Diploma thesis

Determination of the hepatitis C virus genotype/subtype: evaluation of a new assay based on primer-specific real-time polymerase chain reaction

submitted by

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Abstract

Background: Anti-hepatitis C virus treatment has undergone major progress recently through introduction of effective direct-acting antivirals. The hepatitis C virus (HCV) genotype and HCV subtypes 1a and 1b must be determined prior to treatment initiation as it is still essential for the choice of therapy.

Objectives: The aim of this study was to evaluate the performance of the new cobas® HCV GT (Roche Molecular Systems) assay and to compare it to alternative assays used in the routine diagnostic laboratory.

Methods: Accuracy was tested using the Quality Control for Molecular Diagnostics (QCMD) HCV Genotyping Proficiency Program 2014 and 2015 panels. For clinical evaluation, 183 residual routine clinical samples were tested and results were compared to those obtained by the VERSANT® HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics) and the TRUGENE® HCV 5'NC Genotyping Kit (Siemens). All samples showing discrepant results were additionally investigated by home-brew NS5B sequencing. In addition, total times for completion including hands-on times were compared.

Results: When accuracy was tested, the cobas® HCV GT assay reported correct results for panel members containing HCV genotype 3, HCV genotype 4, and HCV genotype 1 subtypes 1a and 1b but the assay could not detect low titer panel members containing HCV genotype 5 correctly. When the clinical performance of the cobas® HCV GT was investigated, 160 out of 183 clinical samples showed concordant results when compared with alternative assays. Seven samples showed indeterminate results with the cobas® HCV GT and 16 samples showed discordant results with at least one of the comparator assays. When times-to-results were compared, the cobas® HCV GT could be completed faster than the comparator assays but hands-on time was similar for all assays.

Conclusions: The new cobas® HCV GT assay showed a good clinical performance and proved to be suitable for use in the routine diagnostic laboratory. The new assay allows for accurate determination of the HCV genotypes 2, 3, and 4 and it is able to discriminate HCV genotype 1 subtype 1a and 1b.

Kurzfassung

Hintergrund: Durch die Einführung neuer Kombinationstherapien ist die Heilung der chronischen Hepatitis C möglich geworden. Für das optimale individuelle Therapiekonzept ist die Bestimmung des HCV-Genotyps und der HCV-Subtypen 1a und 1b vor Therapiebeginn obligatorisch.

Ziele: In dieser Studie wurde die Leistung des neuen cobas® HCV GT Test (Roche Molecular Systems) evaluiert und mit alternativen Tests, die in diagnostischen Routinelabors eingesetzt werden, verglichen.

Methoden: Die Richtigkeit des neuen Test wurde mit Proben der *Quality Control for Molecular Diagnostics* (QCMD) *HCV Genotyping Profiency* Programme 2014 und 2015 ermittelt. Für die klinische Beurteilung des Tests wurden 183 HCV-positive Proben getestet und die Ergebnisse mit denen alternativer Tests, dem VERSANT® HCV Genotyping 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics) und dem TRUGENE® HCV 5'NC Genotyping Kit (Siemens), verglichen. Alle Proben, die diskrepante Ergebnisse mit mindestens einem der Vergleichstests lieferten, wurden zusätzlich mittels *home-brew* NS5B-Sequenzierung getestet. Außerdem wurden die Gesamtzeiten zur Durchführung der Tests, einschließlich des manuellen Aufwandes, verglichen.

Ergebnisse: Bei der Beurteilung der Richtigkeit zeigte der cobas® HCV GT Test korrekte Ergebnisse bei Proben, die HCV-Genotyp 3, HCV-Genotyp 4 und HCV-Genotyp 1 Subtyp 1a und 1b enthielten. Bei Proben mit niedrigen Viruskonzentrationen, die HCV-Genotyp 5 enthielten, konnte mit dem Test der korrekte HCV-Genotyp jedoch nicht bestimmt werden. Bei der klinischen Beurteilung des Tests zeigten 160 von 183 Proben übereinstimmende Ergebnisse. In 7 Proben konnte mit dem cobas® HCV GT Test zwar das Vorhandensein von HCV RNA nachgewiesen werden, es konnte jedoch kein Genotyp bzw. Subtyp bestimmt werden. 16 Proben zeigten diskordante Ergebnisse mit mindestens einem der beiden Vergleichstests. Beim Vergleich der benötigten Gesamtanalysenzeit zur Durchführung konnte gezeigt werden, dass der cobas® HCV GT Test die kürzeste Gesamtanalysenzeit aufweist, der manuelle Aufwand ist jedoch bei allen drei Tests ähnlich.

Fazit: Der neue cobas® HCV GT Test zeigte eine gute klinische Leistung und erwies sich als tauglich für den Gebrauch im diagnostischen Routinelabor.

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1. Introduction

1.1. Epidemiology

The hepatitis C virus (HCV) infection is a major global health issue. More than 185 million people are infected with HCV worldwide. Estimates indicate that three to four million people are newly infected each year. 170 million people are chronically infected with the risk of developing liver disease including cirrhosis and liver cancer. 350 000 deaths occur each year due to all HCV-related causes (1).
HCV has spread all over the world occurring among persons of all ages, genders and races of the world. Eighty percent of patients with acute HCV will develop a chronic HCV infection (2).
Most studies on global HCV epidemiology rely on HCV seroprevalence studies. These studies are often undertaken in selected populations (e.g. blood donors or patients with chronic liver disease) which do not represent the community or region in which they live. However, for several years, the World Health Organization (WHO) has reported data on the worldwide prevalence of HCV infection based on both published studies and submitted data (3).
Although HCV is endemic worldwide, the prevalence shows a large geographical variability (Fig. 1). Countries with the highest prevalence reported are located in Africa and Asia. Areas with lower prevalence rates include the industrialized nations in North America, Northern and Western Europe, and Australia (3). Considering the large population in Asia, the South Asia and East Asia regions have by far the largest number of persons living with HCV infection. The seroprevalence of HCV in Asia ranges between 3.8% in Central Asia (e.g. Armenia, Georgia, Mongolia), 2.0% in Southeast Asia (e.g. Thailand, Vietnam, and Indonesia) and 1.4% in Asian countries with high income (e.g. Japan, South Korea). In Central and Western Europe (e.g. Austria, Germany, Italy), the prevalence of HCV is reported to be 2.4%, whereas it is 2.9% in Eastern Europe (e.g. Lithuania, Moldova, Russia, Ukraine). In North African and Middle East countries (e.g. Egypt, Turkey), the prevalence of HCV is reported to be 3.6 % (1).
The prevalence in Latin America ranges between 1.6% in Central Latin America (e.g. Nicaragua, Costa Rica, Panama), 1.6% in Southern Latin America (e.g.

Argentina, Chile, Uruguay), and 1.2% in Tropical Latin America (Brazil, Paraguay). North American countries (e.g. USA, Canada) show a prevalence of only 1.3% (1).

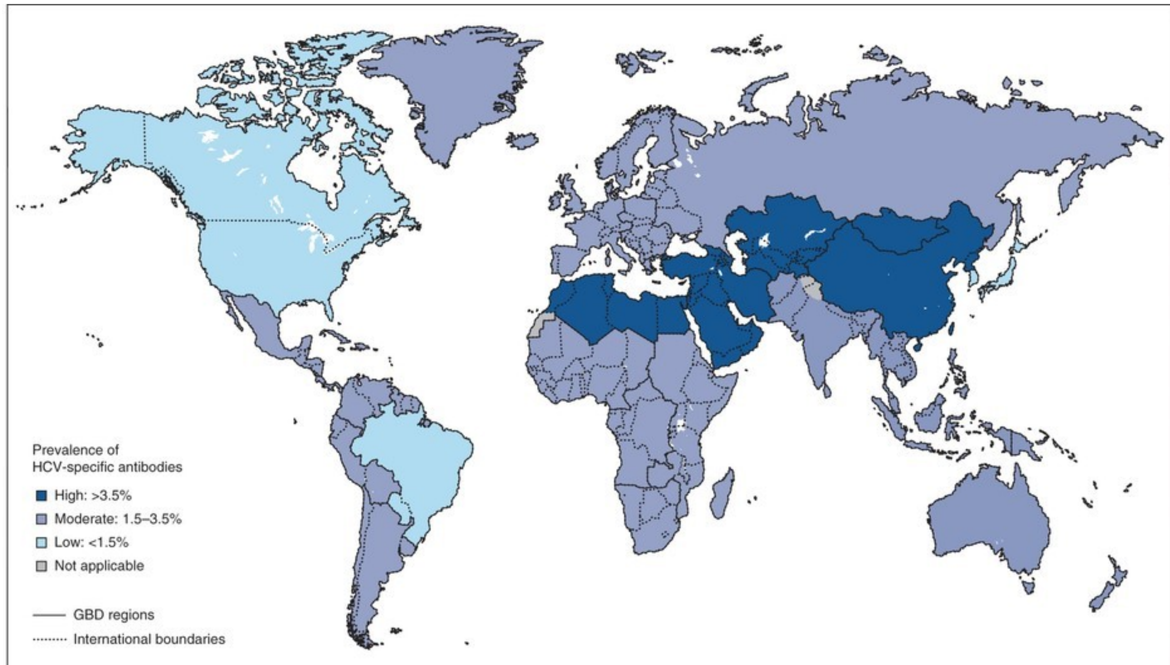


Fig. 1. Prevalence of HCV-specific antibodies.

Besides geographical differences in prevalence rates of HCV infection, there are also certain groups within a population that are at higher risk of infection.
According to the WHO Guidelines for the screening, care and treatment of persons with hepatitis C infection, following groups show an increased rate of HCV infection (4):

- Persons who inject drugs
- Recipients of infected blood products or invasive procedures in health-care facilities with inadequate infection control practices (HCV prevalence among these persons depends on the frequency of medical procedures and level of infection-control practices).
- Children born to mothers infected with HCV (HCV transmission risk ranges 4 to 25% depending on an HIV co-infection of the mother).
- People with sexual partners who are infected with HCV (risk of sexual transmission strongly linked to pre-existing HIV infection).

