

Diploma thesis

**Detection of hepatitis C virus (HCV) RNA: a comparison of
automated platforms**

**Genotype Impact on HCV RNA levels determined with the VERSANT
HCV RNA 1.0 Assay (kPCR)**

Submitted by

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Herewith I, Madeleine Stübler, declare that I have written the present diploma thesis fully on my own and without any assistance from third parties.
Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, 15.04.2014

Madeleine Stübler

Index

1. INTRODUCTION	4
1.1 Morphology of the hepatitis C virus (HCV)	4
1.2 Prevalence of HCV infection.....	7
1.3 Genome, proteins, replication	9
1.4 HCV genotypes and subgenotypes.....	12
1.5 Diagnostics.....	14
1.5.1 Serologic assays	14
1.5.2 Molecular assays	14
1.6 Natural progress of HCV disease	16
1.6.1 Acute hepatitis C (AHC).....	16
1.6.2 Chronic hepatitis C (CHC).....	16
1.7 Treatment.....	17
1.8 Objectives	20
2. MATERIALS AND METHODS.....	21
2.1 Reference material	21
2.2 Clinical specimens.....	21
2.3 HCV RNA quantitation.....	22
2.3.1 Molecular assays used in this study	22
2.3.2 Direct sequencing	26
3. Results.....	28
4. Discussion.....	37

References 40

List of Figures 48

List of tables 50

1. INTRODUCTION

1.1 Morphology of the hepatitis C virus (HCV)

The hepatitis C Virus (HCV) is a small, single stranded 9600-nucleotide RNA molecule of positive polarity, covered by an icosahedral protein capsid and a lipid envelope (Op De Beek et al., 2003) (Fig.1/2/3).

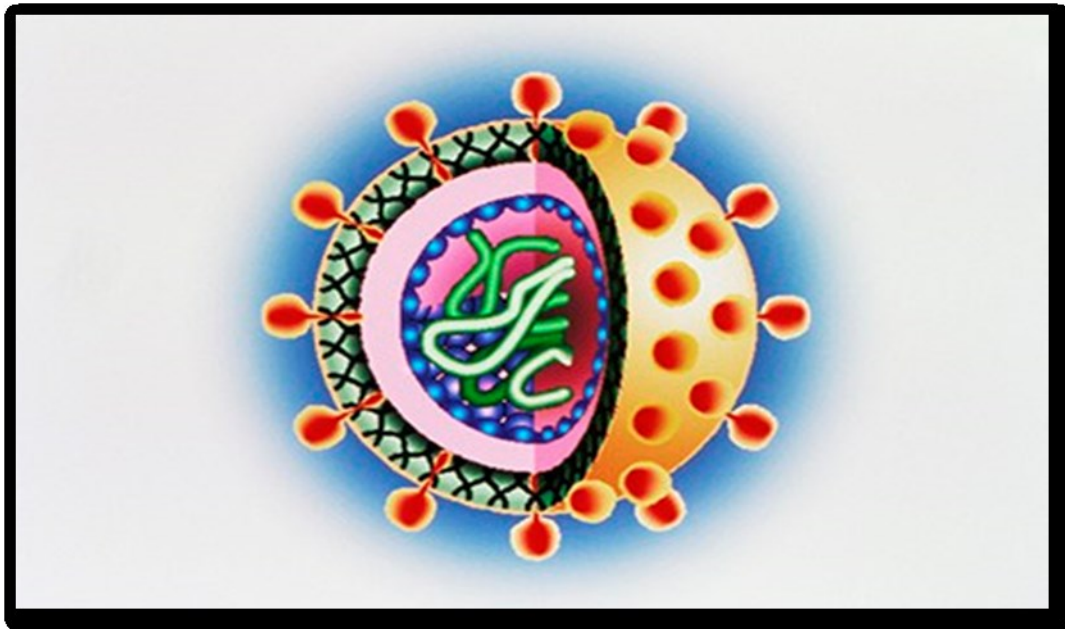


Fig. 1: The structure of HCV.

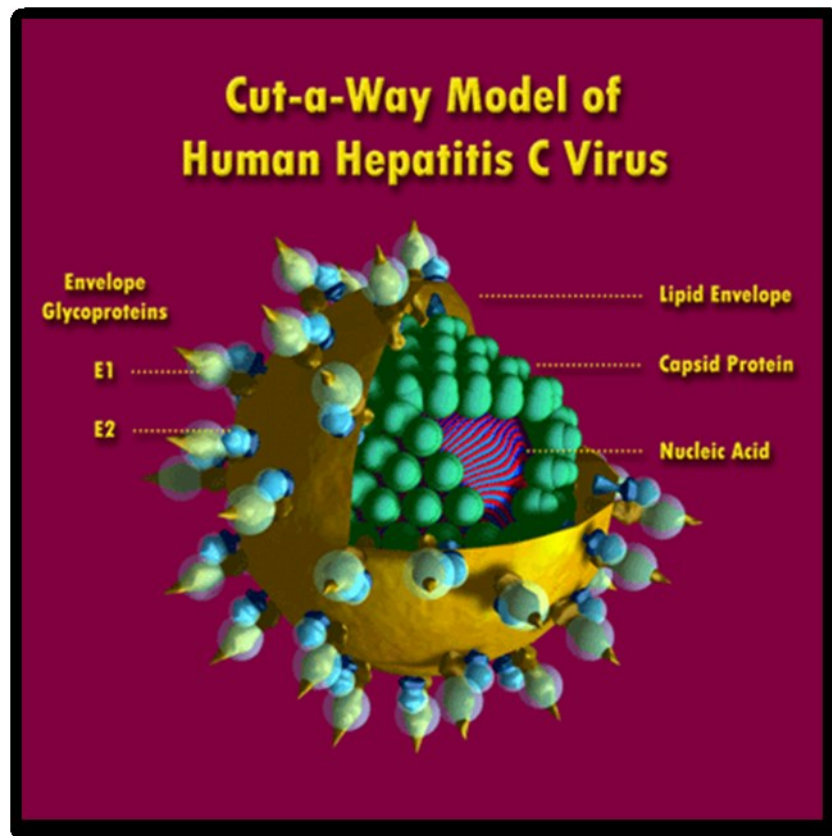


Fig. 2: Detailed structure of HCV.

The HCV belongs to the group of *FLAVIVIRIDAE*; it was first identified in the USA in 1989. Prior to the identification of the causative agent, infectious hepatic disease of unknown origin had been defined as “non A, non B hepatitis”.

The characterization of HCV led to the understanding of its primary role as post-transfusion hepatitis and its pathogenic tendency to induce persistent chronic liver infection leading to cirrhosis and liver cancer.

EM of HCV produced in cell culture

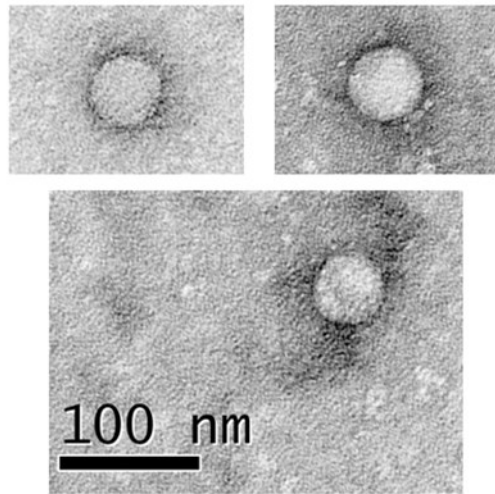


Fig. 3: Electron microscopy of HCV.

The HCV is a very infectious virus and there is no vaccination against it available. It is a very important reason for chronic liver disease leading to liver cirrhosis and liver cancer.

1.2 Prevalence of HCV infection

The HCV infection is endemic in most parts of the world representing a major global health care problem. With more than 170 million people infected with HCV worldwide, that means, nearly 3% of the world's population, the World Health Organization (WHO) has declared HCV infection a major public health challenge with heterogeneous geographical and temporal relevance (WHO, 1999) (Fig.4).

Countries with high rates of chronic infection are Egypt (22%), Pakistan (4.8%), and China (3.2%). The major mode of transmission in these countries is attributed to unsafe injections, with contaminated equipment (WHO, 2012).

In Europe, the estimated prevalence of HCV infection ranges between 0.1 and 1.2% in Northern and Central Europe and between 2.5 and 3.5% in Southern Europe (Esteban et al., 2008).

However, in certain areas such as Southern Italy, the prevalence of HCV infection appears to be significantly higher with up to 26% (Raffaele et al., 2001).

Prevalence of HCV infection in Eastern European countries has been reported to be between 0.9 to 4.9% among blood donors, 1 to 10% among health care workers, 13 to 48% among hemodialysis patients, and up to 92% in hemophilic patients (Naoumov et al., 1999).

In the United States, national survey data suggests that the prevalence is about 1.8% making it the country's most common chronic blood born infection (Brown et al., 1999).

