Hepatitis B Virus Genotyping: Is the Time Ripe for Routine Clinical Use?

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Hepatitis B is one of the major causes of end-stage liver disease and liver cancer worldwide. A number of host and viral factors influence the disease course and outcomes. One such viral factor is hepatitis B virus (HBV) genotypes. There are eight major HBV genotypes described from various geographic regions of the world. Although direct sequencing appears to be the gold standard for HBV genotyping, it is expensive and labor-intensive and therefore cannot be applied for routine clinical use. The newer molecular methods including serotyping have made genotyping easier and simple to apply to large number of samples rapidly. The data collected mainly over the last decade have suggested that HBV genotypes may have a bearing over the natural course of the disease and its response to therapy. This review summarizes the available literature and highlights how genotyping could be incorporated into routine clinical practice in order to improve delivery of care to HBV-infected individuals. (J CLIN EXP HEPATOL 2012;2:174–180)

Hepatitis B virus (HBV) is one of the major global public health problems. About 30% of the world's population has serological evidence of current or past infection with HBV. Of these, an estimated 350 million are chronically infected with HBV and approximately 1 million persons die annually from HBV-related chronic liver diseases (CLD), including severe complications such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC).¹ India falls in the intermediate endemicity zone for HBV prevalence. The reported hepatitis B surface antigen (HBsAg) prevalence among the general population in India ranges from 0.1% to 11.7%. However, more recent studies have reported a lower prevalence rate with the accepted national average ranging between 2% and 4%.² In India, the predominant mode of transmission appears to be early childhood horizontal transmission, in contrast to the vertical perinatal transmission in Southeast Asia and adult horizontal transmission in the West.

The natural history of HBV-infection spans over four phases (immune-tolerant, immune-reconstitution or immune-clearance, inactive carrier, and reactivation) and the most important factor determining the disease outcomes is the host immune response.³ Recently, several viral factors have also been implicated in modifying the disease course and severity. One such viral factor seems to be the HBV genotype. Based on >8% structural variability in the whole genome sequence, HBV can be divided into eight different genotypes (A–H) which are distributed in different geographical regions of the world (Tables 1 and 2).⁴ Recently, two new genotypes have also been reported: genotypes I and J. Genotype I is a recombinant between genotypes A, C, and G which has been isolated from Laos and Vietnam.⁵ Genotype J has been reported from the Ryukyu islands, Japan.⁶

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Abbreviations: CHB: chronic hepatitis B; CLD: chronic liver diseases; DNA: deoxyribonucleic acid; ELISA: enzyme-linked immunosorbent assay; GC: guanine–cytosine; HAI: histological activity index; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; IFN: interferon; LC: liver cirrhosis; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism; SVR: sustained viral response

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Table 1 Geographical distribution of the various hepatitis B virus genotypes.

Genotype	Geographical distribution
A	USA, Northwestern Europe, India
B	Northern and Southeast Asia
C	Asia and Asia Pacific
D	Mediterranean, Middle-East, North America, India
E	Africa, Tunisia
F	Central and South America
G	France, Germany, USA
H	Central and South America, Mexico

Techniques for Hepatitis B Virus Genotyping

Till date, HBV genotyping has been used as a research tool and different genotyping methods have been used by various researchers. The common techniques for HBV genotyping are summarized in Table 3. These methods are described below in brief.

Direct Sequencing

Direct sequencing of the whole genome is considered the gold standard for determining HBV genotypes. First, the deoxyribonucleic acid (DNA) is extracted by polymerase chain reaction (PCR) and the nucleic acid thus amplified is sequenced. The sequence is then compared with the available reference sequences for the different genotypes. A more cost-effective approach is to sequence a single gene (e.g., the surface gene) instead of the whole genome.⁷ Though sequencing appears to be the gold standard and has the advantage of being able to detect new genotypes, it is time consuming and more expensive.

