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► **To cite this version:**

Stéphane Chevaliez, Christophe Rodriguez, Jean-Michel Pawlotsky. New virologic tools for management of chronic hepatitis B and C.: New Virological Tools. Gastroenterology, WB Saunders, 2012, 142 (6), pp.1303-1313.e1. <10.1053/j.gastro.2012.02.027>. <inserm-00699942>

HAL Id: inserm-00699942

<http://www.hal.inserm.fr/inserm-00699942>

Submitted on 22 May 2012

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New Virologic Tools for Management of Chronic Hepatitis B and C

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SHORT TITLE: New Virological Tools

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Abbreviations: HBV: hepatitis B virus; HCV: hepatitis C virus; NGS: next-generation sequencing; TMA: transcription-mediated amplification; IU: international unit; LLOD: lower limit of detection; LLOQ: lower limit of quantification; HBsAg: hepatitis B surface antigen; DBS: dried blood spot; dNTP: deoxynucleotide; ddNTP: dideoxynucleotide; GWAS: genome-wide association studies; GS: genome sequencer; emPCR: emulsion PCR; indel: insertions or deletions; ALT: alanine aminotransferase; ULN: upper limit of normal; HBeAg: hepatitis B e antigen; IFN: interferon; DAA: direct acting antiviral; SVR: sustained virologic response

Disclosures: The authors have collectively received research grants from Gilead and Roche. Stéphane Chevaliez has served as an advisor for Gilead and Janssen. Jean-Michel Pawlotsky has served as an advisor for Abbott, Abbott Molecular, Achillion, Anadys, Biotica, Boehringer-Ingelheim, Bristol-Myers Squibb, DebioPharm, Gen-Probe, Gilead, Glaxo-SmithKline, Idenix, Inhibitex, Janssen, Madaus-Rottapharm, Sanofi-Aventis, Schering-Plough/Merck, Novartis, Pfizer, Roche, Vertex and Virco.

ABSTRACT

Molecular biology techniques are routinely used to diagnose and monitor treatment of patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. These tools can detect and quantify viral genomes, and analyze their sequence, in order to determine their genotype or subtype and to identify nucleotide or amino acid substitutions associated with resistance to antiviral drugs. They include real-time target amplification methods, which have been standardized and are widely used in clinical practice to diagnose and monitor HBV and HCV infections, and next-generation sequencing techniques, which are still restricted to research laboratories. In addition, new enzyme immunoassays can quantify hepatitis B surface and hepatitis C core antigens, and point-of-care tests and alternatives to biologic tests that require whole-blood samples obtained by venipuncture have been developed. We review these new virologic methods and their clinical and research applications to HBV and HCV infections.

Keywords: diagnostic assay, real-time PCR, real-time TMA, next-generation sequencing, antigen quantification, point-of-care tests

Virology techniques developed over the past 20 years are routinely used to diagnose and monitor chronic viral infections, such as those caused by hepatitis B virus (HBV) and hepatitis C virus (HCV). Molecular biology tools can be used to detect and quantify viral genomes, sequence them, assign them to a phylogenic clade or subclade (genotype or subtype), and identify clinically relevant nucleotide or amino acid substitutions, such as those associated with resistance to antiviral drugs. Recent advances include real-time target amplification methods for detecting and quantifying viral genomes and next-generation sequencing (NGS) techniques. Other new assays detect and quantify viral antigens, whereas point-of-care tests and alternatives to biologic tests that require whole-blood samples have been developed. We review these methods and how they might be used to diagnose, treat, and manage patients with HBV or HCV infection.

NEW METHODS FOR QUANTIFYING HBV DNA AND HCV RNA

HCV and HBV genomes must be detected and quantified before treatment decisions can be made and to monitor antiviral therapy. The viral genomes can be detected and quantified using target amplification methods, such as the PCR and transcription-mediated amplification (TMA), and with signal amplification methods, such as hybrid-capture and the branched DNA assay. Recently developed real-time target amplification methods have improved viral genome detection and quantification for clinical and research purposes.

Target Amplification Methods

First-generation target amplification techniques have been widely used to diagnose HBV and HCV infections and to monitor responses to antiviral therapies. In

PCR or TMA assays, amplicons are detected at the end of the amplification process by their specific hybridization to immobilized oligonucleotide probes; the amplicon–probe hybrids are detected by an enzymatic reaction. They are quantified based on competitive amplification of the viral template with a known amount of synthetic standard added to each reaction tube; the relative amounts of viral template and standard amplicons are measured and the results are interpreted with a standard curve established in parallel.

Because amplification reactions are saturable, these methods have a narrow dynamic range of quantification. As a result, high levels of virus are not always accurately quantified and require reanalysis after samples are diluted, whereas low levels of virus (such as in patients receiving antiviral therapy) are often not detectable. This problem was solved by development of real-time target amplification techniques in which quantification takes place during the exponential phase of the amplification reaction. In addition, the reaction is run in a closed system, which prevents carryover contamination and improves specificity.

Real-Time Target Amplification

Real-Time PCR

PCR uses several temperatures and a thermostable DNA polymerase to generate double-stranded DNA amplicons. In assays for HBV, nucleic acids are isolated from a sample and the HBV DNA is directly amplified by PCR. Assays for HCV require reverse transcription of HCV RNA, to synthesize complementary DNA, which is used as template in the PCR reaction. Each complete PCR cycle doubles the number of DNA copies. The principle of real-time PCR is to detect amplicon synthesis during the PCR reaction and thereby deduce the starting amount of viral genome in a clinical sample. A fluorescent

probe is linked to a quencher and annealed to the target sequence, between the sense and antisense PCR primers. During each PCR reaction, as the DNA polymerase extends the primer, its intrinsic nuclease activity degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle and detected by the system is proportional to the amount of amplicons generated in each PCR cycle. Software is used to calculate the threshold cycle in each reaction (Ct), which has a linear relationship with the initial amount of nucleic acid. In each PCR run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification.

Real-Time TMA

The TMA reaction is isothermal and uses 2 enzymes—a reverse transcriptase and a T7 RNA polymerase. The amplicons consist of single-stranded RNA. After lysis of the viral envelope, the viral genome (HBV DNA or HCV RNA) is captured by oligonucleotide probes and bound to magnetic microparticles. Amplification involves autocatalytic isothermal production of RNA transcripts with the 2 enzymes. Each newly synthesized RNA re-enters the TMA process and serves as a template for the next round of replication, resulting in exponential amplification of the target RNA. The amplicons created during amplification are detected in real time, using probes that contain a reporter dye (fluorophore) and a quencher. In the absence of amplicons, these probes exist in a closed configuration, and the fluorescence output of the fluorophore is quenched because it is in close proximity to the quencher. During amplification, when these probes bind to amplicons, the fluorophore and quencher are separated and the fluorescent signal is generated. Software is used to calculate the amount of viral genome in the initial sample by comparison with a panel of quantified standards.

