Review

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HCV core antigen comes of age: a new opportunity for the diagnosis of hepatitis C virus infection

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Abstract: The diagnosis of hepatitis C virus (HCV) infection has been traditionally based on the detection of the host antibody response. Although antibody assays are available in different formats and are fairly accurate, they cannot distinguish between an ongoing infection with HCV replicative activity and a past infection where HCV has been cleared, spontaneously or after a successful therapy. As a chronic infection is mostly asymptomatic until the late clinical stages, there is a compelling need to detect active HCV infection by simple and reproducible methods. On this purpose, the clinical guidelines have suggested to search for the HCV ribonucleic acid (HCV-RNA) after anti-HCV has been detected, but this second step carries several limitations especially for population screening. The availability of fast and automated serological assays for the hepatitis C core antigen (HCVAg) has prompted an update of the guidelines that now encompass the use of HCVAg as a practical alternative to HCV-RNA, both for screening and monitoring purposes. In this paper, we summarize the features, benefits and limitations of HCVAg testing and provide an updated compendium of the evidences on its clinical utility and on the indications for use.

Keywords: diagnosis; HCV core antigen assay; hepatitis C virus; viral infection.

Introduction

The diagnosis of acute or chronic hepatitis due to the hepatitis C virus (HCV) infection relies on the presence of anti-HCV antibodies, the raise of aminotransferases level (mainly ALT) and on the finding of circulating HCV ribonucleic acid (HCV-RNA). As the disease is almost always asymptomatic, in the acute and especially in the chronic, non-complicated phase [1] until the very late stages, the diagnosis if often posed after a positive screening result for anti-HCV for blood donation, high-risk settings, surgical or hospital screening or other reasons, or following the casual finding of raised ALT levels during check-up.

Diagnostic assays for HCV may be categorized in (a) first-level (enzyme immunoassays with different format) and second-level (immunoblot) serological assays for HCV antibodies, which detect the host immune response to an ongoing or past infection, and (b) “virological” assays that allow to establish an active infection by detecting HCV-RNA or, more recently, the hepatitis C core antigen (HCVAg) [2].

According to current guidelines, the first step both for screening and clinical diagnosis of an HCV infection is the research for anti-HCV antibodies [3–5]. When screening assays are used on subjects with a normal immune response, the relative sensitivity and specificity, leading potentially to false-negative or false-positive results, may vary according to the type of assay used but are generally low. In particular, false-positive results between 0.2% and 1% according to the population tested may be estimated [6]. The diagnostic limitation of antibody assays is that a positive result will not allow to differentiate the stage of HCV infection (inactive, active and for the latter if acute or chronic) as it only indicates the previous exposure to HCV, without providing additional information on the current state of the infection. Anti-HCV antibodies are still detectable as anamnestic response in subjects that have already cleared the infection and do not carry the virus anymore, and are not useful to detect those with an ongoing viral replication and possibly with an HCV-related disease. For this purpose, the direct viral assays such as HCV-RNA are needed. Furthermore, by analyzing the presence of anti-HCV antibodies in a general population at an unknown risk, it is fairly common to detect low-level anti-HCV antibodies positivity, with no definite classification by the additional tests [6].

Determination of a HCV infection in subjects with antibody positivity is therefore an essential time for proper
clinical framing and therapeutic strategy. The presence of a current infection is usually established with HCV-RNA research in serum or plasma [3–5]. This analysis, which currently uses high sensitive methods, requires the use of specific technology such as PCR or other RNA amplification methods and instrumentation, and the availability of a further sample or withdrawal with a time of response that can come to several days.

The use of quantitative methods for HCV-RNA allows a single test to determine the presence and simultaneous quantification of HCV-RNA, enabling both strictly diagnostic requirements to be evaluated and the status of HCV related disease activity, setting the therapeutic strategy and monitoring the response to antiviral treatment by providing accurate information on circulating HCV levels, commonly defined as the viral load. Clinical guidelines indicate the quantitative determination of HCV-RNA to determine the pattern and duration of therapy and to define the response to it, by means of traditional schemes and associations (PEG-Interferon + Ribavirin) or, more recently, with use of direct-acting antiviral drugs (DAAs) [4, 7]. Table 1 provides a synopsis of the various combinations of anti-HCV and HCV-RNA/HCVAg results.

