Use of molecular tools to optimize treatment for HCV

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EASL Recommendations on Treatment of Hepatitis C 2016*

European Association for the Study of the Liver*

Recommendations for Testing, Managing, and Treating Hepatitis C

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HCV screening: What to test?

Anti-HCV antibodies are the first line diagnostic test for HCV infection (A1).

An anti-HCV test is recommended for HCV testing ... (I,A).
Early phase of HCV infection

Transfusion settings

High-risk groups
(IVDU, hemodialysis patients, HIV positive individuals, organ donors)

Timely initiation of treatment → high SVR rate
In the case of suspected acute hepatitis C or in immunocompromised patients, HCV RNA testing should be part of the initial evaluation (A1).

Among persons with a negative anti-HCV test who are suspected of having liver disease, testing for HCV RNA or follow-up testing for HCV antibody is recommended if exposure to HCV occurred within the past six months; testing for HCV RNA can also be considered in persons who are immunocompromised (I, C).

Among persons at risk of reinfection after previous spontaneous or treatment-related viral clearance, initial HCV-RNA testing is recommended because an anti-HCV test is expected to be positive (I,C).
Short communication

Twenty-four mini-pool HCV RNA screening outside a blood transfusion setting: Results of a 2-year prospective study

Katja Seme a, Tina Močilnik a, Kristina Fujis a, Dunja Z. Babič a, Aleksandra Todorović b, Tamara Fras-Stefani b, Mario Poljak a *


Short communication

Twenty-four mini-pool HCV RNA screening in a routine clinical virology laboratory setting: A six-year prospective study

Katja Seme, Tina Močilnik, Mario Poljak *

Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia
24 mini-pool HCV RNA screening
routine diagnostic laboratory setting

58,299 anti-HCV negative specimens (2,881 mini-pools) tested by HCV RNA 24 mini-pool screening strategy between 1 June 2004 and 31 May 2016 (12 years)

61 (1:959) anti-HCV negative/HCV RNA positive samples detected

43 anti-HCV negative/HCV RNA positive patients detected
24 mini-pool HCV RNA screening
routine diagnostic laboratory setting

43 anti-HCV negative/HCV RNA positive patients

Immediately after the recognition of an anti-HCV negative/HCV RNA positive patient, the responsible physician was contacted, informed of results and asked for follow-up samples

37 patients responded to the invitation for follow-up testing
1 patient was traced coincidentally
5 patients were lost to follow-up
Hepatitis C?

anti-HCV + HCV RNA
HCV screening: What to test?

How to proceed after anti-HCV positive result?

If anti-HCV antibodies are detected, HCV RNA should be determined by a sensitive molecular method (A1).

An anti-HCV test is recommended for HCV testing, and if the result is positive, current infection should be confirmed by a HCV RNA test (I,A).
No RIBA necessary anymore!

A positive HCV antibody screening test should be followed by reflex RNA testing!
HCV screening: What to test?

HCV core antigen

HCV core antigen is a surrogate marker of HCV replication and can be used instead of HCV RNA to diagnose acute or chronic infection when HCV RNA assays are not available or not affordable (core antigen assays are slightly less sensitive than HCV RNA assays for detection of viral replication) (A1).
HCV core Ag assays

ARCHITECT HCV Ag assay (Abbott Diagnostics)

Ortho HCV cAg enzyme immunoassay (Ortho Clinical Diagnostic)

HCV Ag ELISA (Hunan Jynda Bioengineering Group)

Lumipulse Ortho HCV Ag (Fujirebio)

Lumispot HCV Ag assay (Eiken)
Hepatitis C Core Antigen Testing for Diagnosis of Hepatitis C Virus Infection: A Systematic Review and Meta-analysis

J. Morgan Freiman, MD; Trang M. Tran, BA; Samuel G. Schumacher, MSc, PhD; Laura F. White, PhD; Stefano Ongarello, PhD; Jennifer Cohn, MD, MPH; Philippa J. Easterbrook, MD, MPH; Benjamin P. Linas, MD, MPH; and Claudia M. Denkinger, MD, PhD


Data Synthesis: 44 studies evaluated 5 index tests. Studies for the Abbott ARCHITECT HCV Ag assay had the highest quality, whereas those for the Ortho HCV Ag enzyme-linked immunosorbent assay (ELISA) had the lowest quality. From bivariate analyses, the sensitivity and specificity of the assays were as follows: Abbott ARCHITECT, 93.4% (95% CI, 90.1% to 96.4%) and 98.8% (CI, 97.4% to 99.5%); Ortho ELISA, 93.2% (CI, 81.5% to 97.7%) and 99.2% (CI, 87.9% to 100%); and Hunan Jynda Bioengineering Group HCV Ag ELISA, 59.9% (CI, 46.0% to 71.7%) and 82.9% (CI, 58.6% to 94.3%). Insufficient data were available for a meta-analysis about the Fujirebio Lumipulse Ortho HCV Ag and Eiken Lumispot HCV Ag assays. In 3 quantitative studies using Abbott ARCHITECT, HCVcAg correlated closely with HCV RNA levels greater than 3000 IU/mL.

Limitations: Insufficient data were available on covariates, such as HIV or hepatitis B virus status, for subgroup analyses. Few studies reported genotypes of isolates, and data for genotypes 4, 5, and 6 were scant. Most studies were conducted in high-resource settings and reference laboratories.

Conclusion: The HCVcAg assays with signal amplification have high sensitivity, high specificity, and good correlation with HCV RNA levels greater than 3000 IU/mL and have the potential to replace NAT in settings with high HCV prevalence.
significant reduction of the number of tests performed... potential cost savings ...

