Molecular Methods for Diagnosis of Hepatitis C Virus Infection: Where do we stand?

Molekulare Methoden zur Diagnostik der Hepatitis C Virus Infektion: Derzeitiger Stand

Evelyn Stelzl, H. H. Kessler

Summary: Infections with hepatitis C virus (HCV) are pandemic, and the WHO estimates a worldwide prevalence of 3 %. Diagnosis of anti-HCV antibodies is a marker for prior exposure to HCV infection. Molecular techniques are gaining increasing importance in the diagnosis and monitoring of hepatitis C. They have been useful for direct detection of infectious agents from clinical samples. Because of laborious and time-consuming protocols together with strict precautions to avoid carryover contamination, molecular assays for detection of HCV have originally been done only in especially dedicated laboratories. Over the past several years, novel technologies and automation have been incorporated that minimize contamination risk and employ standard laboratory techniques. These developments have brought molecular assays for detection of HCV into routine diagnostic laboratories and have made them widely used.

Keywords: Hepatitis C Virus; molecular diagnostics; routine diagnostic laboratory; automation; sample preparation.

Zusammenfassung: Infektionen mit dem Hepatitis C Virus (HCV) werden als pandemisch eingestuft. Die WHO schätzt die weltweite Verbreitung auf 3 %. Die Diagnostik von anti-HCV Antikörpern wird als Marker für eine erfolgte HCV Infektion eingesetzt. Molekulare Techniken werden immer bedeutender für Diagnose und Monitoring der Hepatitis C Infektion. Sie sind für den direkten Nachweis der HCV RNA aus der Patientenprobe geeignet. Aufgrund von arbeitsaufwändigen und zeitintensiven Protokollen, verbunden mit sehr strikten Vorkehrungen um Kontaminationen zu vermeiden, wurden anfangs molekulare Methoden für den Nachweis von HCV nur in speziellen Laboratorien durchgeführt. In den letzten Jahren wurden jedoch neue Technologien und verstärkt automatisierte Verfahren entwickelt, um das Kontaminationsrisiko zu verringern und die Durchführung molekularer Tests zu standardisieren. Diese Weiterentwicklungen ermöglichten den Einsatz im klinischen Routinelabor und führten dadurch zu einer weiten Verbreitung.

Schlüsselwörter: Hepatitis C Virus; Molekulare Diagnostik; Klinisch-chemisches Laboratorium; Automatisierung; Probenvorbereitung, Amplifikation, Detektion.

Hepatitis C virus (HCV) is considered to be the most important agent of post-transfusion and community-acquired non-A, non-B hepatitis [1]. HCV is a single-stranded, positive RNA virus with a genome of approximately 10.000 nucleotides. As a blood-borne virus, HCV can be transmitted from blood and blood products. The presence of anti-HCV antibodies in patients infected with HCV has led to the development of immunoserological assays that are specific for these antibodies. The presence of anti-HCV antibodies, however, is only a measure of prior exposure to HCV infection, but cannot be considered a marker for current infection. The measurement of alanine aminotransferase levels (ALT) is considered to be a surrogate marker of HCV infection but not a direct measure of viremia. Therefore, direct detection of HCV is preferable. Because there is presently no routine culture system for detection of HCV available, molecular techniques have been introduced. With polymerase chain reaction (PCR), it has been possible to detect viremia prior to seroconversion [2]. Commercially available molecular assays have recently been introduced for both, qualitative and quantitative determination [3–7].

Sample preparation

Prior to amplification and detection, HCV RNA has to be extracted from the specimen. Extraction of HCV RNA includes lysis of the nucleic acid-containing specimen and removal of substances, which might inhibit subsequent steps of reverse transcription (RT) and/or amplification while protecting the RNA or cDNA from degradation. Furthermore, the risk of contamination and potential hazards caused by microorganisms and toxic reagents should be kept to a minimum during specimen preparation.

