Virology and Diagnosis of the Hepatitis C Virus

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1 Introduction

Worldwide, approximately 170 million people are infected with the hepatitis C virus (HCV), which gives a global prevalence of about 3%[1,5]. HCV was in 1989 identified as the infectious agent for non-A non-B hepatitis, being the first pathogen that was purely identified by molecular techniques[1,6]. Chronic HCV infections are identified as the major cause for chronic liver disease, cirrhosis, hepatocellular carcinoma and liver transplantation[1,7]. The majority of HCV infections remain asymptomatic for many years, which leads to spread of the virus and late treatment[1]. The predominant mode of transmission in many Western countries is injecting drug abuse[7].

1.1 Classification

The hepatitis C virus is the only member of the genus *Hepacivirus* which belongs to the family of Flaviridae[8]. Other members of this family are the classical flaviviruses like dengue virus and yellow fever virus and the pestiviruses such as bovine diarrhea and GB viruses[6]. HCV is a genetically variable RNA virus which can be phylogenetically classified into six major genotypes which are depicted by the numbers 1 to 6. The genotypes can be further divided in subtypes depicted by letters. The genomes of the genotypes differ from each other by 31-33%, whereas the subtypes differ by 20-25%. The natural history and response to treatment varies between the different genotypes and subtypes, and therefore, a proper classification prior to therapy is important[9].

1.2 Epidemiology

HCV infection is a worldwide problem, but the prevalence of hepatitis C infection shows differences in the geographical distribution. Since not all countries register data concerning HCV infection, estimations are rather based on regions than on countries[7]. Northern-Europe has a prevalence rate of less than 1.0 %, while the highest prevalence rate can be found in Northern Africa (above 2.9%). Especially Egypt has a very high prevalence rate of 15-20%, France has a prevalence rate of about 0.8% and the lowest prevalence rate can be found in the United Kingdom and Scandinavian countries (0.1-0.01%)[7,10]. The HCV genotypes show geographically different distributions as well. Genotype 1a is the most common genotype in the US and Northern Europe, whereas genotype 1b is found worldwide. In Mediterranean countries and the Far East, genotype 2a and 2b occur most frequent, but this genotype has a worldwide distribution as well. Genotype 3 is prevalent in the region around India, but is also widely distributed among intravenous drug users in Europe and the US. Genotype 4 is common in the Middle East and Africa. The genotypes 5 and 6 occur infrequently, but can be located in South Africa and Hong Kong/Vietnam/Australia respectively[9,11].

Many HCV infections are asymptomatic and the currently used diagnostic tests for HCV do not distinguish between a resolved or chronic infection. Additionally, many countries do not systematically collect data and therefore the incidence of HCV infection is difficult to determine[7]. According to estimations, annually 1 to 3 new symptomatic infections per 1 million persons occur, but given that the majority of new infections are asymptomatic, the actual incidence of infection is probably much higher[5].

The main transmission pathway of HCV is direct percutaneous exposure to blood. Examples are blood transfusions and transplantations from infected donors or unsafe therapeutic injections. Since the routine usage of HCV screening in donor blood the risk of being infected by HCV by a blood transfusion is almost eliminated. However, the reusage of syringes and needles for therapeutic injections still forms a high risk factor for infection by HCV. The highest risk factor for acquiring HCV in most countries including the US is injecting drug abuse[7].

Other modes of transmission than by blood exposure are less efficient. These are occupational, perinatal and high-risk sexual exposures. Occupational exposures are for example accidental needle-sticks or contact via mucous membranes or injured skin. During birth given by an infected mother, the prevalence of transmission is about 4-7%[7]. The risk of transmission is much smaller if the mother has a viral load lower than $1x10^5$ to $1x10^6$ copies/ml, but with about 19% much larger if the mother is also HIV co-infected. It is suggested that the HIV co-infection related higher risk of perinatal HCV transmission can be lowered if the mother receives antiretroviral treatment during pregnancy[12]. The rate of sexual transmission is still not completely elucidated, but with a risk of 0.1%-0.3% very low[13]. The percentage appears to be higher during an acute HCV infection[7]. Sexual practices with a high risk of mucosal trauma are associated with a higher transmission risk as well[14].

Since the main transmission pathways are the same, HIV-HCV co-infections occur frequently. In the US and Europe, about 35% of the HIV infected individuals have also a HCV infection, the percentage among intravenous drug users is with about 80-90% even

higher[13,14]. In general, the progression of HCV related liver disease is faster and the success rate of antiviral therapy smaller among HIV-HCV co-infected patients[14].

1.3 Morphology

The hepatitis C virion contains a nucleocapsid which is built up from several copies of the core protein. The nucleocapsid is surrounded by an outer envelope that holds two different glycoproteins which are required for attachment and entry into the host cell. The size of the virion varies from 30 to 80 nm[6].

The viral genome is located inside the nucleocapsid. The genome is built up from a single, positive sense RNA strand with an approximate size of 9.6 kilobases. The RNA contains one open reading frame flanked by 3' and 5' untranslated regions (UTR). The open reading frame encodes a polyprotein which is co- and posttranslationally processed into four structural and six non-structural proteins (figure 1) [6,15] .

Structural proteins

Core protein. The core or capsid (C) protein has a hydrophobic C-terminal and a highly basic N-terminal and forms the viral nucleocapsid by oligomerization. It is a well conserved protein which makes it a suitable marker for serological testing[6,16]. Besides its structural function, the C protein is also involved in regulatory functions of the viral life cycle[6].