Evolving strategy for HCV testing in an Italian tertiary care hospital

Maria Cristina Medici, Carlo Chezzi, Flora De Conto, Francesca Ferraglia, Federica Pinardi, Maria Cristina Arcangeletti, Daniela Bernasconi, Claudio Galli, Adriana Calderaro

Unit of Microbiology and Virology, Department of Clinical and Experimental Medicine, University of Parma, 43126 Parma, Italy
Abbott Diagnostics Division, 00144 Roma, Italy

Journal of Clinical Virology 2016;77:92-98

Hepatitis C Core Antigen Testing: A Reliable, Quick, and Potentially Cost-effective Alternative to Hepatitis C Polymerase Chain Reaction in Diagnosing Acute Hepatitis C Virus Infection

Fiona V. Cresswell, Martin Fisher, Daniel J. Hughes, Simon G. Shaw, Gary Homer, and Mohammed O. Hassan-Ibrahim

Clinical Infectious Diseases 2015;60:263-266
HCV proteins expressed in all six HCV genotypes

simultaneously detects HCV-Ags through a standard EIA platform

HCV-Ags EIA detects all four HCV proteins in serum specimens

lowest limit of detection equivalent to serum HCV RNA levels of 150-250 IU/mL
HCV Ab/Ag combo assays

Monolisa HCV Antigen-Antibody Ultra (Bio-Rad Laboratories)

Murex HCV Antigen/Antibody Combination Test (DiaSorin)
HCV screening: With what to test?

Rapid tests?

Rapid diagnostic tests using serum, plasma, fingerstick whole blood or crevicular fluid (saliva) as matrices can be used instead of classical enzyme immunoassays to facilitate anti-HCV antibody screening and improve access to care (A1).

Among FDA-approved, Commercially Available Anti-HCV Screening Assays is OraQuick HCV Rapid Antibody Test (OraSure Technologies, Inc, Bethlehem, PA, USA).
Rapid anti-HCV tests

ASSURE HCV Rapid Test (MP Diagnostics)
Bioeasy HCV Rapid Test (BioeasyDiagnóstica)
First Response HCV Card Test (Premier Medical Corporation)
GENEDIA HCV Rapid (Green Cross)
HCV TRI-DOT (J. Mitra)
ImmuNoComb II HCV Kit (Alere)
Imuno-Rápido HCV Kit (WAMA Diagnóstica)
Multiplo Rapid HBV/HIV/HCV antibody test (MedMira)
MultiSure HCV Antibody Assay (MP Diagnostics)
OraQuick HCV Rapid Antibody Test (OraSure Technologies)
Rapidtest (Cortez Diagnostics)
SD Bioline HCV (Standard Diagnostics)
Signal HCV Kit (Span Diagnostics)
30 different tests evaluated

substantial heterogeneity between studies

performances varied widely among individual point-of-care tests

overall pooled sensitivity 97.4% (95% CI: 95.9-98.4)

overall pooled specificity 99.5% (99.2-99.7)

OraQuick had the highest test sensitivity and specificity and showed better performance than a third generation enzyme immunoassay in seroconversion panels
HCV pre-treatment assessment
HCV pre-treatment assessment

HCV RNA load?

HCV RNA detection and quantification should be made by a sensitive assay with a lower limit of detection of ≤15 IU/ml (A1).

Quantitative HCV-RNA testing is recommended prior to the initiation of antiviral therapy to document the baseline level of viremia (ie, baseline viral load) (I,A).
Applicability of Hepatitis C Virus RNA Viral Load Thresholds for 8-Week Treatments in Patients With Chronic Hepatitis C Virus Genotype 1 Infection

Johannes Vermehren,1,a Benjamin Maasoumy,2,a Raoul Maan,3,4 Gavin Cloherty,5 Caterina Berkhovski,1 Jordan J. Feld,2 Markus Cornberg,2 Jean-Michel Pawlotsky,6,7 Stefan Zeuzem,1 Michael P. Manns,2 Christoph Sarrazin,6,8 and Henner Wedemeyer2,a

1Medizinische Klinik 1, Universitätsspital Zürich, Switzerland; 2Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany; 3Toronto Centre for Liver Disease, Sandra Rotman Centre for Global Health, University of Toronto, Toronto, Canada; 4Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, The Netherlands; 5Abbott Diagnostics, Abbott Park, Illinois; 6National Reference Center for Viral Hepatitis B, C and D, Hôpital Henri Mondor, Université Paris-Est, and 7INSERM U955, Créteil, France

Background. Interferon-free treatment of chronic hepatitis C virus (HCV) genotype 1 infection may be shortened to 8 weeks in treatment-naive, noncirrhotic patients with baseline HCV RNA levels of <4 or <6 million (M) IU/mL based on post-hoc analyses of phase 3 trial data. The applicability of these viral load thresholds in clinical practice is unknown.

Methods. Pretreatment and on-treatment serum samples (n = 740) from patients with HCV genotype 1 infection were included for HCV RNA analysis with 2 widely used assays, Cobas AmpliPrep/Cobas TaqMan (CAP/CTM) and Abbott RealTime HCV (ART) assays.

Results. HCV RNA levels were significantly higher with CAP/CTM than with ART (overall difference, +0.11 log10 IU/mL; P < .001). In treatment-naive, noncirrhotic patients, discordance rates around the clinical cutoffs at 4M and 6M IU/mL were 23% and 18%, respectively. The mean differences between assays in discordant samples were 0.38 (4M) and 0.41 (6M) log10 IU/mL, respectively. Overall, 87% and 95% of treatment-naive, noncirrhotic patients, respectively, had baseline HCV RNA levels below 4M and 6M IU/mL with ART. These rates were significantly higher than those measured with CAP/CTM (64% and 78%, respectively; P < .001). Finally, discordance rates around the proposed thresholds in 2 consecutive samples of the same patient were in the range of 1%-2% for ART and 13%-17% for CAP/CTM.

