

Current focus

Current and future hepatitis C virus diagnostic testing: problems and advancements

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Abstract

Serological antibody assays used in hepatitis C virus diagnosis have improved in sensitivity and specificity. However, detection of active viremia or monitoring levels of virus during or after patient treatment is most commonly undertaken using nucleic acid-based technologies. Advancements in diagnostic technologies and implications for managing patients with hepatitis C in various clinical settings are discussed. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Introduction

It has been estimated that 170 million people worldwide are currently infected with the hepatitis C virus (HCV) [1]. In the US alone, 3.9 million people are infected with HCV and up to 10 000 deaths a year are due to HCV-associated chronic liver disease. Transmission is mainly associated with infected blood products or intravenous drug abuse, although other less common routes such as vertical or sexual transmission are reported [2]. Serological testing of HCV antibody in individuals has improved in both sensitivity and specificity in recent years (discussed below). However, the risk of post-transfusion HCV in the USA and Western Europe is still estimated at one per 100 000 and per 400 000, respectively, and is attributed to the seronegative 'window' period of HCV infection.

The HCV genome is composed of a large open reading frame (ORF) encoding a polyprotein of 3011 amino acids. Computer analysis of nucleotide and deduced polyprotein sequence demonstrated regional genomic amino acid sequence homologies to Flaviviruses and Pestiviruses. Upstream of the ORF, a 341-nucleotide 5'-untranslated region

(5'UTR) has been shown to be similar in length and putative secondary structure to an analogous region in Pestiviruses. The most conserved region of the HCV genome with almost 90% sequence similarity amongst HCV genotypes, the 5'UTR has been a focus of interest for HCV molecular diagnostic technologies. Downstream of this region three HCV structural proteins and several non-structural proteins are processed from the polyprotein by host and viral proteinases, respectively. Comparisons of HCV isolate sequences through the generation of hydrophilicity profiles and Kernel density analysis located conserved antigenic regions, revealing up to 14 candidate B-cell epitopes along the genome [3]. The immunogenic potential of these epitopes was tested against patient sera using short synthetic peptide-based enzyme-linked immunoassay (EIA), and through the expression of recombinant proteins. The development of these findings into commercial HCV antibody screening (anti-HCV) and supplemental antibody assays is outlined in this review. Anti-HCV assays are useful for diagnosing exposure to virus, but provide no evidence of active viremia or identification of infected individuals in the antibody-negative phase. The need to diagnose active viremia and monitor levels of virus during or after patient treatment is undertaken using nucleic acid-based technologies. This review examines advancements in serological and nucleic acid-based assays used in HCV diagnosis and therapeutic monitoring. The implications for managing patients with hepatitis C in various clinical settings are discussed.

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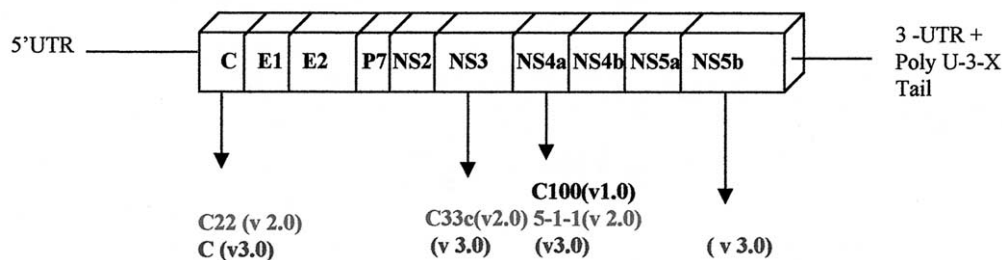


Fig. 1. Outline of the HCV genome and location of recombinant antigens used in EIAs. The genomic origins of recombinant proteins used in HCV antibody testing by EIA and supplemental RIBA are shown. The first-generation tests (v1.0) contained only a single antigen (C100). The implementation of multiple antigens in second- (v2.0) and third-generation (v3.0) assays improved the sensitivity and specificity of HCV antibody detection.

2. Humoral responses to HCV proteins and serological diagnosis

The first HCV serological diagnostic test contained an epitope (c100-3) from the non-structural 4 (NS4) region (Fig. 1). Antibodies to c100-3 (anti-c100-3) were detected in a standard enzyme immunoassay (EIA) format, and in 80% of patients with post-transfusion hepatitis. The delay for detecting anti-c100-3 antibodies ranged from 6 weeks to 6 months after the onset of acute hepatitis, and antibodies persisted during chronic hepatitis [4]. This first-generation test was extremely useful in reducing the risk of post-transfusion-associated HCV infection. However, the lengthy seronegative window period in some donors resulted in transfusion-associated transmission of HCV in antibody-screened blood.

