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Molecular Diagnosis of Viral Hepatitis

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Molecular biology–based assays are invaluable tools for the management of chronic viral hepatitis. They can be used to test blood donations, diagnose active infection, help to establish the prognosis, guide treatment decisions, and assess the virological response to therapy. This article reviews current molecular biology–based techniques and assays, and their practical use in the management of hepatitis B and C virus infection.

Chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) affects over 500 million people worldwide.[1,2] Chronic viral hepatitis is associated with significant morbidity and mortality and is considered a major public health problem in most areas of the world, raising the issues of their diagnosis, treatment, and prevention. In the past decade, improvements in molecular biology–based techniques have yielded highly valuable tools for use in this setting. This article reviews current molecular biology–based techniques and assays, and their practical use in the management of HBV and HCV infection.

Molecular Biology–Based Techniques

Detection and Quantification of Viral Genomes

Viral genomes are generally present in relatively small amounts in body fluids of infected patients, hindering their detection by simple molecular hybridization–based techniques. Thus, their detection and quantification require a preliminary "amplification" step. This can be achieved by using 2 categories of molecular biology–based techniques, namely target amplification and signal amplification.

Target amplification techniques. The principle of target amplification techniques is to synthesize a large number of copies of the viral genome (amplicons) in a cyclic enzymatic reaction. The amplicons can then be detected by various methods, and the amount of viral genomes in the clinical sample can be quantified.

The polymerase chain reaction (PCR) method[3] uses several temperatures and one enzyme, a thermostable DNA polymerase. The amplicons are double-stranded DNA (Figure 1). PCR can be applied to HBV DNA directly, after extraction of nucleic acids or lysis of the viral envelope. In contrast, a reverse transcription step is required for HCV RNA, to synthesize a complementary double-stranded DNA (cDNA) for use as template in the PCR reaction. Each complete PCR cycle doubles the number of DNA copies; after n cycles, 2^n copies of each DNA molecule present at the beginning of the reaction are theoretically synthesized. In fact, the reaction is saturable and reaches a plateau, generally after 35 to 45 cycles.

In transcription-mediated amplification (TMA),[4] the reaction is isothermal and uses 2 enzymes, a reverse transcriptase and a T7 RNA polymerase. The amplicons are single-stranded RNA (Figure 2). 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. TMA reactions also reach a plateau after a certain number of cycles.

Detection of PCR and TMA amplicons is classically based on specific hybridization to immobilized oligonucleotide probes. Amplicon-probe hybrids are revealed in an enzymatic reaction, followed by detection of a colored or luminescent signal. Quantification is 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 at the end of the procedure, and the results are read from a standard curve established in parallel.

Abbreviations used in this paper: bDNA, branched DNA; IFN-α, interferon alfa; PCR, polymerase chain reaction; PEG, pegylated; TMA, transcription-mediated amplification.

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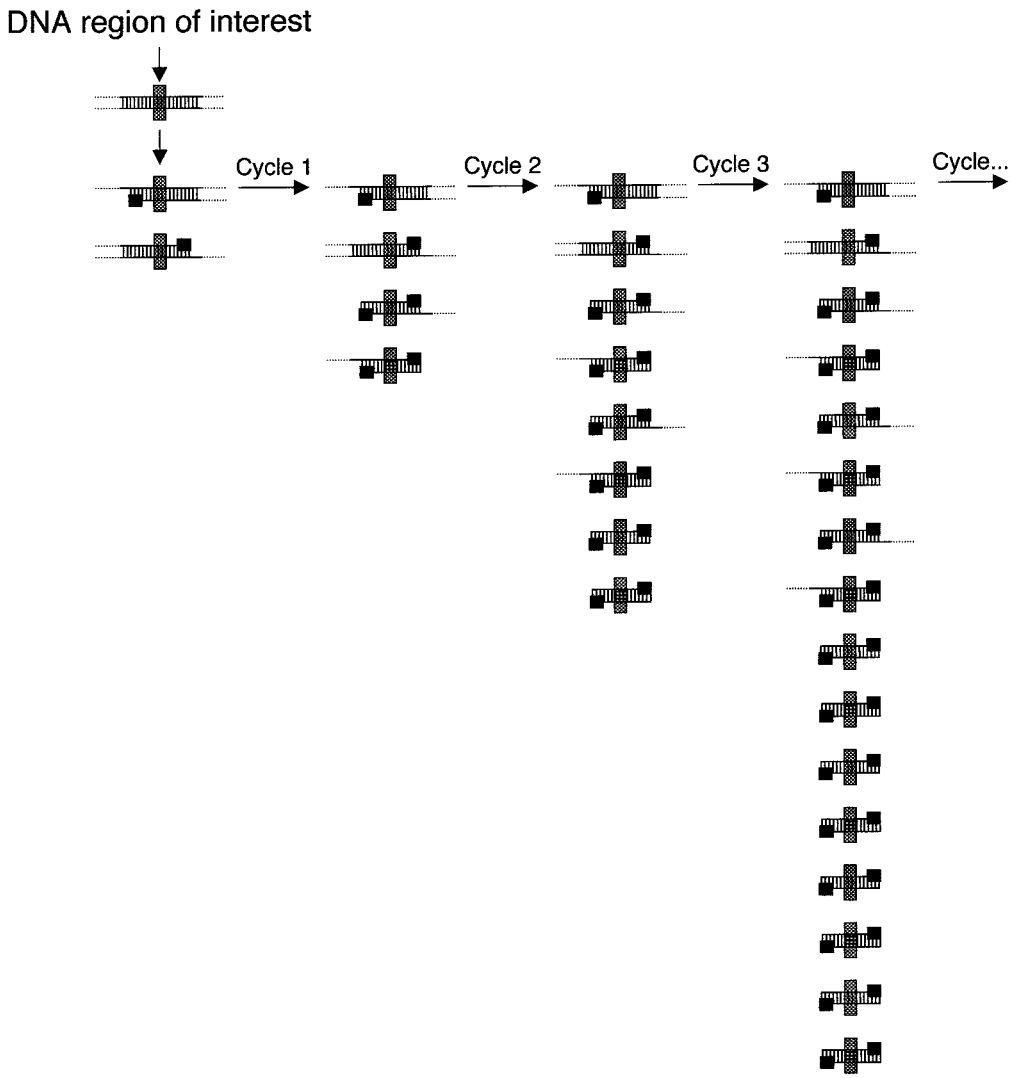

Figure 1. Principle of PCR-based target amplification. During each cycle, DNA is denatured at 90–95°C to separate the 2 strands; specific primers (■) then attach to each strand at a melting temperature depending on the primer sequence and length (annealing); and a new DNA strand is synthesized behind the primers at 72°C on each template strand (elongation). Further amplification cycles swiftly produce large numbers of identical fragments, each of which contains the DNA region of interest.

More recently, "real-time" PCR techniques have been developed. The principle is to detect amplicon synthesis and to deduce the amount of viral genomes in the starting clinical sample during rather than at the end of the PCR reaction.[5] These methods are theoretically more sensitive than classical target amplification techniques and are not prone to carryover contamination. Their dynamic range of quantification is consistently wider, making them particularly useful for quantifying the full range of viral loads observed in untreated and treated patients with HCV and HBV infection.[6–13] Two amplification, detection, and interpretation platforms are currently available: ABI Prism Real-Time PCR devices (Applied BioSystems, Foster City, CA), and the LightCycler device (Roche Molecular Systems, Pleasanton, CA). The LightCycler system can be coupled with an automated viral nucleic acid extraction platform (MagnaPure; Roche Molecular Systems), and similar equipment is being developed for coupling to the ABI Prism system. Several methods can be used in these systems, but need to be adapted to HCV RNA and HBV DNA detection and quantification though appropriate probe design.

In the TaqMan technology,[14] a probe labeled with a "reporter" fluorochrome and a "quencher" fluorochrome is designed to anneal to the target sequence between the sense and antisense PCR primers. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence emission by the reporter. During each PCR reaction, as the DNA polymerase extends the primer, its intrinsic nuclease activity degrades the probe, releasing the reporter fluorochrome. Thus, 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.

