



OCTOBER
11-12, 2024
MILAN, ITALY

DeltaCure
3rd International Meeting

HDV RNA assays: performances and clinical challenges

Maria Paola Anolli, M.D.

Division of Gastroenterology and Hepatology

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

University of Milan, Milan, Italy

Maria Paola Anolli - COIs

- Nothing to disclose



Outline

- HDV RNA quantification significantly differs according to extraction methods in untreated patients
 - HDV RNA quantification significantly differs according to assays in untreated patients
 - HDV RNA quantification differences during therapy
 - Beyond RT-qPCR: new approaches
-

Background

- HDV RNA is considered a key marker of hepatic disease progression and has been established as a surrogate endpoint of treatment efficacy by FDA and joint EASL-AASLD conference.
- A “virological response” has been defined as at least 2 Log HDV RNA reduction or HDV RNA undetectability, compared to baseline.
- Accurate quantification of serum/plasma HDV RNA is crucial for management of treated and untreated CHD patients.
- However, broad variability exists in terms of HDV RNA quantification assays and a WHO International Standard for HDV RNA is only available for HDV genotype 1.

FDA: Food and Drug Administration; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; CHD: chronic hepatitis Delta

Romeo R, et al. Gastroenterology 2009; Keskin O, et al. Clin Gastroenterol Hepatol 2015; Kamal H, et al. Hepatol 2020; Cornberg M, et al. J Hepatol. 2020; Yurdaydin C, et al. J Hepatol 2019

Pitfalls in HDV RNA quantification: many available kits

Assay name	Manufacturer/provider	Type
EurobioPlex HDV Kit EBX 004	Eurobio Scientific	Commercial kit
RoboGene HDV RNA Quantification Kit 2.0	Roboscreen GmbH	Commercial kit
AltoStar HDV RT-PCR Kit 1.5	altona Diagnostics GmbH	Commercial kit
RealStar® HDV RT-PCR Kit 1.0 RUO	altona Diagnostics GmbH	Commercial kit
AmpliSens HDV-FRT	Federal Budget Institute of Science	Commercial kit
Fluorion HDV QNP 2.1 Real-Time PCR Kit	Iontec	Commercial kit
SYSTAAQ HDV Real-Time PCR Kit	SYSTAAQ	Commercial kit
LIPSGENE HDV Kit	VL-Diagnostics GmbH	commercial kit
Bosphore HDV Quantification-Detection Kit v1	Anatolia Geneworks	commercial kit
genesig Real-time PCR Detection Kit for HDV	Primerdesign	Commercial kit
HDV Real-TM Qual Real-time PCR Test	Sacace Biotechnologies	Commercial kit
LightMix Kit HDV ^a	TibMolBio/Roche	commercial kit
HDV Real-time RT-PCR Kit	Creative Biogene	commercial kit
HDV Quantitation Real-Time PCR kit	Dia.Pro Diagnostic Bioprobes s.r.l	Commercial kit
ViroReal Kit HDV ^a	Ingenetix	Commercial kit
HDV Real-Time RT-PCR Kit	Liferiver	Commercial kit
Hepatitis Delta virus One-Step RT-qPCR Kit ^a	NZYTech	Commercial kit
PCRmax LtdTM qPCR test Hepatitis Delta	PCRmax	Commercial kit

- **LLOQ (Lower limit of quantification)**
 <LLOQ: HDV RNA not quantifiable or HDV RNA undetectable
- **LOD (Lower limit of detection)**
 Lowest concentration where 95% of all replicates test positive
- **TND (Target not detected)**
 HDV RNA undetectable in the sample

LLOQ: lower limit of quantification; LOD: lower limit of detection; TND: target not detected