The implementation of newer generation multi-antigen EIA (versions 2.0 and 3.0) and recombinant immunoblot assay (RIBA) resulted in improvements in the sensitivity and specificity of HCV antibody testing [5]. The EIA-2 and RIBA-2 tests contained recombinant polypeptides from the immunodominant regions of core, NS3 and NS4. The long antibody-negative period associated with the first-generation test was reduced by an average of 5 weeks in post-transfusion patients by using EIA-2. On closer examination of the immunodominant regions of the core, NS3 and NS4 using a semi-automated dot blot system, it was shown that IgM anti-core is elevated during the acute phase in post-transfusion hepatitis C [6]. However, anti-HCV IgM is also elevated in a large proportion of patients with chronic hepatitis C, making this test not useful for diagnosis of acute infection. On average, anti-core was detected at 4–8 weeks, 1–3 weeks earlier than NS3 or NS4 following acute infection.

The RIBA-2 assays contained the same HCV antigens as in the EIA screening tests, and therefore, they were not considered to be confirmatory assays, but rather supplemental to EIAs. This immunoblot assay is described in detail elsewhere [7]. In the EIA version 3.0 test, reconfigured core antigen has led to a very modest increase in sensitivity, although in the majority of cases there was no difference in time to seroconversion using EIA3.0 compared to EIA 2.0 [5].

The core protein is the most conserved viral antigen amongst HCV types and, was therefore, a likely antigenic probe in the EIA. Studies have demonstrated two major B-cell epitopes at the N-terminus of core: amino acids (aa) 5–23 and 39–74 using peptide reactivity to sera from patients with chronic HCV [8]. Additional antibody binding sites were described via fine specificity studies in humans and chimpanzees, lying within the N-terminal aa 25–42 and at aa 107–114 [9]. Epitope mapping using branched oligopeptides revealed two major antigenic determinants in NS4: amino acid residues (aa) 1691–1708 and 1710–1728. There was only 50–60% aa sequence homology between types and limited serological cross-reactivity; therefore, type-specific reactivity formed the principle component of the natural humoral response to NS4.

The NS3 recombinant protein included in EIA-2 (c33c) is an antigen covering the carboxyl-terminal two-thirds of the NS3 protease region. Peptide studies could not determine anti-NS3 responses as defined for NS4 or core, suggesting that B-cell responses to NS3 were to conformational or discontinuous epitopes [10]. The reactivity of monoclonal antibodies to the C-terminal one-third of NS3 illustrated a close association with viremia as assessed by genomic detection of HCV RNA by the polymerase chain reaction (PCR). However, isolated reactivity to NS5 antigen has not been associated with detection of HCV RNA (Gretch et al., unpublished data).

2.1. Clinical significance

The EIA tests are considered highly sensitive for detecting active HCV infection. In high-prevalence settings, the EIA-2 test correctly identifies 95% of specimens from patients with active HCV viremia, compared to 97% sensitivity for EIA3.0 [5]. Furthermore, 93% of specimens positive in the EIA-2 test are also positive in the RIBA-2 test, with 6% indeterminate and 1% negative (compared to 83, 7 and 10%, respectively, for EIA-1). False negative EIA testing is primarily associated with immunosuppression in high prevalence settings. This high positive predictive value and specificity of the EIA in high-risk populations has eliminated the requirement for RIBA supplemental testing. However, in low-risk populations, 40–50% of EIA-2-

Table 1
Diagnostic assays for HCV RNA detection

Assay	Use	Sensitivity	Upper range
RT-PCR (in-house)	Qualitative, confirming viremia	100 copies per ml (40 IU/ml)	Not applicable
Roche Amplicor (v 2.0)	Qualitative	100 copies per ml (40 IU/ml)	Not applicable
Roche Monitor (v 2.0)	Quantitative	700 copies per ml (300 IU/ml)	500 000 copies per ml (200 000 IU/ml)
Roche COBAS 2.0 System	(i) Qualitative (ii) Quantitative	(i) 100 copies per ml (40 IU/ml) (ii) 1500 copies per ml (600 IU/ml) ^a	(ii) 12.5×10^5 copies per ml (500 000 IU/ml) ^a
Quantiplex bDNA v 2.0 (Bayer)	Quantitative	200 000 eq./ml (33 000 IU/ml)	120 000 000 eq./ml (20 000 000 IU/ml)

Listed are commonly used tests to detect HCV RNA. Qualitative testing is highly sensitive and used to confirm active viremia. Quantification of serum or plasma viral load is dependent upon quantitative testing. Assays can differ in sensitivity and upper range. The limits shown in the above table are based upon findings in our laboratory unless otherwise stated. The conversion factors between copies per ml and IU/ml are given in the text.

^a According to manufacturer's protocol.

positive specimens are still RIBA-2-negative. Supplemental testing by RIBA may be warranted in low-risk groups to eliminate false positive testing by EIA. However, confirmatory HCV RNA testing (see below) is a rational and cost-effective alternative given the high correlation between RIBA positivity and PCR positivity, and the fact that HCV RNA status defines state of infection.

In the blood donor setting, supplemental RIBA testing is routinely performed on all anti-HCV-positive patients. In this setting, RIBA-3 is more specific than RIBA-2, since it gives less indeterminate results and correlates better with detection of HCV RNA by PCR. In an attempt to further improve the safety of the blood supply, routine screening of blood donors by reverse transcription-PCR (RT-PCR) has recently been incorporated and has identified viremic blood donors (0.001%) who lack antibodies to HCV using current serological tests [11]. This lack of anti-HCV may be due to non-seroconversion or sero-reversion (for example in patients with HIV infection or in immunosuppressed transplant recipients) or to undetermined host or viral genetic factors.