In the fluorescence resonance energy transfer method,[15–17] 2 specific oligonucleotide probes labeled with fluorescent dyes are used in addition to the usual PCR reagents. Their sequences are selected such that they hybridize to

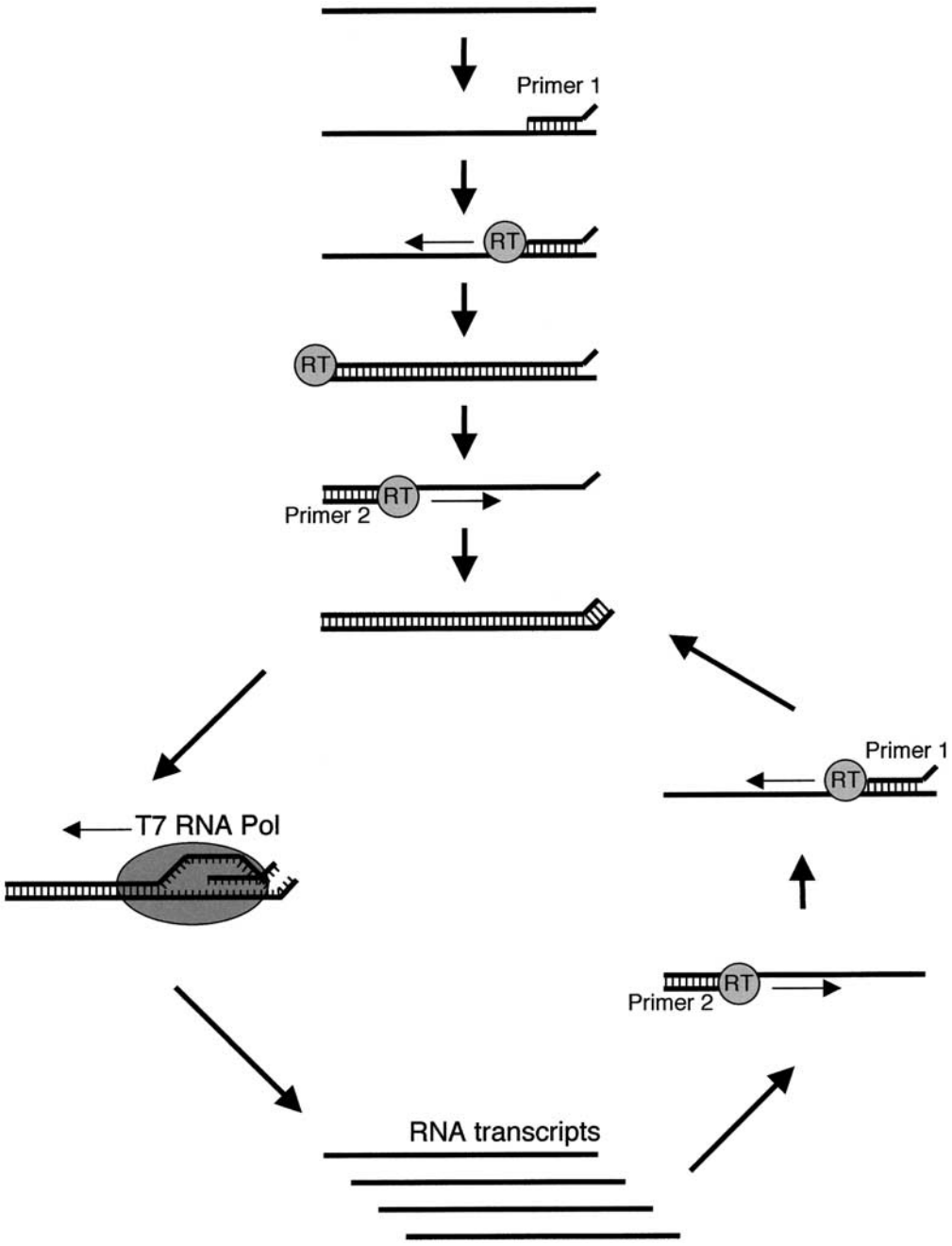

Figure 2. Principle of TMA-based target amplification. The reaction starts with primer 1 (including a T7-promoter sequence) binding to the viral genome target strand. The reverse transcriptase (with the help of primer 2) creates a double-stranded cDNA template including the T7-promoter sequence. Then, T7 RNA polymerase transcribes RNA from the DNA template, producing 100 to 1000 copies of antisense RNA amplicons per template molecule. Double-stranded cDNA synthesis is reinitiated by primer 2 binding to RNA amplicons, and the newly formed cDNAs serve as templates for RNA transcription, resulting in exponential amplification of the target RNA.

the amplified DNA fragment in a head-to-tail arrangement, meaning that the 2 fluorescence dyes are positioned in close proximity to each other. This permits highly efficient energy transfer between the probes, which can be detected as fluorescence emission. As in the TaqMan method, the measured fluorescence is proportional to the amount of DNA amplicons generated during the ongoing PCR process.

In the SYBR Green I dye method,[18,19] the fluorescent dye SYBR Green I produces a minimal background fluorescence signal when unbound. During each PCR cycle, a few molecules bind nonspecifically to the double-stranded DNA amplicons. DNA binding results in a marked increase in SYBR Green I fluorescence emission, which is proportional to the amount of DNA amplicons generated.

Other methods and probes can be used, such as molecular beacons.[20–22]

Whatever the technique used, software is used to calculate the threshold cycle (CT in ABI Prism, Cp in LightCycler) in each reaction with which there is a linear relationship with the initial amount of DNA. In each run, parallel processing of a panel of quantified standards is used to establish a standard curve for quantification.

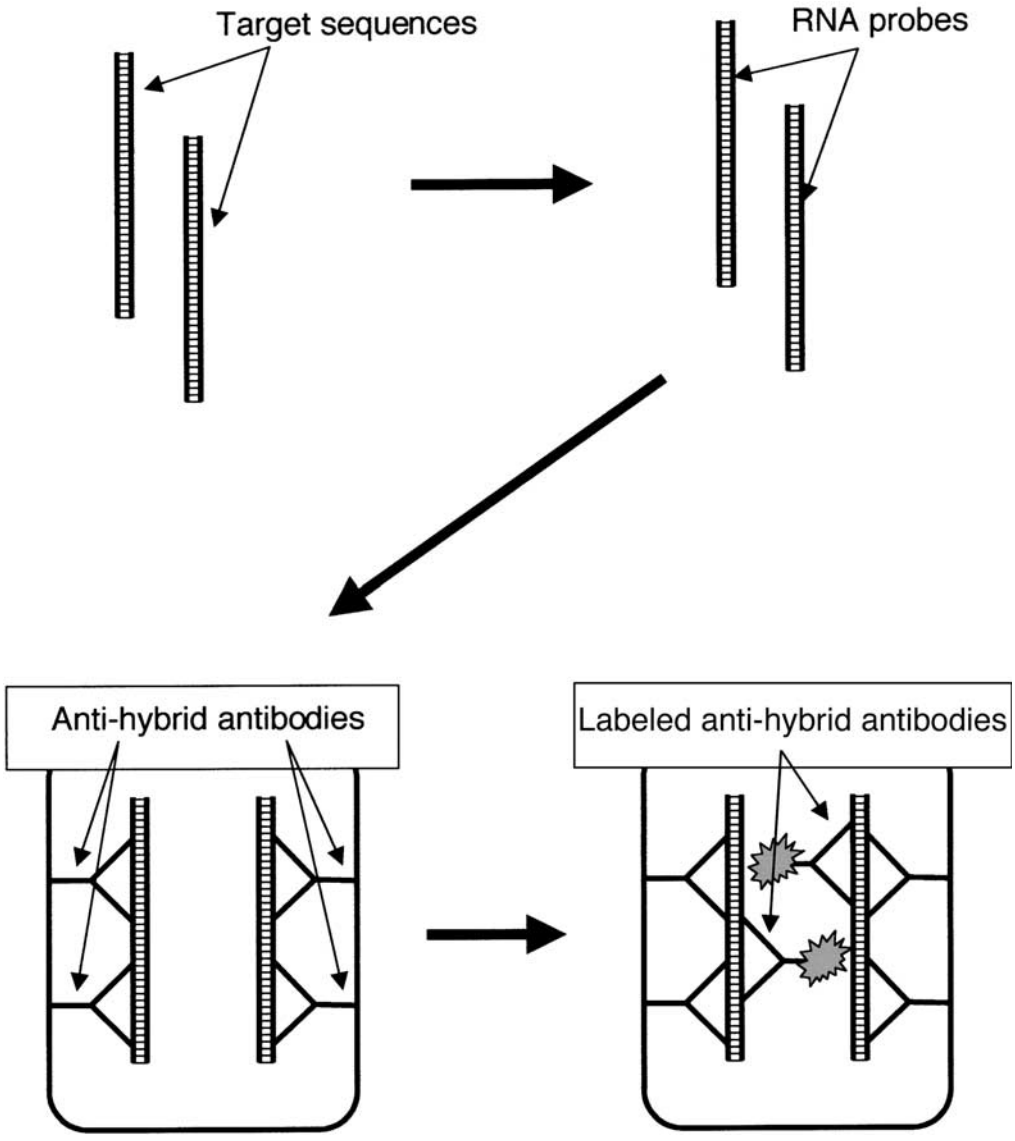

Figure 3. Principle of hybrid-capture signal amplification assay. The target sequence is double-stranded HBV DNA. Hybridization to specific RNA probes creates RNA-DNA hybrids, which are captured on a solid phase (a tube in the first-generation assay, microplate wells in the second-generation assay) by means of universal capture antibodies specific for RNA-DNA hybrids. Detection is performed after signal amplification with multiple antibodies conjugated to a revelation system based on chemiluminescence. Light emission is measured and compared with a standard curve generated simultaneously with known standards.

Signal amplification techniques. In signal amplification techniques, the viral genomes are first hybridized to a holder, by means of specific "capture" oligonucleotide probes. Then, the signal emitted by the hybrids is amplified for detection and measurement.

