Advances in the Molecular Diagnosis of Hepatitis B Infection: Providing Insight into the Next Generation of Disease

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Abstract

Hepatitis B virus (HBV) infection continues to represent a significant health threat, affecting over 400 million people worldwide. Historically, the diagnosis and treatment of chronic hepatitis B (CHB) relied in detection of the hepatitis B surface antigen (HBsAg) and more recently realtime polymerase chain reaction (PCR) analysis. The advent of novel technologies and equipment for the identification and staging of the different stages of HBV infection has resulted in dramatic changes to patient monitoring and management. Through the use of rapid, quantitative HBsAg immunoassays, it is now possible to predict the likelihood of patient response to treatment as well as the clinical course of disease. Ultradeep sequencing technologies (also known as next-generation sequencing) overcome many of the traditional limitations associated with population-based sequencing approaches, and have provided significant insight into the viral response to therapeutic intervention and the molecular pathogenesis of CHB. The authors discuss recent developments in the molecular diagnosis of HBV infection, as well as potential advantages and caveats resultant of this rapid progression of technology.

Keywords

► hepatitis B virus (HBV)
► chronic infection
► diagnosis
► hepatitis B surface antigen (HBsAg)
► next-generation sequencing

Background

Viral evolution occurs at a rate unparalleled by any organic entity. Selective pressures, including host immune response, antiviral treatment and vaccination, as well as viral replicative fitness, all contribute to the rapid diversification of a single virion into multiple quasispecies. These factors in turn play a significant role in determining the clinical course of infection, an increasingly important consideration, given the progression of medicine toward individualization of patient treatment.

The discovery by Blumberg and colleagues of an atypical serum protein, later shown to be hepatitis B surface antigen (HBsAg), in the blood of an Australian aborigine was a milestone in public health safety.[1] It led to the use of the excess HBsAg found in the blood of patients with chronic hepatitis B (CHB) as the first hepatitis B vaccine and provided the impetus for the development of assays for the diagnosis of hepatitis B virus (HBV) infection.

Over the past 25 years, there have been significant advancements in molecular biology and antiviral therapy, perhaps most notably in the treatment of viral infection. In the following review, the recent advances in the molecular diagnosis of HBV infection (through testing of HBsAg) and evolution of infection and drug resistance (through ultradeep

sequencing) in the context of antiviral treatment will be discussed.

Molecular Diagnosis of HBV Infection

The detection of serum hepatitis B surface antigen (HBsAg) represents the serological hallmark of HBV infection. It is an early serological indicator of acute HBV infection, and is the diagnostic marker for defining CHB when present in serum for longer than 6 months. HBsAg seroclearance and anti-HBs seroconversion with undetectable HBV DNA by polymerase chain reaction (PCR) represents successful resolution of infection and is the goal of antiviral therapy. It is believed to represent immunologic control of HBV, and is associated with an improved long-term outcome.[2–4] Automated enzyme immunoassays for detection of HBsAg are now routinely used, but recently sensitive and reliable commercial assays for the quantification of serum HBsAg have become available. Consequently, they have become a focus for translational and clinical research.

HBsAg: Transcription and Translation

HBsAg is predominantly derived from transcriptionally active HBV covalently closed circular DNA (cccDNA). The HBV genome encodes four overlapping open-reading frames (ORFs), including an envelope/surface ORF. The envelope ORF contains three in-frame start codons that further divide it into preS1, preS2, and ORFs domains (►Fig. 1). Three structurally related envelope proteins are formed from the envelope messenger RNA (mRNA) transcripts: small (S ORF), medium (preS2 + S ORF), and large (preS1 + preS2 + S ORF).