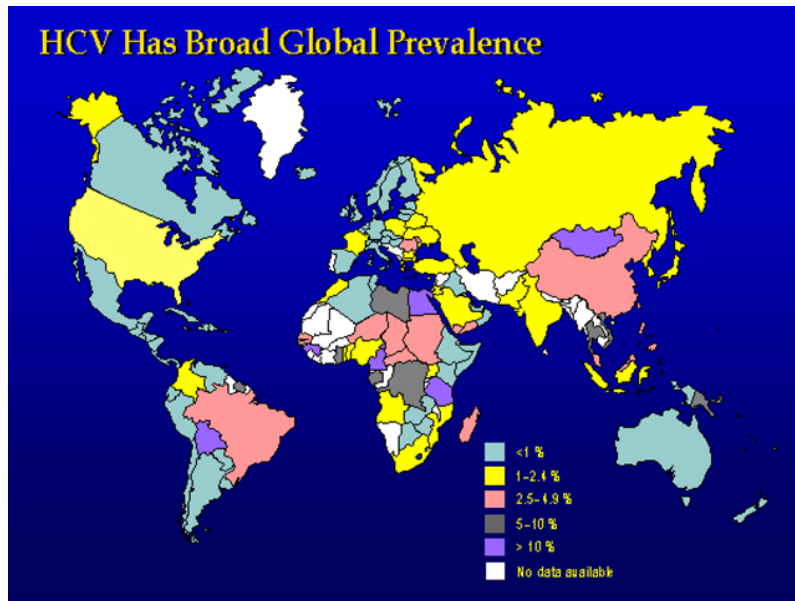


Fig. 4: The global distribution of HCV infection.

In countries with lower socioeconomic status, the HCV prevalence is higher than in those with a higher socioeconomic status.

The HCV is transmitted through blood; in Africa, people are commonly infected with HCV because of lack of control of blood transfusions. In Europe and the USA, HCV is transmitted mainly through intravenous drug abuse nowadays. In contrast to hepatitis B virus (HBV), transmission of HCV through sexual contact or horizontal transmission in childhood are not that common.

The risk to health care workers acquiring HCV in health care settings is well recognized. In fact, aggregate serological data suggest that health care workers show a prevalence of HCV infection similar to that of the general population (Centers for Disease Control and Prevention, 1998). The worldwide annual incidence of HCV infections among health care workers is estimated to reach 16,000 infections due to occupational exposure to percutaneous injuries (Esteban et al., 1996). It is assumed that three million health care workers experience the stressful event of a percutaneous injury with a contaminated sharp object each year.

1.3 Genome, proteins, replication

The HCV genome has a single large open reading frame that encodes for a virus polyprotein of nearly 3000 amino acids. The protein is subsequently processed into functional proteins and there are three types of regions (Fig.5):

- Highly conserved regions (e.g., the 5'untranslated region)
- Variable regions (e.g., envelope 1 [E1] and nonstructural 5b [NS5b])
- Hypervariable regions (HVR) (e.g., HVR1 and HVR2 in E2)

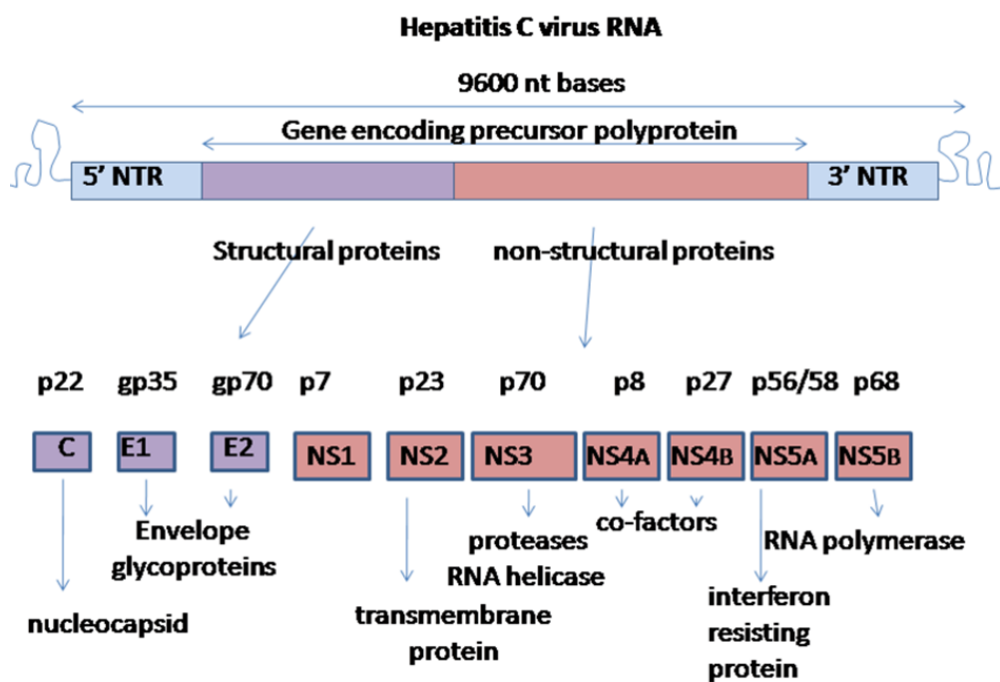


Fig. 5: The HCV genome and proteins encoded.

Towards the 5'end of the genome, the E1 and E2 envelope glycoprotein region encoding the highly variable envelope antigens, the C region encoding the nucleocapsid protein, and the 5'noncoding region (5'NC, untranslated region = UTR) have been described (Fauci et al., 2008).

Towards the 3' end of the HCV genome, the NS2, NS3, NS4a, NS4b, NS5A and NS5B, regions encoding the genes for 6 non-structural proteins and the 3' noncoding region have been described (Fauci et al., 2008).

The non-structural proteins encode replication proteins, such as the helicase, the serine protease, and the RNA-dependent RNA polymerase (Fig.6).

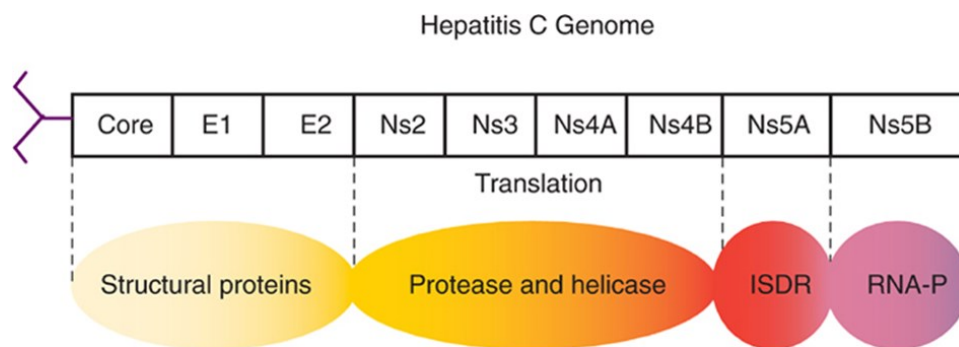


Fig. 6: The HCV genome and its regions.

Not all mechanisms about the HCV lifecycle are known exactly. The envelope glycoproteins are supposed to be the ligand for receptors on the hepatocytes. Several possible receptors for HCV have been identified, such as CD 81, the scavenger receptor class B type 1, and the mannose binding lectins DC-Sign and L-Sign (Pawlotsky et al., 2004).

The HCV not only infects hepatocytes but also B-lymphocytes. Endocytosis of HCV in these cells is believed to be dependent on lipoproteins and mediated by the formation of HCV: lipoprotein complex that contains Apo lipoprotein (Burlone et al., 2009).

Because of the HCV dependence on lipoproteins, viral cell entry is dose dependently reduced and endocytosis of HCV is competitively inhibited by low density lipoproteins (LDL) and very-low-density-lipoprotein, however not by high-density-lipoprotein (Agnello et al., 1999).

That means that the lipid metabolism is an important factor for HCV endocytosis and it has also been shown, that HCV infection contributes to up-regulation of many genes, which related to lipid metabolism (Di Bisceglie, 2009).

Studies have demonstrated that the HCV envelope glycoprotein 2 binds to the CD 81 on the cell surface. It has also been shown that the HCV entry is inhibited in the presence of anti-CD81 or when CD 81 expression is down-regulated in hepatic cells (Morikawa et al., 2011)

It has been suggested that that HCV lipoproteins enable HCV to be transferred by CD 81 to tight junction proteins leading to viral cell entry via endocytosis mediated by the envelope glycoproteins (Burlone et al., 2009).

After receptor mediated endocytosis and fusion of the virus envelope with the endosomal membrane, the positive-strand HCV is thought to be uncoated and translated in the cytoplasm. It seems that processing of the polyprotein, assembly of the replicase, and synthesis of the negative-strand RNA, occur in association with the endoplasmic reticulum, outside of the nucleus of the host cell (Sclan et al., 2009) (Fig. 7).