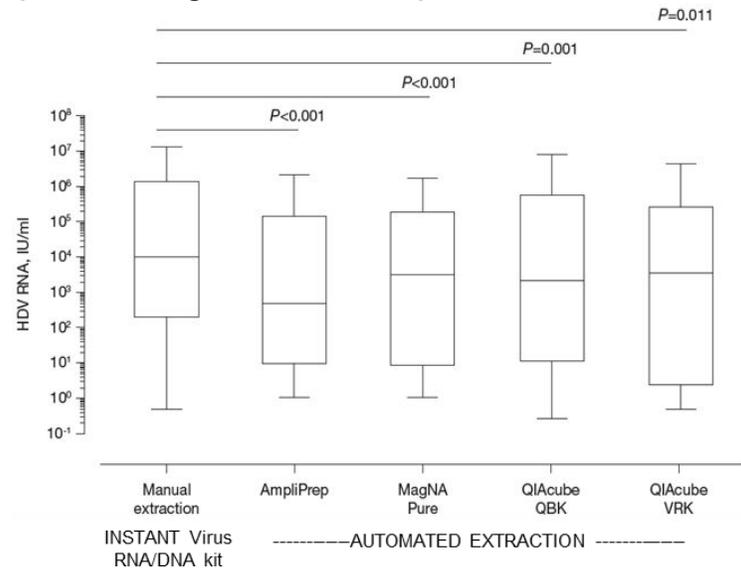
Outline

- HDV RNA quantification significantly differs according to extraction methods in untreated patients
 - HDV RNA quantification significantly differs according to assays in untreated patients
 - HDV RNA quantification differences during therapy
 - Beyond RT-qPCR: new approaches
-

HDV RNA quantification significantly differs according to extraction methods in the same assay (I)

Robogene HDV RNA Quantification Kit 2.0 and different extraction methods (manual vs. automated)

Multicenter German study
18 samples 6 PegIFN treated patients



Multicenter European study
31 samples



Calculated CF and LOD for protocols included in this study.

Center	Nucleic acid extraction	Amplification/detection device	CF ^a	LOD (IU/mL; 95% confidence interval)	Sample equivalent (µL)
A	MagNa Pure 96	Rotor-Gene Q	1870	111.1 (101.5 – 120.7)	10
B1	eMAG	LC480II	922	449.5 (426.0 – 472.9)	18
B2	MagNA Pure 24	LC480II	190	234.6 (217.1 – 252.1)	20
C1	INSTANT Virus RNA/DNA Kit	LC480II	20	4.0 (3.3 – 4.7)	33
C2	QiaCube	LC480II	550	81.8 (74.3 – 89.3)	10
D	INSTANT Virus RNA/DNA Kit	Rotor-Gene Q	40	3.7 (3.0 – 4.4)	33
E	InnuPure C16 touch	qTOWER3	185	23.9 (21.8 – 25.9)	33
F	EZ1 instrument	CFX96	297	21.6 (18.0 – 25.2)	33

- Automated extraction significantly underestimated HDV RNA
- Different extraction methods have different starting and elution volumes
- Protocol specific CF must be determined

CF, correction factor

HDV RNA quantification significantly differs according to extraction methods in different assays (I)