Table 2 Hepatitis B virus subgenotypes and their geographical distribution.

Subgenotypes	Geographical distribution
A1	Sub-Saharan Africa
A2	Northern Europe
A3	Western Africa
B1	Japan
B2–5	China (mainland and Taiwan), South East Asia, East Asia
B6	Alaska, Northern Canada, Greenland
C1–3	China, Korea, South East Asia, Taiwan
C4	Australia
C5	Vietnam, Philippines
D1–5	India, Africa, Mediterranean, Europe
F1–4	Central and South America

Table 3 Common techniques for hepatitis B virus genotyping.⁷

Technique	Pros	Cons
Direct sequencing	Gold standard Only technique that can detect new genotypes	Expensive and time consuming
INNO-LiPA	Simple, cheap, can detect mixtures	More expensive than ELISA and PCR SNPs at the primary site may affect detection
RFLP	Simple, cheap, can detect mixtures	SNPs at the primary site may affect detection
Type-specific ELISA	Simple, cheap and can be applied to large number of samples, no PCR required	Indeterminate results may be as high as 23%
Multiplex PCR	Highly sensitive, rapid, high throughput	SNPs at the primary site may affect detection
Real-time PCR	Highly sensitive, rapid and low-risk of cross-contamination	SNPs at the primary site may affect detection
DNA microarray chip	Highly sensitive, rapid, high throughput, can simultaneously detect mutations	Expensive SNPs at the primary site may affect detection

DNA: deoxyribonucleic acid; ELISA: enzyme-linked immunosorbent assay; polymerase chain reaction; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism.

INNO-line Probe Assay

This is a reverse hybridization technique, also known as line probe assay (LiPA). The PCR-amplified product from the S/pre-S region is hybridized onto specific DNA probes immobilized on a membrane. The hybridization is detected as colored lines. The detected lines are specific for different genotypes or a mixture of genotypes. The modified version of this test evaluated recently demonstrated a success rate of $\geq 94\%$.⁸ Pas et al⁹ evaluated INNO-LiPA HBV genotyping (Innogenetics, Belgium) and an HBV-DNA-Chip (bioMérieux, France) and compared them with the gold standard sequencing of the S gene, using a cohort of 275 patient samples. They demonstrated that INNO-LiPA was an easy and quick tool with a sensitivity and specificity of 99% and 100%, respectively.

Restriction Fragment Length Polymorphism

In this method, the PCR-amplified product is broken down (digested) into a number of fragments with the help of restriction enzymes. These restriction enzymes have a defined site or breakpoint in a genome. The fragments thus obtained are detected by means of electrophoresis. A definite combination of restriction enzymes gives a definite pattern of digested sequences. This pattern of digested sequences is specific for different genotypes.¹⁰ But the traditional restriction fragment length polymorphism (RFLP) methods can identify only genotypes A–F. Zeng et al¹¹ carried out an RFLP using digestion of enzymes BsrI, StyI, DpnI, HpaII, and EaeI, in 240 HBV S gene isolates (obtained from GeneBank) and demonstrated all eight genotypes. Chattopadhyay et al¹² demonstrated the RFLP and PCR using type-specific primers for genotyping agreed well with sequencing among 100 HBV-DNA-positive North Indian patients.

Type-specific Enzyme-linked Immunosorbent Assay

Monoclonal anti-HB antibodies are used in this enzyme-linked immunosorbent assay (ELISA) to detect specific

epitopes from the pre-S2 region of the genome (which defines a genotype).¹³ Serological assays are easy to perform and appear most cost-effective and therefore more likely to become part of routine clinical screening. Another important advantage of this method is that it can also be used in HBsAg-positive and HBV-DNA-negative patients or those who have very low level of viremia. This ELISA detects specific serotypes which are made up of the surface gene products known as a determinant (which is constant) and two variable components (d/y or w/r). So, a serotype is determined by a combination of these three determinants (a + d or y + w or r). Based on this, there can be nine different serotypes detectable by ELISA (ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adr_q+, and adr_q) which either alone or in combination correspond to different genotypes.¹⁴

