Laboratory Diagnostics for Hepatitis C Virus Infection

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Identification of prevalent infection by hepatitis C virus (HCV) is based serologically on detecting anti-HCV immunoglobulin G, using immunoassays, immunoblot assays, and, more recently, immunochromatography-based rapid tests. None discriminate between active and resolved HCV infection. Tests for detecting HCV RNA identify active HCV infection but are costly. Serologic assays for HCV antigens have been developed and show potential for diagnosis of active HCV infection, and their performance characteristics are undergoing evaluation. The diagnosis of acute HCV infection without the demonstration of seroconversion remains elusive.

Hepatitis C virus (HCV) is a positive-strand RNA virus belonging to the *Hepacivirus* genus in the family Flaviviridae [1]. Its 9.6-kb-long viral genome is flanked by 2 untranslated regions at its 5′ and 3′ ends and contains a single open reading frame that encodes a polyprotein of approximately 3000 amino acids. The polyprotein is cleaved into 10 single proteins by a host signal peptidase in the structural region and by viral-encoded proteases in the nonstructural region. Structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. Nonstructural proteins include the p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins. Owing to robust viral replication, an estimated 10 trillion virion particles can be produced per day during the active phase of infection [2]. HCV isolates are classified into 6 genotypes that differ in their nucleotide sequence by 30%–35% and into multiple subtypes that differ in their nucleotide sequence by 20%–25% [3]. Genotypes 1a and 1b are the most prevalent genotypes in the United States and western Europe, followed by genotypes 2 and 3. By contrast, genotype 4 is common in Egypt, genotype 5 in South Africa, and genotype 6 in Southeast Asia.

Following the cloning of the HCV genome, antigenic regions and B-cell epitopes were identified [4]. Recombinant proteins and synthetic peptides containing these immunodominant epitopes were used as antigens in immunodiagnostic assays, leading to the development of commercially available screening and supplemental assays for anti-HCV immunoglobulin G (IgG) [5, 6]. Recently, a rapid anti-HCV IgG assay was approved by the Food and Drug Administration (FDA) for clinical use in the United States [7]. These assays, however, cannot identify whether an antibody-positive person has active HCV infection, since anti-HCV IgG may be detectable in persons who have resolved infection and are no longer viremic.

Nucleic acid testing (NAT) for the detection of HCV RNA remains the gold standard for diagnosing active HCV infections. However, the laboratory setup for performing NAT requires expert technical staff, expensive equipment and reagents, dedicated procedure areas, and availability of pristine serum or plasma samples. Because of these requirements, NAT is not routinely performed in many clinical laboratories. Availability of a serologic assay not based on NAT but indicative of active infection should further facilitate identification of HCV-infected patients and enable referral to care.
DETECTION OF ANTI-HCV IgG

Immunoassays
A number of immunoassays have been developed to detect anti-HCV IgG in serum or plasma specimens. First-generation assays were based on a yeast-expressed recombinant protein containing an epitope from the NS4 region (C100-3) of the HCV genome. Although these assays identified anti-HCV IgG in approximately 80% of patients with posttransfusion hepatitis and led to the substantial reduction in transfusion-associated HCV infections, they lacked sensitivity and specificity [9]. Second- and third-generation assays used a multiantigen format and included antigens from the core, NS3, and NS4 regions (Figure 1); these modifications markedly improved sensitivity and specificity [6]. The difference between the second- and third-generation assays is the inclusion of an additional antigen from the NS5 region [10]. These assays reduced the window period observed in first-generation assays by an average of 5 weeks and permitted anti-HCV to be detected as early as 10 weeks after exposure. The diagnostic specificity of third-generation assays is >99% [11]. Nonetheless, third-generation enzyme immunoassays (EIAs) can yield false-negative results in patients who are undergoing hemodialysis or who are immunocompromised [12]. Furthermore, these assays can yield low positive predictive values among populations with a low (<10%) prevalence of HCV infection [13]. False-positive results can have unintended consequences for the tested person, resulting in psychological harm and unnecessary medical visits. The US Centers for Disease Control and Prevention (CDC) recommended that a person be considered to have serologic evidence of HCV infection if a positive result of anti-HCV screening is confirmed by positive results of a further test, using either a recombinant immunoblot antibody assay (RIBA) or NAT to detect HCV RNA. However, despite these recommendations, reflex supplemental testing has not been widely performed by many laboratories for various reasons, which include complexity of the assays, long turnaround time of test results, and high cost. The CDC issued another set of guidelines that expand earlier recommendations by including an option to use signal-to-cutoff ratios to limit the number of samples needing supplemental testing [14]. These guidelines were based on a study of serum samples (n = 25,000) from US populations with an anti-HCV IgG prevalence ranging from 0.8% to 25%, using FDA-licensed or approved anti-HCV screening kits (enzyme-linked or chemiluminescence immunoassays), RIBA, and NAT. The study established specific signal-to-cutoff ratios for several assays that predicted a true antibody positive result >95% of the time, regardless of the anti-HCV prevalence or characteristics of the population tested [14]. The evaluated assays and their specific signal-to-cutoff ratios are listed in Table 1. It is important to note that these signal-to-cutoff ratios are assay specific and cannot be applied to assays that have not been evaluated.

