



Hepatitis B Virus Genotypes : Clinical Implications

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. Approximately 400 million people in the world are chronic HBV carriers.¹ In India, 10% (40 million) of the total carrier population resides.² Liver injury in HBV infection is predominantly immune-mediated and therefore virus-host interaction is the major determinant of HBV-induced liver disease.³ Host immune system varies from individual to individual and is dependent on many factors, such as, age of infection, genetic susceptibility, inherent immunological competence and environmental factors.⁴ The virus characteristics may also vary and therefore events determined by host-virus interaction may be extremely heterogeneous causing heterogeneous disease characteristics. HBV is the smallest DNA virus with 3200 base pairs, which contains four overlapping genes (Open Reading Frames or ORFs) encoding the viral envelope (S and pre S), nucleocapsid (Pre-core and Core), polymerase with reverse transcriptase enzyme and X proteins.¹ HBV is also known to replicate through RNA intermediates on which reverse-transcriptase enzyme acts to produce complementary DNA (c-DNA) for further replication through DNA-polymerase enzyme.¹ Reverse transcriptase is a poor proofreader and therefore nuclear substitution during HBV replication is a spontaneous event. Approximately $1.4 - 3.2 \times 10^{-5}$ nucleotide substitution per year occurs in the HBV genome during its replication.⁵ Due to such substitutions the antigenic characteristics of HBV peptides (predominantly documented in the envelope region) may change. Therefore, after a long time of evolution four major HBV serotypes (*adw*, *adr*, *ayw*, *ayr*) with subtypes have been described.³⁻⁴ The major group specific antigen 'a' remains similar in all the serotypes, however, the minor variation in the amino acid profile of envelope protein leading to different serotypes of the virus have been associated with epidemiological or distribution characteristics of the virus. Such change in the envelope peptides has been associated with nucleotide changes in HBV genome. Sequence analysis of full length HBV genome of different isolates by now have revealed at least eight different HBV isolates which has variation from each other in more than 8% of genomic sequences.⁶ These isolates with > 8% variation in nucleotide sequence homology are called 'HBV genotypes' and have been alphabetically named from 'A' through 'H'. The relationship between serotypes and genotypes has been elucidated.⁶ The serotype *ayw* occurs in all genotypes except in 'C'. Serotype *adw* have been associated with all genotype except

'D' and 'E'. Where as *adr* and *ayr* subtypes are encountered with genotype 'C'.

The clinical relevance of such genotype is yet unclear. However, because the HBV-induced disease is the resultant of virus-host interaction, the disease characteristics may be influenced by the genotypes of the virus. Interaction between hepatocyte genome and HBV genome may also vary according to the prevalent genotype (It is established that prolonged HBV infection may alter or effect the proto-oncogenes and tumor suppressor genes in hepatocytes).⁷

CLASSIFICATION AND GEOGRAPHICAL DISTRIBUTION

The gold standard for classifying any virus is the sequence divergence in the entire genome. In 1988, based on sequence divergence of more than 8% in 18 HBV isolates, four genotypes of HBV were proposed (A-D).⁸ In 1994, two more HBV genotypes (E,F) satisfying the same sequence divergence criteria were added to the list of HBV genotypes⁹ and Genotype G and H were recently identified after 2000.^{10,11} The ORFs among these genotypes (particularly among A-D) have been found to be different; the inter-typing difference being maximum in Pre S/S region and minimal in PreC/C region. Among all genotypes, genotype F has the maximal divergence in which nucleotide divergence upto 15.5% have been documented.⁶ The recently described genotype G has a peculiar difference from the other genotypes. This genotype has a 36 bp insertion in the core gene and two stop codon in the precore gene causing arrest of translation of HBeAg. However, genotype A invariably co-infects along with genotype G⁵ and is responsible for presence of HBeAg in sera of such patients.⁶ Such observation have raised the possibility whether, genotype G is replication in competent and requires coinfection of other HBV genotypes?

Partial correlation between HBV genotypes and serotypes have been established, however discrepancy between genotypes and serotypes have been observed, which is due to change of serotypes from *d* to *y* or *w* to *r* by single point mutation from A to G at nt 365 and nt 479 respectively,⁶ which converts codon 122 and 160 from lysine to arginine.⁶

The geographical distribution, serotypic correlation, and types of clinically recognized mutated HBV among various genotypes are summarized in Table 1.

