

## MINIREVIEW

# Laboratory Assays for Diagnosis and Management of Hepatitis C Virus Infection

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Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to the family *Flaviviridae* (6). HCV was initially recognized as non-A, non-B hepatitis virus (NANBH) in 1974 until cloning of the etiologic agent in 1989 (2, 10). The global prevalence of HCV infection is approximately 3% (170 million people) (21). In the United States, approximately 3.9 million people (1.8% of the population) are HCV seropositive (19).

The primary mode of HCV transmission is exposure to infected human blood via intravenous drug use or unscreened transfusions (21). The practice of screening donors for HCV antibodies in developed countries since 1990 has substantially lowered the risk of acquiring HCV infection from a transfusion to approximately 1 in 263,000 (39). This is based on results from nucleic amplification testing (NAT) of pooled donor samples that has been performed in North America since March 1999, in which by July 2000, 62 donations from 16.3 million seronegative donors were identified by NAT to be positive for HCV (39). Nosocomial HCV transmission during dialysis, colonoscopy, and surgery has also been reported (21). The rate of HCV seroconversion among health care workers after a needlestick injury is 0 to 7% (9). Perinatal and sexual transmission of the virus is inefficient, but occurs more frequently if the HCV-infected mother or sexual partner is also infected with human immunodeficiency virus type 1 (HIV-1) (9, 43).

Most people with acute HCV infection are asymptomatic or have mild symptoms (fatigue, nausea, jaundice) but are unable to clear the virus, leading to chronic infection in approximately 80% of cases (21). Chronic HCV infection progresses at a variable rate to cirrhosis in 15 to 20% of patients, who then have a 1 to 4% annual risk of developing hepatocellular carcinoma (21). HCV-associated end-stage liver disease is the leading indication for liver transplantation in American adults (19).

Screening of the general population for HCV infection is not recommended. In addition to blood donors, diagnostic testing should be performed for individuals with risk factors for HCV infection who may need medical care (9). Detailed recommendations for identifying those individuals have been outlined by the Centers for Disease Control and Prevention (9).

Although advances have been made, a reliable culture system for HCV is not yet available (6). Laboratory assays that are available for the diagnosis and management of HCV infection

include (i) serologic tests to detect HCV antibodies, (ii) molecular tests to detect and quantitate HCV RNA, and (iii) genotyping techniques. Assays to detect and quantify HCV core antigen have also been developed. Performance characteristics and clinical use of these assays will be discussed.

### SEROLOGIC ASSAYS

**Screening EIAs.** The initial test used to diagnose HCV is an enzyme immunoassay (EIA) for anti-HCV immunoglobulin G (IgG). The HCV genome encodes a polyprotein of 3,011 to 3,033 amino acids that is processed into 10 structural and nonstructural (NS) proteins (6). Three generations of screening EIAs have been developed to detect antibodies against various epitopes of these proteins (Fig. 1).

First-generation EIAs (EIAs 1.0) used the c100-3 epitope of an NS protein (NS4) (20). The sensitivities of these EIAs were low for a high-prevalence population (approximately 80%), and the fraction of positive results that were false positive was as high as 70% for a low-prevalence population (blood donors) (15). This led to the development of more sensitive and specific second-generation EIAs (EIAs 2.0) that incorporated additional antigens from NS (c33c) and structural (c22-3) proteins that were approved for use by the Food and Drug Administration (FDA) in 1992. Second-generation assays detect HCV antibodies in 20% more patients with acute NANBH and in 10% more patients with chronic cases of infection than EIAs 1.0 do and detect HCV antibodies 30 to 90 days sooner than EIAs 1.0 do (3). The mean window of seroconversion was reduced from 16 weeks with EIAs 1.0 to 10 weeks with EIAs 2.0 (15). The sensitivities of EIAs 2.0 in a high-prevalence population are approximately 95% (based on HCV RNA detection by PCR) (15).

In 1996, FDA approved a third-generation EIA (EIA 3.0) that added a fourth antigen (NS5) to those in EIAs 2.0. EIA 3.0 detected antibodies an average of 26 days earlier in 5 of 21 individuals with transfusion-transmitted HCV (4), and the sensitivity is slightly better than that of EIA 2.0 in a high-prevalence population (as high as 97%) (18). Both the Abbott HCV EIA (version 2.0; Abbott Laboratories, Abbott Park, Ill.) and the Ortho HCV enzyme-linked immunosorbent assay (ELISA; version 3.0; Ortho-Clinical Diagnostics, Inc., Rochester, N.Y.) are used in the United States.

