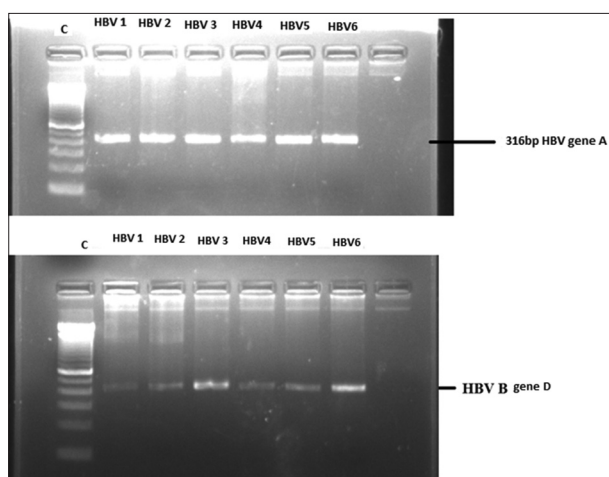


Fig. 1: Polymerase chain reaction amplification results. (a and b) Hepatitis B virus (HBV) genotyping results, where an amplicon of 316 bp represents HBV gene A. The HBV were purified by gel extraction and subjected to direct sequencing for genotype determination on comparison with the standard NCBI representative sequences for hepatitis C virus and hepatitis E virus

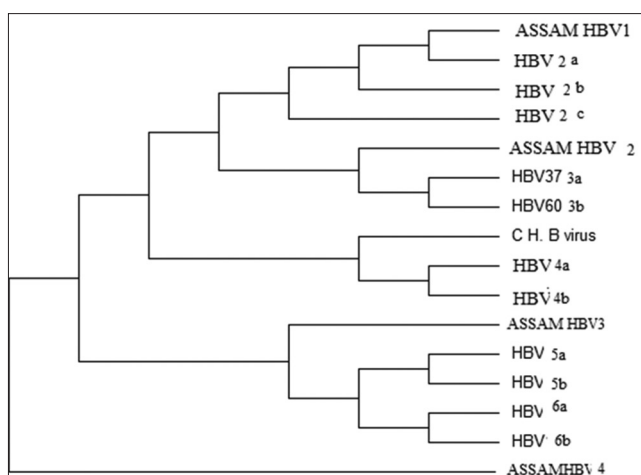


Fig. 2: Phylogenetic analysis using the ExPasy software tool. Randomly sequenced hepatitis B virus (HBV) cases representing HBV genotype D (ASSMHBV1) to (ASSMHBV4) by multiplex polymerase chain reaction genotyping, therefore confirming and validating our genotyping results

Sequencing of the surface, basal core, precore and the core region of HBV

Representative cases of the PCR amplified product of core promoter, pre-core and core region (742p fragment) and surface region (416) will be purified using the gel extraction method (Qiagen gel extraction kit) and will be subjected to sequencing. The sequencing will be done for checking the presence of viral mutations in the surface and basal core promoter, precore, and the core region of HBV as well as for the validation of the genotyping results by multiplex PCR.

RESULTS AND DISCUSSION

Blood samples were collected from patients with liver disease who were receiving care in regional referral hospitals in Guwahati. These patients had a median age of 40 ± 16 years and showed a male to female ratio of 10:1. The majority of the liver disease patients were male (31/50, 62%). The hepatitis virus infection spectrum analyzed based on IgM ELISA results was HBV (19/50, 38%) and coinfection (3/50, 6%).

Others had alcoholic (12/50, 24%) and cryptogenic (16/50, 32%) liver disease.

Viral genotyping

Viral genotyping was performed for HBV samples. Genotyping was performed by multiplex PCR. HBV genotype D (16/19, 59.1%) was the most prevalent in the HBV-positive cases (Fig. 1). A few of the randomly selected genotyped samples were crosschecked and validated by direct sequencing of the core region of HBV followed by phylogenetic analysis. The nucleotides that were sequenced by direct sequencing were aligned using ClustalW, and version 1.6 of the tree view program from MEGA (6) was then used to construct an unrooted phylogenetic tree (Fig. 2). After comparison with known sequences from the NCBI Genbank database, the distribution of the genotypes based 4 isolated HBV (ASSAMHBV1, ASSAMHBV2, ASSAMHBV3, and ASSAMHBV4) was found to be one each from genotypes.

DISCUSSION

Along with age, sex and viral factors, alterations in host genetic factors and environmental factors are also considered important in the development of liver disease, but unfortunately, there are very limited data available on these important aspects in liver disease patients from Northeast India, who are ethnically distinct from those in other parts of the country. The highest prevalence of fecal-oral infection occurs in regions where standards of sanitation and higher alcohol consumption low. Alcohol consumption is well entrenched in the social fabric of many adult populations, virtually constituting a behavioral norm. The level of alcohol consumption in a population is an important determinant of health and social well-being (Rehm *et al.*, 2009). Widespread and increasing use of alcohol is drawing attention to the health consequences of alcohol consumption. Alcohol-related liver disease is a significant burden on health, with alcohol consumption accounting for an estimated 3.8% of global mortality [46]. Alcohol is a major cause of liver cirrhosis in the Western world. Alcoholic cirrhosis is increasingly seen in countries such as Japan and India, which traditionally had a low prevalence of the disease [47]. In our studies, we found that the adult who are more exposed to poor sanitation and use more alcohol the chances of spread of HBV is more (21.43%). The burden of alcohol related liver disease is likely to be higher in the future as indicated by our results which showed that an alcohol related liver disease cases are predominant in the younger age group of 11-40 years, since the consumption of alcohol is been taken up in the early phase of life in northeast India; moreover, it is customary to consume alcohol in several tribal groups of Assam and northeast India. Larger studies in various populations are required to explore further the link between the innate and adaptive immune systems in relation to HBV infection, in an attempt to identify additional susceptibility loci and indications of the initiator of the disease.

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