- People with HIV infection (persons with HIV infection, in particular men having sex with men are at increased risk of HCV infection through unprotected sex).
- People who have used intranasal drugs.
- People who have had tattoos or piercings.

1.2. Transmission

The HCV is transmitted primarily parenterally, especially through contaminated blood. Up to the early 1990’s, blood transfusion from infectious donors was considered to be the predominant mode of HCV transmission. Since then, intravenous drug abuse has become the major route of transmission. However, in approximately 20% of HCV infections, the path of transmission remains unknown (5). With the discovery of HCV in 1989 and the availability of blood donor anti-HCV screening, the residual risk of transmission though blood transfusion has decreased significantly in developed countries (6). In countries with low socioeconomic status, HCV infection is still mainly caused by unsafe medical treatment such as unsafe injection practices, renal dialysis, and unscreened blood transfusions. According to the WHO, between 8 and 12 billion injections are administered yearly around the world and 50% of these are considered to be unsafe (4). In countries with high socioeconomic status, the main path of transmission is intravenous drug abuse with contaminated needles nowadays. Globally, approximately two-third of intravenous drug abusers are anti-HCV positive (7).

Another path of transmission is the perinatal HCV infection. The transmission rate ranges between 0 and 6% among only HCV-positive mothers and up to 25% among mothers with an HIV/HCV coinfection (8).

Studies have shown that HCV transmission through heterosexual intercourse is very unlikely while transmission in HIV-positive men having sex with men is occurring more frequently (9,10).

Furthermore, there exist a variety of activities that involve a potential exposure to blood or blood-derived body fluids with the possibility of HCV transmission. These include tattooing, body-piercing, intranasal drug use, acupuncture, circumcision, manicure and pedicure, and bloody dental procedures (5,11).

1.3. HCV Disease

1.3.1. Acute hepatitis C

Acute hepatitis C (AHC) is defined as the presence of HCV up to six months after infection (4). The incubation period ranges between 6 to 10 weeks (12). Due to the fact that acute illness is clinically mild and typically unrecognized, it is diagnosed infrequently. The initial signs of acute infection may be non-specific including malaise, weakness, and anorexia. Only a minority of individuals show more specific symptoms such as jaundice, dark urine, aversion to smoking among smokers, and abdominal discomfort (13,14). 80% of individuals do not show any symptoms at all (12). Because of the silent onset, studies regarding the clearance of AHC are difficult to carry out. It has been reported that 15 to 45% of infected individuals clear HCV spontaneously (4,13).

1.3.2. Chronic hepatitis C

The transition from AHC to chronic hepatitis C (CHC) is usually sub-clinical (13). CHC is defined as liver inflammation caused by HCV that is lasting six month or more. About 45 to 85% of patients with AHC proceed to CHC with the percentage being influenced by many factors including the age at time of infection, gender, ethnicity, and the development of jaundice during acute infection (14).
The majority of patients with CHC does not show any symptoms or suffer from unspecific symptoms such as fatigue and malaise in the first years of disease. Therefore, it is common for patients to remain undiagnosed with hepatitis C until they present with complications of end stage liver disease (12,13). CHC may cause liver cirrhosis, liver failure, and hepatocellular carcinoma (4). Estimates indicate that 16% of chronically infected individuals develop liver cirrhosis during the following 20 years after infection (15). Once cirrhosis has developed, there is an annual risk of 1-5% for hepatocellular carcinoma and an annual risk of 3-6% for hepatic decompensation (13).
Several co-factors have been revealed which increase the risk of a chronically HCV infected individual towards development of fibrosis or cirrhosis. These include male sex, heavy alcohol consumption, elevated serum ALT levels and high-grade necroinflammatory activity (16).

1.4. Diagnostics

1.4.1. Testing of liver enzymes

The elevation of liver enzymes such as serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) may be the first hint for an HCV infection. Liver enzyme alteration may either be found in patients with signs of a liver disease or as an unexpected finding in asymptomatic patients (17).
ALT, a marker for hepatocyte necrosis, begins to increase 2 to 8 weeks after exposure and may reach a level of greater than 10 times the upper limit of normal (14). However, ALT levels show a high grade of fluctuation during progression of hepatitis C disease and up to 40% of infected patients may show a normal aminotransferase level (18,19).

1.4.2. Serologic antibody assays

Anti-HCV antibodies are the first-line specific diagnostic test for HCV infection (20). Serologic antibody assays are indirect tests to detect IgG antibodies induced by viral infection. Anti-HCV antibodies can be detected in the serum or plasma with a number of immunoassays (21). Screening for anti-HCV antibodies is usually performed with an enzyme immune assay (EIA), as this test is highly sensitive, cheap, and easy to use (19,22). The serologic window ranges 4 to 10 weeks or more. 90% of individuals show positive serologic assays 3 month after infection (23). Anti-HCV antibodies persist lifelong in patients with CHC (19). A positive serological antibody assay indicates either a current HCV infection or a cured HCV infection and therefore needs to be confirmed by a nucleic acid test (12).

1.4.3. Detection of viral RNA

Today, the main marker to confirm active hepatitis C is the detection of HCV RNA in the peripheral blood. In the case of suspected AHC or in immunocompromised patients, HCV RNA testing must be part of the initial evaluation (20). Two technologies are routinely used for HCV RNA testing. These include reverse transcription followed by real-time polymerase chain reaction (qPCR) and transcription-mediated amplification (TMA) (24). Anti-HCV-positive, HCV RNA negative individuals should be retested for HCV RNA three to six months later to confirm true convalescence (20).

1.5. Treatment

The aim of HCV treatment is to cure the infection in order to prevent the complications of HCV-related diseases. A sustained virological response (SVR) is the pursued endpoint of a successful therapy. The SVR is defined as undetectable HCV RNA 12 (SVR12) or 24 (SVR24) weeks after treatment completion. The infection is cured in more than 99% of patients who achieve an SVR (20).

As shown in Fig. 2, the standard treatment of HCV infection has made great progress over the last decades (25). The combination of pegylated interferon (PegINF)-alfa and ribavirin for 24-48 weeks was the standard treatment until 2011 (26). SVR with this therapy was ranging 40 to 50% among patients infected with genotype 1 and up to about 80% among patients infected with genotype 2, 3, 4, 5, and 6 (20).

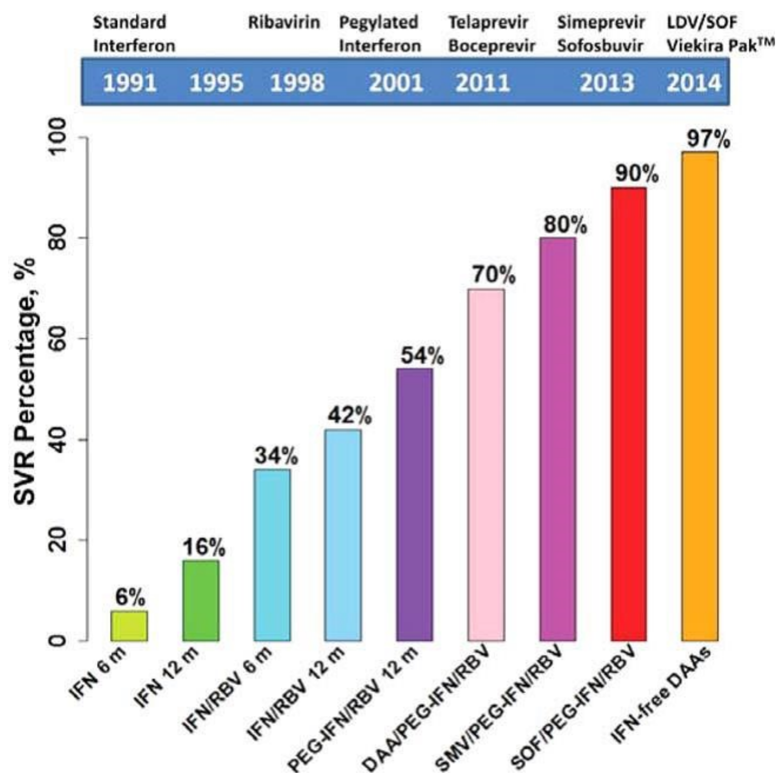


Fig. 2. Increase of SVR rates according to improved anti-HCV therapy between 1991 and 2014 (25).

Recently, direct acting antivirals (DAA's) have been introduced for anti-HCV therapy. DAA's target viral non-structural proteins and inhibit specific steps in virus replication (27).

In 2011, the first-generation DAA's telaprevir and boceprevir were approved for treatment of HCV genotype 1 infection. Telaprevir and boceprevir are protease inhibitors that target the HCV NS3-4A serine protease and must be administered in combination with PegINF-alfa and ribavirin. However, the side effects and costs prevented them to be used in patients infected with genotype 1 (20).

In 2014, three new DAA's were approved: sofosbuvir, a pangenotypic nucleotide analogue inhibitor of the HCV RNA-dependent RNA polymerase, simeprevir, a NS3/4A protease inhibitor active against genotypes 1 and 4, and daclastavir, a pangenotypic NS5A inhibitor. With the new HVC DAA's licensed, INF-free combinations have been broadly used across Europe from 2014 onwards. In general, HCV DAA's can be classified into four groups based on their mechanism of action. NS3/4A protease inhibitors such as simeprevir and paritaprevir inhibit the viral NS3/4A serine protease, while NS5A inhibitors such as daclatasvir, ledipasvir, and ombitasvir target the viral NS5A protein. Nucleoside analog NS5B polymerase inhibitors as, for example, sofosbuvir interact with the active site of the viral NS5B RNA-dependent RNA polymerase. Non-nucleoside NS5B polymerase inhibitors as, for example, Dasabuvir bind outside of the active site of the NS5B polymerase (20,25).