E1 and E2 glycoproteins. The structural proteins E1 and E2 are glycoproteins located at the surface of the viral envelope. They form a heterodimer by non-covalent interactions, a complex which is essential for viral entry. The E2 glycoprotein contains a hypervariable

region (HVR) and can interact with several cellular receptors, whereas the E1 glycoprotein most likely mediates membrane fusion. E1 and E2 are essential for viral attachment and cell entry[17,18].

p7 protein. The p7 protein is a small protein which is classified as a viroporin since it can oligomerize to form a cationic channel *in vitro*. p7 can localize in the membranes of the endoplasmatic reticulum (ER) and is a critical protein for virus particle formation and secretion *in vitro.* It is required for HCV replication in chimpanzees as well[19].

Non-structural proteins

NS 2. The NS2 protein is an integral membrane protein that possesses a dimeric cysteine protease that cleaves the NS2-3 junction. It is not essential for HCV RNA replication, but nevertheless crucial for the formation of infectious virus particles[6].

NS3-4A complex. The NS3 protein is a rather hydrophobic protein containing a serine protease and a helicase-NTPase domain. It is bound non-covalently to the NS4A co-factor. Although the NS4A co-factor does not contribute to the catalytic triad, its central position is important for the processing of the non-structural proteins as it takes part in the shallow binding pocket of the complex. Another function of the complex is contribution to the viral RNA replication. The helicase-NTPase domain of NS3 has the ability to unwind double stranded RNA in the 3' to 5' direction. Translocation on the nucleic acid occurs under usage of the energy obtained by NTP hydrolysis[6]. The NS3-4A complex is able to cleave host signal proteins as well and interferes in this way with the host immune response[3]. NS3-4A protease activity is needed for the release of the NS5B polymerase and hence crucial for viral replication. Therefore, the inhibition of NS3-4A is an emerging target for HCV specific antiviral therapy[20].

NS4B. NS4B is a transmembrane protein located in the ER membrane. It functions as an inducer of the formation of the membranous web which is required for RNA replication[21].

NS5A. NS5A is membrane-anchored phosphoprotein whose function is still not fully elucidated. It likely forms a dimer that includes a putative RNA binding groove. Due to the fact that it contains an unconventional zinc-coordinating motif, it is hypothesized that the protein interacts with viral and cellular proteins, membranes and RNA. It is believed that NS5A is essential for RNA replication[6,22].

NS5B. NS5B is a membrane-anchored protein that functions as a viral RNA-dependent RNA polymerase (RdRp) and is therefore essential for replication of the viral genome. RNA replication occurs either primer-dependent or by *de novo* initiation mechanisms. Because of its importance in the viral life cycle, NS5B is a target for the development of new anti-HCV drugs as well[23].

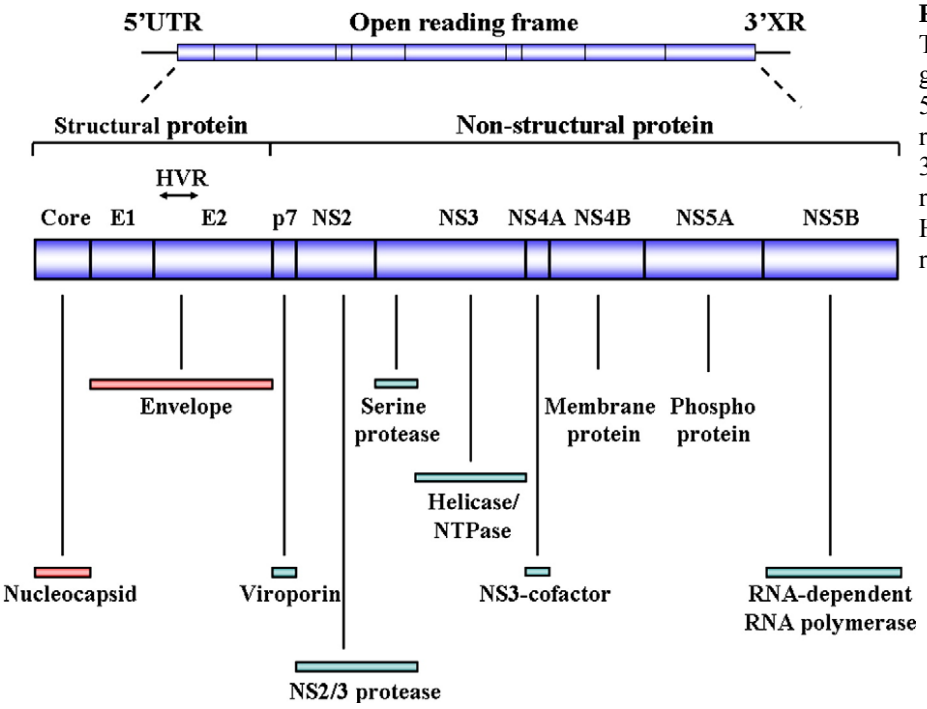

Figure 1[3]
The HCV genome and gene products.
5'UTR: 5' untranslated region
3'XR: 3' untranslated region
HVR: hypervariable region

1.4 Viral life cycle

The viral life cycle begins with the infection of a suitable cell of the host, which is in the majority of the cases a hepatocyte. The attachment and cell entry of HCV is facilitated by the envelope by which it is surrounded[8]. Since it is complicated to culture HCV, it is difficult to make definitive statements concerning the viral receptors. However, research with model systems gives strong evidence for a number of putative HCV receptors that are bound by the E2 glycoprotein[17].

The first putative HCV receptor discovered was the tetraspanin CD81. The exact role of CD81 is not clear yet. The tetraspanin family is involved in membrane fusion, but since the expression of CD81 only is not sufficient for cell entry, also other cellular receptors must be involved. It is proposed that CD81 is rather bound after attachment to another receptor[17]. Another putative HCV receptor is the human scavenger receptor class B type I (SR-BI) which can be found in a large variety of tissues, but is especially high expressed in the liver and steroidogenic tissue. A natural ligand for SR-BI is high density lipoprotein. Since model systems have shown that the co-expression of CD81 and SR-BI is not sufficient for HCV infection, there appears to be at least one other receptor crucial for HCV entry[17]. Molecules that are possibly involved in the cell entry of HCV are the C-type lectins like L-SIGN and DC-SIGN, the LDL receptor, glycosaminoglycans and the tight junction component claudin-1[6,17].