Recombinant Immunoblot Assays
RIBAs such as RIBA-3 (Chiron) and strip immunoblot assays, including INNO-LIA Ab III (Innogenetics), DECISCANT HCV (Sanofi-Pasteur), and Lia Tek HCV (Organon), are intended for use as additional, more specific tests for detection of anti-HCV IgG in serum or plasma specimens that have been found reactive in anti-HCV screening assays. Detection of anti-HCV by RIBA is based on immobilization of HCV recombinant antigens and synthetic peptides from core, NS3, and NS5 proteins (Figure 1) as individual bands onto a

| Table 1. Signal-to-Cutoff Ratios Established for Food and Drug Administration–Approved, Commercially Available Screening Assays for Detecting Anti–Hepatitis C Virus Immunoglobulin G |
|---|---|---|---|
| **Assay** | **Manufacturer** | **Method** | **Ratio** |
| Ortho HCV Version 3.0 EIA | Ortho | EIA (manual) | ≥3.8 |
| Abbott HCV EIA 2.0 | Abbott | EIA (manual) | ≥3.8 |
| VITROS Anti-HCV | Ortho | CIA (automated) | ≥8.0 |
| AxSYM Anti-HCV | Abbott | MEIA (automated) | ≥10.0 |
| ARCHITECT Anti-HCV | Abbott | CMIA (automated) | ≥5.0 |
| Advia Centaur HCV | Siemens | CIA (automated) | ≥11.0 |

Ratios are predictive of true-positive results >95% of the time. Abbreviations: CIA, chemiluminescence immunoassay; CMIA, chemiluminescent microparticle immunoassay; EIA, enzyme immunoassay; MEIA, microparticle enzyme immunoassay.
membrane. Reactivity with ≥2 proteins indicates a positive result; the test result is considered to be indeterminate if reactivity to only 1 protein is detected. Indeterminate results may be due to nonspecific cross-reacting antibodies or indicative of recent infection that has not yet triggered a broad humoral response. In HCV-infected individuals, assay results are generally indeterminate during the first week of infection and become fully positive 1–6 months later [15]. False-negative RIBA results may be observed in immunocompromised patients or in individuals who have resolved infection in whom anti-HCV levels are waning [14]. The advantages of RIBAs are that they are serologic assays and can be performed on the same sample used in the screening assay and that they are highly specific. However, these assays are not amenable to routine use, in view of their relative decreased sensitivity, high cost, hands-on complexity, and long duration of the procedure. Furthermore, a true-positive RIBA result only indicates the presence of anti-HCV and may reflect past infection with spontaneous clearance; confirmation of active infection still requires testing for HCV RNA.