More recently, 'window-phase' blood donations have been evaluated using prototype antigen-based EIA for the core protein [12]. Sera that were EIA-3-negative but RNA-positive were reacted to monoclonal antibodies to core antigen coated on microtiter plates. Core antigen was detected in 94% of window-phase plasma donations. The

antigen was detectable 1–2 days after the appearance of RNA and during the antibody-negative period of infection. These results highlight the potential usefulness of antigen-based EIA to complement traditional serological assays, but further clinical evaluations are required.

3. Molecular diagnostics

Molecular diagnostic approaches have utilized the discovery of genetic enzyme systems involved in nucleic acid replication and repair. As an example, the discovery of the PCR became recognized as a highly sensitive technique for detection and amplification of target sequences in nucleic acids. The extremely high affinity and specificity of nucleic acid hybridization have allowed for the development of nucleic acid assays that exceed the sensitivity of antibody-based technologies. The linking of these assays with appropriate detection systems, therefore, makes them highly desirable for detecting HCV RNA in patient samples. Tables 1 and 2 summarize current and new HCV molecular diagnostic assays, respectively.

3.1. Qualitative nucleic acid diagnostics for HCV RNA

HCV RNA detection by RT-PCR is widely used to confirm HCV diagnosis and for assessing viremia in pa-

Table 2
New technologies for HCV RNA quantification

Assay	Use	Sensitivity	Upper range
Quantiplex bDNA v 3.0 (Bayer)	Quantitative	E4800 copies per ml (800 IU/ml)	48 000 000 eq./ml (8 000 000 IU/ml)
TMA	Qualitative	10 copies per ml	Not Applied
TaqMan™ Real-Time PCR System	Quantitative	Adaptable down to 10 copies per ml	10^9 copies per ml
LightCycler™ Real-Time PCR	Quantitative	1000 copies per ml (SYBER I)	10^7 copies per ml (SYBER I)
NASBA	Quantitative	3000 copies per ml	

The above technologies have been described recently for detection of HCV RNA. Studies with these tests have demonstrated increased limits of sensitivity and also, for real-time PCR assays, wider linear ranges of RNA quantification. The Quantiplex bDNA v3.0 has significantly improved in sensitivity compared to bDNA 2.0 and results can be reported in copies per ml or IU/ml. The sensitivity and upper range shown above for bDNA 3.0 are based on findings in our laboratory.

tients during and following antiviral therapy. Laboratories and manufacturers have designed individual assays comprising RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification and detection of PCR amplicons. The most sensitive and optimized PCR assays report detection of HCV RNA in patient's serum at concentrations of less than 100 copies per ml. Optimization and performance of RT-PCR assays have been reviewed in depth previously [13].

One area where significant progress has been made is in the isolation of viral RNA from patient serum. Commercial kits have been designed which attempt to ease the isolation and purification of viral nucleic acids in the clinical setting. Chaotropic solutions are used to release nucleic acid from virions and denature proteins, a process often augmented by proteinases. The separation phase of nucleic acid from other materials is achieved by temporary non-specific adsorption to a matrix of silica particles (often in the form of disposable spin columns). High salt concentration washes are used to remove proteins and low-molecular-weight compounds, and the subsequent application of a low salt concentration buffer elutes the purified nucleic acid. The recovery rates of extraction and purification of HCV RNA using commercial kits are variable. The efficiency of extraction is improved using proteolytic enzymes, carrier nucleic acid and RNase-free reagents [14].

Recently, oligonucleotide-based capture technologies have been described which appear to be faster, more efficient and more suitable for automation [15]. Generally, these capture methods use magnetic particles coated with oligonucleotides to capture specific nucleic acid targets. A commercial kit designed by Gen-Probe utilizes multiple oligonucleotides complementary to the 5'UTR of HCV as well as the pol and 3' long-terminal repeat regions of HIV. Multiplex systems detecting more than one virus in a patient sample are of particular interest in the blood-screening setting.

The HCV Amplicor test kit (Roche Molecular Diagnostics) was recently approved by the FDA and is a widely used automated qualitative assay for diagnosing HCV RNA in patient sera. RNA is isolated and subjected to RT-PCR in a single tube. One of the primer pairs is labeled with biotin, and this allows for amplicon hybridization to HCV-specific probe coated on microwell plates, followed by colorimetric signal detection. Version 1.0 of this test had a lower analytical sensitivity (700 copies per ml as described by the manufacturer) compared to the best in-house assays. However, Amplicor 2.0 compares better in sensitivity to in-house assays (100 copies per ml). This improved sensitivity is suggested to be as a result of optimized RT-PCR master mix, which ensures better denaturation of the target, and more efficient annealing and extension of primers. Roche has now developed a fully automated Amplicor system termed the complete bioanalytical system (COBAS) for HCV RNA testing. Compared to the manual Amplicor test, there was comparable sensitivity, specificity, positive/

negative predictive values and a significant reduction in labor time.