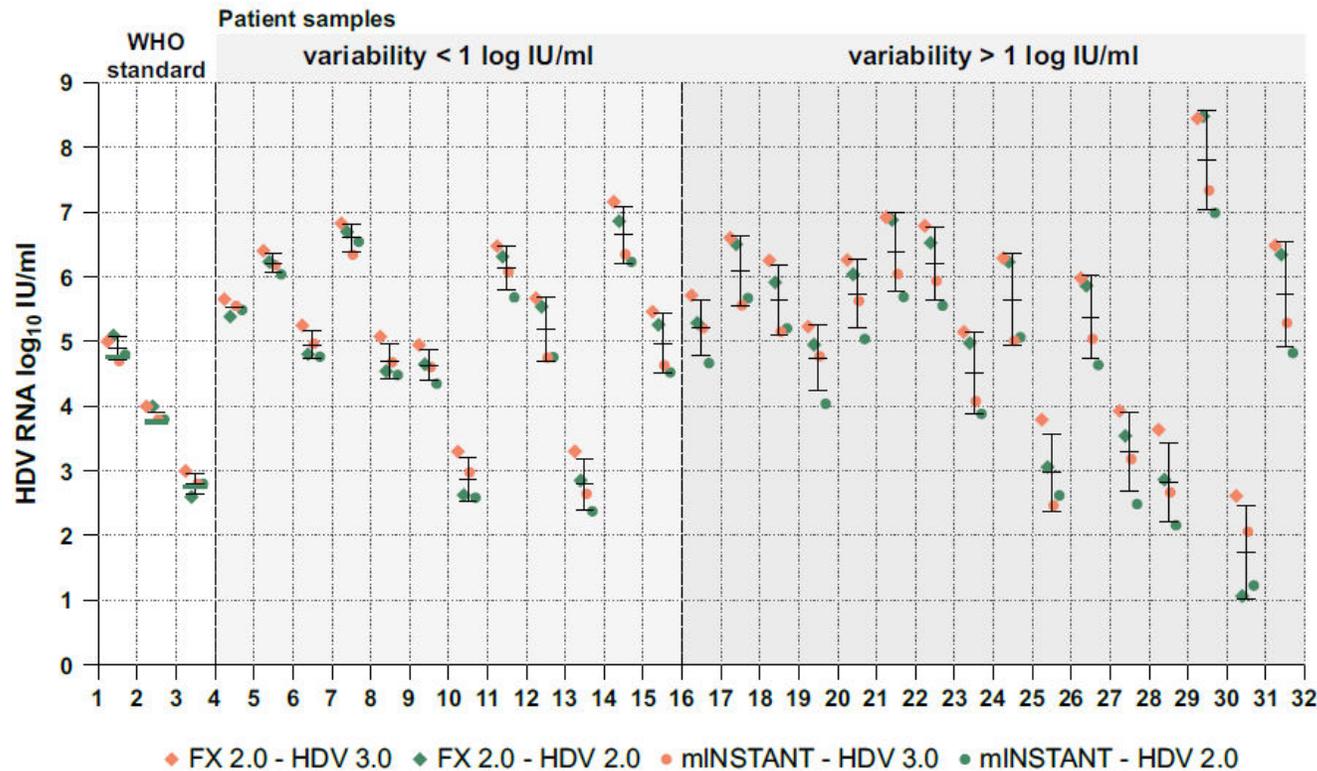


Single center German Study

Robogene 2.0 Quantification Kit vs. Robogene 3.0 Quantification Kit

Manual (INSTANT Virus RNA/DNA kit) vs. Automated (INSTANT Virus RNA/DNA Kit – FX 2.0) extraction

32 samples (28 real-life, 4 WHO IS dilutions)



- HDV RNA levels varied by >1 log IU/mL in 57% (n=16/28) of real-life plasma samples
- Manual extraction is not comparable to automated INSTANT FX 2.0 extraction regardless of the quantification kit

WHO IS, WHO International Standard

Outline

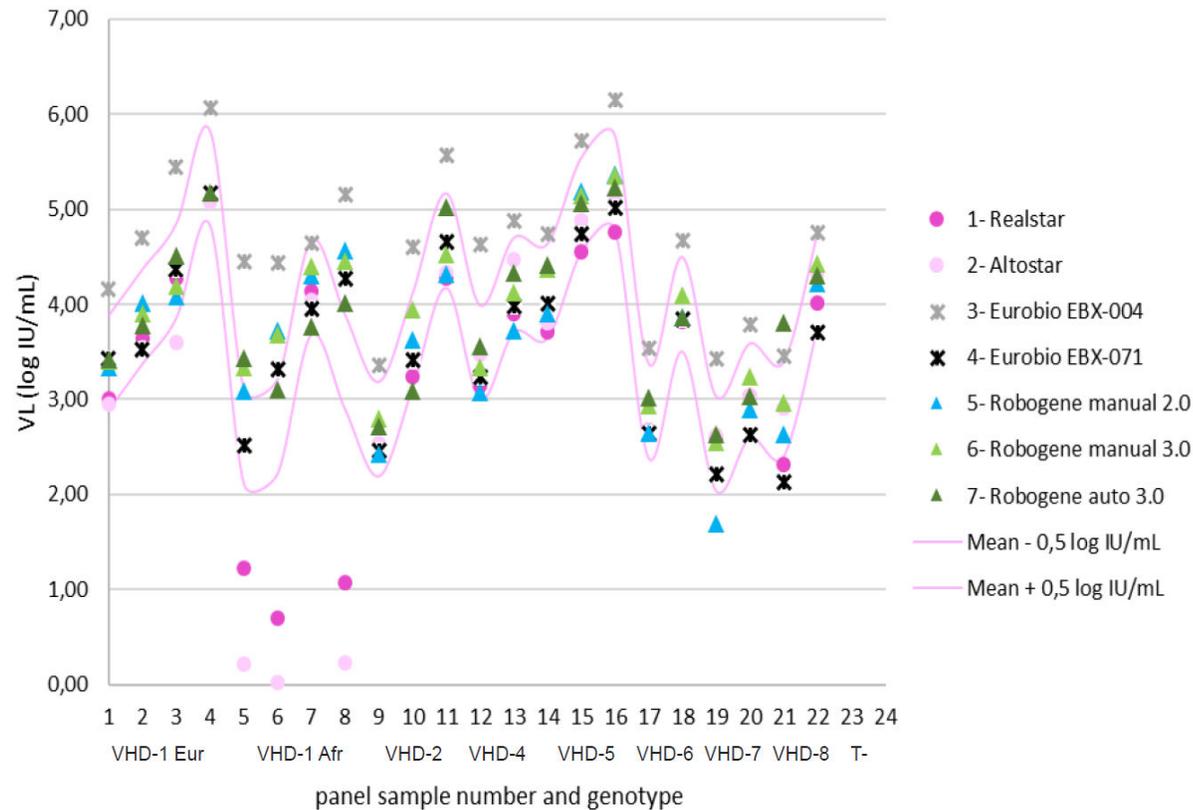
- HDV RNA quantification significantly differs according to extraction methods in untreated patients
 - HDV RNA quantification significantly differs according to assays in untreated patients
 - HDV RNA quantification differences during therapy
 - Beyond RT-qPCR: new approaches
-