**Confirmatory anti-HCV assays.** Chiron Corporation (Emeryville, Calif.) developed a strip immunoassay to help resolve true-positive from false-positive EIA results. FDA approved

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Assay	HCV Polyprotein <sup>a</sup>						
	Core	E1	E2/NS1	NS2	NS3	NS4	NS5
<b>ORTHO ELISA<sup>b</sup></b>							
1 <sup>st</sup> generation					c100-3 (a.a.1569-1931)		
2 <sup>nd</sup> generation	c22-3 (a.a.2-120)				c200 (a.a.1182-1931)		
3 <sup>rd</sup> generation	c22-3 (a.a.2-120)				c200 (a.a.1182-1931)		NS5 (a.a.2054-2995)
<b>CHIRON SIA<sup>c</sup></b>							
3 <sup>rd</sup> generation	c22p <sup>d</sup> (a.a.10-53)				c33c (a.a.1192-1457)	5-1-1p <sup>d</sup> (a.a.1694-1735)	NS5 (a.a.2054-2995)
						c100p <sup>d</sup> (a.a.1920-1935)	
<b>ABBOTT EIA<sup>e</sup></b>							
2 <sup>nd</sup> generation	HC-34 (a.a.1-150)				HC-31 (a.a.1192-1457 and 1676-1931)		
						c100-3 (a.a.1569-1931)	

FIG. 1. HCV antigens used for serologic assays. a, E, envelope; NS, nonstructural protein; a.a., amino acid sequence of recombinant protein or synthetic peptide antigen. b, Ortho HCV ELISA (version 3.0; Ortho-Clinical Diagnostics, Inc.). c, Chiron RIBA HCV strip immunoassay (SIA; version 3.0; Chiron Corporation). d, p, synthetic peptide. e, Abbott HCV EIA (version 2.0; Abbott Laboratories).

the second-generation recombinant immunoblot assay (RIBA) in 1993, followed by approval of the third-generation RIBA in 1999. The strip immunoassays include the EIA antigens and human superoxide dismutase (hSOD). Because the recombinant antigens c33c and NS5 used for EIA are made as fusion proteins with hSOD, recombinant hSOD is included on the RIBA strip to detect nonspecific antibodies. RIBA is considered positive if there are reactions with at least two antigens with intensities greater than or equal to that for the weak IgG control and no reactivity with hSOD. Indeterminate RIBAs are those in which there are reactions with only one antigen or the hSOD plus one or more HCV antigens. Replacement of the c100 and c22 recombinant proteins with synthetic peptides in the version 3.0 RIBA has significantly reduced the number of indeterminate RIBA results (31, 32).

**Use of serologic assays.** Of the 25 to 35% of patients with acute infection who develop symptoms, only 50 to 70% will have detectable antibodies at that time, but 90% will have measurable antibodies after 3 months (29). Serologic assays detect HCV antibodies that indicate present or previous infection, but they cannot discriminate acute from chronic or resolved infection. Anti-HCV IgM antibodies can be detected in 50 to 93% of patients with acute HCV infections and 50 to 70% of chronic cases, so they are not a reliable indicator of acute infection (30).

Confirmation by RIBA is needed only for low-risk patients (healthy blood donors) or if a high-risk patient is HCV RNA negative (25, 33). Confirmation by RIBA has not been very useful for resolving weakly positive samples (optical density ratios between 1 and 2), and molecular HCV RNA detection is recommended instead (33). Individuals with indeterminate RIBA results should be evaluated by a sensitive HCV RNA detection test (29).

Patients with acute hepatitis of uncertain origin and negative hepatitis serology panels should undergo qualitative HCV RNA testing (12). Occasionally, immunocompromised patients, patients undergoing hemodialysis, and patients with mixed cryoglobulinemia have false-negative serology results and may require HCV RNA testing for diagnosis (13, 21, 42). Passively transferred maternal anti-HCV antibodies may be detected in the children of HCV-infected mothers for up to 1 year; however, defined diagnostic criteria for HCV RNA detection are not available (9, 43).

