

Updated Molecular Diagnosis of Chronic Hepatitis C

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INTRODUCTION

Hepatitis C is an infectious liver disease caused by the hepatitis C virus (HCV). It is transmitted through contact with the blood of an infected person or by secondary infection after treatment. HCV has a global prevalence of 2.35%, affecting 160 million individuals^[1]. Failing of HCV eradication led to lifelong chronic conditions. Many estimated group samples reported 55%-85% of newly infected cases progress to chronicity; of those, 20%-30% will develop liver fibrosis, cirrhosis, and liver failure and 2%-5% will advance to hepatocellular carcinoma^[2,3].

HCV contribute to mortality from liver disease, thereby mortality is around 1 in 8 and twice as many will develop liver cirrhosis. Estimation of 8 000 to 10 000 deaths/year are ascribed to HCV infection. Therefore, of total infected worldwide, 20 million HCV will be related deaths in the coming decades^[4,5]. Early diagnosis and evaluation of HCV cases is very helpful for the management and the control of the disease. To increase the depth of understanding strategies HCV control related to health care, it is absolutely urgent that medical specialists in all areas will hold discussions to generate cooperation for getting précised estimations for endemic areas. Diagnostic tests for HCV can be divided into two general categories: (1) serologic assays which detect anti-HCV antibodies, and (2) molecular assays which detect, quantify, and/or characterize HCV RNA genomes within an infected patient. Serologic assays have been subdivided into screening tests for anti-HCV and supplemental antibody tests. Molecular assays can be divided into qualitative tests for HCV RNA, quantitative tests for assessing viral RNA levels, and HCV genotype tests. Most immunological tools-for immunological assay- were developed for: (1) getting an early diagnosis for HCV infections in estimated areas; (2) increasing the chances of successful treatment; (3) increasing impact of essential life style changes; (4) limitation of cross-infection and; (5) reduction of the long period of HCV seronegativity^[6-11].

SEROLOGICAL ASSAY

The principles of serological assay are based on detection of anti-HCV antibodies in plasma or serum. Because hepatitis C virus

ABSTRACT

The scope of this review allows an exhaustive analysis of updated molecular diagnosis of hepatitis C in endemic estimates representing examples to illustrate the use of molecular techniques for the HCV diagnosis. Hepatitis C is an emerging infection that threatens for economic and psychosocial disaster in estimated endemic areas worldwide. It is a global Human calamity requiring integrity of all researchers' efforts with strong governmental aids. Findings of current review article focused on the utility of measuring HCV core antigen levels in the clinical setting, as a substitution and/or conformational for measuring HCV RNA levels, especially in endemic areas with low economics. Currently, molecular assays based RT PCR allows the monitoring and generation of PCR products simultaneous with the amplification process. Quantitative assessment of HCV RNA levels, via signal amplification and quantitative PCR, are valuable tools in the clinical management of patients before, during and after therapy. Fortunately, the most promising approach is fully automated real-time PCR assays, which are faster, more sensitive than classical target amplification techniques and are not prone to carryover contamination.

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infects liver cells, reside, replicate and translate proteins, the immune system produces antibodies against circulating viral products. Immune system takes 8-12 weeks to develop enough antibodies. Three generations of screening anti-HCV test have been designed to detect antibodies against various epitopes of these proteins. The 1st generation anti-HCV test (EIA- I) contained a single HCV recombinant antigen derived from the nonstructural (NS4) gene, designated Cl00-3. It lacked optimal sensitivity and specificity and gave false positive results. The 2nd modified EIAs contains multi recombinant antigen: NS (c33c) and structural (c22-3) from the core and NS3 and NS4 genes antigen. The 2nd modified EIA assays was more sensitive than 1st generation for several reasons: (1) high-prevalence rates for estimated population based HCV polymerase chain reaction (PCR) as a golden standard test; (2) more earlier detection for HCV antibodies from 30-90 days; (3) detecting more estimated acute cases and chronic cases that may exceed 20% and 80% respectively; (4) reduction the window of seroconversion from 16 weeks with EIAs 1 to 10 weeks with EIAs 2.0^[12-14]. The 3rd modified EIA (EIA 3) added a fourth antigen (NS5) to those in EIAs 2. 3rd modified EIA detected antibodies in earlier times than former antibody assays. Data diverge between first, second, and third generation assays were found only for HCV RNA negative samples and for donors who showed no biochemical or clinical evidence of liver disease and had no history of exposure to HCV^[15,16]. Table 1 summarize the Sensitivity and Accuracy of the three Anti-HCV Screening Tests^[6,17]