After release of the viral genome to the host cell, the viral RNA is translated into a single polyprotein. The RNA strand of HCV possesses an internal ribosome entry site (IRES) located at the 5' end and is CAP-independent. The IRES can bind to the 40S subunit of the host ribosome, which then interacts with the viral RNA and subsequently binds the

60S subunit to form the 80S complex. Host factors and viral proteins modulate and regulate the transcription of the polyprotein. For example, the presence of the HCV core protein inhibits translation. After translation, the polyprotein is processed. The cleavage into the several viral proteins is achieved by viral proteases and host proteases as well[6].

The replication of the viral RNA begins with the synthesis of a negative stranded RNA template which is used to make several copies of positive sensed single-stranded RNA. Both processes are catalyzed by the RdRp NS5B. However, other viral and host factors take part in the replication complex as well to guarantee template specificity and fidelity. Replication takes place in the membranous web which is localized in the ER[6].

After successful translation and replication, the capsid protein multimerizes to form the nucleocapsid. It is supposed that during this process, the C protein interacts with the viral RNA which might be crucial to terminate RNA replication and initiate viral packaging. Once the nucleocapsid is formed, the virus acquires an envelope derived from an intracellular membrane by budding. HCV release takes most likely place through the secretory pathway[6].

The hepatitis C virus has a high turnover rate. The productively infected cells have an *in vivo* half life of 1 to 70 days, whereas the free HCV virion has an *in vivo* half-life of a few hours[24].

2 Clinical features and pathogenesis

2.1 Pathogenesis

Hepatitis C is a blood-borne pathogen that naturally only infects humans. HCV mainly infects hepatocytes but the cellular tropism appears to be much larger. The virus has been

found in various other cell, like in T-cell and B-cells, antigen presenting cells, other blood mononuclear cells, epithelial gut cells and in the brain as well, but it is at present unclear whether the virus can truly replicate in these cells[3,4,24].

HCV infection has an average incubation period of 6-8 weeks and it takes about 8-9 weeks until anti-HCV antibodies can be detected[24]. HCV RNA is present in the peripheral blood 1-2 weeks after exposure to the virus[3].

Host immune response

In the early phase of infection, the presence of the virus in the host cell triggers the expression of type 1 interferon (IFN) α/β and IFN-induced genes like IFN-regulatory factor-3 and double-stranded RNA-dependent protein kinase[3,15]. This up regulation has the purpose to inhibit viral replication and to induce apoptosis in infected hepatocytes. The expression of HCV antigens on the cell surface might be enforced by IFN as well[3].

Moreover, natural killer (NK) cells are abundant in the liver in patients with an acute infection. These cells control viral replication by cytolysis of infected cells and the production of replication inhibiting cytokines. NK cells also activate dendritic cells and T-cells. Dendritic cells (DC) are mainly important as link between innate and adaptive immunity through its function as antigen presenting cells. They also produce cytokines that stimulate other immune cells[3].

However, HCV is capable to escape the host innate immune response which facilitates chronic infection[15]. For example, the NS3/4A serine protease blocks the endogenous IFN production, but also the HCV core protein and NS5A can interfere with the intracellular signalling of innate immune cells[3].

Within several weeks after the primary infection, anti-HCV antibodies can be detected. During the acute phase of infection, HCV specific CD4+ and CD8+ T-cells are present. The T-cell response is associated with a decreasing HCV RNA titer and spontaneous HCV clearance co-incidences with a strong and sustained CD4+ and/or CD8+ response[3]. Contrary, if the T-cell response is impaired, chronic infection occurs more often[15]. It is supposed that the failure of the T-cell response can be due to high viral load during early infection or dysfunction of dendritic cells, but still remains to be fully elucidated[3,15]. Other mechanisms by which the virus can evade the immune system are the high mutational rate due to lack of proof reading of the RdRp, persisting of viral particles in extrahepatic tissue and masking by covering the virus with lipids[3,24].

2.2 Clinical manifestations

Acute (primary) hepatitis C

The majority of the patients with acute hepatitis C infection are asymptomatic. In symptomatic patients, fatigue, jaundice, dyspepsia and abdominal pain can be observed. However, these symptoms are rather unspecific which makes a diagnosis in the acute phase unlikely. Elevated alanine aminotransferase (ALT) levels, which represent the damage of hepatocytes, can first be detected 4-12 weeks after HCV exposure[25]. The acute phase of infection is further characterized by low anti-HCV antibody and high HCV RNA levels[24]. The rate of spontaneous resolution in the first three month after infection is averaged 26% but has a wide range of about 14-46% (figure 2)[4,25]. If after 6 month still HCV RNA can be detected, the patient is defined to be chronically infected with HCV. The outcome of infection is influenced by both host and viral factors, but can still not be

predicted accurately[25]. The main predicting factor is the genotype. The most common genotype 1 is at the same time the one which responds least to therapy, the genotypes 2 and 3 respond the best. Although the data about genotypes 4, 5 and 6 are limited, the same therapy strategy as for genotype 1 is recommended[26]. It appears that spontaneous resolution occurs more often in patients with a symptomatic acute infection[25]. Moreover, patients who already receive treatment during the early phase (8 to 12 weeks) of acute hepatitis C infection are associated with a higher rate of spontaneous resolution compared to patients who are treated later during acute infection or first during chronic infection[27].