In the Hybrid-Capture system (Digene Corp., Gaithersburg, MD) (Figure 3),[23] HBV DNA is hybridized to specific RNA probes to create RNA-DNA hybrids. The hybrids are captured on a solid phase coated with universal capture antibodies specific for RNA-DNA hybrids, and are detected with multiple antibodies (resulting in signal amplification) conjugated to a revelation system based on chemiluminescence.

In the "branched DNA" (bDNA) assay (Figure 4),[24–29] viral genomes are specifically captured on microwells by hybridization with oligonucleotide probes. Synthetic bDNA amplifier molecules are hybridized to immobilized target hybrids in the microwells (first-generation HBV DNA and HCV RNA assays, and second-generation HCV RNA assays). A preamplifier molecule is added to the bDNA complex in the third-generation HCV and HBV assays to augment the signal amplification (i.e., assay sensitivity). Signal amplification is achieved through the multiple repeat sequences within each bDNA amplifier molecule that serve as sites for hybridization with alkaline phosphatase–conjugated oligonucleotide probes. Detection is based on alkaline phosphatase–catalyzed chemiluminescence emission from a substrate. Quantification is based on a standard curve generated simultaneously with known standards.

Analysis of the Viral Genome Sequence

Viral genome sequence analysis is aimed at identifying signature sequences and/or amino acid substitu-

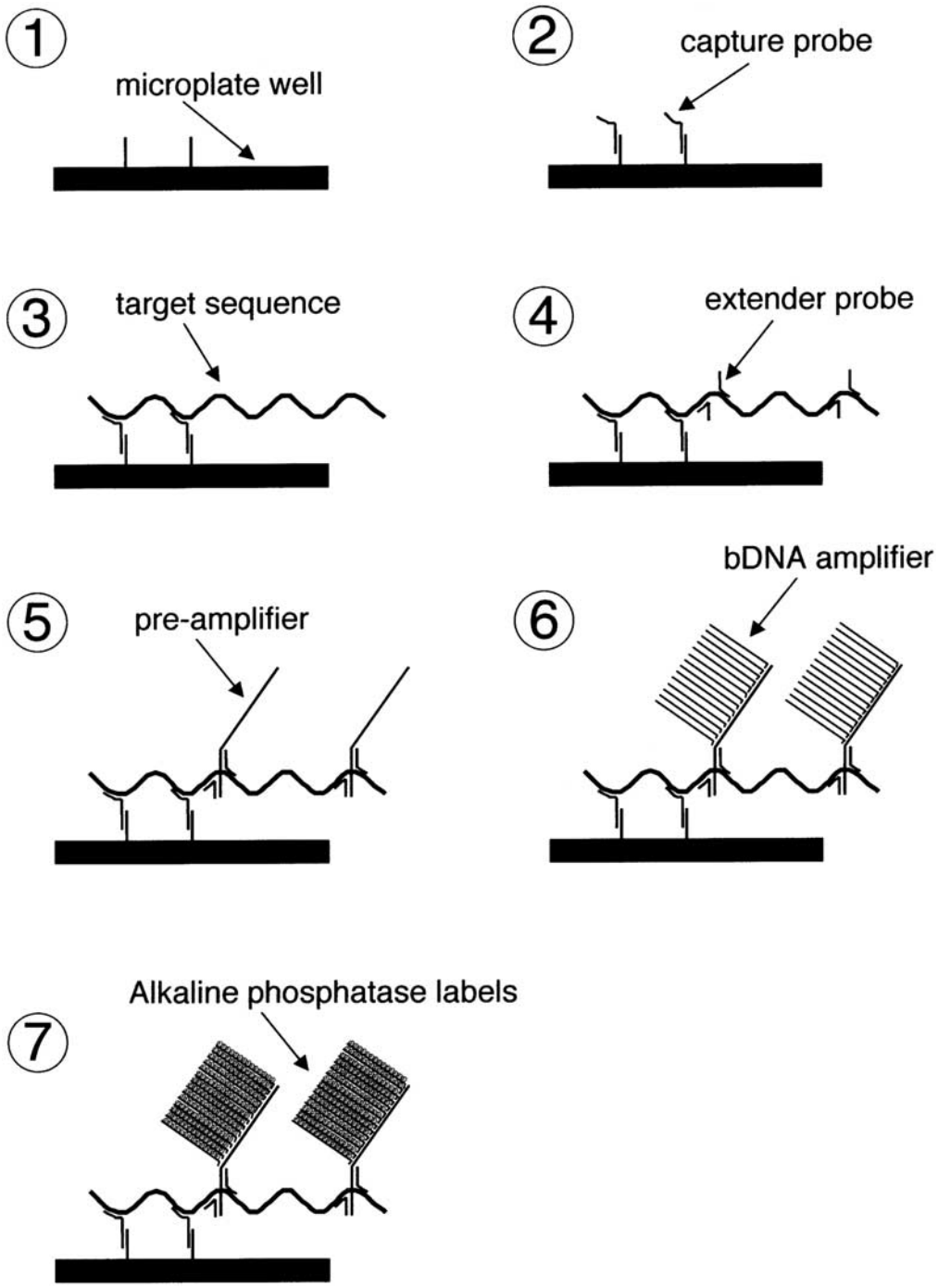

Figure 4. Principle of bDNA signal amplification assay. The target sequences are captured on the wells of a microtiter plate by means of specific "capture probes." "Extender probes" are used to hybridize synthetic bDNA amplifier molecules (in first-generation HBV DNA and HCV RNA assays, and in second-generation HCV RNA assays) or, as shown in the Figure, preamplifier molecules that in turn hybridize bDNA molecules (third-generation HCV and HBV assays). The multiple repeat sequences within each bDNA molecule serve as sites for hybridization to alkaline phosphatase–conjugated oligonucleotide probes. Alkaline phosphatase catalyzes chemiluminescence emission from a substrate, which is measured and compared with a standard curve generated simultaneously with known standards.

tions at specific positions. In practice, signature sequences are used to classify viral strains into phylogenetic groups of clinical interest, called genotypes (including HBV and HCV types, and HCV subtypes),[30,31] and amino acid substitutions known to be associated with viral resistance to antiviral therapy (essentially HBV resistance to specific polymerase inhibitors[27,32,33]) can also be identified. The entire genome of HBV or HCV cannot be routinely analyzed, and available techniques therefore study particular genomic regions. Genome sequence analysis is based on direct sequencing, which provides the full sequence of the analyzed fragment, or on alternative techniques that identify specific sequences at given positions.

Direct sequencing. Direct sequencing of PCR amplicons in an automated DNA sequencer can be used to determine the exact nucleotide and deduced amino acid sequence of the analyzed fragment. It can be applied to the detection of known clinically relevant motifs or mutations, and can also be used for "blind" analysis

aimed at detecting new motifs or mutations. The sequences must be analyzed and interpreted carefully, because the coexistence of variant viral populations in the same blood sample (quasispecies distribution of viral genomes) may lead to sequence ambiguities.[34–37] Identification of amino acid substitutions at specific positions relies on direct examination of the generated sequences, whereas genotype determination is based on phylogenetic analysis of the generated sequences relative to reference sequences. Direct sequencing is the gold standard for genomic sequence analysis.

Alternative techniques. As direct sequencing is labor-intensive and only available in specialized laboratories, alternative techniques have been developed for routine clinical use. These techniques necessitate prior knowledge of the target motifs or mutations. Both "home-made" PCR amplification with sequence-specific primers[38] and restriction fragment length polymorphism analysis of PCR amplicons[39] can be used to determine viral genotypes or to detect point mutations. However, by far the most widely used assays are based on reverse hybridization of PCR amplicons.[40–46] In these assays, PCR amplification of the region of interest is followed by stringent hybridization with oligonucleotide probes fixed to a solid phase; the probes are designed to be complementary to the various possible sequences. Revelation is based on a colorimetric reaction. Hybridization to a particular probe means that the analyzed fragment has the complementary sequence.

Molecular Diagnosis of HCV Infection

Available Assays

HCV RNA detection and quantification. Table 1 lists the commercial assays that can currently be used to detect or quantify HCV RNA. Approval of each test for diagnostic purposes or research use only varies from one country to another at the time of writing.

Qualitative, nonquantitative assays can detect the presence of HCV RNA, but they cannot measure HCV viral load. They are based on target amplification, i.e., PCR or TMA. Table 1 shows the lower detection cutoffs of the available tests, as stated by the manufacturers. Qualitative assays remain clinically useful because they are more sensitive for HCV RNA detection than most of the currently available quantitative assays. Quantitative HCV RNA assays are based on target or signal amplification. The manufacturers' stated dynamic ranges of quantification of the currently available tests are shown in Table 1. The dynamic range of quantification can be extended by diluting and retesting high-viral-load samples, but the dilution step may affect accuracy. All currently available assays have satisfactory specificity: their stated specificity is higher than 95% in all instances, and reaches 98%–99% in most cases. They accurately quantify HCV RNA within their respective dynamic ranges of quantification, with no difference among the various HCV genotypes[27,47–53] (Pawlotsky et al., unpublished results). However, it is recommended not to take into account HCV RNA load variations of less than 3-fold (i.e., ± 0.5 $\log_{10}$), which may be related to the intrinsic variability of the assays. In contrast, variations of more than 3-fold (i.e., 0.5 $\log_{10}$) can reliably be considered to reflect significant differences in HCV RNA load.