**HCVAg assay**

The HCVAg encoded by the region 5′ terminal of the RNA sequence proposed as a viral genome of HCV is a proteic structure of 191 amino acid residues and can be divided into three “domains”: residues 1–117, basically basic and with two short hydrophobic regions; residues 118–174, most hydrophobic, with site C terminal at the end of p21; the highly hydrophobic domain 175–191, which represents the signal sequence for the E1 coating protein [8]. The sequence of HCVAg is highly conserved among all the different viral genotypes, and the presence of circulating antigen is associated with complete viral particles but is also due to the presence of antigen/antibody complexes and a release of “RNA free” antigen by hepatocytes that go into processes of immune-mediated lysis or apoptosis [9]. Like HCV-RNA, HCVAg is also detected in the serum or plasma long before antibodies to HCV: the antibody window can be extended up to 12 weeks, whereas HCVAg is usually detectable within 2–3 weeks of infection, almost simultaneously with HCV-RNA detection with molecular biology techniques [10]. A positive result for HCVAg confirms the viral replication activity.

The first diagnostic availability for HCVAg at the beginning of this century [11] was by qualitative methods for screening purposes. Subsequently, quantitative methods were made available. Those, although intrinsically suitable for monitoring, required manual preparation of the samples and were much less sensitive to HCV-RNA assays, and hence after some initial positive evaluations [12], they were considered to be of poor clinical utility and applicability. Recently, a new-generation test for HCVAg became available [13]. This assay is fully

<table>
<thead>
<tr>
<th>Combination of results</th>
<th>1 – Most likely interpretation</th>
<th>2 – Alternative interpretation</th>
<th>3 – Least likely interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HCV positive</td>
<td>Inactive HCV infection. Past infection with residual anti-HCV; HCV clearance after treatment</td>
<td>False-positive anti-HCV (if not confirmed by supplemental assays)</td>
<td>Passively transferred anti-HCV antibodies (mother to infant; intravenous gamma-globulins; invasive procedures)</td>
</tr>
<tr>
<td>HCV-RNA/HCVAg negative</td>
<td></td>
<td></td>
<td>Combined false positive due to carryover or other preanalytical errors</td>
</tr>
<tr>
<td>Anti-HCV positive</td>
<td>Active HCV infection, either acute or chronic</td>
<td>HCV reactivation in “silent” carrier or HCV reinfection in a patient that had previously cleared the virus</td>
<td>“Occult” HCV infection – virus sequences may be found in liver and/or other tissues without any serological marker</td>
</tr>
<tr>
<td>HCV-RNA/HCVAg positive</td>
<td>Naive subject – no exposure to HCV</td>
<td>Full seroreversion after HCV clearance, either spontaneous or therapy-induced</td>
<td>False positive or contamination due to carryover or other preanalytical errors</td>
</tr>
<tr>
<td>Anti-HCV negative</td>
<td>Early stages of an acute primary HCV infection (antibody “window”). If only HCV-RNA positive, very early primary infection</td>
<td>Acute or chronic HCV infection in immunosuppressed/ immunocompromised patients</td>
<td></td>
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<tr>
<td>HCV-RNA/HCVAg negative</td>
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The alternative and the least likely interpretation may be switched according to the prevalence and incidence in different geographical areas and in specific settings or risk groups.
automated and includes a preanalytic step based on the use of urea, hydrochloric acid and detergents that results in the lysis of circulating viral particles and dissolution of the Ab/Ag complexes. This process is consistent among sessions, also contributing to ensure a high analytical sensitivity, corresponding to about $10^3$ mIU/mL of HCV-RNA, with a range between 500 and 3000 IU/mL in various experiences [13–15]. Figure 1 compares the analytical sensitivity of this assay with previously available methods for the qualitative or quantitative determination of HCVAg [16–18]. This chemiluminescent microparticle immunoassay uses microparticles coated with anti-HCV monoclonal antibodies for capture and similar antibodies for the detection of HCVAg. The assay can be performed on the same sample and the same instrumentation used for antibody detection, provided the respective instrument is available in the lab, thus allowing a quick response. The running time of a single test is 30 min, from the presentation of the sample to the analytical system to the data output and the instrumentation on which the test is feasible is more widespread than molecular biology systems, making information more accessible to clinicians and consequently to their patients.