Chronic hepatitis C

Conversely, 54-86% of the patients can not clear the infection and develop chronic hepatitis C (figure 2)[4]. Since chronic hepatitis C remains often subclinical as well, routine examinations are important to guarantee optimal treatment[24]. HCV RNA and high levels of anti-HCV antibodies are detectable during chronic hepatitis C infection. Both normal and elevated serum ALT levels can be present. In general, three groups of chronic hepatitis C patients can be defined[24]:

- Patients with normal ALT levels are usually associated with an asymptomatic mild hepatitis. Although the long term perspectives are very good for this group, in about 10% of the cases, cirrhosis can occur as well[24].
- Another group which takes about 50% of the newly diagnosed hepatitis C patients in account shows elevated ALT levels which might fluctuate in the course of the disease. This coincides with a mild chronic hepatitis, which is mainly asymptomatic. In general, a liver biopsy shows mild necro-inflammatory lesions

with little fibrosis. The progress of disease is slow in this group, as the risk of liver cirrhosis is as well[24].

- The third group of approximately 25% of all newly diagnosed patients is difficult to distinguish from the second group. They have elevated ALT levels, but this does not correlate with the severity or symptoms of the hepatitis. In a progressed state of disease, gamma glutamyltranspeptidase, ferritin and gamma globulin levels can be used as disease marker. However, the best way to determine this form of hepatitis is a liver biopsy, which in general shows necro-inflammatory lesions and extensive fibrosis or cirrhosis[24].

Both the stage of fibrosis (ranging from mild fibrosis to cirrhosis) and necro-inflammatory activity (ranging from minimal chronic hepatitis to severe chronic hepatitis) can be scored to assess the response to therapy and/or likelihood for cirrhosis. Some clinical features of chronic HCV infection are aggregates in the portal tracks comprised from inflammatory infiltrates or necrotic tissue in the periportal area. Another manifestation is steatosis, which is the presence of large lipid droplets in hepatocytes[24].

Cirrhosis and hepatocellular carcinoma

In 20% of the HCV cases, the patient will develop liver cirrhosis. Cirrhosis caused by HCV is often asymptomatic in the early stage of disease but can be discovered by a liver biopsy. In 20-25% of those cases, a further progression to hepatocellular carcinoma (HCC) takes place, a process which can take between 10 to 30 years[24]. The mortality of HCV related cirrhosis, including due to cirrhosis caused HCC, is about 0-4% per year[4].

Since the main part of hepatitis C infections remains asymptomatic, it is difficult to determine the morbidity of the disease. Estimated 34% of all people infected with HCV develop the worst outcome of the infection, defined as the development of cirrhosis, with eventually ascites, hepatic insufficiency and HCC[24].

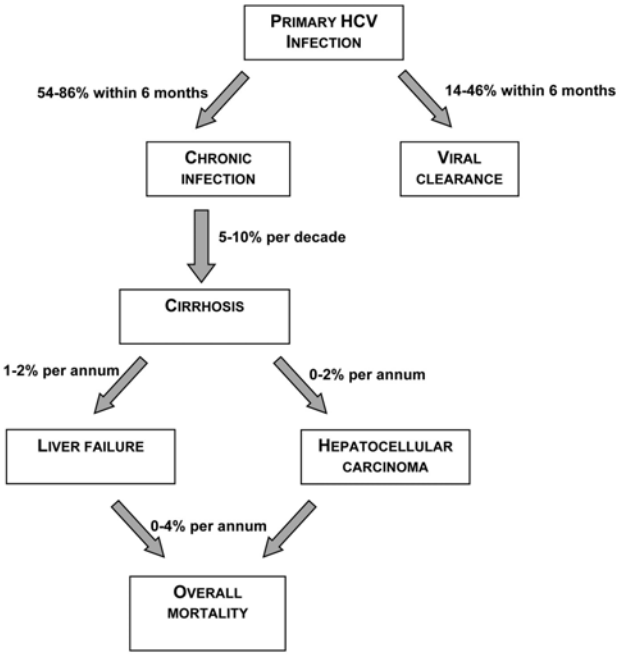

Figure 2[4]
The natural history of HCV infection.

2.3 Current treatment methods

Currently, the standard antiviral therapy against HCV consists of a combination from the nucleoside analogue ribavirin and pegylated or conventional IFN-α. Sustained virological response can be achieved in approximately 40-50% (genotype 1) or 70-80% (genotypes 2 and 3) of the cases[8]. Patients with genotype 2 or 3 respond better to antiviral therapy than the other genotypes[1]. In case of infection with these genotypes, the patient receives 24 weeks the combined antiviral therapy. If infected with the genotypes 1, 4, 5 and 6, twelve weeks after the start of the antiviral therapy the viral load is quantified. The therapy is stopped if no early virological response has occurred. Otherwise, the therapy is continued for a total of 48 weeks[8]. The current therapy can have unwanted side effects like haemolytic anaemia, nausea, diarrhoea and cough, but moreover also depression and impairment of quality of life[8,28].

2.4 Other than hepatic manifestations

There is a wide spectrum of extrahepatic manifestations that are caused by HCV infection. A disease in which the role of HCV is well-investigated, is mixed cryoglobulinemia, a B-cell lymphoproliferative disorder. During this disease, immunoglobulins which precipitate below 37°C defined as cryoglobulins are formed, which cause symptoms like weakness, arthralgias and palpable purpura in the lower extremities[29]. Mixed cryoglobulins can be found in about 50% of all HCV patients, but cryoglobulinemia vasculitis occurs only in 5-10% of the cases[30]. It is supposed that the chronic infection with HCV may overstimulate B cell polyclonal expansion, which favors mutations that can induce mixed cryoglobulinemia. For the same reason, also malignant lymphomas like non-hodgkin lymphoma are associated with HCV infection. Other extrahepatic manifestations that might occur due to HCV infection are neurologic disorders and nephropathies[29].