Available Methods

Table 1 shows the performance of current and upcoming commercial real-time PCR and TMA assays. These assays accurately quantify HBV DNA and HCV RNA in clinical practice.¹⁻⁵ The first-generation Cobas Ampliprep®/Cobas Taqman® assay substantially underestimated levels of HCV RNA in approximately 30% of patients infected with HCV genotype 4, and occasionally failed even to detect this genotype, due to nucleotide polymorphisms at the hybridization site of the PCR primers or TaqMan probe (in the 5' non-coding region of the HCV genome).^{1, 3, 6, 7} This issue has been resolved in the second-generation assay, scheduled for release in 2012.⁸

These assays avoid false-positive that result from carryover contamination. They are fully or partly automated (in the latter case, the extract has to be transferred for automated PCR amplification and quantification, after automated extraction). These techniques are recommended to quantify HBV DNA and HCV RNA in international liver society guidelines.⁹⁻¹⁴

Future Needs

The advent of real-time PCR and TMA methods has resolved many of the problems of quantifying HBV DNA and HCV RNA in clinical practice. HBV DNA assays need further improvement, to increase their upper limit of quantification—clinicians frequently encounter patients (particularly those that are immunotolerant) with levels of HBV DNA much higher than 10⁸ international units (IU)/mL.

There were confusing results from recent phase 2 and 3 clinical trials of direct acting antiviral (DAA) agents against HCV, administered with or without pegylated IFN- α and ribavirin, according to the use of the lower limit of detection (LLOD) or the lower

limit of quantification (LLOQ) defined by the assay manufacturers to assess the on-treatment virologic response and to predict sustained virologic response (SVR). In trials of pegylated IFN- α , ribavirin, and telaprevir or boceprevir, HCV RNA levels below the LLOD (at weeks 4, 8, or 12, depending on the protease inhibitor) were reported to be more predictive of SVR than HCV RNA levels below the LLOQ.^{15, 16} However, the LLOQ is, by definition, accurate and reproducible, because it is within the dynamic range of quantification, whereas the LLOD is defined statistically and the actual amount of HCV RNA it indicates varies among patients and samples. Therefore, the LLOD is not suitable for making individual therapeutic decisions. Future assays should have identical LLOD and LLOQ values, to ensure accurate definition of undetectable levels of HCV RNA. In the meantime, new time points should be defined for accurate assessment using the LLOQ.

NEW SEROLOGIC METHODS

HBsAg Quantification

The level of hepatitis B surface antigen (HBsAg) has been proposed as a marker of infected liver mass or the amount of covalently closed circular DNA, the episomal form of HBV, which persists in hepatocytes. Combined analysis of HBsAg and HBV DNA levels can be used to identify inactive carriers and monitor response to therapy.¹⁷⁻²⁰ Two commercial enzyme immunoassays are available; they have narrow dynamic ranges of quantification, so samples that contain high levels of HBsAg must often be retested after being diluted. The HBsAg assay on Architect[®] system (Abbott Diagnostics; Abbott Park, Illinois) has a dynamic range of quantification of 0.05–250.00 IU/mL (maximum authorized dilution, 1:999), whereas the HBsAg II Quant assay on Elecsys[®] or Cobas[®] e devices (Roche Diagnostics GmbH; Mannheim, Germany) has a dynamic range of quantification of 0.05–130.00 IU/mL (no recommendation for maximum dilution). Both

assays are European Conformity (CE)-marked, but they have not been approved by the FDA and are available for research use only in the United States. The assays are inexpensive, easy to perform, and accurately quantify HBsAg in all HBV genotypes.^{21, 22}

HCV Core Antigen Detection and Quantification

Levels of HCV core antigen correlate with levels of HCV RNA in patients with chronic HCV infection.²³⁻²⁶ Assays that quantify HCV core antigen can therefore be used as alternatives to those that measure levels of HCV RNA, as shown in various populations of patients.^{23, 27-29} The Architect® HCV Ag assay (Abbott Diagnostics) is commercially available. Its dynamic range of quantification is 3.0–20,000 fmol/L. This assay is CE-marked but has not been approved by the FDA and is available for research use only in the United States. It is less expensive and easier to use than current HCV RNA tests for diagnosis of chronic hepatitis C and monitoring of antiviral therapy. However, the HCV Ag assay's LLOD corresponds to HCV RNA levels of 500–3000 IU/mL, depending on the HCV genotype.³⁰ Assays for HCV core antigen are therefore not suitable for response-guided therapy, according to current guidelines.

POINT-OF-CARE TESTS AND ALTERNATIVES TO BIOLOGIC TESTS THAT REQUIRE WHOLE-BLOOD SAMPLES

Point-of-care tests are used directly at the site of patient care, outside of the biology laboratory. They include immunoassays based on agglutination, immunochromatography or immunofiltration, as well as assays for nucleic acid detection and quantification. Point-of-care tests can be particularly useful for large-scale screening and improving access to care in regions without molecular biology laboratories.

Alternatives to biologic tests that require whole-blood samples, obtained by venipuncture, are being developed. They can use original specimen matrices, such as oral fluids or blood collected from a finger stick into a capillary tube. Blood can be collected on filter paper, known as the dried blood spot (DBS) method, which makes it possible to store desiccated samples for transport as non-hazardous material via regular mail or courier services. DBS collection and storage is easy and, although standard procedures must be carefully followed, little training is required for preparation. HBV DNA and HCV RNA can be detected and quantified from DBS by classic or real-time PCR methods, although at a cost of reduced analytical sensitivity, compared to the whole blood-based methods described above. The reduction in sensitivity results from small input volumes, less-efficient nucleic acid extraction, and nucleic acid degradation in extreme storage conditions. One study reported that the limit of detection of HBV DNA from DBS was approximately 1 Log₁₀ higher than from serum samples. There was a high level of concordance in HBV genotype analysis of DBS and serum samples, and also in detection of precore mutants and drug-resistant variants.³¹ In another report, the detection limit for HCV RNA in DBS was about 1000 IU/mL. Concordant results were also obtained from HCV quantification and genotype analyses of serum and DBS.³² In theory, the analytical sensitivity of viral genome detection from DBS could reach levels similar to those obtained from serum and plasma samples, as recently shown for HIV.³³ Any serum marker, including HBsAg and HCV core antigen, could also be detected and quantified from DBS. However, this has not been tested yet.

NGS

Viral genome sequence analysis can be used to determine HBV or HCV genotype, assess mutations in the HBV core or core promoter, and identify amino acid

substitutions responsible for resistance to antiviral agents. DNA sequence analysis has been based for many years on the chain termination method, described by Sanger et al.³⁴ NGS methods offer new opportunities but also create new challenges, because they generate large amounts of data and their results can often be difficult to interpret.

Sequencing Techniques

The Sanger method has been improved by the inclusion of fluorescent labels and capillary electrophoresis.^{35,36} This technique involves DNA denaturation, annealing, and elongation. During elongation, DNA polymerase randomly incorporates deoxynucleotides (dNTP) or dideoxynucleotides (ddNTP) that have been labeled with different fluorochromes, resulting in chain elongation or termination, respectively. Chains of different lengths are therefore generated, each ending with a labeled nucleotide. The amplicons are then migrated in a capillary system that separates them according to their lengths. Bioinformatic software measures the fluorescence peak at each position of the analyzed sequence and identifies the corresponding ddNTP to determine a consensus sequence. The presence of multiple peaks at the same position indicates a mixed sequence.

