Diplomarbeit

Detection of recombinant hepatitis C virus strains

eingereicht von

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zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

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Graz, 22. März 2018

I 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, March 22, 2018 *Viktoria Handler eh*

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Zusammenfassung

Hintergrund: Entsprechend den aktuellen EASL Guidelines (European Association for the Study of the Liver) sollten der Genotyp und bei Genotyp 1 der Subtyp (1a oder 1b) des Hepatitis C Virus (HCV) vor Behandlungsbeginn bestimmt werden, da dieser Einfluss sowohl auf die Wahl als auch auf die Dauer der Therapie hat. Zurzeit sind sieben HCV Genotypen und über 67 verschiedene Subtypen bekannt. Zusätzlich wurden einige intra- und intergenotypische HCV-Rekombinationen identifiziert. Von diesen ist die sogenannte „St. Petersburg - Variante“ die häufigste.

Zielsetzung: Vergleich der Ergebnisse von drei Assays, basierend auf unterschiedlichen molekularen Methoden um den HCV Genotyp und Subtyp zu bestimmen.

Materialien und Methoden: In dieser Studie wurden insgesamt 279 Proben von Patientinnen und Patienten mit chronischer Hepatitis C untersucht. Der HCV Genotyp und Subtyp wurde mit 3 verschiedenen molekularen Techniken bestimmt: Sequenzierung (HCV core, NS2/NS3 Verbindung, NS3, NS5A und die NS5B Regionen), primer-spezifische Real-Time PCR mit dem cobas® HCV GT Assay (Roche) (5’UTR, Core, NS5B Regionen) und mit dem VERSANT® HCV Genotype 2.0 Assay (Siemens, LiPa) basierend auf reverser Hybridisierung (5’UTR und Core Regionen).

Ergebnisse: Von insgesamt 279 Proben wurden 53 als Genotyp 1, Subtyp 1b, 177 als Genotyp 2 und 48 als rekombinante HCV Formen bestehend aus Genotyp 2 und Genotyp 1 mittels Sequenzierung identifiziert (2k/1b, n=46; 2b/1a, n=1; 2a/1b, n=1). Bei einem Patienten wurde eine Doppelinfektion (2b/1b) gefunden. Alle 53 Proben mit dem Subtyp 1b konnten korrekt mit dem VERSANT® HCV Genotype 2.0 Assay identifiziert werden und bei 51 von 53 Proben konnte mit dem cobas® HCV GT Assay der richtige Subtyp zugeordnet werden (2 Proben ergaben Genotyp 1, ohne Angabe des Subtyps).

Bei den Genotyp 2 Proben wurden alle 177 mit dem VERSANT® HCV Genotype 2.0 Assay korrekt identifiziert, 174 von 177 Proben mit dem cobas® HCV GT Assay (3 Proben ergaben mit dem cobas® HCV GT Assay ein invalides Ergebnis). Der VERSANT® HCV Genotype 2.0 Assay bewertete alle 48 rekombinanten Proben als Genotyp 2. Der cobas® HCV GT Assay identifizierte 43 Proben als Genotyp 2 und Subtyp 1b (Doppelinfektion nicht auszuschließen) und 5 Proben als Genotyp 2. Eine Probe wurde basierend auf den Ergebnissen aller Geräte als Doppelinfektion aus Genotyp 1 und Genotyp 2 identifiziert.

Schlussfolgerung: Für die Untersuchung von Patientenproben, die keine rekombinanten Stämme enthalten, ist eine Analyse im Bereich der 5'UTR und Core Regionen ausreichend. Um rekombinante HCV Formen zu identifizieren muss eine zusätzliche Region nahe dem 3'-Ende des HCV-Genoms analysiert werden.

Abstract

Background: According to the most recent EASL recommendations (European Association for the Study of the Liver) on the management of hepatitis C virus (HCV) infection, HCV genotypes and genotype 1 subtypes (1a or 1b) should be assessed prior to treatment initiation, as this will impact the choice and duration of direct-antiviral treatment regimens. Currently, HCV is classified into 7 different genotypes and 67 subtypes. Additionally, several natural intra- and intergenotypic recombinants of HCV have been identified. Among these, the so-called “St Petersburg variant”, a genotype 2k/1b recombinant, is the most prevalent worldwide.

Objectives: To compare results obtained by assays using different molecular techniques for determination of HCV genotypes 1 and 2 as well as recombinant forms between these two genotypes.

Materials and methods: In this study, 279 samples derived from patients with chronic HCV infection were investigated. HCV genotypes and subtypes were determined by the reverse hybridization-based VERSANT® HCV Genotype 2.0 assay (LiPA; Siemens Healthcare) that targets the 5´UTR and HCV core regions, and the real-time PCR based cobas® HCV GT (cobas; Roche Molecular Systems) assay that targets the 5´UTR, core, and NS5B regions. Assay results were compared to direct sequencing of the HCV core, NS2/NS3 junction, NS3, NS5A, and the NS5B regions as the reference standard.

Results: In total, 53 patients had subtype 1b, 177 patients had genotype 2, and 48 patients had genotype 2/genotype 1 recombinants according to direct sequencing (2k/1b, n=46; 2b/1a, n=1; 2a/1b, n=1). Finally, one patient had a mixed genotype 1b + genotype 2b infection. All 53 samples with subtype 1b were correctly subtyped by the VERSANT® HCV Genotype 2.0 assay, and 51/53 samples were subtyped with the cobas® HCV GT, respectively (two samples yielded genotype 1 results only). Among genotype 2 samples, 177/177 and 174/177 samples were correctly reported as genotype 2 by the VERSANT® HCV Genotype 2.0 assay and the cobas® HCV GT, respectively (three samples yielded invalid results with the

cobas® HCV GT). The VERSANT® HCV Genotype 2.0 assay reported all 48 genotype 2/genotype 1 recombinants as genotype 2, whereas the cobas® HCV GT assay reported 43 as genotype 1b/genotype 2 (which cannot rule out double infection) and five samples were reported as genotype 2 only. One sample was classified as mixed genotype 1+ genotype 2 infection when results obtained by all assays were taken into consideration.

Conclusions: When analyzing HCV patient samples containing strains without recombination, analysis of the 5´UTR and core regions is sufficient, while identification of HCV recombinant forms requires inclusion of an additional region close to the 3´ end of the HCV genome.

1 Introduction

The hepatitis C virus (HCV) is a small, blood borne RNA virus (Fig. 1). The HCV infection is one of the main causes of liver disease worldwide, resulting in hepatic inflammation with symptoms ranging from a mild illness up to a severe disease, with a high risk of advancing over time into fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). Approximately over 170 million people are infected worldwide, 64 to 103 million people suffer from chronic hepatitis C and approximately 700,000 people die from hepatitis C-related liver failure and HCC each year (2,3).

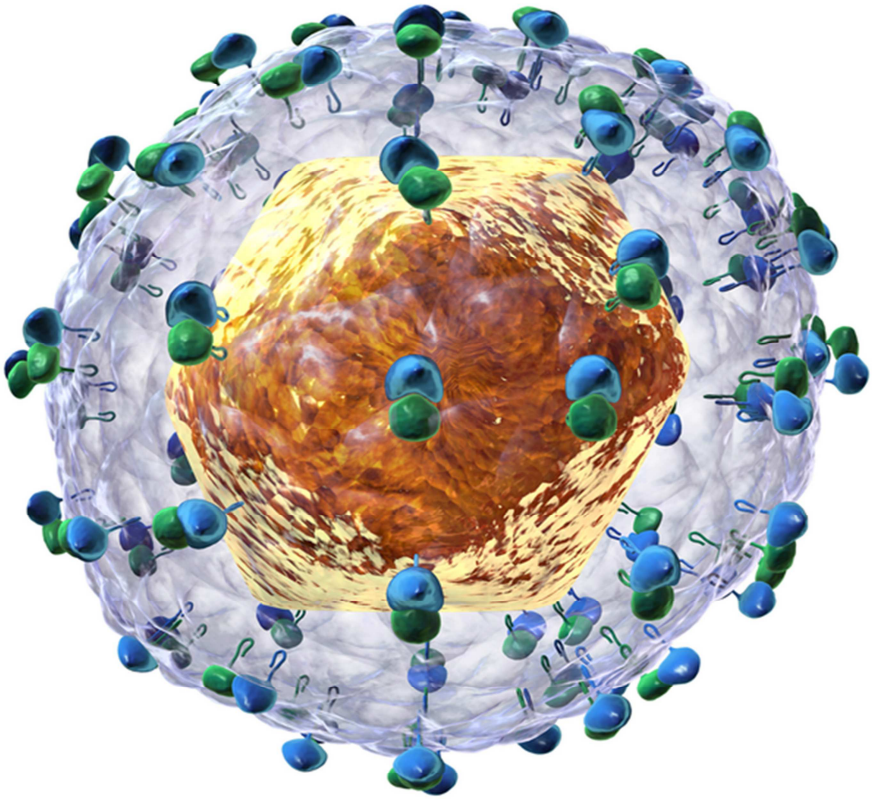

Fig. 1. The HCV (https://commons.wikimedia.org/w/index.php?curid=44967236).

1.1 Prevalence and transmission of HCV

The HCV infection is found worldwide. The most affected regions are Central Asia with an anti-HCV prevalence of 5.4%, Sub-Saharan/Central Africa with 4.2%, North Africa with 3.1%, and Eastern Europe with 3.3%. In Central Europe, the anti-HCV prevalence has been reported to be 0.8% (2).

The HCV is a blood-borne virus. Infection is highly associated with health inequity. In middle- and high income countries, most HCV infections concern people who inject drugs (PWID), using and sharing material that is not sterilized and may thus be contaminated with blood (4). In low-income countries, people get infected mainly by unsafe medical injections and procedures. This includes, for example, medical and dental procedures without safe infection control, unscreened blood products, hemodialysis as well as scarification, and circumcision routines (5–7).