**Applications of the HCVAg test**

The quantitative determination of HCV core (HCV core antigen or HCVAg) antigen in serum or plasma may be proposed as an alternative or complementary to testing for HCV-RNA in the following clinical situations:

- Diagnosing an acute hepatitis C
- Diagnosis of an active HCV infection
- Monitoring of high-risk subjects
- Therapy monitoring and follow-up of treated patients.

**Acute infection by HCV**

According to the recent WHO definitions [19], the diagnosis of acute hepatitis C may be made, subject to compatible clinical conditions, under the following conditions: (a) seroconversion for anti-HCV; (b) presence of HCV RNA in the absence of anti-HCV; (c) positivity for anti-HCV and negativity for anti-HBc, anti-HAV and anti-HEV IgM. The second (b) option is certainly the most accurate as anti-HCV antibodies are detectable in the vast majority of patients with HCV infection but may be negative in the early stages of acute hepatitis and in immunosuppressed or immune depressed patients [4]. Determination of HCV-RNA therefore allows timely diagnosis, particularly useful in high-risk groups such as injecting drug users (IDUs), and in patients with reduced immune function such as patients undergoing hemodialysis or with HIV co-infection. An HCVAg assay, although of inferior analytical sensitivity to HCV-RNA assays, is capable of detecting acute HCV infection with similar clinical sensitivity. In the early stages of infection, the increase in the concentration of HCV-RNA in the bloodstream is sudden (ramp-up phase) and very rapidly reaches very high levels. Then the objective of assessing an acute HCV infection may be achieved either by HCV-RNA or by HCVAg testing. In order to simplify the screening process, it is also possible to combine HCVAg with anti-HCV detection in a single immunoassay, similarly to what has been done for HIV. Some HCVAg/Ab combination assays are already available [20, 21].

![Figure 1: Sensitivity of the different generations of assays for the determination of the HCV core antigen (HCVAg). The values are expressed in pg/mL; alternatively, HCVAg may be measured in femtomoles (fmol)/L; the equivalency is 1 fmol/L = 0.02 pg/mL [13].](image-url)
limitations are that HCVAg may be detected only in the early stages because a process to disrupt Ag/Ab complexes is not included, and the sensitivity for HCVAg is lower than the one achieved by the current HCVAg assay, thus reducing the clinical sensitivity in the antibody window phase. This has been recently highlighted in a retrospective evaluation of 337 blood donations obtained at the initial stage of infection. In this study, only 8% of cases showed HCV-RNA levels <10 IU/mL and overall HCVAg was positive in 92.4% of cases, whereas the sensitivity of two HCVAg/Ab assays was 38.3% and 47.5%, respectively [10].

Further evaluations conducted on groups of subjects at high risk of infection such as injecting drug users (IDUs) and hemodialysis subjects verified that the determination of the core antigen could significantly anticipate the diagnosis of acute hepatitis C with similar accuracy to HCV-RNA [22, 23]. Upon a clinical suspicion of viral hepatitis of likely viral origin, HCVAg may then be the first-line assay instead of anti-HCV (Figure 2).

Diagnosing active HCV infection

Over the last years, we have assisted to a scaling up of prevention interventions that have reduced the incidence of new infections. Nevertheless, treating those already infected is necessary to achieve reductions in HCV-related mortality [24]. The detection of people with HCV active infection in the absence of clinical manifestations is the first, crucial step in preventing the further spread of the infection and improving the health status of people already infected [19, 23]. A very recent collaborative study has assessed the worldwide prevalence of active HCV infections at the end of 2015 to be 1.0% (95% uncertainty interval 0.8–1.1), corresponding to 71.1 million (62.5–79.4) people [25]. These figures are quite lower compared to previous estimates, and the authors underline the fact that many earlier papers did consider the antibody prevalence that, as indicated above, does discriminate against a previous infection, a rather frequent occurrence as the elimination of the infection can occur not only through effective therapy but also spontaneously [1, 4, 7]. Some recent evidence derived from population studies conducted in European Hospitals has shown how the frequency of HCV active infection in anti-HCV positive asymptomatic subjects was less than 50% [26–28].