More recently, Bayer Diagnostic laboratories have applied transcription-mediated amplification (TMA) to detect HCV RNA (Fig. 2). The TMA technique combines target-capture, the amplification of RNA and detection in a single tube. In the first step, released RNA is captured by a capture-oligonucleotide, and the viral-probe complex is bound to magnetic microparticles. RNA is then purified through several washes and aspirations to remove potential assay inhibitors. The isolated RNA is subjected to TMA, an isothermal, autocatalytic target amplification method that makes use of reverse transcription and amplification of RNA transcripts by T7 RNA polymerase (T7 RNA pol). A primer containing a T7 promoter sequence hybridizes to a target RNA (or internal control RNA), and cDNA is synthesized by reverse transcription. Another primer binds to the cDNA-containing promoter sequence, and a second DNA strand is synthesized by reverse transcriptase. The T7 RNA pol binds its promoter sequence in the double-stranded DNA molecule, and numerous RNA transcripts are synthesized. These RNA molecules serve as templates in the next round of TMA replication, and the result is an exponential amplification of RNA molecules. Detection is carried out using a hybridization protection assay with amplicon-specific acridinium ester-labeled DNA probes. DNA probe hybridized to RNA is protected from alkaline hydrolysis and retains its specific chemiluminescence measured in a luminometer. Two separate acridinium ester chemiluminescent signals are used; one emits a 'flash signal' and is linked to the internal control probe, while the target probe emits a 'glow signal'. The increased clinical sensitivity of this assay was reported in a study, which detected HCV RNA in patients who had a complete virologic response to interferon treatment as defined by PCR [16]. HCV RNA was detected by TMA in end-of-treatment serum samples from patients who were HCV-negative by Amplicor and had later relapsed. The TMA detection limit was 10–50 HCV RNA copies per ml. This sensitivity may in part be due to the extraction of larger volumes of serum (500 μ l in the TMA assay compared with 200 μ l in Amplicor HCV 2.0).

3.2. Quantitative molecular tests

The level of HCV RNA circulating in the serum or plasma of a patient is termed the serum (or plasma) viral load. A variety of technologies have been developed and reviewed previously in detail [5,7,17]. These assays differ in the level of sensitivity, linear range of quantification of viral load, specificity, reproducibility and bias to HCV genotype. Current commercial assays vary in performance but in general have advanced to meet most of the needs for monitoring viremia.

The quantification of HCV RNA in patient sera was previously complicated by the lack of an international reference standard. Assays reported HCV levels in different

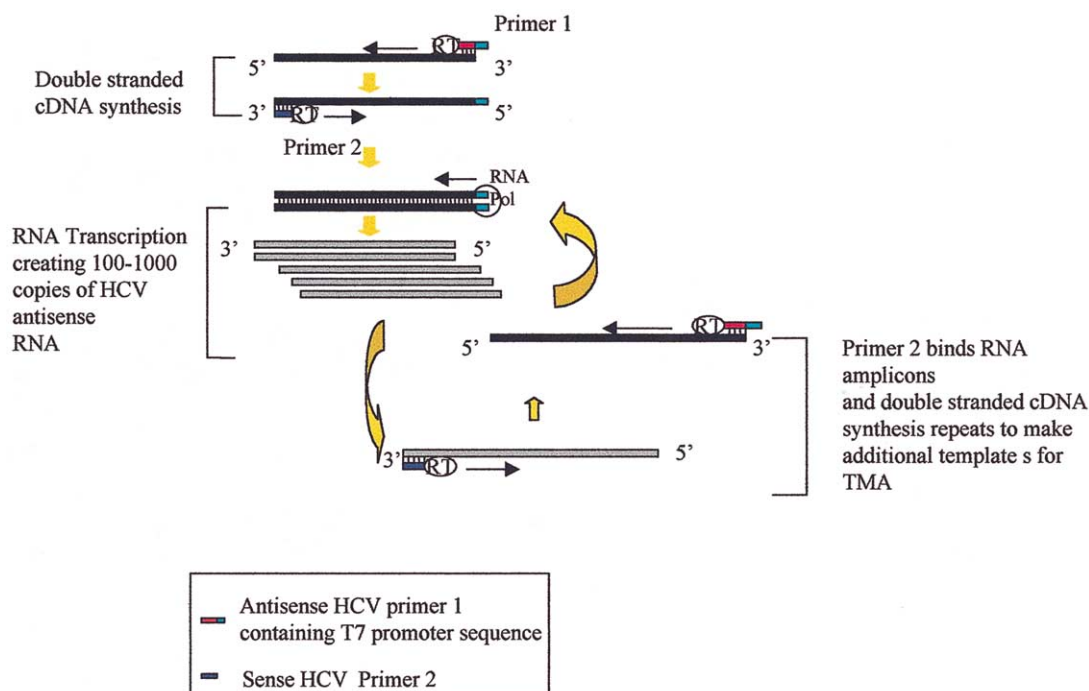


Fig. 2. Schematic representation of transcription-mediated amplification (TMA). The molecular processes for HCV RNA TMA amplification are demonstrated. Reverse transcription of extracted HCV RNA results in a double-stranded complementary DNA (cDNA) molecule synthesized using an HCV antisense primer (containing a T7 promoter sequence), and a second sense HCV primer. The enzyme T7 RNA polymerase (RNAPol) binds to its promoter sequence, and numerous RNA transcripts are synthesized, serving as templates for next round of TMA amplification. In current test formats, the amplified RNA and internal control RNA are detected by hybridization to acridinium ester-labeled DNA probes. Hybridized products are protected from alkaline hydrolysis, and the DNA probes retain their chemiluminescence 'signal type' measured in a luminometer.