The HCV can also be transmitted from unprotected sex, especially in HIV-positive men who have sex with men (MSM). In heterosexual couples, HCV infection is reported infrequently (8,9). The transmission from mother to child ranges from 3-10%. Unfortunately, there are no proven interventions to reduce the risk of vertical transmission (10).

Table 1
Regional prevalence and number of infected individuals (all ages), modified from (2).

Regions	**Anti HCV prevalence**	**Viremic HCV prevalence**	**Viremic rate**	**2013 population (millions)**	**Anti-HCV infected (millions)**	**Viremic HCV infected (millions)**
Asia Pacific, high income	1.1% (0.5-1.7%)	0.8% (0.4-1.2%)	74%	182	2.0 (0.9-3.0)	1.5 (0.6-2.2)
Asia, Central	5.4% (3.5-6.8%)	2.3% (1.5-3.0%)	43%	84	4.5 (2.9-5.7)	1.9 (1.3-2.5)
Asia, East	1.2% (0.4-1.8%)	0.7% (0.3-1.1%)	60%	1434	16.6 (6.3-25.3)	10.0 (3.9-15.1)
Asia, South	1.1% (0.7-1.5%)	0.9% (0.5-1.2%)	81%	1650	18.8 (11.3-24.5)	15.2 (8.9-19.8)
Asia, Southeast	1.0% (0.8-1.8%)	0.7% (0.5-1.1%)	63%	635	6.6 (5.3-11.3)	4.2 (3.4-7.2)
Australasia	1.4% (1.0-1.5%)	1.0% (0.8-1.1%)	75%	28	0.4 (0.3-0.4)	0.3 (0.2-0.3)
Caribbean	0.8% (0.2-1.3%)	0.6% (0.1-0.9%)	70%	39	0.3 (0.1-0.5)	0.2 (0.0-0.4)
Europe, Central	1.3% (1.1-1.6%)	1.0% (0.9-1.2%)	80%	119	1.5 (1.3-1.9)	1.2 (1.1-1.5)
Europe, Eastern	3.3% (1.6-4.5%)	2.3% (1.1-3.0%)	69%	207	6.8 (3.4-9.3)	4.7 (2.4-6.3)
Europe, Western	0.9% (0.7-1.5%)	0.6% (0.5-1.0%)	70%	425	3.7 (3.0-6.3)	2.6 (2.1-4.4)
Latin America, Andean	0.9% (0.4-1.3%)	0.6% (0.3-0.9%)	70%	57	0.5 (0.2-0.7)	0.4 (0.2-0.5)
Latin America, Central	1.0% (0.8-1.4%)	0.8% (0.6-1.1%)	75%	246	2.6 (1.9-3.5)	1.9 (1.4-2.6)
Latin America, Southern	1.2% (0.5-2.1%)	0.9% (0.4-1.6%)	79%	62	0.8 (0.3-1.3)	0.6 (0.2-1.0)
Latin America, Tropical	1.2% (0.9-1.2%)	1,0% (0.7-1.0%)	80%	207	2.5 (1.9-2.6)	2.0 (1.5-2.1)
North Africa/Middle East	3.1% (2.5-3.9%)	2.1% (1.7-2.6%)	66%	469	14.6 (11.9-18.2)	9.7 (7.8-12.1)
North America, high income	1.0% (1.0-1.9%)	0.8% (0.7-1.4%)	76%	355	3.7 (3.4-6.7)	2.8 (2.6-5.0)
Oceania	0.1% (0.1-0.6%)	0.1% (0.1-0.4%)	69%	10	0.0 (0.0-0.1)	0.0 (0.0-0.0)
Sub-Saharan Africa, Central	4.2% (2.4-9.2%)	2.6% (1.5-5.5%)	61%	100	4.3 (2.4-9.2)	2.6 (1.5-5.5)
Sub-Saharan Africa, East	1.0% (0.6-3.1%)	0.6% (0.4-2.0%)	62%	385	3.9 (2.4-12.1)	2.4 (1.6-7.9)
Sub-Saharan Africa, Southern	1.3% (0.8-2.5%)	0.9% (0.6-1.7%)	69%	75	1.0 (0.6-1.9)	0.7 (0.4-1.3)
Sub-Saharan Africa, West	5.3% (2.9-9.1%)	4.1% (2.3-6.7%)	77%	367	19.3 (10.5-33.3)	14.9 (8.5-24.6)
Other	1.9% (1.0-3.4%)	1.3% (0.7-2.4%)	69%	27	0.5 (0.3-0.9)	0.4 (0.2-0.7)
Total	**1.6% (1.3-2.1%)**	**1.1% (0.9-1.4%)**	**70%**	**7162**	**114.9 (91.9-148.7)**	**80.2 (64.4-102.9)**

1.2 The HCV genome

The hepatitis C virus is a Hepacivirus, belonging to the *Flaviviridae* family. It is a positive single-stranded RNA-virus with a length of 9.6 kb nucleotides and one single open reading frame, encoding one single virus polyprotein of 3011 amino acids, flanked by untranslated regions (UTR's) at both sides. It is subdivided into three structural proteins (core, E1, E2) and seven nonstructural proteins (p7, NS2/3, NS4A/B, and NS5A/B). NS2 and p7 are essential for producing infectious virions, the remaining non-structural proteins for the replication of the HCV (1,11).

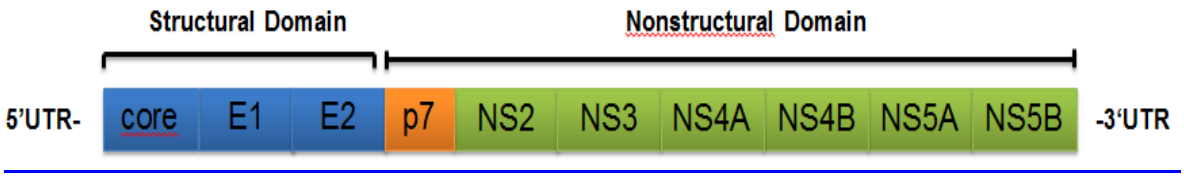

Fig. 2. Genome of HCV (modified from: https://upload.wikimedia.org/wikipedia/commons/c/c0/HCV_genome.png).

1.3 Diagnostics

The acute HCV infection is usually asymptomatic. Due to this, only few people get tested and diagnosed during the acute infection. Chronic HCV infection may also remain asymptomatic for decades and only be revealed by symptoms of secondary liver damage or serious liver disease (12).

To establish the diagnosis of an HCV infection, a special algorithm is required. In the first step, an enzyme linked immunosorbent assay (ELISA) to identify specific anti-HCV antibodies is used (13,14). If this test shows a positive result, detection and quantitation of HCV RNA must be done to confirm the result obtained by ELISA testing. Interpretation of results is shown in Table 2.

Table 2
Interpretation of results obtained by assays useful for diagnostics of HCV infection.

Anti-HCV	HCV RNA	Interpretation
+	+	Acute or chronic HCV infection
+	-	Resolution of HCV infection; false positive result*
-	+	Early acute HCV infection; CHC in setting of immunosuppression
-	-	Absence of HCV infection

*Note that an immunoblot assay may reveal unspecific reactivity of the immunoassay; if positive, repetition of HCV RNA testing within 6-12 months is suggested.

Detection and quantitation of HCV RNA is performed by nucleic acid amplification testing (NAT), utilizing real time polymerase chain reaction (qPCR) which is also considered the “gold standard”. Additionally, detection and quantitation of HCV RNA plays an important role in the follow-up of patients under anti-HCV treatment (15–17).

1.4 Acute Hepatitis C (AHC)

The incubation time of hepatitis C infection is 6-10 weeks. Specific HCV antibodies usually appear not earlier than 12-24 weeks after infection. Detection of HCV antibodies to diagnose acute hepatitis C infection is thus unreliable. In contrast, polymerase chain reaction (PCR) for detection and quantitation of HCV RNA is usually possible within 10-14 days after infection (14).

Typical symptoms of hepatic inflammation are often less distinctive. Due to its asymptomatic nature, the diagnosis is often set up late (11,18).

