Laboratory Testing for Hepatitis C

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Serological Detection of Hepatitis C Virus

Serological diagnosis of patients infected with the hepatitis C virus (HCV) can be performed using two categories of tests: indirect tests, which detect antibodies against HCV; and direct tests, which detect, quantify, or characterize components of the viral particle, such as HCV RNA testing and testing for detection of the HCV core antigen.

Anti-HCV antibodies are usually detected using third- and fourth-generation immunoenzymatic assays – enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA) 3 and EIA/ELISA 4, respectively – which contain HCV core antigens and HCV nonstructural genes. The specificity of the EIA tests available on the market that detect anti-HCV was determined to be higher than 99%, whereas their sensitivity, which was more difficult to determine due to the lack of gold standard tests with high sensitivity, was 95-99% [1]. However, false-positive results for anti-HCV can occasionally occur, especially in populations with prevalence rates below 10% [2-4].

There are many reasons why laboratories do not routinely use a supplementary test based on immunoblot analysis, such as the recombinant immunoblot assay, to complement the diagnosis of HCV infection. In addition to the high cost of such a test, the lack of laboratory standards that can evaluate its performance and interpretation, in conjunction with its actual accuracy, is among the principal reasons. Furthermore, this type of test does not distinguish past from present infection, and its use is only indicated for confirmation of EIA results.

In contrast, the use of nucleic acid testing (NAT) makes it possible to differentiate between viremic and nonviremic individuals by detection of HCV RNA, allowing the clinician a differentiated approach to anti-HCV-positive individuals. However, there can be situations in which HCV RNA is not detected (negative HCV RNA) and the individual has active infection with HCV. This can occur in individuals in whom anti-HCV antibody titers are high and RNA titers are low [5]. Therefore, HCV RNA might not be detectable in certain individuals in the acute phase of the disease. However, these findings are transient, and chronic infection can develop [6]. In addition, HCV RNA intermittent positivity has been observed in individuals chronically infected with HCV [6-8]. Negativity of HCV RNA results can indicate resolved infection. In 15 to 25% of those anti-HCV positive individuals who acquired the infection after 45 years of age, the infection resolves spontaneously. This percentage increases to 40-45% in those who acquired the HCV infection in childhood or young adulthood [9].

Different tests based on polymerase chain reaction (PCR) have been developed to directly detect the viral particle. One characteristic of real-time PCR is amplification coupled with detection, which allows the evaluation of the number of viral genomes at the onset of and throughout the reaction. Qualitative detection of HCV RNA by reverse transcriptase (RT)-PCR is generally accepted as the most sensitive and standardized test to date [10,11]. Nevertheless, there is variability among the results from different laboratories, as evidenced by the use of international panels of proficiency. The accuracy and reliability of the results are directly related to the laboratory procedures adopted in the performance of the tests [12]. The lack of preliminary care in sample collection, in conjunction with the time involved in preparing and separating the samples, can result in incorrect results. It is extremely important that all laboratory procedures comply with Good Laboratory Practice and strictly follow the protocols standardized by the manufacturers of the diagnostic kits and reagents.

The gold standard consists of the careful use of NAT, standardized for detection of HCV RNA, together with EIAs (specificity in conjunction with sensitivity).

An alternative to aid diagnosis is the use of the ratio between optical density and cut-off value (OD/COV) or the sample/cut-off ratio as an indicator of the true positivity of the test. Studies carried out in Brazil show that, in EIAs, reagents with OD/COV greater than 3 are repeatedly associated with 100% true-positive results (positive predictive value) and present approximately 92% positivity for HCV RNA by RT-PCR [13]. In terms of the population studied, the positive predictive value is increased when accompanied by risk factors, high levels of alanine aminotransferase (ALT), or liver disease.

In immunocompetent patients, EIAs present excellent reproducibility; however, in hemodialyzed or immunocompromised patients, EIA sensitivity is significantly reduced [14].

In low-risk populations, such as blood donors, or in random population screening, i.e., in populations that do not present risk factors for the acquisition of HCV infection, negative EIA results are sufficient to rule out the presence of HCV. However, false-positive results can occur in these populations. In such cases, a qualitative study of HCV RNA should be performed to confirm the diagnosis.

In high-risk populations, when there is clinical suspicion of HCV infection, positive EIA results confirm the exposure to HCV. A qualitative study of HCV RNA should be performed to distinguish individuals with chronic infection from those who have eliminated the HCV spontaneously.

In patients with chronic hepatitis of unknown cause and negative anti-HCV EIA results, especially in immunocompromised patients [14], a qualitative study of HCV RNA should be performed. The presence of HCV RNA confirms the diagnosis, although a negative result does not rule out HCV infection. In such cases, it is recommended that a new HCV RNA study be performed six months after the first study. Detection of the HCV core antigen by EIA can be an alternative for early diagnosis of HCV infection.

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The HCV core antigen ELISA was developed to be used as a serological screening test to detect the HCV core antigen, especially during the immunological window period, when antibodies are not detected. This assay was found to have sensitivity close to that of NAT, with a mean difference in detection of one to two days [15].

Based on this assay, a new assay was developed to detect and quantify HCV core antigen. The modifications made to this new assay, such as the dissociation of immune complexes, which allows the detection of free antigens and core antigen antibodies, and the change in the signal amplification, through the modification of the conjugate, have increased the sensitivity of the test. Studies have demonstrated that this test can reduce the immunological window by 3.3 days in comparison with the previous test (i.e., the HCV core antigen ELISA). This increase in sensitivity has led to a significant (58-day) decrease in the size of the immunological window. The difference between this EIA and PCR was only 0.24 days [16].