**Rapid Assays**

Despite the excellent sensitivity and specificity of third-generation EIAs, the turnaround time for reporting test results is at least 1 day, thereby making it difficult to deliver the results to tested individuals at first visit. Rapid tests are formatted such that they do not require complicated instrumentation or testing by skilled technical staff. They potentially generate results within an hour and therefore may be used for point-of-care testing. The CDC recently completed evaluation of 3 rapid tests for detecting anti-HCV IgG (OraSure, Chembio, and Medmira) in laboratory and field settings. These assays, which are based on recombinant antigens derived from core, NS3, NS4, and NS5 proteins in an immunochromatographic format, were found to exhibit a high specificity of >99%, with sensitivity ranging from 86% to 99% [7, 16].

The OraQuick HCV Rapid Antibody Test for the detection of anti-HCV IgG was recently approved by the FDA for use with fingerstick, whole-blood, and venous blood specimens from individuals aged ≥15 years and at risk for infection with HCV and from persons with signs and symptoms of hepatitis. The test also received an FDA waiver under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) [17]. Since facilities that can perform CLIA-waived tests outnumber the laboratories performing screening EIAs, the test has the potential to be used far more widely, such as in physician offices, outreach clinics, and community-based organizations.

Rapid tests are typically more expensive than conventional immunoassays and are not designed for testing large batches of specimens. However, in nonclinical settings and laboratories that conduct low-volume testing, adoption of rapid testing can be cost-effective. CDC guidelines formulated for confirming screening anti-HCV results remain to be refined to accommodate rapid anti-HCV testing. It is important to emphasize that OraQuick HCV test has not been approved for general screening. A positive result of a rapid anti-HCV positive test is indicative of the presence of anti-HCV and, again, does not indicate active infection.

**DETECTION OF VIRUS-SPECIFIC MOLECULES**

**NATs for HCV RNA Detection**

HCV RNA is detectable in serum or plasma as early as 1 week after exposure and therefore remains the most reliable marker and gold standard for diagnosis of active HCV infection [18]. NATs currently in routine use for detecting HCV RNA are based on polymerase chain reaction (PCR), branched DNA signal amplification, and transcription-mediated amplification. Qualitative and quantitative PCR assays for HCV RNA testing that have been approved for clinical use by regulatory authorities in the United States and Europe are listed in Table 2. With the advent of ultrasensitive quantitative NATs that have a broad dynamic range and can detect as little as 5 IU/mL of HCV RNA, the use of qualitative methods is expected to decline. Until 1997, HCV RNA titers measured by different quantitative NATs were expressed in different units. The development of an international HCV RNA standard by the World Health Organization provided a common standard unit of measurement, the international unit (IU) [19], which is now used in all commercial assays. All NATs exhibit high sensitivities of up to 99% across all 6 genotypes of HCV. The availability of ultrasensitive quantitative NATs combined with reporting of viral titers in similar unitage across different assays has been crucial for effectively monitoring response to therapy during anti-viral treatment of HCV infected patients.

In addition to NATs for qualitative and quantitative HCV RNA, assays for HCV genotyping are also available; these are based variously on direct sequencing, reverse hybridization to genotype-specific oligonucleotide probes, and restriction fragment length polymorphism analysis [18]. HCV genotyping has clinical applications, since infection by certain genotypes is associated with differential outcomes of treatment, which currently are based on interferon and ribavirin [20].

**Immunoassays for HCV Core Antigen**

The HCV core or nucleocapsid protein is a phosphoprotein of 191 amino acids in length. Antigenic characterization showed the potential of HCV core antigen as a diagnostic marker, and several assays that rely on detecting the presence of the HCV core antigen alone or in combination with anti-HCV in serum
or plasma have been developed [21–23]. A quantitative antigen assay, the Architect HCV Ag assay (Abbott), is now commercially available in Europe. It adopts an automated platform and is a chemiluminescence-based immunoassay in which microparticles are coated with a monoclonal antibody to the HCV core antigen [21]. Detection of HCV core antigen within the first 2 weeks of acute infection has been reported in studies involving blood donors. It has been shown to have a sensitivity ranging from 80% to 99% and a specificity ranging from 96% to 100%. A 100% sensitivity in the window period of infection has also been reported in other studies involving blood donors [8]. Although the assay has been shown to have equivalent sensitivity across all HCV genotypes, detection of genotype 1 (1 pg/mL HCV core antigen = 8000 IU/mL HCV RNA) is slightly better than genotype 3 (1 pg/mL HCV core antigen = 6711 IU/mL of HCV RNA) [8]. The advantages of the HCV core antigen assay are that it is an immunoassay, it does not require sample processing as in molecular assays, and a positive result confirms active infection. One limitation of the HCV core antigen assay is that it has a lower sensitivity than NAT; its lower limit of detection is approximately 1000 IU/mL of HCV RNA. The Architect HCV Antigen assay is not yet commercially available in the United States.

