

Evelyn Stelzl, Hannah M. Appel, Rochak Mehta, Ed G. Marins, Jörg Berg, Christian Paar, Hanna Zurl, Brigitte I. Santner and Harald H. Kessler*

Evaluation of the new cobas® HCV genotyping test based on real-time PCRs of three different HCV genome regions

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Abstract

Background: Determination of the hepatitis C virus (HCV) genotype and discrimination between HCV subtypes 1a and 1b is still mandatory prior to anti-HCV treatment initiation. The aim of this study was to evaluate the performance of the recently introduced cobas® HCV GT assay (Roche) and to compare it to two comparator assays.

Methods: The cobas® HCV GT assay is based on primer-specific real-time polymerase chain reaction (PCR). For comparison, the TRUGENE® HCV 5'NC Genotyping Kit (Siemens) and the VERSANT® HCV Genotype 2.0 Assay (Siemens) were employed. Accuracy of the new assay was determined using proficiency panels. For clinical evaluation, 183 residual clinical samples obtained from patients with chronic hepatitis C infection were included.

Results: When accuracy was tested, panel members containing HCV subtypes 1a, 1b, and 3a were identified as expected; however, the new assay failed to identify low titer panel members containing HCV subtype 5a correctly. Of 183 clinical samples, 160 gave concordant results. For seven samples, an indeterminate result was reported with the cobas® HCV GT assay and the remaining 16 samples were found discordant with one of the comparator assays. When time-to-results of the assays were compared, the

new assay showed shorter total time and similar hands-on time per sample.

Conclusions: The cobas® HCV GT assay showed a good performance and proved to be suitable for use in the routine diagnostic laboratory. Due to the high level of automation, fast and reliable results are obtained with short hands-on time.

Keywords: cobas® HCV GT; genotype; hepatitis C virus (HCV); real-time PCR; subtype.

Introduction

Testing for the hepatitis C virus (HCV) genotype has been important in understanding HCV classification, epidemiology, evolution, transmission clustering, treatment response and natural history [1]. According to the latest version of the European Association for the Study of the Liver (EASL) guideline, the HCV genotype/subtype should be determined with an assay that accurately discriminates HCV subtype 1a from 1b [2].

For determination of the HCV genotype/subtype, different molecular techniques may be used including population sequencing, reverse hybridization, and the newly-developed primer-specific real-time polymerase chain reaction (PCR) [3, 4]. The new cobas® HCV GT assay (Roche Molecular Systems, Pleasanton, CA, USA) based on primer-specific real-time PCR (RT-PCR) was brought on the market recently. This assay is based on nucleic acid amplification for identification of HCV genotypes 1 to 6 and subtypes 1a and 1b using the cobas® 4800 system. This system consists of two instruments: the cobas x 480 instrument which is used for automated specimen processing followed by automated amplification and detection on the cobas z 480 analyzer. The assay includes genotype and subtype specific primers for amplification of parts of the 5'UTR, Core, and NS5B regions of the HCV genome and fluorescent dye-labeled oligonucleotide probes for differentiation of

*Corresponding author: Harald H. Kessler, Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Universitätsplatz 4, 8010 Graz, Austria, Phone: +43 316 380 4380, Fax: +43 316 380 9656, E-mail: harald.kessler@medunigraz.at

Evelyn Stelzl, Hannah M. Appel, Hanna Zurl and Brigitte I. Santner: Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz, Austria

Rochak Mehta and Ed G. Marins: Roche Molecular Diagnostics, Pleasanton, CA, USA

Jörg Berg and Christian Paar: Institute of Laboratory Medicine, Kepler Universitätsklinikum, Medical Campus III, Linz, Austria

HCV geno- and subtypes. Additionally, an internal control monitors the sample preparation and RT-PCR process.

The aim of this study was to evaluate the performance of the new cobas[®] HCV GT assay and to compare results with those obtained by the VERSANT[®] HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) and the TRUGENE[®] HCV 5'NC Genotyping Kit (Siemens).

Materials and methods

Molecular assays

Characteristics of HCV genotyping assays used in this study are shown in Table 1. Briefly, the cobas[®] HCV GT (Roche) was performed according to the manufacturer's package insert instructions. This HCV genotyping test is for use on the cobas[®] 4800 system. After automated specimen processing, each sample is amplified in three RT-PCR reactions using genotype and subtype specific primers and fluorescent dye-labeled oligonucleotide probes. 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. The cobas[®] HCV GT assay has been in vitro diagnostics (IVD)/Conformité Européenne (CE)-labeled but not FDA approved.

The TRUGENE[®] HCV 5'NC Genotyping Kit (Siemens) and the VERSANT[®] HCV Genotype 2.0 Assay (Siemens) were performed according to the manufacturer's package insert instructions. For both of the assays, HCV RNA was extracted using the generic protocol of the NucliSENS[®] easyMAG[®] instrument (bioMérieux, Marcy-l'Etoile, France) with an input volume of 500 µL and an elution volume of 50 µL.