Anti-HCV treatment has undergone a major progress in recent years moving from long treatment duration, severe adverse events, and low SVR rates to short treatment duration, higher efficacy, and more tolerable (IFN-free) treatment options (25).

1.6. Morphology of the HCV

The HCV, which was first identified in 1989, is a member of the genus Hepacivirus in the family of *Flaviviridae* (28).

Similar to all members of the *Flaviviridae* family, the HCV is enveloped with a lipid bilayer in which two or more envelope glycoproteins are anchored (Fig. 3). The envelope surrounds the capsid protein (also known as core) which contains the RNA genome (29).

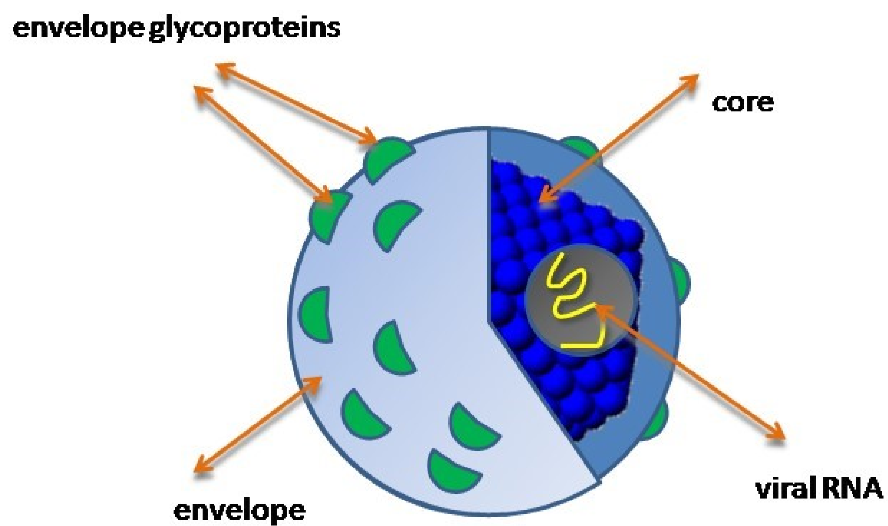


Fig. 3. Morphology of the HCV.

1.7. The HCV genome

The HCV is a positive sense, single stranded RNA virus that contains about 9000 nucleotides. The sequence has a single large open reading frame encoding a polyprotein of 3000 amino acids (19). The genome of HCV consists of a coding region, which is flanked by two non-coding (NC) regions, the 5' and the 3' NC region (Fig. 4). The coding region encodes a single polyprotein that is split into structural proteins and non-structural proteins (29).

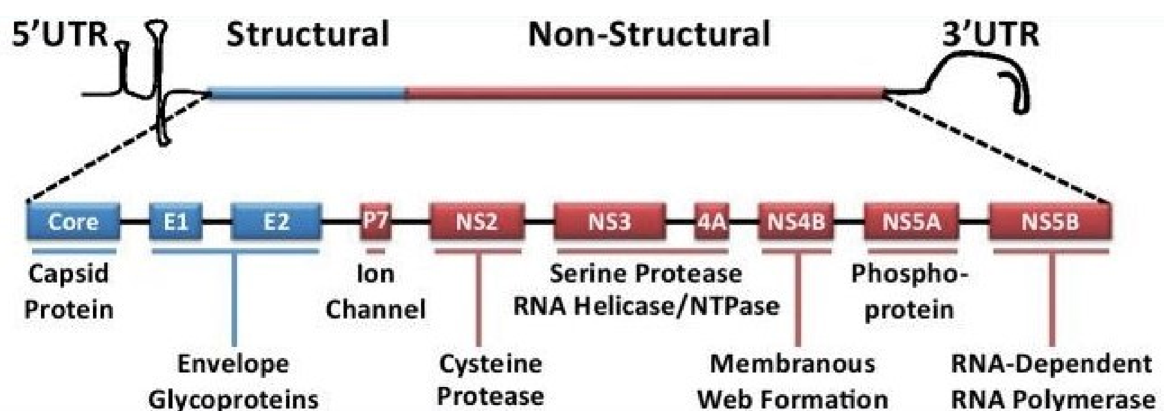


Fig. 4. Structure of the HCV genome.

The 5' and 3' NC regions may be essential for polyprotein translation and genome replication. The 5' NC region is the most conserved region of the genome and contains the internal ribosome entry side that binds to the ribosome and constitutes the first step of HCV polyprotein translation. The HCV open reading frame encodes several proteins including 3 structural proteins (core, E1 and E2) and 6 non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The core protein is a highly conserved, RNA-binding protein, which forms the viral capsid (Fig. 4). The two highly variable transmembrane envelope glycoproteins, E1 and E2, are essential components of the virion envelope and necessary for viral entry and fusion. The non-structural proteins NS2 to NS5B are involved in polyprotein processing and viral replication (Fig. 5). NS2 constitutes together with NS3 the NS2-NS3 protease, a zinc-dependent metalloprotease that cleaves the

site between NS2 and NS3. The viral protein NS3 contains a serine protease domain and a helicase/NTPase domain. NS4A is a cofactor of NS3 protease activity. The NS3-NS4A protease is essential for the HCV lifecycle as it catalyzes the cleavage of the HCV polyprotein at the NS3/NS4A, NS4A/NS4B, NS4B/NS5a and NS5A/NS5B junctions. NS4B is an integral membrane protein and NS5A a polyphosphorylated protein that regulates HCV replication. NS5B is the RNA-dependent RNA polymerase, which catalyzes the replication of the virus (29,30).

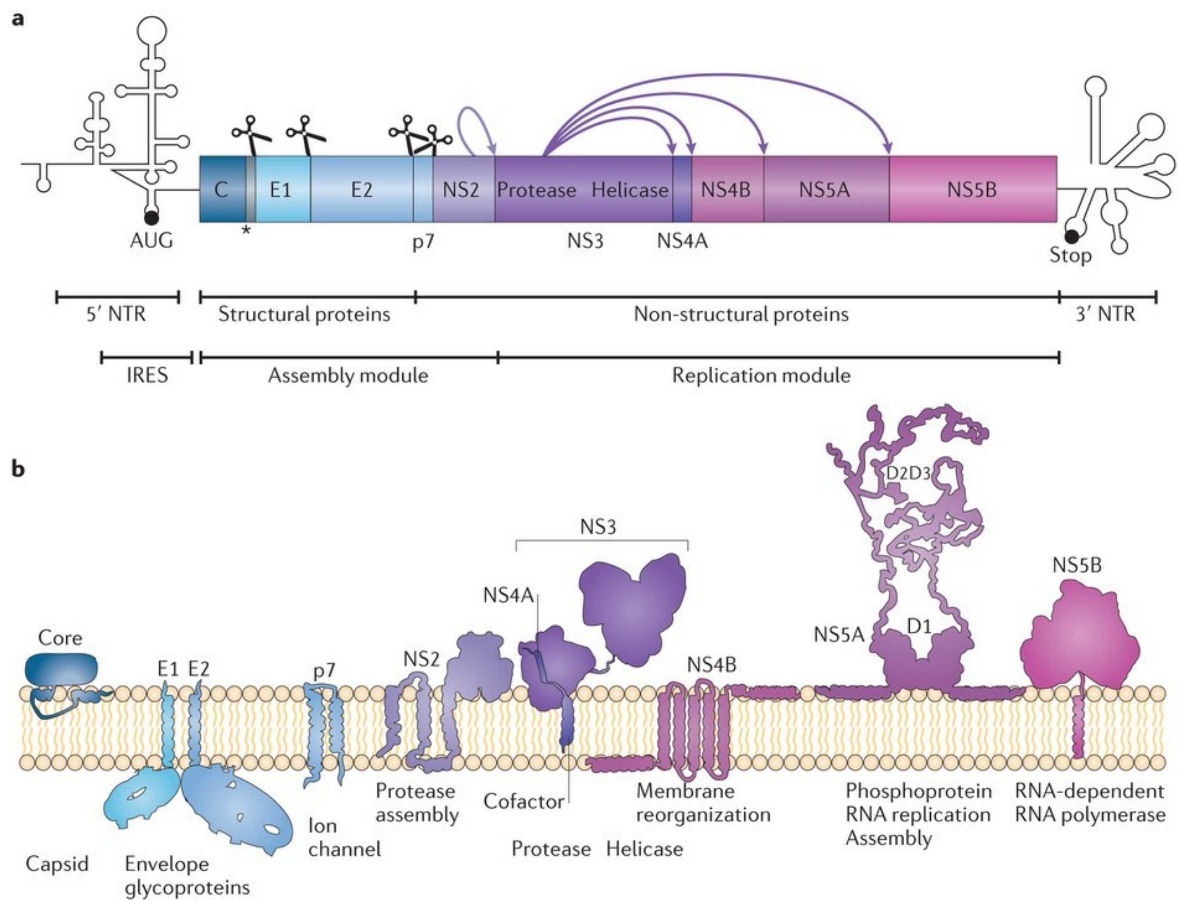


Fig. 5. a, HCV genome organization; b, membrane structure and major functions of the HCV polyprotein.

1.8. HCV Genotypes

Due to the combination of a lack of proof-reading activity by the RNA dependent RNA polymerase and a high level of viral replication, the genome of the HCV shows a high genetic diversity. Only 39% of all amino acid positions are pan-genotypic conserved or highly conserved across all HCV genotypes. The best conserved regions of the genome are the 5' and 3' non-coding regions and the region encoding the capsid. The most variable regions are encoding for the envelope glycoproteins E1 and E2 (31,32).