The international guidelines indicate the HCV-RNA amplification assay for HCV active infection detection [3–5]. The "classical" diagnostic sequence [3] involves performing the assay for HCV-RNA on antibody-reactive screening anti-HCV samples and possibly using second-level antibody test on negative RNA samples to confirm
the presence of antibodies. An alternative is the implementation of HCVAg instead of HCV-RNA, limiting the execution of this test to the samples that are negative for HCVAg [29]. This hypothesis is supported by the recent WHO definition that indicated that a HCV chronic infection diagnosis could be posed by either HCV-RNA or HCVAg positivity [5], which in fact equals the two dosages from a clinical point of view. HCV-RNA and HCVAg equivalency for diagnostic purposes were also sanctioned by the European Association for the Study of the Liver (EASL) [4] and the latest diagnostic algorithms proposed by the WHO [5, 19]. Consequently, the determination of HCVAg may be proposed instead of the research of HCV-RNA to identify the infections in progress [29–31] (Figure 2). The limitation of this strategy may be represented by lower sensitivity of HCVAg in respect of HCV-RNA [12–14]. Indeed, among subjects with chronic untreated HCV infection, the levels of viremia are usually greater than 10^5 IU/mL [1, 28], and the frequency of levels of HCV-RNA below the detection limit of the HCVAg test has been observed to vary between <0.5% and 7% depending on the method used [32, 33]. This apparent limitation is counterbalanced by the greater stability of HCVAg in biological samples [34], which becomes relevant when biological samples are to be sent to a core laboratory [29]. There is already evidence of HCVAg application in population screening. Kuo et al. [35] demonstrated that a strategy that combines anti-HCV and HCVAg determination would have a sensitivity of 96.8% and 100% specificity when considering only high-signal antibody responsiveness. More recently, Furliniet al. [28] demonstrated substantial equivalence between HCV-RNA and HCVAg in a hospital population screening: over 700 positive subjects for anti-HCV had an active infection rate of 46.6%, and concordance between the two markers of infection was very high (kappa statistics: 0.95). It should be noted that although most discordances were on samples that resulted as positive for HCV-RNA and negative for HCVAg, an HCVAg positivity and negativity for HCV-RNA also occurred as noted also by other authors [14, 15, 36]. In their recent review of 23 papers, Khan et al. [37] have reported the negativity for HCVAg in 3.52% of over 3600 samples positive for HCV-RNA and, conversely, the lack of detectable HCV-RNA in 0.52% of over 4500 samples positive for HCVAg. As the specificity of HCVAg was 99.98% on 5394 samples negative for HCV RNA and anti-HCV [13] and 100% in other cohorts of HCV-negative subjects [14, 15], it is likely that the presence of HCVAg in the apparent absence of HCV RNA is not due to non-specific reactions but may be ascribed to a possible degradation of nucleic acid in the specimens under examination or to the non-homogeneous distribution of viral RNA in the biological matrix, as all the discordant samples had a very low concentration of HCVAg. The high overall agreement between HCV-RNA and HCVAg suggests therefore the direct use of HCVAg as a single marker for HCV screening [38], and the suitability of this approach has been recently confirmed in a high prevalence setting [39] (Figure 2).

Monitoring of high-risk subjects

The risk of acquiring HCV is especially high in several patient groups, namely, IDUs and patients undergoing hemodialysis. Among IDUs, the rate of infection is historically very high [40–42], and recent data from Europe report national estimates of over 40% [26] and may attain 80%. Superinfection or reinfection is also common [43]. As a consequence, the incidence of HCV is also high, and surveillance by means of assays that spot an active infection is needed and HCVAg has proven useful for this purpose [19] as well an in HIV-positive individuals, who are also at a very high risk of HCV coinfection [44, 45]. Hemodialyzed patients still bear a high risk of acquiring HCV [42, 46] mostly due to inappropriate procedures and not to the dialysis process itself [47], and also in this setting, the search for HCVAg instead of HCV-RNA has proven effective to verify HCV incidence [34]. The use of HCVAg in the hemodialysis sector has been recommended in EIRE since 2011 [48] both for the initial screening of patients on hemodialysis and for the follow-up measures to ensure that the patients do not acquire HCV over time while on treatment (Figure 2).