Multiplex Polymerase Chain Reaction

In this method, apart from the standard nested PCR (wherein, universal primers are used for round one and round two of PCR), genotype-specific primers are also used in addition to the internal primers in round 2. The products are then detected as bands on agarose gel electrophoresis. The bands thus obtained are specific for the genotypes.¹⁵ A recent modification of primers could enable the detection of genotypes A–G instead of A–F (with earlier versions).¹⁶ Multiplex PCR has demonstrated a sensitivity of 94–100% and a specificity of 92–97.5% and can also accurately determine mixed genotypes.

Real-time Polymerase Chain Reaction

The melting curve analysis during real-time PCR is used to determine the HBV genotype. Because of the presence of different guanine–cytosine (GC) content of and/or degree of complementarity between the probe and target, in different genotypes the melting temperature analysis can identify the genotypes. This method has the advantage of simultaneously determining the HBV-DNA load as well as genotype and its concordance with direct sequencing has been found to be close to 92%.¹⁷ One study ($n=126$ chronic hepatitis B (CHB) patient samples) compared the performance of real-time PCR, DNA microarray chip, and sequencing. It found that though DNA microarray technology was the most sensitive method, real-time PCR was more sensitive than other methods for detection of mixture of genotypes. It was also the cheapest and fastest among the three for this purpose.¹⁸ Another important advantage of this technique is a very low-risk of cross-contamination.

Microarray Chips

Genotype-specific oligonucleotide probes on a slide are used to capture genotype-specific sequences from an amplified PCR product from the pre-S region. This can detect single as well as mixture of genotypes with high sensitivity. Song et al¹⁹ using 15 oligonucleotide probes demonstrated

100% concordance with RFLP and sequencing data for HBV genotyping. One advantage of a microarray technology is that it can detect not only genotypes but a large number of other mutations (such as pre-core, core promoter, and drug-associated mutations) in the HBV genome during the same analysis.²⁰ In essence, it is a high throughput technology, which can give a large volume of information in a short time.

If HBV genotyping has to become a clinically routine procedure and not remain only as a research tool for all patients infected with hepatitis B, then the simple, cheaper, and reliable techniques such as type-specific ELISA, real-time PCR, INNO-LiPA, and RFLP will have to become the techniques of choice.

Clinical Relevance of Hepatitis B Virus Genotypes

Because of the geographical sequestration of various genotypes, direct comparison of the clinical features between all the genotypes is limited. However, there is information regarding the clinical behavior of genotype B vs C and genotype A vs D, since these genotypes are clustered together in a geographical area (Table 1). We shall present here the available data on the clinical implications of the various HBV genotypes.

Hepatitis B Virus Genotypes and Severity of Illness

Among 126 Chinese patients infected with HBV, 61% of whom had active CHB (21 mild, 30 moderately severe, and 10 severe CHB), genotype B was commoner in the mild CHB group than in the moderate ($P=0.0001$) or in severe groups ($P=0.001$).²¹ Among 258 histologically confirmed HBV-related patients, advanced fibrosis was more frequent in genotype C-infected (74/224) patients than in genotype B (4/30)-infected. Genotype B patients were also more likely to be hepatitis B e-antigen (HBeAg)-negative than genotype C patients.²² A large study from the risk evaluation of viral load elevation and associated liver disease (REVEAL) group demonstrated that in Taiwanese HBV carriers ($n=2762$; 33,847 person years of follow-up), the incidence of HCC was significantly higher among genotype C-infected patients than among genotype B-infected ones (785.8/100,000 person years vs 305.8/100,000 person years). The hazard ratio for the development of HCC for genotype C was 1.76 (1.19–2.61) vs genotype B.²³ However, in a previous study, comparing 100 HBV carriers and 170 patients who are HBV-infected with cirrhosis and HCC demonstrated that though genotype C was more commonly detected among cirrhotics (60% vs 23%; $P<0.001$) and liver cancer (415% vs 15%; $P=0.005$) patients >50 years of age, genotype B was more frequent among patients with HCC <50 years of age (80% vs 52%; $P=0.03$).²⁴ Thus, it is clear that HBV genotype C is associated with more advanced liver disease with increased risk of developing HCC as compared with genotype B.

