Practical use of hepatitis C and B molecular tools : Belgian Guidelines

M. Adler[1], P. Goubau[2], G. Leroux-Roels[3], D. Sprengers[4], J.-M. Pawlotsky[5]

(1) Department of Gastroenterology and Hepatopancreatology, Hôpital Erasme, ULB, Brussels ; (2) AIDS Reference Laboratory, Université Catholique de Louvain, Brussels ; (3) Centre for Vaccinology, Ghent University and Hospital, Ghent ; (4) Division of Hepatogastroenterology, St Augustinus Ziekenhuis, Wilrijk ; (5) Department of Virology, INSERM U635, Hôpital Henri Mondor, Université Paris 12, Créteil, France.

Abstract

This article discusses the use of virologic assays in the diagnosis and management of hepatitis C virus (HCV) and hepatitis B (HBV)infection. The use of virologic tests has become essential in the management of HCV and HBV infection to diagnose viral infection, guide treatment decisions, and assess the virologic response to antiviral therapy. The continuing development of test systems accompanied by new antiviral drugs and novel therapeutic approaches should lead to an optimization of the treatment of HCV infection.

Molecular methods for viral testing have become an integral part of the diagnostic and therapeutic management of infections with hepatitis C virus (HCV) and hepatitis B virus (HBV). (Acta gastroenterol. belg., **2005**, 68, **308-313**).

Key words : hepatitis C virus, hepatitis B virus, HCV RNA quantification, DNA quantification, HCV markers, molecular diagnosis HCV, HBV.

I. Hepatitis C virus infection

Chronic HCV infection affects approximately 180 million subjects worldwide, i.e. 3% of the world population. It is recognized as a slowly progressive disease, culminating in cirrhosis in as many as 20% of cases after twenty of more years of infection, and in hepatocellular carcinoma in 1 to 4% per year of those with cirrhosis (1). It results in death in 1 to 3% of those infected and represents 30% of the indications of liver transplantation in Belgium (2).

A) *HCV virological tools*

1. HCV serological assays

The detection of anti-HCV antibodies in plasma or serum is based on the use of third-generation EIAs that detect mixtures of antibodies directed against various HCV epitopes located in the core, non-structural 3 (NS3), NS4 and NS5 proteins. The procedure can be fully automated and is well adapted to large volume testing. The optical density (OD) ratio of the reaction (sample OD/internal control OD) is proportional to the amount of antibodies in the serum or plasma sample. The sensitivity of third-generation EIAs is difficult to determine, given the lack of a gold standard method. It is excellent in non-immunodepressed patients with chronic hepatitis C, but false negatives can be observed in some haemodialysis patients and in patients with profound immune depression. In addition, antibodies may appear late in the course of acute hepatitis C. The specificity of third-generation EIAs for anti-HCV is greater than 99%. Among immunocompetent populations with a low anti-HCV prevalence (such as subjects undergoing systematic screening : volunteer blood donors, persons in the general population, health care workers), the proportion of false-positive results may be higher (3). In these cases, an OD ratio ≥ 3.8 is highly predictive of the presence of HCV RNA (4), whereas in the case of a low OD ratio, it is impossible to discriminate among a false positive result or a low antibody titer in a patient who previously recovered from acute hepatitis C.

Immunoblot assays, such as RIBA® (Chiron) or INNO-LIA®, have been used as confirmatory assays for anti-HCV reactivity identified by EIA (5). In case of indeterminate immunoblot assay result, another serum sample is requested after 1 month for repeated immunoblot assay or HCV RNA testing. Negative immunoblot assay is interpreted as a false-positive screening test result. Given the good performance of third-generation anti-HCV EIAs and the availability of molecular methods for HCV RNA detection, most laboratories have abandoned this approach. Some laboratories confirm the results of a first positive anti-HCV test by performing the same ELISA on a second sample obtained at a later moment from the same subject or use an alternative EIA on the same sample (6).

EIA-based serological assays can also be used to determine the HCV genotype, by seeking for antibodies directed to genotype-specific HCV epitopes in the NS4 protein (7). The assay (Murex HCV Serotyping 1-6 HC02, Abbott Laboratories, North Chicago, Illinois) is interpretable in approximately 90% of chronically infected immunocompetent patients and identifies the HCV type (1 to 6), but does not discriminate among the subtypes.