Newly synthesized envelope protein interacts with mature HBV nucleocapsids at the endoplasmic reticulum prior to secretion from the hepatocyte. HBsAg production greatly exceeds that required for virion assembly, and excess envelope proteins also combine to form noninfectious filamentous and spherical subviral HBsAg particles.[5] Such subviral particles are secreted into the systemic circulation, and typically exceed HBV virions by a variable factor of 10^2- to 10^5- fold.[6] The role that these subviral particles play is difficult to define; they may help in evading or subverting the host immune response,[7] and can also coexist with anti-HBs as part of circulating immune complexes.[8] HBsAg can also be independently produced from integrated viral DNA sequences formed by illegitimate recombination of double-stranded linear HBV DNA into host DNA.[9,10]

Assays for HBsAg Quantification

The two most commonly reported assays for the quantification of serum HBsAg are the ARCHITECT HBsAg QT (Abbott Laboratories, Rungis, France)[11] and the Elecsys HBsAg II Quant (Roche Diagnostics, Penzberg, Germany.[12,13] Both immunoassays are relatively inexpensive, are fully automated with a high throughput capacity and express HBsAg titers in IU/mL (standardized against the World Health Organization international standard). Given that most serum HBsAg titers are above the dynamic range of the assays, samples are usually initially diluted prior to testing. The Elecsys HBsAg II Quant assay has an on-board dilution system that minimizes retesting,[13] and reduces the potential for error. These two assays demonstrate a strong correlation across a range of HBsAg titers.[14,15] Both of these assays detect all forms of circulating HBsAg: virion-associated as well as subviral filamentous and spherical particles.

HBsAg and Intrahepatic HBV Markers

The limiting factor in clearing HBV infection is believed to be the pool of HBV cccDNA in the nucleus of infected hepatocytes. It has been proposed that the cccDNA level may also represent the infected liver cell burden.[16] The most common method for quantifying HBV cccDNA requires liver tissue, and the technique itself is not standardized and is restricted to specialized research centers. HBsAg titers have been proposed as a surrogate marker for transcriptionally active HBV cccDNA. A recent detailed study of HBsAg levels in a large cohort of treatment naïve patients with CHB demonstrated an
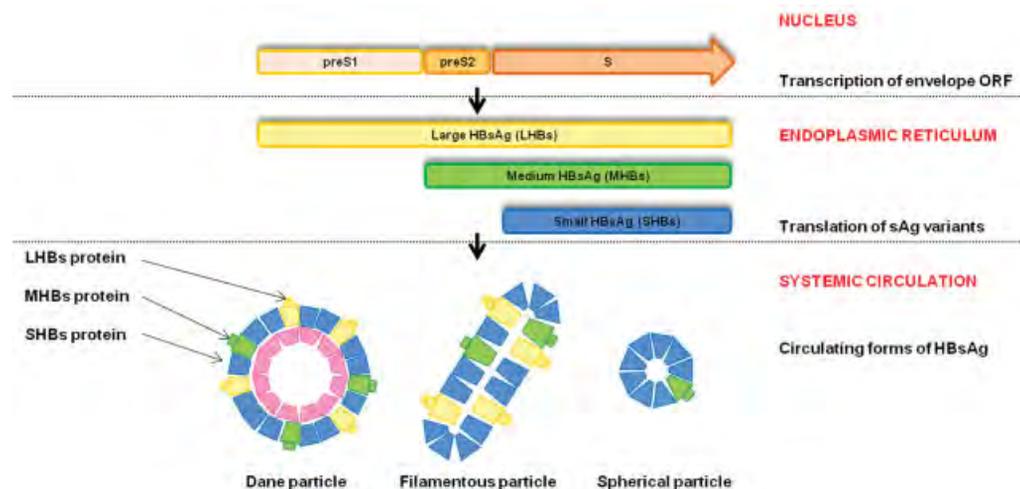
Fig. 1 HBsAg transcription, translation, and physical forms in the systemic circulation. HBsAg, Hepatitis B surface antigen; ORF, open reading frame.

association between HBsAg titers, cccDNA, and total intra-hepatic HBV DNA, but only in hepatitis B e antigen-positive (HBeAg-positive) patients.[17] This may be because of the relative preservation of HBsAg production in comparison to HBV replication in HBeAg-negative CHB.[18] The relationship between HBsAg titers and intrahepatic HBV markers is complex, and is likely affected by both virological and host immunologic factors. Further research is required to elucidate the utility of HBsAg as a practical biomarker for intrahepatic HBV DNA replicative forms.