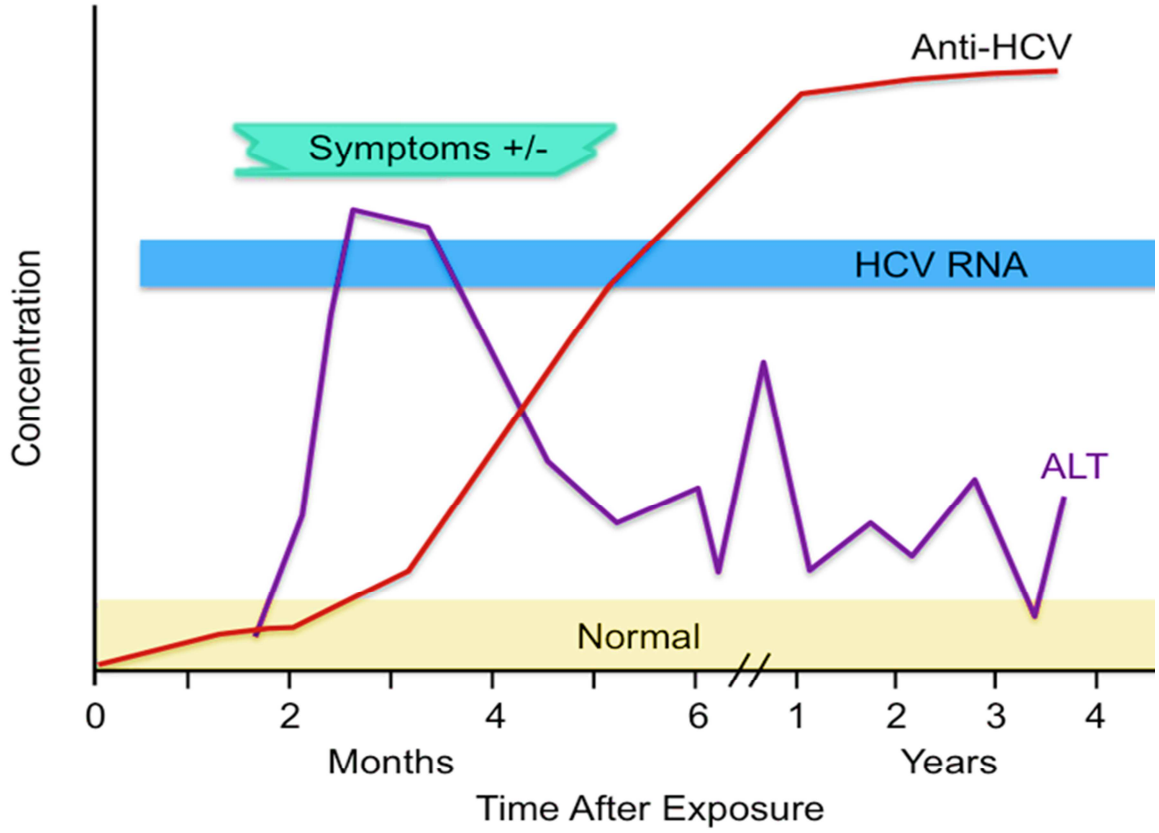

Fig. 3. Serological pattern of acute HCV infection modified from (19).

1.5 Chronic Hepatitis C (CHC)

An HCV infection that is persisting for more than 6 months is called chronic HCV infection. Depending on the age, up to 80% of the patients infected with HCV will develop CHC. The CHC can be asymptomatic for years but may lead to a severe liver disease and complications such as cirrhosis and HCC.

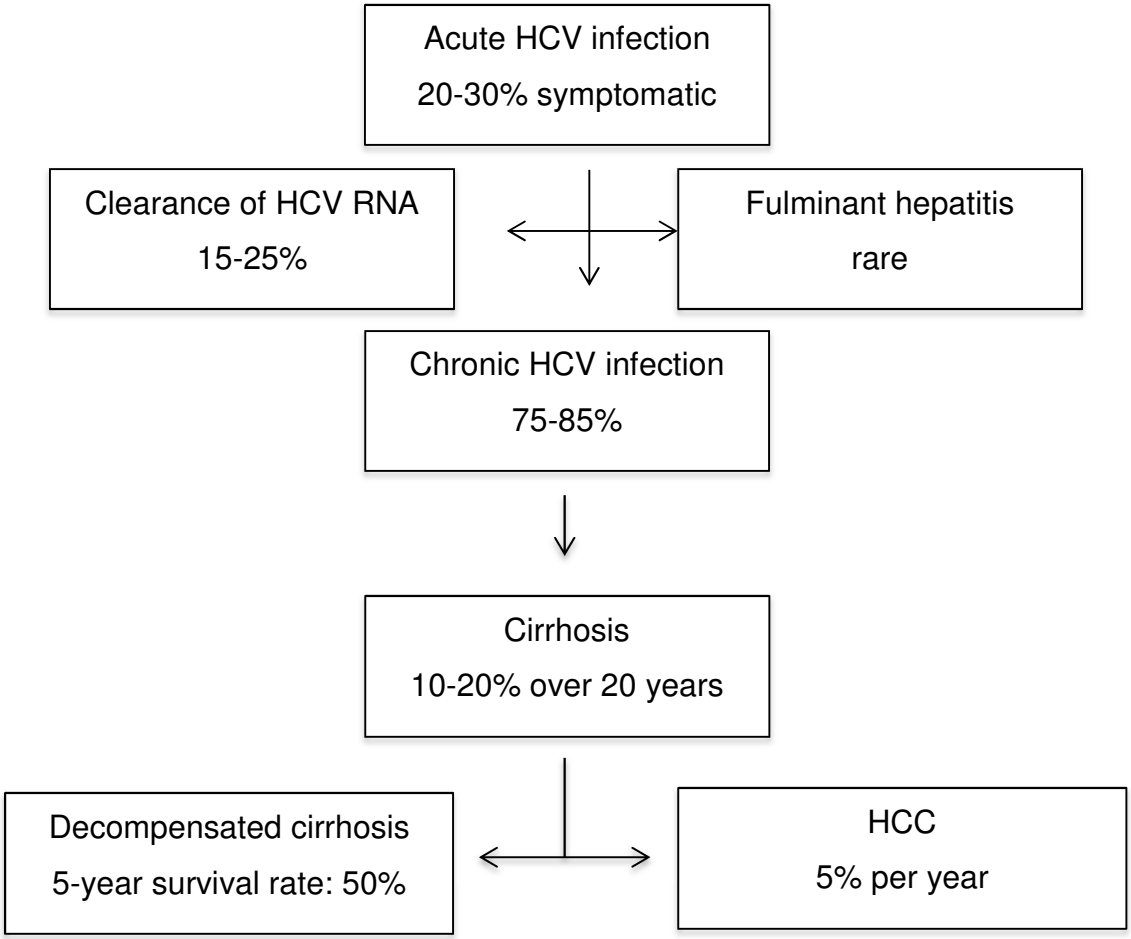

Fig. 4. Natural history of HCV infection (modified from http://www.medsci.org/v03p0047.htm).

1.6 HCV genotypes and subtypes

Currently, HCV is classified into 7 genotypes and 67 different subtypes (Fig. 4). Subtypes 1a, 1b, and 3 are in charge of most of the HCV infections in Western countries (19).

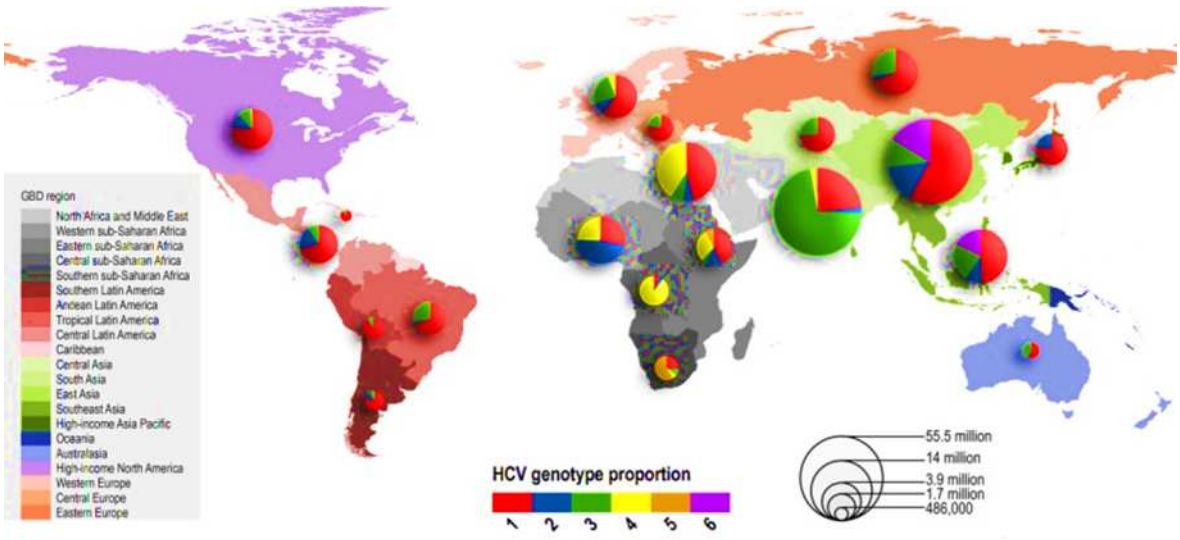

Fig. 5. Global distribution of HCV genotypes (20).

Testing for the HCV genotype has been important in understanding HCV classification, epidemiology, evolution, transmission clustering, treatment response, and natural history. HCV exhibits more than 30% nucleotide sequence divergence at the genotype level and 20% divergence at the subtype level (19).

The genetic diversity of HCV is the result of a high error rate of the RNA-dependent RNA polymerase and the pressure that is exposed by the host immune system. The genetic diversity is unequally distributed. While the regions responsible for important viral functions (such as translation and replication) are most conserved, the maximum variable region is that one coding for the glycoproteins E1 and E2, responsible for the HCV envelope (20–22).