As a consequence, HCV genotypes and subtypes have been classified. Similar sequences have been clustered into three groups. Sequences with a similarity of 65-70% have been defined as genotypes. Sequences with a similarity of more than 85% have been defined as subtypes and those with a similarity of at least 90% as quasispecies (33,34). Phylogenetic tree analysis and comparison of HCV sequences from core, E1 and NS5 regions revealed seven genotypes and 67 subtypes (Fig. 6) (34).

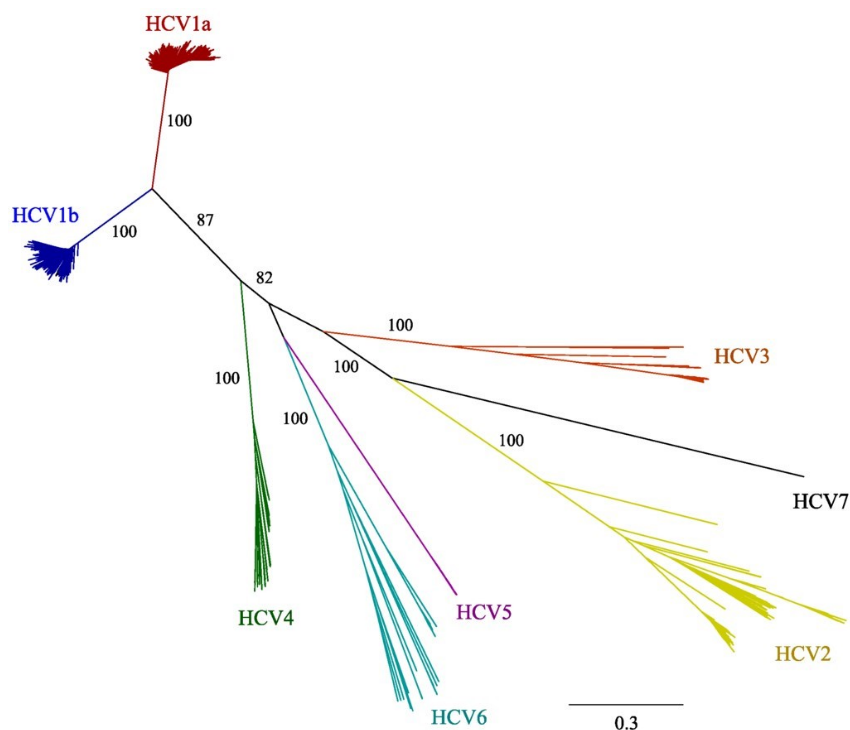


Fig. 6. Phylogenetic tree of HCV.

The HCV genotype 1 shows the highest prevalence worldwide, representing 46.2% of all HCV infections. The second most common HCV genotype is genotype 3 with 30.1% of all cases worldwide. HCV genotypes 2, 4, and 6 are responsible for 22.8% and genotype 5 for less than 1% of all HCV infections (35).
It has been shown that HCV subtypes such as subtypes 1a, 1b, 2a, and 3a are widely distributed across the globe whereas certain HCV genotypes are more locally found including genotype 2, 4, 5 and 6 (35).
Figure 7 shows the relative prevalence of each HCV genotype in different regions of the world. HCV genotype 2 predominates in West Africa, HCV genotype 4 in Egypt and Middle East, HCV genotype 5 in South Africa, and HCV genotype 6 in Southeast Asia (34,36).

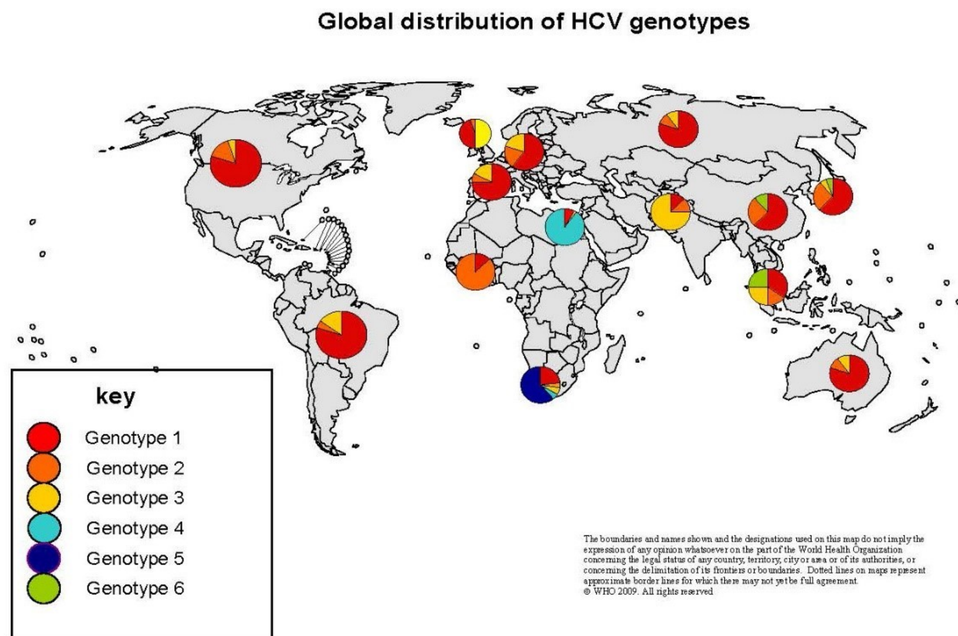


Fig. 7. Global distribution of HCV genotypes.

1.9. Molecular techniques for detection/quantitation and identification of HCV genotypes/subtypes

1.9.1. Real time polymerase chain reaction (qPCR)

The real time polymerase chain reaction (qPCR) has become the method of choice for the detection/quantitation of nucleic acids. In the routine diagnostic laboratory, molecular assays based on qPCR have largely replaced those based on conventional PCR. Numerous qPCR and reverse transcription qPCR (RT-qPCR) assays have been developed to allow the detection/quantitation of viruses and bacteria. Detection formats include hybridization probes, hydrolysis probes, molecular beacons, and scorpions (37).

The hydrolysis probe format uses an oligonucleotide with a fluorescent label (reporter dye) at its 5'-end and a quencher dye, which lacks native fluorescence at its 3'-end. When probes are intact, the fluorescence of the reporter dyes is suppressed by the quencher dye. During PCR amplification, the probes hybridize to their target sites between the primer binding sites, and DNA polymerase extends the primers. During elongation, the 5' exonuclease activity of the polymerase cleaves the probe, separating the reporter dye from the quencher. Consequently, emission of fluorescent light from the reporter dye can be detected during the data acquisition step (37).

The use of genotype and subtype specific primers and fluorescent dye-labeled probes makes it possible to utilize RT-qPCR for identification of HCV genotypes/ subtypes.

1.9.2. Reverse hybridization

The principle of reverse hybridization is used for identification of HCV genotypes/subtypes. Immobilized oligonucleotide probes are bound to a solid phase that usually consists of nitrocellulose. After PCR amplification of certain regions of nucleic acids and denaturation of the PCR products, biotinylated PCR products are hybridized to the complementary oligonucleotide probes. After the hybridization step, non-hybridized PCR products are washed away, while alkaline phosphatase labeled streptavidin is bound to the biotinylated hybrid. Thereafter, a special chromogen reacts with the streptavidin-alkaline phosphatase complex forming a purple/brown precipitate, which results in a visible banding pattern on the strip (Fig. 8.) (38).

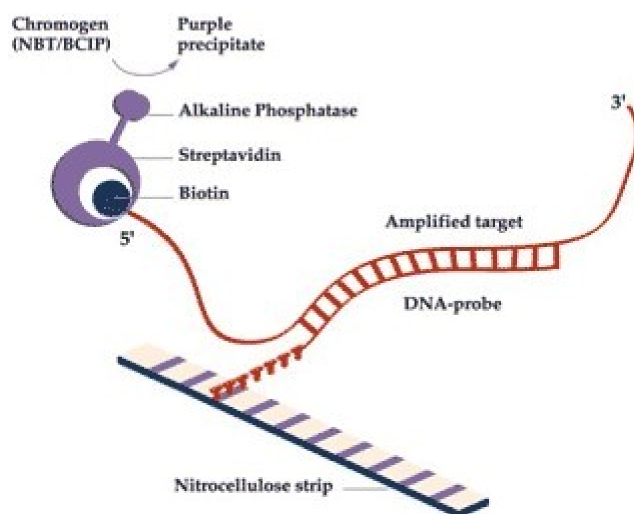


Fig. 8. Principle of reverse hybridization.

1.9.3. Population Sequencing

Sequencing methods that provide detailed information about the nucleotide sequence of nucleic acids have become an important tool in molecular diagnostics. The basic principle of sequencing was introduced by Frederick Sanger ('Sanger Sequencing') 30 years ago and is, with light modifications, still used in molecular diagnostics (38).

The principle of Sanger sequencing relies on base-specific chain termination in four separate reactions ('A','G','C', and 'T'- reaction) during PCR. Each reaction mixture contains sample amplified DNA, primers, and DNA polymerase. In addition, each reaction contains 2'-deoxynucleotidetriphosphates (dATP, dGTP, dCTP, and dTTP) and, for the specific reactions, one of the four 2',3'-dideoxynucleotidetriphosphates (ddATP, ddGTP, ddCTP, and ddTTP), for example ddATP for the 'A' reaction, ddGTP for the 'G' reaction, etc. The extension of a newly synthesized DNA strand terminates as soon as the corresponding ddNTP is incorporated. All of the four ddNTPs are labeled with different fluorescence dyes. The fluorescence dyes are excited by a laser and detected during the electrophoretic separation. The DNA bands move according to their masses. The nucleotide sequence of the extension product can be detected with a computer program (38,39).

1.10. Why is determination of genotypes still important?

According to the 2015 EASL Recommendations of Treatment of Hepatitis C, anti-HCV treatment depends on the HCV genotype and the HCV subtypes 1a and 1b and must therefore be assessed prior to treatment initiation (20) (Fig. 9).