Most of these studies comparing the severity of the disease in relation to genotypes B and C come from South East Asia, where the prevalence of other genotypes is limited. There are, however, studies from the Indian subcontinent (where genotypes D and A predominate) (Table 4)²⁵⁻³⁰ which have looked at the effect of these genotypes on disease severity. The studies comparing the clinical outcomes and clinical severity of the disease between genotypes A and D have presented conflicting results.

Thakur et al²⁵ from India studied 130 HBV carriers, all of whom had histological evaluation. The patients were divided into three groups: incidentally detected asymptomatic HBsAg-positive subjects (IDAHs; $n=52$), LC ($n=48$), and HCC ($n=30$). Only genotypes A and D and mixture of the two were found. Although there was no difference in the genotype distribution between these three groups, patients in the IDAHs group who were infected with genotype D had a histological activity index (HAI) >4 . Genotype D was significantly more frequent in IDAHs patients with HAI >4 , as compared with genotype A (53% vs 32%; $P<0.05$). This was in contrast from another study from North India, which demonstrated that genotype A may be associated with more severe liver disease than genotype D. In this study, 70 patients were evaluated of which 53% were genotype A, 46% were genotype D, and 1% were genotype C. Patients with genotype A more often had alanine aminotransferase (ALT) levels higher than 1.5 times upper limit of normal than those with genotype D (81% vs 58%; $P<0.05$). Although there was no difference in the genotype prevalence between patients with CH and LC, in the subgroup of >25 years of age, genotype A-infected patients more often had LC than genotype D-infected patients (98.2% vs 57%; $P<0.05$).²⁶ Subsequent large studies have not been able to demonstrate any influence of genotype A or D on the clinical severity of HBV disease from India. Among 247 patients from North India, 68.7% of whom were infected with genotype D, no difference in the prevalence of genotypes was found between any disease states (acute hepatitis, asymptomatic carriers, CH, LC, or HCC). Further, of the 87 patients who had liver biopsy available, the HAI and fibrosis stage were similar between the genotypes.²⁷ Chattopadhyay et al²⁸ compared the genotype distribution between asymptomatic carriers, acute hepatitis, and fulminant hepatitis and also could not find

any difference. Another recent study from North India evaluated 273 HBV carriers [entire spectrum of HBV diseases was included: acute hepatitis (50), fulminant hepatic failure (24), CHB (84), cirrhosis (24), HCC (35), and inactive carriers (61)] and again could not demonstrate any difference in severity between the different genotypes.³⁰

A study from Uzbekistan in Central Asia also could not demonstrate any difference in ALT levels or e-antigen status between genotype A- and D-infected patients.³¹ Toan et al³² evaluated 86 asymptomatic carriers, 92 patients with LC, and 84 with HCC in Vietnam and demonstrated that genotype A was more prevalent among asymptomatic carriers.

In a study from Spain, liver-related deaths were more frequent in genotype F as compared with genotype A ($P=0.02$) or genotype D ($P=0.002$), but the sample size in genotype F group was very small and genotype D was more frequent among LC patients.³³

Thus, although the results of studies evaluating genotypes A and D are conflicting, there is some suggestion that genotype D may be associated with more severe liver disease than genotype A. But this needs to be confirmed by more robust prospective data.