The Sanger sequencing method is well suited for detecting small numbers of substitutions in known genes, but it cannot be used to sequence large portions of plant or animal genomes for genome-wide association studies (GWAS),³⁷⁻³⁹ or to sequence large amounts of targets for gene expression profiling (RNA-Seq).⁴⁰

Viruses that establish chronic infections, such as HIV, HBV, and HCV, behave as quasispecies—complex mixtures of genetically related but distinct viral populations in equilibrium in a given replicative environment (Figure 1). For this reason, at any given time, patients carry a large number of different viral genome sequences. Classic

sequencing techniques can only identify the consensus sequence of a viral quasispecies at a given time point, whereas most intermediate and minor viral populations go undetected. In addition, this approach cannot link different substitutions present in the same viral variant or distinguish substitutions present in different variants. PCR products can be cloned and sequenced to identify intermediate virus populations and linked substitutions, but this approach is cumbersome and time-consuming and has therefore been restricted to the research setting.

Principles of NGS

NGS methods have been recently developed to increase sequencing capacity while generating clonal sequences. Current NGS methods use a 3-step sequencing process: library preparation, DNA capture and enrichment, and sequencing (Figure 2).⁴¹ In library preparation, DNAs of appropriate lengths are prepared and labeled with the primers required for subsequent isolation and sequencing. DNA is then captured and clonal DNA is enriched (this step is not required with Pacific Biosciences technology). The DNA is then sequenced, via polymerization combined with detection based on pyrophosphate (pyrosequencing), H⁺ ions (pH variation) or fluorescence, or ligation combined with fluorescence detection. The detected signals are transformed into readable sequences by an integrated browser and specific software, to produce biologically relevant information.

NGS Methods

NGS technologies developed by various manufacturers (Table 2) include high-capacity systems, such as the Genome Analyzer, HiSeq sequencers (Illumina Inc., San Diego, California), and 5500 series sequencers, which use SOLiD technology (Applied

Biosystems, Carlsbad, California) and long-read sequencers, such as the Genome Sequencer (GS) FLX or Junior (454 Life Sciences, Roche Diagnostics Corp., Branford, Connecticut), Ion Torrent (Applied Biosystems) and PacBio RS (Pacific Biosciences, GenProbe, Menlo Park, California).

The Illumina technology fragments DNA and then tags the fragments with primers that are complementary to probes, to allow fixation on a flow cell. Clonal DNA is then enriched by PCR, to generate clusters of amplicons, and sequenced, through polymerization with fluorescent dNTPs.⁴² The SOLiD system (Applied Biosciences) uses emulsion PCR (emPCR) to enrich clonal DNAs fixed to microbeads. The beads are then transferred to a flow chip and DNA is sequenced by ligation with fluorescent primers.⁴³ These 2 technologies generate millions of short reads (Table 2). They are well suited to resequencing when a reference sequence is available (human genome or transcriptome) and the read length of 100–150 bp is sufficient to identify a known gene, for example in GWAS or RNA-seq studies. These technologies can also be used to identify individual substitutions in viral genes, such as those associated with drug resistance. Analysis of a large number of sequences (corresponding to the depth of the reaction) ensures high levels of sensitivity for minor viral variants. However, short reads cannot be used to establish linkages between substitutions located on the same viral variants, which is required for viral resistance studies.

The long-read sequencers have more limited sequencing capacities but generate longer sequences. In the GS-based technology, DNA is labeled and clonally enriched by emPCR at the surface of microbeads, which are then loaded in picotiter plates containing over a million wells. During the pyrosequencing reaction, light is emitted every time a new base is inserted by polymerization.⁴⁴ The Ion Torrent technology is technically close to the GS technique, but emPCR amplicons are loaded on a semiconductor sequencing

chip; the addition of nucleotides during the polymerization reaction generates H⁺ ions, which induce local pH changes that are detected and measured.⁴⁵ The PacBio RS technology is substantially different. After preparation of the DNA library, unamplified DNAs are transferred to an optical system, called a zero-mode waveguide, which amplifies fluorescence emitted during polymerization. In this technique, fluorescent signals from each clonal DNA molecule, processed by thousands of polymerase molecules fixed to the zero-mode waveguide, are detected without the need for prior amplification.⁴⁶ This group of sequencers generates smaller numbers of sequences, but their length is suitable for linkage studies and de novo characterization of genomic sequences for which no reference sequence is available. They are therefore well suited to virology studies.

Limitations of NGS Methods

All existing methods are susceptible to generating sequencing errors. Evaluations of the Genome Analyzer, 5500 sequencer and GS FLX have calculated accuracy values of 96.7%–100%—similar to those of the classic Sanger method (Table 2).⁴⁷ However, there is controversy about the actual error rates of these methods, so prospective assessments are required by independent investigators.

NGS methods generate unreadable sequences (called junk data) at variable rates. For instance, these rates appear to be high with SOLiD technology on the 5500 sequencer.⁴⁷⁻⁵⁰ This is associated with an overcost but not with lower-quality final results, because these sequences can be easily identified and removed. False-positive or false-negative results for substitutions have been reported at higher rates with Illumina technology than the SOLiD and GS FLX technologies.^{47, 50} They are easily corrected when

the sequencing depth is high, as in GWAS or gene expression profiling studies, but are problematic for studies of rare variants.

Performance can be affected by AT- or GC-rich regions, especially with the Illumina and SOLiD technologies, leading to low overall resolution of genome sequences.⁵¹ For the detection of insertions or deletions (indels), the GS-FLX system has been shown to provide the best performance,^{47, 52} but it performs less well in homopolymeric regions, where it has high rates of error.^{51, 53}

Analyzing NGS Data

In the first step of NGS data analysis, signals generated by the sequencing reaction are converted into readable sequences by proprietary base-caller software. At the end of this step, data are composed of sequences in FASTA format, along with quality scores for each nucleotide of each sequence. The most frequently used format is the PHRED quality score,⁵⁴⁻⁵⁶ which is based on base-calling error probabilities.

In the second part of the analysis, the large number of generated sequences must be processed into comprehensible biologic data by software, through 3 steps that depend on the type of study and sample analyzed.⁵⁷ The goal of the first step is to classify and format sequences into readable data. In the second step, unreliable sequences are eliminated, including sequences with a Phred quality score below 20 (1% risk of base-caller error)⁵⁸ and excessively short sequences (the precise cut-off depends on the technology used). The third step, sequence alignment, is critical and particularly difficult for viral sequences, because of the level of natural genetic variation (numerous substitutions or indels, relative to reference sequences) and the coexistence of numerous viral populations with different sequences. A number of alignment algorithms have been developed but were found to generate different alignments and generally fail

to take into consideration the specificities of viral infections.⁵⁹⁻⁶⁴ Additional alignment quality filters are therefore needed. This part of the analysis must ultimately generate a set of formatted sequences that can be used with confidence for specific analyses and to generate clinically meaningful information.

We recently developed in-house software to model the dynamics of viral populations during and after antiviral therapy and characterize drug resistance.⁶⁴⁻⁶⁶ This software package has been used to demonstrate the preexistence of amino acid substitutions that confer HBV and HCV resistance to specific antiviral drugs. It has also been used to characterize the complex dynamics of sensitive and resistant viral populations in patients with chronic hepatitis B treated with adefovir and in patients with chronic hepatitis C treated with telaprevir combined with pegylated IFN- α , with or without ribavirin.^{64, 65}

Future Needs

The main advantage of NGS techniques for studying chronic viral infections is their improved depth, relative to the Sanger method—this ensures that minor viral populations that are of possible clinical significance are sequenced. No further improvements in depth appear to be needed, because existing technologies can detect minor viral populations in complex quasispecies mixtures. However, the clinical significance of small viral populations is not clear. In addition, it is still difficult to distinguish between true substitutions and errors generated by the sequencing reaction, so specificity needs to be improved.