HBsAg in the Different Phases of Chronic Hepatitis B

Several independent studies have demonstrated that the serum HBsAg titers differ between the four phases of CHB (►Table 1).[19–22] In the context of the natural history of CHB, the correlation between HBsAg titers and HBV DNA is complex, and studies have demonstrated conflicting associations. However, similar to HBV DNA levels, HBsAg titers are typically higher in the blood of patients with HBeAg-positive compared with HBeAg-negative CHB. The highest and lowest median HBsAg titers are evident in the immune-tolerant (IT) and low-replicative (LR) or immune-control (IC) phases, respectively (►Table 1). It is important to note that the IC phase is characterized by the highest HBsAg/HBV DNA ratio.[20,22] The apparent "disconnect" between HBsAg and HBV DNA at different phases may possibly be due to the expression of HBsAg from integrated viral envelope DNA, instead of HBsAg production off mRNA derived from the HBV cccDNA template. Another possible explanation is a difference in the immune regulation of viral replication during different phases of infection, resulting in altered ratios of HBV virion to subviral HBsAg particles.[5] Finally, very recent work has shown that the presence of preS/S variants, commonly found in patients with CHB, can influence the levels of circulating HBsAg without impacting significantly on serum HBV DNA load.[23]

Combining HBsAg titers and HBV DNA load to provide complementary information during the natural history of CHB has been likened to the assessment of a ship's "longitude and latitude" position in the ocean.[24] Several groups have proposed HBsAg and HBV DNA titer cutoffs to more accurately identify patients in the IC phase. The proposed HBV DNA cutoff level that defines this phase has been the source of much debate and the measurement of HBsAg levels may have a clinical utility in differentiating patients who are likely to remain in the IC phase.[25,26] This is of clinical relevance, as a single point test of serum alanine aminotransferase and HBV DNA can potentially misclassify patients in the IC and HBeAg-negative hepatitis phases, respectively. A DNA level of < 2,000 IU/mL and an HBsAg of < 1,000 IU/mL typically defines the LR phase.[25,26]

Antiviral Therapy: Effect on HBsAg Titers

Retrospective studies of CHB patients treated with pegylated interferon have shown that there may be a role for HBsAg quantification in the initial identification of potential responders at baseline and also in predicting response for patients on therapy.[27–29]

In HBeAg-positive patients, reduction of HBsAg levels to < 1,500 IU/mL at week 12 of pegylated-interferon (PEG-IFN) therapy has been shown to be a favorable indicator for subsequent HBsAg seroconversion. More than half of the patients who attained this level at week 12 had HBeAg seroconversion 6 months posttreatment and nearly 20% of these achieved subsequent HBsAg clearance at 6 months posttreatment. In contrast, HBsAg levels of >20,000 IU/mL at week 12 was a strong negative predictor of response and could become a potential stopping rule.[29,30] Combining threshold levels of HBsAg with HBsAg decline on therapy may offer even higher predictive values. The study of Chan and colleagues showed very high rates of response (HBeAg seroconversion and HBV DNA < 2,000 IU/mL) in patients having both > 1 $\log_{10}$ decline in HBsAg as well as HBsAg levels < 300 IU/mL at 24 weeks of PEG-IFN therapy (75%) compared with patients not achieving these combined values (15%; $p < 0.001$).[31]

An early decline in levels of HBsAg in HBeAg-negative patients treated with PEG-IFN was also shown to have a high predictive value of a sustained response. A decline of 0.5 and 1 $\log_{10}$ HBsAg at weeks 12 and 24 of therapy, respectively, had a high predictive value of achieving an undetectable viral load at 24 weeks posttherapy (12 weeks—negative predictive value [NPV] 90%, positive predictive value [PPV] 89%; 24 weeks—NPV 97%, PPV 92%).[28] Consistent with this, a retrospective analysis of HBsAg levels from the Roche registration study for PEG IFN-2a showed that patients who achieved HBsAg decline ≥ 10% from baseline at week 12 of treatment, almost 50% achieved a sustained response at 1-year posttreatment and 40% of these individuals achieved HBsAg clearance at 5-years posttreatment. Rijckborst and colleagues

Table 1 Distribution of HBsAg titers in the different phases of chronic hepatitis B