Table 1

Low-Prevalence (Blood Donor)			High-Prevalence (Liver Clinic)		
Test	Sensitivity	Accuracy	Test	Sensitivity	Accuracy
EIA-1	95-98%	30-50%	EIA-1	60-80%	70-85%
EIA-2	99.7-99.8%	50-61%	EIA-2	92-95%	88-95%
EIA-3	99.7-99.9%	96%	EIA-3	97%	96%

It is noted in endemic estimates, antibody assay does not differentiate between acute, active, or resolved infection. It may yield false negative results in the gap period between exposure and seroconversion or in patients with immunologic disorders that impair production of antibody or due to cross-reactivity with other antigens. The false positivity rate of the EIA increases as the prevalence of hepatitis C antibody positivity decreases in a population. Early stages of the infection are missed because the antibodies develop only after one and half months of infection. Thereby, antibody test may be negative in the initial period (diagnostic window period) before the seroconversion phase that may last up to 2 months in immunocompetent subjects and as long as 6 to 12 months in immunodeficient patients^[17].

MOLECULAR ASSAY

The principle of molecular assay based on detecting and quantifying HCV RNA and determines the HCV. Molecular assays for hepatitis C can be divided into three general categories: (1) tests that detect the presence or absence of the HCV RNA genome in patient plasma or serum (eg, qualitative HCV RNA tests); (2) tests that assess the quantity of HCV RNA in the blood, otherwise known as the viral load (eg, quantitative HCV RNA tests); and (3) tests that determines the genetic nature of HCV (eg, HCV genotype tests). Patients who test positive for HCV RNA in the blood are known to be actively infected by HCV and are at increased risk for developing significant liver disease^[6]. Viral genomes are generally present in relatively small amounts in body fluids of infected patients, hindering their detection by simple molecular hybridization-based techniques. Thus, their detection and quantification require a preliminary “amplification”

step. This can be achieved by using 2 categories of molecular biology-based techniques, namely target amplification and signal amplification. Qualitative detection assays are based on the principle of target amplification using “classic” polymerase chain reaction (PCR) or “real-time” PCR. Abbott RealTime (ART) and Roche Cobas TaqMan viral load assays for quantification of HCV genotypes in patient specimens were evaluated in previous studies. The ART HCV assay was a more sensitive and precise tool for accurate HCV viral load quantification across the HCV genotypes tested, especially genotype 1b. Recently, a new serologic test for the detection of the HCV core antigen in anti-HCV-negative serum or plasma has been developed and is now commercially available^[18,19].

Detection of HCV core antigen (Ag) assay

HCV has a number of structural and non-structural proteins including: 2 envelope glycoproteins (E1 and E2), the nucleocapsid protein (core-C) and several nonstructural (from NS2 to NS5) proteins. The mature HCV core protein is a 21-kd phosphoprotein made of the 191 first amino acids of the polyprotein. In the cytoplasm of infected cells, it is located in close vicinity to the perinuclear membranes and the endoplasmic reticulum, where it polymerizes in the presence of genomic RNA to form viral capsid. HCV core protein is antigenic, interacts with numerous cellular proteins and induces specific cellular and humoral responses and is detected in peripheral blood of HCV patients^[20,21]. The HCV Ag was detected prior to the appearance of anti-HCV in the patients' sera to reduce of the window period by 23 d or even longer. The window period between infection and seroconversion remains a dramatic problem in the transfusional and diagnostic setting. Therefore, numerous of bioassays were developed for detection of free HCV core antigens especially for those with negative antibody test among acute cases of HCV^[22,23].

Many studies devoted HCV core Ag assay is useful as a screening strategy of HCV infection, distinguishes between current and cleared HCV infections, detect anti-HCV-negative with positive HCV RNA cases and mark HCV replication. Prior to seroconversion, core antigen is generally detected 1 or 2 d later than HCV RNA^[24]. One of the problematic views for HCV core antigen detection, structural amino acid changes may alter the antigenic properties of the proteins, thus allowing the virus to escape neutralizing antibodies^[25].

An automated chemiluminescent immunoassay was developed to quantify and determine HCV core antigen (Ag) in human sera or plasma to clarify the relationship between HCV Ag and RNA. HCV Ag was determined by the Abbott Architect i2000SR analyzer using HCV Ag assay kit, it was easy testing, overcome shortcomings of the conventional core Ag assays, reduce the period between exposure and detection of 35.8 d^[26]. Such Ag assays are required in future to direct diagnosis for HCV infection, to monitor therapeutic response, and to evaluate the control mass drug treatment decisions. HCV core antigen was detected with an HCV Ag ELISA assay. This assay is based on a two-step sandwich principle. Briefly, murine monoclonal antibodies recognizing different epitopes of HCV core antigen were coated on microwells of the solid phase and were conjugated with horseradish peroxidase^[18].