3 Diagnosis

The detection of hepatitis C or anti-HCV antibodies is important for both the first diagnosis and the monitoring of the treatment. Since it is difficult to propagate the virus in culture routinely, for clinical purposes only serological and molecular techniques are used[1].

The conventional serological tests include anti-HCV antibody and HCV antigen tests[16,31]. If genotype-specific HCV epitopes are used for the immunoassay, this technique can also be used to determine the HCV genotype[31]. Another serological marker than anti-HCV antibodies is the HCV core protein. This antigen is present in the serum even before

seroconversion occurs and thus of special importance in the serological detection of early infections[16]. The molecular techniques consist from several methods of Nucleic Acid Testing (NAT) and genotype testing. NATs include qualitative reverse transcriptase polymerase chain reaction (RT-PCR) and transcription-mediated amplification (TMA), which are used for detection; and branched-chain DNA (bDNA) amplification, quantitative RT-PCR and Real-Time RT-PCR, which are used for quantification. The usage of NATs is currently the preferred method to confirm a HCV infection[1]. However, serological tests are still used mainly in developing countries because of the relative low costs compared to molecular techniques[16].

3.1 Conventional techniques

3.1.1 Immunoassays and immunoblots

Enzyme immunoassays (EIA). The first technique for detecting HCV antibodies was an enzyme immunoassay (EIA) launched in 1989[32]. This assay used the recombinant antigen c100-3 which is coded by NS4[32,33]. An immunoblot was used as confirmatory test. Because of the low sensitivity, in 1991 the second-generation EIA was developed. This assay contained antigens from NS3 and NS4 and the core protein[32]. Additionally to the increased sensitivity and specificity, the second-generation had also a shorter average seroconversion period[33]. Again immunoblot against the antigens was used a confirmatory test[32]. The third and last generation EIA which is still used was marketed in 1993[32,34]. In addition to the antigens of the second generation EIA, these tests contain also the NS5 antigen[32]. As confirmational test, an immunoblot or RT-PCR test is carried out. However, recently it is proposed to run the third-generation EIAs in duplo to confirm positive

testing because of the high specificity and low costs of this test[34]. A disadvantage of EIAs is the presence of a time window where a negative result is measured despite of a HCV infection. This is due to the seroconversion period of about 56 days[33]. Immunocompromised patients like transplant recipients and HIV patients, hemodialyse patients and mixed cryoglobulinemia patients are more likely to be tested false negative[34,35]. Moreover, EIAs have a high rate of false positive results, which make confirmations tests obligate[33]. EIAs are based on the enzyme-linked immunosorbent assay (ELISA) technique. The third generation EIAs use the c100-3 epitope of NS4, the non-structural antigen c33c, the structural antigen c22-3 and the NS5 antigen[35].

EIAs can also be used to discriminate between the six different genotypes. For this purpose, the assays currently used identify the genotype by competitive EIA that detects genotype-specific anti-NS4 antibodies[31,36]. These tests can not determine the subtype, are susceptible for spontaneous mutations which result in mistyping and deliver in only 90% of the immunocompetent patients interpretable results[31,36]. The sensitivity among hemodialysis, transplantation and oncology patients is poor[37]. However, they are still used because they are less expensive and faster than the molecular methods[31].

Chemiluminescence immunoassays. Since the available EIAs are still not sensitive and specific enough and because of the time window where no antigens can be detected, the chemiluminescence immunoassay (CLIA) was developed[33]. Beside of the CLIA, also fully automated chemiluminescent microparticle immunoassays (CMIA) are available[38]. The advantage of these assays is that they have a high through-put, great precision and that fully automated analyzers are available[33]. Moreover, the frequency of false-positive

results is lower when chemiluminescence assays are used, which leads to a higher specificity and a better positive predictive value. The reason why the chemiluminescent assays perfom better are not entirely clear. A possible reason could be the different sample processing[39]. Chemiluminescent immunoassays work with HCV NS3, NS4 and core protein derived capture antigens[38]. If an antigen-antibody reaction occurs, a light signal proportional to the amount of antibody is created[40]. This reaction is read out as a signal-to-cutoff ratio[33].

Immunoblot assays. Because of its high specificity and low costs, recombinant immunoblot assays (RIBA) are widely used as a confirmatory test for immunoassays with a positive test result[32,39]. Currently, automated third generation RIBAs are used[41]. To avoid a false-positive test result in both EIA and RIBA, the immunoblot assay usually uses another set of antigens than the immunoassay to detect anti-HCV antibodies[38]. However, since the recently available EIAs have a high specificity and sensitivity and NATs are becoming more common, the usage of RIBA is decreasing[31].

RIBAs are strip immunoassays which contain bands with 4 different immobilized HCV antigens, human superoxide dismutase (hSOD) and both low and high concentration immunoglobulin G (IgG) as a control[41]. The hSOD strip is involved because the c33c and NS5 recombinant antigens used in the EIAs are fusion proteins with hSOD. In this way, nonspecific antibodies can be detected[35]. If antibodies bind, luminescence can be measured. Fully automated systems are available that calculate the relative intensity of reflection on each band and compare them with the IgG control[41]. If the reaction with at

least two antigens has a greater intensity than the low concentration IgG, the RIBA is considered to be positive[35].

Another widely used immunoblot assay is the line immunoblot (LIA). The LIA works in general in the same way as the RIBA, but makes use of another set of total six recombinant HCV antigens. LIAs contain beside of the three IgG control lines a streptavidin control line[42].