Patients	PegIFN-α, RBV and sofosbuvir	PegIFN-α, RBV and simeprevir	Sofosbuvir and RBV	Sofosbuvir and ledipasvir	Ritonavir-boosted paritaprevir, ombit-asvir and dasabuvir	Ritonavir-boosted paritaprevir, and ombitasvir	Sofosbuvir and simeprevir	Sofosbuvir and daclatasvir
Genotype 1a	12 wk	12 wk, then PegIFN-α and RBV 12 wk (treatment-naïve or relapsers) or 36 wk (partial or null responders)	No	8-12 wk, without RBV	12 wk with RBV	No	12 wk without RBV	12 wk without RBV
Genotype 1b					12 wk without RBV			
Genotype 2	12 wk	No	12 wk	No	No	No	No	12 wk without RBV
Genotype 3	12 wk	No	24 wk	No	No	No	No	12 wk without RBV
Genotype 4	12 wk	12 wk, then PegIFN-α and RBV 12 wk (treatment-naïve or relapsers) or 36 wk (partial or null responders)	No	12 wk without RBV	No	12 wk with RBV	12 wk without RBV	12 wk without RBV
Genotype 5 or 6	12 wk	No	No	12 wk without RBV	No	No	No	12 weeks without RBV

Fig. 9. Treatment recommendations for HCV-monoinfected or HCV/HIV coinfected patients with chronic hepatitis C without cirrhosis, including treatment-naïve patients and patients who failed on a treatment based on PegIFN-α and ribavirin (RBV).

The HCV genotype plays an important role in guiding the use of currently available treatment options as DAA's do not necessarily act pangenotypic (25). As shown in Fig. 8., there are drugs on the market with highly different efficacy against different HCV genotypes. Knowledge of the HCV genotype is necessary to determine the duration of treatment and to estimate the probability of treatment response (20).
Before introduction of DAA's, the standard anti-HCV treatment was based on a pegylated INFα/ribavirin combination. With this treatment, patients infected with HCV genotype 1 had a significantly lower response rate to anti-HCV treatment compared to patients infected with HCV genotype 2 and 3 (40). With the

introduction of DAA’s, the treatment response rates in patients infected with HCV genotype 1 increased significantly (25).

The new anti-HCV treatments demonstrate increased response rates in genotypes 1 and 2 but have limited activity against genotype 3. Besides being the most difficult to treat, genotype 3 shows the highest rate of steatosis among all HCV genotypes and an increased risk of hepatocellular carcinoma (41).

2. Objectives

The aim of this study was to evaluate the performance of the new cobas® HCV GT assay (Roche Molecular Systems, Pleasanton, CA, USA). Accuracy was tested using two panels, the Quality Control for Molecular Diagnostics (QCMD) 2014 and 2015 proficiency panels. For clinical evaluation of the new assay, 183 residual routine clinical samples were tested and the results were compared to those obtained by the VERSANT® HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics Inc., Tarrytown, NJ, USA) and the TRUGENE® HCV 5'NC Genotyping Kit (Siemens). All samples showing discrepant results were additionally investigated by home-brew NS5B sequencing. Additionally, times-to-result of the assays including hands-on times were compared.

3. Materials and Methods

3.1. Study design

3.1.1. Accuracy (QCMP Panels)

The accuracy of the cobas® HCV GT assay was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2014 and 2015 Hepatitis C Virus Genotyping EQA Program panels (www.qcmd.org). Both panels consisted of seven plasma samples with various genotypes of HCV and one sample negative for HCV RNA.

3.1.2. Clinical samples

A total of 183 consecutive residual clinical samples obtained from female and male patients with chronic HCV infection were included for evaluation of the new assay. Clinical samples had been analyzed in the routine diagnostic laboratory utilizing the TRUGENE® HCV 5´NC Genotyping Kit. The number of samples containing HCV genotypes 2, 3, and 4 as well as subtypes 1a and 1b was virtually equal (1a: n=27; 1b: n=38; 2: n=37; 3: n=47; 4: n=34). Furthermore, one sample containing HCV genotype 6 was included.

All clinical samples were tested with the Roche cobas® HCV GT, the VERSANT® HCV Genotype 2.0 Assay (LiPA), and the TRUGENE® HCV 5´NC Genotyping Kit. If discrepant results were obtained, samples were additionally investigated with home-brew NS5B sequencing.

3.2. Methods

Molecular assays used in this study are described in Table 1.

Table 1: Features of HCV genotyping assays used in this study.

	Manufacturer			
Characteristics	Roche Molecular Systems	Siemens Healthcare Diagnostics	Siemens Healthcare Diagnostics	Home-brew assay
Assay name	Roche cobas® HCV GT	VERSANT® HCV Genotype 2.0 Assay (LiPA)	TRUGENE® HCV 5´NC Genotyping Kit	Home-brew NS5B sequencing
Target sequence(s)	5´UTR + core + NS5B	5´UTR + core	5´UTR	NS5B
Detection method	RT-qPCR	Reverse hybridization	Sequencing	Sequencing

UTR, untranslated region; NS5B, non-structural 5 B region; RT-qPCR, reverse transcription real-time PCR.

3.2.1. The cobas® HCV GT assay

The cobas® HCV GT assay is based on RT-qPCR. This assay was performed according to the manufacturer's package insert. The cobas® HCV GT assay has been designed for the identification of HCV genotypes 1 to 6 and genotype 1 subtype 1a and 1b in human plasma or serum from individuals with chronic HCV infection. This HCV genotyping test is for use on the cobas® 4800 system (Roche) which consists of the cobas® x 480 instrument (Roche) and the cobas® z 480 analyzer. Automated specimen processing is performed on the the cobas® x 480 instrument (Fig. 10.). Specimen processing starts with the release of nucleic acids from patient samples and external controls by addition of proteinase and a chaotropic lysis reagent to the sample. The released nucleic acids bind to the silica surface of magnet glass particles. Unbound substances and impurities, such as denatured proteins, cellular debris, and potential PCR inhibitors are removed with subsequent washing steps. The purified nucleic acids are eluted from the magnetic glass particles with elution buffer at elevated temperature (42).

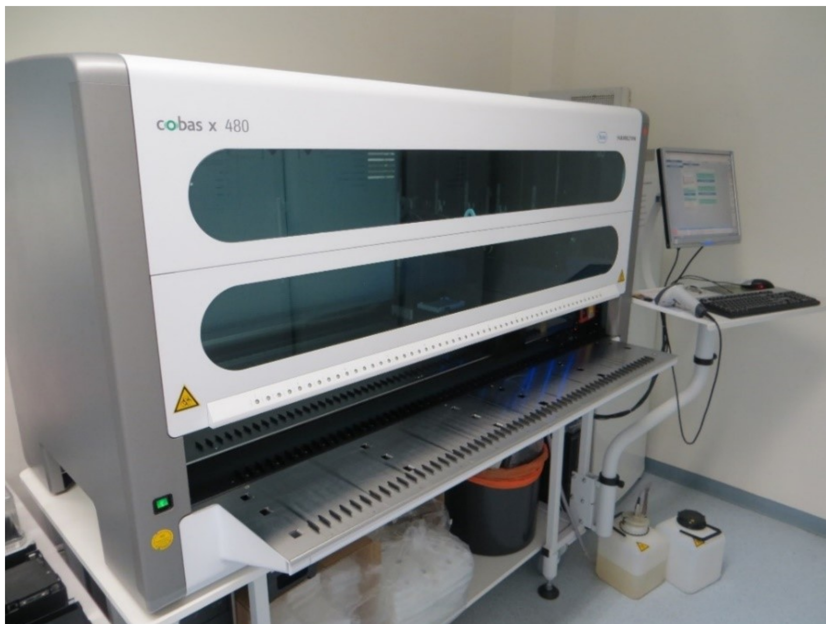


Fig. 10. The cobas® x 480 instrument.

After nucleic acid extraction, automated amplification and detection is performed on the cobas® z 480 analyzer (Fig. 11.). With the cobas® HCV GT assay, each sample is amplified in three real-time RT-PCR reactions using genotype and subtype specific primers and fluorescent dye-labeled oligonucleotide probes. The 5'UTR, core, and NS5B regions serve as targets. In addition, each reaction includes an internal control, which is amplified and detected using primers and probes for a highly conserved region of the HCV genome, regardless of the HCV genotype. The probes are labeled with four different fluorescent reporter dyes allowing simultaneous detection of HCV and up to three genotypes or subtypes in each reaction. After completion of each PCR cycle, the fluorescence of the released reporter dye is measured. Data are continuously calculated by the cobas® 4800 software (42).

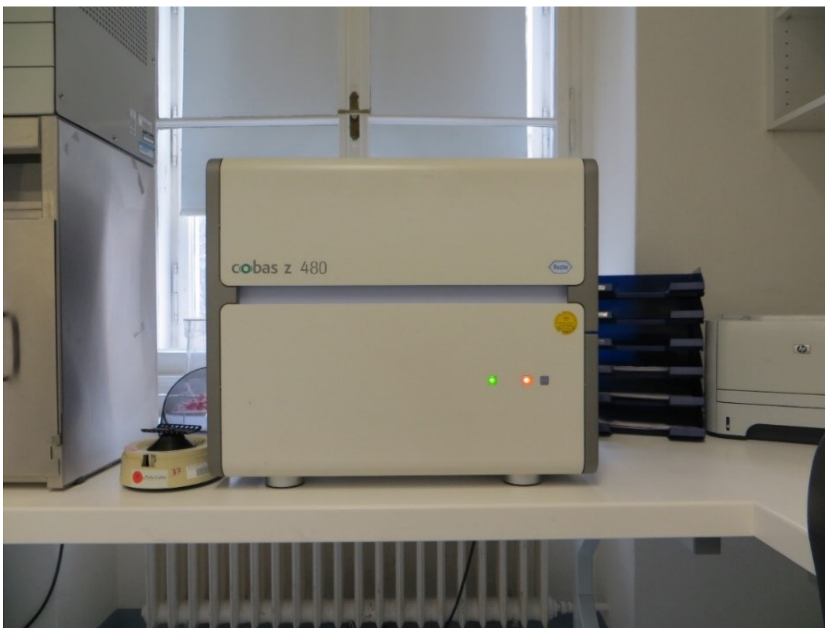


Fig. 11. The cobas® z 480 analyzer.