HCV immunoblot assays

Recombinant immunoblot assays (RIBA) consist of recombinant or synthetic HCV proteins coated onto plastic strips that are then exposed to the patient's serum. RIBA can discriminate between true and false- positive results for samples repeatedly reactive by enzyme-linked immunosorbent assay (ELISA). These strips may also contain non-viral antigens that may be co-expressed during the synthetic

process for getting antigens to capture the anti- HCV to control false positive ELISA^[27]. Different generations RIBA test cannot distinguish between a current or past infection. The recombinant immunoblot assay take part in establishment of the cause of a positive anti-HCV immunoassay in a person with undetectable HCV RNA. The ability of serum antibodies to recognize all HCV quasi species is restricted, and neutralizing anti-HCV antibodies have not yet been identified^[28,29]. Genome analyses are needed to facilitate understanding viral diversification with respect to disease mechanisms.

Nucleic acid amplification technology (NAT):

Detecting HCV RNA levels is urgent for integration of diagnosis and management programs for (1) reducing "window period" for detection of HCV and getting further and earlier screening for HCV patients (2) evaluation of patients with positive anti-HCV antibodies categories to confirm the early HCV infection (3) marking response or relapses for treatment programs (4) measurement of the viral load during the start of treatment programs (5) qualitative detection of HCV RNA specially at the end of treatment programs^[30].

The NAT is directed to be qualitative assays or quantitative tests. The qualitative and quantitative tests used the same procedures for hybridization and colorimetric detection of the hybridized amplification products^[31].

Qualitative PCR assays of HCV NAT

Qualitative NAT detects the presence or absence of HCV-RNA for reporting as positive or negative in serum or plasma within 1–2 weeks after exposure to the virus and much sooner than serologic tests. Qualitative NATs are also up to 100 folds more sensitive than techniques in the last decades. Assays were developed for direct capture of 5' UTR HCV RNA in clinical samples. They use magnetic particles coated with oligonucleotides to capture specific nucleic acid targets^[32].

The most common methods available for detecting and quantifying HCV RNA based on PCR, signal amplification technique (branched DNA and Versant HCV RNA. Two semi-automated PCR assays, the complete bioanalytical system (COBAS Monitor Amplicor HCV 2.0) and the LCx HCV RNA assay (Abbott), are commonly used by many laboratories.

COBAS AMPLICOR HCV 2.0

A semi-automated, PCR-based assay for HCV RNA detection and marketed as the "complete bioanalytical system" (COBAS). It is an *in vitro* diagnostic nucleic acid amplification test for the qualitative detection of HCV RNA in human serum or plasma. This test is run on the COBAS® AMPLICOR Analyzer, which automates PCR amplification and detection of HCV RNA. Amplicor HCV version 2.0 assay has a reported low limits of detection of 50 IU/mL. It is recommended for patients who have evidence of liver disease with positive antibody test and who are suspected of being actively infected with HCV. Positive results indicates HCV replication and therefore is an evidence for active HCV infection. The performance of the second version of this assay ("HCV Monitor 2.0 assay") have been analyzed with the dynamic With the dynamic quantification up to 500 000 mL

Siemens VERSANT HCV RNA Qualitative Assay

It based transcription-mediated amplification (TMA) as an isothermal nucleic-acid-based method and can amplify HCV-RNA targets a billion-fold in less than one hour's time RNA in clinical samples. The

TMA consists of three steps (1) target capture (2) target amplification (3) specific detection of target amplicons by hybridization protection assay. It relies on the enzymes reverse transcriptase and T7 RNA polymerase to generate detectable levels of RNA product from either an RNA or a DNA template. The TMA is able to detect HCV RNA in some patients who had no detectable HCV RNA by the 1st and 2nd versions of HCV PCR) at the end of treatment and subsequently experienced virologic relapse^[34,35]. In PCR-negative samples, positive TMA results may indicate the presence of low levels of HCV RNA. Patients with positive TMA results may achieve sustained virologic response, management decisions during therapy should not be based upon a single positive TMA test result^[36].

Real-time quantitative reverse PCR

RT-PCR allows the quantification on the early exponential phase of the reaction. It relies on threshold cycle (Ct) values determined during the exponential phase of PCR rather than endpoint. Thereby, RT PCR detects amplicon synthesis and deduce the number of viral genomes in the starting clinical sample during the PCR rather than at the end^[37]. A pair of primers and a probe was designed for the recognition of a highly conservative 5'- non-coding region (5' -NCR) in HCV genome. The antisense primer contains a biotin residue at the 59-terminus to facilitate nonradioactive detection of amplification products. HCV RT-PCR assay had a sensitivity of 1000 RNA copies per reaction, with a dynamic range of detection between 103 and 107 RNA copies.