Table 1 : HBV Genotypes: Distributions, Serotypes, Mutant Forms

Genotypes	Subtypes	Frequency of Mutation		Geographical Distribution
		Pre-core Mutants	Core Promoter Mutants	
A	adw2, ayw1	Uncommon	Common	India, Western Europe, USA, Central Africa
B	adw2, ayw1	Common	Common	South East Asia, Japan, USA, China
C	adr, ayr, adw2	Common	Common	Same as above
D	ayw	Common	Common	India, Mediterranean, USA
E	ayw	Not known	Not known	West Africa
F	adw, ayw	Uncommon	Not known	Central and South America, Polynesia
G	adw	Common	Not known	Europe, USA
H	adw	Common	Not known	Central America

METHODS USED FOR GENOTYPING

Genotyping of viruses by nucleotide sequencing and subsequent homology comparison of phylogenetic tree analysis is cumbersome and labor intensive and therefore cannot be applied to large number of samples. With recent advances in molecular techniques several novel genotyping methods have been described. These methods can be divided into two broad categories.

1. Genotyping based on study of nucleotide sequences
2. Serological methods for genotyping HBV

These methods usually targets to analysis of preS / S region (as it is most heterogenous among the genotypes) and pre C/C region (as it is least heterogenous and analysis of sequence in this region identifies core promoter or pre-core mutation which is correlated with the genotypes. The above mentioned mutants have been associated with occurrence of liver cancers and progressive liver disease. Therefore such analysis have tried to associate genotype, mutant HBV and severity of liver disease).⁶

1. Genotyping by nucleotide sequence analysis :-

There are four described methods for nucleotide sequence study in HBV.

- a. PCR-RFLP¹² (polymerase chain reaction – restriction fragment length polymorphism)

This method was described by Mizokami et al¹² in which he identified the restriction site in the genome of 'S' region for each genotype, and developed RFLP for each genotype. In this method, first PCR amplification for the S region is performed. The PCR product is then subjected restriction enzyme (endonucleases) digestion specific for each genotype and then the digested product is subjected to agar-gel electrophoresis, and staining. Digested nucleotide specific for each genotype is then identified.
- b. Line probe assay¹³

Sequence specific oligomers for each genotype are immortalized on a paper strip, to which PCR amplified test samples are hybridized (reverse hybridization).
- c. PCR using type Specific Primers¹⁴

In this method genotype specific primers for Pre S/S region are used on the test sample, which would

result in amplification of particular genotype of HBV depending upon the corresponding specific primer.

- d. Genotype Specific Probe assay¹⁵

In this method nucleotide sequences corresponding to Pre S region are amplified by PCR with a predesigned Pre S₁ primer labeled with Biotin. These PCR amplicons are then delivered into wells on a microplate on which complementary sequences specific to one or other genotypes have been immortalized. Thereafter hybridized HBV DNA amplified from test samples in these wells are identified by colorimetric method. This test kit is available commercially (Smitest HBV genotyping Kit, Genome Science, Fukushima, Japan)

2. Serological method for HBV genotyping (ELISA)^{16,17}

Usuda et al^{16,17} identified seven distinct antigenic epitopes on Pre S₂ envelope peptides (Pre S₂ is the most divergent region in HBV genome) against which monoclonal antibodies were developed. These epitopes have been named as, a (common antigenic determinant), b (specific for Pre S₂ and present in all genotypes), k, m, s, u, g (these epitopes in various distinct combinations for each genotype are present on Pre S₂).

In the first step, HBsAg present in test sera sample are immobilized on a well of microlitre plate coated with antibody to common antigenic determinant of HBsAg (a). Captured HBsAg then is tested binding with monoclonals against b, k, m, s and u. The epitope b is ubiquitously present on Pre S₂ across all genotypes and ensures presence of Pre S₂ on the microtitre wells. Genotypes are then distinguished by presence of combinations of various epitopes: *bsu* for genotype A, *bm* for genotype B, *bks* for genotype D and E and *bk* for F. To distinguish between genotype D and E monoclonals against another cryptic epitope 'g' which is expressed with HBsAg is used. For this, on a solid phase monoclonals to 'a' common antigenic determinant and 'g' epitope labeled with an enzyme are immobilized, to which test sera is added. HBsAg along with monoclonals against g epitope (sandwich ELISA) detect presence of g epitope indicating presence of *bks* (g), i.e. genotype D.