units, making comparisons of results from different laboratories impossible. Recently, a WHO international standard for HCV RNA quantification has been established and will be used to calibrate reagents for HCV RNA quantitative testing [18]. This standard was prepared from pooled patient serum and was assigned a concentration value of 50 000 IU/ml. Lyophilized aliquots of this HCV standard are made available in an attempt to provide a reference standard whereby various tests can report in comparable units. One IU of HCV RNA is equal to approximately 6 eq. using the Bayer bDNA (2.0) technology described below (Gretch, unpublished) or 2.5 copies using quantitative PCR assays [19].

3.2.1. HCV Quantiplex bDNA assay (Bayer Diagnostics)

A commonly used automated signal amplification method for viral nucleic acid quantification is the HCV branched DNA assay (bDNA). This assay has now gone through three generations with increased clinical sensitivity, linearity and genotype independence at each phase [20,21]. RNA isolation is based on crude lysis of patient plasma or serum using proteinase K digestion. The released viral nucleic acid is hybridized to oligonucleotide target capture probes that are homologous to the 5'UTR and core region of the HCV genome. These hybrids are captured onto oligonucleotide-modified microwells, followed by further hybridization to bDNA amplifier molecules. Signal of the

captured HCV RNA is enhanced by binding of multiple alkaline phosphatase-labeled oligonucleotide probes to the bDNA amplifier molecules. Chemiluminescent detection is measured on a plate luminometer using a dioxetane substrate. The concentration of HCV RNA is measured against a standard curve using RNA standards. The sensitivity of the second-generation bDNA assay (bDNA 2.0) was limited to approximately 200 000 eq./ml, which is roughly 33 000 IU/ml, and an upper limit of 120 000 000 eq./ml or 20 000 000 IU/ml. Intra-assay and inter-assay coefficients of variation were less than 10 and 25%, respectively [22]. Preliminary studies in our laboratory with bDNA 3.0 confirmed an increased sensitivity down to 800 IU/ml, with an upper limit of 8 million IU/ml and with greatly improved specificity at the low end. The manufacturer reports that the newer generation bDNA 3.0 is accurate for all six HCV types; however, this needs independent confirmation. The sensitivity (800 IU/ml) of bDNA 3.0 still limits its use for detection of low-level viremia, which requires RT-PCR- or TMA-based strategies.

3.2.2. Quantitative target amplification methodologies based on PCR

Quantitative in-house RT-PCR assays have been described in detail [13,17]. In endpoint dilution, samples (serum, RNA or cDNA) are diluted in series and tested by qualitative PCR or RT-PCR. The last positive dilution

(endpoint) is used to calculate starting nucleic acid concentration. This highly sensitive methodology can be used to detect single-copy or absolute cDNA. However, in practice, endpoint PCR is subject to assay variables, making it inappropriate for high-throughput clinical settings. Reliability of results requires a broad dilution series of samples tested in multiple, thereby making the assay labor intensive and costly. In addition the assumption that all steps are equally as efficient is likely not to hold in practice; inefficient cDNA synthesis may underestimate starting RNA concentrations.

Quantitative competitive PCR (QC-PCR) is a technique in which a known amount of internal control (DNA or RNA) is added to the specimen prior to testing. The reagent competitor has some overlap with the target sequence, but is designed in a way (for example different molecular weight) so that it can be distinguished from wild-type HCV RNA. A major problem with QC-PCR is the fact that size or sequence of the competitor RNA or cDNA may not be equal, resulting in differences in amplification efficiencies. In addition, DNA competitors do not account for differences in both extraction and reverse transcription efficiencies of target RNA. These in-house methodologies have high analytical sensitivities, but their high variability and lack of standardization has limited their use in clinical settings.

3.2.3. *Amplicor Monitor (Roche Diagnostics)*

The Roche Amplicor Monitor assay is a standardized quantitative PCR (Q-PCR) kit available commercially for HCV, which is currently in its second version (v 2.0). A quantitation standard (QS) is co-amplified with target to monitor the efficiencies of extraction and amplification reactions. The non-infectious QS transcript contains identical primer binding sites as the HCV RNA, but has a unique probe binding site that allows distinction from HCV amplicon. RT-PCR of both target and QS occurs simultaneously in a single tube using the enzyme *Thermus thermophilus* DNA polymerase (rTth pol), which performs both reverse transcription and PCR. Both the sample DNA and competitor are detected by hybridization to biotin-labeled probe, followed by incubation with enzymatically labeled streptavidin and detection in a colorimetric assay.