Conclusions. Selection of patients for 8-week regimens on the basis of a single HCV RNA determination may not be reliable because viral load levels around the proposed clinical thresholds show significant interassay and intrapatient variability.

Keywords. hepatitis C virus; HCV RNA assay; baseline viral load; Cobas AmpliPrep/Cobas TaqMan; Abbott RealTime HCV.
Rates of patients suitable for 8 weeks of direct-acting antiviral (DAA) combination therapy based on baseline viral load determination.
HCV pre-treatment assessment

HCV core antigen concentration?

If HCV RNA testing is not available or not affordable, HCV core antigen detection and quantification by EIA can be used as a surrogate marker of HCV replication (A1).
HCV pre-treatment assessment

HCV genotype?

The HCV genotype and genotype 1 subtype (1a or 1b) must be assessed prior to treatment initiation and will determine the choice of therapy, among other parameters (A1).

Testing for HCV genotype is recommended to guide selection of the most appropriate antiviral regimen (I,A).

The following laboratory testing is recommended at any time prior to starting antiviral therapy:
- HCV genotype and subtype
- Quantitative HCV RNA (HCV viral load) (I,C).
HCV genotyping problems

- incorrect HCV genotyping and subtyping baseline
- mixed infections baseline
- mixed infections due reinfection
- reinfection/relaps
- recombinant forms/chimeras
Incorrect HCV genotyping and subtyping

<table>
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<tr>
<th>Commercial assay result</th>
<th>NS5B DNA Sequencing</th>
<th>Trugene [n (%)]</th>
<th>VERSANT [n (%)]</th>
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<tr>
<td>1b</td>
<td>1a</td>
<td>13 (10%)</td>
<td>5 (5%)</td>
</tr>
<tr>
<td>1a</td>
<td>1b</td>
<td>5 (4%)</td>
<td>-</td>
</tr>
<tr>
<td>1b</td>
<td>3a</td>
<td>1 (0.7%)</td>
<td>-</td>
</tr>
<tr>
<td>3a</td>
<td>1b</td>
<td>1 (0.7%)</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>4d</td>
<td>-</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>4c</td>
<td>1b</td>
<td>1 (0.7%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td>21 (16%)</td>
<td>6 (6%)</td>
</tr>
</tbody>
</table>

Short Communication

The need for a sequencing-based assay to supplement the Abbott m2000 RealTime HCV Genotype II assay: A 1 year analysis

Marlin Benedet a, Dena Adachi a, Anita Wong b, Sallene Wong b, Kanti Pabbaraju b, Raymond Tellier b,c, Julian W. Tang a,d,*

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b Alberta Provincial Laboratory for Public Health, Calgary, Alberta, Canada
c Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada
d Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada

ABSTRACT

Background: Hepatitis C (HCV) genotyping is important for treatment planning. The Abbott m2000 Real-Time HCV Genotype II assay is a PCR-based assay targeting specific regions of the 5’ NCR gene for genotypes 1–6, and the NS5b gene for subgenotypes 1a/1b. However, not all genotypes can be resolved, with results being reported as: ‘indeterminate’, ‘mixed’, ‘genotype X reactivity with Y’, or just the major genotype 1 alone.

Objectives and study design: To assess the supplementary testing required for these unresolved HCV genotypes, these samples were tested further using an in-house core/E1 sequencing assay. The resulting genotypes/subgenotypes were assigned using phylogenetic analysis with reference HCV genotype sequences. Additional testing was conducted using the INNO-LiPA HCV II assay for truly mixed genotypes.

Results: Out of 1052 samples tested, 89 (8.5%) underwent further sequencing to determine the HCV genotype: 16 that were ‘indeterminate’ on the m2000, were mostly genotype 2s and 3s by sequencing; 12 that were ‘mixed’, were mostly one of the genotypes reported in the mixture; 7 that were ‘X reactivity with Y’, were usually genotype X; 54 that gave just a major genotype 1 result were mostly 1a, with some 6 and 1b, and a few 1c. For three truly mixed genotypes, additional testing using the VERSANT® HCV Genotype Assay (LiPA) 2.0, showed two mixed 1 and 3, and one indistinguishable 6c–6l genotypes.

Conclusions: The Abbott m2000 RealTime HCV Genotype II assay can resolve most (∼90%) HCV genotypes. However, in 9–10% of cases, to fully resolve the genotype, additional testing is required.
Mixed infections baseline

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Direct NS5B sequencing</th>
<th>Versant HCV Genotype 2.0</th>
<th>Abbott Real time HCV genotype II.</th>
<th>UDP'S HCV Subtyping 454/GS-Junior*</th>
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<tr>
<td>P77</td>
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<td>1b</td>
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<td>1b 43% / 3a 35% / 1a 20%</td>
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<tr>
<td>P-IND-29</td>
<td>4d</td>
<td>Ind</td>
<td>4</td>
<td>4d (72%) + 1a (28%)</td>
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<tr>
<td>P-IND-30</td>
<td>2j</td>
<td>2</td>
<td>ND</td>
<td>2j (95%) + 4f (5%)</td>
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<tr>
<td>P-IND-31</td>
<td>4p</td>
<td>Ind</td>
<td>3 + 4</td>
<td>4p (53%) + 3a (47%)</td>
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<tr>
<td>P-IND-32</td>
<td>4d</td>
<td>Ind</td>
<td>1a + 4</td>
<td>4d (87%) + 1a (13%)</td>
</tr>
</tbody>
</table>