Newly produced positive-strand RNA and structural proteins probably gain an envelope by budding into the endoplasmic reticulum before being released by transport through the Golgi apparatus (Di Bisceglie, 2009).

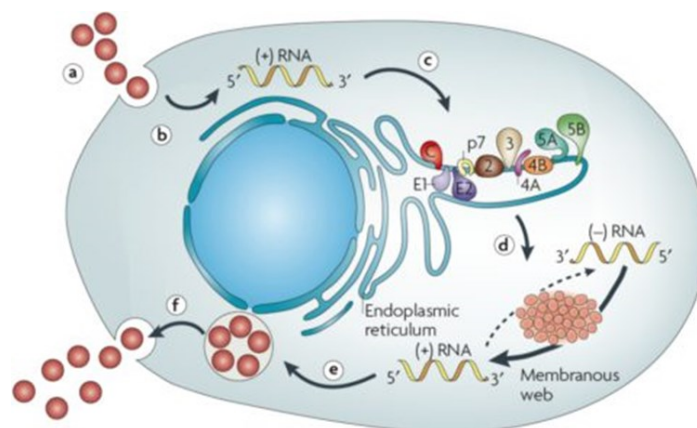


Fig.7: HCV lifecycle

1.4 HCV genotypes and subgenotypes

The HCV is generally classified into 6 major genotypes, which differ one from another in sequence homology. The diversity of HCV genomes results from mutations due to higher error rates in RNA replication because of the poor fidelity of the RNA polymerase and is associated with effective humoral immunity (Fig.8). Neutralizing antibodies to HCV have been described but they are not an important factor for immunity against HCV infection.

The distribution of HCV genotypes and subgenotypes varies in different parts of the world. Several subgenotypes such as 1a, 1b, 2a, and 2b are ubiquitous, while others are confined to specific geographic areas.

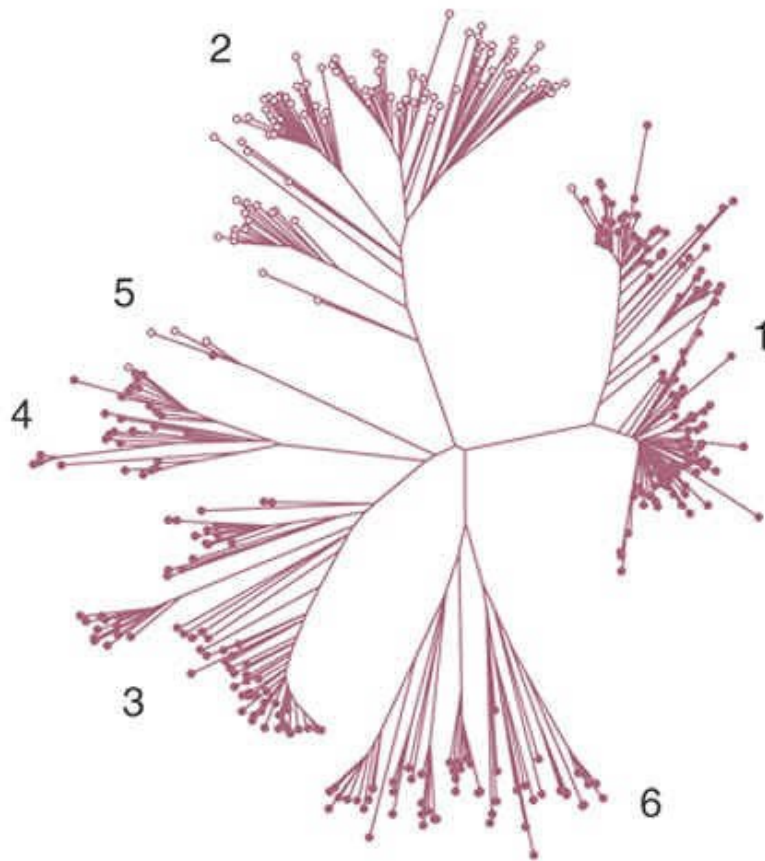
Certain genotypes are more prevalent in some geographical areas:

- Genotype 1 in Europe and the USA
- Genotype 4 in the Middle East and in Northern and Central Africa
- Genotype 5 in Southern Africa

The most common genotype is HCV genotype 1. In a recent Austrian study, HCV genotype 1 was found to be responsible for HCV infection in approximately 65% of patients investigated (Ferenci et al., 2008).

Several studies showed consistency with the hypothesis that the relative risk of developing liver cirrhosis or hepatocellular carcinoma may be significantly higher in patients with HCV subgenotype 1b (Bellentani et al., 1999; Roffi et al., 1998).

Due to different antigenic epitopes in different HCV genotypes and subgenotypes, it is almost impossible to produce an effective HCV vaccine. Cross protection is also very unlikely because the genotypes differ too much with antigen presentation.



Phylogenetic analysis of HCV
(P.Simmonds, J.Hepatol. 1999, 31, suppl. 1, 54-60)

Fig.8: Phylogenetic tree of HCV.

1.5 Diagnostics

For HCV screening, serological testing for anti-HCV antibodies is done. It is possible that false-positives may emerge due to unspecific reactions; therefore, testing for HCV-RNA is required for confirmation. For detection of antibodies, serum is collected, for detection of HCV RNA, plasma or serum can be used; in Europe, serum is preferred. If plasma is employed, EDTA whole blood is preferred because heparinized blood is not suitable due to inhibitory effects of heparin may inhibit PCR reactions (Hoffmann et al., 2012).

1.5.1 Serologic assays

Serologic assays are designed for antibody screening. Patients with suspected chronic hepatitis C (CHC) are identified by an enzyme-linked immunosorbent assay (ELISA) (Di Bisceglie, 2009).

The ELISA detects a mixture of antibodies directed against different epitopes on the HCV core and non-structural proteins (Di Bisceglie, 2009). With the latest generation of assays, antibodies can be detected from 4 weeks after infection onwards.

If the anti-HCV ELISA shows a positive result, it is necessary to check for HCV RNA.

1.5.2 Molecular assays

Molecular assays are used for detection of HCV RNA, HCV genotyping, testing on IL28B polymorphism, and for detection of drug resistance.

For quantitation of HCV RNA, real time polymerase chain reaction (qPCR) is employed. It provides information about the presence of HCV RNA and its concentration in the blood. Precise determination of the viral load also provides information about the risk of transmission.

Most commonly, a part of the 5'UTR is used for amplification. The viral load is expressed as International Units (IU)/mL.

For HCV genotyping two major methods, direct sequencing and assays based on the reverse hybridization principle are employed. Direct sequencing allows for analysis of new genotypes.

1.6 Natural progress of HCV disease

1.6.1 Acute hepatitis C (AHC)

After an incubation period ranging from 2 to 12 weeks with an average of 6 to 8 weeks, symptoms develop which are usually mild and non-specific. Symptoms may include fatigue, mild right upper body pain, nausea, lack of appetite, and muscle pain. Additionally, non-hepatic symptoms such as fever, rash, and arthralgia may occur. In most patients, only mild enlargement of the liver is observed (Di Bisceglie, 2009).

Acute HCV may progress to chronic hepatitis C (CHC) in 60 to 80% of elder patients (Fig. 9).

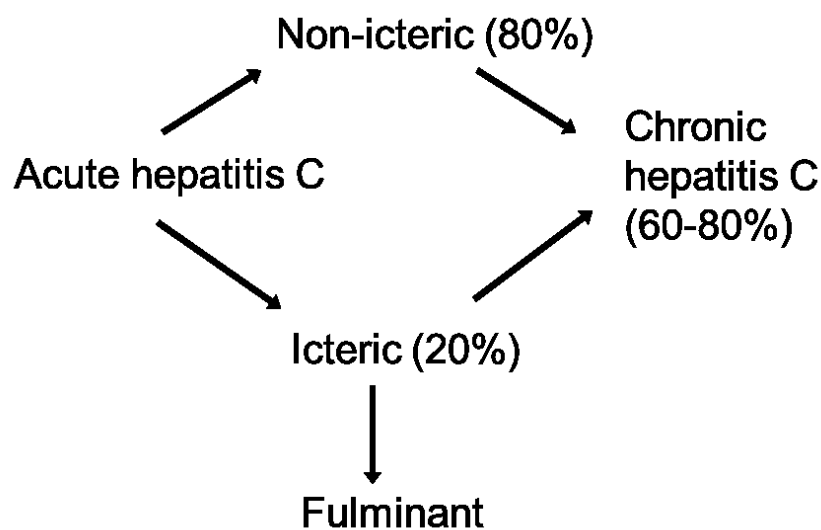


Fig. 9: Progression of HCV infection.