#### HCV RNA DETECTION AND QUANTITATION

The presence of HCV RNA in plasma defines active infection, and HCV RNA can be detected 1 to 3 weeks postexposure (29). A single negative HCV RNA assay result does not

TABLE 1. Commercially available HCV RNA detection assays<sup>a</sup>

Test	Method	Manufacturer <sup>a</sup>	Lower limit of detection (IU/ml)	Linear range of quantification (IU/ml)	Primary application
Qualitative					
AMPLICOR HCV test, version 2.0	RT-PCR	Roche	50		Evidence of active infection and response to therapy
VERSANT HCV RNA qualitative assay	TMA	Bayer	5		Evidence of active infection and response to therapy
Ampliscreen HCV test, version 2.0	RT-PCR	Roche	<50		Blood screening
Procleix HIV-1/HCV assay	TMA	Chiron	<50		Blood screening
Quantitative					
AMPLICOR HCV MONITOR, version 2.0	RT-PCR	Roche	600	600–500,000	Determination of viral load and length of therapy
VERSANT HCV RNA assay, version 3.0	bDNA	Bayer	615	520–8,300,000	Determination of viral load and length of therapy

<sup>a</sup> Roche, Roche Diagnostics; Bayer, Bayer Diagnostics; Chiron, Chiron Corporation.

exclude the possibility of active infection with a transient drop in the level of viremia below the assay's limit of detection (29).

Laboratories detect HCV RNA with commercially available assay kits (Table 1) or by in-house home-brewed methods. Because of the limited amount of HCV RNA in infected individuals, a target or signal amplification step is needed. Reverse transcriptase (RT) PCR (RT-PCR) and transcription-mediated amplification (TMA) are target amplification methods. The branched DNA (bDNA) assay is a signal amplification technique.

For RT-PCR, an RT step converts RNA to cDNA, which is used as a template for the PCR (41). Primers whose sequences correspond to the 5' untranslated region (5' UTR) are commonly used because this is the most conserved region of the genome (8). The Roche AMPLICOR HCV test (Roche Diagnostics, Branchburg, N.J.) includes 37 amplification cycles followed by hybridization to an HCV-specific oligonucleotide probe. The semiautomated version of the AMPLICOR HCV test uses the COBAS instrument to reduce the hands-on time required for detection and calculation by the technologist (1). The qualitative AMPLICOR HCV test (version 2.0) received FDA approval in 2001 and has a lower limit of detection of 50 IU/ml (23). The less sensitive quantitative HCV RNA assays lack FDA approval and are available only for research purposes.

TMA involves a more complex set of reactions with T7 RNA polymerase and RT under isothermal conditions to form detectable levels of RNA (41). TMA uses primers that contain a T7 RNA polymerase binding site so that RT synthesizes cDNA that becomes a template from which T7 RNA polymerase can synthesize numerous copies of RNA. The RNA amplicons reenter the TMA cycle and become templates for the next replication cycle. The TMA-based VERSANT HCV RNA qualitative assay (Bayer Diagnostics, Tarrytown, N.Y.) is not approved by FDA, but it is able to detect very low levels of HCV RNA (5 IU/ml) that are undetectable with RT-PCR systems (37, 38). The Procleix HIV-1/HCV assay also uses TMA technology and was approved by FDA in February 2002 for the screening of blood donations to identify HCV-positive donors who are antibody negative (17). A PCR-based blood

screening assay (Ampliscreen HCV Test, version 2.0) is also expected to attain FDA approval.

The bDNA method of RNA detection uses solid-phase oligonucleotide probes that capture target RNA, followed by hybridization of a branched secondary (bDNA) probe (41). The bDNA amplifiers bind to enzyme-conjugated tertiary probes, and after substrate is added, the chemiluminescence produced is proportional to the amount of target RNA. The VERSANT HCV RNA assay (version 3.0; Bayer Diagnostics, Tarrytown, N.Y.) is a semiautomated quantitative bDNA test with a sensitivity better than that of the previous manual Quantiplex HCV RNA assay (version 2.0) that is attributed to improved probe numbers and an improved design. There is also a lower level of background activity due to redesigned label extenders and the use of nonnatural synthetic nucleotides. A study comparing the COBAS AMPLICOR HCV MONITOR assay (version 2.0) to the VERSANT HCV RNA assay (version 3.0) reported a correlation coefficient of 0.941 (5). It was reported that the AMPLICOR test had a higher level of quantitation than the VERSANT assay for samples with 500 to 100,000 IU/ml and underestimated the amount of RNA in specimens with >100,000 IU/ml (5). Another comparative study reported overall substantial agreement between the results of the VERSANT and AMPLICOR assays but reported significant differences when the assays were used to determine whether the quantity was above or below the 800,000-IU/ml threshold that has been proposed for adjustment of the length of treatment with combination agents (14).