There are now two variations of the assay, the Monitor version 2.0 test and a semi-automated COBAS Monitor version 2.0 test. Initial studies looking at the first-generation Monitor assay illustrated a sensitivity of approximately 1000 copies per ml (300 IU/ml). However, version 1.0 Monitor underestimated viral loads in genotype 2 and 3 patients when compared to genotype 1 [23]; this genotype bias has been corrected in the version 2.0 test (Gretch, unpublished).

The dynamic range for both versions of the Amplicor Monitor test extends from about 300 IU/ml for the lower range to 200 000 IU/ml for the higher range, an approximately 3 log linear range [24]. The assay is non-linear at

viral titers above 500 000 copies per ml, requiring routine test specimens to be diluted 100-fold prior to quantitative PCR.

The automation of Monitor test has improved its coefficient of variation (CV), with intra-assay and inter-assay variabilities of 22 and 35%, respectively, compared to 20–60% CVs shown by the first-generation manual version [25]. The Monitor Q-PCR assays are more sensitive than bDNA, but the inherent limitation is the lower dynamic range and high experimental variability even with automated versions. In the COBAS format, the amplification step, amplicon dilution, detection and quantitation are automatically performed by the COBAS analyzer. Although there have been improvements in master mix to optimize reaction processes, the quantification of final PCR products may be influenced by plateauing or saturation effects of reagents influencing final read-out.

3.2.4. *TaqMan™ real-time quantitative RT-PCR*

The TaqMan chemistry and ABI Prism 7700 (Perkin-Elmer Corporation and Applied Biosystems) has been described for quantification of HCV RNA [26,27]. RT-PCR detection is achieved with an oligonucleotide probe labeled with a fluorophore and fluorescence quencher that, upon binding to the PCR product, undergoes quencher removal by the 5'-3' exonuclease activity of *Taq* DNA polymerase. During the PCR, the reporter signal increases above a background fluorescence (normalized by a passive reference dye) termed the cycle threshold. This point is represented by the exponential log phase of PCR product accumulation. Standard curves can be designed using known amounts of RNA or DNA to which test samples can be compared and viral load calculated.

Initial studies have illustrated significant improvements compared to available bDNA 2.0 and Roche Monitor 2.0 testing; higher sensitivities, wider linear ranges of quantification, simplicity and reproducibility make the system ideal for high-throughput screening. The wide dynamic range makes the assay suitable for monitoring viral load before and during therapy. Martell et al. describe a 5 log dynamic range (10^3 – 10^7) with CVs of 1 and 6% for intra- and inter-assay reproducibility, respectively.

We have also examined the use of real-time PCR in the clinical setting, using the rTth DNA pol, which has dual activities of reverse transcription and PCR. This offers increased RT efficiency and increases the potential sensitivity of the PCR as compared with *Taq* DNA amplification alone. There was an excellent correlation coefficient between RNA quantity and cycle threshold ($r = 0.99$). The assay was highly sensitive (10 copies per ml), had a wide linear range (10^1 – 10^9 copies per ml) for quantification of synthetic RNA standards, and a 6 log linear range using dilutions of patient sera. Quantitative values correlated well with bDNA 2.0 testing ($r = 0.747$). As in any PCR format, essential negative controls are required to ensure that there is no contamination; low positive cycle thresholds can be

checked using additional software functions that dissect the spectra of all dyes (multi-component) in a reaction. The assay, is therefore, highly sensitive, extremely dynamic and adaptable for high-throughput monitoring of patient viral titers during management.

3.2.5. SYBER green I dye and LightCycler™ fluorimeter real-time PCR technology

As in the TaqMan™ technology, the LightCycler™ (Roche Diagnostics) is designed to decrease the time needed to achieve PCR results by monitoring amplification of target sequences in real-time by a fluorimetric assay. There are two detection systems; SYBER green I dye which binds double-stranded DNA and the use of probes labeled with different fluorophores. Probe hybridization to target results in an increase in fluorescence resonance energy transfer, which is measured by the fluorimeter. Studies with HCV RNA quantification have utilized the SYBER I green system [14,28,29]. Standard curves using 10-fold dilutions of synthetic RNA transcripts had dynamic ranges of 3.57–9.57 log RNA copies per ml [28]. Diluted sera ranging from 10³ to 10⁷ used to generate standard curves demonstrated the potential to quantify clinical specimens ranging from 10³ to 7 × 10⁶ copies per ml [29].

An important factor in confirming clinical specificity is the requirement to carry out a melting point analysis after the final PCR cycle of all samples and controls. This is to distinguish between target amplicon and any primer dimers or contamination in controls, since SYBER I green will bind double-stranded DNA non-specifically. Although further studies are required, this system offers a cheaper alternative to the TaqMan™ system. However, in a high-throughput setting, the TaqMan™ system may be more efficient, since after RNA isolation RT-PCR can be carried out in a single tube using the same rTth DNA pol reagents. The SYBER I format requires cDNA to be synthesized and then added to a PCR reaction. Furthermore, results must be checked for non-specific SYBER I binding.