43 studies of HCV mono-infected “low-risk” patients (n = 7969)
- 5-year recurrence risk of 0.95% (95% CI, .35%-1.69%)

14 studies of HCV monoinfected “high-risk” patients (n = 771)
- 5-year recurrence risk of 10.67% (95% CI, 6.38%-15.66%)

4 studies of HIV/HCV coinfected patients
- 5-year recurrence risk of 15.02% (95% CI, .00%-48.26%)
Origin and Evolution of the Unique Hepatitis C Virus Circulating Recombinant Form 2k/1b

Jayna Raghwani,a Xiomara V. Thomas,b Sylvie M. Koekkoek,b Janke Schinkel,b Richard Molenkamp,b Thijs J. van de Laar,c Yutaka Takebe,a Yasuhiro Tanaka,a Masashi Mizokami,a Andrew Rambaut,e and Oliver G. Pybusb

Institute of Evolutionary Biology, University of Edinburgh, Ashworth Laboratories, Edinburgh, United Kingdom; Academic Medical Center, Department of Medical Microbiology, Section of Clinical Virology, Amsterdam, The Netherlands; VU University Medical Centre, Department of Medical Microbiology and Infection Control, Amsterdam, The Netherlands; AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan; Fogarty International Center, National Institutes of Health, Bethesda, Maryland, USA; and Department of Zoology, University of Oxford, Oxford, United Kingdom

Since its initial identification in St. Petersburg, Russia, the recombinant hepatitis C virus (HCV) 2k/1b strain has been isolated from several countries throughout Eurasia. The 2k/1b strain is the only recombinant HCV to have spread widely, raising questions about the epidemiological background in which it first appeared. In order to further understand the circumstances by which HCV recombinants might be formed and spread, we estimated the date of the recombination event that generated the 2k/1b strain using a Bayesian phylogenetic approach. Our study incorporates newly isolated 2k/1b strains from Amsterdam, The Netherlands, and has employed a hierarchical Bayesian framework to combine information from different genomic regions. We estimate that 2k/1b originated sometime between 1923 and 1956, substantially before the first detection of the strain in 1999. The timescale and the geographic spread of 2k/1b suggest that it originated in the former Soviet Union at about the time that the world’s first centralized national blood transfusion and storage service was being established. We also reconstructed the epidemic history of 2k/1b using coalescent theory-based methods, matching patterns previously reported for other epidemic HCV subtypes. This study demonstrates the practicality of jointly estimating dates of recombination from flanking regions of the breakpoint and further illustrates that rare genetic-exchange events can be particularly informative about the underlying epidemiological processes.
43 studies of HCV mono-infected "low-risk" patients (n = 7969)
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4 studies of HIV/HCV coinfected patients
- 5-year recurrence risk of 15.02% (95% CI, 0.00%–48.26%)

HCV recombinant forms/chimeras

Susser S, EASL 2016
HCV pre-treatment assessment

IL-28?
Hepatitis C Pharmacogenetics: State of the Art in 2010

Nezam H. Afdhal,1 John G. McHutchison,2 Stefan Zeuzem,3 Alessandra Mangia,4 Jean-Michel Pawlotsky,6 Jeffrey S. Murray,7 Kevin V. Shianna,3 Yasuhiro Tanaka,8 David L. Thomas,9 David R. Booth,10 and David B. Goldstein,5 for the Pharmacogenetics and Hepatitis C Meeting Participants

In 2009, a correlated set of polymorphisms in the region of the interleukin-28B (*IL28B*) gene were associated with clearance of genotype 1 hepatitis C virus (HCV) in patients treated with pegylated interferon-alfa and ribavirin. The same polymorphisms were subsequently associated with spontaneous clearance of HCV in untreated patients. The link between *IL28B* genotype and HCV clearance may impact decisions regarding initiation of current therapy, the design and interpretation of clinical studies, the economics of treatment, and the process of regulatory approval for new anti-HCV therapeutic agents. (Hepatology 2011;53:336-345)

- spontaneous clearance of HCV in untreated patients
- response to standard-of-care treatment
Relative predictivity of patient and viral characteristics for sustained virological response

- CC IL28B genotype vs. non-CC: P < 0.0001
- HCV RNA < 600,000 vs. > 600,000IU/mL: P < 0.0001
- Caucasian vs. AA ethnicity: P < 0.0001
- Hispanic vs. AA ethnicity: P = 0.0041
- Metavir F0-2 vs. F3-4: P < 0.0001
- Fasting blood glucose < 5.6 vs. ≥ 5.6 mmol/L: P < 0.0001

Lai M and Afdhal NH, Hepatology 2012; 56: 367-372
HCV pre-treatment assessment

HCV resistance?
HCV pre-treatment assessment

HCV resistance?

REVIEW IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

Hepatitis C Virus Resistance to Direct-Acting Antiviral Drugs in Interferon-Free Regimens

Jean-Michel Pawlotsky¹,²

¹National Reference Center for Viral Hepatitis B, C and D, Department of Virology, Hôpital Henri Mondor, Université Paris-Est, Créteil, and ²INSERM Unité 955, Créteil, France
HCV pre-treatment assessment

HCV resistance?