HDV RNA quantification significantly differs according to assays (II)



Single center French study

RealStar 1.0, AltoStar 1.5, EurobioPlex EBX004, EurobioPlex EBX071, Robogene 2.0, Robogene 3.0 manual vs. automated
24 samples



- Similar qualitative and quantitative results for the 7 assays overall
- Systematic over-quantification by EurobioPlex EBX004
- Quantification problem on some strains of African HDV-1 genotype for Altona kits

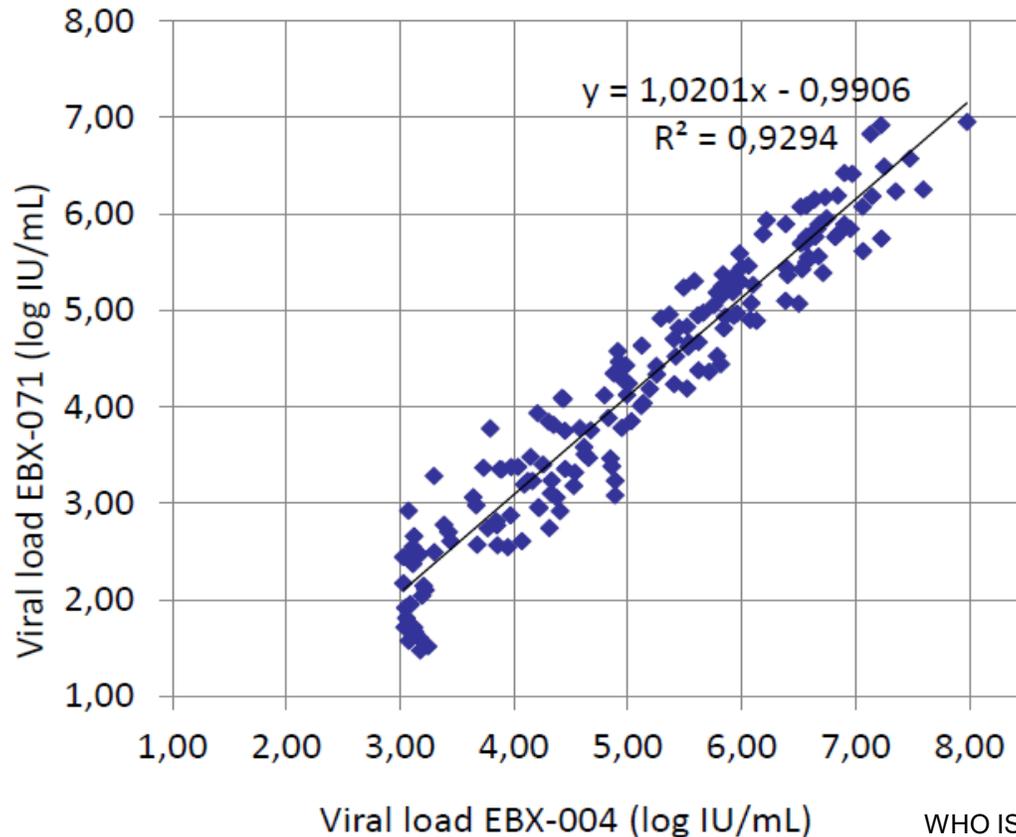
HDV RNA quantification significantly differs according to assays (III)