Until recently, the quantitative units used in the various assays did not represent the same amount of HCV RNA in a clinical sample. The World Health Organization (WHO) has now established an international standard for universal standardization of HCV RNA quantification units. An HCV RNA international unit has been defined.[54,55] It is currently used in all commercial HCV RNA quantitative assays and should be preferred to any other quantitative unit.[50,56,57] Indeed, the use of standardized international units for HCV RNA quantification allows recommendations and guidelines to be derived from clinical trials and applied in clinical practice with any quantitative HCV RNA assay.

HCV genotype determination. Table 2 shows the commercial assays based on molecular methods that can be used to determine the HCV genotype. Again, current approval for diagnostic purposes or research use only varies from one country to another. All these assays target the 5′ non-coding region of HCV genome. Relative to genotyping in the NS5B region—considered the gold standard for genotype determination—mis-typing seems to be rare, whereas mis-subtyping is frequent (approaching 10% of cases). This has no clinical consequences, as clinical decisions are not currently based on the HCV subtype. The HCV genotype can also be determined by means of serological methods based on the detection of genotype-specific antibodies.

Practical Use

Screening of blood donations for HCV RNA. Routine screening of blood donations for anti-HCV antibodies has drastically reduced the risk of post-transfusion hepatitis C.[58] However, the "serologic window" between HCV infection and the detection of specific antibodies varies from one patient to the next (on average, 7 to 8 weeks on the basis of current assays). The resulting residual risk was calculated to be approximately 1 of 233,000 donations in the 1997–1999 period in the

Table 1. Available HCV RNA Detection and Quantification Assays

Assay	Manufacturer	Method	Lower detection cutoff	Dynamic range of quantification[a]	Setting	Availability
Amplicor HCV v2.0	Roche Molecular Systems, Pleasanton, California	Manual qualitative RT-PCR	50 IU/mL	None	Diagnosis	Available kit
Cobas Amplicor HCV v2.0	Roche Molecular Systems, Pleasanton, California	Semi-automated qualitative RT-PCR	50 IU/mL	None	Diagnosis	Available kit
Ampliscreen HCV	Roche Molecular Systems, Pleasanton, California	Semi-automated qualitative RT-PCR	<50 IU/mL[b]	None	Blood screening	Available kit
Amplicor HCV Monitor v2.0	Roche Molecular Systems, Pleasanton, California	Manual quantitative RT-PCR	600 IU/mL	600–500,000 IU/mL	Diagnosis	Available kit
Cobas Amplicor HCV Monitor v2.0	Roche Molecular Systems, Pleasanton, California	Semi-automated quantitative RT-PCR	600 IU/mL	600–500,000 IU/mL	Diagnosis	Available kit
Versant HCV RNA Qualitative Assay	Bayer Corporation, Diagnostics Division, Tarrytown, New York	Manual qualitative TMA	10 IU/mL	None	Diagnosis	Available kit
Versant HCV RNA 2.0 Assay (bDNA)	Bayer Corporation, Diagnostics Division, Tarrytown, New York	Manual branched DNA signal amplification	200,000 genome equivalents/mL	200,000–120,000,000 genome equivalents/mL	Diagnosis	Available kit
Versant HCV RNA 3.0 Assay (bDNA)	Bayer Corporation, Diagnostics Division, Tarrytown, New York	Semi-automated branched DNA signal amplification	615 IU/mL	615–7,700,000 IU/mL	Diagnosis	Available kit
LCx HCV RNA Quantitative Assay	Abbott Diagnostic, Chicago, Illinois	Semi-automated quantitative RT-PCR	25 IU/mL	25–2,630,000 IU/mL	Diagnosis	Kit in development
SuperQuant	National Genetics Institute, Los Angeles, California	Semi-automated quantitative RT-PCR	30 IU/mL	30–1,470,000 IU/mL	Diagnosis	No kit available: samples tested by the manufacturer in a "home-made" procedure
Procleix HIV-1/HCV Assay	Chiron Corp., Emeryville, California	Manual qualitative TMA	<50 IU/mL[b]	None	Blood screening	Available kit

RT-PCR, reverse transcriptase–polymerase chain reaction.
[a]Quantitative assays only.
[b]The given lower detection cutoff is based on individual determination and may vary when the assay is used in plasma pools of various sizes and according to the extraction procedure.

United States (1 of 700,000 donations in France during the same period). HCV RNA detection in pooled or individual donations by molecular biology techniques (e.g., qualitative PCR or TMA assays) has recently been implemented in blood banks in the European Union and the United States to reduce this "window period" and to improve the viral safety of blood products. Despite a certain heterogeneity among centers in terms of the size of the plasma pools tested and the sensitivity of the anti-HCV antibody assays used for screening, the 2-year experience with HCV RNA testing of blood donations confirms initial estimates. Indeed, in the United States, 29,253,815 donations were tested between March–April 1999 and June 2001, and 113 of them (i.e., 1 of 259,000) were found to be HCV RNA–positive and anti-HCV antibody–negative (SL Stramer, personal communication, September 2001). The rate of false-positive results causing loss of the donation was 1 of 15,800 in the first year of testing (SL Stramer, personal communication, September 2001). Interestingly, 80% of anti-HCV antibody–positive donations were found to be HCV RNA–positive, suggesting that the vast majority of the remaining 20% of donors had resolved the infection.[59]

Diagnosis of HCV infection. HCV RNA assays are useful in several diagnostic settings. In all instances, qualitative assays are used because they are more sensitive for HCV RNA detection than the current quantitative

Table 2. Available HCV Genotyping Assays

Assay	Manufacturer	Method	Ability to identify types	Ability to identify subtypes	Availability
Inno-LiPA HCV II	Innogenetics, Ghent, Belgium	Reverse hybridization of PCR amplicons	1, 2, 3, 4, 5, 6	1a, 1b, 1a/1b, 1, 2a/2c, 2b, 2, 3a, 3b, 3c, 3, 4a, 4b, 4c/4d, 4e, 4f, 4h, 4, 5a, 6a, 10a	Available kit
Trugene HCV 5′NC Genotyping kit	Visible Genetics Inc., Toronto, Ontario	Direct sequencing of the 5′ noncoding region of HCV genome followed by sequence comparison with an HCV genotype sequence database	1, 2, 3, 4, 5, 6	1a, 1b, 1c, 2a, 2b, 2c, 2d, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 5a, 6a, 6b	Available kit
Trugene HCV NS5B Genotyping kit	Visible Genetics Inc., Toronto, Ontario	Direct sequencing of the NS5B region of HCV genome followed by sequence comparison with an HCV genotype sequence database	Theoretically all types	Theoretically all subtypes	Kit in development

assays (Table 1). Nevertheless, a quantitative HCV RNA assay with equal sensitivity could be used for diagnostic purpose. It is likely that sensitive quantitative assays will replace purely qualitative assays in the future.

Acute hepatitis C. In acute HCV infection, anti-HCV antibodies are detected in only 50% to 70% of patients at the onset of symptoms; in the remaining patients, anti-HCV antibodies usually emerge after 3 to 6 weeks.[60–62] Thus, HCV RNA must be sought with a sensitive technique in patients with acute hepatitis who have no serological markers of viral hepatitis (including anti-HCV antibody negativity).[63] HCV RNA detection strongly suggests acute hepatitis C, which is confirmed by subsequent antibody seroconversion. In contrast, negative HCV RNA detection makes this diagnosis very unlikely. When both anti-HCV antibodies and HCV RNA are present during acute hepatitis, it may be difficult to discriminate among acute hepatitis C, an acute exacerbation of chronic hepatitis C, and acute hepatitis of another cause in a patient with chronic hepatitis C.

Chronic hepatitis C. In patients with symptoms of chronic hepatitis, HCV RNA testing must be performed when anti-HCV antibodies are present, to assess viral replication and to confirm the diagnosis of HCV infection.[63,64] HCV RNA testing should also be performed when no antibodies are found and hepatitis C is suspected in hemodialyzed or immunosuppressed patients who may (occasionally) have seronegative chronic hepatitis C, especially if the immunodepression is profound.[63]

Mother-to-infant transmission. Babies born to HCV-infected mothers usually carry HCV antibodies for between a few months and 1 year, because of passive transfer from the mother.[65–68] The diagnosis of HCV infection in the baby is thus based on HCV RNA detection.[63] The dates at which the test should ideally be performed are not precisely known. In case of HCV RNA negativity, the lack of transmission is confirmed by the gradual disappearance of HCV antibodies. If the baby is infected, HCV RNA detection may be positive at birth or only later during the first year of life, whereas anti-HCV antibodies persist.

Accidental exposure. HCV RNA can generally be detected in serum within a few days to a few weeks after accidental exposure to HCV-infected blood. Elevated alanine aminotransferase (ALT) activity and seroconversion are observed a few weeks later in the absence of therapy. Antiviral therapy, which prevents progression to chronicity in a large proportion of cases, is not an emergency and may be started once the diagnosis of acute hepatitis C is made.