Hepatitis B Virus Genotypes and Disease Progression

It has been demonstrated in a study from Japan that genotype A patients more often have a tendency to persist as a CH after an episode of acute hepatitis B.³⁴ In this particular study, 97 acute hepatitis patients were studied and 7/33 (23%) infected with genotype A persisted as CH whereas only 11% and 7% of genotype B- and C-infected patients, respectively, developed CH. Another study from China demonstrated that patients with acute hepatitis B due to genotype C2 were more likely to progress to CH than those with genotype B2.³⁵

Between 1988 and 1992, 4841 Taiwanese HBV carrier men were followed and 154 developed new HCC. Genotyping was done in these 154 and 316 controls (those who did not develop HCC). The adjusted OR for developing HCC for genotype C was 5.11 (3.2–8.18) vs other genotypes. The effect of DNA load was additive to genotype C in increasing the risk of developing HCC in this study.³⁶

One of the early studies comparing genotypes A and D was published from Spain, wherein 258 Spanish HBV

Table 4 Prevalence of hepatitis B virus genotypes from India.

Study	n	Genotype A (%)	Genotype D (%)	Genotype C (%)	Mixed (%)
Thakur et al ²⁵	130	46	48		6
Kumar et al ²⁶	70	53	46	1	
Chattopadhyay et al ²⁸	160	7.5	92.5		
Banerjee et al ²⁹	230	25	57	18	
Madan et al ²⁷	247	25.7	68.3	5.9	
Sharma et al ³⁰	273	28.6	67.4	0.7	3% had genotype B

carriers were followed up. The prevalence of genotypes A, D, and F was 52%, 35%, and 7%, respectively. Genotype A was demonstrated to be associated with favorable disease outcomes than genotype C or F. The biochemical remission and clearance of HBV-DNA was more frequent in genotype A-infected patients ($P=0.002$) and so was HBsAg clearance ($P=0.03$).³³

More studies are needed which will prospectively follow-up patients of different genotypes in order to definitely answer the question about the influence of genotypes on disease progression over time.

Hepatitis B Virus Genotypes and Response to Interferon Therapy

The influence of genotypes on the response to interferon (IFN) therapy may soon become one reason for HBV genotyping becoming part of clinical care of HBV-infected patients. A number of studies have now demonstrated that the response to both standard IFN and pegylated IFN may be influenced by HBV genotypes.

Erhardt et al,³⁷ in a retrospective analysis (among 144 CHB patients treated with standard IFN), demonstrated that the sustained virological response was significantly higher among genotype A-infected patients as compared with genotype D (49% vs 26%; $P<0.005$). Genotypes B, C, and E were excluded from the final analysis because of small numbers of such patients in the population. Between genotype B- and C-infected patients, genotype B is associated with higher sustained viral response (SVR) as compared with genotype C. Among e-antigen-positive patients treated with standard IFNs, the SVR was 39% and 17% among genotype B- and genotype C-infected patients, respectively. Low pre-treatment HBV-DNA load and genotype B were independent factors associated with SVR.³⁸

Data pooled from two large multinational pegylated IFN trials in patients with CHB demonstrated that there was a gradation of e-antigen clearance/e-antigen seroconversion response according to the genotypes: SVR was 44.3%, 31.5%, 29.1%, and 24.4% among genotypes A-D-infected patients, respectively.^{39,40} In addition, long-term HBsAg loss/seroconversion was also clearly significantly higher among genotype A infected CHB patients (both e-antigen-positive (15.3%, 1.7%, 2.2%, and 1.6% for genotypes A-D, respectively) and e-antigen-negative (20%, 6%, 9%, and 6% for genotypes A-D, respectively) when treated with pegylated IFNs.^{41,42} Among 221 e-antigen-positive patients undergoing treatment with pegylated IFN- α 2b, it was demonstrated that the on-treatment HBsAg decline was significantly more common in genotypes A and B vs genotypes C and D.⁴³