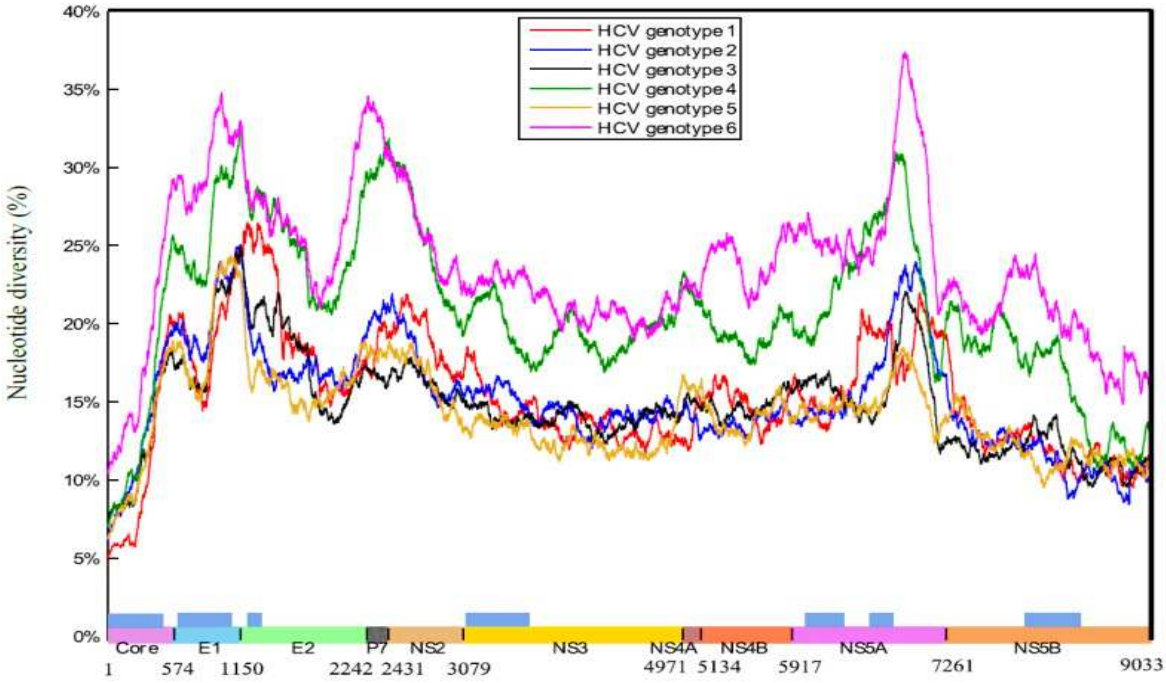

Fig. 6. Genetic diversity of HCV genotypes (24).

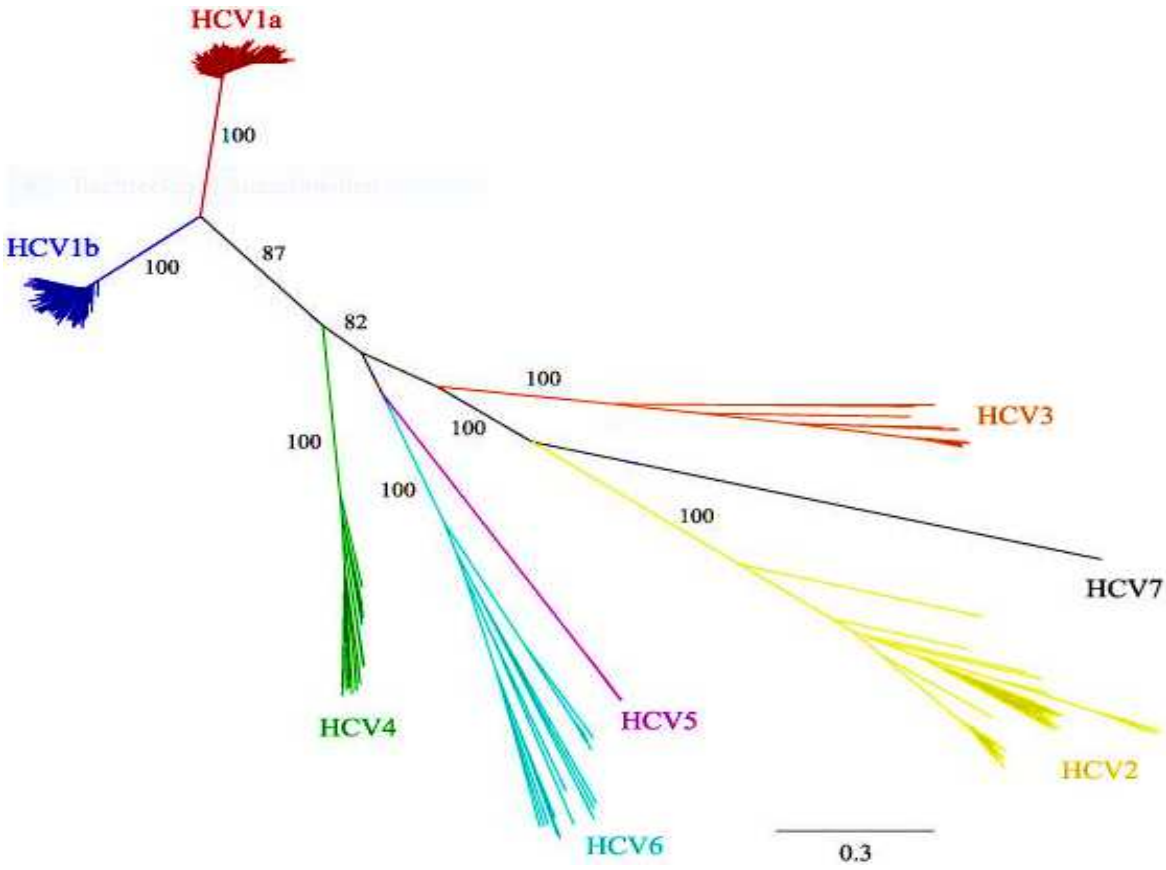

Fig. 7. Phylogenetic tree of genotypes (24).

1.7 HCV genotyping

For HCV genotyping, three different molecular techniques can be used: nucleic acid sequencing, reverse hybridization, and primer-specific real-time PCR (qPCR).

1.7.1 Nucleic acid sequencing

Nucleic acid sequencing was developed by Fred Sanger in Cambridge, more than 30 years ago. This technique was the first one to be used routinely for DNA sequencing in laboratories and has been modified from DNA replication. A specific primer hybridizes to the target sequence on the template strand. The polymerase incorporates nucleotides to create a complimentary copy of the template. Four sequencing reactions are carried out in separate tubes. In each tube, a small quantity of a 2'3'-dideoxy-nucleotide-triphosphate (ddATP, ddGTP, ddCTP, ddTTP) is added. The polymerase does not differentiate deoxy-nucleoside-triphosphates between dideoxy-nucleoside-triphosphates. To make the chain grow, a dNTP is added, to terminate it, a ddNTP is joined. If a ddNTP is added, there is no 3′-hydroxyl group to interact with an incoming nucleoside triphosphate and no further nucleosides can be added. In each tube is now a mixture of oligonucleotides, all terminating with the one particular ddNTP that was added before. Using electrophoresis can separate the oligonucleotides and the oligonucleotide primer, labeled at the 5′-end with 32P, shows an image that can be used to read the reverse complement of the template – the DNA sequence (23).

Recently, next generation sequencing (NGS), also known as "second generation", "massive parallel", or "deep" sequencing, has been introduced enhancing "first generation sequencing" techniques such as the Sanger sequencing technique. NGS provides three to four magnitudes more information than Sanger sequencing. However, the extremely high expenses have largely impaired its use in the routine diagnostic laboratory until now.

The concept of NGS is similar to Sanger sequencing, but NGS is able to process thousands of fragments in a massive parallel fashion. There are four main steps: library preparation, cluster generation, DNA sequencing, and data analysis.

For library preparation, DNA or cDNA samples are randomly fragmented and followed by 5' and 3' adapter ligation. The adapter-ligated fragments are then PCR amplified and purified. Subsequently, the library is loaded into a flow cell where fragments are captured on surface-bound, complementary oligonucleotides. Each fragment is then amplified into distinct, clonal clusters. When cluster generation is complete, the templates are ready for sequencing. Single bases are detected as they are incorporated into DNA template strands. During data analysis, the newly identified sequence is aligned to a reference genome. Following alignment, many variations of analysis are possible such as single nucleotide polymorphism, insert deletion identification, and phylogenetic and metagenomics analysis (23).

1.7.2 Reverse hybridization

For identification of HCV genotypes/subtypes, reverse hybridization can also be used. After extraction of nucleic acids, PCR amplification is performed. The biotinylated amplification products are then denatured and hybridized to complementary oligonucleotides (oligonucleotide probes), bound to a solid carrier, usually a nitrocellulose strip, followed by stringent washing steps. A special reaction forms a purple/brown precipitate. On the reverse hybridization strip, this results in a visible hybridization pattern.

Hybridization is highly specific, allowing single nucleotide mismatch detection. The read-out can be performed manually or automated. The test requires 8 hours including nucleic acid extraction and PCR amplification (24,25).

1.7.3 Primer-specific real-time PCR

For identification of HCV genotypes and subtypes, primer-specific real-time PCR (qPCR) can be employed (26). qPCR has become the widest used method of choice to detect and quantitate viruses in the world. Molecular assays based on qPCR have replaced those based on conventional PCR in the routine diagnostic laboratory. qPCR combines amplification and detection in a single step. Fluorescent dyes are used to label PCR products during thermal cycling. The accumulation of the fluorescent signal is measured during the exponential phase of the reaction. Detection formats include hydrolysis probes, hybridization probes, molecular beacons, and scorpions. The hydrolysis probe format uses a specially designed oligonucleotide with a fluorescent label at its 5' end (reporter dye) and a quencher lacking native fluorescence at its 3' end. During PCR amplification, probes hybridize to their target sites between primer binding sites. DNA polymerase extends the primers. The 5'exonuclease activity of the DNA polymerase cleaves the probe and separates the reporter dye from the quencher dye. In consequence, emission of fluorescent light from reporter dye can be detected.

1.8 Treatment of HCV

The aim of HCV therapy is to cure the infection and prevent complications of HCV-related liver and extra-hepatic diseases. In recent years, therapy of HCV infection has changed significantly. Pegylated interferon-α has been replaced by direct acting antivirals (DAA). There are four classes of DAAs available: NS3/4A protease inhibitors, NS5A inhibitors, nucleoside and nucleotide NS5B polymerase inhibitors, and non-nucleoside NS5B polymerase inhibitors. Combinations among different classes of DAAs are used for anti-HCV treatment today. In special cases, ribavirin, a synthetic nucleoside analogue, may be used additionally (30).