1.6.2 Chronic hepatitis C (CHC)

The CHC can be asymptomatic for many years. Living with CHC over 25 to 30 years may lead to severe complications such as decompensate liver cirrhosis and hepatocellular carcinoma (HCC) (Centers of Disease Control and Prevention (CDC)).

1.7 Treatment

The main goal of HCV therapy is to prevent the progression of liver disease. The decision for treatment depends on liver disease severity, risk factors for disease progression, and the HCV genotype (Di Bisceglie, 2009).

All treatment-naïve patients with compensated liver disease and detectable HCV RNA should be considered for anti-HCV therapy (European Association of the Study of the Liver 2011; Ghany 2009; Sarrazin 2010).

The end point for anti-HCV therapy is the sustained virologic response, characterized by undetectable HCV RNA at week 24 after end of treatment.

Accurate HCV RNA quantification is essential for the management of chronic hepatitis C therapy. Monitoring of the HCV RNA concentration during antiviral combination treatment with pegylated interferon alpha (PEG-IFNa) and ribavirin (RBV) is key to assess virologic response, guide treatment duration, and decide futility (Chevaliez et al., 2012).

In patients infected with HCV genotype 1, treatment is currently based on a triple combination treatment with PEG-IFNa, RBV, and one of two protease inhibitors, either telaprevir or boceprevir. The rapid virologic response (defined as undetectable HCV RNA after the first 4 weeks on triple therapy) is a strong predictor of sustained viral response (Fried et al. 2011; Jacobson et al., 2011/ 2012).

Futility rules have been established in order to prevent unnecessary exposure to the protease inhibitor and to avoid adverse events, the emergence of viral resistance, and useless costs (Jacobson et al., 2001). Recently, new direct-acting antiviral drugs (DAAs) have reached early to late clinical development. Several trials are on-going in treatment-naïve and treatment-experienced patients infected with HCV genotypes 1 to 4 (Sarrazin et al., 2012).

New DAAs for treatment of chronic hepatitis C include HCV protease inhibitors, NS5B polymerase inhibitors (nucleosides/nucleotides and non-nucleosides), and NS5A inhibitors.

Numerous inhibitors of the NS3/4A protease are in clinical development, aiming to replace boceprevir and telaprevir. HCV NS3/4A protease inhibitors can be divided

into linear compounds, such as boceprevir and telaprevir, and macrocyclic compounds. Protease inhibitors often have high antiviral potency, but specific viral mutations that confer resistance have been reported for all compounds in development and there is widespread cross resistance within both linear and macrocyclic groups (Sarrazin et al., 2010). Upcoming “second-wave” protease inhibitors have one or more incremental advantages over the first-wave compounds (boceprevir, telaprevir). These include higher potency, higher genetic barriers to resistance and more favorable pharmacokinetic profiles, including once-daily dosing (Ciesek et al.2011). Simeprevir is a second-wave, macrocyclic, once-daily protease inhibitor that has activity against several genotypes; although it is currently being developed primarily for genotype 1 infection. Further second-wave protease inhibitors include asunaprevir, danoprevir, faldaprevir, sovalprevir, ABT-450, and GS-9451. It seems likely that one or more second-wave protease inhibitors will reach the market by 2014 and be used in the context of either IFN-containing or all oral combinations (Manns and von Hahn, 2013). Nucleoside and nucleotide inhibitors of the NS5B RNA-dependent RNA polymerase bind to the enzyme active site and lead to premature chain termination of the nascent viral genome. The NSB active site is well-conserved across HCV genotypes as amino acid substitutions in this location are generally poorly tolerated and result in a significant loss of viral fitness (Pawlotsky et al., 2012). Therefore, nucleoside and nucleotide polymerase inhibitors tend to have good activity against a broad range of genotypes and have a high genetic barrier to resistance. Three nucleoside or nucleotide NS5B polymerase inhibitors currently are in clinical development: mericitabine, sofosbuvir and VX-135. Among those, sofosbuvir and VX-135 seem to have the highest antiviral activity. Sofosbuvir, a nucleotide analog, has recently been approved by both the FDA and the EMA and is therefore the most advanced in this class. Regarding the new drug application submitted to the FDA, two combinations are most likely to be approved: sofosbuvir with PEG-INFa plus RBV for HCV genotype 1 and sofosbuvir with RBV for HCV genotypes 2 and 3. Overall, their high potency and high barrier to resistance make nucleoside and nucleotide polymerase inhibitors a highly promising class that may become part of first-choice IFN-containing and all-oral regimens, a so-called backbone of future anti-HCV therapies (Manns and von Hahn, 2013).

Non-nucleoside NS5B polymerase inhibitors bind the NS5B protein outside the active site, thereby causing a conformational change that inhibits the polymerase activity (Manns and von Hahn, 2013).

Drugs in clinical development targeting the NS5A RNA-binding protein include daclatasvir, ledipasvir, ABT-267, ACH-3102, MK-8742 and PPI-668. NS5A is essential for viral replication; however, its precise function and, therefore, the mechanism of action of NS5A inhibitors are unclear. Nevertheless, the above compounds induce a rapid decline in viral load. Currently, NS5A inhibitors look like promising combination partners both for the PEG-INF plus RBV regimen and all-oral regimens although they may be less useful in subgenotype 1a compared with other genotypes (Manns and von Hahn, 2013) (Fig.10).

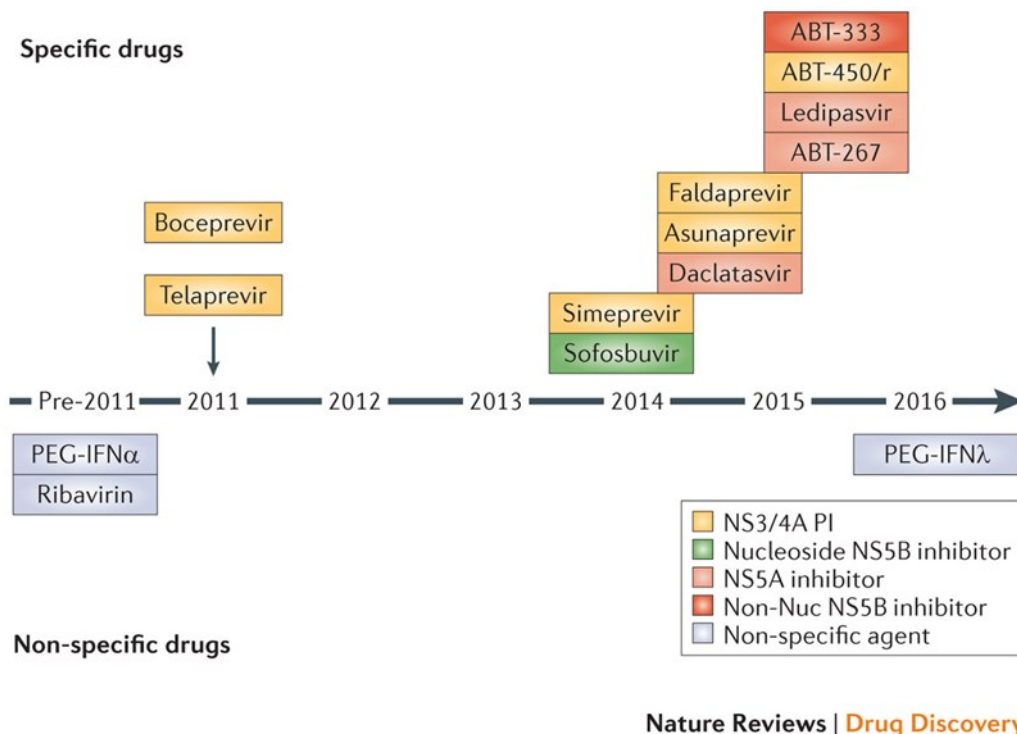


Fig.10: Present and future anti-HCV therapy.

1.8 Objectives

With new anti-HCV therapies, monitoring of the HCV RNA concentration is and will remain the most useful parameter to assess treatment response and guide treatment decisions. Recently, new automated IVD/CE-labeled test systems for quantification of HCV RNA were introduced. The VERSANT HCV RNA 1.0 Assay (kPCR) (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) includes sample preparation and PCR mix setup on an automated platform followed by real-time PCR. The COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test (Roche Molecular Systems, Inc., Branchburg, NJ), version 2.0, is performed on a fully automated platform including the sample preparation, PCR set up, and real-time PCR procedures.

The aim of this study was to compare the genotype-dependent efficiency of the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0. For experiments, commercially available reference material and clinical samples were used.

2. MATERIALS AND METHODS

2.1 Reference material

Five members of the Worldwide HCV Performance Panel WWHV302(M) (SeraCare Life Sciences, Milford, MA) consisting of naturally occurring plasma specimens from diverse geographic locations were employed. Members included HCV subgenotypes 1a, 1b, 2a, 3b, and 4a. Of each member, a 1:100-dilution was prepared using human plasma collected from healthy individuals without detectable HCV antibodies and HCV RNA. Of each dilution, 8 aliquots were prepared. Four aliquots each were analyzed with the Siemens assay and the Roche assay in parallel.