	IT (log₁₀ IU/mL)	IC (log₁₀ IU/mL)	LR (log₁₀ IU/mL)	ENH (log₁₀IU/mL)
Chan et al 2010[a]	4.97	3.78	2.24	2.98
Jaroszewicz et al 2010[19,20]	4.96	4.37	3.09	3.87
Kim et al 2011[21]	4.29	3.64	2.05	3.23
Nguyen et al 2010[22]	4.53	4.03	2.86	3.35

Abbreviations: IT, immune tolerant; IC, immune clearance; LR, low replicative or immune control; ENH, HBeAg-negative CHB.
[a]This was a longitudinal study; HBsAg titers taken from the first visit.

proposed a clinically useful algorithm in HBeAg-negative CHB that any HBsAg decline at week 12 with a 2 log$_{10}$ drop or more in HBV DNA could predict almost 40% of sustained responders in their cohort.[32] Patients not achieving any HBsAg decline or only having a < 2 log$_{10}$ drop in HBV DNA did not respond.

The effects of nucleos(t)ide analogue therapy on HBsAg levels during treatment has not been comprehensively evaluated. Preliminary data suggest that the decline of HBsAg is slower and seroconversion is less frequent compared with PEG-IFN treatment. Very recent mathematical modeling, based on a patient population with an average follow-up on treatment of over 8 years, suggested that even if HBV replication was controlled that HBsAg clearance was unlikely to occur in the patient's lifetime.[33] Over such a long period, issues of compliance and development of antiviral resistance would also need to be considered. However, further studies of patients on very long form therapy are required.

Ultradeep Sequencing of the HBV Genome

Historically, the genetic diversity of HBV quasispecies was estimated through laborious and expensive PCR amplification and cloning of individual amplicons, followed by population-based Sanger sequencing. This situation has been revolutionized with the introduction of the "next generation" of ultradeep-sequencing platforms. Ultradeep sequencing overcomes the major caveats of clonal Sanger sequencing, allowing direct sequencing of the mixed population sample and relative quantification of individual mutations with extremely high coverage (up to 10^5 reads per base pair) over a relatively short time frame.[34,35]

There are an increasing number of ultradeep-sequencing platforms commercially available and although the underlying technologies differ in several aspects (see below and recent reviews[35,36]), all utilize the primary steps of DNA library (or PCR amplicon) preparation, amplification, and subsequent sequencing by synthesis.

Sequencing by synthesis involves sequencing of a single strand of DNA through synthesis of the complementary strand, one base at a time, and the detection of the individual nucleotide incorporated at each step.[37] Fluorescence (Illumina-based) or light (pyrosequencing: Roche and QIAGEN-based) is only emitted when the nucleotide solution complements the first unpaired base in the template DNA strand. This enables reliable sequence detection in "real time." In instances when the expected genetic sequence is known, bases may be added in a predetermined order, allowing longer frame reads.[35,38] Where the reference sequence is unknown, or expected to deviate significantly from the wild-type (WT) sequence, it may be preferable to add nucleotides in a cyclical fashion.

Because these signals are obtained by synthesizing new copies of DNA template, the results provide unambiguous information that can be used for extremely reliable investigation of viral mutations. Furthermore, unlike population-based approaches, a large number of samples can be sequenced simultaneously through the inclusion of unique identifier tags, ligated to a universal, conserved primer sequence.[36]

454 Sequencing (Roche Diagnostics)

Despite relatively high costs and labor times compared with other ultradeep-sequencing technologies,[39] 454 sequencing remains the most commonly used platform for amplicon sequencing, owing primarily to its longer read length (►Table 2).

454 sequencing relies on amplification of the DNA library (or PCR amplicon) via emulsion PCR. Emulsion PCR uses water droplets within an oil phase as microreactors for single strands of DNA. Each water droplet contains a single-DNA-template strand and all of the necessary reagents for clonal amplification of the target.[36,40] Prior to sequencing, each microreactor (along with the clonally amplified DNA) is deposited into a separate well on a glass slide and then overlain with the sequencing enzymes. A pyrosequencing reaction is used to generate light as each nucleotide is incorporated into the nascent DNA strand.[40] This approach is highly sensitive with multiple reports estimating detection of single point mutations down to a level of $< 2\%$.[41–43]