2. HCV molecular assays

Molecular techniques can be used on the one hand to determine the HCV genotype, on the other hand to detect and quantify HCV RNA.

Correspondence : M. Adler, Service Médico-Chirurgical de Gastro-Entérologie et d'Hépato-Pancréatologie, Hôpital Erasme, 808 route de Lennik, 1070 Brussels, Belgium.
E-mail : madler@ulb.ac.be.

The reference method for HCV *genotype* determination is direct sequencing of the NS5B or E1 regions of the HCV genome, which must be used in molecular epidemiology studies, where exact subtyping is mandatory. In clinical practice (7) HCV genotype can be determined by various commercial kits, using direct sequence analysis of the 5' noncoding region (Trugene® 5'NC HCV Genotyping Kit, Bayer HealthCare, Diagnostics Division, Tarrytown, New York) or reverse hybridization analysis by means of genotype-specific probes located in the 5' noncoding region (commercialized as INNO-LiPA HCV II, Innogenetics, Ghent, Belgium, or Versant® HCV Genotyping Assay, Bayer HealthCare). Mistyping is rare with these techniques, but mis-subtyping may occur in 10 to 25% of cases, related to the studied region. These errors have no clinical consequences, because only the type is used for therapeutic decision-making.

Qualitative HCV RNA detection assays are based on the principle of target amplification using either "classic" polymerase chain reaction (PCR), "real-time" PCR or transcription-mediated amplification (TMA). Qualitative detection assays must be sensitive, i.e. able to detect 50 HCV RNA international units (IU/ml) or less, and have equal sensitivity for the detection of all HCV genotypes. The lower limit of detection of the qualitative, non quantitative reverse-transcriptase PCR-based assay Amplicor® HCV v2.0, or of its semi-automated version Cobas® Amplicor® HCV v2.0 (Roche Molecular Systems, Pleasanton, California) is 50 IU/ml, whereas that of the TMA-based assay Versant® HCV RNA Qualitative Assay (Bayer HealthCare) is 10 IU/ml. Real-time PCR assays, which are also able to quantify HCV RNA, have lower limits of detection, of the order of 5-30 IU/ml.

The HCV RNA level can be *quantified* by means of target amplification techniques (competitive PCR or real-time PCR) or signal amplification techniques (branched DNA assay). "Home-made" assays must be avoided and commercially available standardized assays must be used in practice in order to ensure accurate HCV RNA quantification. Four assays are currently available. Two of them are based on competitive PCR : Amplicor HCV Monitor® v2.0 and its semi-automated version Cobas® Amplicor HCV Monitor® v2.0 (Roche Molecular Systems), and LCx® HCV RNA Quantitative Assay (Abbott Laboratories) ; the third one is based on third-generation bDNA technology, Versant® HCV RNA 3.0 Assay (Bayer Healthcare) ; and the fourth is based on Taqman real-time PCR amplification (Cobas® TaqMan from Roche and HCV Quant ASR from Abbott). These TaqMan assays are highly sensitive (8), have a broad dynamic range and provide precise quantification of viral load. A single test with sensitivity similar to the qualitative tests, that accurately quantify high viral load would be beneficial for clinical laboratories, if feasible for financial reasons.

B) *Practical use of HCV virological tools*

1. Diagnosis

Acute hepatitis

Seroconversion may be delayed by several weeks (usually 4-10 weeks) after exposure and therefore the diagnosis of acute hepatitis C must rely on the qualitative detection of HCV RNA by a sensitive test (lower limit of detection of 50 IU/ml), which may appear as early as 1-2 weeks after exposure (7), quickly followed by a gross elevation of transaminases (9).