For resolution of discrepant results, sequencing of the HCV NS5B region was done as described earlier [5]. 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 GAY ACC CGC TGY

TTT GAC TC -3' and HCV-PR5 5'-GCN GAR TAY CTV 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>).

Study design

The accuracy of the cobas[®] HCV GT 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 containing different genotypes of HCV with HCV RNA concentrations exceeding 1000 IU/mL and one sample negative for HCV RNA.

For evaluation of the clinical performance, 183 consecutive residual serum samples obtained from patients with chronic HCV infection were included. 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 subtype 1a and 1b was virtually equal. Furthermore, one sample containing HCV genotype 6 was included. All results obtained with the cobas[®] HCV GT were compared with those obtained by the comparator assays. If a sample revealed a discordance regarding genotype or subtype 1a/1b, this sample was investigated additionally by home-brew NS5B sequencing.

Results

When eight members of the QCMD 2014 Hepatitis C Virus Genotyping EQA program were tested with the cobas[®] HCV GT, five of seven members positive for HCV RNA gave genotype and subtype results as expected (Table 2). Of two members containing HCV subtype 5a, one was classified as HCV genotype 4 and the other one tested indeterminate with the cobas[®] HCV GT. The HCV RNA negative member

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

Characteristics	Manufacturer			
	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 region(s)	5'UTR + core + NS5B	5'UTR + core	5'UTR	NS5B
Detection method	Real-time PCR	Line probe assay	Sequencing	Sequencing

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

^aHCV RNA not detected. ^bHCV RNA detected but no genotype or subtype identified.

showed an invalid result indicating that no HCV RNA was detectable. The same results were found when samples from the QCMD 2015 Hepatitis C Virus Genotyping EQA program were analyzed (Table 3).

When 183 clinical samples were tested with the cobas® HCV GT and results obtained were compared with those obtained with the TRUGENE® HCV 5'NC Genotyping Kit and the VERSANT® HCV Genotype 2.0 Assay, 160 samples revealed concordant results. Seven of 183 (3.8%) samples showed indeterminate results with the cobas® HCV GT indicating detection of HCV RNA without identification of the HCV genotype or subtype. Corresponding results obtained with comparator assays are shown in Table 4. Viral loads of these samples ranged from 1.5E+04 IU/mL to 1.4E+06 IU/mL. Of 183 samples, 16 (8.7%) showed discordant results with at least one comparator assay (Table 5). Seven samples were found to contain subtype 1a with the cobas® HCV GT but revealed

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

^aHCV RNA not detected. ^bHCV RNA detected but no genotype or subtype identified.

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

^aHCV RNA detected but no genotype or subtype identified.

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	Homebrew 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; 4	4e	4o	4o
16	1; 1a; 1b	1	1a	1a

^aINP, interpretation not possible.

subtype 1b with one of the comparison assays. Vice versa, one sample was found to contain subtype 1b with the cobas® HCV GT but revealed genotyped 1a with one of the comparison assays. Two samples revealed genotype 1 with the cobas® HCV GT but no subtype could be determined. In five samples, the cobas® HCV GT reported two different genotypes (1 and 2 resp. 1 and 4). For one sample, the cobas® HCV GT reported subtypes 1a and 1b. Additional results obtained by NS5B sequencing are shown in Table 5.

When a single sample was tested with the cobas® HCV GT, the overall time required was 200 min per sample

Table 6: Calculation of times per single sample required for assays used in this study.

Times per 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

(Table 6). The TRUGENE® HCV 5'NC Genotyping Kit could be performed within 255 min. The total time required for the VERSANT® HCV Genotype 2.0 Assay was 405 min. Hands-on work was found to be similar for all of the assays.

Discussion

Determination of the HCV genotype is part of pre-therapeutic assessment and recommended with a genotyping assay that determines genotypes and discriminates subtype 1a and 1b accurately. The cobas® HCV GT is based on primer-specific real-time PCR for identification of HCV genotypes 1 to 6 and subtypes 1a and 1b. In this study, the cobas® HCV GT was evaluated and compared with the VERSANT® HCV Genotype 2.0 Assay (LiPA) and the TRUGENE® HCV 5'NC Genotyping Kit.

When members of the QCMD 2014 and 2015 Hepatitis C Virus Genotyping EQA program were tested, all members except those with HCV genotype 5a were reported as expected. According to the package insert instructions of the cobas® HCV GT, the limit of detection for genotype 5 samples is 1000 IU/mL for plasma samples. For all other genotypes, the limit of detection is 250 IU/mL or even lower. Viral concentrations of the members included in the proficiency panels were found to be very close to this limit. As low viral concentrations are rarely found in the clinical routine, it might be better to include samples with higher HCV concentrations in the panel. Further investigations regarding HCV genotype 5 could not be performed because samples from patients with HCV genotype 5 were not available. In Europe, the prevalence of HCV genotype 5 is extremely low; HCV genotype 1 is predominant followed by HCV genotypes 2 and 3 [6].