Sequence length is an important factor in identifying linkages between different substitutions located on the same viral strand. Full-length viral genome analysis will improve our understanding of the dynamics of viral populations in different clinical

circumstances. Reading lengths will need to be extended considerably to meet this need (the HBV genome contains approximately 3000 bp, the HCV genome 10,000 bp).

Currently, the main need is for bioinformatic tools to analyze NGS sequences in the context of viral infections. Complex tools might be suitable for research purposes, but clinicians will require bioinformatic tools that can translate millions of generated sequences into simple, clinically relevant information on which therapeutic decisions can be made.

CLINICAL USE OF VIROLOGIC TOOLS

HBV Infection

Tests that are useful for patients with HBV infection include enzyme immunoassays that detect HBV antigen and antibodies, assays that detect and quantify HBV DNA, and methods that identify amino acid substitutions associated with HBV resistance to nucleoside/nucleotide analogues. There is debate over the value of assays that quantify HBsAg. Tools that determine HBV genotype or identify core and core promoter mutants are valuable for research but have no clinical use.

Diagnosis and Prognosis

Chronic HBV carriers are defined by the persistence of HBsAg in their blood for more than 6 months. Chronic hepatitis B is characterized by a level of HBV DNA >2000–20,000 IU/mL (i.e. >3.3–4.3 Log₁₀ IU/mL), a persistent or intermittent increase in level of aminotransferase, and specific features of liver biopsies.^{9, 13, 14} Patients with viral loads below 2000 IU/mL are generally considered to be inactive carriers, although there is controversy over this value.

The level of HBV DNA has prognostic value. A chronically high level of HBV DNA

is associated with a significant risk of progression to cirrhosis and hepatocellular carcinoma^{67,68}. However, no HBV DNA threshold has been established that has a strong predictive value for the occurrence of clinical outcomes, at the individual level.

Decision to Treat

The decision to treat chronic hepatitis B is based on multiple parameters, including the level of HBV DNA. The European Association for the Study of the Liver Clinical Practice Guidelines state that HBV therapy is indicated for non-cirrhotic patients with an HBV DNA level >2000 IU/mL (>3.3 Log₁₀ IU/mL), serum levels of alanine aminotransferase (ALT) activity above the upper limit of normal (ULN), and a liver biopsy (or a non-invasive test) that indicates moderate to severe activity (METAVIR grade ≥A2) and/or fibrosis (METAVIR stage ≥F2).⁹ Patients with compensated cirrhosis and detectable HBV DNA should be considered for treatment and patients with decompensated cirrhosis require urgent antiviral treatment, whatever the level of HBV replication.⁹ The Clinical Practice Guidelines of the American Association for the Study of Liver Diseases and the Asian-Pacific Association for the Study of the Liver state that antiviral treatment should be considered for non-cirrhotic patients with HBV DNA titers >20,000 IU/mL (>4.3 Log₁₀ IU/mL) if they are HBeAg positive, or >2000 IU/mL (>3.3 Log₁₀ IU/mL) if they are HBeAg negative, and if they have increased serum levels of ALT activity (>2 x ULN), and/or a liver biopsy showing inflammation and/or fibrosis.^{13, 14} Patients should not be tested for drug resistance before therapy.

In patients with normal ALT activity, serum levels of HBV DNA and ALT should be measured at 3- to 6-month intervals. More frequent monitoring should be considered during the first year for hepatitis B e antigen (HBeAg)-negative patients with normal

levels of ALT and a level of HBV DNA <2000 IU/mL (<3.3 Log₁₀ IU/mL), to confirm their status as inactive carriers.

Choice of Therapy

Pegylated interferon (IFN)- α is considered to be the first-line treatment option for HBeAg-positive patients without cirrhosis who have increased levels of ALT and a low level of HBV DNA (<2 x 10⁶ IU/mL, such as <6.3 Log₁₀ IU/mL). This treatment is more controversial for HBeAg-negative patients with the same characteristics.⁹ The HBV genotype is a significant predictor of the response to pegylated IFN- α . However, the individual predictive value of the HBV genotype for the outcome of pegylated IFN- α therapy is weak and could be biased by the relationship between genotype and ethnicity.^{69, 70}

Most patients are treated with nucleoside or nucleotide analogues. The international liver society guidelines recommend either tenofovir or entecavir monotherapy as the first-line treatment for chronic hepatitis B.^{9, 13, 14, 71} The HBV genotype has no influence on the effectiveness of nucleoside/nucleotide analogues.⁷⁰

Monitoring Treatment

The ideal endpoint of therapy is a sustained loss of HBsAg, with or without seroconversion to anti-HBs.⁹ However, this is rarely achieved with current drugs. The practical aim is therefore to reduce levels of HBV DNA as far as possible (ideally below 10–20 IU/mL), to achieve biochemical remission and histologic improvement and prevent HBV-related liver disease.^{9, 13, 14} Treatment of HBeAg-positive patients is therefore monitored by assays for HBV DNA and ALT every 3 to 6 months and HBeAg and anti-HBe every 6 months.

Treatment of HBeAg-positive patients can be stopped after a consolidation period of 6 to 12 months following HBe seroconversion.^{9, 13, 14} However, durable HBe seroconversion, in association with low levels of HBV replication, is rare following treatment with nucleoside/nucleotide analogues. In HBeAg-positive patients who do not develop anti-HBe antibodies, and in HBe-negative patients, nucleoside/nucleotide analogues must be administered for life, with the aim of achieving profound and durable HBV DNA suppression. If HBV DNA is still detectable (>10–20 IU/mL) after 48 weeks of therapy, current guidelines, based on studies of modestly potent nucleoside/nucleotide analogues with low barriers to resistance, recommend adding a second drug with no viral cross-resistance to the first to prevent emergence of resistant variants. However, this is not necessary with entecavir or tenofovir if the HBV DNA level continues to decrease after week 48, because it is likely to become undetectable thereafter.⁷² Serial measurements of HBsAg levels could help identify patients who will subsequently clear HBsAg.

In patients who adhere and respond to treatment, selection of resistant HBV variants should be suspected if virologic breakthrough occurs, defined as a re-increase in the HBV DNA level by 1 Log₁₀ or more above the nadir, or as HBV DNA that becomes detectable after being undetectable.^{14, 73} A blip (single detection of a low-level of HBV DNA, preceded and followed by undetectable HBV DNA), should not be considered a virologic breakthrough. If resistance occurs, the only effective strategy is to add a second drug against which the selected HBV variant is not cross-resistant.^{9, 14} Such drug combinations include tenofovir and a nucleoside analogue. Identification of amino acid substitutions associated with resistance has little clinical value, because this information rarely influences the choice of therapy. Thus, resistance testing by means of classic sequencing or reverse hybridization is generally not recommended. The only exception

is HBV with the rtA181V/T substitution—it has full sensitivity to only entecavir, which must be combined with tenofovir.