3.2.6. Nucleic acid sequence-based amplification (NASBA)

Quantitative techniques based on isothermal amplification of HCV RNA and competitor standard RNAs have also been described [30]. NASBA (Organon) combines the qualitative TMA and quantitative competitive PCR strategies. The assay is formatted so that amplicons and standards are hybridized to oligonucleotide probe immobilized on streptavidin-coated paramagnetic beads by a biotin–avidin reaction. Wild-type and competitor RNAs are distinguished by hybridization with electrochemiluminescence probes labeled with ruthenium molecules and detected by a semi-automated electrochemiluminescence detection instrument. The amount of HCV RNA is automatically calculated from a standard curve obtained using three calibrators. The detection limit using 100 µl of serum is 3000 RNA molecules [23], which is not as sensitive as Q-PCR systems or real-time PCR.

4. HCV genotypes

The isolation, sequencing and comparison of HCV genomes from different parts of the world has demonstrated HCV genotypes that have up to 30% genetic variability and subtypes with 20–25% genomic diversity within isolates [31]. Several typing schemes have led to a consensus nomenclature describing six major HCV genotypes and multiple subtypes. The clinical significance of HCV genotypes is evidenced by the fact that patients with HCV genotype 1 respond less well to interferon therapy than patients with HCV genotype 2 or 3. Thus, determination of HCV genotype has become an important test for assisting clinicians with decisions regarding therapy.

4.1. Genotyping techniques

The gold standard for HCV genotyping is to amplify viral nucleic acid and perform direct nucleic acid sequence analysis of the region of the viral genome to be classified. Computer analysis can then be used to deduce phylogenetic relationships between viral isolates. Using this approach, identical results were achieved regardless of sequencing in the 5'UTR, core, E1, NS3 or NS5 genes. However, the expense and complexity of sequencing limits the technique on a large scale or routine clinical basis. Several alternative methods for HCV genotype determination have been described.

Genotyping HCV by PCR has proven somewhat useful for types and subtypes using type-specific oligonucleotide primers for core, NS5 and E1. However, the technique is prone to technical issues related to conventional qualitative PCR: contamination, optimization for sensitivity, specificity and finally standardization.

Restriction fragment length polymorphism (RFLP) analysis on the PCR products of 5'UTR and NS5 has also been employed to genotype HCV. Specific restriction endonucleases are used to cleave amplicons containing conserved mutations in these regions relative to other HCV genotypes. Therefore, only certain genotypes will be cleaved by specific restriction enzymes, and unique RFLP patterns will be observed on ethidium bromide-stained gels. Although useful in the high-throughput setting, limitations of the technique include PCR dependency and potential partial digestions of amplicons (and therefore, unclear RFLP patterns).

The INNO-LiPA HCV II (Innogenetics) is a DNA hybridization test which utilizes the binding of biotinylated PCR amplicons with type-specific DNA probes immobilized on nitrocellulose strips. Positive reactions are identified using a streptavidin-colorimetric reaction. The assay has improved in sensitivity compared to version 1 and is capable of discriminating between all six HCV genotypes and most subtypes. Clinical studies assessing reproducibility and specificity are ongoing.

Sequencing remains the only confirmatory gold standard for HCV genotyping, and a commercial direct-sequencing assay (Trugene 5'NC HCV Genotyping kit; Visible Genetics, Toronto, Canada) based on the 5'UTR has been developed. Initial studies suggest concordant results with Inno LiPA between types and a slight increase in the ability to discriminate between subtypes [32].

5. Monitoring HCV viremia: clinical applications

5.1. Establishing the diagnosis of hepatitis C

In the clinical setting, the diagnosis of chronic hepatitis C is often initiated by identification of a risk factor in a patient or by observation of an elevated serum aminotransferase level on routine screening. Alternatively, a patient may present with symptoms of acute hepatitis, or more alarmingly with symptoms of advanced chronic liver disease, such as ascites or variceal bleeding. In all cases, the first step in establishing the diagnosis of hepatitis C is anti-HCV testing by EIA. If the EIA is positive, and the patient has either a risk factor, an elevated aminotransferase level, or symptoms of hepatitis, confirmatory testing for HCV viremia by PCR or an equivalent test is indicated.

Supplemental testing by RIBA is non-informative in this setting, since 99% of cases are either RIBA-positive (93%) or indeterminate (6%). If the patient initially tests negative for anti-HCV by EIA, HCV RNA testing may be indicated if the patient is immunosuppressed or if there is no other identifiable cause for hepatitis.

In our experience at a University Hospital setting, approximately 5% of HCV RNA-positive specimens test negative in the screening EIA. On the other hand, 80% of EIA-positive specimens test positive for HCV RNA by PCR, while the other 20% presumably represent resolved infections. Typically, once chronic HCV infection is established, patients remain RNA-positive in serum on a continuous basis over long periods of time. It should be noted that 30–40% of patients with active HCV infection will have normal serum aminotransferase levels despite histological evidence of liver disease [33,34].

5.2. Pre-treatment evaluation of HCV RNA

Currently, the primary goals of treatment for hepatitis C are a sustained virologic response defined as persistently HCV RNA-negative following antiviral treatment which is clearly associated with improved liver histology. Present treatments for HCV infection include interferon (IFN) monotherapy, combination therapy with ribavirin and modified long-acting IFN-based drugs (Pegylated IFN) alone or in combination with ribavirin. Presently with IFN monotherapy, a virologic end-of-treatment response is achieved in

approximately 30–50% of patients. After discontinuation of treatment, a considerable proportion relapse, with sustained response rates at 24 weeks after the end of treatment being less than 20% [35]. Response rates are significantly improved with combination therapy, especially when pegylated IFN is combined with ribavirin.