Therapy monitoring and follow-up of the treated patients

Guidelines for the treatment of chronic liver disease caused by HCV identify HCV-RNA and viral genotype for the choice of therapeutic regimen and the duration of treatment [4, 49]. In addition, the outcome of the therapy is based on viral RNA kinetics, and a sustained long-term response (SVR) is identified by the absence of circulating HCV-RNA at 3–6 months after the end of the treatment [4]. The evaluations conducted on patients treated with “standard” therapy (combination of PEG-Interferon [IFN] and Ribavirin) concurrently established the substantial equivalence of HCV-RNA and HCVAg [50–52], with an equal prediction based on the variations in concentration if response to viremia or antigenemia decreased within 2 weeks after beginning treatment [53]. It has also been shown that even faster kinetics, both
for RNA and for antigen, have a high predictive value for SVR [51, 52] and that HCVAg shows similar sensitivity and specificity in recurrence identification with respect to HCV-RNA [53].

Different is the situation with regard to direct-acting antiviral drugs (DAAs) that, unlike PEG-IFN + Ribavirin, target the key passages in the viral replication mechanism and provide a complete response with eradication of the infection in over 90% of patients treated [4, 7]. The high efficacy of DAAs associated with their extreme safety questions the real need for monitoring during treatment, as is the case with PEG-IFN-based regimens, also because there are no indications for alternative therapy based on virological response [4, 53]. It may therefore be assumed that quantitative determination of HCVAg can be a viable option to simplify monitoring with a method that does not require specialized technicians and provides a faster response time. Early reports with protease inhibitors have proven HCVAg measurement as a potential alternative for monitoring treatment response during DAA-based regimens [54], and this option is also covered in recent EASL guidelines [4]. Conversely, the American Association for the Study of Liver Disease guidelines recommend quantitative HCV viral load testing after 4 weeks of therapy [55], and the most recent Asian Pacific Association for the Study of the Liver guidelines indicate the relevance of monitoring of HCV loads during treatment, without specifying the method(s) to be used, for response-guided therapy to determine futility, treatment protocol and duration [56].

The clinical validity of HCVAg testing in patients treated with the currently available DAAs has been demonstrated in several recent studies [57–59]. Moreover, Aghemo et al. [57] showed similar predictivity for SVR of the two tests and Chevaliez et al. [58] found a >99% concordance between HCV-RNA and HCVAg on a cohort of over 600 DAA-treated patients. The clinical validity of the use of HCVAg has also been demonstrated in follow-up after therapy [58, 59] and is recommended by EASL guidelines [4]; in this situation, HCVAg was equivalent to HCV-RNA to identify recurrences [58, 59].

Organizational and economic evaluations

A first element to consider is the redeemability for each of the HCV diagnostic tests, which is currently foreseen both for first and second HCV tests and for HCV-RNA and HCVAg. The HCVAg assay is beneficial compared to the additional tests to confirm anti-HCV reactivity: the positive relapse of the HCVAg assay as an alternative method has been demonstrated both in terms of time (90-min report) and cost, with a reduction of direct and indirect costs of 479% and economic savings for the health service [60]. In acute HCV infections, it has been demonstrated that HCVAg testing instead of HCV-RNA attains the same sensitivity, would save a few thousand dollars and delivers results on the same day, beginning then with immediate therapy, if indicated, and facilitating the activities of contact tracing and partner notification [19]. In the field of population diagnosis, Chapko et al. [61] compared antibody-based algorithms with conventional laboratory tests or rapid tests and researching HCV-RNA with qualitative or quantitative methods, concluding that strategies that verify the presence of HCV infection by a single visit are more effective than those requiring a second visit, which is also considered by WHO to be less effective in screening as the compliance will be low [5]. A significant advantage is the possibility of performing an HCVAg test in the same laboratory sector and possibly with the same analytical system used for the determination of anti-HCV. Thus, positive antibody specimens can be immediately analyzed for HCVAg, and any additional HCV-RNA test may be required only in case of antigen negativity [27], limiting the execution of the confirmatory antibody test only to samples negative by both HCVAg and HCV-RNA. This sequential approach (antibody, antigen and RNA) is also beneficial in cost-effective perspective [31, 62], ensuring the best relationship between invested resources and identification of subjects with active infection over the conventional approach of testing for antibodies and then for HCV-RNA [63]. Although an in-depth health economics analysis is beyond the scope of this article, it is worth mentioning that several recent papers have evaluated the cost-effectiveness and long-term impact of HCV screening policies [62–68], also envisioning the use of HCVAg [62, 63].

In conclusion, the new serological assay for HCVAg has the requisites for an adoption as a primary tool in HCV screening and diagnosis.

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