The endpoint of therapy is sustained virologic response (SVR), defined by undetectable HCV RNA in blood 12 weeks (SVR12) or 24 weeks (SVR24) after completion of the treatment. The SVR should be assessed by a sensitive molecular method with a lower level of detection ≤15 IU/ml (27). SVR is accompanied by normalization of the liver enzymes and improvement or suspension of liver inflammation and fibrosis. In patients with advanced fibrosis and cirrhosis, the eradication of HCV reduces the rate of decompensation and the risk of developing HCC. Nowadays, HCV infection can be cured in 99% of patients achieving SVR. For an optimal choice of anti-HCV therapy, the HCV genotype/subtype should be identified with an assay that is able to determine accurately the HCV genotype and to discriminate HCV subtype 1a from 1b according to the latest version of the EASL guidelines (27). Genotyping/subtyping should be performed with an assay that is able to analyze the sequences of the 5' untranslated region and a part of another genomic region, generally the NS5B-coding or the core coding regions (27).

Therapy for HCV infection advanced in both non-cirrhotic patients and those with liver cirrhosis depending on the HCV genotype and the HCV genotype 1 subtypes 1a and 1b (Tables 3 and 4). However, treatment of HCV genotype 3 infection is still challenging. HCV genotype 3 infection is a highly prevalent infection, very common in intravenous drug users (IVDU). Its natural history shows a rapid progression of liver disease, a high incidence of HCC and high relapse rates. HCV genotype 3 is more than any other HCV genotype associated with

steatosis (up to 70%) and causes alterations in lipid metabolism, which lead to insulin resistance, diabetes, and thus an increased cardiovascular risk (28–30). The magnitude of HCV genotype 3 infections correlates with the level of viral replication and disappears after successful antiviral therapy suggesting a cause and effect relationship between genotype and natural history (31–34). In patients with HCV cirrhosis, HCV genotype 3 has been associated with a higher risk of HCC (35).These special characteristics might explain partly the suboptimal response to treatment (36). Several studies also showed, that achieving a SVR improves the insulin resistance and declines the incidence of diabetes (33,34).

Table 3
Treatment recommendations for HCV-monoinfected or HCV/HIV coinfected patients with CHC without cirrhosis, including treatment-naïve patients and patients who failed on a treatment based on pegylated IFN-α and ribavirin (treatment-experienced, DAA-naïve patients) (27).

Patients	Treatment-naïve or -experienced	Sofosbuvir/ledipasvir	Sofosbuvir/velpatasvir	Ombitasvir/paritaprevir/ritonavir and dasabuvir	Ombitasvir/paritaprevir/ritonavir	Grazoprevir/elbasvir	Sofosbuvir and daclatasvir	Sofosbuvir and simeprevir
Genotype 1a	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	12 wk with ribavirin	No	12 wk, no ribavirin if HCV RNA ≤ 800.000 (5.9log) IU/ml or 16 wk with ribavirin if HCV RNA > 800.000 (5.9log) IU/ml[2]	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin[1] or 24 wk, no ribavirin					12 wk with ribavirin[1] or 24 wk, no ribavirin	
Genotype 1b	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	8-12 wk, no ribavirin	No	12 wk, no ribavirin	12 wk, no ribavirin	No
	Treatment-experienced	12 wk, no ribavirin		12 wk, no ribavirin				
Genotype 2	Both	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
Genotype 3	Treatment-naïve	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced		12 wk with ribavirin[3] or 24 wk, no ribavirin				12 wk with ribavirin[3] or 24 wk, no ribavirin	
Genotype 4	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	12 wk with Ribavirin	12 wk, no ribavirin	12 wk, no Ribavirin	12 wk, no ribavirin
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin				12 wk, no ribavirin if HCV RNA ≤ 800.000 (5.9log) IU/ml or 16 wk with ribavirin if HCV RNA > 800.000 (5.9log) IU/ml	12 wk with ribavirin or 24 wk, no ribavirin	12 wk with ribavirin or 24 wk, no ribavirin
Genotype 5 or 6	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin					12 wk with ribavirin or 24 wk, no ribavirin	

[1] Add Ribavirin only in patients with RASs that confer high-level resistance to NS5A inhibitors at baseline if RAS testing available

[2] Prolong to 16 weeks and add Ribavirin only in patients with RASs that confer resistance to elbasvir at baseline if RAS testing available

[3] Add ribavirin only in patients with NS5A RASs Y93H at baseline if RAS testing available.

Table 4
Treatment recommendations for HCV-monoinfected or HCV/HIV coinfected patients with CHC with compensated (Child-Pugh A) cirrhosis, including treatment-naïve patients and patients who failed on a treatment based on pegylated IFN-α and ribavirin (treatment-experienced, DAA-naïve patients) (27).

Patients	Treatment-naïve or -experienced	Sofosbuvir/ ledipasvir	Sofosbuvir/ velpatasvir	Ombitasvir/ paritaprevir/ ritonavir and dasabuvir	Ombitasvir/ paritaprevir/ ritonavir	Grazoprevir/elbasvir	Sofosbuvir and daclatasvir	Sofosbuvir and simeprevir
Genotype 1a	Treatment-naïve	8-12 wk, no ribavirin	12 wk, no ribavirin	24 wk with ribavirin	No	12 wk, no ribavirin if HCV RNA ≤ 800.000 (5.9log) IU/ml or 16 wk with ribavirin if HCV RNA > 800.000 (5.9log) IU/ml[2]	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin[1] or 24 wk, no ribavirin					12 wk with ribavirin[1] or 24 wk, no ribavirin	
Genotype 1b	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	12 wk, no ribavirin	No	12wk, no ribavirin	12 wk, no ribavirin	No
	Treatment-experienced							
Genotype 2	Both	No	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
Genotype 3	Treatment-naïve	No	12 wk with ribavirin[3] or 24 wk, no ribavirin	No	No	No	24 wk with ribavirin	No
	Treatment-experienced							
Genotype 4	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	12 wk with Ribavirin	12 wk, no ribavirin	12 wk, no Ribavirin	12 wk, no ribavirin
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin				12 wk, no ribavirin if HCV RNA ≤ 800.000 (5.9log) IU/ml or 16 wk with ribavirin if HCV RNA > 800.000 (5.9log) IU/ml	12 wk with ribavirin or 24 wk, no ribavirin	12 wk with ribavirin or 24 wk, no ribavirin
Genotype 5 or 6	Treatment-naïve	12 wk, no ribavirin	12 wk, no ribavirin	No	No	No	12 wk, no ribavirin	No
	Treatment-experienced	12 wk with ribavirin or 24 wk, no ribavirin					12 wk with ribavirin or 24 wk, no ribavirin	

[1] Add Ribavirin only in patients with RASs that confer high-level resistance to NS5A inhibitors at baseline if RAS testing available

[2] Prolong to 16 weeks and add Ribavirin only in patients with RASs that confer resistance to elbasvir at baseline if RAS testing available

[3] Add ribavirin only in patients with NS5A RAS Y93H at baseline if RAS testing available.

1.9 HCV recombinant forms

The first HCV recombinant form was reported by KALININA et al., Saint Petersburg, in 2002 (37). In that article, HCV subtypes 2k and 1b were found to form the recombinant (RF1_2k/1b). Meanwhile, this recombination has been identified in many parts of the world (Table 5). Recombination between genotypes 2 and 1 (strain RF1_2k/1b) was identified in Ireland, France, Cyprus, Estonia and Germany and between genotypes 2 and 5 (strain R1) in France (38–43).

In a recent study, four patients living in Austria were found to be probably infected with a recombinant HCV strain. In that study, a new HCV genotype test, the cobas® HCV GT (Roche Molecular Systems, Pleasanton, CA, USA) was evaluated. This assay is based on real time PCR, using regions at both ends of the HCV genome and results obtained with the Roche assay were compared to those obtained with the TRUGENE® HCV 5'NC Genotyping Kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) and the VERSANT® HCV Genotype 2.0 Assay (Siemens). Among 183 consecutive residual serum samples, four were identified to be possibly infected with a recombinant strain of genotype 2 and subtype 1b. In that study, comparison assays using the 5'end of the virus (core region and/or 5'UTR) for determination of the HCV genotype/subtype revealed HCV subtype 2a and 2a/c. Contrarily, a home-brew NS5B sequencing assay, which uses part of the 3'end of the viruses, revealed subtype 1b (44). In a second study, full-genome next-generation sequencing using the Illumina MiSeq v2 300 cycle kit (Illumina, San Diego, CA, USA) was performed to clarify the results. The point of recombination was found within the NS2 gene between nucleotide positions 3189-3200, based on H77 numbering (45).

Table 5
Summary and main features of the published cases of recombination in HCV, modified from (46) (NJ: neighbour-joining, ML: maximum likelihood; K2P: Kimura 2 parameter, TN: Tamura-Nei; GTR: general time reversible, G: gamma distribution for heterogeneity among sites).