Assessment of disease severity and prognosis. No virological parameters can be used to assess the severity of HCV-related liver disease (which is currently evaluated by means of liver biopsy), or to establish the prognosis. In particular, neither the HCV genotype nor HCV viral load at the time of liver biopsy seems to correlate with necro-inflammatory activity or the extent of fibrosis, or to predict subsequent outcome. Therefore, repeated HCV RNA determinations are not needed during follow-up of untreated patients.

Treatment of HCV infection. The reference therapy for chronic hepatitis C is now a combination of weekly pegylated (PEG) interferon alfa (IFN-α) and daily ribavirin administration.[69] Molecular biology–based techniques are particularly useful for managing the successive phases of treatment.

Decision to treat. In the absence of contraindications to antiviral therapy, treatment of chronic hepatitis C is indicated for patients with elevated serum ALT activity, chronic hepatitis on liver biopsy (with or without cirrhosis), and HCV RNA positivity.[63,70] Patients with severe HCV-related extrahepatic disorders (e.g., symptomatic cryoglobulinemia-associated vasculitis or glomerulonephritis) and detectable HCV RNA should also be treated.[63,70] For patients with repeatedly normal ALT activity and/or mild hepatitis on liver biopsy but HCV RNA positivity, the strategy varies according to the HCV genotype. The patients infected with HCV genotypes 2 or 3 should be treated, because more than 80% of them will achieve a sustained virological response (i.e., HCV RNA clearance maintained for 24 weeks after treatment withdrawal), which is the endpoint of therapy.[69] In the patients infected with other genotypes, the decision to treat is based on the assessment of the natural prognosis of HCV-related liver disease on liver biopsy.[63]

Selection of optimal therapy. With the previous reference treatment, based on recombinant IFN-α administered 3 times weekly in combination with daily ribavirin, both the HCV genotype and HCV viral load were significant predictors of sustained virological response.[71,72] Based on these results, 24 weeks of treatment was recommended for patients infected by genotypes 2 and 3 (whatever their viral load); and for patients infected by genotypes 1, 4, and 5 who had a low pretreatment viral load (i.e., <800,000 IU/mL) and no other predictors of nonresponse (such as male gender, age over 40 years, and fibrosis on liver biopsy).[57,63,73] In contrast, patients infected by genotypes 1, 4, or 5 who had a high pretreatment viral load (i.e., >800,000 IU/mL) needed 48 weeks of treatment, provided that they were virological responders (i.e., HCV RNA–negative) at week 24 of therapy.[57,63,73] Both the HCV genotype and HCV viral load, together with body weight, age, and the presence of bridging fibrosis/cirrhosis on liver biopsy, were recently identified as significant predictors of a sustained virological response to the PEG–IFN-α–ribavirin combination.[69] However, available data are inadequate to make recommendations on how to tailor treatment to pretreatment virological parameters. In practice, HCV genotype determination must be performed before PEG–IFN-α–ribavirin treatment. It is indeed very likely, based on the results of recombinant IFN-α–ribavirin combination therapy[71,72] and the better efficacy of PEG–IFN-α–ribavirin,[69] that patients infected by genotypes 2 and 3 do not need more than 24 weeks of treatment; this question is being addressed in ongoing clinical trials. In contrast, patients infected by genotype 1 (and, by extension, those infected by genotypes 4, 5, or 6) should receive 48 weeks of treatment. Whether or not the treatment duration and/or the doses of PEG–IFN-α and ribavirin should be modulated according to baseline viral load (for reasons of cost/safety/efficacy) remains to be determined.

Treatment monitoring. Qualitative HCV RNA detection assays must still be used to assess the virological response of chronic hepatitis C to therapy, because they are more sensitive than current quantitative assays (Table 1). In patients who receive PEG–IFN-α and ribavirin for 48 weeks, the probability of a sustained virological response is extremely low when HCV RNA is still detectable at week 24.[69] Thus, HCV RNA determination at week 24 can help with the decision-making process, according to pretreatment liver biopsy findings and overall prognosis, as follows: when negative, continue therapy; when positive, stop therapy or continue to improve liver histology and slow liver disease progression without clearing the infection.

In the patients who complete treatment, the end-of-treatment virological response is evaluated at week 24 or 48 according to treatment duration. HCV RNA detection at this latter time point is highly predictive of a subsequent relapse. The main endpoint of treatment is a sustained virological response, characterized by normal ALT activity and HCV RNA negativity 24 weeks after treatment cessation. Long-term follow-up of sustained virological responders to antiviral therapy suggests that viral clearance 24 weeks after treatment withdrawal is highly predictive of long-term viral eradication.[74–76] This needs to be confirmed in sustained virological responders to the PEG–IFN-α–ribavirin combination.

Controversy persists over whether HCV RNA quantification should be used to assess the virological response and to make clinical decisions during therapy. It has recently been suggested that a 2-log fall in viral load (i.e., a baseline viral load divided by 100) or HCV RNA negativity (patients whose baseline viral load was less than 2 log above the detection cutoff of the assay) at week 12 has poor positive predictive value but excellent negative predictive value for a subsequent sustained virological response to PEG–IFN-α and ribavirin therapy (Fried et al., unpublished data). This means that, in the absence of such changes, a patient has a minimal chance of a sustained virological response. If confirmed, these results would allow the decision to stop or continue therapy to be made as early as week 12 of treatment. Monitoring of viral replication dynamics based on frequent blood sampling during the first days to weeks of treatment may

allow patients to be categorized according to their actual virological response.[77,78] Such categorization might be useful to optimize treatment efficacy and to determine the best indications of future new treatments. Ongoing studies, such as the European Commission–granted multicenter DITTO trial, are assessing the clinical relevance of monitoring HCV RNA dynamics during therapy.

Molecular Diagnosis of HBV Infection

Available Assays

HBV DNA detection and quantification. Table 3 shows the commercial assays that can currently be used to detect and quantify HBV DNA. For each of them, current approval for diagnostic purposes or research use only varies from one country to another.

Table 3 shows the dynamic ranges of quantification stated by the manufacturers. The dynamic range of quantification can be extended to higher values by diluting and re-testing high-viral-load samples, but the dilution step may affect accuracy. These assays have been shown to be specific and accurate within their respective dynamic ranges of quantification.[23,25,28,29,79–84] The possible influence of the HBV genotype on quantification has not been extensively studied. As with HCV, it is recommended not to take into account HBV DNA load variations of less than 3-fold (i.e., ± 0.5 $\log_{10}$), whereas variations of more than 3-fold (i.e., 0.5 $\log_{10}$) can reliably be considered to reflect significant changes. HBV DNA quantitative units currently used in the various assays do not represent the same actual amount of HBV DNA in a given clinical sample. WHO has established an international standard for universal standardization of HBV DNA quantification units, and an HBV DNA international unit has been defined.[85] This international unit must be preferred to any other quantitative unit and should now be implemented in all commercial HBV DNA quantitative assays. This will be particularly useful for recently developed highly sensitive HBV DNA assays, as well as for establishing clinically relevant thresholds and recommendations for clinical decisions based on HBV DNA load.

HBV genotype determination and identification of resistance mutations. No commercial assays can be used to determine the HBV genotype, the clinical relevance of which remains unknown. A line probe assay has recently been developed (Innogenetics, Ghent, Belgium) to identify HBV DNA polymerase mutations associated with HBV resistance to lamivudine.[43] It is based on PCR amplification of the HBV reverse transcriptase gene and reverse hybridization to oligonucleotide probes bearing various resistance-associated mutations coated on a nitrocellulose strip.[43]

Table 3. Available HBV DNA Detection and Quantification Assays

Assay	Manufacturer	Method	Lower detection cutoff[a]	Dynamic range of quantification[a]	Setting	Availability
HBV Digene Hybrid-Capture I	Digene Corp., Gaithersburg, Maryland	Hybrid capture signal amplification in tubes	700,000 copies/mL	700,000–560,000,000 copies/mL	Diagnosis	Available kit
HBV Digene Hybrid-Capture II	Digene Corp., Gaithersburg, Maryland	Hybrid capture signal amplification in microplates	142,000 copies/mL	142,000–1,700,000,000 copies/mL	Diagnosis	Available kit
Ultra-sensitive HBV Digene Hybrid-Capture II	Digene Corp., Gaithersburg, Maryland	Hybrid capture signal amplification in microplates after centrifugation	4,700 copies/mL	4,700–57,000,000 copies/mL	Diagnosis	Available kit
Amplicor HBV Monitor	Roche Molecular Systems, Pleasanton, California	Manual quantitative RT-PCR	1,000 copies/mL	1,000–4,000,000 copies/mL	Diagnosis	Available kit
Cobas Amplicor HBV Monitor	Roche Molecular Systems, Pleasanton, California	Semi-automated quantitative RT-PCR	200 copies/mL	200–200,000 copies/mL	Diagnosis	Available kit
Versant HBV DNA 1.0 Assay (bDNA)	Bayer Corporation, Diagnostics Division, Tarrytown, New York	Manual branched DNA signal amplification	700,000 genome equivalents/mL	700,000–5,000,000,000 genome equivalents/mL	Diagnosis	Available kit[b]

[a] The lower detection cutoffs and dynamic ranges of quantification are given in nonstandardized units, making them difficult to compare.
[b] A more sensitive version is in development.