Clinical Research

Cohort studies, surveillance studies, and clinical trials of new therapeutic reagents or strategies require serial measurements of HBV DNA. In research studies, tests for resistant variants should be systematically performed when antiviral therapy fails, to identify the resistant variant and characterize its nature and dynamics. Sensitive sequencing methods such as NGS are particularly useful for characterizing the dynamics of sensitive and resistant virus populations over time. Information about HBV genotype and core and core promoter mutations might be used in epidemiology and pathophysiology studies.

HCV Infection

Assays that detect antibodies against HCV, detect and quantify HCV RNA, or determine HCV genotype are useful for diagnosis and treatment of HCV infection. Assays that detect and quantify HCV core antigen are alternatives to tests for HCV RNA, but are not sensitive enough to use in treatment monitoring or response-guided therapy. Resistance testing is not useful for patient management but is widely used in clinical trials of DAA or host-targeted agents, in case of treatment failure.

Diagnosis and Decision to Treat

Patients are diagnosed with chronic HCV infection based on the detection of anti-HCV antibodies by enzyme immunoassays and HCV RNA by a sensitive molecular biology-based technique that has a detection limit of about 10 to 15 IU/mL.

All treatment-naïve patients with compensated liver disease and detectable HCV RNA should be considered for therapy.^{10, 12} Assessment of liver disease severity is important for decision making.^{10, 12} Patients infected with HCV genotype 1, in whom pegylated IFN- α and ribavirin combination therapy failed to eradicate the virus, should be considered for retreatment, because they might benefit from the triple combination of pegylated IFN- α , ribavirin, and a protease inhibitor.

Selecting Therapy

The HCV genotype should be determined before treatment is started. Patients infected with HCV genotypes other than type 1 should be treated with pegylated IFN- α and ribavirin alone. For patients infected with genotypes 2 or 3, the dose of ribavirin is 0.8 g/day and the treatment duration is 24 weeks; patients with genotypes 2 and 3 with baseline factors that indicate low responsiveness should receive weight-based ribavirin.¹⁰ For patients infected with genotypes 5 and 6, the dose of ribavirin is based on body weight, and treatment should last up to 72 weeks, depending on the virologic response during treatment.

Patients infected with HCV genotype 1 who qualify for therapy should receive the triple combination of pegylated IFN- α , ribavirin, and either telaprevir or boceprevir. The subtype (1a or 1b) does not need to be determined for patients that receive the combination of pegylated IFN- α and ribavirin, or triple drug combinations that include a protease inhibitor, because genotype 1 subtypes do not affect treatment decisions with these therapeutic strategies.⁷⁴ Further trials are needed to improve the selection of patients infected with HCV genotype 1 who might not need protease inhibitors, such as young patients with mild fibrosis, a low baseline level of HCV RNA, or the CC *IL28B* genotype.⁷⁵

Monitoring Treatment

The endpoint for HCV therapy is a SVR, characterized by undetectable HCV RNA (<10–15 IU/mL) 24 weeks after the end of treatment—this corresponds with viral eradication in more than 99% of cases.

Monitoring of HCV RNA levels during treatment is key in determining virologic response, in guiding duration of treatment, and in deciding futility of treatment. This is particularly important when direct acting antivirals are used, as reviewed by Barritt and Fried in this issue.

Failure of triple combination therapy to clear HCV is associated with outgrowth of variant populations that are resistant to the protease inhibitor used. However, resistance testing based on HCV sequence analysis of the protease region has no utility in clinical practice.⁷⁶ Resistant variants are present in almost all infected patients before therapy begins, but technologies available in clinical virology laboratories are not sensitive enough to detect them;⁶⁵ negative results are therefore uninterpretable. In addition, the profile of HCV variants at the start of therapy does not appear to affect the outcome of treatment when the protease inhibitor is combined with pegylated IFN- α and ribavirin. At the time of treatment failure (virologic breakthrough or relapse), the resistant population is always enriched, and phase 2 and 3 trials have reported that that it is dominant in 50% to 70% of patients.^{77,78} Resistance testing is still not indicated at this time because the results will have no impact on subsequent treatment decisions.⁷⁶

Clinical Research

In cohort studies, surveillance studies, and clinical trials of HCV DAA or host-targeting agents, given alone or in combination, serial HCV RNA testing is needed to

characterize the virologic response and detect virologic breakthrough and relapse. Resistance variants should always be analyzed in patients who fail to respond to therapy, to characterize the nature and dynamics of the selected viral populations and identify amino acid substitutions selected by the DAAs. When several antiviral drugs with different target sites and mechanisms of action are combined, all the target sites should be studied. Sensitive sequencing methods such as NGS are particularly useful for characterizing the dynamics of sensitive and resistant virus populations over time.

CONCLUSION

New technologies are available for detecting and quantifying viral antigens and genomes, and for analyzing viral genome nucleotide and amino acid sequences. Real-time target amplification (PCR or TMA) methods are well standardized and widely used in clinical practice to diagnose and monitor HBV and HCV infection. In contrast, NGS techniques are largely restricted to research laboratories. These generate large quantities of data; software is needed (and is being developed) that can provide clinicians with meaningful information.

REFERENCES

1. Chevaliez S, Bouvier-Alias M, Brillet R, Pawlotsky JM. Overestimation and underestimation of hepatitis C virus RNA levels in a widely used real-time polymerase chain reaction-based method. *Hepatology* 2007;46:22-31.
2. Chevaliez S, Bouvier-Alias M, Laperche S, Hezode C, Pawlotsky JM. Performance of version 2.0 of the Cobas AmpliPrep/Cobas TaqMan real-time PCR assay for hepatitis B virus DNA quantification. *J Clin Microbiol* 2010;48:3641-7.
3. Chevaliez S, Bouvier-Alias M, Pawlotsky JM. Performance of the Abbott real-time PCR assay using m2000sp and m2000rt for hepatitis C virus RNA quantification. *J Clin Microbiol* 2009;47:1726-32.
4. Paba P, Fabeni L, Perno CF, Ciotti M. Performance evaluation of the Artus hepatitis C virus QS-RGQ assay. *J Virol Methods* 2012;179:77-80.
5. Thibault V, Pichoud C, Mullen C, Rhoads J, Smith JB, Bitbol A, Thamm S, Zoulim F. Characterization of a new sensitive PCR assay for quantification of viral DNA isolated from patients with hepatitis B virus infections. *J Clin Microbiol* 2007;45:3948-53.
6. Chevaliez S, Bouvier-Alias M, Castera L, Pawlotsky JM. The Cobas AmpliPrep-Cobas TaqMan real-time polymerase chain reaction assay fails to detect hepatitis C virus RNA in highly viremic genotype 4 clinical samples. *Hepatology* 2009;49:1397-8.
7. Chevaliez S, Fix J, Soulier A, Pawlotsky JM. Underestimation of hepatitis C virus genotype 4 RNA levels by the CobasAmpliPrep/CobasTaqMan assay. *Hepatology* 2009;50:1681.