HCV ≠ HIV
HCV ≠ HBV

"resistance-associated variants" (RAVs)

"resistance associated substitutions" (RASs)

viral variants that carry RASs must be called "resistant variants"
HCV pre-treatment assessment
HCV resistance - methodological and general issues

- choice of resistant variants detection method
- differences in the genetic barrier of the drug
- differences in RAS persistence/clearance time
- HCV genotype/subtype
- special patient populations (cirrhosis.....)
HCV pre-treatment assessment

HCV resistance - methodological and general issues

• choice of resistant variants detection method
• differences in the genetic barrier of the drug
• differences in RAS persistence/clearance time
• HCV genotype/subtype
• special patient populations (cirrhosis.....)
Intermediate viral populations, detected by population sequencing (if >5% of the quasispecies) or by cloning and sequencing

Major viral populations, detected by population sequencing

Minor viral populations, detected by next-generation sequencing techniques
HCV pre-treatment assessment
HCV resistance - methodological and general issues

- choice of resistant variants detection method
- differences in the genetic barrier of the drug
- differences in RAS persistence/clearance time
- HCV genotype/subtype
- special patient populations (cirrhosis.....)
HCV pre-treatment assessment

HCV resistance - methodological and general issues

- choice of resistant variants detection method
- differences in the genetic barrier of the drug
- differences in RAS persistence/clearance time
- HCV genotype/subtype
- special patient populations (cirrhosis.....)
Estimated clearance time for resistant variants

Soriano V et al. AIDS Rev 2016; 18: 81-8
HCV pre-treatment assessment

HCV resistance - methodological and general issues

• choice of resistant variants detection method
• differences in the genetic barrier of the drug
• differences in RAS persistence/clearance time
• HCV genotype/subtype
• special patient populations (cirrhosis.....)
## HCV genotype/subtype matters

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<td>L31M/V</td>
<td>Y93H/N</td>
<td>L31V</td>
<td>Y93H/N</td>
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<td>&gt;100x</td>
<td>&gt;100x</td>
<td>&gt;1,000x</td>
<td>&gt;1,000x</td>
<td>-1</td>
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<td>Ombitasvir</td>
<td>&gt;1,000x</td>
<td>&gt;100x</td>
<td>&lt;3x</td>
<td>&gt;10,000x</td>
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<td>&gt;50x</td>
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<td>Daclatasvir</td>
<td>&gt;100x</td>
<td>&gt;1,000x</td>
<td>&gt;100x</td>
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<td>&gt;10,000x</td>
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</table>

EC₅₀ fold-change compared to WT replicon

Cheng et al., EASL 2012; Wong et al., AAC 2013; Krishnan et al., AAC 2015; Fridell et al., Hepatology 2011; Liu et al., AAC 2015; Cheng et al., EASL 2013; Patel et al., EASL 2015; Ng et al., CROI 2014; Asante-Appiah et al., AASLD 2014
HCV pre-treatment assessment

HCV resistance - methodological and general issues

- choice of resistant variants detection method
- differences in the genetic barrier of the drug
- differences in RAS persistence/clearance time
- HCV genotype/subtype
- special patient populations (cirrhosis.....)
Impact of Treatment Exp, Q80K Depends on Cirrhosis (12 Wks’ SMV + SOF in GT1)

No Cirrhosis (OPTIMIST-1\textsuperscript{[1]})

- All pts: 150/155 (97%)
- Naive: 112/115 (97%)
- Exp’d: 44/46 (95%)
- 1a + Q80K: 68/70 (96%)
- 1a no Q80K: 83/103 (83%)

Cirrhosis (OPTIMIST-2\textsuperscript{[2]})

- All pts: 86/103 (83%)
- Naive: 44/50 (88%)
- Exp’d: 42/53 (79%)
- 1a + Q80K: 25/34 (74%)
- 1a no Q80K: 35/38 (92%)

HCV pre-treatment assessment

HCV resistance?

Systematic testing for HCV resistance prior to treatment is not recommended.

Indeed, this obligation would seriously limit access to care and treatment regimens can be optimized without this information (B1).

The presence of baseline RAVs in treatment-naïve persons does not preclude achieving an SVR with a combination direct-acting antiviral regimen.

Furthermore, RAVs are often not detectable with routine (population sequencing) detection methods, nor with more sensitive tests of HCV variants, after patients are followed up for several months.
HCV pre-treatment assessment

HCV resistance ... in NS3?

1. ...for those patients whose prior treatment regimen containing an NS5A inhibitor failed and who have cirrhosis or require urgent retreatment, testing for RAVs that confer decreased susceptibility to NS3 protease inhibitors (e.g. Q80K) and to NS5A inhibitors should be performed using commercially available assays.

2. ...for treatment-naïve patients or those experienced with PEG-IFN/ribavirin who have HCV genotype 1a infection and cirrhosis, testing for the Q80K NS3 RAV is recommended when simeprevir and sofosbuvir are being considered as treatment.
Physicians who have easy access to a reliable test assessing HCV resistance to **NS5A inhibitors** (spanning amino acids 24 to 93) can use these results to guide their decisions, as specified in these recommendations. The test should be based on population sequencing (reporting RASs as “present” or “absent”) or deep sequencing with a cut-off of 15% (only RASs that are present in more than 15% of the sequences generated must be considered) (B1).

---

1. ...those treatment-naïve or PEGIFN/ribavirin-experienced persons with genotype 1a HCV who are being treated with elbasvir/grazoprevir, the presence of baseline NS5A RAVs significantly reduces rates of SVR 12.

2. ...those patients whose prior treatment regimen containing an NS5A inhibitor failed and who have cirrhosis or require urgent retreatment, testing for RAVs that confer decreased susceptibility to NS3 protease inhibitors (eg, Q80K) and to NS5A inhibitors should be performed using commercially available assays.