Practical Use

Screening of blood donations for HBV. Routine screening of blood donations for hepatitis B surface antigen (HBsAg) and total anti–hepatitis B core antibodies has drastically reduced the risk of post-transfusion hepatitis B, which was estimated to be approximately 1 of 137,000 donations in the 1997–1999 period in the United States (1 of 475,000 donations in France during the same period), i.e., about twice the residual risk of HCV transmission before the implementation of HCV RNA testing. HBV DNA can be detected on average 21 days before the appearance of HBsAg, even when HBsAg is assayed with the most sensitive tests. However, no extra donors would be rejected on the basis of pooled HBV DNA testing than by using the most sensitive HBsAg tests on an individual basis (SL Stramer, personal communication, September 2001). At the time of writing, HBV DNA screening by molecular biology techniques has not been implemented in blood banks in Europe and the United States.

Diagnosis of HBV infection. No molecular biology–based assays are necessary for the diagnosis of acute hepatitis B, which is based on serological testing. Chronic hepatitis B is defined by HBsAg persistence in serum for more than 6 months. In this setting, HBV DNA detection-quantification is necessary to determine whether or not HBV is replicating.[2] When HBV DNA is detected, the quantitative result should be interpreted according to hepatitis B e antigen (HBeAg) status, ALT activity, and histologic status, including the activity and degree of fibrosis. In the presence of HBeAg, the diagnosis of replicating chronic hepatitis B can be made whatever the viral load.[2] In contrast, the interpretation of HBV DNA quantification is difficult in HBeAg-negative/anti–HBeAg-positive patients (pre-core mutant HBV), who generally have lower replication levels than HBeAg-positive patients. Indeed, the recent development of highly sensitive assays based on target amplification, which can detect HBV DNA in the majority of HBsAg carriers, made it necessary to define a clinically relevant replication threshold. It has been suggested that an HBV DNA load of less than 10^5 copies/mL is associated with an "inactive carrier state," whereas HBV DNA loads higher than 10^5 copies/mL should be considered as clinically significant.[2] In fact, the best discriminatory threshold (or interval, as overlaps may exist) remains to be established in appropriate clinical studies using highly sensitive and accurate HBV DNA assays, and standardized international units.

Assessment of disease severity and prognosis. Histologic examination of liver biopsy material is still the best way of assessing the severity of chronic hepatitis B and establishing the prognosis. HBV DNA detection also provides valuable prognostic information. Indeed, active HBV replication is associated with a significant risk of progression to chronic hepatitis B complications (including cirrhosis and hepatocellular carcinoma).[2] This risk is low in the absence of detectable HBV DNA, except in patients with cirrhosis, who may subsequently develop hepatocellular carcinoma despite the absence of HBV replication. The possible prognostic significance of HBV DNA load, and informative thresholds, remains to be determined in appropriate clinical studies.

Treatment of HBV infection. HBV DNA detection and quantification are key tools for treatment monitoring in chronic hepatitis B.

Decision to treat. The decision to treat patients with chronic hepatitis B must be taken individually, on the basis of precisely weighted parameters. Elevated serum ALT activity, a liver biopsy showing chronic hepatitis with or without cirrhosis, and the presence of significant levels of HBV DNA are strong arguments for initiating antiviral therapy.[2] As stated above, no precise clinically relevant HBV DNA thresholds are known, and prospective trials are needed to determine HBV DNA loads (in international unit/milliliter) above which patients with chronic hepatitis B should be treated (and below which they should not be treated).

Selection of optimal therapy. The treatment of chronic hepatitis B is based on IFN-α administration at a dose of 5 to 10 million units 3 times a week subcutaneously for 16 to 32 weeks, or lamivudine at a dose of 100 mg per day orally for 48 weeks or longer (150 mg twice daily in human immunodeficiency virus–coinfected patients).[86–91] Which of these 2 drugs should be chosen for first-line treatment for chronic hepatitis B is controversial.[2] The patients with a low HBV DNA level are more likely than those with a high HBV DNA level to have a sustained response (HBe seroconversion) to IFN-α. HBV DNA quantification could thus help selecting optimal therapy. Again, the precise HBV DNA cutoff that discriminates between "low" and "high" pretreatment replication needs to be determined, using standardized quantification units.

Treatment monitoring. HBV DNA quantification, together with repeated ALT determinations and HBeAg/anti-HBe antibody assessments in HBeAg-positive patients, is critical in treatment monitoring.[2] Nonresponders to IFN-α have little or no change in HBV DNA load during therapy, whereas responders show a significant decrease. Successful IFN-α treatment is char-

acterized by HBe seroconversion and a reduction in HBV DNA load below the detection cutoff of signal amplification assays. Small amounts of HBV DNA may be still detectable in HBe seroconverters with target amplification assays. In contrast, HBV DNA is never detected after HBs seroconversion.

In patients receiving lamivudine monotherapy, the potent specific antiviral effect of the drug leads to a significant and rapid decrease in viral load. HBV DNA becomes undetectable in signal amplification assays within a few days to a few weeks in the vast majority of patients, but low-level replication may remain detectable with target amplification assays. HBe seroconversion can occur in HBeAg-positive patients.

Assessment of HBV resistance to lamivudine. HBV resistance to lamivudine is frequent, occurring in 14% to 32% of cases after 1 year of treatment, and 38% to 58% of cases after 2 years of treatment. It is characterized by a relapse of HBV replication during therapy, at levels that may be lower than those seen before treatment.[92–102] HBV resistance to lamivudine is related to the selection of HBV mutants bearing amino acid substitutions located within or close to the catalytic site of HBV DNA polymerase.[103] These mutations can be detected by direct sequencing or reverse hybridization.[43] These techniques currently have no routine indications. Indeed, HBV resistance is shown by HBV DNA load monitoring, and the identification of lamivudine resistance mutations is clinically irrelevant: patients with these mutations are generally kept on lamivudine or may be switched to nucleotide analogs, such as adefovir dipivoxil or tenofovir, in case of rapidly progressing liver disease. Identification of antiviral resistance mutations might be useful in the future when several combination therapies become available.

Conclusion

In the past decade, the introduction and constant improvement of molecular biology–based techniques have provided invaluable tools for the management of chronic viral hepatitis. They can now be used to test blood donations, diagnose active infection, help to establish the prognosis, guide treatment decisions, and assess the virological response to therapy. Further work is required to fully standardize assays and quantification units, improve automation, and better define clinically relevant thresholds that can be used to establish universal recommendations for patient care. The development of increasingly sensitive and accurate assays for detection and quantification will improve the assessment of the response to antiviral therapy and permit earlier detection of viral resistance. Techniques able to detect minor viral populations within a given patient's quasispecies will permit the detection of resistant mutants before or early during therapy. Our understanding of the mechanisms underlying antiviral resistance will benefit from new technologies, models, and strategies, such as in vitro replication or culture models, in vivo infection models, viral quasispecies analysis strategies, viral kinetics/dynamics monitoring, DNA microarray development, and widespread use of in vitro models to study viral protein functions and virus-host interactions. Together with the development of new antiviral drugs and novel therapeutic approaches, these improvements will allow us to optimize the treatment of chronic viral hepatitis and improve the global results.

References

1. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000;20:1–16.
2. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology 2001; 120:1828–1853.
3. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 1986;324:163–166.
4. Compton J. Nucleic acid sequence-based amplification. Nature 1991;350:91–92.
5. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y) 1993;11:1026–1030.
6. Enomoto M, Nishiguchi S, Shiomi S, Tanaka M, Fukuda K, Ueda T, Tamori A, Habu D, Takeda T, Yano Y, Otani S. Comparison of real-time quantitative polymerase chain reaction with three other assays for quantitation of hepatitis C virus. J Gastroenterol Hepatol 2001;16:904–909.
7. Martell M, Gomez J, Esteban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J Clin Microbiol 1999;37:327–332.
8. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.
9. Chen RW, Piiparinen H, Seppanen M, Koskela P, Sarna S, Lappalainen M. Real-time PCR for detection and quantitation of hepatitis B virus DNA. J Med Virol 2001;65:250–256.
10. Brechtbuehl K, Whalley SA, Dusheiko GM, Saunders NA. A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. J Virol Methods 2001;93:105–113.
11. Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J Clin Microbiol 2000;38:2897–2901.
12. Loeb KR, Jerome KR, Goddard J, Huang M, Cent A, Corey L. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. Hepatology 2000;32:626–629.
13. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, Yoshiba M, Kohara M. Quantitation of hepatitis B

virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899–2903.
14. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–994.
15. Chen X, Zehnbauer B, Gnirke A, Kwok PY. Fluorescence energy transfer detection as a homogeneous DNA diagnostic method. Proc Natl Acad Sci U S A 1997;94:10756–10761.
16. Andrus A, Cox S, Beavers S, Parker A, Anuskiewicz J, Mullah B. High-throughput synthesis of functionalized oligonucleotides. Nucleic Acids Symp Ser 1997:37:317–318.
17. Parks SB, Popovich BW, Press RD. Real-time polymerase chain reaction with fluorescent hybridization probes for the detection of prevalent mutations causing common thrombophilic and iron overload phenotypes. Am J Clin Pathol 2001;115:439–447.
18. Cane PA, Cook P, Ratcliffe D, Mutimer D, Pillay D. Use of real-time PCR and fluorimetry to detect lamivudine resistance-associated mutations in hepatitis B virus. Antimicrob Agents Chemother 1999;43:1600–1608.
19. Woo TH, Patel BK, Cinco M, Smythe LD, Symonds ML, Norris MA, Dohnt MF. Real-time homogeneous assay of rapid cycle polymerase chain reaction product for identification of Leptonema illini. Anal Biochem 1998;259:112–117.
20. Lewin SR, Vesanen M, Kostrikis L, Hurley A, Duran M, Zhang L, Ho DD, Markowitz M. Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy. J Virol 1999;73:6099–6103.
21. Chen W, Martinez G, Mulchandani A. Molecular beacons: a real-time polymerase chain reaction assay for detecting Salmonella. Anal Biochem 2000;280:166–172.
22. McKillip JL, Drake M. Molecular beacon polymerase chain reaction detection of Escherichia coli O157:H7 in milk. J Food Prot 2000;63:855–859.
23. Barlet V, Cohard M, Thelu MA, Chaix MJ, Baccard C, Zarski JP, Seigneurin JM. Quantitative detection of hepatitis B virus DNA in serum using chemiluminescence: comparison with radioactive solution hybridization assay. J Virol Methods 1994;49:141–151.
24. Urdea MS, Horn T, Fultz TJ, Anderson M, Running JA, Hamren S, Ahle D, Chang CA. Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. Nucleic Acids Symp Ser 1991:24:197–200.
25. Hendricks DA, Stowe BJ, Hoo BS, Kolberg J, Irvine BD, Neuwald PD, Urdea MS, Perrillo RP. Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. Am J Clin Pathol 1995;104:537–546.
26. Davis GL, Lau JY, Urdea MS, Neuwald PD, Wilber JC, Lindsay K, Perrillo RP, Albrecht J. Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. Hepatology 1994;19:1337–1341.
27. Pawlotsky JM, Martinot-Peignoux M, Poveda JD, Bastie A, Le Breton V, Darthuy F, Remire J, Erlinger S, Dhumeaux D, Marcellin P. Quantification of hepatitis C virus RNA in serum by branched DNA-based signal amplification assays. J Virol Methods 1999;79:227–235.
28. Pawlotsky JM, Bastie A, Lonjon I, Remire J, Darthuy F, Soussy CJ, Dhumeaux D. What technique should be used for routine detection and quantification of HBV DNA in clinical samples? J Virol Methods 1997;65:245–253.
29. Pawlotsky JM, Bastie A, Hezode C, Lonjon I, Darthuy F, Remire J, Dhumeaux D. Routine detection and quantification of hepatitis B virus DNA in clinical laboratories: performance of three commercial assays. J Virol Methods 2000;85:11–21.
30. Simmonds P. Viral heterogeneity of the hepatitis C virus. J Hepatol 1999;31(suppl 1):54–60.
31. Bollyky PL, Holmes EC. Reconstructing the complex evolutionary history of hepatitis B virus. J Mol Evol 1999;49:130–141.
32. Mutimer D. Hepatitis B virus antiviral drug resistance: from the laboratory to the patient. Antivir Ther 1998;3:243–246.
33. Zoulim F, Trepo C. New antiviral agents for the therapy of chronic hepatitis B virus infection. Intervirology 1999;42:125–144.
34. Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, Guardia J, Gomez J. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J Virol 1992;66:3225–3229.
35. Weiner AJ, Brauer MJ, Rosenblatt J, Richman KH, Tung J, Crawford K, Bonino F, Saracco G, Choo QL, Houghton M. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology 1991;180:842–848.
36. Pawlotsky JM, Germanidis G, Frainais PO, Bouvier M, Soulier A, Pellerin M, Dhumeaux D. Evolution of the hepatitis C virus second envelope protein hypervariable region in chronically infected patients receiving alpha interferon therapy. J Virol 1999; 73:6490–6499.
37. Pawlotsky JM, Germanidis G, Neumann AU, Pellerin M, Frainais PO, Dhumeaux D. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. J Virol 1998;72:2795–2805.
38. Okamoto H, Kobata S, Tokita H, Inoue T, Woodfield GD, Holland PV, Al-Knawy BA, Uzunalimoglu O, Miyakawa Y, Mayumi M. A second-generation method of genotyping hepatitis C virus by the polymerase chain reaction with sense and antisense primers deduced from the core gene. J Virol Methods 1996;57:31–45.
39. Nakao T, Enomoto N, Takada N, Takada A, Date T. Typing of hepatitis C virus genomes by restriction fragment length polymorphism. J Gen Virol 1991;72:2105–2112.
40. Stuyver L, Rossau R, Wyseur A, Duhamel M, Vanderborght B, Van Heuverswyn H, Maertens G. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J Gen Virol 1993;74:1093–1102.
41. Stuyver L, Wyseur A, van Arnhem W, Lunel F, Laurent-Puig P, Pawlotsky JM, Kleter B, Bassit L, Nkengasong J, van Doorn LJ, Maertens G. Hepatitis C virus genotyping by means of 5′-UR/core line probe assays and molecular analysis of untypeable samples. Virus Res 1995;38:137–157.
42. Stuyver L, Wyseur A, van Arnhem W, Hernandez F, Maertens G. Second-generation line probe assay for hepatitis C virus genotyping. J Clin Microbiol 1996;34:2259–2266.
43. Stuyver L, Van Geyt C, De Gendt S, Van Reybroeck G, Zoulim F, Leroux-Roels G, Rossau R. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. J Clin Microbiol 2000;38:702–707.
44. Teles SA, Martins RM, Vanderborght B, Stuyver L, Gaspar AM, Yoshida CF. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. Artif Organs 1999;23: 1074–1078.
45. Viazov S, Zibert A, Ramakrishnan K, Widell A, Cavicchini A, Schreier E, Roggendorf M. Typing of hepatitis C virus isolates by DNA enzyme immunoassay. J Virol Methods 1994;48:81–91.
46. Mantero G, Zonaro A, Albertini A, Bertolo P, Primi D. DNA enzyme immunoassay: general method for detecting products of polymerase chain reaction. Clin Chem 1991;37:422–429.
47. Yu ML, Chuang WL, Dai CY, Chen SC, Lin ZY, Hsieh MY, Wang LY, Chang WY. Clinical evaluation of the automated COBAS AMPLICOR HCV MONITOR test version 2.0 for quantifying serum hepatitis C virus RNA and comparison to the quantiplex HCV version 2.0 test. J Clin Microbiol 2000;38:2933–2939.
48. Gerken G, Rothaar T, Rumi MG, Soffredini R, Trippler M, Blunk MJ, Butcher A, Soviero S, Colucci G. Performance of the COBAS