A systematic review reported that irrespective of e-antigen status, genotype A was associated with significantly higher response rates to IFNs as compared with genotype D, whereas genotype B was associated with higher response rates as compared with genotype C in e-antigen-positive

patients only.⁴⁴ Therefore, in future, decision regarding therapy with IFNs may involve the determination of genotype of the infecting strain, and only patients with favorable genotypes (A or B) in addition to other favorable factors (low viral load, high pre-treatment ALT levels, and younger age) will be offered therapy with IFNs. Patients infected with genotype D, irrespective of viral load or ALT levels, may not be good candidates for IFN-based therapy.⁴⁵

Hepatitis B Virus Genotypes and Response to Nucleos(t)ide Therapy

Unlike the clear association of genotypes with response to IFN therapy, the association of genotypes with response to nucleos(t)ide therapy in patients with CHB is more controversial. The meta-analysis cited above analyzing 20 studies did not find any association between HBV genotypes and response to nucleos(t)ide analog (lamivudine, adefovir, entecavir, or telbivudine) therapy.⁴⁴ Other studies also have not been able to show any effect of genotypes on the response rates to telbivudine, adefovir, or entecavir.⁴⁶⁻⁴⁸

Recently, however, data are accumulating that patients infected with genotype B HBV are more likely to develop YMDD (tyrosine-methionine-aspartate-aspartate) mutations while on lamivudine therapy. In a study from China, 319 patients with CHB who received lamivudine for >1 year were evaluated. The rate of development of YMDD mutations was higher in genotype B-infected patients than in genotype C-infected (36.5% vs 24.1%; $P=0.05$).⁴⁹

Hepatitis B Virus Subgenotypes and their Clinical Implications

In a study from Eastern China, Yin et al⁵⁰ evaluated 462 HCC, 425 asymptomatic carriers, and 234 patients with CH. The prevalence of HBV subgenotypes was C2 subtype in 72.9%, B2 subtype in 13.4%, and mixture of genotypes was 10.6%. Hepatitis B virus C2 was found to be more prevalent among patients with HCC whereas HBV B2 was more frequently associated with HCC recurrence after resection. Patients infected with genotype mixtures were more likely to have higher viral load.

A large study from Eastern India evaluated 109 HBV genotype D-infected patients (41 had occult HBV infection; 28 had CHB and 40 were asymptomatic carriers). The distribution of subtypes of HBV D was D1-17%, D2-29%, D3-34%, and D5-20%. Genotype D1 was significantly associated with CLD and was more commonly associated with pre-core mutation (A1896). Occult HBV was more commonly associated with HBV genotype D3.⁵¹

Kew et al⁵² have demonstrated that the African subtype of genotype A (genotype A1) has been associated with higher rates of HCC especially in younger individuals than in other genotypes.

The subtype of genotype A from India has been reported to be the 'Aa' subtype, which is associated with lower viral loads and lower in vitro replication capacity as compared with subtype Ae and genotype D virus.^{53,54} This may also explain the clinically milder disease associated with genotype A-infected strains described earlier from India.

CONCLUSION

Hepatitis B virus genotyping which started as a research tool, mainly to study the evolutionary pattern of HBV, has now become available at clinical laboratories, thanks to the newer molecular biology techniques. The techniques for genotyping have evolved to simpler and cheaper versions which agree very well with the gold standard of direct sequencing. This availability of simple genotyping methods, along with the understanding of the fact that certain genotypes may influence disease severity, clinical outcomes, and therapeutic response, will definitely bring this tool to the door step of hepatology clinics. The most important clinical use of HBV genotype determination lies in the prediction of the development of HCC (along with other variables) and the prediction of response to IFN therapy. It can open an era of tailored treatment for hepatitis B infected patients. Although larger prospective studies are still needed to evaluate the role of genotypes in determining the progression of disease, we feel that HBV genotyping has reached the stage where it should be incorporated in routine clinical hepatology practice at least for therapeutic decisions.

CONFLICTS OF INTEREST

All authors have none to declare.

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