3.1.2 Core antigen detection

Another serological method to detect a hepatitis C infection is the detection of the HCV core protein. This method is very useful for identifying patients with an early infection. After the exposure to HCV, two serological time windows are defined: During the first phase, no antigen or nucleic acid can be detected, but during the second phase, these two can be detected while antibody detection is not possible, because seroconversion has not occurred yet[16]. This time window has an average period of 56 days, and during the second phase, the patient is infectious[16,33]. The commercial available assays can detect the core antigen 1 to 2 days after HCV RNA is detectable[43]. Although in developed countries the screening of blood donors by nucleic acid testing is the standard procedure to avoid false-negative results during this time window, in less developed countries the core antigen detection is an attractive alternative to prevent posttransfusion HCV infections[16]. The currently available HCV core assays are more specific, but less sensitive than NATs or anti-HCV antibody tests. They are not able to detect all genotypes even well, and even a combined antigen-antibody test which is also available has a lower sensitivity compared to NATs. Moreover, the core antigen can only be detected at a viral

load higher than 2 x 10^4 IU/ml[44]. Thus, the core antigen assays are a good way to avoid posttransfusional HCV infection in countries where NATs are economically or technically not available, but they represent not the preferred method for donor blood screening[16].

Core antigen detection takes place by means of an anticore antigen monoclonal antibody ELISA[43].

3.2 Molecular techniques

Currently, nucleic acid testing (NAT) is used to detect HCV RNA and is the common confirmatory test after a positive anti-HCV antibody test. During acute HCV infection the NAT test will be positive 1 to 3 weeks earlier than the serological test. NATs can be used for both quantification and qualification[1]. For all assays, the World Health Organisation (WHO) First International Standard for HCV RNA is used which expresses the test results in International Units per milliliter (IU/ml). This standardization was introduced by the WHO Collaborative Study Group and the WHO Expert Committee on Biological Standardization to make it possible to compare the HCV viremia level even if different assays were used. Moreover, the Standard states that the assays have to quantify HCV RNA independently from the RNA genotype[2]. To make a dilution of the samples even at high viral loads dispensable, a linear range from 50 IU/ml to 6 or 7 log IU/ml is required. The tests have to be applicable on treated, untreated and relapsing patients as well. Furthermore, it is important that all tests are reproducible and repeatable to simplify the follow-up of infected patients[2]. An overview of the currently available tests is given in Table 1.

For HCV RNA measurements, either plasma or serum from blood obtained by venous puncture can be used. If the test can not be carried out immediately, the samples have to be stored preferably at -60 °C or lower[2].

Table 1[1,2] Overview of the currently available molecular HCV tests

Assay Results	Assay Method	Commercial Name	Supplier	Lower limit of detection of Range, IU/ml	Note
Qualitative	RT-PCR	AMPLICOR HCV v2.0/COBAS Amplicor v2.0/Ampliscreen	Roche Diagnostics	50	Ampliscreen used for blood and organ donation screening
	RT-PCR	Ultra-Qual	National Genetics Institute	40	Reference in-house laboratory test
	TMA	VERSANT HCV Qualitative Assay	Siemens Diagnostics	10-615	
	TMA	Procleix HIV-1/HCV assay	Chiron	10	Used for blood and organ donation screening
Quantitative	bDNA	VERSANT HCV RNA 3.0 Assay	Siemens Diagnostics	$6.15x10^2$ -$7.7x10^6$	
	RT-PCR	LCX HCV RNA Quantitative Assay	Abbott Molecular Diagnostics	25-$2.63x10^6$	
	RT-PCR	AMPLICOR HCV Monitor v2.0/ COBAS Amplicor Monitor 2.0	Roche Diagnostics	$6x10^2$-$5x10^5$	
Quantitative detection	Real Time RT-PCR	COBAS Taqman HCV test	Roche Diagnostics	43-$6.9x10^7$	
Genotyping	Sequence Analysis	Trugene 5'NC HCV Genotyping Assay	Bayer HealthCare		
	Reverse hybridization	INNO-LiPA HCV II	Innogenetics		
	Reverse hybridization	Versant HCV Genotyping Assay 2.0	Innogenetics		

3.2.1 Qualitative tests

Qualitative tests are used to determine if the virus is absent or present in the sample. They have a higher analytical sensitivity than quantitative tests which is equal for all HCV genotypes[31,45]. If HCV RNA can be detected, the patient is certainly infected with hepatitis C, a diagnosis which is independent from ALT levels or antibody testing[1]. Qualitative testing is also applied to check if the HCV infection is resolved and is widely used to screen blood and organ donations[1,45]. Since the viral load in the sample is usually too low to be detected directly by simple molecular hybridization methods, amplification methods are needed. For the detection, the target, in this case the HCV RNA, is amplified by the assays[45]. Currently, two different assays are available. The most common test uses

the reverse transcription polymerase chain reaction (RT-PCR). Another, more recent qualitative test is working with transcription mediated amplification (TMA)[1].

Reverse transcriptase polymerase chain reaction (RT-PCR)

The most prevalent method to detect, but also quantify HCV RNA is reverse transcription PCR (RT-PCR). The commercial available tests for detection are used for diagnosis, therapeutic monitoring and blood or organ screening and have a lower limit of detection of 50 IU/ml. Moreover, a reference *in-house* laboratory test with a lower limit of detection of 40 IU/ml can be used. These tests have a sensitivity of more than 96% and a specificity of more than 99%[1].

The RT-PCR method is used for both detection and quantitation. The commercially available assay works with a competitive RT-PCR that possesses an internal control. First, the HCV RNA is extracted and precipitated. After the adding of a standard, amplification takes place by usage of a reverse transcriptase and a DNA polymerase. The primers used for amplification are coding for the 5' UTR of the HCV genome and for the synthetic RNA used in the standard. After hybridization with magnetic beads that are coated with amplicon-specific oligonucleotides, detection takes place by adding conjugate avidine-horseradish peroxydase and its substrate, which leads to a colorimetric reaction[2].

Transcription mediated amplification (TMA)

Tests working with transcription mediated amplification (TMA) have with more than 98% a higher sensitivity than the common RT-PCR assays. They also have a lower limit

of detection of about 10 IU/ml[45]. The commercial available tests are both used for diagnosis and blood and organ screening[1].

