Evaluation of assay methods and false positive results in the laboratory diagnosis of hepatitis C virus infection.

Recep Kesli

Abstract

Diagnosis of hepatitis C virus (HCV) infection routinely performed in the microbiology laboratories is based on the detection of serum antibodies against hepatitis C virus (anti-HCV) by two main methods: enzyme immunoassay (EIA) and chemiluminescence immunoassay (CIA). Different reagents built on these two methods were manufactured and are still in use for the diagnosis of HCV infections. The most common problem in the laboratory screening assay of anti-HCV is the false positivity of low titers. A confirmatory test is needed to discriminate the false positive results from the accurate ones. A lot of discussions focused on deciding a reliable, easy-to-use and cost-effective confirmative test method in order to check and confirm the results of anti-HCV reagents routinely used in the detection of serum anti-HCV level.

Key Words: Hepatitis C virus, Laboratory diagnosis, False positivity.

Hepatitis C virus

In the mid-1970s, a new disease entity termed “non-A, non-B” (NANB) hepatitis was first described, and in the following years leading to the discovery of the virus, posttransfusion and community-acquired NANB hepatitis increasingly became a potentially serious disease, resulting in cirrhosis and/or hepatocellular carcinoma [1, 2]. Hepatitis C virus (HCV) is a single-stranded RNA virus with a genome of about 10,000 nucleotides containing a single large, continuous open reading frame and with an organization most closely resembling the Flaviviridae [3]. Hepatitis C virus infection is a global health problem. The World Health Organization (WHO) estimates that at least 170 million people are infected with HCV worldwide, and most of the patients are concentrated in developing countries [4].

Methods used at laboratory diagnosis of hepatitis C virus infection

Diagnostic procedures of hepatitis C virus infection in laboratories are principally based on the detection of antibodies (IgG) against recombinant HCV polypeptides by two main methods: Enzyme immunoassay (EIA) and Chemiluminescence immunoassay (CIA). Non-structural and recombinant antigens have been used in the production of immunoassay reagents. Serologic and virologic markers of past or present HCV infection include IgG antibodies (anti-HCV). An assay for Ig M anti-HCV is available, but it does not distinguish between acute and chronic HCV infection [5].

Three different generations of HCV kits have been developed to date. The first generation HCV enzyme immunoassay detected only antibodies against non-structural region 4 (NS4) with recombinant antigen c100-3. First-generation anti-HCV reagents had relatively poor specificity and sensitivity. In the development of second-generation tests, additional antigens from the core region (c22-3), the NS3 region (c33c) and a part of c100-3 from the NS4 region were used. Second-generation reagents show increased sensitivity and specificity. Third-generation EIA includes an additional antigen from the NS5 protein and a reconfiguration of the core and NS3 antigens [6].

Antibodies to HCV are detectable an average of 8 weeks later after an exposure. Antibodies to NS3 and core antigen generally appear a few weeks before the antibodies to NS4 and NS5-antigen. Whereas anti-NS3 is almost always detectable, anti-core antibody may initially be absent, especially in the case of self-limiting or asymptomatic infections, and in up to one third of the cases does not appear until the chronic stage. Total serum HCV core antigen, a surrogate marker of HCV replication, can also be detected and quantified and commercial assay is available . HCV core antigen can be detected on average 1 to 2 days after HCV RNA during the pre-seroconversion period. HCV Ag test presented as a “Direct marker for diagnosis of HCV infection”. Sensitivity of HCV core antigen test is lower than HCV-RNA assay [7, 8].

Diagnostic tests may be divided into three groups:
1- Screening test (anti-HCV tests based on EIA or CIA)
2- Supplemental test (recombinant immunoblot assay-RIBA)
3- Confirmatory test (HCV RNA).
**False positivity of anti-HCV tests**

Although present tests have better sensitivity and specificity rates than their predecessors, there still exists a high prevalence of false-positive results, especially among low risk group, immunocompromised patients or populations without liver diseases, leading to unnecessary cost-effective health expenditures and confusing diagnostic challenges. The most common problem in the laboratory screening assay of anti-HCV is the false positivity of low titers. Among immunocompetent populations with anti-HCV prevalence less than 10 % (e.g., volunteer blood donors, military personnel, general population, health care workers, or clients attending sexually transmitted diseases clinics ) the proportion of false positive results averages approximately 35 % (range: 15 % to 60 %) [9,10].

Among immunocompromised populations (e.g., hemodialysis patients) average rate of false-positive results approximately: 15 %. False positive anti-HCV results obtained from both CIA and EIA based reactives can be explained with by the fact that no structural antigens and proteins have been derived from HCV up to now. HCV has not to be cultured, natural viral proteins were unlikely obtained. A lot of discussions focused on deciding a reliable, easy-to-use and cost-effective confirmative test method in order to check and confirm the results of anti-HCV reactives routinely used. A confirmatory test is needed to discriminate the false positive results from the accurate ones [11].