Chronic hepatitis

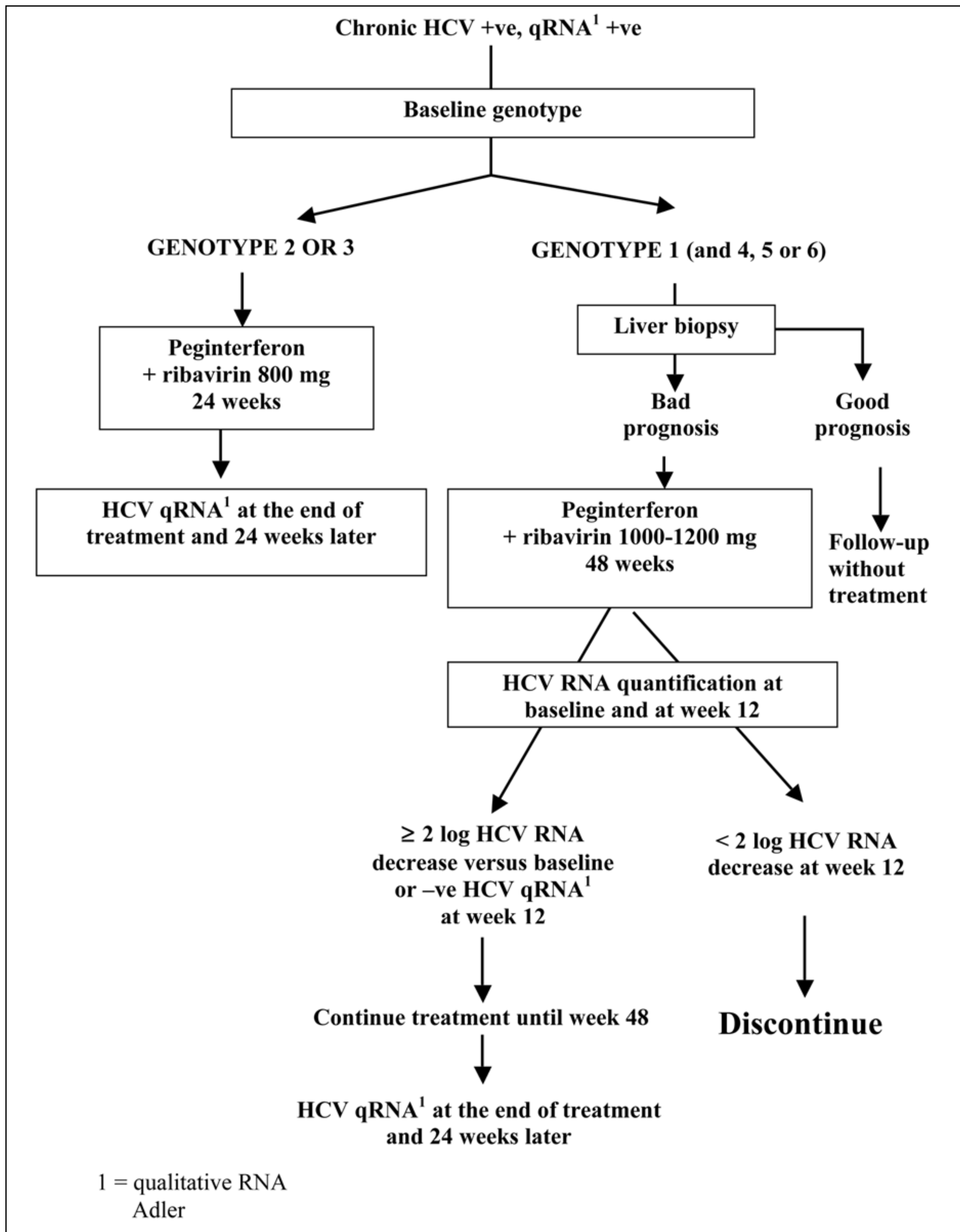
In patients with clinical or biological signs of chronic liver disease, chronic hepatitis C is certain when both anti-HCV antibodies and HCV RNA are present. In patients who have no indication for therapy or have a contra-indication to the use of antiviral drugs, virological tests have no prognostic value (10).

2. Management of antiviral therapy

The current standard treatment for chronic hepatitis C is the combination of pegylated interferon (IFN) alfa and ribavirin. The efficacy endpoint of hepatitis C treatment is the "sustained virological response" (SVR), defined by the absence of detectable HCV RNA in serum 24 weeks after the end of treatment. Only patients with detectable HCV RNA in a sensitive assay (> 50 IU/ml) should be considered for therapy. The HCV genotype should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure.