3. ...in persons with genotype 3 HCV who are considering treatment with sofosbuvir/velpatasvir or daclatasvir/sofosbuvir-based regimens...only recommended for treatment approaches for treatment-naïve patients with cirrhosis or treatment-experienced patients without cirrhosis.
HCV pre-treatment assessment

HCV resistance ... in NS5A?

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HCV pre-treatment assessment
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HCV pre-treatment assessment

HCV resistance ... in NS5B?
**NS3/4A HCV resistance - commercially available assays?**

### HCV GenoSure NS3/4A Drug Resistance Assay

**HCV GenoSure NS3/4A Drug Resistance Assay**

- Analyzes the genetic sequence for the nonstructural proteins NS3 and NS4A of HCV genotypes 1a and 1b.
- **Identifies the Q80K polymorphism**
- Detects mutations in NS3 and NS4A and identifies drug-resistant variants for protease inhibitors grazoprevir, paritaprevir, and sofosbuvir.
- Sensitivity to detect minor variant levels as low as 10% provides a detailed understanding of the patient's viral population for informed treatment decisions.

**Download a sample report**

<table>
<thead>
<tr>
<th>Test Name</th>
<th>HCV GenoSure NS3/N4</th>
</tr>
</thead>
<tbody>
<tr>
<td>LabCorp Test Number</td>
<td>330940</td>
</tr>
<tr>
<td>Limitation</td>
<td>For patients with HCV genotype (subtype) 1a or 1b and a viral load ≥ 2000 IU/mL</td>
</tr>
<tr>
<td>Specimen Collection</td>
<td>3 mL plasma, EDTA or PPT tube, shipped frozen</td>
</tr>
<tr>
<td>Turnaround Time</td>
<td>7 to 10 days</td>
</tr>
</tbody>
</table>

Pharmaceutical companies are using our HCV genotypic and phenotypic resistance assays to support all phases of research and clinical development for several new HCV compounds. As new compounds reach the clinic, Monogram's assays will continue to provide useful information for patient management. For more information about our full line of HIV, HCV, or pharmaceutical services, visit our Pharma Collaborations page.
NS5 HCV resistance - commercially available assays?

The NS5A Drug Resistance Assay is a genotypic (sequencing) resistance assay that analyzes the nonstructural NS5A region of hepatitis C virus (HCV) genotypes 1a or 1b to using next-generation sequencing (NGS) techniques. Amino acid substitutions in the NS5A region are identified, and a viral susceptibility call for the direct-acting agents (DAAs) that inhibit the NS5A proteins is reported as either resistance possible, or "none/undetermined". Monogram offers NS5A testing for both HCV genotype 1 and HCV genotype 3 viruses.

Features of HCV NS5A Drug Resistance Assay

- Assessment of HCV susceptibility for currently available NS5A DAAs, including daclatasvir, elbasvir, ledipasvir and ommitasvir for HCV genotype 1 viruses.
- Assessment of daclatasvir susceptibility for HCV genotype 3 patients.
- Identification of amino acid variants within the NS5A protein of HCV genotypes 1a or 1b, or genotype 3
- Detection of mixtures of wild-type and drug-resistant variants when present at levels as low as 10% of the total population.
**Sentosa® SQ HCV Genotyping Assay**

The Sentosa® SQ HCV Genotyping Assay is a Next-Generation Sequencing (NGS) solution for genotyping and resistance associated variant (RAV) calling for Hepatitis C Virus (HCV) clinical samples. The workflow enables automated sample extraction, library & template preparation, sequencing, automated data analysis and reporting. This allows to rapidly detect and differentiate HCV genotypes 1 to 6 (including subtypes 1a and 1b).

**Sample Processing Time**
- Automated HCV genotyping in 2.5 days (less than 2h hands-on time)
- Combines genotyping with RAV calling in one single run
- Detect and differentiate HCV genotypes 1 to 6 (including subtypes 1a and 1b)
- Seamless sample ID traceability and IT connectivity with Sentosa® Link
- Automatic data analysis and report generation with Sentosa® SQ Reporter
- UDG to eliminate carry-over contamination

**HCV Genotypes and Subtypes**

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>No. of Amplicons</th>
<th>Target Genotypes and Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS5B</td>
<td>1</td>
<td>1a, 1b, 2, 3, 4, 5, 6</td>
</tr>
<tr>
<td>NS3</td>
<td>1</td>
<td>1a, 1b</td>
</tr>
<tr>
<td>NS5A</td>
<td>1</td>
<td>1a, 1b</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>3</strong></td>
<td></td>
</tr>
</tbody>
</table>

RAV calling for subtypes 1a and 1b only

**Sentosa® SQ HCV Genotyping Assay Specifications**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Input</td>
<td>530 μL serum, plasma</td>
</tr>
<tr>
<td>Sample Throughput</td>
<td>15 samples/ run</td>
</tr>
<tr>
<td>Controls</td>
<td>1 system control, 1 extraction control</td>
</tr>
<tr>
<td>Coverage / Target</td>
<td>≥ 200 X for genotyping; ≥ 500 X for variant calling</td>
</tr>
<tr>
<td>Analytical Sensitivity</td>
<td>≥ 1,000 HCV IU/mL for genotypes 1a, 1b, 2, 3 and 4</td>
</tr>
<tr>
<td></td>
<td>≥ 2,000 HCV IU/mL for genotypes 5 and 6</td>
</tr>
<tr>
<td>Analytical Specificity</td>
<td>No cross-reactivity with HAV, HBV, HIV, CMV, EBV, BKV, Dengue virus or genomic DNA</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>99.2% (95% CI: 97.20% – 99.79%)</td>
</tr>
<tr>
<td>Turn-Around Time (TAT)</td>
<td>~ 2 days (from sample to result)</td>
</tr>
</tbody>
</table>

* Data based on feasibility study
Assays

Standardized Genotyping Kit Generating Amplifiers suited for Sanger and Next Generation Sequencing (NGS) platforms.