2.2 Clinical specimens

For evaluation of the clinical performance, 183 anonymized clinical residual samples obtained from patients with chronic hepatitis C infection were investigated. Peripheral venous blood had been collected in 8.0-ml Vacuette tubes containing separation gel (Greiner Bio-One, Kremsmünster, Austria). Within 6 h of drawing the blood, tubes had been centrifuged at 1200×g for 12min at room temperature. After centrifugation, serum aliquots had been prepared and immediately frozen at -70°C until tested. In the routine diagnostic laboratory, an aliquot of each sample had been previously analyzed using the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test (Roche), version 1.0 and found to be within the analytical measuring range. Samples included different HCV genotypes: 1 (n= 61), 2 (n= 32), 3 (n = 39), and 4 (n = 51). Due to the large sample input volume required for this study, samples were diluted 1:10 with human serum collected from healthy individuals without detectable HCV antibodies and HCV RNA prior to testing. Following preparation of 2 aliquots, samples were analyzed with the Siemens assay and the Roche assay in parallel.

2.3 HCV RNA quantitation

2.3.1 Molecular assays used in this study

Basic features of the molecular assays used in this study are shown in Table 1.

The VERSANT HCV RNA 1.0 (kPCR) assay is based on HCV RNA extraction and real-time polymerase chain reaction for quantitative detection of human HCV RNA in plasma or serum of HCV-infected individuals. This assay is run on the VERSANT kPCR Molecular System which consists of the Sample Preparation (SP) Module designed for automated nucleic acids extraction and PCR mixture preparation and the Amplification/Detection (AD) Module designed for real-time PCR and detection (Fig.11). The assay is intended to be used in conjunction with clinical presentation and other laboratory markers of disease status to aid in the management of HCV-infected individuals undergoing antiviral therapy. The analytic measuring range of the system is from 1.5×10^1 (equaling the limit of detection) to 1.0×10^8 IU/mL (<http://www.healthcare.siemens.de/moleculardiagnosics>).

The COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, v2.0 is a nucleic acid amplification test for the quantitation of Hepatitis C Virus (HCV) RNA genotypes 1 to 6 in human EDTA plasma or serum. This assay is run on a fully automated platform consisting of the COBAS AmpliPrep instrument designed for automated nucleic acids extraction and PCR mixture preparation connected with the COBAS TaqMan instrument designed for real-time PCR and detection (Fig. 12). The test is intended for use in the management of patients with chronic HCV in conjunction with clinical and laboratory markers of infection. The analytic measuring range of the Roche assay is from 1.5×10^1 (equaling the limit of detection) to 1.0×10^8 IU/mL (COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0, Roche Compendium).

For the present study, both of the molecular assays were performed according to the manufacturer's package insert instructions. All experiments were done with single kit lots of the molecular assays. Runs were considered valid and patient results reported if all conditions outlined in the manufacturer's package insert instructions occurred. For both of the assays, the limit of detection (LOD) and the lower limit of

quantification (LLQ) are identical with 15 HCV RNA IU/mL according to the assay package inserts. Valid results were reported quantitatively (IU/mL), as “positive <LOD” (if the value obtained was under the LOD), or as “not detectable” (Fig.13).

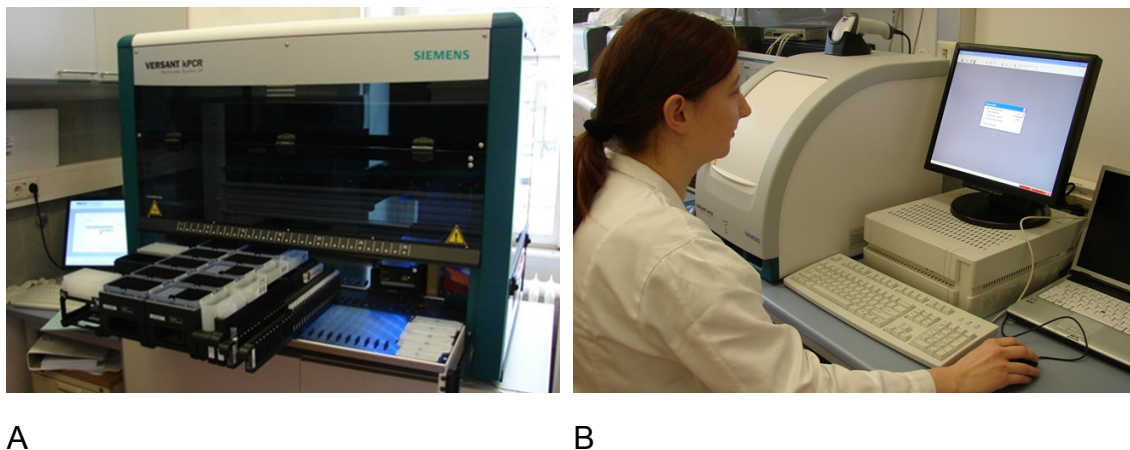


Fig.11: The VERSANT kPCR Molecular System consisting of the Sample Preparation (SP) Module for nucleic acids extraction and PCR mixture preparation (A) and the Amplification/Detection Module (B).



Fig.12: Cobas Ampliprep/Cobas Taq Man for HCV RNA quantitation. The fully automated platform consists of a nucleic acids extraction / PCR mixture preparation device (left) and an amplification / detection device based on the real-time PCR technology.

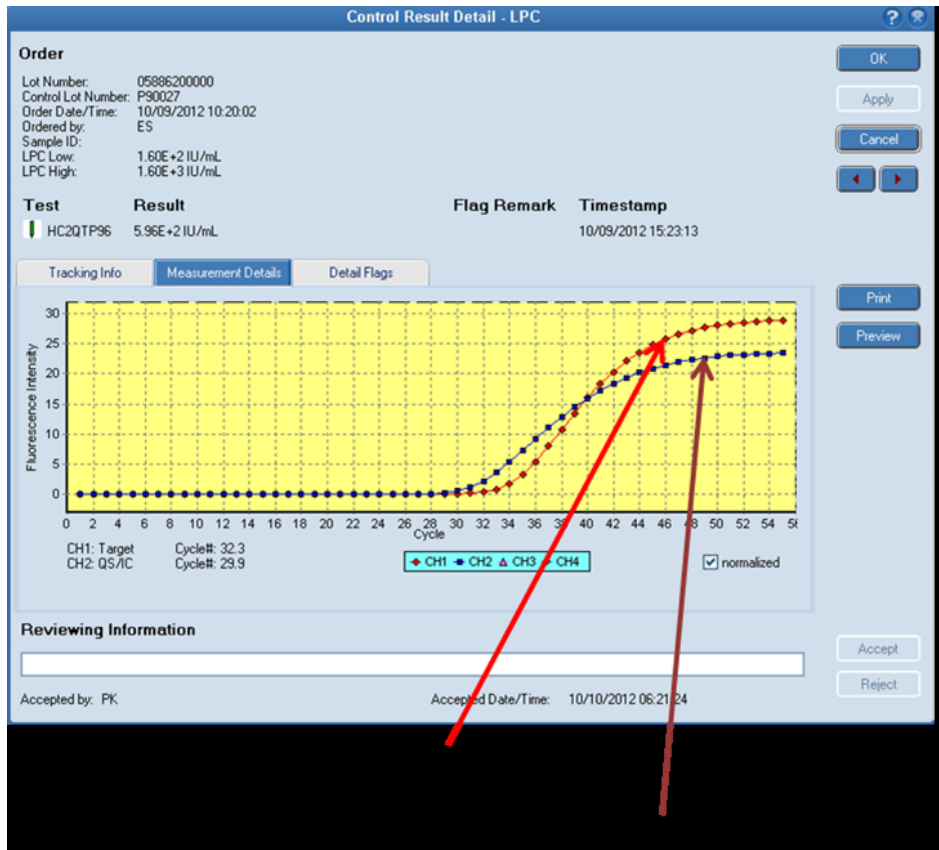


Fig.13: Amplification curves obtained with the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, v2.0. This example shows the amplification curves obtained from a low-titer HCV sample and that from the internal quantitation standard serving also as internal control.

Table 1.

Features of the molecular test systems used in this study.