Low pre-treatment HCV RNA levels, usually measured at a single baseline time-point, have consistently been shown to be an independent predictor of sustained response to interferon monotherapy [36]. The prediction appears to be also upheld with interferon plus ribavirin therapy. For example, sustained virologic responses were achieved in 50–94% of patients who had 2×10^4 copies per ml by Q-PCR but only in 0–25% of patients with more than 2×10^5 copies per ml of HCV RNA [37]. Higher levels of HCV RNA appear to lower the chance of sustained response to treatment. Despite differences in assay performance, this phenomenon appears true regardless of the type of assay used for the quantification HCV RNA. Further standardization of HCV quantitative assays in international units will help strengthen decision-making regarding baseline viral load thresholds and treatment regimens.

HCV genotype is a second independent viral predictor for treatment response. Numerous studies have shown that patients with HCV genotype 1 have a significantly lower chance of obtaining a sustained response to therapy than those infected with HCV genotype 2 or 3. Furthermore, patients with genotype 2 or 3 require a shorter duration of therapy to achieve a sustained response than do patients with genotype 1 [38].

5.3. Monitoring HCV RNA during therapy

Many studies have confirmed the utility of monitoring HCV RNA response during therapy, reviewed in [5]. For IFN monotherapy, the recommendation of the National Institutes of Health Consensus Conference is to test for HCV RNA at 3 months, continuing therapy in patients who are HCV-negative and consider discontinuation or alternative therapies for patients remaining positive [39]. This rule is extended to 6 months for patients being treated with interferon plus ribavirin. Persistence of HCV RNA is a strong predictor of treatment failure, while the eradication of HCV RNA in serum at 6 months corresponds to a sustained response in most, but not all, patients. Presently, there is no way to predict which responders will relapse, although increased assay sensitivity at either 6 months or end of treatment is one option to explore. The majority of patients who remain negative for HCV RNA at 6 months after completion of therapy will have no detectable HCV RNA in liver or serum during long-term follow-up evaluation [40]. Other studies suggest that early clearance of viremia is also a predictor of sustained response. In one study, determination of HCV RNA at 4 and 12 weeks after initiation of IFN therapy appeared to be a more accurate predictor of treatment outcome than baseline viral load [41].

The value of persistent viremia for early prediction of no response was 97% when there were similar RNA titers at weeks 4 and 12.

Investigators have studied the kinetics of HCV during treatment in responders and non-responders [42,43]. It has been estimated that HCV virion half-life is on average 2.7 h, with pretreatment production and clearance rates of 10^{12} virions per day. During therapy it has been shown that after a 9 h delay (due to IFN α pharmacokinetics) the decline in viremia is characterized by a concave shape. In the first phase (day 1) there is a rapid dose-dependent decline, and in the second phase (day 2) a much slower decline. Non-responders have been characterized by no further decline during the second phase [42]. The implications of kinetics are then to consider more aggressive dosing regimens, optimizing treatments not only to pre-treatment factors but also to initial decline of viral load. However, these studies rely on the assumption that eradication of virus will depend upon half-life of virions in serum and that this is equal to replication in the liver. Clinical trials are needed to assess the efficiency and practicality of kinetic-based therapeutic monitoring using current and future treatments for hepatitis C.

6. HCV viremia and disease

Histological examination remains the gold standard for the assessment of liver injury, but liver biopsy data is often limited. The asymptomatic nature of hepatitis C and the fact that most patients with HCV viremia have chronic liver disease regardless of serum alanine aminotransferase (ALT) levels means that clinical or biochemical parameters are not reliable for assessing disease status. Attempts have been made to correlate HCV viral load and severity of liver disease. The reports are conflicting; while some retrospective cross-sectional analyses showed no significant association between RNA titer and severity of disease, other studies (both cross-sectional and longitudinal) suggest a relationship between viral load and either ALT levels or liver disease [44,45].

Studies in our laboratory have demonstrated higher viral loads in patients with advanced liver disease than in asymptomatic carriers, but there was no correlation with histopathology [34]. Presently, long-term longitudinal studies in defined patient cohorts with known genotype, duration of infection and other risk factors for liver injury, such as alcohol intake, are ongoing. Interestingly, preliminary findings support the relationship between fluctuations in serum viral load from one time point to another and progression of liver disease, as has been observed previously with regard to ALT values [46]. However, at present, there is no role for monitoring levels of viremia in untreated patients with hepatitis C.

7. Summary

Technologies for assessment of HCV antibody and RNA levels have improved remarkably, as has our understanding of how to best use these tests in patient management. Improvements in the sensitivity of qualitative RNA assays may prove useful for predicting relapse in treatment responders; this area requires careful evaluation. Further standardization of quantitative RNA tests and incorporation into therapeutic trials may lead to additional advances in terms of predicting response at an early time point, giving the clinician better tools for the management of hepatitis C.

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