3.2.2. The VERSANT® HCV Genotype 2.0 Assay (LiPA)

The VERSANT® HCV Genotype 2.0 Assay (LiPA) is based on the reverse hybridization principle. This assay was performed according to the manufacturer's package insert. HCV RNA was extracted using the NucliSENS® easyMAG® instrument (bioMérieux, Marcy-l´Etoile, France) (Fig. 12.). The generic protocol with an input volume of 500 µl and an elution volume of 50 µl was employed.

Fig. 12. The NucliSENS® easyMAG® instrument.

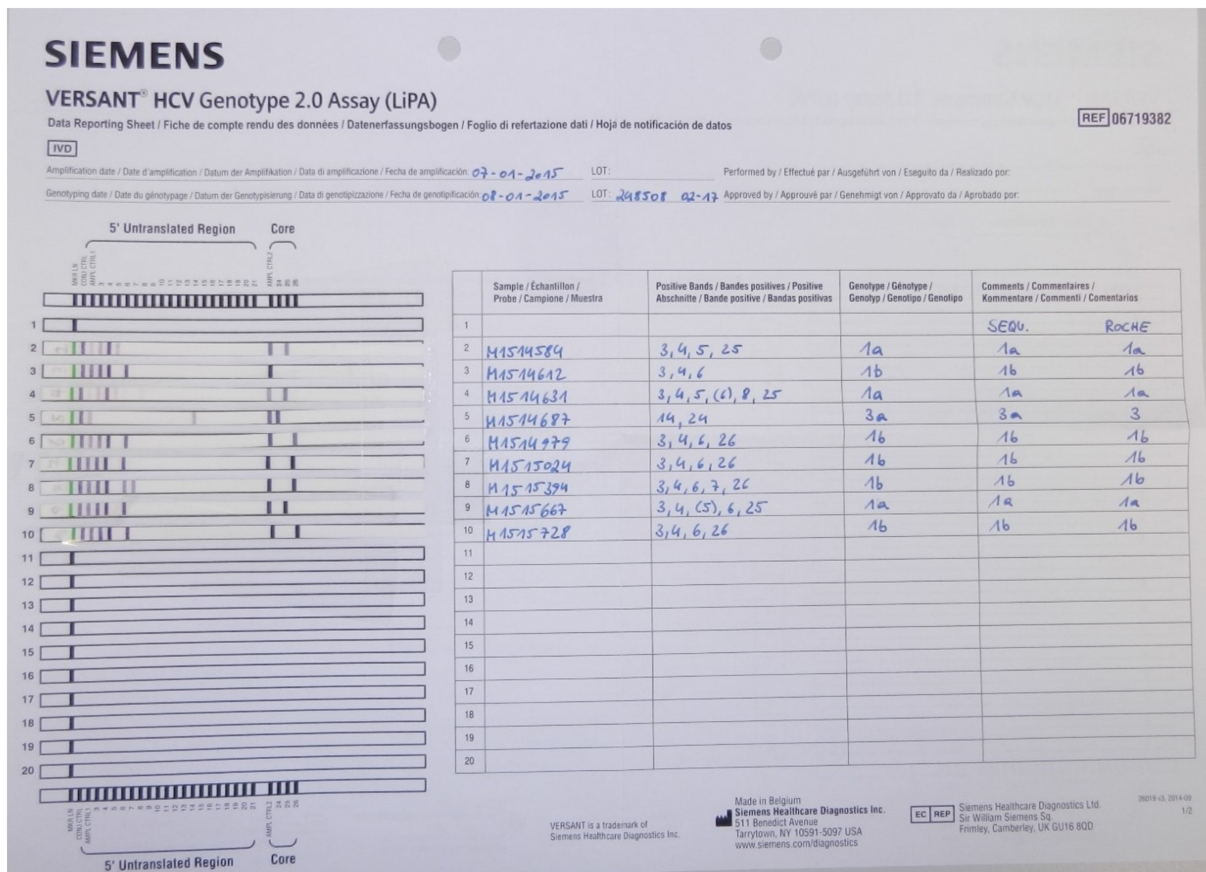
After extraction, HCV RNA was amplified using the VERSANT® HCV Amplification 2.0 Kit (Siemens) on the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. HCV genotypes were determined using the VERSANT® HCV Genotype 2.0 Assay (LiPA) on a fully automated strip processing instrument, the AutoBlot 3000 (MedTech Inc., Chapel Hill, NC, USA) (Fig. 13.).

Fig. 13. The AutoBlot 3000 processing instrument.

This test utilizes the principle of reverse hybridization described above (section 1.9.2., p.16). After several automated pipetting and washing steps, lines develop on the nitrocellulose strips (Fig. 14).

Fig. 14. Banding pattern developed on the nitrocellulose strips.

The VERSANT® HCV Genotype 2.0 Assay (LiPA) has been designed for identification of HCV genotypes 1 to 6 and subtypes 1a and 1b in human serum or EDTA plasma samples. In the majority of cases, additional subtype information is available. The probes on the nitrocellulose strip are specific for the 5'UTR and the core regions of different HCV genotypes. After completion of the assay, the banding pattern is visible on the strip (Fig. 15). HCV genotypes/subtypes are identified visually by the aid of a reading card and an interpretation chart, both included in the kit package. The reading card helps to determine the position of the strip. The result is then compared to an interpretation chart in order to determine the HCV genotype/subtype (43).



SIEMENS

VERSANT® HCV Genotype 2.0 Assay (LiPA)

Data Reporting Sheet / Fiche de compte rendu des données / Datenerfassungsbogen / Foglio di refertazione dati / Hoja de notificación de datos

REF 06719382

IVD

Amplification date / Date d'amplification / Datum der Amplifikation / Data di amplificazione / Fecha de amplificación: 07-01-2015 LOT: Performed by / Effectué par / Ausgeführt von / Eseguito da / Realizado por:

Genotyping date / Date du génotypage / Datum der Genotypisierung / Data di genotipizzazione / Fecha de genotipificación: 08-01-2015 LOT: 248508 02-17 Approved by / Approuvé par / Genehmigt von / Approvato da / Aprobado por:

5' Untranslated Region Core

	Sample / Échantillon / Probe / Campione / Muestra	Positive Bands / Bandes positives / Positive Abschnitte / Bande positive / Bandas positivas	Genotype / Génotype / Genotyp / Genotipo / Genotipo	Comments / Commentaires / Kommentare / Commenti / Comentarios	
1				SEQU.	ROCHE
2	M1514584	3, 4, 5, 25	1a	1a	1a
3	M1514612	3, 4, 6	1b	1b	1b
4	M1514631	3, 4, 5, (6), 8, 25	1a	1a	1a
5	M1514687	14, 24	3a	3a	3
6	M1514979	3, 4, 6, 26	1b	1b	1b
7	M1515024	3, 4, 6, 26	1b	1b	1b
8	M1515394	3, 4, 6, 7, 26	1b	1b	1b
9	M1515667	3, 4, (5), 6, 25	1a	1a	1a
10	M1515728	3, 4, 6, 26	1b	1b	1b
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

5' Untranslated Region Core

VERSANT is a trademark of Siemens Healthcare Diagnostics Inc.

Made in Belgium
Siemens Healthcare Diagnostics Inc.
511 Benedict Avenue
Tarrytown, NY 10591-5097 USA
www.siemens.com/diagnostics

EC REP Siemens Healthcare Diagnostics Ltd.
Sir William Siemens Sq.
Frimley, Camberley, UK GU16 8QD

26019 v3, 2014-09
1/2

Fig. 15. Interpretation of the banding pattern on the nitrocellulose strip.

3.2.3. The TRUGENE® HCV 5’NC Genotyping Kit

The TRUGENE® HCV 5’NC Genotyping Kit is based on detection of a specific region of the HCV genome by Sanger sequencing as described above (section 1.9.3., p.17). HCV RNA was extracted using the generic protocol of the NucliSENS® easyMAG® instrument (Fig. 12) with an input volume of 500 µl and an elution volume of 50 µl according to the manufacturer’s instructions. After extraction, HCV RNA was amplified with the VERSANT® HCV Amplification 2.0 Kit (Siemens) using the Gene Amp® PCR System 9700 (Applied Biosystems). Amplification products were generated from the 5’NC region of the HCV RNA genome. The TRUGENE® HCV 5´NC Genotyping Kit was employed and performed according to the manufacturer’s package insert. CLIP sequencing (Siemens) allows both directions of the target amplicon to be sequenced simultaneously in the same tube using two different dye-labeled primers (Cy5 and Cy5.5) for each reaction. The TRUGENE® HCV 5’NC Genotyping Kit uses amplification products from nucleotides 254 to 378 of the HCV genome. After preparation of the master mix, 4 µl of each purified amplification product sample was added to its respective master mix tube. Four wells filled with termination mix (A, C, G, T) were prepared for each sample. 5 µl of the CLIP Master Mix with the first sample amplification products were transferred to the first set of A,C,G, and T wells. This was repeated for the remaining samples. When all samples had been transferred, the CLIP cycling program was started. After completion of the reaction, samples were denatured. For preparation of electrophoresis, the acrylamide gel was polymerized in the Gel Toaster (Siemens) (Fig. 16) (44).