Characteristics	Manufacturer and details	
	Siemens Healthcare Diagnostics	Roche Molecular Systems
Assay name	VERSANT HCV RNA 1.0 Assay (kPCR)	COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, v2.0
Target sequence	5`NCR	5`NCR
Sample volume	700 μ L or more depending on type of sample tube	650 μ L
Detection method	TaqMan	TaqMan
Internal control	Heterologous	Homologous
Standards	Two external quantitation standards	One internal quantitation standard
Range of linearity	$1.5 \times 10^1 - 1.0 \times 10^8$ IU/mL	1.5×10^1 IU/mL- 1.0×10^8 IU/mL

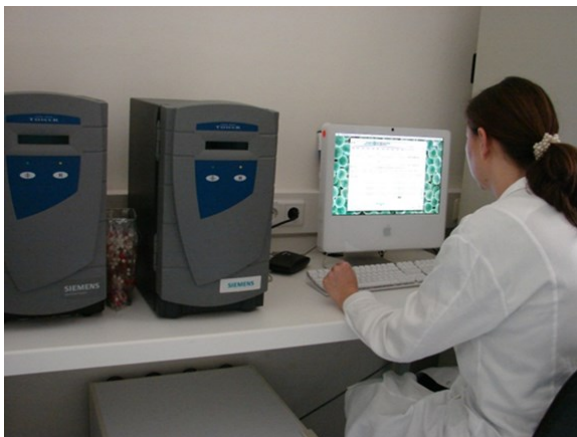
2.3.2 Direct sequencing

HCV genotypes were determined by using the TRUGENE HCV Genotyping Kit (Siemens) according to the manufacturer's package insert instructions. The HCV genotype was generated by means of the TRUGENE HCV 5'NC Module of the OpenGene software system (Siemens) (Fig.14). In addition to the HCV genotype, the Librarian tool of the OpenGene software contains 286 sequences for genotype and sub-genotype calls and states the HCV subgenotype and the closest homology to a certain HCV isolate analyzed including the Genbank accession number.

Sequence analysis of the almost full-length 5' NCR and part of the core region was performed on the remaining HCV genotype 2 aliquots. Reverse transcription and PCR were performed by using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Prior to reverse transcription, RNA was treated for 5 min at 65°C. For the single-round reverse transcription PCR, external forward and reverse primers were used as described recently to amplify a 447-base pair fragment.¹⁵ Reverse transcription PCR was carried out with a 50- μ l volume containing 10 μ l sample RNA, 0.2 μ M forward primer, 0.2 μ M reverse primer, and 400 μ M each deoxynucleoside triphosphate. Buffer Q solution and the reverse transcription enzyme mixture were added as detailed in the manufacturer's instructions. Reverse transcription PCR was run on an ABI9700 thermocycler (Applied Biosystems, Foster City, CA) with the following temperature program: 30 min at 50°C and 15 min at 95°C, followed by 45 cycles with 30 s at 94°C, 30 s at 54°C, and 45 s at 72°C and a final extension for 5 min at 72°C. After reverse transcription PCR, cycle sequencing was performed by using the Thermo Sequenase Primer Cycler Sequencing Kit (GE Healthcare, Little Chalfont, UK). The four Cycle sequencing reaction mixtures contained reagents included in the kit, 0.2 μ M forward primer (as used for reverse transcription PCR but 5'-Cy5 labeled), 0.2 μ M reverse primer (as used for reverse transcription PCR but 5'-Cy5.5 labeled), and 1.25 μ L amplification product. The cycle sequencing program consisted of 25 cycles with 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C. After cycle sequencing, 6 μ L of formamide loading dye was added. Samples were heated at 72°C for 3 min and incubated for a minimum of 30 min on ice. Fragments (1.8 μ l/lane) were separated on a TRUGENE tower (Siemens) at 60°C, 2000 V, 50% laser power, and a sampling rate of 0.5 s for 60 min with a size 500 polyacrylamide gel. The sequencing covered a

408-bp stretch of the amplification product, corresponding to nucleotides 37 of the 5'NCR through 102 of the core region (available from <http://hcv.lanl.gov/cgi-bin/LOCATE/locate.cgi>). The forward and reverse sequences were searched and combined using the OpenGene software module GeneObjects (version 4.1; Siemens) and read against a newly created HCV subgenotype 2a-specific library. This library was generated with the OpenGene software program (Siemens) by importing the respective sequences into the library file in an aligned fashion as detailed in the manufacturer's instructions. Sequences were aligned with ClustalW.

All experiments were done in an International Standard organization (ISO 9001:2008)-certified laboratory, the Molecular Diagnostics Laboratory, IHMEM.



A



B

Fig.14: Two TRUGENE towers for direct sequencing according to the Sanger principle and the personal computer containing the OpenGene software module GeneObjects for evaluation of data generated (A). HCV sequencing data obtained by the OpenGene software system (B).

3. Results

When dilutions of five members of the Worldwide HCV Performance Panel WWHV302(M) were tested with the Roche and the Siemens assays, mean HCV RNA concentrations were found to be $<0.5 \log_{10}$ unit for HCV subgenotypes 1a, 1b, 3b, and 4a (Table 2). The mean HCV concentration for the member containing HCV subgenotype 2a was $0.76 \log_{10}$ unit lower when tested with the Siemens assay than that obtained with the Roche assay. Genetic analysis of this member by using the TRUGENE HCV Genotyping Kit revealed a 100%-homology to HCV isolate HCJ5.

Of 183 clinically referred samples, 177 tested positive with both of the assays, 176 of them within the analytical measuring range and one positive $<LLQ$ (Table 3). In two samples, HCV RNA was not detectable with both of the assays. With four samples, discrepant results were obtained: three samples (two HCV genotype 2 samples and one HCV genotype 4 sample) were positive under LLQ with the Roche assay and not detectable with the Siemens assay and one sample (HCV genotype 1) was positive under LLQ with the Roche assay while the Siemens assay resulted in an HCV RNA concentration of 20 IU/mL.

Table 2.

Results obtained from 1:100-dilutions analyzed four times in parallel with the Siemens and the Roche assays using the WWHV302(M) panel members 01, 02, 04, 06, and 11

Panel member	HCV subtype	Mean of HCV RNA IU/mL measured (standard deviation)		Mean log ₁₀ unit difference between assays
		VERSANT HCV RNA 1.0 Assay (kPCR)	COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0	
WWHV302(M)-01	1b	8795 (1240)	8898 (834)	-0.01
WWHV302(M)-02	1a	1817 (144)	1590 (159)	0.06
WWHV302(M)-04	2a	1208 (144)	6890 (306)	-0.76
WWHV302(M)-06	3b	48133 (2479)	34200 (2121)	0.15
WWHV302(M)-11	4a	302 (72)	306 (76)	-0.01

Table 3.

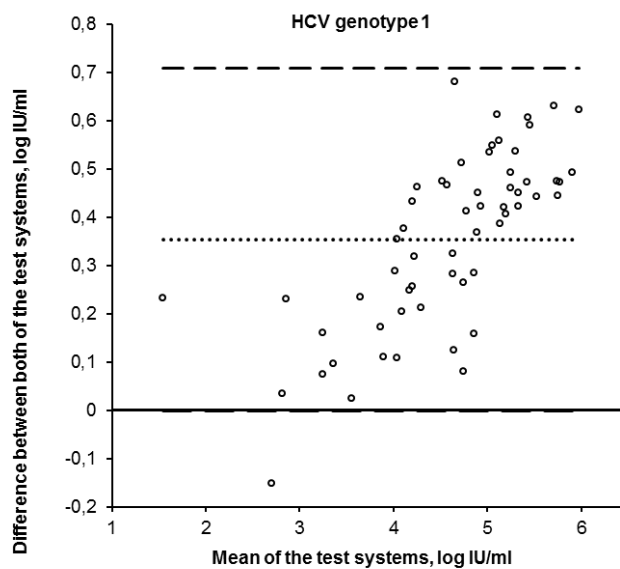
Comparison of results from clinically referred samples obtained by the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Test, version 2.0

	VERSANT HCV RNA 1.0 Assay (kPCR)			
	Positive	Positive<LLQ	TND	
COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0	Positive	176	0	0
	Positive<LLQ	1	1	3
	TND	0	0	2

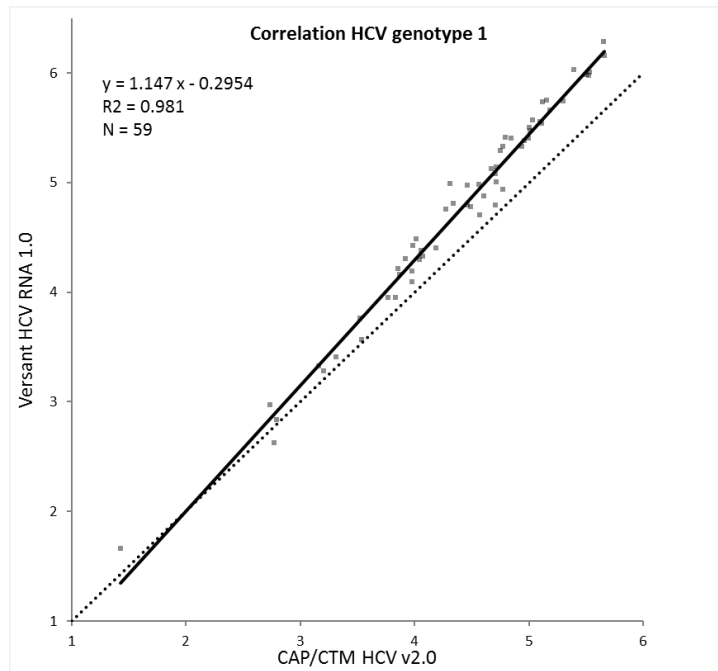
LLQ, limit of quantitation; TND, target not detectable

When results of 176 clinical samples which gave HCV RNA concentrations within the analytic measuring range with both of the assays were compared, 132 were found to be within 0.5 log₁₀ unit and 27 were found to be within 0.5 log₁₀ unit and 1.0 log₁₀ unit. Results of 17 samples showed deviations of more than 1.0 log₁₀ unit.