8. Vermehren J, Colucci G, Gohl P, Hamdi N, Abdelaziz AI, Karey U, Thamke D, Zitzer H, Zeuzem S, Sarrazin C. Development of a second version of the Cobas AmpliPrep/Cobas TaqMan hepatitis C virus quantitative test with improved genotype inclusivity. *J Clin Microbiol* 2011;49:3309-15.
9. European Association for the Study of the Liver. EASL Clinical Practice Guidelines: management of chronic hepatitis B. *J Hepatol* 2009;50:227-42.
10. European Association for the Study of the Liver. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. *J Hepatol* 2011;55:245-64.
11. Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011;54:1433-44.
12. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009;49:1335-74.
13. Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008;2:263-283.
14. Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009;50:661-2.
15. Lawitz E, Poordad F, Bronowicki JP, Marcellin P, Feinman VS, Kwo PY, Guyader D, Davis M, Harrison SA, Pedicone L, Deng W, Burroughs M, Brass CA, Albrecht JK, Zeuzem S. The effect of using lower limit of quantitation (LLQ) vs lower limit of detection (LLD) for the definition of undetectable HCV RNA: data from the RESPOND-2 and SPRINT-2 trials. *Hepatology* 2011;54:442A.

16. Harrington PR, Zeng W, Naeger LK. Clinical relevance of detectable but not quantifiable hepatitis C virus RNA during boceprevir or telaprevir treatment. *Hepatology* 2012; in press.
17. Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Luo K, Wang Y, Hadziyannis S, Wolf E, McCloud P, Batrla R, Marcellin P. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009;49:1141-50.
18. Moucari R, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, Dauvergne A, Asselah T, Boyer N, Bedossa P, Valla D, Vidaud M, Nicolas-Chanoine MH, Marcellin P. Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009;49:1151-7.
19. Wursthorn K, Jung M, Riva A, Goodman ZD, Lopez P, Bao W, Manns MP, Wedemeyer H, Naoumov NV. Kinetics of hepatitis B surface antigen decline during 3 years of telbivudine treatment in hepatitis B e antigen-positive patients. *Hepatology* 2010;52:1611-20.
20. Chan HL, Thompson A, Martinot-Peignoux M, Piratvisuth T, Cornberg M, Brunetto MR, Tillmann HL, Kao JH, Jia JD, Wedemeyer H, Locarnini S, Janssen HL, Marcellin P. Hepatitis B surface antigen quantification: why and how to use it in 2011. A core group report. *J Hepatol* 2011;55:1121-31.
21. Sonneveld MJ, Rijckborst V, Boucher CA, Zwang L, Beersma MF, Hansen BE, Janssen HL. A comparison of two assays for quantification of Hepatitis B surface antigen in patients with chronic hepatitis B. *J Clin Virol* 2011;51:175-8.
22. Wursthorn K, Jaroszewicz J, Zacher BJ, Darnedde M, Raupach R, Mederacke I, Cornberg M, Manns MP, Wedemeyer H. Correlation between the Elecsys HBsAg II

- assay and the Architect assay for the quantification of hepatitis B surface antigen (HBsAg) in the serum. *J Clin Virol* 2011;50:292-6.
23. Bouvier-Alias M, Patel K, Dahari H, Beaucourt S, Larderie P, Blatt L, Hezode C, Picchio G, Dhumeaux D, Neumann AU, McHutchison JG, Pawlotsky JM. Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. *Hepatology* 2002;36:211-8.
 24. Park Y, Lee JH, Kim BS, Kim DY, Han KH, Kim HS. New automated hepatitis C virus (HCV) core antigen assay as an alternative to real-time PCR for HCV RNA quantification. *J Clin Microbiol* 2010;48:2253-6.
 25. Hosseini-Moghaddam S, Iran-Pour E, Rotstein C, Husain S, Lilly L, Renner E, Mazzulli T. Hepatitis C core Ag and its clinical applicability: potential advantages and disadvantages for diagnosis and follow-up? *Rev Med Virol* 2012; in press.
 26. Descamps V, Op de Beeck A, Plassart C, Brochot E, Francois C, Helle F, Adler M, Bourgeois N, Degre D, Duverlie G, Castelain S. Strong correlation between liver and serum levels of hepatitis C virus core antigen and RNA in chronically infected patients. *J Clin Microbiol* 2012;50:465-8.
 27. Moscato GA, Giannelli G, Grandi B, Pieri D, Marsi O, Guarducci I, Batini I, Altomare E, Antonaci S, Capria A, Pellegrini G, Sacco R. Quantitative determination of hepatitis C core antigen in therapy monitoring for chronic hepatitis C. *Intervirology* 2010;54:61-65.
 28. Mederacke I, Potthoff A, Meyer-Olson D, Meier M, Raupach R, Manns MP, Wedemeyer H, Tillmann HL. HCV core antigen testing in HIV- and HBV-coinfected patients, and in HCV-infected patients on hemodialysis. *J Clin Virol* 2012;53:110-5.

29. Miedouge M, Saune K, Kamar N, Rieu M, Rostaing L, Izopet J. Analytical evaluation of HCV core antigen and interest for HCV screening in haemodialysis patients. *J Clin Virol* 2010;48:18-21.
30. Ross RS, Viazov S, Salloum S, Hilgard P, Gerken G, Roggendorf M. Analytical performance characteristics and clinical utility of a novel assay for total hepatitis C virus core antigen quantification. *J Clin Microbiol* 2010;48:1161-8.
31. Jardi R, Rodriguez-Frias F, Buti M, Schaper M, Valdes A, Martinez M, Esteban R, Guardia J. Usefulness of dried blood samples for quantification and molecular characterization of HBV DNA. *Hepatology* 2004;40:133-9.
32. Tuaille E, Mondain AM, Meroueh F, Ottomani L, Picot MC, Nagot N, Van de Perre P, Ducos J. Dried blood spot for hepatitis C virus serology and molecular testing. *Hepatology* 2010;51:752-8.
33. Marconi A, Balestrieri M, Comastri G, Pulvirenti FR, Gennari W, Tagliazucchi S, Pecorari M, Borghi V, Marri D, Zazzi M. Evaluation of the Abbott Real-Time HIV-1 quantitative assay with dried blood spot specimens. *Clin Microbiol Infect* 2009;15:93-7.
34. Sanger F, Air GM, Barrell BG, Brown NL, Coulson AR, Fiddes CA, Hutchison CA, Slocombe PM, Smith M. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* 1977;265:687-95.
35. Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 1987;238:336-41.
36. Smith LM. High-speed DNA sequencing by capillary gel electrophoresis. *Nature* 1991;349:812-3.