Fig. 16. The Gel Toaster.

CLIP sequencing segments were electrophoretically separated on a polymerized acrylamide gel using the Long Read Tower (Siemens) (Fig. 17).

Fig. 17. The Long Read Tower.

The separated CLIP sequencing segments were then detected using the OpenGene® DNA Sequencing System (Siemens) and the sample sequence were reviewed directly on the system screen (Fig. 18) and exported or printed as a report (Fig. 19).

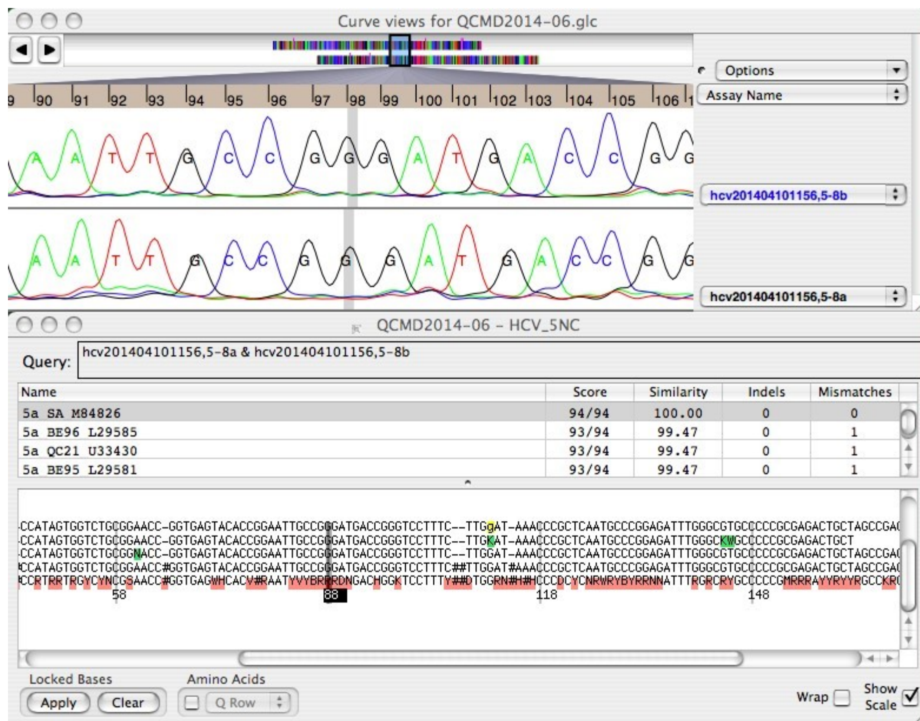


Fig. 18. Screen shot showing HCV RNA sequencing data.

The OpenGene® software system (Siemens) automatically compares the sample sequence to previously characterized isolates in order to determine the HCV genotype of the sample. The resulting genotype/subtype is then reported on the TRUGENE® HCV 5'NC Genotyping Report (Fig. 19).

TRUGENE® HCV 5'NC
Genotyping Report

Sample ID: HCV RV 2011-8
Report Date: 2011/04/13
Case Name: HCV RV 2011-8
Institution: Institute of Hygiene
Universitaetsplatz 4 8010 Graz
Labname : Molecular diagnostics laboratory

HCV 5'NC Genotype: 5a

SA_M84826

100 % homology; Score 66/66

The genotype of the Hepatitis C Virus in the analyzed serum is closest (or identical) to the following 10 isolates:

Subtype	Isolate	Accession	Homology	Score/66
5a	SA	M84826	100	66
5a	QC21	U33430	99.5	65
5a	BE95	L29581	99.5	65
5a	BE96	L29585	99.5	65
5a	FR22	U51767	98.9	64
4a	VGA02005		97.4	61
4a	VGA02006		97.4	61
4a	VGA02009		97.4	61
4a	VGA02017		97.4	61
1a	VGF02013		97.4	61

The genotype obtained using the TRUGENE HCV 5' NC Assay is determined by CLIP™ sequencing and analysis of a portion of the 5' non-coding region of the viral genome.
Two or more isolate sequences can be identical within the sequenced region. It is not always possible to determine the HCV subtype using the 5' non-coding region. In these cases, another region must be used to determine the HCV subtype. The homology is the percentage of identical bases within the sequenced region, between the sample and the isolate. The score indicates the number of identical bases at known polymorphic positions. There are 66 such positions in the library. Accession refers to the Genbank accession number.

Authorized reviewer: *Signature:*__________ *Date:*__________
*Name:*__________ *Title:*__________

5NC_091

HCV Genotype analysis by DNA sequencing.
For Research Use Only. Not for use in diagnostic procedures.
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GeneObjects(TM) 4.1 (2008/05/26) / HCV_5NC_312.gnl Wed Aug 28 2002 05:48 PM

Page 1 of

Fig. 19. The TRUGENE® HCV 5’NC Genotyping Report.

3.2.4. NS5B Sequencing

For home-brew NS5B sequencing, samples were sent to the Institute of Laboratory Medicine at the Kepler University Hospital (Medical Campus III, Linz, Austria). Home-brew sequencing was performed according to an assay published recently (45). Plasma samples (1000 µL) were extracted on the MagNA Pure LC 2.0 (Roche Diagnostics, Penzberg, Germany) using the MagNA Pure LC total NA Isolation Kit LV (Roche) according to the manufacturer's instructions. An elution volume of 50 µl was used. Reverse transcription (RT) was carried out from 3 µL sample eluate by using Superscript III enzyme (invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The primer (final concentration of 2 µM) for RT was 5´-CTCAGGCCTAT-TGGCCTGGAG-3`. Thereafter, PCR from 10 µL of the RT product was performed with GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, WI, USA) according to manufacturer's instructions with the following primers: HCV-PR3: 5´- TAT GA**Y** ACC CGC TG**Y** TTT GAC TC -3` and HCV-PR5 5´-GC**N** GA**R** TA**Y** CT**V** GTC ATA GCC TC -3` at 0.4 µM final concentration each. Cycling was initiated with 95°C for 2 min followed by 10 s at 95°C, 30 s at 58°C, and 30 s at 72°C, and a final extension at 72°C for 5 min.

Amplification products were purified with ExoSAP-IT® (affymetrix, Santa Clara, CA, USA) reagent and, then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Sequencing products were separated on a 3130 ABI sequencing unit (Applied Biosystems) according to manufacturer's instructions. Forward and reverse sequences were aligned and analyzed with the SeqScape v2.6 analysis software (Applied Biosystems). HCV genotypes were determined utilizing the online analysis tool geno2pheno (http://hcv.geno2pheno.org/index.php).

4. Results

Eight members of the Quality Control for Molecular Diagnostics (QCMD) HCV Genotyping Proficiency Program 2014 were tested with the cobas® HCV GT. The panel included 7 HCV RNA positive members and 1 HCV RNA negative member. Results are shown in Table 2. Five of the 7 HCV RNA positive members showed the expected HCV genotype and the HCV 1a or 1b subtype, respectively. Both of the 2 discrepant results occurred in members containing HCV genotype/subtype 5a. One of the 2 members containing genotype/subtype 5a was classified as genotype 4 and the other one tested indeterminate, indicating that HCV RNA was detected but no genotype and subtype could be identified. The HCV RNA negative member tested invalid, indicating that no HCV RNA could be detected in this member.

Table 2: Accuracy testing utilizing the Quality Control for Molecular Diagnostics HCV Genotyping Proficiency Program 2014.

Vial no.	HCV RNA concentration (IU/ml)	HCV subgenotype expected	HCV (sub)genotype obtained by the cobas® HCV GT
1	5.00E+03	4a	4
2	1.00E+04	1b	1; 1b
3	Negative	Negative	Invalid[a]
4	5.00E+03	5a	4
5	1.00E+04	1b	1; 1b
6	5.00E+03	5a	Indeterminate[b]
7	1.00+E04	3a	3
8	5.00E+03	1a	1; 1a

[a]HCV RNA not detected.
[b]HCV RNA detected but no genotype or subtype identified.

When 8 members of the QCMD HCV Genotyping Proficiency Program 2015 were tested, identical results as those obtained with members of the QCMD HCV Genotyping Proficiency Program 2014 (i.e., HCV genotype 5 samples could not be detected correctly) were found (Table 3).

Table 3: Accuracy testing utilizing the Quality Control for Molecular Diagnostics HCV Genotyping Proficiency Program 2015.

Vial no.	HCV RNA concentration (IU/ml)	HCV subgenotype expected	HCV (sub)genotype obtained by the cobas® HCV GT
1	> 1.00E+03	5a	4
2	> 1.00E+03	1b	1; 1b
3	> 1.00E+03	4a	4
4	Negative	Negative	Invalid[a]
5	> 1.00E+03	3a	3
6	> 1.00E+03	1a	1; 1a
7	> 1.00E+03	5a	Indeterminate[b]
8	> 1.00E+03	1b	1; 1b

[a]HCV RNA not detected.
[b]HCV RNA detected but no genotype or subtype identified.

When the clinical performance of the cobas® HCV GT was investigated, 183 clinical samples were tested and results were compared to those obtained by the TRUGENE® HCV 5’NC Genotyping Kit and the VERSANT® HCV Genotype 2.0 Assay. 160 samples (87.4%) showed concordant results. Seven samples (3.8%) showed indeterminate results with the cobas® HCV GT, indicating detection of HCV RNA without identification of the HCV genotype or subtype. Results for these 7 samples are shown in Table 4.

Table 4: Resolution of indeterminate results.[a]

Sample no.	**HCV (sub)genotype result obtained by**		
	Roche cobas® HCV GT	VERSANT® HCV Genotype 2.0 Assay (LiPA)	TRUGENE® HCV 5´NC Genotyping Kit
1	Indeterminate	1a	1a
2	Indeterminate	3a	3a
3	Indeterminate	Interpretation not possible	3k
4	Indeterminate	4	4a
5	Indeterminate	4a/c/d	4c
6	Indeterminate	4a/c/d	4c
7	Indeterminate	6	6

[a]HCV RNA detected but no genotype or subtype identified.