When HCV RNA concentrations obtained by the Siemens assay were compared to those obtained by the Roche assay, the Siemens assay revealed a 0.35 log₁₀ units higher measurement in samples collected from patients with HCV genotype 1 (Fig. 15). Of 59 HCV genotype 1 samples, 58 (98.3%) revealed higher HCV RNA concentrations by the Siemens assay, including nine within 0.5 log₁₀ unit and 1.0 log₁₀ unit higher. For all HCV genotype 1 samples, the correlation coefficient (r²) was 0.98 indicating a good correlation (Fig.15). However, at the high end, HCV RNA concentrations were found to be around 0.5 log₁₀ higher with the Siemens assay when compared to the Roche assay.



A



B

Fig. 15: Results for 59 clinical samples obtained from patients with HCV genotype 1 infection tested by the Siemens and the Roche assays. Agreement between results. Bland-Altman plot (A): the x-axis shows the mean value of each sample obtained by the assays. The y-axis shows the difference between the values obtained with the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0. Solid line, zero bias; dotted line, mean difference between the values; dashed lines, mean difference plus or minus 1.96 SD (95% limits of agreement). Correlation between the results (log IU/mL) obtained with the Siemens and the Roche assays (B). Solid line, regression curve; dotted line, identity line.

Of 28 samples containing HCV genotype 2, 13 showed an HCV RNA concentration within 0.5 log₁₀ unit and 1.0 log₁₀ unit lower when tested with the Siemens assay and an additional of 4 samples more than 1.0 log₁₀ unit lower (Fig. 16). All of those 17 samples were found to contain HCV subgenotype 2a while the remaining 11 samples showing less than 0.5 log₁₀ unit difference were found to contain HCV subgenotype 2b (Table 4).

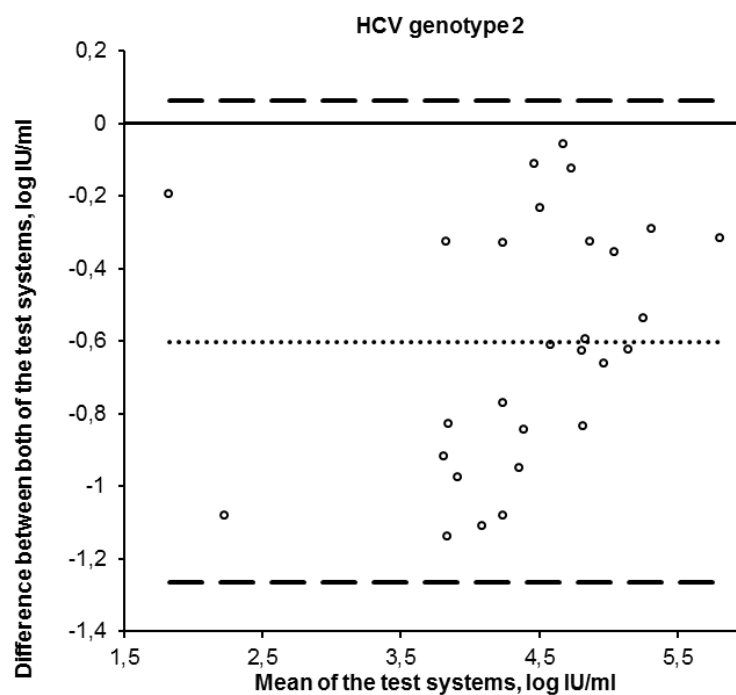


Fig.16: Results for 28 clinical samples obtained from patients with HCV genotype 2 infection tested by the Siemens and the Roche assays. Agreement between results. Bland-Altman plot: the x-axis shows the mean value of each sample obtained by the assays. The y-axis shows the difference between the values obtained with the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0. Solid line, zero bias; dotted line, mean difference between the values; dashed lines, mean difference plus or minus 1.96 SD (95% limits of agreement).

Table 4

HCV isolates showing similar and those showing divergent HCV RNA concentrations when tested with the Roche and the Siemens assays. HCV isolates were obtained with and designated as stated in the genotyping report of the standard TRUGENE HCV Genotyping test system.

Subgenotype (<i>n</i>)	Isolates (<i>n</i>)		
	Similar (<0.5 log ₁₀ unit) HCV RNA concentration obtained by both of the assays	0.5 – 1.0 log ₁₀ lower HCV RNA concentration obtained with the Siemens assay	>1.0 log ₁₀ lower HCV RNA concentration obtained with the Siemens assay
2a (17)	-	HCJ5 (12), HCJ6 (1)	HCJ5 (4)
2b (11)	FU99023VGI (7), VGA02002 (2), VGA02030 (2)	-	-
3a (39)	TH85 (10), VD (9), FU99013VGI (4), NZL1 (4), K3a (3), T1787 (3), 16252 (1)	K3a (1) ^a	QC175 (2), FU99024VGI (1), QC195 (1)
4a (28)	QC27 (10), VGA02009 (3), VGA02007 (2), VGA02008 (2)	-	VGA02032 (9), VGA02018 (2)
4b (1)	FR26 (1)	-	-
4c (19)	GB358 (10), QC137 (9)	-	-
4e (2)	GB113 (2)	-	-

^aThe HCV RNA concentration of this sample was found to be close to the lower limit of detection (190 IU/mL when determined with the Roche assay and 40 IU/mL when determined with the Siemens assay).

All samples with HCV genotype 3 contained HCV subgenotype 3a. Four of 39 samples with HCV subgenotype 3a showed a significantly lower (>1.96 SD) HCV RNA concentration (more than $1.0 \log_{10}$ unit lower) when tested with the Siemens assay (Fig. 17). Those samples were found to contain a 100%-homology to three different isolates. One sample showed an HCV RNA concentration within $0.5 \log_{10}$ unit and $1.0 \log_{10}$ unit lower when tested with the Siemens assay containing isolate K3a. The HCV RNA concentration of this sample was found to be at the low end revealing 40 IU/mL when determined with the Siemens assay and 190 IU/mL with the Roche assay. The remaining 34 samples with less than $0.5 \log_{10}$ unit difference were found to contain a 100%-homology to seven different isolates.

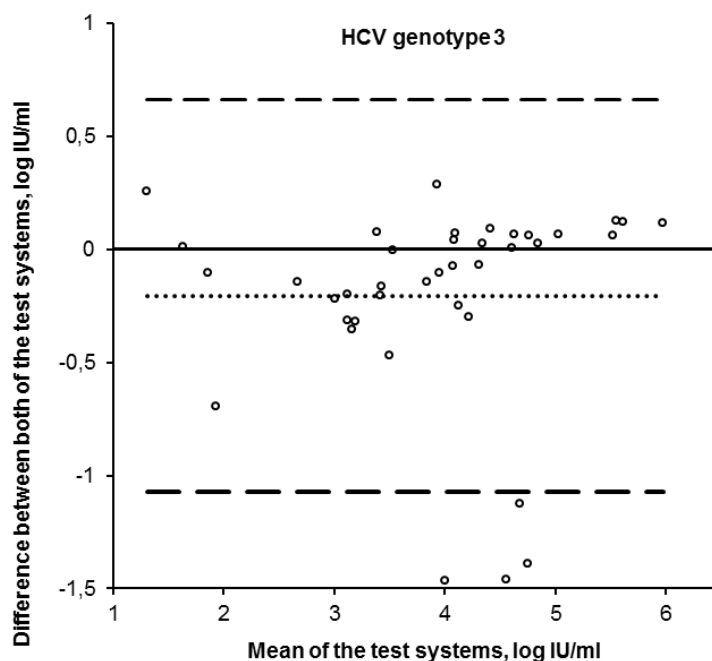


Fig.17: Results for 39 clinical samples obtained from patients with HCV genotype 3 infection tested by the Siemens and the Roche assays. Agreement between results. Bland-Altman plot: the x-axis shows the mean value of each sample obtained by the assays. The y-axis shows the difference between the values obtained with the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0. Solid line, zero bias; dotted line, mean difference between the values; dashed lines, mean difference plus or minus 1.96 SD (95% limits of agreement).