The qualitative TMA assays detect HCV RNA after amplification of the target. The oligonucleotides used as a probe are complementary for the HCV 5'UTR. After sample lysis, the HCV RNA is captured by the oligonucleotides which are bound to magnetic particles. An internal RNA control is added to each sample as well. Primers, reverse transcriptase and T7 RNA polymerase are added and during an isothermal TMA process[46]. Detection of the anti-sense single-stranded RNA product takes place with a chemiluminescent signal[2].

3.2.2 Quantitative tests

Quantitative NATs are used to determine the viral load of a patient, which is important for the control of the treatment and the management of HCV infection. The aim of HCV treatment is to achieve a sustained virological response, which is defined as being HCV RNA negative 6 month after the termination of antiviral therapy. If the outcome of the first quantitative NAT (prior antiviral therapy) is greater than $8x10^5$ IU/ml, the patient has a high viral load, a value smaller than $8x10^5$ IU/ml is called a low viral load. The chance to achieve a sustained virological response is greater in patients with a low viral load is better compared to the group with a high viral load[1].

Beside this pretreatment measurement, quantitative NATs are used for monitoring hepatitis C viremia (figure 3). The predictive parameter is the rate of virological response. Four and twelve weeks after start of therapy, the HCV viral load should be measured to make a prediction about the chance to achieve a sustained virological response. If the

patient is tested HCV RNA negative after 4 weeks which is defined as a rapid virological response, with a chance of 75% of a sustained virological response can be achieved. If the patient is initially tested HCV RNA negative after 12 weeks which is defined as an early virological response, he has a chance of 67% to achieve a sustained virological response[1]. A third use of quantitative testing is to make a decision about the continuation of therapy. If there is no decline of at least 2 log IU/ml after 12 weeks of therapy, cessation of therapy should be considered because in this case a sustained virological response is very unlikely[1]. For quantitative tests, the WHO standard requires a minimum sensitivity of 50 IU/ml. Methods used for quantitation work with either amplification of the signal as used in the branched-chain bDNA technique or with amplification of the target, as used in RT-PCR and Real-Time RT-PCR. For all of these methods, assays are commercially available[2].

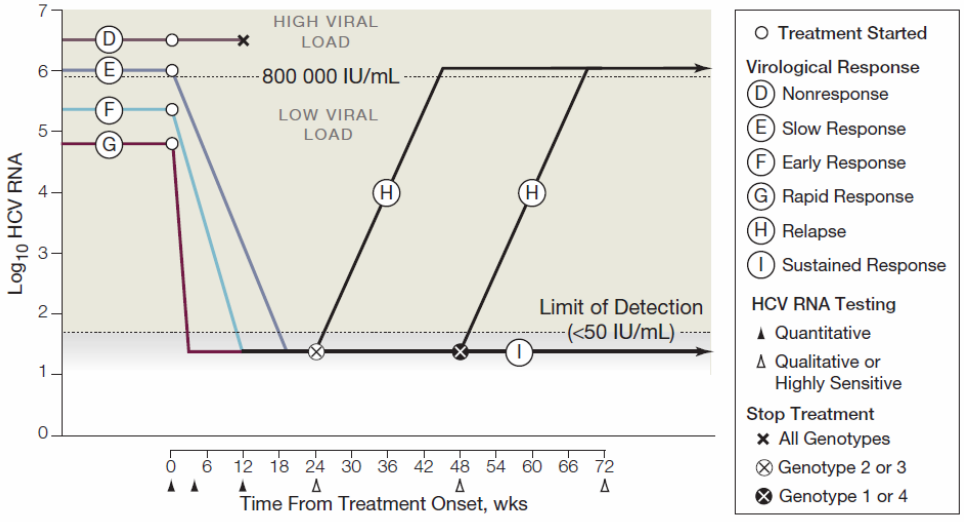

Figure 3[1]
Monitoring treatment response with molecular testing of patients with chronic Hepatitis C Virus Infection

Branched-chain DNA (bDNA) amplification

Currently, an assay with the branched-chain (bDNA) technique is widely used. This test has a range of detection of $6.15x10^2$-$7.7x10^5$ IU/ml, a good accuracy for all genotypes and is high reproducible and standardized. The specificity ranges from 96%-98.8%[1,2]. Disadvantages of this assay is a low sensitivity and the use of an external control[2]. bDNA assays also need a long incubation period[47].

Assays that use the branched DNA technique work with an amplification of the signal[2]. The technique is based on the hybridization of the HCV RNA to oligonucleotide capture and target probes and works in a sandwich-manner. The probes consist from a set of sequences complementary to the highly conserved 5' UTR and the 5' third of the core antigen[2,48]. After extraction of the HCV RNA by chemical lysis, it is directly hybridized in wells coated with the specific capture oligonucleotides. Then sequentially a target probe, a preamplifier probe and finally an amplifier probe is added, which one after another hybridize and form a branched DNA complex. This is the signal amplifying process. The DNA complex can be detected by adding alkaline phosphatase labeled probes and subsequent incubation with a chemiluminescent substrate. The assay uses an external control[2].

Quantitative RT-PCR

Beside of the application for detection purposes, the reverse transcriptase PCR (RT-PCR) technique is also used in a variety of quantitation assays. The commercially available assays have, depending on the type, a range of quantification of $6x10^2$-$5x10^5$ IU/ml or 25-$2.63x10^6$ IU/ml. Comparable results can be provided by an *in-house* test, which has a

range of 40-2x10^6 IU/ml. The assays used for quantitative RT-PCR work the same way as the assays used for qualitative RT-PCR[1,2].