Genotype G described recently is determined by the combination of Pre S₂ serotype for genotype D i.e. *bks* (g) along with HBsAg-serotype *adw* (it is the characteristics of this genotype). The results of ELISA genotyping have been

validated by RFLP. ELISA kits for serological genotyping are commercially available (HBV genotype EIA, Institute of Immunology, Tokyo, Japan)

CLINICAL RELEVANCE OF HBV GENOTYPES:

1. HBV genotype and Pre core mutants

G 1896 A mutation at nucleotide 1896 in the Pre core region of HBV genome converts codon 28 from tryptophan to a stop codon, as a result of which translation of HBeAg precursor is aborted with a consequent HBeAg-ve, yet replicating HBV which is classically known as pre core mutant (PCM). HBV DNA, is present in a double helix form with base pairing. The nucleotide 1896 in HBV DNA, pairs with the nucleotide 1858. Usually base pairing such as A-T or C-G are stable and viable.

Nucleotide variability (T or C) at position 1858 has been commonly observed. The relationship between HBV genotype, type of Pre core mutation and nucleotide variability at position 1858 has been studied^{5,6,18}. Genotypes other than 'A' usually have a T at nucleotide 1858 (T-1858), which makes a wobble or loose pairing with G-1896, in the stem of the 'ε' encapsidation signal. The mutation of G to A in 1896 resulting in A 1896 (Pre-core stop codon mutant) tightens the stem structure by making a T-A pair between nucleotide position of 1858 and 1896. In contrast, genotype 'A' possesses a C 1858 making a stable C-G pair with G 1896. If G 1896 A mutation occurs in genotype A HBV, then A 1896 would pair with C 1858 making a C-A wobbly base pair which would not provide encapsidation signal and therefore would be non-viable. G 1896 A mutation in genotype A HBV then would be possible, if a simultaneous mutation T 1858C would occur. Therefore pre core mutant only occurs in HBV strains with T1858 and rarely occurs with C1858 HBV strains. On the other hand core promoter mutation such as A 1762T and G1764A, associated with reduced production of Pre core transcripts and reduced HBeAg production resulting in HBeAg -ve HBV infection with replication have been well documented among genotype A HBV infection.^{5,6,18}

2. Recombination of genomes of distinct HBV genotype and coinfection of HBV genotypes

Mixed infections of distinct genotype have been well recognized.⁶ During replication of HBV of each genotype in the same host, genome recombination of two or more HBV genotype is possible. Indeed, recombination of B and C,¹⁹ A and D,²⁰ A and G²¹ have been reported. Interestingly, sites of recombination among genotype B and 'C' have been constant and located between nucleotides 1731 to 1838 and 2437 to 2486.⁶ These insertion sites belonged to Pre core / Core region of HBV DNA genome.¹⁹ Further, more often combination of core region of 'C' genotype with B genotype have been described.¹⁹ One recent study¹⁹ which analysed 70 HBV sequences from Japan and South east Asian countries, reported that genotype 'B' prevalent in Japan was pure without any recombination, where as genotype B isolated from the latter countries had recombination of genotype 'C'.

Based on this observation, the authors suggested to classify genotype B further to B_j (Japan) and B_a (Asia) respectively. As mentioned earlier genotype 'G' does not possess ability to produce HBeAg, due to presence of two stop codons in the pre core region. Therefore, genotype G is usually co-infected with genotype A which is competent to synthesize HBeAg. A/G recombination in few patient have also been reported.⁶ It is by now believed that 10% of HBV isolates may have inter genotypic recombination, which obviously cannot occur without mixed genotypic infection.⁶

Superinfection of one genotype over other may hepatitis flare has also been reported,⁵ and may account for 2% of the flare documented among patients with chronic HBV infection.⁵

3. Disease and virological characteristics

Interestingly, genotype 'B' and C are highly prevalent in hyperendemic countries for HBV, such as in China, Taiwan, Vietnam, Indonesia and rarely in low/intermediate endemic countries like Japan. In contrast, genotype A and D are distributed in low or intermediate endemic countries like USA, Europe, and India. Even though, occurrence of HCC has not been documented to be associated with any particular genotype, it is more prevalent in the countries with genotype B or C HBV infection in comparison to similar prevalence in countries with genotype A and D HBV infection.