Illumina Sequencing (Illumina/Solexa)

Illumina has developed an ultradeep-sequencing platform that utilizes a solid-phase flow cell for clonal amplification and sequencing by synthesis. Sheared DNA (or PCR amplicons) is ligated to universal adaptors that hybridize to complementary sequences on a glass slide coated with millions of primers. Clonal amplification occurs through denaturation of the hybridized DNA and generation of the complementary DNA strand via extension from the 3′ end of the ligated adaptor/primer sequence.[36,44] The 3′ end of the newly generated sequence then anneals to an adjacent, complementary surface-bound primer, forming a bridge and a new site for DNA synthesis in the next PCR cycle. The final sequencing by synthesis reaction is performed using fluorescently labeled reversible terminators[44] with sensitivity reportedly comparable to that of the 454 platform.[35,36,45]

Table 2 Summary of relevant ultradeep sequencing technologies

Method	Amplification	Separation	Sequencing	Read length
Roche 454	Emulsion PCR	Microbeads and picotiter plate	Pyrosequencing	Up to 700 bp
Illumina	In situ bridge amplification	Glass slide hybridization	Reversible terminators	Up to 500 bp
Pyromark	Standard PCR	Biotinylation	Pyrosequencing	~ 20 bp

Source: Table adapted from Radford et al[35]

Although the operating cost of this system is reduced in comparison to other ultradeep platforms, the main limitation to this system is read length,[35] although this has increased in recent years (►Table 2).

Pyromark Sequencing (QIAGEN)

Whereas 454 and Illumina sequencing platforms are both suitable for de novo sequencing, Pyromark is ideal for rapid turnaround on presence/absence of previously characterized mutations of interest.

In this platform, the primary PCR is performed using a biotinylated reverse primer and the double-stranded PCR product is bound to streptavidin beads. Following denaturation, a regular sequencing primer (complementary to the reverse strand) is added in a rapid hybridization, followed by pyrosequencing of the nucleotides immediately 3′ to the primer. In this manner, Pyromark sequencing provides accurate sequence data and proportions of specific mutant genotypes within an individual sample for $\sim$ 20 bases, over a time frame of $\sim$ 15 minutes, down to a sensitivity of $<$ 5%.[46,47]

Detailed specifics of these sequencing technologies (as well as several others) have been the subject of elegant reviews by Metzker[36] and Radford.[35] As such, the following text will focus primarily on the application of ultradeep-sequencing techniques to the sequencing of HBV.

Applications for Ultradeep Sequencing in HBV Research: The Current State of Play

HBV represents a unique setting for the application of ultradeep sequencing. Although characterized as a DNA virus, the partially double-stranded nature of the 3.2 kB HBV genome requires replication via a pregenomic RNA intermediate using a virally encoded reverse transcriptase. Akin to RNA virus reverse transcriptases, the HBV polymerase lacks proofreading ability, resulting in transcriptional error rates similar to other RNA viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV).[48] High transcriptional error rates result in the rapid generation of multiple viral quasispecies within a single individual which has major implications on the clinical course of disease.

As early as 2004, preliminary ultradeep-sequencing studies of HBV adopted a cross-sectional approach, enabling direct comparison of traditional population-based studies with the newly available ultradeep techniques.[47] More recently, these studies have become geared toward the longitudinal assessment of viral sequences in patient samples, with relevant clinical data allowing for a more detailed investigation of the HBV viral pool.[49,50]

The spectrum of currently available data generated using ultradeep sequencing in studies of HBV is discussed in the context of three clinically relevant categories: viral discovery/ viral quasispecies analysis, disease pathogenesis, and response to therapy and immune pressure/antiviral resistance detection.

Viral Discovery/Viral Quasispecies Analysis

Traditionally, the identification of novel viruses was painstakingly slow involving initial identification via electron microscopy, in vitro culturing of the infectious agent, followed by nucleic acid purification and amplification, cloning and only then, viral sequencing. Despite the success of this approach, a major caveat remains the preferential amplification of the dominant infectious species.