Quantitative assays of nucleic acid amplification

Quantitative NATs give HCV RNA loads in international units with appropriate calibrated panels; 800 000 IU/ML corresponds to 2 million viral genome copies/mL. The National Institute for Biological Standards and Controls and WHO have established an international standard for universal standardization of all HCV RNA quantification units for detection and evaluations in international units (IU). Standardization of HCV RNA assays to IU is based on genotype 1 panels. Several assays have been developed for quantitation of HCV RNA including competitive reverse transcription PCR. Estimated field trails between 1999-2011 showed quantitative tests are used in evaluation of patients being considered for therapy programs and to assess early response to therapy. Viral load testing has been used in early assessment of treatment response^[38-43].

Advance in practical Approach RT-PCR for HCV

Greater sensitivity is required, because some chronic HCV patients have viral loads below 1 000 copies/ml. Nevertheless, new real-time PCR-based assays have been developed for a broad dynamic range for quantification. This sense clinical laboratories to use RT PCR-based methods for estimation of HCV infection based on many criteria; (1) they can detect very minute amounts of HCV RNA (down to 10 (IU)/mL); (2) accuracy of quantitation of HCV RNA reaches levels up to approximately 107 IU/mL; (3) the dynamic range of quantification adequately covers most clinical needs for diagnosis and monitoring programs; (4) specificity, precision and reproducibility are effective and (5) they offer opportunities for high throughput and automation^[44]. Two standardized HCV RNA quantitative assays based on real-time PCR were recently developed for lab diagnosis (Abbott Real Time HCV and Roche COBAS Taq Man HCV)^[45,46]. Both tests target the 5'-NTR in all genotyping with the exception of Abbott HCV RNA ASR test, in which 1a and 1b are differentiated using SNPs within NS5B region^[47].

Abbott Real Time HCV assay

It gives a wider dynamic linear range of quantification 12 IU/mL to 100 million IU/mL. Principally, this test is used with the Abbott m2000sp and m2000rt automated analyzer. TaqMan measurements for most samples and showed that the RT PCR assay is able to detect all genotypes with no bias. The primers and probe sequences target conserved regions in the 5' untranslated region of the HCV genome. The assay uses a linear probe with a fluorophore at the 5' end and a quencher at the 3' end^[48]. In the absence of target, the probe self-quenches by random coiling. In the presence of target, the probe anneals, separating the fluorophore from the quencher. Fluorescence is generated primarily by probe hybridization, since the read temperature for detection is quite low (35°C). A significant step forward with the RT PCR system includes increased quality measures to aid in the assessment of the accuracy and reliability of viral load determinations at the time of verification and as a long-term tool to assess clinical testing with no significant genotype bias. The Real Time assay uses novel data reduction algorithms that allow analysis of results without user interaction or inspection. The data reduction encompasses several validity checks and transformations, including raw fluorescence thresholds, baseline correction, slope correction, normalization, internal control evaluation, reaction efficiency evaluation, and an innovative method of determining the crossing threshold. Real Time HCV assay proved to be suitable for use in the routine diagnostic laboratory^[49].

COBAS TaqMan HCV test

The TaqMan HCV Test is a real-time nucleic acid amplification assay for the quantitative detection of HCV RNA in human serum or plasma. It is fully automated nucleic acid isolation and quantification, and ensures a broad dynamic quantification range (43 to 69 000 000 IU/mL). It is composed of two products, Roche COBAS AmpliPrep and COBAS TaqMan HCV assay (CAP/CTM). It based automated reverse transcription (RT)-PCR amplification and real-time detection of cleaved dual fluorescent dye-labeled oligonucleotide (TaqMan) probes that allow the specific and simultaneous detection and quantitation of the target sequence as well as an internal HCV quantitation standard with the CTM 48. The TaqMan-based CAP/CTM test has been successfully used in clinical practice and in drug development trials and has been approved by European and U.S. authorities for the monitoring of anti-HCV therapy^[50]. This has prompted the development of an upgraded, second-generation assay that could provide an improved genotype inclusivity and workflow. The CAP/CTM v2 accurately quantifies HCV RNA across all HCV genotypes, including specimens with rare polymorphisms previously associated with under quantification^[51].

CONCLUSION

It is the time to unite the effort and expertise in this area of urgent work to provide spending money and wasting time to fight the HCV disease and its risk. Estimated endemic area requires real monitoring programs for diagnosis of HCV supported by the health authorities donor. It has become an urgent need for unique of molecular techniques used RT-PCR for detection of HCV RNA and immunological assays based hepatitis C core Ag detection tools. Recent well designed studies have explored the applicability of the hepatitis C core Ag, as a substitute and/or conformational for measuring HCV RNA levels.

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