Strain	Genotype	Country	Recombination breakpoint(s)	Phylogenetic analyses
Intergenotype				
RF1_2k/1b	2k/1b	Russia, Ireland, Uzbekistan, Georgia/France, Cyprus	NS2, positions 3175-3176	NJ-??-?boot; Simplot, Bootscan
D3	2i/6p	Vietnam	NS2/NS3 junction, between positions 3405 and 3464	NJ-K2P-1000boot; Simplot, Bootscan
SE-03-07-1689	RF3_2b/1b	Philippines	NS3, positions 3466-3467	NJ-??-?boot (CLUSTALW); Similarity plot (RAT)
R1	2/5	France	NS2/NS3 junction, between residues 3420 and 3440	NJ-TN+G-100boot (MEGA); RDP
D177	RF_2b/6w	Taiwan	NS2/NS3 junction, position 3429	NJ-K2P-1000boot; Simplot
	RF_3a/1b	Taiwan	Undetermined	NJ-K2P-1000boot; Simplot
	RF_2a/1a	Taiwan	Undetermined	NJ-K2P-1000boot; Simplot
HC10-0804	2b/1b	Japan	NS2/NS3 junction, positions 3443-3444	NJ-K2P-1000boot; Simplot, Bootscan
JF779679	2b/1a	USA	NS2/NS3 junction, positions 3405-3416	NJ-??-?boot (CLUSTAL W); Simplot
Intersubtype	Subtypes			
PE22	RF2_1b/1a	Peru	NS5B, position 8321	NJ-K2P-1000boot (MEGA); Simplot, LARD
HC-J1	1a/1c	Japan	2 sites in E1-E2, at positions 1407 and 2050	No PhylTree, Simplot, Bootscan
Khajal	1a/1c	India	5 sites, from core to NS3, at positions 801, 1261, 2181, 3041, and 3781	ML (Modeltest), 5000 boot (NJ); Simplot, Bootscan
H23	1b/1a	Uruguay	core, at position 387	ML-GTR+G, aLRT (Phyml); GARD, LARD
R49	4a/4d	Portugal	undetermined	NJ-K2P-1000boot
Intrapatient	**Subtype**			
	1b	Spain	NS5B, at the residue 286	No PhylTree, Simplot, Bootscan
	1a, 1b, 3a	Spain	1 or 2 sites within E1E2 or NS5A	ML, GTR+G, 1000boot (Phyml); RDP3 (at least 3+); SH + ELW (Tree Puzzle)

2 Objectives

HCV recombinant forms may have an impact on treatment decisions. The aim of this study was to compare results obtained by assays using different molecular techniques for determination of HCV genotypes 1 and 2 as well as recombinant forms between these two genotypes. Assays based on primer-specific real-time PCR, reverse hybridization, and Sanger sequencing were employed.

3 Materials and methods

3.1 Specimens

In this study, 279 anonymized residual plasma samples obtained from patients with chronic HCV infection were investigated. Blood had been collected in 3.5-ml K-EDTA tubes. After centrifugation, plasma aliquots were frozen at –70 C until testing.

3.2 Molecular assays used in this study

Assays employed in this study included the cobas® HCV GT (Roche) based on primer-specific real-time PCR, the VERSANT® HCV Genotype 2.0 assay (Siemens) based on the reverse hybridization principle, and Sanger sequencing.

3.2.1 The cobas® HCV genotyping test

The cobas® HCV genotyping test (GT) (Roche) is based on primer-specific real time PCR. This assay was used according to the manufacturer's package insert instructions. Results were managed by the cobas® 4800 software, assigning results for all tests as one or more genotypes and subtypes, indeterminate (HCV RNA detected, but no genotype or subtype identified) or invalid (no HCV RNA detected). Results were reviewed directly on the computer screen, exported, and printed.

The cobas® HCV GT has been designed for qualitative identification of HCV genotypes 1 to 6 and genotype 1 subtypes 1a and 1b in human plasma or serum obtained from patients with chronic HCV infection.

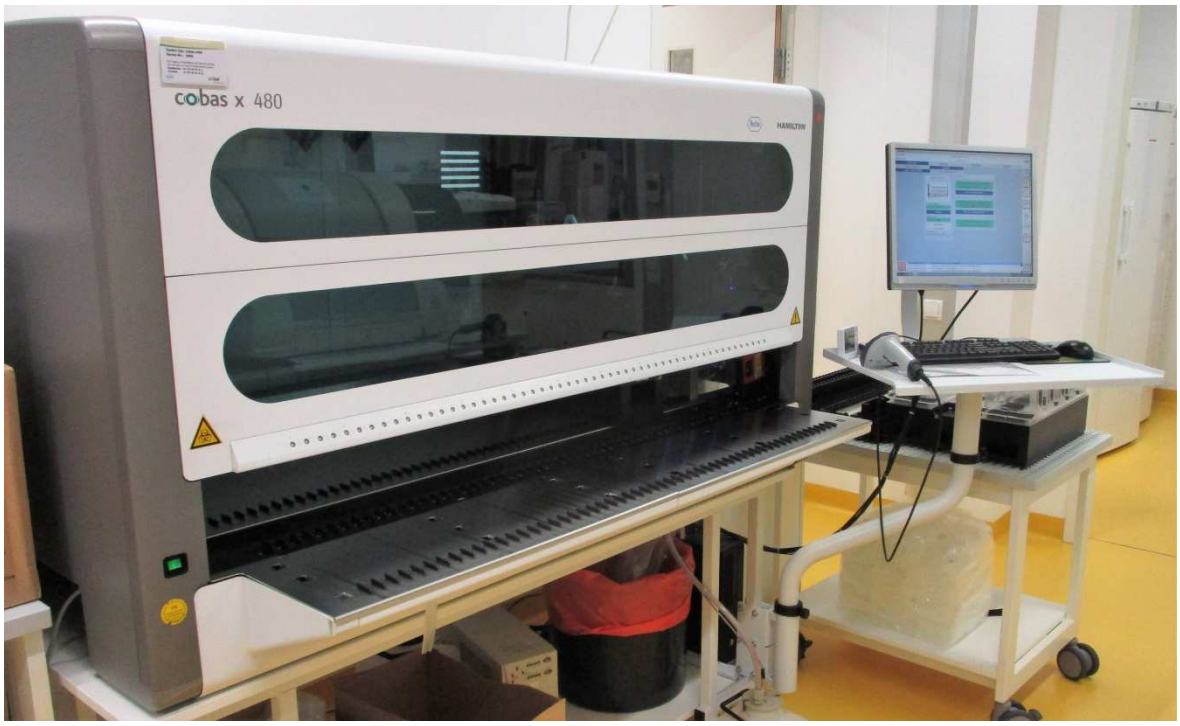

Fig. 8. The cobas ® x 480 platform.

This assay utilizes the cobas® x 480 platform for fully automated sample preparation (nucleic acid extraction and purification) and the cobas® z 480 analyzer for qPCR amplification and detection. Three different target regions in the HCV genome (5'UTR, Core, NS5B) are used to achieve genotyping and subtyping accuracy. Furthermore, the cobas® HCV GT includes two external controls (negative control and positive control). The negative control consists of normal human plasma (non-reactive by in vitro diagnostics (IVD)/Conformité Européenne (CE labeled) and/or Food and Drug Admission (FDA)-approved tests for antibodies to HIV 1/2, antibodies to HCV, HBsAg, and antibodies to HBc). The positive control includes normal human plasma and <0.001% synthetic (armored) HCV RNA, encapsuled in MS2 bacteriophage coat protein. In addition, each master mix includes primers and probes for the 5'UTR region for amplification and detection of HCV RNA, independent of the HCV genotype/subtype, used as an Internal Control to monitor both the extraction and the amplification/detection procedures.

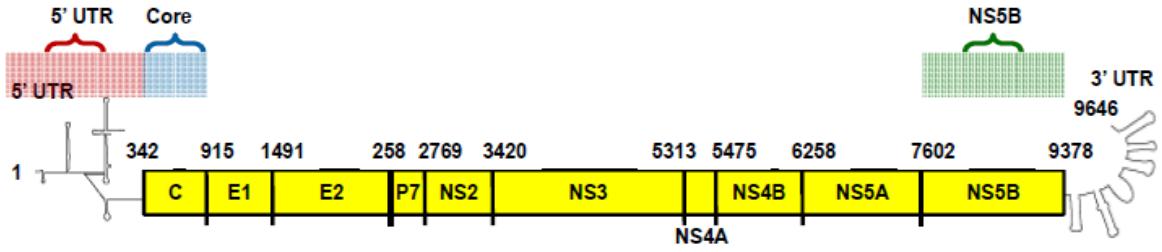

Fig. 9. 5'UTR, core and NS5B region used by cobas® HCV GT to determine HCV genotypes and subtypes (taken from cobas® 4800 Assay Overview August 2016/Version 1.2)

The cobas® x 480 platform requires a minimum sample input volume of 800 µl. (In this study, 1000 µl of plasma were provided to the platform.) The platform transfers 400 µl of each sample into a specific well of the deepwell plate and adds proteinase and chaotropic lysis reagent to release the nucleic acids then. The released nucleic acids bind to silica surface of magnetic glass particles. After several washing steps, all unbound substances (denatured proteins, cellular debris, and potential PCR inhibitors) are removed to purify the samples. The nucleic acids are then removed from the magnetic glass particles with elution puffer at elevated temperature.

Following extraction, PCR mixes are prepared in a way that each sample is amplified in three RT-PCRs. For this, three specific master mixes were added to three separate wells. The master mixes include deoxyuridine triphosphate (dUTP) instead of deoxythymidine triphosphate (dTTP), which is incorporated into the newly synthesized DNA, the amplicon. AmpErase, catalyzing the removal of uracil from DNA, inactivates any contaminating amplicons as PCR templates from previous PCR runs. AmpErase has though no activity in RNA or naturally occurring DNA, which does not contain uracil. Due to annealing and denaturation temperatures of PCR, AmpErase is disabled and not able to inactivate amplicons formed during subsequent cycles of PCR. Each master mix contains genotype and subtype specific primers and fluorescent dye-labeled probes. Thus, RT-PCR for genotype and subtype is achieved and detection of genotypes 1 to 6 and subtypes 1a and 1b is feasible. Depending on the genotype, different primers for different regions are used. For detection of HCV genotypes 2, 3 and 6, specific primers for

5’UTR are used. To provide evidence of HCV genotypes 1, 4, and 5, specific primers for core region are used. For detection of genotype 1 subtypes 1a and 1b, specific primers for NS5B are used.