Patients infected with HCV *genotype 1* should receive a high dose of ribavirin, i.e. 1,000 to 1,200 mg daily (based on body weight less than or greater than 75 kg), and they require 48 weeks of treatment. HCV RNA quantification should be performed at baseline and after 12 weeks of treatment with the same technique (Table I). Treatment must be continued until week 48 when there is at least a 2-log drop in HCV RNA level, i.e. when baseline HCV RNA level is divided by 100 or more, or when qualitative HCV RNA is undetectable at week 12. This defines a positive early virological response. Despite having a $\geq$ 2 log drop in HCV RNA, some rare patients are still HCV RNA positive by PCR. They should be retested at week 24 and therapy is continued only if the result is negative. This practice, proposed by Davis *et al.* (11), does not appear in most consensus statements related to the management of hepatitis C (1,2,12) and is not included in the therapeutic algorithm proposed by the Belgian Commission for Reimbursement of drugs but it may be applicable for a small number of patients. The SVR must be assessed 24 weeks after the end of therapy. In the absence of a 2 log drop at week 12, treatment can be stopped, or continued with the only aim to slow down liver disease progression without clearing the virus (13,14). One should,

Table I. — **Viral testing algorithm for naive patients with chronic HCV treated by Peginterferon/Ribavirin**



however, be prudent to stop therapy when HCV RNA reduction is between 1.8 and 2.0 log. This "stopping rule", based on monitoring of HCV RNA load reduction at week 12, also applies to patients co-infected with HCV and human immunodeficiency virus (15).

Treatment-naïve, immunocompetent patients with genotype 1 can expect the following results, if treated according the algorithm described above : a SVR in 46 to 52%, a relapse (i.e. HCV RNA negative at the end of treatment and positive 24 weeks later) in 15% and non response (HCV RNA positive at the end of therapy) in 30 to 35% (16,17,18).

Patients infected with HCV *genotypes 2 or 3* should be treated with a low dose of ribavirin, i.e. 800 mg per

day, independent of body weight. No monitoring of HCV RNA level changes during therapy is recommended. Like in HCV genotype 1-infected patients, the SVR must be assessed by means of a sensitive HCV RNA assay 24 weeks after the end of therapy. This SVR occurs in 78-80% (16,17,18).

In the absence of any clinical trial including a sufficient number of patients, patients infected with HCV *genotypes 4, 5 or 6* should be treated like those infected with HCV genotype 1, i.e. with a high dose of ribavirin (1000-1200 mg per day, according to body weight less or greater than 75 kg). In the absence of published data, no stopping rules have been defined and it is recommended to treat these patients for a total of 48 weeks. The SVR must be assessed by means of a sensitive HCV RNA assay 24 weeks after the end of therapy.

II. Hepatitis B virus infection

Approximately 350 million people worldwide are infected with HBV. Although a significant number of them have inactive carriage, others will develop chronic hepatitis and 15 to 20% of them will progress to cirrhosis within 5 years. The annual incidence of hepatocellular carcinoma in the presence of cirrhosis is approximately 6% per year (19).

A) *HBV virological tools*

1. HBV serological assays

EIAs have been developed to detect both direct markers of infection (HBV antigens : HBs antigen and HBe antigen) and indirect markers of infection (anti-HBV antibodies : anti-HBs antibodies, total anti-HBc antibodies, anti-HBc IgM, anti-HBe antibodies. All of these techniques are both sensitive and specific. However, amino acid substitutions in the HBs antigen sequence can rarely lead to false negative results of HBs antigen detection. The new generations of assays have better sensitivity for HBs antigen mutants. In addition, total anti-HBc antibody detection assays have a poorer specificity than other HBV serological assays and may be falsely positive in patients who were never infected with HBV. These patterns can generally be resolved by the analysis of the other HBV markers in the same patient.

2. HBV molecular assays

Molecular techniques can be used on the one hand to detect and quantify HBV DNA levels, on the other hand to identify HBV variants (20).

Detection and quantification of HBV DNA can be based on the use of liquid hybridization (HBV Digene Hybrid-Capture®, Digene Corp., Gaithersburg, Maryland), competitive reverse-transcriptase PCR (Amplicor HBV Monitor® and the semi-automated Cobas® Amplicor HBV Monitor®, Roche Molecular Systems) ; branched DNA-based assay (Versant HBV DNA 3.0 Assay, Bayer HealthCare) and real-time PCR (Cobas TaqMan HBV Test, Roche Molecular Systems) that can be coupled with automated extraction with the Cobas Ampliprep®. These assays have been shown to be specific and accurate within their respective dynamic ranges of quantification. However, these ranges of quantification are very different from each other and generally poorly overlap. This makes it difficult to compare HBV DNA levels measured with different techniques. Laboratory values are now given in IU/ml, the number of copies is given by dividing the IU by 5 or 6 according to the method used[21]. In addition, the possible influence of the HBV genotype on quantification has not been extensively studied. Real-time PCR assays are those that offer the broadest dynamic range of quantification. The clinical value of low levels of HBV DNA in infected patients is often a matter of debate (see below).