Key Benefits:
- Easy-to-use and automated workflow with pre-loaded microwell plates
- Designed for Sanger & Next Generation Sequencing
- High-throughput (ISO 13485) Assay for 8 to 24 samples
- GMP-manufactured
- Validated and validated protocols for Sanger and NGS genotyping
- Compatible with DeepChek and ViroScore® Sanger Suite Software
- Suitable for nucleic acids
  - HIV (HIV-1, HVT, V3)
  - HCV (HCV-1, 2b, 3a, W)
  - Other (HEV, IEV, CMV, HPV)

DeepChek®

DeepChek® Combined with NGS Platforms Aids in the Generation of Meaningful Genotyping Reports for Personalized Healthcare.

Key Benefits:
- Compatible with most NGS platforms
- Extended portfolio of applications (HIV -> CE-IVD marked, HBV, HCV, CMV, HPV, ...)
- Many types of analyses (subtyping, variant calling, drug resistance assessment, ...)
- Reporting directly suited for clinical use and research activities
- Well-validated and reliable platform

Please, send me details about this product

Less
Not all RASs are equal

<table>
<thead>
<tr>
<th>RAS</th>
<th>GT 1a M28T</th>
<th>GT 1a Q30R</th>
<th>GT 1a L31M/V</th>
<th>GT 1a Y93H/N</th>
<th>GT 1b L31V</th>
<th>GT 1b Y93H/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ledipasvir</td>
<td>&gt;20x</td>
<td>&gt;100x</td>
<td>&gt;100x</td>
<td>&gt;1,000x</td>
<td></td>
<td>&gt;1,000x/-</td>
</tr>
<tr>
<td>Ombitasvir</td>
<td>&gt;1,000x</td>
<td>&gt;100x</td>
<td>&lt;3x</td>
<td>&gt;10,000x</td>
<td>&lt;10x</td>
<td>&gt;50x</td>
</tr>
<tr>
<td>Daclatasvir</td>
<td>&gt;100x</td>
<td>&gt;1,000x</td>
<td>&gt;100x</td>
<td>&gt;1,000x</td>
<td>&gt;20x</td>
<td>&gt;20x</td>
</tr>
<tr>
<td>Elbasvir</td>
<td>&gt;10x</td>
<td>&gt;10x</td>
<td>&gt;10x</td>
<td>&gt;100x</td>
<td>&lt;10x</td>
<td>&gt;10x/-</td>
</tr>
<tr>
<td>Velpatasvir</td>
<td>&lt;10x</td>
<td>&lt;3x</td>
<td>&gt;20x</td>
<td>&gt;100x</td>
<td>&lt;3x/-</td>
<td>&lt;3x</td>
</tr>
<tr>
<td>Odalasvir</td>
<td>&gt;20x</td>
<td>&lt;10x</td>
<td>&lt;3x</td>
<td>&gt;1,000x</td>
<td>&lt;3x</td>
<td>&lt;3x</td>
</tr>
<tr>
<td>ABT-530</td>
<td>&lt;3x</td>
<td>&lt;3x</td>
<td>&lt;3x</td>
<td>&lt;10x</td>
<td>&lt;3x</td>
<td>&lt;10x</td>
</tr>
<tr>
<td>MK-8408</td>
<td>&lt;10x</td>
<td>&lt;10x</td>
<td>&lt;10x</td>
<td>&lt;10x</td>
<td>&lt;10x</td>
<td>&lt;10x</td>
</tr>
</tbody>
</table>

EC₅₀ fold-change compared to WT replicon

Cheng et al., EASL 2012; Wong et al., AAC 2013; Krishnan et al., AAC 2015; Fridell et al., Hepatology 2011; Liu et al., AAC 2015; Cheng et al., EASL 2013; Patel et al., EASL 2015; Ng et al., CROI 2014; Asante-Appiah et al., AASLD 2014
NS5A cross-resistance

**Clinically relevant RASs** which when detected at baseline by means of either population sequencing or deep sequencing with a cut-off of 15%, may influence the choice of first-line treatment regimen.

<table>
<thead>
<tr>
<th>NS5A amino acid position</th>
<th>Ledipasvir RASs (Genotype 1a)</th>
<th>Elbasvir RASs (Genotype 1a)</th>
<th>NS5A RASs (Genotype 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q30</td>
<td>Q30E, Q30G, Q30H, Q30K, Q30R</td>
<td>Q30B, Q30E, Q30G, Q30H, Q30K, Q30L, Q30R</td>
<td></td>
</tr>
<tr>
<td>L31</td>
<td>L31M, L31V</td>
<td>L31F, L31M, L31V</td>
<td></td>
</tr>
<tr>
<td>P32</td>
<td>P32L, P32S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H58</td>
<td>H58D</td>
<td>H58D</td>
<td></td>
</tr>
<tr>
<td>Y93</td>
<td>Y93C, Y93H, Y93N, Y93S</td>
<td>Y93C, Y93H, Y93N, Y93S</td>
<td></td>
</tr>
</tbody>
</table>

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EASL Guidelines 2016
Physicians who have easy access to a reliable test assessing HCV resistance to NS5A inhibitors...the test should be based on population sequencing (reporting RASs as “present” or “absent”) or deep sequencing with a cut-off of 15% (only RASs that are present in more than 15% of the sequences generated must be considered) (B1).
Due to high genetic diversity of HCV, Sanger based sequencing requires amplification of target genes (NS3, NS5A or NS5B) using primers that are genotype and subtype specific.
HCV Sanger based sequencing