Fig. 10. The cobas ® z 480 instrument.

A thermostable DNA polymerase is used for both PCR amplification and reverse transcription. The probes are labeled with 4 different fluorescent reporter dyes, for simultaneously detection of HCV and up to 3 genotypes or subtypes per reaction. Each of the oligonucleotide probes in the master mixes is labeled with a non-fluorescent quencher dye and a fluorescent reporter dye. The fluorescence of the reporter dye is suppressed by the quencher dye when probes are intact. During PCR amplification, probes hybridize to their target sites between the primer binding sites and DNA polymerase extends the primers. The 5’-to-3’ nuclease activity of the DNA polymerase cleaves the hybridized probes, resulting in separating the reporter and quencher dyes and generates a fluorescent signal. With each PCR cycle, increasing amounts of cleaved probes are generated and the cumulative signal of the reporter dye increases. By measuring the fluorescence of the released reporter dyes in each cycle, real-time detection and discrimination of PCR products is achieved.

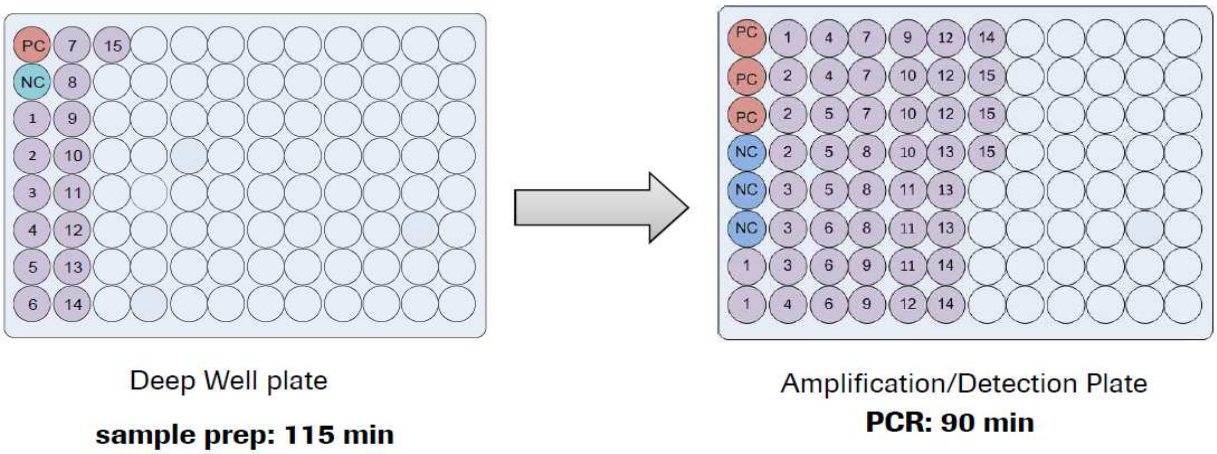

Fig. 11. Pipetting step from deepwell plate to microwell plate. The eluate extracted from external positive control (PC), external negative control (NC) and each patient (1-15) is aliquoted into three separate wells of a microwell plate (taken from cobas® 4800 Assay Overview August 2016/Version 1.2.).

After addition of all reagents needed for qPCR, the cobas® x 480 platform adds the eluates. Additionally, external controls are included in each run. After completion of PCR mix preparation, the microwell plate must be covered by hand with sealing foil and transferred to the cobas® z 480 instrument, where amplification and detection of the HCV genotype/subtype is performed. Nucleic acid extraction and preparation of PCR mixes (also called sample preparation) requires 115 min, while qPCR requires 90 min.

3.2.2 Determination of HCV genotypes/subtypes with the VERSANT® HCV Genotype 2.0 assay

HCV RNA was isolated from 500 µl plasma with the NucliSens® easyMAG® (bioMérieux SA, Marcy-l'Etoile, France) platform and reagents. The principle of this method is based on binding nucleic acids from biological samples to magnetic silica particles. The lysis buffer, containing a chaotropic agent, is added to the specimen. The chaotropic agent is responsible for the disruption of any cellular particle including viruses, bacteria, or fungi eventually present in the specimen. In parallel, nucleic acids are released. Any nucleases present in the specimen are inactivated by the lysis buffer. Adding magnetic silica particles to the lysed specimen is the initial step for the isolation process. Under high salt concentration, the nucleic acids bind to the magnetic silica. Then, the magnetic silica particles are washed several times with two different wash buffers. After the washing steps, elution buffer is added. The nucleic acids are separated from the magnetic silica particles by elevating the temperature and use of a magnetic device.

Fig. 12. The Nuclisens® easyMAG® nucleic acids extraction platform (bioMérieux).

Extracted HCV RNA was amplified using the HCV Amplification 2.0 Kit (Siemens). With this procedure, the reverse transcription (RT) and polymerase chain reaction (PCR) amplification are performed one after the other in the same tube. The reaction tube does not need to be opened or manipulated during the procedure, as all reagents needed are added prior to initiation of the reaction. Extracted HCV RNA is added to a reaction tube in a final step. The reverse transcription into complementary DNA (cDNA) is performed using HCV-specific primers. For PCR amplification, the reaction mixture is heated to activate the DNA polymerase and simultaneously inactivate the reverse transcriptase. Portions of the 5'UTR and core regions of the HVC genome are co-amplified from the cDNA using two pairs of biotinylated primers to produce two distinct biotinylated DNA fragments of 240 and 270 base pairs. The RT-PCR master mix was prepared in a sterile tube. The RT-PCR master mix was vortexed and centrifuged briefly before 30 µl were added to each reaction tube. Then, 20 µl of extracted RNA were added and stored at room temperature for 10 minutes to allow degradation of any contaminating uracil-containing DNA. After RT-PCR, tubes were removed from the thermal cycler and stored at -20°C until further use.

The VERSANT® HCV Genotype 2.0 assay (Siemens) based on the reverse hybridization principle was used according to the manufacturer's package insert instruction. This test is a line probe assay for *in vitro*-diagnostic use, which identifies HCV genotypes 1 to 6 and genotype 1 subtypes 1a and 1b in human serum or EDTA plasma. The assay was performed on the AutoBlot 3000H according to the manufacturer's application sheet. All test materials needed were taken from the refrigerator to adjust to room temperature (20° to 25°C) before use. The volumes needed for the hybridization and stringent wash solutions were determined as recommended in the manufacturer's instructions and solutions were preheated on the AutoBlot 3000H heated bottle plate (37-50°C) for approximately 60 minutes. All reagents were diluted according to the manufacturer's application sheet and the strips (one per sample) were labeled above the green marker line with a waterproof pencil. The strips were loaded in the tray with the marker lines facing up, using a steril forceps. When the system preheating was completed, flexible tubes were put into the appropriate reagent bottles.

Fig. 13. The Autoblot 3000H instrument (Siemens).

For denaturation, 10 µl of denaturation solution was pipetted into the lower end of each through. After thawing tubes containing amplification products, 10 µl of extracted RNA were added and mixed by pipetting up and down with the denaturation solution in the appropriate through. Before the tray was loaded on the AutoBlot 3000H to be processed automatically, it was incubated at room temperature for 5 minutes to denature samples. After automated completion of the assay, strips were removed from each through and placed on absorbent paper. When the strips had dried completely, they were fixed on a data-reporting sheet and read using the interpretation chart.

Each VERSANT® HCV Genotype 2.0 Assay (LiPa) strip has 3 control lines and 22 parallel DNA probe lines containing sequences specific for HCV genotypes/subtypes. The conjugate control line monitors the color development reaction. One of the amplification controls contains universal probes that hybridize to the PCR product from the 5'UTR, the other amplification control contains

universal probes that hybridize to the PCR product from the core region. HCV genotypes/subtypes are determined by aligning the assay strips with the reading card and comparing the line pattern from each assay strip with the patterns shown on the interpretation sheet.

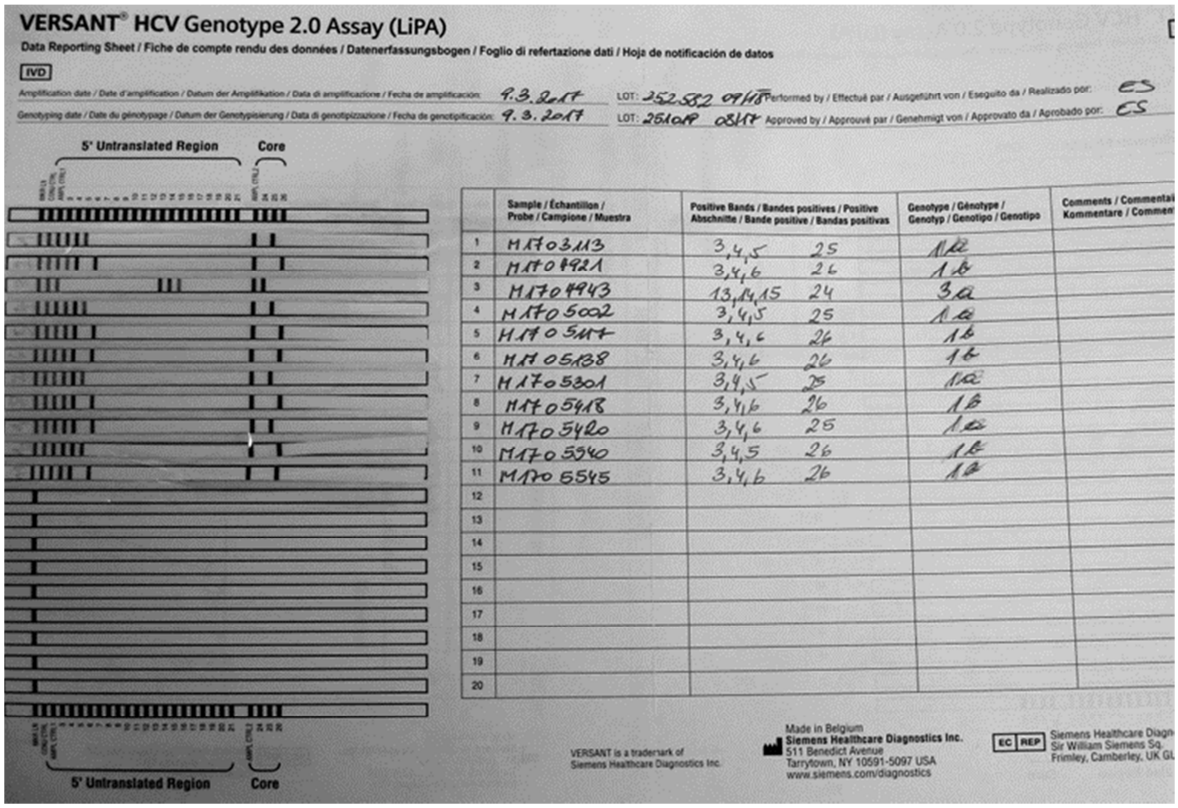

Fig. 14. Interpretation of the visible banding pattern on the nitrocellulose strip.