Emerging Technologies for HCV Detection

Recent advances in molecular technologies have produced promising new tools that have the potential for use in the development of new and improved assays for the diagnosis of HCV infection. Prototype nanoparticle-based diagnostic assays have been developed for detection of biomarkers in various diseases, including hepatitis C. The most commonly used nanoparticles are quantum dots (QDs) and gold nanoparticles [24]. QDs are nanoparticles made of semiconductor materials that emit light upon excitation at different spectra, depending on their size, which greatly increases the ability to multiplex [25]. A sensitivity of 1 ng/mL was achieved in a biochip-based assay in which a QD-conjugated RNA oligonucleotide probe targeted the HCV NS5B [26]. A multiplex platform using a microfluidic chip and antigen-coated QDs embedded in polystyrene beads has been reported to detect antibodies to HBV, HCV, and HIV with a sensitivity in the picomolar range [27, 28]. Gold nanoparticles, usually 2–50 nM in size, have been successfully used in various assay formats to detect anti-HCV, as well as HCV RNA [29].

Among other novel technologies currently being evaluated for their diagnostic potential in HCV infections is the use of aptamers as capture molecules. Aptamers are short, single-stranded oligonucleotides that can fold into specific 3-dimensional structures and recognize target molecules such as small chemicals, proteins, and even cells [30]. They have been used for various diagnostic applications because of their ability to bind their targets with high affinity and specificity. Furthermore, these molecules are generated by an in vitro selection process, which eliminates the need for generation of

### Table 2. Qualitative and Quantitative Hepatitis C Virus RNA Assays Available for Clinical Use

<table>
<thead>
<tr>
<th>Assay</th>
<th>Manufacturer</th>
<th>Method</th>
<th>Lower Limit of Detection,(^a) (Dynamic Range) IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Qualitative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplicor HCV v2.0</td>
<td>Roche</td>
<td>RT-PCR (manual)</td>
<td>50</td>
</tr>
<tr>
<td>COBAS Amplicor HCV v2.0</td>
<td>Roche</td>
<td>RT-PCR (semiautomated)</td>
<td>50</td>
</tr>
<tr>
<td>Ampliscreen(^b)</td>
<td>Roche</td>
<td>RT-PCR (semiautomated)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Versant HCV RNA</td>
<td>Gen-Probe</td>
<td>TMA (manual)</td>
<td>10</td>
</tr>
<tr>
<td>UltraQual HCV RT-PCR</td>
<td>National Genetics</td>
<td>RT-PCR</td>
<td>10</td>
</tr>
<tr>
<td>Procleix HIV-1/HCV(^b)</td>
<td>Gen-Probe</td>
<td>TMA (manual)</td>
<td>&lt;50</td>
</tr>
<tr>
<td><strong>Quantitative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplicor HCV Monitor</td>
<td>Roche</td>
<td>RT-PCR (manual)</td>
<td>(600–700 000)</td>
</tr>
<tr>
<td>COBAS Amplicor HCV Monitor v2.0</td>
<td>Roche</td>
<td>RT-PCR (semiautomated)</td>
<td>(600–700 000)</td>
</tr>
<tr>
<td>Versant HCV RNA 3.0</td>
<td>Siemens</td>
<td>bDNA (semiautomated)</td>
<td>615 (615–7 700 000)</td>
</tr>
<tr>
<td>COBAS Ampliprep/TaqMan</td>
<td>Roche</td>
<td>qPCR (semiautomated)</td>
<td>18 (43–69 000 000)</td>
</tr>
<tr>
<td>Real Time HCV/m2000sp/m2000rt</td>
<td>Abbott</td>
<td>qPCR (semiautomated)</td>
<td>12 (12–100 000 000)</td>
</tr>
<tr>
<td>HCV SuperQuant</td>
<td>National Genetics</td>
<td>RT-PCR (semiautomated)</td>
<td>20 (20–1 000 000)</td>
</tr>
<tr>
<td>LCx HCV RNA-Quantitatie(^b)</td>
<td>Abbott</td>
<td>RT-PCR (manual)</td>
<td>25 (25–2 630 000)</td>
</tr>
</tbody>
</table>