Ultradeep sequencing methods have proven superior in the analysis of minor viral variants. Although Sanger sequencing approaches such as the workflow described above may detect only those quasispecies representing more than 20% of all viral isolates,[47,51] ultradeep sequencing has repeatedly demonstrated the ability to detect minor isolates comprising less than 2% of the total population.[42,50,52] A recent study by Margeridon-Thermet et al demonstrated this sensitivity, detecting coinfection of a small number of patients with HBV genotypes A and G.[42] In several instances, viral recombination events between these genotypes were also identified. Although the consequences of HBV coinfection with HIV, HCV, and delta agent (HDV) have been well characterized, the implications of dual HBV genotype infection remain relatively unknown. Ultradeep sequencing represents an opportunity to investigate and further define not only the frequency of HBV genotype coinfections and recombination events, but how such phenomena may impact on the pathogenesis of HBV-associated disease.

A recent study by Nishijima et al examined potential differences between intrahepatic HBV variants compared with those present circulating in the serum.[45] This study, performed using the Illumina Genome Analyzer II, hypothesized that those HBV isolates present in the serum may possess a replicative advantage compared with those quasispecies present in the liver parenchyma (which would be more likely to remain in the inactive carrier state). Although the results failed to find any appreciable differences in the HBV quasispecies present at these sites, a range of drug-resistant HBV variants were identified in treatment naive patients, suggesting that preexisting minor mutants may occur naturally (see below). This finding has significant implications for the prospective monitoring and individualized therapy of HBV-infected patients and the progression of their clinical disease.

Disease Pathogenesis

Chronic infection with HBV is temporally linked with the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Several multifactorial models (also known as risk calculators) have been developed with the aim of identifying those patients at increased risk of developing HCC. In addition to the widely recognized risks of increased and sustained serum HBV load; there is evidence to suggest that genotypic differences as well as particular mutations in the HBV genome (including basal core promoter and pre-S deletions), may also be important in HBV-disease progression.

Among Asian countries, HBV-genotype C infection tends to result in more severe liver disease (including fibrosis and HCC) than infection with genotype B.[53] Likewise, among those of European ethnicity, genotype D infection is typically associated with more severe disease than genotype A, possibly due to greater heterogeneity within genotype D

quasispecies.[54] This is further confounded by the identification of subtypes within HBV genotypes, some of which are the result of intergenotypic recombination events. For example, the Ba (B2) subgenotype of HBV-genotype B, prevalent throughout most of Asia and believed to have arisen from a recombination event between HBV-genotypes B and C has been linked to the development of more severe liver disease than other genotype-B viruses such as genotype Bj (B1) virus seen in Japan.[55,56] Likewise, the African HBV-subtype A1 is associated with rapid disease progression and a higher incidence of HCC, compared with the European genotype A2.[57] Although yet to be determined, increasing evidence suggests that concurrent mutations within the HBV promoter and core regions, affecting production of the HBeAg, may play a role in determining such disease progression.[41,58] Ultradeep sequencing platforms provide the potential to monitor genotypic recombination on a previously unmatched scale.

Response to Therapy and Immune Pressure/Antiviral Resistance Detection

Clinical interest in establishing more effective treatment regimens for chronic HBV has meant that the majority of ultradeep-sequencing studies in this setting have focused on the emergence of antiviral resistance and viral response to host immune pressure.

Infection with HBV during early childhood frequently results in chronicity, often requiring lifelong adherence to antiviral treatments to curtail liver disease and progression to HCC (see above). A direct consequence of such treatment is the emergence of antiviral resistance. In general, there are two theories on the emergence of antiviral resistance in HBV infection. The first is that resistant HBV quasispecies arise naturally during the course of infection, owing to the lack of fidelity of the HBV reverse transcriptase. These resistant quasispecies circulate as minor variants within a larger population.[48] The second is that treatment with antiviral drugs directly places the HBV under selective pressure to mutate as a means of survival, resulting in the emergence of treatment-resistant mutants.[42]