This test can be considered a viable alternative to detecting viremia directly when NAT cannot be used for reasons of cost, organization, emergency, or logistic difficulties. Tests that allow simultaneous or combined detection of HCV core antigen and antibody in a single assay are currently available on the market. These tests, known as HCV Ag/Ab combo assays, have high sensitivity and specificity, reducing the duration of the immunological window (during which antibodies are not detected) by up to 12 days [17]. Studies carried out using this assay showed sensitivity close to that of NAT, with a mean difference in detection of 1 to 2 days [18]. The use of NAT in the diagnosis of HCV infection makes it possible to distinguish viremic from nonviremic individuals through the detection of HCV RNA.

Therefore, these tests can be considered a plausible future solution in the screening of blood donors, organ transplantation programs, and cases of occupational exposure, in which a rapid and low-cost diagnosis is necessary.

In order to standardize the tests, the World Health Organization and the United States National Institute for Biological Standards and Controls have established a standard measure known as the international unit (IU). Assays for qualitative detection of HCV RNA are important tools because they are significantly more sensitive than are most quantitative tests. Qualitative assays are based on the principle of target amplification using either PCR or transcription-mediated amplification. The cut-off value of the lower limit of detection of HCV RNA of these commercial assays is 50 IU/mL and 6 IU/mL, respectively [19]. The specificity of these essays exceeds 99%. A positive test for HCV RNA confirms active replication of HCV. Clinical and laboratory follow-up with study of HCV RNS should be performed to confirm the absence of active replication of HCV. Once HCV infection is confirmed, performing further qualitative tests for HCV RNA in patients submitted to clinical follow-up evaluation but not receiving treatment has no diagnostic utility.

The quantification of HCV RNA can be performed by target amplification using PCR or by signal amplification using branched DNA (bDNA). In these commercial assays, the cut-off value of the lower limit of quantification of HCV RNA ranges from 600 to 615 IU/mL, and the upper linear limit ranges from 850,000 to 7,700,000 IU/mL [20]. The standardization in IU does not represent the actual number of viral particles in the preparation. There are significant variations among commercial assays. The dynamics of each assay should be observed, and appropriate dilutions of the material being analyzed should be performed to ensure the accuracy of the quantification.

The ideal assay for HCV RNA should have a lower detection limit of 5 to 50 IU/mL and a linearity curve of 6 to 7 $\log_{10}$. Traditional assays for detection of viral load, such as bDNA and Roche Monitor, present detection limits of 615 IU/mL and 600 IU/mL respectively [21,22], which are inadequate to define end-of-treatment response or sustained virological response. Real-time PCR assays are a promising tool due to their sensitivity and broad range of linearity. Cobas Taqman 48 HCV assay is a quantitative assay that has a detection limit of 10 to 100 IU/mL, which makes it well suited for use in follow-up treatment (at the initiation and at week 12) [23].

Acute Infection and Cutting/Piercing Accidents

After exposure to HCV, anti-HCV antibodies can be detected by EIA in 50 to 70% of the patients at the onset of symptoms, this percentage increasing to approximately 90% after 3 months. Routinely, HCV RNA can be detected between post-exposure weeks 1 and 3, remaining at detectable levels when symptom onset occurs. From post-infection week 2 to post-infection week 8, levels of ALT rise, and this increase is accompanied by the appearance of hepatocytic lesions.

Vertical Transmission

An important question is that of exactly how mother-to-child transmission of the HCV infection is defined. In many children born to mothers with chronic hepatitis C, anti-HCV (IgG) is detectable in the blood. These antibodies are acquired through passive transplacental transfer. These passively acquired antibodies will remain detectable for the first 12 to 15 months of life. Therefore, the criterion to identify mother-to-child transmission of HCV infection is the detection of anti-HCV and HCV RNA in the blood of the child after the age of 18 months.

Chronic Infection

In patients with chronic hepatitis C, the diagnosis of chronicity is based on the detection of anti-HCV and HCV RNA in the blood, using techniques of high sensitivity, and is confirmed through liver biopsy.

Loss of anti-HCV and isolated presence of HCV RNA are uncommon in immunocompetent patients with chronic hepatitis C. However, these findings can occur in hemodialyzed patients and in severely immunocompromised patients

Follow-Up Treatment

Some patients with detectable HCV RNA should be considered for treatment. Genotyping should be performed at the initiation of treatment in order to define treatment duration, since, according to treatment protocols, patients infected with genotype 2 or 3 should be treated for 24

weeks, whereas those infected with genotype 1 should be treated for 48 weeks [24].

A considerable limitation in the evaluation of patients with chronic infection with HCV has been the lack of standardization of the tests for detection of HCV RNA. A significant difference has been observed in the assays used, both in terms of sensitivity (upper limit of detection) and in terms of dynamics. These differences are observed not only among the different assays but also among different laboratories performing a given assay. Therefore, it is important that, throughout the clinical follow-up of a patient receiving specific treatment, the same assays and, if possible, the same laboratory always be used [25-28].

Quantification of HCV RNA should be performed in the pretreatment sample and in the week-12 sample in order to evaluate the predictive value of the treatment response.

Since the qualitative study of HCV RNA presents a lower limit of detection of 50 IU/mL, it should be used at week 4 of treatment, as a predictor of sustained virologic response (SVR), then again, to detect the SVR, at the end of treatment and at 6 months after the end of treatment. Therefore, presenting negative PCR results by week 4 of treatment has a high predictive value for achieving an SVR.

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