37. Wang WY, Barratt BJ, Clayton DG, Todd JA. Genome-wide association studies: theoretical and practical concerns. *Nature Rev Genet* 2005;6:109-18.
38. Hardy J, Singleton A. Genomewide association studies and human disease. *N Engl J Med* 2009;360:1759-68.
39. Ku CS, Loy EY, Pawitan Y, Chia KS. The pursuit of genome-wide association studies: where are we now? *J Hum Genet* 2010;55:195-206.
40. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009;10:57-63.
41. Metzker ML. Sequencing technologies: the next generation. *Nat Rev Genet* 2010;11:31-46.
42. Bennett S. Solexa Ltd. *Pharmacogenomics* 2004;5:433-8.
43. Next-generation genome sequencing. Towards personalized medicine. Pandey V, Nutter R, Prediger E, eds. Wiley, Hoboken, New jersey, 2008.
44. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005;437:376-80.
45. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, Leamon JH, Johnson K, Milgrew MJ, Edwards M, Hoon J, Simons JF, Marran D, Myers JW, Davidson JF, Branting A, Nobile JR, Puc BP, Light D, Clark TA, Huber M,

- Branciforte JT, Stoner IB, Cawley SE, Lyons M, Fu Y, Homer N, Sedova M, Miao X, Reed B, Sabina J, Feierstein E, Schorn M, Alanjary M, Dimalanta E, Dressman D, Kasinskas R, Sokolsky T, Fidanza JA, Namsaraev E, McKernan KJ, Williams A, Roth GT, Bustillo J. An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 2011;475:348-52.
46. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B, Bibillo A, Bjornson K, Chaudhuri B, Christians F, Cicero R, Clark S, Dalal R, Dewinter A, Dixon J, Foquet M, Gaertner A, Hardenbol P, Heiner C, Hester K, Holden D, Kearns G, Kong X, Kuse R, Lacroix Y, Lin S, Lundquist P, Ma C, Marks P, Maxham M, Murphy D, Park I, Pham T, Phillips M, Roy J, Sebra R, Shen G, Sorenson J, Tomaney A, Travers K, Trulson M, Vieceli J, Wegener J, Wu D, Yang A, Zaccarin D, Zhao P, Zhong F, Korlach J, Turner S. Real-time DNA sequencing from single polymerase molecules. *Science* 2009;323:133-8.
47. Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, Schork NJ, Murray SS, Topol EJ, Levy S, Frazer KA. Evaluation of next-generation sequencing platforms for population targeted sequencing studies. *Genome Biol* 2009;10:R32.
48. Shen Y, Sarin S, Liu Y, Hobert O, Pe'er I. Comparing platforms for *C. elegans* mutant identification using high-throughput whole-genome sequencing. *PloS One* 2008;3:e4012.
49. Walter NA, Bottomly D, Laderas T, Mooney MA, Darakjian P, Searles RP, Harrington CA, McWeeney SK, Hitzemann R, Buck KJ. High throughput sequencing in mice: a platform comparison identifies a preponderance of cryptic SNPs. *BMC Genomics* 2009;10:379.

50. Suzuki S, Ono N, Furusawa C, Ying BW, Yomo T. Comparison of sequence reads obtained from three next-generation sequencing platforms. *PloS One* 2011;6:e19534.
51. Dohm JC, Lottaz C, Borodina T, Himmelbauer H. Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Res* 2008;36:e105.
52. Hoppman-Chaney N, Peterson LM, Klee EW, Middha S, Courteau LK, Ferber MJ. Evaluation of oligonucleotide sequence capture arrays and comparison of next-generation sequencing platforms for use in molecular diagnostics. *Clin Chem* 2010;56:1297-306.
53. Gilles A, Meglec E, Pech N, Ferreira S, Malausa T, Martin JF. Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing. *BMC Genomics* 2011;12:245.
54. Ewing B, Green P. Base-calling of automated sequencer traces using Phred. II. Error probabilities. *Genome Res* 1998;8:186-94.
55. Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. *Genome Res* 1998;8:175-85.
56. Richterich P. Estimation of errors in "raw" DNA sequences: a validation study. *Genome Res* 1998;8:251-9.
57. Richter BG, Sexton DP. Managing and analyzing next-generation sequence data. *PLoS Computat Biol* 2009;5:e1000369.
58. Altshuler D, Pollara VJ, Cowles CR, Van Etten WJ, Baldwin J, Linton L, Lander ES. An SNP map of the human genome generated by reduced representation shotgun sequencing. *Nature* 2000;407:513-6.

59. Wang C, Mitsuya Y, Gharizadeh B, Ronaghi M, Shafer RW. Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. *Genome Res* 2007;17:1195-201.
60. Tsibris AM, Korber B, Arnaout R, Russ C, Lo CC, Leitner T, Gaschen B, Theiler J, Paredes R, Su Z, Hughes MD, Gulick RM, Greaves W, Coakley E, Flexner C, Nusbaum C, Kuritzkes DR. Quantitative deep sequencing reveals dynamic HIV-1 escape and large population shifts during CCR5 antagonist therapy in vivo. *PLoS One* 2009;4:e5683.
61. Archer J, Rambaut A, Taillon BE, Harrigan PR, Lewis M, Robertson DL. The evolutionary analysis of emerging low frequency HIV-1 CXCR4 using variants through time-an ultra-deep approach. *PLoS Comput Biol* 2010;6:e1001022.
62. Poon AF, Swenson LC, Dong WW, Deng W, Kosakovsky Pond SL, Brumme ZL, Mullins JI, Richman DD, Harrigan PR, Frost SD. Phylogenetic analysis of population-based and deep sequencing data to identify coevolving sites in the nef gene of HIV-1. *Molecular Biol Evol* 2010;27:819-32.
63. Gianella S, Delport W, Pacold ME, Young JA, Choi JY, Little SJ, Richman DD, Kosakovsky Pond SL, Smith DM. Detection of minority resistance during early HIV-1 infection: natural variation and spurious detection rather than transmission and evolution of multiple viral variants. *J Virol* 2011;85:8359-67.
64. Rodriguez C, Chevaliez C, Bensadoun P, Pawlotsky JM. Ultra-deep pyrosequencing, a powerful new tool to study HBV Resistance to nucleoside/nucleotide analogues, identifies pre-existing HBV variants bearing resistance mutations and characterizes their on-treatment kinetics. *Hepatology* 2011;52:117A.

65. Chevaliez S, Rodriguez C, Soulier A, Ahmed-Belkacem A, Hezode C, Pawlotsky JM. Molecular characterization of HCV resistance to telaprevir by means of ultra-deep pyrosequencing: preexisting resistant variants and dynamics of resistant populations. *J Hepatol* 2011;54:S30.
66. Rodriguez C, Soulie C, Marcelin A, Calvez V, Descamps D, Charpentier C, Flandre P, Bellecave P, Lee G, Harrigan P, Pawlotsky JM, Masquelier B. HIV-1 coreceptor usage prediction by “ultra-deep pyrosequencing” increases the rate of patients treated with success by CCR5 antagonists. *Antiviral Ther* 2011;16:A100.
67. Chen CF, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, Su J, Hsiao CK, Wang LY, You SL, Lu SN, Chen CJ. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. *Gastroenterology* 2011;141:1240-1248.
68. Chen CJ, Yang HI, Iloeje UH. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. *Hepatology* 2009;49:S72-84.
69. Buster EH, Janssen HL. The importance of HBV genotype in HBeAg-positive chronic hepatitis B patients in whom sustained response is pursued. *J Hepatol* 2011;54:395-6.
70. Raimondi S, Maisonneuve P, Bruno S, Mondelli MU. Is response to antiviral treatment influenced by hepatitis B virus genotype? *J Hepatol* 2010;52:441-9.
71. Woo G, Tomlinson G, Nishikawa Y, Kowgier M, Sherman M, Wong DK, Pham B, Ungar WJ, Einarson TR, Heathcote EJ, Krahn M. Tenofovir and entecavir are the most effective antiviral agents for chronic hepatitis B: a systematic review and Bayesian meta-analyses. *Gastroenterology* 2010;139:1218-29.
72. Zoutendijk R, Reijnders JG, Brown A, Zoulim F, Mutimer D, Deterding K, Petersen J, Hofmann WP, Buti M, Santantonio T, van Bommel F, Pradat P, Oo Y,