Sixteen samples (8.7%) showed discordant results with one or both of the comparator assays (Table 5). Seven samples were found to contain subtype 1a with the cobas® HCV GT, the VERSANT® HCV Genotype 2.0 Assay (LiPA), and home-brew NS5B sequencing but subtype 1b with the TRUGENE® HCV 5’NC Genotyping Kit. In one of these seven samples, interpretation with the VERSANT® HCV Genotype 2.0 Assay was not possible. One sample was found to contain subtype 1b with the cobas® HCV GT, the VERSANT® HCV Genotype 2.0 Assay (LiPA), and home-brew NS5B sequencing but subtype 1a with the TRUGENE® HCV 5’NC Genotyping Kit.

Two further discrepant samples were reported as HCV genotype 1 with the cobas® HCV GT without determination of subtypes. In four samples, the cobas® HCV GT reported an infection with HCV genotype 1 subtype 1b and HCV genotype 2, while the VERSANT® HCV Genotype 2.0 Assay (LiPA) reported HCV subtype 2a or 2c, the TRUGENE® HCV 5´NC Genotyping Kit HCV subtype 2a, and home-brew NS5B sequencing subtype 1b. Finally, in two samples, the cobas® HCV GT reported a double infection (HCV subtype 1a and HCV subtype 1b; HCV genotype 1 without determination of the subtype and HCV genotype 4).

Table 5: Resolution of discordant results.

Sample no.	HCV (sub)genotype result obtained by			
	Roche cobas® HCV GT	VERSANT® HCV Genotype 2.0 Assay (LiPA)	TRUGENE® HCV 5´NC Genotyping Kit	Home-brew NS5B sequencing
1	1; 1a	1a	1b	1a
2	1; 1a	1a	1b	1a
3	1; 1a	1a	1b	1a
4	1; 1a	1a	1b	1a
5	1; 1a	1a	1b	1a
6	1; 1a	1a	1b	1a
7	1; 1a	INP[a]	1b	1a
8	1; 1b	1b	1a	1b
9	1	1a	1a	1a
10	1	1b	1b	1b
11	1b; 2	2a/c	2a	1b
12	1b; 2	2a/c	2a	1b
13	1b; 2	2a/c	2a	1b
14	1b; 2	2a/c	2a	1b
15	1; 1a; 1b	1	1a	1a
16	1; 4	4e	4o	4o

[a]INP, interpretation not possible.

When overall times required for the completion of assays were compared, the cobas® HCV GT was found to be the fastest assay with a total time of 200 minutes required (Table 6). Hands-on times were found to be similar for all assays compared in this study.

Table 6: Comparison of times required for assays used in this study.

Times/sample required	**Assays**		
	Roche cobas® HCV GT	VERSANT® HCV Genotype 2.0 Assay (LiPA)	TRUGENE® HCV 5´NC Genotyping Kit
Hands-on time (min)	30	40	30
Total time (min)	200	405	255

5. Discussion

According to the latest version of the European Association for the Study of the Liver clinical practice guideline on treatment of hepatitis C, the HCV genotype and the HCV genotype 1 subtype (1a/1b) must still be assessed prior to treatment initiation and will determine the choice of therapy (20). An HCV genotyping/genotype 1 subtyping assay that determines HCV genotypes and discriminates HCV subtypes 1a and 1b accurately is still of paramount importance.

The cobas® HCV GT assay is based on primer-specific real-time PCR and has been designed for the identification of HCV genotypes 1 to 6 and HCV genotype 1 subtypes 1a and 1b in human plasma or serum from individuals with chronic HCV infection. This assay was evaluated in this study and results were compared to those obtained with the VERSANT® HCV Genotype 2.0 Assay (LiPA) and the TRUGENE® HCV 5'NC Genotyping Kit. Discrepant results were additionally investigated with a home-brew NS5B sequencing assay.

When members of the Quality Control for Molecular Diagnostics HCV Genotyping proficiency panels 2014 and 2015 were tested, all members except those with HCV genotype 5a were reported as expected. According to the manufacturer's package insert, there is a large difference in the limit of detection for HCV genotype 5 compared to all other HCV genotypes. The limit of detection for plasma samples of HCV genotype 1 subtype 1a, HCV genotype 2, HCV genotype 3, and HCV genotype 4 is 125 IU/ml, for HCV genotype 1 subtype 1b 250 IU/ml, and for HCV genotype 5 1000 IU/ml. HCV RNA concentrations of members included in the QCMD Proficiency Panels and containing HCV genotype 5 were found to be very close to this limit. In clinical routine diagnostics, low HCV RNA concentrations are rarely found and it thus might be better to include samples with higher HCV concentrations in future panels. Further investigations regarding HCV genotype 5 could not be performed because samples from patients with HCV genotype 5 were not available as the prevalence of this genotype is very low in Europe (35).

When 183 clinical samples were tested with the cobas® HCV GT, 160 samples (87.4%) showed corresponding results with the comparator assays. In 7 samples (3.8%), indeterminate results were found with the cobas® HCV GT indicating detection of HCV RNA without identification of the HCV genotype or subtype. In this assay, genotype and subtype specific primers and fluorescent dye-labeled oligonucleotide probes target the 5'UTR, core, and NS5B region. In addition, an internal control is included using primers and probes for a highly conserved region of the HCV genome. The indeterminate results of the cobas® HCV GT assay may be caused by oligonucleotide mismatches in the target regions. Due to the internal control, HCV RNA can be detected but the HCV genotype/subtype cannot be identified.

In 16 samples (8.7%), discordant results were obtained by at least one comparator assay. In seven samples, the cobas® HCV GT reported subtype 1a but was found to contain subtype 1b with the TRUGENE® HCV 5'NC Genotyping Kit. Contrary, in one sample the cobas® HCV GT reported subtype 1b and the TRUGENE® HCV 5'NC Genotyping Kit reported subtype 1a. In these 8 samples, the home-brew NS5B sequencing assay could confirm the result obtained by the cobas® HCV GT.

Two of the 16 samples with discordant results were found to contain genotype 1 with the cobas® HCV GT but the assay could not determine the subtype. This may be caused by oligonucleotide mismatches in the target region.

In four samples, the cobas® HCV GT reported the presence of 2 different HCV genotypes, HCV genotype 1 subtype 1b and HCV genotype 2. The target regions of the cobas® HCV GT are 5'UTR, core, and NS5B. In contrast, the VERSANT® HCV Genotype 2.0 Assay (LiPA) targets the 5' UTR and the core region and reported HCV genotype 2 either subtype 2a or 2c. The TRUGENE® HCV 5'NC targets only the 5'UTR region and reported HCV genotype 2 subtype 2a. Additional results obtained with the home-brew NS5B sequencing, targeting the NS5B region, indicated HCV genotype 1 subtype 1b. The reason for these discordant results might be the presence of a recombinant HCV strain. It is known that recombination plays a significant role in the evolution of RNA viruses by creating genetic variations (46). Regarding HCV, intergenotypic, intragenotypic

and intra quasispecies recombination have been reported in populations in different geographic locations (47). The first recombinant strain of HCV, a intergenotypic recombinant of HCV subtypes 2k and 1b, was reported in St. Petersburg in 2002 (48). Since then, the same recombinant strain has been reported in other countries, such as Ireland, Usbekistan, Cyprus, France, and Estonia. Additionally, at least ten other different intergenotypic recombinant forms of HCV have been described. All HCV genotypes except HCV genotype 4 and 7 have been found within these recombinant strains. HCV genotype 2 is present in the majority of the intergenotypic recombinants found until today, which might suggest a critical role in the process of recombination and for the stability and functionality of the resulting recombinant genome (47).

In one sample, the cobas® HCV GT detected a double infection with HCV genotype 1 subtypes 1a and 1b. With the comparator assays, only a single infection could be detected. The VERSANT® HCV Genotype 2.0 Assay (LiPA) detected HCV genotype 1 only and no HCV subtype could be determined. Both, the TRUGENE® HCV 5'NC Genotyping Kit and the home-brew NS5B sequencing assay detected HCV genotype 1 subtype 1a only. In another sample, the cobas® HCV GT reported the presence of HCV genotype 1 and HCV genotype 4. The two comparator assays and the home-brew NS5B sequencing assay could only detect HCV genotype 4. These discordant results may be explained by a second HCV strain present in the patient sample but with a lower viral concentration.

When the times-to-result per single sample were compared, the cobas® HCV GT was found to be the fastest assay with a total time of 200 minutes required in comparison to the VERSANT® HCV Genotype 2.0 Assay (LiPA) with 405 minutes, and the TRUGENE® HCV 5'NC Genotyping Kit with 255 minutes. Hands-on times were found to be similar for all three assays: 30 minutes with the cobas® HCV GT and the TRUGENE® HCV 5'NC Genotyping Kit and 40 minutes with the VERSANT® HCV Genotype 2.0 Assay (LiPA).

In conclusion, the new cobas® HCV GT assay showed a good performance. It proved to be suitable for use in the routine diagnostic laboratory. The new assay allows the accurate determination of the HCV genotype and it is able to

discriminate HCV genotype 1 subtype 1a and 1b. With this assay, reliable results are obtained quickly without the need for especially trained laboratory staff.

6. Annex

Results obtained from this study were presented at the ISMD2016 - Eleventh International Symposium on Molecular Diagnostics, Graz, May 26–28, 2016 (Appel HM, Stelzl E, Berg J, Paar C, Zurl H, Santner BI, Kessler HH: Evaluation of the new cobas® HCV Genotyping Test based on real-time PCRs of three different HCV genome regions).

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