Institute of Hygiene, Karl-Franzens-University Graz, Austria.
Correspondence: Evelyn Stelzl, Institute of Hygiene, KF-University Graz, Universitätsplatz 4, 8010 Graz, Austria.
E-mail: evelyn.stelzl@uni-graz.at

Sample preparation is currently considered the major weakness in molecular detection of HCV RNA. Conventional sample preparation protocols are usually time consuming, labor-intensive and susceptible to contamination. It has been demonstrated that the probability of false-positive results because of contamination increases in relation to the number of manipulations involved in sample processing [8, 9]. To save time and labor, automated extraction protocols with less manipulation steps have recently been brought to the market. One of those, the Cobas AmpliPrep™ analyzer (Roche Molecular Systems, Pleasanton, Calif.) proved to be suitable for the routine-diagnostic laboratory [10]. Compared to manual assays, it was shown to save hands-on work and to be easy to use. However, pipetting steps for aliquoting the mastermix and addition of the extracted samples have still to be done manually. Furthermore, this analyzer only allows HCV RNA extraction from serum or plasma samples. For extraction of HCV RNA from specimens such as whole blood, the MagNA Pure instrument (Roche Applied Systems, Mannheim, Germany) could be used [11]. With this instrument, even automated pipetting of PCR reaction mixture is possible.

Amplification and detection techniques

Amplification techniques are classified into those based on target amplification and those based on signal amplification. Four target amplification techniques (polymerase chain reaction, ligase chain reaction, nucleic acid sequence-based amplification, and transcription-mediated amplification) and one signal amplification technique (branched DNA technique) have gained more or less importance for routine molecular diagnostics of infectious diseases. Based on data of the European Union Quality Control Concerted Action (EU QCCA) for detection of HCV RNA in serum, mainly two commercial kits based on different amplification techniques are currently used in Europe (Table 1).

Nucleic Acid Target Amplification: Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

The PCR technology was discovered by Kary Mullis in the mid-eighties [12, 13]. The principle of PCR includes repeated cycles of amplifying selected nucleic acid sequences. The whole procedure is carried out in a programmable thermal cycler and results in a theoretically exponential increase in the total number of DNA copies synthesized [14, 15].

In 1993, a new easy-to-handle assay (Amplicor™ HCV Test, Roche) for detection of HCV RNA in serum was introduced [2]. For amplification of HCV RNA targets, RNA must be converted to cDNA by reverse transcription (RT) prior to PCR. Conventional RT-PCR is a two-step process requiring separate enzymes and buffer conditions. The reopening of the reaction tubes was recognized as the major contamination source. Therefore, the DNA polymerase of the thermophilic bacterium *Thermus thermophilus*, which possesses enhanced reverse transcriptase activity in the presence of manganese ions, was introduced [16]. RT is carried out at 60 °C, which increases specificity as well as efficiency by destabilizing secondary structures in the RNA template. Furthermore, there is no need to change or add reagents between the RT and PCR steps. The reaction tubes remain closed during the entire procedure, which minimizes the contamination risk and reduces the hands-on work required. When using the Amplicor™ HCV Test, Roche, PCR amplification products for the target (HCV) and the Internal Control (IC) are denatured with sodium hydroxide to form single-stranded DNA. After this, they are hybridized to specific capture probes for each of the products (HCV and IC, respectively) followed by colorimetric detection [17, 18].

To meet the needs of the routine diagnostic laboratory, the Cobas Amplicor™ instrument, which allows the automation of the amplification and detection steps of a PCR test, has been developed [19, 20]. It allows significant reduction of manual steps and can be used as walk-away system. The Cobas Amplicor™ has been found to be an easy, quick, and reliable way to perform high-volume PCR [21–23]. For HCV testing, there are presently both qualitative (Cobas Amplicor™ HCV Test) and quantitative (Cobas Amplicor™ HCV Monitor

Table 1 Methods used with the EU QCCA HCV proficiency panels [modified from 49, 50]

	Methods 1999		Methods 2000	
	Qualitative	Quantitative	Qualitative	Quantitative
	n=44 (%)	n=35 (%)	n=71 (%)	n=48 (%)
RT-PCR (Roche)	28 (64)	23 (65)	52 (73)	34 (71)
bDNA (Bayer)	0	8 (23)	0	11 (23)
Home-brew	15 (34)	3 (9)	19 (27)	2 (4)
Other	1 (2)	1 (3)	0	1 (2)

Test) versions available. Similar to other assays for quantitation of pathogens, the Cobas Amplicor™ HCV Monitor Test employs a well-defined quantitation standard (QS) and enables quantitation of target RNA in a HCV RNA positive specimen [18, 24, 25]. The HCV quantitation standard is standardized against the WHO HCV International Standard 96/790, and the results are expressed in IU/ml [26]. According to the manufacturer's package insert, the detection limit of the Cobas Amplicor™ HCV Monitor Test, v2.0 is 600 IU/ml.