Because of the higher sensitivities of commercially available qualitative assays in comparison to those of quantitative assays (Table 1), the value of quantitative assays has been limited to pretreatment evaluations. Commercial assays no longer report results in numbers of copies of RNA per milliliter, which represent different amounts of RNA, depending on the assay. The World Health Organization international standard has provided a common unit of measure that allows comparison between results from different assays (35). Qualitative assays should be used to confirm viremia and assess the therapeutic response until quantitative assays with comparable sensitivities are available.

## GENOTYPING

On the basis of its extensive genetic heterogeneity, HCV has been divided into six major genotypes (represented by Arabic numerals) and at least 80 subtypes (represented by lowercase letters) (11). Different genotypes share approximately 65% sequence homology (11). Genotypes 1, 2, and 3 are found throughout the world; but the other genotypes are common in particular geographic regions (genotype 4 is common in North Africa and the Middle East, genotype 5 is common in South Africa, and genotype 6 is common in Southeast Asia) (44). The predominant genotype in patients with chronic HCV infection in the United States is genotype 1 (72% of patients), followed by genotype 2 (16%) and genotype 3 (10%) (28).

Molecular and serologic methods may be used to determine HCV genotypes. Commercial assays are available, but none are approved by FDA. The predominant molecular techniques used (hybridization and direct DNA sequencing) are based on nucleotide differences in the highly conserved 5' UTR among genotypes.

The widely used INNO-LiPA HCV II assay (Innogenetics, Ghent, Belgium) uses PCR products from the 5' UTR that hybridize to type-specific probes embedded on a nitrocellulose strip (40). The Trugene HCV 5' noncoding region genotyping kit (Visible Genetics, Inc., Toronto, Ontario, Canada) determines genotypes by direct sequencing of the 5' UTR (16). The Trugene and INNO-LiPA assays can be performed with amplification products obtained from the Roche AMPLICOR assays, and a recent comparison reported that the two assays have similar abilities to determine genotypes (16). The two assays have difficulty differentiating subtypes, with accuracies of 76 and 74%, respectively, compared to that of the "gold standard" method that sequences a different region of the genome, NS5B (16). Since management decisions have been made on the basis of genotype, not subtype, the overall accuracies of both assays have been considered acceptable.

Serologic methods rely on the detection of antibodies to genotype-specific epitopes in the NS4 or core region. Compared to molecular assays, serologic genotyping is easier to perform and less expensive, but it has lower sensitivity and specificity (22, 34).

## THERAPY

The results of HCV RNA detection and genotyping assays have been incorporated into algorithms that can be used to make management decisions, but guidelines must be reassessed as new therapeutic agents become available. Indications for the treatment of patients with chronic HCV infection include detectable HCV, increased alanine aminotransferase levels, and histologic evidence of liver fibrosis and moderate inflammation (29). The efficacy of therapy is measured by whether a sustained virologic response (SVR) occurs. SVR is defined as undetectable HCV RNA 24 weeks after the cessation of treatment (12). Treatment for chronic HCV infection has evolved from interferon monotherapy, which results in a disappointing SVR rate of 10 to 20% (29), to combination therapy with interferon plus ribavirin, which is associated with a higher SVR rate of nearly 40% (27, 36). Optimal therapy is now considered to be weekly pegylated interferon plus daily

ribavirin, with SVRs reported in more than half of the patients treated (SVR rate, 54%) (26). Infection with a non-genotype 1 strain has been identified as the strongest independent predictor of SVRs for all of these therapeutic regimens (24).