- Luetgehetmann M, Berg T, Hansen BE, Wedemeyer H, Janssen HL. Entecavir treatment for chronic hepatitis B: adaptation is not needed for the majority of naive patients with a partial virological response. *Hepatology* 2011;54:443-51.
73. Pawlotsky JM, Dusheiko G, Hatzakis A, Lau D, Lau G, Liang TJ, Locarnini S, Martin P, Richman DD, Zoulim F. Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. *Gastroenterology* 2008;134:405-15.
74. Chevaliez S, Bouvier-Alias M, Brillet R, Pawlotsky JM. Hepatitis C virus (HCV) genotype 1 subtype identification in new HCV drug development and future clinical practice. *PLoS One* 2009;4:e8209.
75. Clark PJ, Thompson AJ. Host genomics and HCV treatment response. *J Gastroenterol Hepatol* 2012;27:212-22.
76. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology* 2011;53:1742-51.
77. Sullivan JC, De Meyer S, Bartels DJ, Dierynck I, Zhang E, Spanks J, Tigges A, Adda N, Martin EC, Beumont M, Jacobson IM, Sherman KE, Zeuzem S, Picchio G, Kieffer TL. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. *J Hepatol* 2011;54:S4.
78. Zeuzem S, Barnard RJ, Howe JA, Ogert RA, Ralston R, Boparai N, Brass CA, Albrecht JK, Burroughs M, Sniukiene V, Poordad F, Gordon SC, Jacobson IM. Boceprevir resistance-associated variants (RAVS) are observed more frequently in HCV (GT1)-infected patients with poor response to peginterferon alpha-2b/ribavirin. *J Hepatol* 2011;54:S4-5.

FIGURE LEGENDS

Figure 1. Quasispecies distribution of HBV and HCV genomes and methods to detect major (>10% of the viral quasispecies), intermediate, and minor (<1% of the viral quasispecies) viral populations.

Figure 2. Current workflow for NGS methods, including (A) library preparation, (B) DNA capture and clonal DNA enrichment, and (C) high-throughput sequencing that can be achieved by polymerization, combined with detection based on pyrophosphate (pyrosequencing), H⁺ ions (pH variation) or fluorescence, or by ligation combined with fluorescence detection. The detected signals are transformed into readable sequences by integrated browser and specific software in order to yield biologically relevant information.

Table 1. Commercially Available Real-Time Target Amplification Assays for HBV DNA and HCV RNA Detection and Quantification

Assay	Manufacturer	Method	Automated extraction device	Amplification device	Volume required (μL)	LLOD (IU/mL)	Dynamic range of quantification (UI/mL)
HBV DNA							
COBAS® TaqMan® HBV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	Manual with High Pure System Viral Nucleic Acid Kit	COBAS® TaqMan	650 μL	6-10 IU/mL	29 (1.5 Log_{10})- 1×10^8 (8.0 Log_{10}) IU/mL
COBAS® Ampliprep· COBAS® TaqMan® (CAP/CTM) HBV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	COBAS® Ampliprep®	COBAS® TaqMan	650 μL	20 IU/mL	20 (1.3 Log_{10})- 1.7×10^8 (8.2 Log_{10}) IU/mL
RealTime™ HBV	Abbott Molecular, Des Plaines, IL	Real-time PCR	<i>m2000</i> _{SP}	<i>m2000</i> _{RT}	200 or 500 μL	10 IU/mL (for 500 μL) 15 IU/mL (for 200 μL)	10 (1.0 Log_{10})- 1.0×10^9 (9.0 Log_{10}) IU/mL
Artus HBV QS-RGQ Assay*	Qiagen, Hilden, Germany	Real-time PCR	QIASymphony RGQ	Rotor-Gene Q	1000 μL	10.2 IU/mL	31.6 (1.5 Log_{10})- 2×10^7 (7.3 Log_{10}) IU/mL
APTIMA® HBV Quantitative Assay	Gen-Probe, San Diego, CA	Real-time TMA	Panther®		Available in 2012		

HCV RNA							
COBAS® TaqMan® HCV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	Manual with High Pure System Viral Nucleic Acid Kit	COBAS® TaqMan	650 µL	20 IU/mL	25 (1.4 Log ₁₀)-3.0 x 10 ⁸ to 3.9 x 10 ⁸ (8.5-8.6 Log ₁₀) IU/mL
COBAS® Ampliprep- COBAS® TaqMan® (CAP/CTM) HCV Test, v2.0	Roche Molecular Systems, Pleasanton, CA	Real-time PCR	COBAS® AmplipPrep	COBAS® TaqMan	650 µL	15 IU/mL	15 (1.2 Log ₁₀)-1.0 x 10 ⁸ (8.0 Log ₁₀) IU/mL
RealTime™ HCV	Abbott Molecular, Des Plaines, IL	Real-time PCR	m2000 _{SP}	m2000 _{RT}	200 or 500 µL	12 IU/mL (for 500µL) 30 IU/mL (for 200 µL)	12 (1.1 Log ₁₀)-1.0 x 10 ⁸ (8.0 Log ₁₀) IU/mL
Artus HCV QS-RGQ Assay*	Qiagen, Hilden, Germany	Real-time PCR	QIASymphony RGQ	Rotor-Gene Q	1000 µL	36.2 IU/mL	67.6 (1.8 Log ₁₀)-17.7 x 10 ⁶ (7,2 Log ₁₀) IU/mL
VERSANT HCV RNA 1.0 Assay (kPCR)	Siemens Medical Solutions Diagnostics, Tarrytown, NY	Real-time PCR	Sample Preparation (SP) Module	Amplification and Detection (AD) Module	500 µL	15 IU/mL	15 (1.2 Log ₁₀)-1.0 x 10 ⁸ (8.0 Log ₁₀) IU/mL

APTIMA® HCV Quantitative Assays	Gen-Probe, San Diego, CA	Real-time TMA	Panther®	Available in 2012
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*Available only for EDTA-plasma specimens.
IU/mL: international units per milliliter.

Table 2. Characteristics of NGS Techniques

Manufacturer	Sequencing device	Technology (template preparation/ NGS chemistry)	Type	Number of single reads per run* (x 10⁶)	Number of nucleotides per run* (Gb)	Maximum sequence length* (bp)	Accuracy
Applied Biosystems	5500	emPCR/ligation	High throughput	800	9	75	99.6-99.8%
	5500xl		High throughput	1600	15	75	
	Ion Torrent (ChiP 316)	emPCR/real-time sequencing	Long reads	6.2	>1	>400	99.97%*
Illumina	MiSeq	Solid capture/reversible terminator	High throughput	3.4	>1	150	96.7-100%
	Genome Analyzer IIx		High throughput	320	95	150	
	HiSeq 1000		High throughput	1500	300	100	
	HiSeq 2000		High throughput	3000	600	100	
454 Life Sciences/Roche Diagnostics	GS Junior	emPCR/pyrosequencing	Long reads	0.1	0.035	400	99%*
	GS FLX+		Long reads	1	0.7	1000	97.4-99.9%
Pacific Biosciences/Gen-Probe	PacBio RS	Single molecule/real-time sequencing	Long reads	0.035	0.045	1200	99.99%*

*Information provided by the manufacturers; Gb, gigabase; bp, base pair; emPCR, emulsion PCR