Recently, a specially designed software, called Amplilink™, was developed which was designed to permit the control of up to three Cobas Amplicor™ instruments. This software increased convenience, saved labor, and was found to be a very useful addition for clinical laboratories performing molecular testing with the Cobas Amplicor™ system [27].

A serious challenge for a molecular diagnostic facility performing assays based on PCR is to avoid false-positive results due to carryover contamination [28, 29]. Risk of contamination is less of a concern when prepackaged assays are used. However, it is essential always to be aware of the potential for a contamination and to prevent its occurrence rather than to deal with these problems retrospectively. Contamination control can be classified in two basic approaches: physical and enzymatic. Physical approaches to contamination control have been described in detail elsewhere [4]. The introduction of uracil-*N*-glycosylase provides an enzymatic means to prevent carryover contamination. Uracil-*N*-glycosylase inactivates previously amplified DNA that may be present in the new sample so that it can no longer serve as a template for PCR [30–32]. The high denaturation temperature used in PCR conveniently destroys any residual uracil-*N*-glycosylase, thus keeping it from neutralizing the next generation of newly formed amplified products. Uracil-*N*-glycosylase, also known as AmpErase, has been included in all Cobas Amplicor™ assays.

Signal Amplification:
branched DNA technique (bDNA)

The bDNA technique can be used for detection of RNA or DNA. An assay for quantitation of HCV RNA has been developed by Chiron Corporation and is now distributed by Bayer (Bayer Diagnostics, Tarrytown, NY) [33, 34].

In this process, multiple specific synthetic oligonucleotides hybridize to the target and capture the target onto a solid surface. Synthetic bDNA amplifier molecules, which are enzyme-conjugated, branched oligonucleotide probes, are added. Hybridization proceeds between the amplifiers and the immobilized hybrids. After addition of a chemiluminescent substrate, light emission is measured. Because all hybridization reactions occur simultaneously, the observed signal is proportional to the amount of target DNA. By the use of four standards with each batch of specimens, quantitative results can be determined from a calibration curve.

In comparison with target amplification methods the bDNA technique is generally less sensitive but also less likely to have contamination problems because the target molecules themselves are not amplified during the process. The bDNA system does not allow the use of an internal standard. Therefore, it is not known whether interfering substances impair quantitation with the bDNA technique; however, it seems unlikely that PCR inhibitors could affect bDNA hybridizations.

The bDNA method was originally rather laborious and very time-consuming but recently, the HCV RNA 3.0 Quantitative Assay (Bayer) with a dynamic range reported to be more than 4 log units and with up to a 100-fold increased sensitivity compared to the second-generation assay has been introduced and can be run on the Quantiplex™ bDNA System 340 (Bayer), which largely automates the procedure.

HCV Genotyping

The complete HCV genome sequence has been determined in different HCV isolates worldwide and substantial nucleotide sequence variability through the viral genome was found [1]. The comparison of published sequences of HCV has led to the identification of distinct HCV genotypes that may differ from each other by as much as 33 % over the entire viral genome [35]. Regions encoding the putative envelope glycoproteins (E1 and E2/NS1) have been found to be most variable [36]. The 5'noncoding region (5'NCR) is the most conserved with a 92 % homology among different HCV types [37].

The variability within the HCV genome has formed the basis for several genotyping systems for HCV gene families. At the second International Conference of HCV and Related Viruses (August 1994, San Diego, CA), the classification system according to Simmonds *et al.*, based on 5'noncoding region and NS5 sequence analysis, was proposed to be used in future studies related to HCV genotypes [38]. The Simmonds system consists of 6 genotypes and several subtypes: 1a to 1c, 2a to 2d, 3a to 3f, 4a to 4k, 5a, and 6a, but new genotypes and subtypes have been described.

Genotyping and subtyping of HCV is relevant to therapeutical management, severity of liver disease, vaccine development, and epidemiology [39–41]. Differences between genotypes in their response to treatment were observed in recent studies in which a combined treatment including interferon-alpha and ribavirin was administered [39, 40, 42]. Therefore, therapeutical management of chronic HCV infection based on the HCV genotype has been suggested in the Consensus Statement of the European Association of the Study of the Liver EASL Consensus Panel (EASL, 1999). Furthermore, HCV subtype 1b was found significantly more prevalent in patients with decompensated liver disease that required liver transplantation [43]. In liver transplant recipients, HCV subtype 1b was associated

with earlier recurrence and more severe hepatitis and cirrhosis in comparison with other subtypes [44, 45]. HCV genotypes may also pose problems in the development of anti-HCV vaccines. All different HCV isolates should be incorporated into the process of vaccine development to ensure a broad-spectrum effective immunity after vaccination.