**Reasons of false positivity**

The aminoacid sequence and the purity of the HCV antigen used for assay development are significant factors influencing both the specificity and the sensitivity of anti-HCV immunoassays. Because of the high IgG concentration in human blood (>5 mg/ml)-e.g. in paraproteinemia or auto-antibody production or after Ig G denaturation caused by repeated freezing and thawing or by heat-inactivation of serum samples-there is a strong tendency for some of the IgG molecules to be bound to the micro-well surface by direct adsorption or by indirect capture via the surface molecules, and then arouse a signal, giving false-positive results. This problem might be more serious when the samples are from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), portal cirhosis, and some infectious diseases due to the very complicated, higher concentration of immunoglobulin components in their blood [11].

**Supplemental test**

RIBA also a kind of serologic assay may be used as a supplemental test. A positive RIBA result interpreted as anti-HCV positive. A confirmed anti-HCV positive result indicates the need for serial determination of HCV-RNA activity. As a result RIBA only may be a confirmative test for the anti-HCV positivity. But it is not enough to confirm current HCV infection. RIBA has several disadvantages, such as the difficulties of performing the test, high percentage of the indetermined results and high cost-effectiveness causing the less use or preventing the common use in many developing countries, in routine diagnostic laboratory procedures [12].

RIBA HCV 3.0 SIA (Strip Immunoblot Assay) contains recombinant antigens and synthetic peptides (Recombinant c33c and NS5 antigens; Synthetic 5-1-1, c100, and c22 peptides). RIBA is positive when patients exhibit HCV RNA positivity. RIBA may be either positive or indetermined or negative when patients exhibit HCV RNA negativity. Mesurements of HCV-RNA should be especially reserved for the patients with indetermined or positive RIBA results. A negative RIBA is indicative of unspecific reactivity in the anti-HCV screening test. A positive RIBA in many cases is not proof of specific reactivity in the screening test [13]. In patients with a high pretest probability of infection, a positive serological screening test should be “confirmed” by performing a qualitative test for HCV RNA rather than the supplemental recombinant immunoblot assay. This strategy is cost-effective and more informative than using the RIBA to confirm positive antibody screening tests in a diagnostic setting [14].

**Confirmation test is needed**

Although third-generation HCV reactives are more sensitive and specific than older generation assays, they still have a high percentage of false positive reactions, so that it is mandatory to confirm every reactivity, especially with low titers by anti-HCV CIA or EIA with HCV RNA assay (reactives with a lower limit of detection of 50 IU/mL or less) to avoid false positive results. To minimize the likelihood of false-positive anti-HCV results, CDC has recommended the confirmation of all anti-HCV results by either RIBA or HCV-RNA assay [15,16].

HCV RNA is the earliest marker of infection, and a direct indicator of ongoing viral replication. It appears 1 to 2 weeks after infection before any alterations in liver enzyme levels and appearance of anti-HCV antibodies can be detected. If the nucleic acid testing (NAT) result is positive, active HCV infection is confirmed. If NAT result is negative, the HCV antibody or infection status can not be determined. NAT assays used to detect and quantify HCV RNA: the following target amplification methods used qualitative detection of HCV RNA and have lower limits of detection of 5-50 IU/mL: Reverse Transcriptase-PCR (single/dual enzyme RT-PCR, nested RT-PCR), TMA (transcription-mediated amplification), NASBA (isothermal RNA amplification). Quantification of HCV-RNA can be determined by comparative or Real-Time PCR assays, signal amplification methods (bDNA) [14,17-18].

In certain situations HCV RNA result can be negative in persons with active HCV infection. As the titer of anti-HCV increases during acute infection, the level of circulating HCV-RNA de-
TABLE 1. Interpretation of HCV test results.

<table>
<thead>
<tr>
<th>Immunoassay for anti-HCV</th>
<th>Nucleic acid test for HCV RNA</th>
<th>Supplemental test for anti-HCV</th>
<th>Interpretation of HCV status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Never infected with HCV</td>
</tr>
<tr>
<td>Positive</td>
<td>Not done</td>
<td>Not done</td>
<td>Unknown, screening test positive needs confirmation</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive/not done</td>
<td>Active HCV infection</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Not done</td>
<td>Unknown, single negative HCV RNA result cannot determine infection status; perform RIBA to rule out screening test false-positive</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Has been infected with HCV/repeat testing for HCV RNA to rule out active infection as HCV RNA levels may fluctuate</td>
</tr>
<tr>
<td>Positive</td>
<td>Not done</td>
<td>Positive</td>
<td>Has been infected with HCV; follow-up testing for HCV RNA, liver enzymes is indicated to determine current infection status</td>
</tr>
<tr>
<td>Positive</td>
<td>Not done/ Negative</td>
<td>Negative</td>
<td>Never infected with HCV</td>
</tr>
</tbody>
</table>

Chronic hepatitis C

Chronic hepatitis C is certain when both anti-HCV antibodies and HCV-RNA are present. Detectable HCV replication in the absence of anti-HCV antibodies is exceptional with the current enzyme immunoassays, almost exclusively observed in: profoundly immunodepressed patients, hemodialysis patients, agammaglobulinemic subjects [19,20].

Conclusion

The diagnosis of HCV should be as reliable as possible, because a positive result has a deep impact on the life of the affected person. Commonly used anti-HCV CIAs or EIAs produce a high percentage of false positive results, proving the necessity of the use of an easy-to-use, reliable and cost-effective confirmation test, HCV-RNA, in routine diagnostic laboratory procedures.

References