Real-Time RT-PCR

The most recent method used in HCV diagnostic is the Real-Time RT-PCR technique[2]. These tests can quantify HCV RNA even if the viral load is high, but at the same time they are that sensitive that they can be used for detection purposes as well[1]. Currently, a high automated assays is commercially available with a range of quantification of 43-6.9x10^7 IU/ml[2]. Other advantages of Real-Time RT-PCR assays are that they are more economic and faster than other NATs[1].

The advantage of the recent launched Real-Time RT-PCR assays is that they can quantify the amplification during each cycle. The assays extract the viral RNA automatically and capture it depending on the assay on magnetic glass particles or magnetic microparticles. The RT-PCR is carried out using 5'UTR primers and a reverse transcriptase and DNA polymerase. A standard is used for a correct quantification. Detection of the amplification products takes place at each cycle by means of labeled oligonucleotide probes which emit fluorescent light in case of binding to the RT-PCR product. The whole process is carried out automatically in an analyzer[2].

3.2.3 Genotyping

Since the dose, duration and outcome of the antiviral therapy can vary among the different genotypes, the HCV genotype has to be determined after a positive NAT [1]. There are several molecular techniques for genotyping. First of all, *in-house* techniques

like direct sequencing of the NS5B or E1 region, sequence alignment and phylogenetic analysis can be carried out to determine the genotype[31].

The genotype can also be detected by Restriction Fragment Length Polymorphism (RFLP). For identifying the genotype with the RFLP method, first the HCV RNA isolated and used for the synthesis of cDNA, which is amplified by RT-PCR. The RT-PCR product is loaded on an agarose gel, electrophoresed, stained with ethidium bromide and read out with an imaging apparatus. If the sample is positive, the restriction enzymes *AccI*, *MboI* and if necessary for subtyping *EcoRII* are added for digestion. The product is then loaded on agarose gel again, and the restriction pattern is read out as described above. The restriction pattern is genotype specific[49].

However, since *in-house* techniques and the RFLP method are expensive and labour extensive, in the last decades some commercial kits for genotyping have been developed[26]. One method for determining the genotype is a direct sequence analysis. The commercial available assay for identifying the genotype by sequencing analyzes the 5'UTR of the HCV genome. Before sequencing, the HCV RNA is extracted, cDNA is synthesized and amplified by RT-PCR. Sequencing takes place using the sensitive CLIP technique. After analyzing the samples with a DNA sequencer, the results are compared with a database with known HCV isolates[50].

Another technique used in a commercial kit is reverse hybridization with genotype-specific probes which are located in the 5' UTR as well, known as Line Probe assay (LiPA)[26,31]. The current genotype LiPAs use biotinylated cDNA obtained by RT-PCR for reverse hybridization. Only the appropriate fragments from the 5' UTR and the core region are amplified for this purpose. The LiPA consists of a nitrocellulose strip with

immobilized oligonucleotide probes. These probes are genotype specific for the 5'UTR and the core region. After hybridizing, bound cDNA is detected by adding streptavidin and its substrate. If positive for a specific genotype, a visible line occurs on the strip that can be assigned by an interpretation chart[26].

The preferred regions used to determine the genotype are highly conserved. Nevertheless, mistyping still occurs because of the high mutation rate of the virus[26].

4 Conclusions and future perspectives

Because it is difficult to propagate the hepatitis C virus in culture, molecular virological techniques have been important in the research at HCV already since its discovery in 1989. During two decades of research, a multiplicity of serological and molecular detection techniques has been developed[1].

The introduction of standard screening on HCV in blood donations has almost eradicated the incidence of post-transfusion HCV infection. In general, NATs are used for this purpose. NATs are at the same time the best method to quantify HCV RNA, which is important for monitoring and the setting up of antiviral therapy[1]. Especially the new developed Real-Time RT-PCR assays show promise to become the best available technology, since they can be used for both high sensitive detection and quantifying of HCV RNA[2]. Recently it is found that beside of the 5'UTR also parts of the 3'UTR are well conserved and can therefore be used for diagnostics. It is shown that the viral load can be detected with a NAT accurately independent from the genotype using the 3'UTR as diagnostic target, so a new generation of assays could work with this sequence[51].

However, for developing countries NATs are often not feasible or economic. For these countries, an improvement of the common serological tests should be aspirated[16]. The current serological enzyme immunoassays of the third generation are already that sensitive that confirmatory testing after a positive result is actually dispensable[52]. However, to make sure to detect HCV infection even before seroconversion has occurred, core antigen tests are the only available serological method that can be used. This method lags in sensitivity behind the NATs and needs hence further optimalization[16].

Also a proper diagnosis of the genotype is important for the adjustment of therapy and predictions concerning the outcome of the infection. Using the currently available assays, mistyping is rare but still occurs due to the high mutation rate of the virus[26]. The mutation rate of HCV is still also a major problem in both detection and therapy of HCV infection. Acquired drug resistance against the standard combination therapy is an emerging issue in the field of HCV. Therefore, new treatments for HCV have been developed. The Specifically Targeted Antiviral Therapy against hepatitis C virus (STAT-C) shows promise to improve the current therapeutical strategies. New developed compounds are inhibitors of the NS3-4A serine protease, but since rapid resistance due to mutations are pre-existent or can occur *de novo*, a combination with the common therapy is advised[53]. Other novel therapeutic approaches of the STAT-C are inhibition of the HCV polymerase and immune modulation through the IFN pathway, but also RNA interference compounds or antisense oligonucleotides are in development[8].

In conclusion, the current available HCV tests show a high sensitivity and specificity which is important for a optimal therapy and the safety of blood and organ donations. However, HCV infections still remain a global burden due to other modes of

transmission, especially intravenous drug use and contaminated medical instruments. In addition, the high mutation rate of the virus is problematic because drug resistance can occur. Therefore, ongoing research is needed for optimal HCV management.

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