Abbreviations: bDNA, branched DNA; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; TMA, transcription-mediated amplification.

\(^a\) Dynamic ranges are specified in parentheses.

\(^b\) Used for blood screening only.
antibodies in vivo [31]. The role of these molecules in the detection of HCV antigen has been described in preliminary reports [32, 33].

A novel amplification method, loop-mediated isothermal amplification (LAMP), has the potential to be developed into a point-of-care NAT for HCV RNA detection [34–36]. HCV RNA has been reported to be successfully amplified, to a sensitivity of 8 copies/mL. A device that integrates the LAMP methodology on microfluidic chips has been described and is reported to be capable of completing amplification in an hour, using <1 μL of sample, with a sensitivity of 10 fg/μL [37]. These various promising technologies await further evaluation.

DISTINGUISHING ACUTE FROM CHRONIC HCV INFECTION

People who are recently infected by HCV tend to respond better to antiviral therapy than those who are chronically infected [12]. This consideration drives efforts to identify recent or acute HCV infection. An ideal approach to the diagnosis of acute HCV infection is either the demonstration of seroconversion in a patient found at first testing to be positive for HCV RNA or of progression to HCV RNA-positive status in a patient with a recent history of exposure to HCV infection. These approaches are possible for closely monitored select populations such as intravenous drug users who regularly attend a healthcare or needle-exchange center or individuals who have recently been exposed to well-defined risk events, like a needlestick injury or receipt of an HCV-infected organ transplant [38]. The majority of the patients are, however, already positive for HCV RNA and anti-HCV at presentation, so identification of whether the patient is in the early or acute phase of infection is seldom possible. Unlike acute hepatitis B virus infection, in which immunoglobulin M (IgM) antibody to the hepatitis B core antigen is diagnostic of acute infection and precedes the appearance of IgG, for HCV infection the IgM antibody responses are variably detected in both acute and chronic phases [39]. Anti-HCV IgM cannot therefore serve as a diagnostic marker of acute HCV infection. As such, no IgM anti-HCV assays are licensed for clinical use.

In the absence of an IgM anti-HCV assay, various alternative approaches have been explored to distinguish acute from chronic HCV infection. These include monitoring viral load fluctuations [40] or variations in HCV antibody titers [41] and determination of the avidity of IgG antibody to HCV [42, 43]. One such anti-HCV IgG avidity assay is based on use of a mixture of recombinant HCV core, NS3, and NS4 antigens. Evaluation of its performance by use of seroconversion panels and samples from chronically infected blood donors showed a progressive increase in the anti-HCV IgG antibody titers to the antigens as the infection progressed [45]. These promising assays require further evaluation and validation in various clinical settings.

CONCLUSIONS

Serologic testing for prevalent HCV infection based on anti-HCV IgG has become more robust and refined. With the introduction of rapid formats for anti-HCV testing, an even broader base of populations at risk of HCV infection can now be screened with a view to identifying infected individuals for referral to care and treatment. NATs for detecting HCV RNA remain the mainstay for discriminating between people who are actively infected and those who have resolved infection. Immunoassays for HCV core antigen detection can limit the use of costly and labor-intensive NATs. The NATs would need to be reformatted and marketed to be more amenable to point-of-care testing and more affordable.

Notes

Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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