Evidence for the former comes from a recent study of natural mutations within the catalytic domain for the reverse transcriptase (RT; YMDD domain) in Western China. A Pyromark-based study of 270 treatment naive HBV-infected patients demonstrated the presence of the rtM204V/I substitutions in around 15% of individuals at baseline, at frequencies of between 0.5 and 22%.[59] Rodriguez-Frias et al and Homs et al similarly described the presence of common

resistance-associated substitutions—rtA181T, rtV191I, rtA194T, and rtM204I in patients prior to the initiation of lamivudine therapy, albeit at much lower levels than the Chinese study.[41,50] Whether such discrepancies are the result of genotypic differences is unknown and will likely be the focus of future study. Most recently, Sede and colleagues used the 454-sequencing platform to demonstrate evidence of the lamivudine-resistant variant rtL180M 7 years prior to detection of lamivudine resistance via population-based sequencing.[49] Although data regarding the presence of rtL180M at baseline was not available, the presence of this variant at around 1% during the first 12 months of treatment suggests that lower levels may have been circulating within the patient prior to treatment initiation.

Despite this compelling data, the direct effect of antiviral therapy in selecting mutation of the HBV reverse transcriptase cannot be overlooked. In a comprehensive study regarding the effects of antiviral treatment on the emergence of resistant mutants using the 454-ultradeep-sequencing platform, Margeridon-Thermet et al demonstrated a significant increase in the presence of the rtV173L, rtL180M, and rtM204V/I substitutions following treatment with either lamivudine, adefovir, or entecavir.[42] Likewise in the aforementioned studies by Rodriguez-Frias et al and Homs et al, all patients who experienced virological breakthrough or treatment failure returned samples that were dominated by those common resistance substitutions (rtA181T, rtM204V/I) detected at baseline.[41,50]

Ultradeep-sequencing studies have also increased our understanding of the importance of mutational linkage; that is the presence of multiple drug-resistant mutations on the same DNA template. Ultradeep-sequencing data suggest that although resistance-associated substitutions may be present at baseline; these are rarely present in combination.[50] This is in contrast to posttreatment samples in which common resistance variants including rtL180M, rtA181T, and rtM204V/I are present in the same viral sequence.[42,49,50] Ijaz and colleagues further suggest that the rtM204V substitution may be acquired prior to rtL180M in HBV patients on lamivudine monotherapy.[60] The same study also concluded that in some instances there may be a reversion to WT virus while on combination antiviral treatment, reflecting variation of effect due to replicative capacity.[60]

The order of acquisition and emergence of treatment-resistance-associated mutations is further compounded by the overlapping nature of the HBV genome, as the coding sequence for the RT is shared by the HBsAg (►**Fig. 2**).[61]
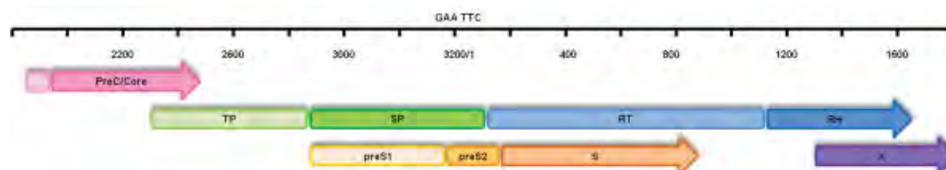
Fig. 2 Linear depiction of the hepatitis B virus (HBV) genome, demonstrating overlapping nature of four major open reading frames. Genome nomenclature is provided according to the EcoR1 restriction enzyme site. TP, terminal protein; SP, spacer; RT, reverse transcriptase; RH, RNase H; S, surface.

Mutations in the RT resulting from antiviral therapy very often alter the overlapping-surface-gene sequence. Data suggest that while the RT is under selective pressure following antiviral treatment, HBsAg is subject to an independent set of selective pressures in the host immune response.[61]

Evidence for this arises from Margeidon-Thermet et al's investigation of HBsAg stop codons in treatment-naive patients. Among 10 separate mutations identified in the HBsAg (nine of which were only detectable via ultradeep sequencing), six generated premature stop codons in the HBsAg, yet the corresponding RT mutation was silent.[42] It has been suggested that truncation of HBsAg may facilitate viral evasion of the immune response through reduction of secreted virus.[61] This finding also emphasizes that the RT sequence is strongly limited by its functionality and that nonsynonymous mutations which do not confer a direct selecting advantage are not tolerated.

