New Virologic Tools for Management of Chronic Hepatitis B and C



Stéphane Chevaliez*,‡ Christophe Rodriguez*,‡ Jean-Michel Pawlotsky*,‡

National Reference Center for Viral Hepatitis B, C, and D, Department of Virology, Hôpital Henri Mondor, Université Paris-Est, Créteil, France; and ‡INSERM U955, Créteil, France

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 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 Polymerase Chain Reaction; Real-Time Transcription-Mediated Amplification; 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 polymerase chain reaction (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 classic 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

Abbreviations used in this paper: ALT, alanine aminotransferase; bp, base pair; DAA, direct-acting antiviral; DBS, dried blood spot; emPCR, emulsion polymerase chain reaction; GS, genome sequencer; GWAS, genome-wide association studies; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; LLOD, lower limit of detection; LLOQ, lower limit of quantification; NGS, next-generation sequencing; PCR, polymerase chain reaction; SVR, sustained virologic response; TMA, transcription-mediated amplification.

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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, 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 (Roche Molecular Systems, Pleasanton, CA) substantially underestimated levels of HCV RNA in approximately 30% of patients infected with HCV genotype 4, and occasionally failed even to detect this genotype, because of nucleotide polymorphisms at the hybridization site of the PCR primers or TaqMan probe (in the 5′ noncoding 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-positives 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 who are immunotolerant) with levels of HBV DNA much higher than 10^8 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 interferon (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.