Eleven of 50 samples with HCV genotype 4 showed an HCV RNA concentration of more than $1.0 \log_{10}$ unit lower when tested with the Siemens assay (Fig. 18). All of those 11 samples were found to contain a 100%-homology to two different isolates. Another 17 samples with HCV subgenotype 4a contained a 100%-homology to four different isolates showing less than $0.5 \log_{10}$ unit difference. The remaining 22 samples with less than $0.5 \log_{10}$ unit difference consisted of 19 HCV subgenotype 4c samples, two subgenotype 4e samples, and one subgenotype 4b sample.

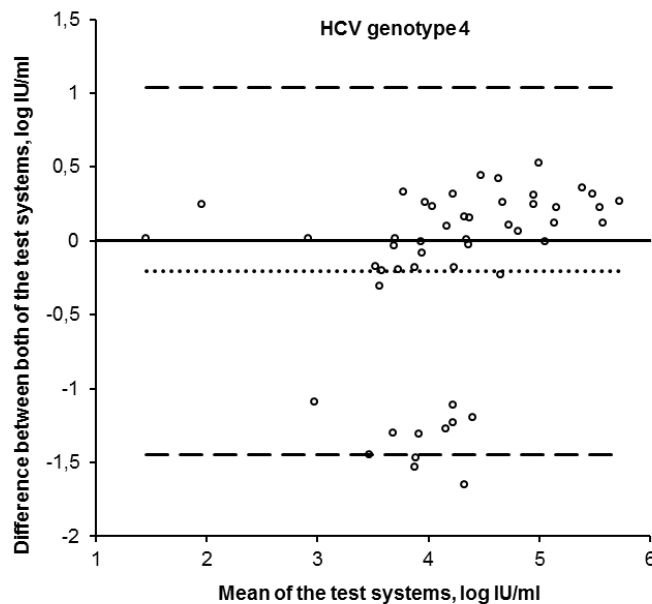


Fig.18: Results for 50 clinical samples obtained from patients with HCV genotype 4 infection tested by the Siemens and the Roche assays. Agreement between results. Bland-Altman plot: the x-axis shows the mean value of each sample obtained by the assays. The y-axis shows the difference between the values obtained with the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test, version 2.0. Solid line, zero bias; dotted line, mean difference between the values; dashed lines, mean difference plus or minus 1.96 SD (95% limits of agreement).

The nearly full-length 5'NCR of the HCV genome was sequenced in HCV subgenotype 2a and 2b samples to determine the role of sequence polymorphisms. Figure 2 shows the alignment of the member of the Worldwide HCV Performance Panel WWHV302(M) containing HCV subgenotype 2a and clinical samples containing HCV subgenotype 2a or 2b. Base substitutions among the different sequences analyzed were found at positions 37, 38, 181, 218, 223, 256, 276, 307, and 340.

4. Discussion

Accurate monitoring of HCV RNA levels during treatment is of paramount importance for the determination of virological response, for the duration of treatment, and for the application of stopping rules. This is of particular importance when direct-acting antivirals are used (Barritt et al., 2012).

For quantification of HCV RNA in the routine diagnostic laboratory, in vitro diagnostics (IVD)/Conformité Européene (CE)-labeled and/or FDA-approved assays for detection and quantitation of HCV RNA have recently been brought on the market. Because assays for the detection, confirmation, and quantitation of HCV are listed in List A of Annex II of the Directive 98/79/EC, a more stringent standardization procedure is required to meet the criteria for the IVD/CE label (Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices). Standardization of those assays is usually based on the WHO International Standard for hepatitis C virus RNA nucleic acid amplification technology-based assays. This standard has been prepared by diluting a high titer genotype 1a HCV isolate in pooled, human plasma (Saldanha et al., 1999; Saldanha et al., 2005).

Despite the stringent standardization procedure, a significant variability in HCV RNA concentration results within different assays was published (Caliendo et al., 2006). Furthermore, an underestimation of HCV RNA concentrations in samples containing HCV genotype 2 or HCV genotype 4 was reported when using the COBAS AmpliPrep/COBAS TaqMan HCV Quantitative Test (Roche), version 1.0, in several studies (Chevaliez et al., 2007; Elkady et al., 2010; Bossler et al., 2011; LaRue et al., 2012). This problem was caused by mismatches with the probe sequence. A second-generation dual probe assay, the version 2.0, was thus developed showing an accurate quantitation over all major HCV genotypes (Vermehren et al., 2011; Wedemeyer et al., 2012; Chevaliez et al., 2013). This new assay was used as comparator assay for evaluation of the genotype impact in the present study.

As no genotype-specific international standard is available, a genotype panel must be used to demonstrate test coverage. In this study, comparison of results obtained

from four members of the Worldwide HCV Performance Panel WWHV302(M) including HCV subgenotypes 1a, 1b, 3b, and 4a were found to be in good agreement; however, the mean HCV concentration for the member containing HCV subgenotype 2a was found to be more than 0.5 log₁₀ unit lower when tested with the Siemens assay than that obtained with the comparator assay. The underestimation of HCV subgenotype 2a was confirmed in 100% of clinically referred samples while the HCV RNA concentration of all of the HCV subgenotype 2b samples tested in this study obtained by the Siemens assay was similar to that obtained by the comparator assay. Furthermore, several isolates of HCV subgenotypes 3a and 4a revealed an underestimation of more than 1.0 log₁₀ unit by the Siemens assay. Underestimation may occur because of poor PCR efficiency due to mismatches between the primers and/or the probes and the target viral sequences. Regarding HCV genotype 2 samples, sequence differences were confirmed in the 5'NCR in this study. When differences between HCV subgenotype 2a and HCV subgenotype 2b samples were compared, several polymorphisms were found. Interestingly, 2 of them (an R/A-to-G substitution at position 181 and a T-to-C substitution at position 218) were found exclusively in HCV subgenotype 2a samples. Nucleotide substitutions may be responsible for mismatches with the primer pair and/or the probes, resulting in the underestimation of the HCV RNA concentration in the sample. Unfortunately, this could not be confirmed as sequences of primers and probes are property of Siemens.

Good agreement is a function of both good correlation and good standardization. In clinical samples containing HCV genotype 1, the Siemens assay showed good correlation ($r^2=0.981$) but revealed about 0.5 log₁₀ higher HCV RNA values than the Roche assay at the high end which may indicate insufficient standardization ($y=1.147$). The clear separation between the HCV subgenotype 2a and 2b results demonstrates poor PCR efficiency for HCV subgenotype 2a, but the HCV subgenotype 2b results obtained with the Siemens assay were also found lower when compared to those obtained with the Roche assay which may be a standardization issue, too. Apart from isolates with poor amplification efficiency, HCV genotype 3 and 4 samples showed sufficient standardization. The underestimation of HCV RNA concentrations in patients infected with HCV subgenotype 2a and those infected with certain isolates of HCV subgenotypes 3a and 4a may be an issue in

management of anti-HCV therapy. Especially, the interpretation of log RNA decline in patients with a starting viral load of less than 1000 IU/mL of HCV RNA may be erroneous and lead to inappropriate therapeutic decisions in the context of stopping rules based on the virologic response.

In conclusion, the VERSANT HCV RNA 1.0 Assay (kPCR) shows a good agreement with quantitation of samples containing HCV genotype 1 except of high end samples. However, this assay substantially underestimates HCV RNA concentrations in HCV subgenotype 2a samples and in HCV subgenotype 3a and 4a samples containing certain isolates. Because this may affect everyday management of anti-HCV therapy this assay should be improved.

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List of figures

Fig. 1: Available from:

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Fig. 2: Available from: <http://www.medikkajournal.com/Hepc.htm>

Fig. 3: Available from: http://en.wikipedia.org/wiki/File:HCV_pictures.png

Fig. 4: Available from: <http://www.medikkajournal.com/Hepc.htm>

Fig. 5: Available from: http://en.wikipedia.org/wiki/File:HCV_genome.png

Fig. 6: Available from:

http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/hepatology/hepatitis-C/images/Hepatitisfig_large1.jpg

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Fig. 9: Kessler HH, Molecular Diagnostics Laboratory

Fig. 10: Manns MP, von Hahn T, Nature Reviews 2013

Fig.11-14: Kessler HH, Molecular Diagnostics Laboratory

Fig.15-18: Genotype impact on HCV RNA levels determined with the Versant HCV RNA 1.0 Assay (kPCR): Kessler HH, Hübner M, Konrad PM, Stelzl E, Stübler MM, Baser MH, Santner BI, Journal of Clinical Virology 2013

List of tables

Table 1:

Features of the molecular test systems used in this study.

Table 2:

Results obtained from 1:100-dilutions analyzed four times in parallel with the Siemens and the Roche assays using the WWHV302(M) panel members 01, 02, 04, 06, and 11.

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Comparison of results from clinically referred samples obtained by the VERSANT HCV RNA 1.0 Assay (kPCR) and the COBAS AmpliPrep/COBAS TaqMan HCV Test, version 2.0.

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