3.2.3 Sanger sequencing

Regions encoding HCV core, NS2/NS3 junction, NS3 protease, NS5A, and the NS5B polymerase gene were amplified by polymerase chain reaction and population based sequencing as described in SUSSER et al. (47).

The following primers were used with the identical method as described in DIETZ et al. (48):

sCore_outer–CCATAGTGGTCTGCGGAAC,

sCore_inner–GCTAGCCGAGTAGCGTTG,

asCore_2a–CATTGGARCAGTCATTRGTC,

asCore_2b/k–GTTCGAGCAATCRTTRGTGG,

asCore_2c–GCTRGAGTTAGAGCAATCG,

NS2-2k-outF–CCGCAGCYTATGGTGGTTG,

NS2-1b-outR–GACTCAACGGGTACAAAKTCC,

NS2-2k-innF–TCCGGGYGTRGTGTTTGAC,

NS2-1b-innR–GGCAGAGCAGYGGACCAC.

All sequencing analyses were performed according to the manufacturer's protocol (Big Dye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Analyses were performed using a standard nucleotide BLAST optimized for highly similar sequences.

4 Results

In this study, 279 anonymized leftover samples from Germany were investigated with the cobas® HCV GT and results were compared to those obtained with the VERSANT® HCV GT 2.0 assay.

Of 279 patients, 53 patients (19.0%) were found to be infected with HCV subtype 1b, 177 (63.4%) with HCV genotype 2, and 48 (17.2%) with an HCV genotype 2/genotype 1 recombinant. With Sanger sequencing, HCV recombinants could further be classified as 2k/1b (n=46), 2b/1a (n=1), and 2a/1b (n=1). Finally, one patient (0.4%) was found to have a mixed 1b+2b infection. Results obtained from 279 samples are shown in Figure 13.

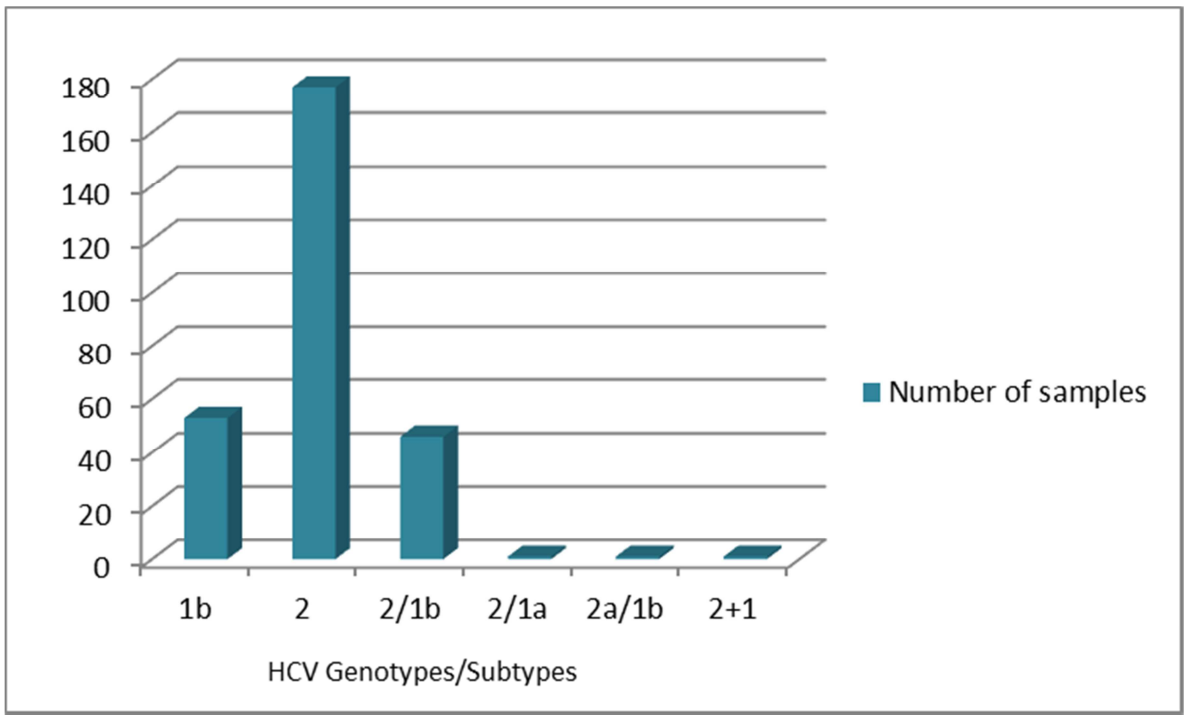

Fig. 15. Results obtained by sequencing 279 samples.

With the cobas® HCV GT, all runs were analyzable. Of all 279 samples, 276 could be analyzed with this assay. Three (of 177 samples with HCV genotype 2) samples were found to be invalid, indicated by a nondetectable Internal Control. Results obtained from two (of 53 samples with HCV subtype 1b) samples were reported as HCV genotype 1 only. Results obtained from five (of 48 samples with HCV recombinantions) samples were reported as HCV genotype 2 only.

The VERSANT® HCV Genotype 2.0 assay identified all 177 samples with HCV genotype 2 correctly. All 53 samples with HCV subtype 1b were identified correctly. All 48 samples with HCV recombinations were reported as HCV genotype 2.

5 Discussion

Determination of HCV genotype is part of pre-therapeutic assessment and recommended with a genotyping assay that is able to identify HCV genotypes and discriminate HCV genotype 1 subtypes 1a and 1b accurately. The cobas® HCV GT is based on primer-specific real-time PCR to identify HCV genotypes 1- 6 and subtypes 1a and 1b. Identification of more than one HCV genotype/subtype may indicate either mixed infection or presence of recombinant HCV strain. In this study, 279 anonymized leftover samples from Germany were investigated with the cobas® HCV GT on the cobas® x 480 platform and the cobas® z 480 instrument and compared to results obtained by the VERSANT® HCV Genotype 2.0 assay. In addition, direct sequencing of the HCV core, NS2/NS3 junction, NS3, NS5A, and the NS5B regions as the reference standard was performed.

In this study, 51 out of 53 samples with HCV subtype 1b were subtyped correctly with the cobas® HCV GT assay. It remains unclear why two samples with HCV subtype 1b yielded an HCV genotype 1 result only. Regarding samples with HCV genotype 2, this assay reported all of them correctly; however, for three of 177 samples invalid results were obtained due to unknown reasons. Of 48 samples with HCV recombinations, the cobas® HCV GT assay identified 43, while five were reported as containing HCV genotype 2 only due to unknown reasons. The VERSANT® HCV Genotype 2.0 assay reported all 53 samples with HCV subtype 1b and all 177 samples with HCV genotype 2 correctly. All 48 samples with HCV recombinations were reported as HCV genotype 2. Considering the design of this assay, it is not possible to detect HCV recombinant forms. One sample was classified as mixed HCV genotype 1 + genotype 2 based on results obtained with the VERSANT® HCV Genotype 2.0 assay. To confirm this, analysis of this sample utilizing a next generation sequencing-based assay is required.

When considering the increasing amount of recombinant HCV strains, this may affect the best choice of anti-HCV therapy because several anti-HCV drugs are still non-pangenotypic. A recent study from Germany revealed, that recombinant HCV genotype 2/genotype 1 infections treated with genotype-1 based regimens, show very effective eradication of recombinant viruses and a sustained response

rate of 96% (47). In that study, a prevalence of 14-25% infected with a genotype 2/subtype 1b recombinant was observed. However, HCV genotype 2/subtype 1 recombinants are typically determined as HCV genotype 2 infections by the routinely used hybridization assays. Most patients in the German study were initially treated with genotype-2 based regimens (Sofosbuvir/Ribavirin for 12 weeks). This treatment was insufficient, with high relapse rates >90%. With the primer-specific real-time PCR, recombinant strains could be detected but mixed infections could not be excluded. Consequently, sequencing of the recombination sites is the only safe way to identify any viral recombination (47).

Recently, four patients living in Austria were found to be infected with recombinant HCV genotype 2/subtype 1b (45). However, the true prevalence of HCV recombinant forms in Austria is not known. More data are therefore required to determine the prevalence of HCV recombinant strains in Austria.

In conclusion, when analyzing HCV patient samples containing strains without recombination, analysis of the 5´UTR and core regions is sufficient, while identification of HCV recombinant forms requires inclusion of an additional region close to the 3´ end of the HCV genome.

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