The duration of standard interferon plus ribavirin therapy has been based on the viral genotype and the pretreatment viral load. The SVR rates for patients infected with genotype 2 or 3 are essentially the same for 24 and 48 weeks of therapy, showing no benefit for the longer course of therapy (28, 36). In 1999, an international consensus panel recommended interferon plus ribavirin therapy for 24 weeks in patients infected with genotype 2 or 3 isolates (12). For patients infected with genotype 1 isolates, the panel recommended 48 weeks of interferon plus ribavirin therapy for those with a high pretreatment viral load (>800,000 IU/ml) and only 24 weeks of therapy for patients with those with a low pretreatment viral load (<800,000 IU/ml) (12, 35). A recent 48-week trial of pegylated interferon plus ribavirin reported a 42% SVR rate for patients with genotype 1 infections and an 82% SVR rate for patients with genotype 2 and 3 infections (26). Trials are under way to assess whether the duration of pegylated interferon therapy can be reduced on the basis of genotype and pretreatment viral load.

The more sensitive qualitative HCV RNA assays are recommended to make an early prediction of whether SVR will ultimately be achieved if patients complete the full course of therapy. For patients receiving interferon monotherapy, American and European consensus conferences recommended the cessation of treatment for patients with detectable HCV RNA after 12 weeks of therapy (12, 29). For patients receiving interferon plus ribavirin, week 24 is considered the most accurate time to assess the response and consider the cessation of therapy, since 10% of patients with detectable HCV RNA at 12 weeks will attain an SVR (27). A recent analysis of patients participating in three trials of pegylated alpha-2a interferon suggested that a decision to stop treatment could be made as early as week 12 if there is detectable HCV RNA or a <2-log<sub>10</sub> drop in RNA levels (24).

## ANTIGEN DETECTION

An accurate and specific ELISA for the detection and quantitation of HCV core antigen (Total HCV Core Ag assay; Ortho-Clinical Diagnostics, Rochester, N.Y.) has recently been developed (7). The assay has better sensitivity than an earlier version because of an immune complex dissociation step prior to antigen detection with a monoclonal antibody (7). The performance of the assay correlates well with those of molecular HCV RNA detection methods, but the lower level of detection (20,000 IU/ml) is significantly higher (7). A more sensitive assay is under development. Antigen detection may be useful in regions where the cost of molecular HCV testing has been prohibitive.

## SUMMARY

It was noted in 1974 that 75% of cases of transfusion-associated hepatitis were caused by NANBH, but 15 years passed before advances in molecular biology led to the cloning of the NANBH agent and anti-HCV assays were developed (2). In a

little more than a decade, our knowledge of and ability to diagnose and treat HCV infections have increased dramatically. In 1990, mass screening of blood donors was implemented in the United States and was estimated to have prevented 40,000 HCV infections in the first year (2). Improvements in EIAs and the performance of NAT on donations prior to transfusion have further lowered the incidence of transfusion-transmitted HCV (39).

Although the incidence of new HCV infections in the United States has declined, the population of individuals infected for  $\geq 20$  years who are at risk for serious complications is projected to increase until about 2015 (19). Improvements in therapy have resulted in better virologic response rates, and molecular HCV tests have proved useful in making management decisions. As new therapeutic options evolve and laboratory assays change, the clinical relevance and use of laboratory testing for HCV will require frequent reassessments. Consensus conferences that include experts from a variety of disciplines provide an ideal environment where these issues can be debated (12, 29).

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## ERRATUM

### Laboratory Assays for Diagnosis and Management of Hepatitis C Virus Infection

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- Volume 40, no. 12, p. 4407–4412, 2002. Page 4407, column 2, line 5 from bottom: “Oviedo, Fla.” should read “Rochester, N.Y.”  
Page 4408, column 1, line 8 from bottom: “0%” should read “90%.”  
Page 4408, column 1, line 2 from bottom: “patients with” should be deleted.  
Page 4408, column 2, line 17: “by using” should read “for.”  
Page 4409, Table 1, footnote *a*: “Bayer Corporation (Tarrytown, N.Y.)” should read “Bayer Diagnostics.”  
Page 4409, column 1, line 7 from bottom, and column 2, line 10: “Emeryville, Calif.” should read “Tarrytown, N.Y.”  
Page 4409, column 2, line 15 from bottom: “80,000-IU/ml” should read “800,000-IU/ml.”  
Page 4410, line 10 of Genotyping: “infecting” should read “in.”  
Page 4410, column 2, line 3 of Antigen Detection: “Ortho Diagnostics, Raritan, N.J.” should read “Ortho-Clinical Diagnostics, Rochester, N.Y.”  
Page 4411, column 2, reference 24: “Prognostic” should read “2002. Prognostic” and “J. Hepatol., in press” should read “J. Hepatol. 37:500–506.”