Genotypes: 1a, 1b, 3a
Region: NS3, NS5A, NS5B

→ $3 \times 2 \times 3 = 18$ PCR primers

Low viral load + high genetic diversity = nested PCR
→ $18 \times 2 = 36$ primers

Long NS5B PCR amplicons → seq primers
→ $36 + 2 \times 3 = 42$ primers

→ endless optimization
detecting all RASs present in three DAA target genome regions (NS3, NS5A, and NS5B) requires sequencing of one-third of the HCV genome.

why not to sequence the entire HCV genome?

whole-genome sequence (WGS)

population sequencing or NGS
HCV whole genome sequencing using NGS

- preamplification of large overlapping fragments with specific primers

- preamplification in single long-range PCR with specific primers

- generation of HCV replicon transcripts coupled with single-primer isothermal amplification

- direct sequencing of RNA extracts (for samples with a high HCV RNA viral load only)
three high-throughput NGS methods compared:
• an unselected HCV RNA metagenomic approach
• preenrichment of HCV RNA by probe capture
• HCV preamplification by PCR

⇒ each NGS method generated near-complete HCV genome sequences from more than 90% of samples

⇒ all NGS methodologies accurately identified mixed-HCV genotype infections

⇒ the method of preenrichment of HCV RNA by probe capture has great potential to be used routinely in clinical practice
NGS provides a rapid method for generating the whole HCV genome to accurately and simultaneously determine HCV genotypes/subtypes, RASs, and quasispecies diversity and to allow comprehensive viral strain analysis.
Next-Generation Sequencing: a Diagnostic One-Stop Shop for Hepatitis C?

Mario Poljak
Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Before starting chronic hepatitis C treatment, the viral genotype/subtype has to be accurately determined and potentially coupled with drug resistance testing. Due to the high genetic variability of the hepatitis C virus, this can be a demanding task that can potentially be streamlined by viral whole-genome sequencing using next-generation sequencing as demonstrated by an article in this issue of the Journal of Clinical Microbiology by E. Thomson, C. L. C. Ip, A. Badhan, M. T. Christiansen, W. Adamson, et al. (J Clin Microbiol. 54:2455–2469, 2016, http://dx.doi.org/10.1128/JCM.00330-16).
All current strategies for obtaining HCV whole genome sequences using NGS have some important limitations!!

- costly
- too laborious, and technically challenging for many laboratories
- the overall success rate of obtaining a reliable consensus sequence is still not adequate in many instances
- reads are too short to enable the detection of the physical linkages of individual single nucleotide variants across the haplotype (quasispecies) of each viral strain present

single molecule sequencing technologies aka third-generation sequencing
Single molecule sequencing technologies aka third-generation sequencing

PacBio RS II platform from Pacific Biosciences (Menlo Park, CA), provides very long, single-molecule reads allowing the full length of each targeted HCV RNA molecule to be continuously sequenced in one contiguous pass so that the linkage information between every individual mutation on the same HCV molecule is well characterized.

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detected low levels of HCV NS5A-resistant variants in treatment-naive patients infected with genotypes 1a and 3a
A real-time PCR-(or TMA)-based assay with a lower limit of detection of \( \leq 15 \text{ IU/mL} \) should be used to monitor HCV RNA levels during and after therapy (A1).

Measurement of HCV core antigen levels by EIA can be used as an alternative to HCV RNA level measurement to monitor treatment efficacy during and after therapy when HCV RNA assays are not available or not affordable (A1).

Assessment of viral response, including documentation of SVR, requires use of an FDA-approved quantitative or qualitative nucleic acid test (NAT) with a detection level of 25 IU/mL or lower.
**HCV treatment monitoring: When to monitor?**

In patients treated with an IFN-free regimen, HCV RNA or HCV core antigen levels should be measured at baseline, between week 2 and 4 for assessment of adherence (optional), at end-of-treatment (week 8, 12, 16 or 24 in patients treated 8, 12, 16 or 24 weeks, respectively), and 12 or 24 weeks after the end of therapy (SVR12 or SVR24, respectively) (A2).

Quantitative HCV-RNA testing is recommended prior to the initiation of antiviral therapy to document the baseline level of viremia (ie, baseline viral load) (I,A).

Quantitative HCV viral load testing is recommended after 4 weeks of therapy and at 12 weeks following completion of therapy. Antiviral drug therapy should NOT be interrupted or discontinued if HCV RNA levels are not performed or available during treatment. Quantitative HCV viral load testing can be considered at the end of treatment and 24 weeks or longer following the completion of therapy (I,B).
HCV treatment monitoring:
When to monitor?

Monitoring of treatment efficacy can be simplified in order to improve access to care by measuring HCV RNA or HCV core antigen levels only at baseline and 12 or 24 weeks after the end of therapy (SVR12 or SVR24, respectively) (A2).

If HCV RNA is detectable at week 4 of treatment, repeat quantitative HCV RNA viral load testing is recommended after 2 additional weeks of treatment (treatment week 6).

If quantitative HCV viral load has increased by greater than 10-fold (>1 log10 IU/mL) on repeat testing at week 6 (or thereafter), then discontinuation of HCV treatment is recommended.

The significance of a positive HCV RNA test result at week 4 that remains positive, but lower, at week 6 or week 8 is unknown. No recommendation to stop therapy or extend therapy can be provided at this time (III,C).
HCV treatment monitoring

HCV resistance during or after therapy?

Monitoring for HCV drug resistance-associated variants during or after therapy is **not recommended** (IIb,C).