Ultradeep sequencing has also been used to study vaccine escape variants, which are an emerging problem particularly in developing countries where the dominant HBV genotype can often differ from the vaccine strain.[62,63] Solmone et al showed that in one treatment-naive patient the classic sG145R vaccine escape variant was detected at a prevalence of over 30%, signifying that immune-selective pressure plays an important role in driving viral mutation.[54] The use of ultradeep sequencing for detection of other vaccine escape mutants that were not detected by conventional population-based sequencing, including sP120T (the concurrent rtT128N mutation displays equal antiviral susceptibility to WT virus), in treatment naive[42] and experienced antiviral patients,[49] further highlights the importance of the newer technologies in detecting pathogenically relevant strains of HBV.

During HBV infection G to A hypermutation is thought to occur as a result of innate immune defense against viral infection. First reported in the setting of HIV infection, specialized host enzymes belonging to the apolipoprotein B RNA-editing catalytic polypeptide-like 3 (APOBEC3) family of cytidine deaminases induce deamination of cytidine bases in negative-stranded DNA during replication, resulting in the replacement of cytidine with uracil; corresponding with a G to A mutation in the positive DNA strand.[64,65]

G to A hypermutation has been observed at low levels in some HBV-infected patients,[66] and it is suggested that it may be immune-induced G to A hypermutation which is responsible for the generation of some antiviral resistant HBV mutants.[43] Ultradeep sequencing has the potential to improve on currently available point mutation assays such as the reverse hybridization LiPA DR (Innogenetics) to investigate not only if a certain mutation is present, but also whether other similar G to A mutations exist in parallel, which may be an indication of hypermutation due to immune response rather than the emergence of antiviral resistance.[54,57] This was evidenced recently by Reuman and colleagues who demonstrated that ∼ 2.9% of HBV sequence reads were classified as hypermutated following sequencing via the Roche platform.[43]

Regardless of their means of emergence, there is a clear need to track all relevant changes in the viral pool over time as it relates to individualization of patient treatment and outcome. The inclusion of baseline data generated via ultradeep sequencing regarding the presence/absence of treatment-resistant mutants and hypermutated variants in antiviral naive patients will allow the clinician to make informed decisions regarding treatment options in these patients and provide an explanation for patients who fare poorly following alterations to the antiviral regimen.

Conclusions and Future Directions: Where to Next?

As with all technological advancement, there are numerous issues to overcome. Emerging data demonstrates that quantification of HBsAg may assist in staging patients, selecting patients for therapy, and subsequent individualization of treatment, particularly for those patients treated with PEG-IFN. The determination of HBsAg titers within the first 3 months of treatment has proven beneficial in identifying those patients who are unlikely to respond to treatment. In this instance, the inclusion of algorithms that combine both HBV DNA load and HBsAg titers will further define those patient populations most likely to benefit from therapy. Here there is a clear requirement for larger, prospective studies that consist of all major HBV genotypes to help realize the full potential of HBsAg quantification.

The implementation of ultradeep sequencing for analysis of HBV genomes within the diagnostic laboratory will have several hurdles to overcome. The predominant limitations of this technology are inherent within the basic techniques employed. The differences between the statistical and biologic/clinical relevance of HBV mutation,[41,52] maximal sequence read length,[35,38] and PCR amplification bias[45,59] are common themes throughout many reports of ultradeep sequencing. In particular with HBV there are the combined issues of potential bias due to primer-binding-site variation, current knowledge of viral mutation (relevant for pyromark sequencing), as well as the sheer volume of data generated in each ultradeep-sequencing run.[67]

The former can only be realized through the completion of additional longitudinal studies and the inclusion of relevant clinical data. The latter are being addressed through the development of the "next" next generation of sequencing methods: third-generation sequencing (reviewed by Schadt[38]). This third generation of sequencing techniques will negate the use of PCR amplification and its innate biases to observe DNA synthesis in vitro in real time.[45,59] In this manner, third-generation sequencing also promises to reduce the lengthy manual handling times associated with current ultradeep-sequencing approaches, decreasing the generation of raw data, but increasing both throughput and read length.[67] In tandem these changes will undoubtedly further increase the utility of ultradeep-sequencing technologies not only in the molecular diagnosis of HBV, but in every facet of medical research.

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