Reports from Japan, Taiwan and China indicates that⁵: a) HBeAg positivity and HBV DNA load among genotype B infected individual may be lower than in genotype C infection, b) Immune clearance phase in genotype C may be longer than in genotype B, therefore liver damage among former group may be more than in the latter patients, c) Due to the latter fact, histological severity among genotype C patient may be greater than genotype B, d) Hepatitis flares have been more frequently documented among genotype 'C' patients than in genotype B patients, and e) genotype B patients responds better to antiviral treatment in comparison to genotype C patients. Therefore initial reports suggest that genotype 'C' infection may be associated with more severe liver disease with poor response to therapy than in patients with genotype 'B' virus infection.

Studies from Taiwan have documented that genotype 'C' was significantly more prevalent among patients with cirrhosis and hepatocellular cancer (HCC) among population older than 50 years of age, in comparison to age-matched asymptomatic carriers, whereas, genotype 'B' was documented more frequently among younger HCC patients (age < 50 years) in comparison to age-matched asymptomatic carriers.²² The latter patients (particularly < 35 years) did not have cirrhosis.²³ In Japan, where both genotype B and C are prevalent, genotype 'C' rather than genotype 'B' was found to be associated more often with cirrhosis and HCC and unlike Taiwan, none of the younger patients (<35 years) with HCC had genotype 'B' infection.²⁴

Reports on genotype A and D have predominantly been from Europe, USA and India. One report has documented preferential association of genotype D with acute hepatitis and A with chronic hepatitis.²⁴ Thakur et al from India

reported that, genotype D was more prevalent than genotype A, among incidentally detected asymptomatic HBsAg positive subjects with a histological activity index of >4 and among cirrhotic patients with higher Child's score.²⁵ The latter study also documented that genotype D was more prevalent among HCC patients younger than 40 years of age. However, a recently conducted study from All India Institute of Medical Sciences²⁵ which included 251 patients, could not identify any difference in clinical presentation, histological severity, replicative status, occurrence of HCC and therapeutic response among patients with genotype A and D infection.

Studies from Europe have however documented that, genotype 'A' may have better response to antiviral treatment than genotype 'D'.²⁷ Unfortunately due to limited information on association of genotypes with disease severity and response to antiviral therapy, clear-cut opinion on these issues would await more prospective data.

4. Indian Perspectives

Data on HBV genotype from Indian subcontinent is scarce. There have been only two reports on the genotypic prevalence of HBV from India.^{25,26} The total number of patients with chronic liver disease included in both these studies were 338. Genotype A was detected in 33% (n=112), D in 61% (n=206), A+ D in 2% (n=2), and C in 13 (4%). As mentioned earlier, while one study²⁵ (n=130) could associate genotype D with more severe liver disease and HCC (below 40 years); other study²⁶ (n=208) could not document any such association. However, a recent study²⁸ shows that patients with genotype A infection among Indian patients with asymptomatic HBV carrier, have lower HBeAg +vity rate, lower HBV DNA level than similar patients with genotype A infection in Europe. The former patients were found to have T1809 and T1812 nucleotide mutation immediately upstream of the precore initiation codon, which could be responsible for decreased viral reapplication in them. It has been suggested that genotype A in Asia to be named as Aa and that in Europe as Ae.²⁸

SUMMARY

Eight HBV genotypes have been described till date based on nucleotide divergence of more than 8% and are alphabetically named as A through H. While genotype B and C are more prevalent in China, Japan and South East Asia where severe liver disease and liver cancer are frequent, genotype A and D are prevalent in Europe, India and USA where liver cancer occurs less frequently. Information on virological characteristics in relation to each genotype, their influence on the liver disease and its progression, response to various antiviral regimens are limited and more prospective studies including large sample size is needed for further clarity on the role of HBV genotypes on liver disease.

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