The reference technique for the identification of HBV mutants remains sequencing of the region of interest. A line probe assay based on reverse hybridization has recently been developed (Innogenetics, Ghent, Belgium) to identify precore and core promoter mutants and determine the HBV genotype. The clinical relevance of these parameters is unknown and thus these assays still do not have an indication in clinical practice. Sequencing-based assays and reverse hybridization assays are also being developed to identify HBV DNA polymerase mutations associated with HBV resistance to anti-HBV drugs. The development of a large number of new drugs might bring usefulness for these assays to tailor therapeutic regimens.

B) *Practical use of HBV virological tools*

1. Diagnosis

No molecular biology–based assays are necessary for the diagnosis of *acute hepatitis B*, which is based on serological testing (presence of both HBs antigen and anti-HBc IgM, or isolated anti-HBc antibodies with the presence of anti-HBc IgM at the recovery phase).

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. When HBV DNA is detected, the quantitative result should be interpreted according to 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. In contrast, the interpretation of HBV DNA quantification is difficult in *HBeAg-negative/anti–HBeAg-positive patients (possible pre-core HBV mutant)*, 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 or 2×10^4 IU/ml is

associated with an "inactive carrier state", whereas higher HBV DNA loads should be considered as clinically significant. In fact, the best discriminatory threshold remains to be established and a "gray zone" between 10^3 and 10^5 copies/mL remains in order to discriminate between inactive carriers and patients with chronic hepatitis B.

2. Management of antiviral therapy

HBV DNA monitoring is a key tool for treatment monitoring in chronic hepatitis B. The decision to treat patients with chronic hepatitis B must be taken individually, on the basis of precisely weighted parameters, including serum ALT activity, liver disease severity assessment, and HBV DNA levels that allow to discriminate inactive from active carriers. There is no consensus on the first-line treatment of chronic hepatitis B. However, it seems reasonable to restrict the administration of pegylated IFN alfa to HBe antigen-positive patients with ALT levels higher than twice the upper limit of normal and a "low" viral load (but no precise threshold is known), whereas the remaining patients and those who failed to achieve a seroconversion upon pegylated IFN therapy should be treated, probably for their full life if they have an indication for therapy, by specific HBV inhibitors such as lamivudine, adefovir or entecavir (22,23,24).

HBV DNA quantification, together with repeated ALT determinations and HBeAg/anti-HBe antibody assessments in HBeAg-positive patients, is critical in treatment monitoring. HBV DNA testing should be performed every 6 months to detect HBV resistance. The virological response is defined by a decrease of HBV DNA of at least 1 log (HBV DNA level divided by at least 10) relative to baseline. The magnitude of the response is measured by the number of logs of the HBV DNA decrease. HBe antigen-positive patients may achieve an HBe seroconversion, defined by the disappearance of HBe antigen and the appearance of anti-HBe antibodies. These patients may, rarely, achieve an HBs seroconversion.

In patients on long-term suppressive therapy with these specific HBV inhibitors, HBV resistance may occur, at various frequencies and dates according to the treatment schedule. This is particularly the case with lamivudine and more rarely with adefovir (25). HBV resistance is defined by a reincrease of HBV DNA level of at least 1 log compared to the nadir which is sustained on at least 2 successive determinations in a patient who remained compliant. Selection of drug-resistance mutations is accompanied by virologic breakthrough and in some patients by biochemical breakthrough, rarely hepatic failure and death. For patients with confirmed lamivudine resistance, options include continuing lamivudine as long as benefit to the patient (based on clinical assessment, ALT and HBV DNA levels) or discontinuing lamivudine with either monitoring hepatic flares, switch to other antiviral agent such as adefovir or maintaining the lamivudine and adefovir for a few months and then stopping lamivudine for patients with underlying cirrhosis or those immunosuppressed.

Conclusion

Virological tests have become invaluable in the management of both HCV and HBV infections to diagnose infection, guide treatment decisions and assess the virological response to therapy. Improvement in the performance of the assays, together with the development of new antiviral drugs and novel therapeutic approaches, will allow major progresses in the treatment of these infections.

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