The HCV genotype can be determined by nucleotide sequencing of the entire genome followed by composition of a phylogenetic tree, which is presently the "gold standard" for the detection and identification of the various HCV genotypes and subtypes [46]. This approach, however, is cumbersome and regarded as impractical for routine clinical laboratory settings [47]. Therefore, more convenient methods have especially been designed for routine laboratory settings. Today, two commercially available assays are commonly used in routine diagnostic laboratories. One of them is based on the reverse-hybridization principle (Inno-LiPA HCV II; Innogenetics, Zwijndrecht, Belgium). This assay allows the determination of six HCV types and their most common subtypes. The HCV type/subtype can be deduced with an interpretation chart, which is included in the kit. The other method is based on sequencing the 5'NCR of the HCV genome (TruGene™ HCV 5'NC Genotyping Kit; Visible Genetics, Toronto, Canada). This assay is based on a nucleotide sequencing reaction of RT-PCR products from the 5'UTR. For both of the assays, the RT-PCR products can conveniently be retrieved from the Cobas Amplicor™ HCV Test or the Cobas Amplicor™ HCV Monitor Test.

Quality control

Quality control is an important issue in the routine diagnostic laboratory. Attention must be paid to internal controls, external controls, and interlaboratory quality control.

Internal controls are positive controls to detect a technical failure of the assay, which could be produced, for example, by the presence of an inhibitor [48]. These competitive controls with a low copy number should be introduced into each reaction at the beginning of the extraction to follow up the whole procedure. The internal control has primer-binding sites identical to those of the target sequence, a randomized internal sequence of similar length and base composition as the target sequence, and a unique probe-binding region that differentiates the internal control amplification product from the target amplification product. Addition of an internal control is obligatory to meet the requirements of a serious quality control in the routine diagnostic laboratory. This principle is also used for quantitative PCR assays, in which the quantitation standard serves for both, quantitation and technical failure of the assay.

External controls include negative and positive controls, which must be included in each run. These controls are often provided from the test kit manufacturer. For quantitative assays positive controls with different concentrations can be used. Similar to internal controls, external controls should be carried through the whole test procedure.

Interlaboratory quality control assesses the results of a certain laboratory in comparison with those of other laboratories. For example, the EU QCCA has provided HCV panels (http://www.qcmd.org/Index2.htm). These panels were established for the evaluation of currently employed nucleic acid amplification methods. Results of two panels have been reported so far [49, 50]. They show a distinct improvement when more automated and standardized assays are employed (Table 2). Genotyping panels are scheduled for distribution from both, EU QCCA and Acrometrix (Benicia, CA) in 2002.

Conclusions

Molecular assays for detecting HCV RNA have been used since the early 1990s. Laborious RNA extraction protocols followed by separate reverse transcription and PCR assays employing nested primer sets were described. At that time, those assays usually lasted for more than 24 h and posed potential problems of false positive results due to crossover contamination. Recently, automated and easy to handle assays, which include also automated sample preparation have been introduced.

Table 2 Quantitative HCV-RNA performance scores, percentages. Other assays include the bDNA technique and home-brew methods. Good/Excellent = 100 % acceptable results; adequate = 80–99 % acceptable results; mediocre = 60–79 % acceptable results; poor = less than 60 % acceptable results

	1999			2000		
	All data sets	Roche assay	Other assays	All data sets	Roche assay	Other assays
Good/Excellent	17	100	0	63	76	29
Adequate	26	100	0	10	15	0
Mediocre	20	71	19	2	3	0
Poor	37	23	77	25	6	71

Advances in automation of molecular assays for detection of HCV RNA help to avoid human error and enable standardization of testing procedures, thus increasing the precision and reproducibility of the results. Furthermore, there are benefits related to technologist training, support, and savings of hands-on time. To guarantee the analytical quality of molecular assays in the routine diagnostic laboratory, only standardized and maximum automated assays should be used.

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