AMPLICOR HCV MONITOR test, version 2.0, an automated reverse transcription-PCR quantitative system for hepatitis C virus load determination. J Clin Microbiol 2000;38:2210–2214.
49. Martinot-Peignoux M, Boyer N, Le Breton V, Le Guludec G, Castelnau C, Akremi R, Marcellin P. A new step toward standardization of serum hepatitis C virus-RNA quantification in patients with chronic hepatitis C. Hepatology 2000;31:726–729.
50. Lee SC, Antony A, Lee N, Leibow J, Yang JQ, Soviero S, Gutekunst K, Rosenstraus M. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J Clin Microbiol 2000;38:4171–4179.
51. Pradat P, Chossegros P, Bailly F, Pontisso P, Saracco G, Sauleda S, Thursz M, Tillmann H, Vlassopoulou H, Alberti A, Braconier JH, Esteban JI, Hadziyannis S, Manns M, Rizzetto M, Thomas HC, Trepo C. Comparison between three quantitative assays in patients with chronic hepatitis C and their relevance in the prediction of response to therapy. J Viral Hepat 2000;7:203–210.
52. Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Comparison of 3 quantitative HCV RNA assays—accuracy of baseline viral load to predict treatment outcome in chronic hepatitis C. Scand J Infect Dis 1998;30:441–446.
53. Sarrazin C, Teuber G, Kokka R, Rabenau H, Zeuzem S. Detection of residual hepatitis C virus RNA by transcription-mediated amplification in patients with complete virologic response according to polymerase chain reaction-based assays. Hepatology 2000;32:818–823.
54. Saldanha J, Lelie N, Heath A, and the WHO Collaborative Study Group. Establishment of the first international standard for nucleic acid amplification technology (NAT) assays for HCV RNA. Vox Sang 1999;76:149–158.
55. Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu M, and the Collaborative Study Group. Calibration of HCV working reagents for NAT assays against the HCV international standard. Vox Sang 2000;78:217–224.
56. Saldanha J. Validation and standardisation of nucleic acid amplification technology (NAT) assays for the detection of viral contamination of blood and blood products. J Clin Virol 2001;20:7–13.
57. Pawlotsky JM, Bouvier-Alias M, Hezode C, Darthuy F, Remire J, Dhumeaux D. Standardization of hepatitis C virus RNA quantification. Hepatology 2000;32:654–659.
58. Glynn SA, Kleinman SH, Schreiber GB, Busch MP, Wright DJ, Smith JW, Nass CC, Williams AE. Trends in incidence and prevalence of major transfusion-transmissible viral infections in US blood donors, 1991 to 1996. Retrovirus Epidemiology Donor Study (REDS). JAMA 2000;284:229–235.
59. Stramer SL, Caglioti S, Strong DM. NAT of the United States and Canadian blood supply. Transfusion 2000;40:1165–1168.
60. Farci P, Alter HJ, Wong D, Miller RH, Shih JW, Jett B, Purcell RH. A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. N Engl J Med 1991;325:98–104.
61. Puoti M, Zonaro A, Ravaggi A, Marin MG, Castelnuovo F, Cariani E. Hepatitis C virus RNA and antibody response in the clinical course of acute hepatitis C virus infection. Hepatology 1992;16:877–881.
62. Hino K, Sainokami S, Shimoda K, Niwa H, Iino S. Clinical course of acute hepatitis C and changes in HCV markers. Dig Dis Sci 1994;39:19–27.
63. European Association for the Study of the Liver. EASL International Consensus Conference on Hepatitis C. Paris, 26-28, February 1999, Consensus Statement. J Hepatol 1999;30:956–961.
64. Pawlotsky JM, Lonjon I, Hezode C, Raynard B, Darthuy F, Remire J, Soussy CJ, Dhumeaux D. What strategy should be used for diagnosis of hepatitis C virus infection in clinical laboratories? Hepatology 1998;27:1700–1702.
65. Wejstal R, Widell A, Mansson AS, Hermodsson S, Norkrans G. Mother-to-infant transmission of hepatitis C virus. Ann Intern Med 1992;117:887–890.
66. Roudot-Thoraval F, Pawlotsky JM, Thiers V, Deforges L, Girollet PP, Guillot F, Huraux C, Aumont P, Brechot C, Dhumeaux D. Lack of mother-to-infant transmission of hepatitis C virus in human immunodeficiency virus-seronegative women: a prospective study with hepatitis C virus RNA testing. Hepatology 1993;17:772–777.
67. Ohto H, Terazawa S, Sasaki N, Hino K, Ishiwata C, Kako M, Ujiie N, Endo C, Matsui A, Okamoto H, Mishiro S. Transmission of hepatitis C virus from mothers to infants. N Engl J Med 1994;330:744–750.
68. Zanetti AR, Tanzi E, Newell ML. Mother-to-infant transmission of hepatitis C virus. J Hepatol 1999;31(suppl 1):96–100.
69. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.
70. National Institutes of Health Consensus Development Conference Panel statement: management of hepatitis C. Hepatology 1997;26(suppl):2S–10S.
71. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J, and the International Hepatitis Interventional Therapy Group (IHIT). Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426–1432.
72. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK, and the Hepatitis Interventional Therapy Group. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 1998;339:1485–1492.
73. Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J, and the ALGOVIRC Project Group. Is an "a la carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? Hepatology 2000;31:211–218.
74. Chemello L, Cavalletto L, Casarin C, Bonetti P, Bernardinello E, Pontisso P, Donada C, Belussi F, Martinelli S, Alberti A, and the TriVeneto Viral Hepatitis Group. Persistent hepatitis C viremia predicts late relapse after sustained response to interferon-alpha in chronic hepatitis C. Ann Intern Med 1996;124:1058–1060.
75. Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, Kilani A, Areias J, Auperin A, Benhamou JP, Degott C, Erlinger S. Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. Ann Intern Med 1997;127:875–881.
76. Lau DT, Kleiner DE, Ghany MG, Park Y, Schmid P, Hoofnagle JH. 10-Year follow-up after interferon-alpha therapy for chronic hepatitis C. Hepatology 1998;28:1121–1127.
77. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998;282:103–107.
78. Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, Colucci G, Roth WK. Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon alpha2a. Gastroenterology 2001;120:1438–1447.
79. Aspinall S, Steele AD, Peenze I, Mphahlele MJ. Detection and

quantitation of hepatitis B virus DNA: comparison of two commercial hybridization assays with polymerase chain reaction. J Viral Hepat 1995;2:107–111.
80. Krajden M, Minor J, Cork L, Comanor L. Multi-measurement method comparison of three commercial hepatitis B virus DNA quantification assays. J Viral Hepat 1998;5:415–422.
81. Chan HL, Leung NW, Lau TC, Wong ML, Sung JJ. Comparison of three different sensitive assays for hepatitis B virus DNA in monitoring of responses to antiviral therapy. J Clin Microbiol 2000;38:3205–3208.
82. Ho SK, Chan TM, Cheng IK, Lai KN. Comparison of the second-generation digene hybrid capture assay with the branched-DNA assay for measurement of hepatitis B virus DNA in serum. J Clin Microbiol 1999;37:2461–2465.
83. Niesters HG, Krajden M, Cork L, de Medina M, Hill M, Fries E, Osterhaus AD. A multicenter study evaluation of the digene hybrid capture II signal amplification technique for detection of hepatitis B virus DNA in serum samples and testing of EUROHEP standards. J Clin Microbiol 2000;38:2150–2155.
84. Poljak M, Marin IJ, Seme K, Brinovec V, Maticic M, Meglic-Volkar J, Lesnicar G, Vince A. Second-generation Hybrid capture test and Amplicor monitor test generate highly correlated hepatitis B virus DNA levels. J Virol Methods 2001;97:165–169.
85. Saldanha J, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. Vox Sang 2001;80: 63–71.
86. Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr., Lindsay K, Payne J, Dienstag JL, O'Brien C, Tamburro C, Jacobson IM, et al. A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. N Engl J Med 1990;323:295–301.
87. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657–1661.
88. Lok AS, Lai CL, Wu PC, Leung EK. Long-term follow-up in a randomised controlled trial of recombinant alpha 2-interferon in Chinese patients with chronic hepatitis B infection. Lancet 1988;2:298–302.
89. Lai CL, Lok AS, Lin HJ, Wu PC, Yeoh EK, Yeung CY. Placebo-controlled trial of recombinant alpha 2-interferon in Chinese HBsAg-carrier children. Lancet 1987;2:877–880.
90. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–1263.
91. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF, and the Asia Hepatitis Lamivudine Study Group. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–68.
92. Zollner B, Petersen J, Schroter M, Laufs R, Schoder V, Feucht HH. 20-fold increase in risk of lamivudine resistance in hepatitis B virus subtype adw. Lancet 2001;357:934–935.
93. Schalm SW. Clinical implications of lamivudine resistance by HBV. Lancet 1997;349:3–4.
94. Yao FY, Terrault NA, Freise C, Maslow L, Bass NM. Lamivudine treatment is beneficial in patients with severely decompensated cirrhosis and actively replicating hepatitis B infection awaiting liver transplantation: a comparative study using a matched, untreated cohort. Hepatology 2001;34:411–416.
95. Lau DT, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, Kleiner DE, Schmid P, Condreay LD, Gauthier J, Kuhns MC, Liang TJ, Hoofnagle JH. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000;32:828–834.
96. Villeneuve JP, Condreay LD, Willems B, Pomier-Layrargues G, Fenyves D, Bilodeau M, Leduc R, Peltekian K, Wong F, Margulies M, Heathcote EJ. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. Hepatology 2000; 31:207–210.
97. Benhamou Y, Bochet M, Thibault V, Di Martino V, Caumes E, Bricaire F, Opolon P, Katlama C, Poynard T. Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. Hepatology 1999;30:1302–1306.
98. Dienstag JL, Schiff ER, Mitchell M, Casey DE Jr., Gitlin N, Lissoos T, Gelb LD, Condreay L, Crowther L, Rubin M, Brown N. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. Hepatology 1999;30:1082–1087.
99. Tillmann HL, Trautwein C, Bock T, Boker KH, Jackel E, Glowienka M, Oldhafer K, Bruns I, Gauthier J, Condreay LD, Raab HR, Manns MP. Mutational pattern of hepatitis B virus on sequential therapy with famciclovir and lamivudine in patients with hepatitis B virus reinfection occurring under HBIg immunoglobulin after liver transplantation. Hepatology 1999;30:244–256.
100. Perrillo R, Rakela J, Dienstag J, Levy G, Martin P, Wright T, Caldwell S, Schiff E, Gish R, Villeneuve JP, Farr G, Anschuetz G, Crowther L, Brown N, and the Lamivudine Transplant Group. Multicenter study of lamivudine therapy for hepatitis B after liver transplantation. Hepatology 1999;29:1581–1586.
101. Ono-Nita SK, Kato N, Shiratori Y, Masaki T, Lan KH, Carrilho FJ, Omata M. YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: a study by in vitro full-length viral DNA transfection. Hepatology 1999;29: 939–945.
102. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714–717.
103. Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 2001;33:751–757.

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