When clinical samples were tested with the cobas® HCV GT, 160/183 (87.4%) showed corresponding results

with the comparator assays based on genotypes and subtypes 1a and 1b. Indeterminate results with the cobas® HCV GT were found in 7/183 (3.8%) of samples indicating detection of HCV RNA without identification of the HCV genotype or subtype that may be caused by oligonucleotide mismatches in the target region. The percentage observed in the present study was found to be similar to that reported with another commercially available assay based on real-time PCR [7].

Of the remaining 16 samples, seven samples were found to contain subtype 1a with the cobas® HCV GT but revealed subtype 1b with one of the comparator assays. Additionally, one sample was found to contain subtype 1b with the cobas® HCV GT but revealed genotyped 1a with one of the comparator assays. For these eight samples, results reported by NS5B sequencing confirmed results reported by the cobas® HCV GT. Two samples revealed genotype 1 with the cobas® HCV GT but the assay failed to determine the subtype probably due to oligonucleotide mismatches in the target region. In four samples, the cobas® HCV GT reported genotypes 1 and 2. When analyzing these samples with the comparator assays and NS5B sequencing, different results were found. HCV genotype 2 was reported with both the VERSANT® HCV Genotype 2.0 Assay (LiPA) using parts of the 5'UTR and core regions and the TRUGENE® HCV 5'NC Genotyping Kit using the 5'UTR only. In contrast, NS5B sequencing reported genotype 1. Based on these results, the presence of a recombinant HCV strain in these patients may be a possible explanation. Several reports about HCV recombinants have been published from 2002 onwards [8, 9]. For one sample, the cobas® HCV GT reported a mixed infection with HCV subtypes 1a and 1b. Another sample was found to contain HCV genotypes 1 and 4. When comparing these results with those of the comparator assays, one subtype or genotype each could be found with the same genotype being reported by NS5B sequencing. These discordant results may be explained by a second HCV strain present in the patient but with a lower viral concentration. For bulk sequencing, it has been known that strains with a proportion below 20% cannot be detected. Next-generation sequencing (NGS) may be a superior alternative; however, NGS protocols are currently not appropriate for the routine diagnostic laboratory because they lack standardization. Furthermore, they are time-consuming, labor- and cost-intensive.

Comparison of times required for the cobas® HCV GT with those required for the VERSANT® HCV Genotype 2.0 Assay (LiPA) and the TRUGENE® HCV 5'NC Genotyping Kit revealed the shortest total time for the cobas® HCV

GT. However, hands-on time was similar for all of the assays.

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 correct determination of the HCV genotypes 1 to 4 and is able to discriminate HCV subtype 1a from 1b. Due to the high level of automation, fast and reliable results are obtained with short hands-on time. While the comparator assays need subjective interpretation of results, the new assay provides automated interpretation and connection to the laboratory information system.

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References

1. Simmonds P. Genetic diversity and evolution of hepatitis C virus—15 years on. *J Gen Virol* 2004;85:3173–88.
2. European Association for the Study of the Liver. EASL recommendations on treatment of hepatitis C 2015. *J Hepatol* 2015;63:199–236.
3. Stelzl E, van der Meer C, Gouw R, Beld M, Grahovac M, Kessler HH, et al. Determination of the hepatitis C virus subtype: comparison of sequencing and reverse hybridization assays. *Clin Chem Lab Med* 2007;45:167–70.
4. Hoffmann D, Michler T, Protzer U. Hepatitis viruses. In: Kessler HH, editor. *Molecular diagnostics of infectious diseases*, 3rd ed. Berlin, Boston: De Gruyter, 2014:113–23.
5. Morice Y, Roulot D, Grando V, Stirnemann J, Gault E, Jeantils V, et al. Phylogenetic analyses confirm the high prevalence of hepatitis C virus (HCV) type 4 in the Seine-Saint-Denis district (France) and indicate seven different HCV-4 subtypes linked to two different epidemiological patterns. *J Gen Virol* 2001;82:1001–12.
6. Manns MP, von Hahn T. Novel therapies for hepatitis C – one pill fits all? *Nat Rev Drug Discov* 2013;12:595–610.
7. McCormick AL, Macartney MJ, Abdi-Abshir I, Labbett W, Smith C, Irish D, et al. Evaluation of sequencing of HCV core/E1, NS5A and NS5B as a genotype predictive tool in comparison with commercial assays targeting 5'UTR. *J Clin Virol* 2015;66:56–9.
8. Gonzalez-Candelas F, Lopez-Labrador FX, Bracho MA. Recombination in hepatitis C virus. *Viruses* 2011;3:2006–24.
9. Kalinina O, Norder H, Mukomolov S, Magnus LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002;76:4034–43.