Single center French study

EurobioPlex HDV EBX 071 vs. EurobioPlex EBX 004 with automated extraction [m2000sp device (Abbott)]

561 samples



- 98.9% concordant (pos/neg) results
- Mean difference 0.89 log IU/mL (EBX004 > EBX071)
- EurobioPlex EBX071: LOD 20 IU/mL, LLOQ 50 IU/mL (vs. 100 IU/mL EurobioPlex EBX004)

WHO IS: World Health Organisation International Standard; LOD: Limit of Detection; LOQ: Lower Limit of Quantification

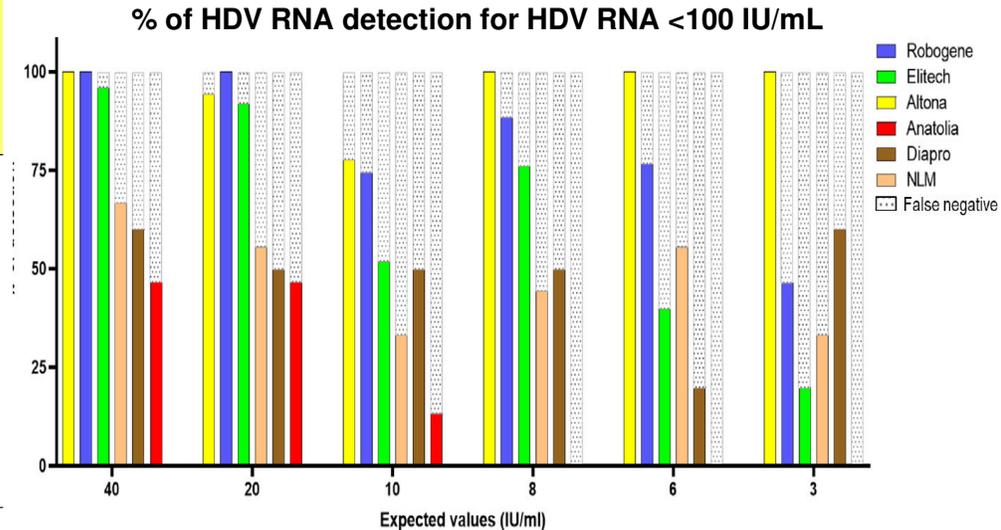
HDV RNA quantification significantly differs according to assays (IV)



Multicenter Italian quality control national study (30 centers)
 9 different assays (6 commercial assays, 3 in-house assays)
 29 samples (21 clinical samples, 8 dilution of WHO IS)

HDV-RNA Quantitative Assays	Centers (N)
RoboGene HDV RNA Quantification Kit 2.0 CE-IVD	9
Elitech HDV-RNA Quantification by Eurobio RUO	7
RealStar/AltoStar HDV RT-PCR Kit Altona RUO	5
Bosphore HDV Real-Time PCR Kit-Anatolia Geneworks CE-IVD	3
Diapro HDV Quantitation Real-Time PCR CE-IVD	2
HDV Real TM Quant Nuclear Laser Medicine RUO	1
Home Made ddPCR or Real-Time PCR	3

Assay	Centers (N)	Limit of detection 95% (LOD 95%)		
		Median LOD 95% (IU/ml) ^a	LOD 95% Range MIN-MAX (IU/ml)	LOD 75% Range (IU/ml)
Robogene	9	31	3-316	3-316
Elitech	7	100	100-316	100-316
Altona RealStar	4	10	3-316	3-100
<i>Altona AltoStar</i>	1	-	3	3
Anatolia	3	316	31-316	10-316
Diapro	2	-	316-1,000	316-1,000
Nuclear Laser Medicine	1	-	31	31
Home Made	3	-	31-316	31-316



- Heterogeneous sensitivity across different assays and across laboratories using the same assay
- Lowest LOD: Altona and Robogene 2.0
- Lowest rates of false negative results with Altona and Robogene 2.0 with HDV RNA <100 IU/mL

Outline

- HDV RNA quantification significantly differs according to extraction methods in untreated patients
 - HDV RNA quantification significantly differs according to assays in untreated patients
 - HDV RNA quantification differences during therapy
 - Beyond RT-qPCR: new approaches
-

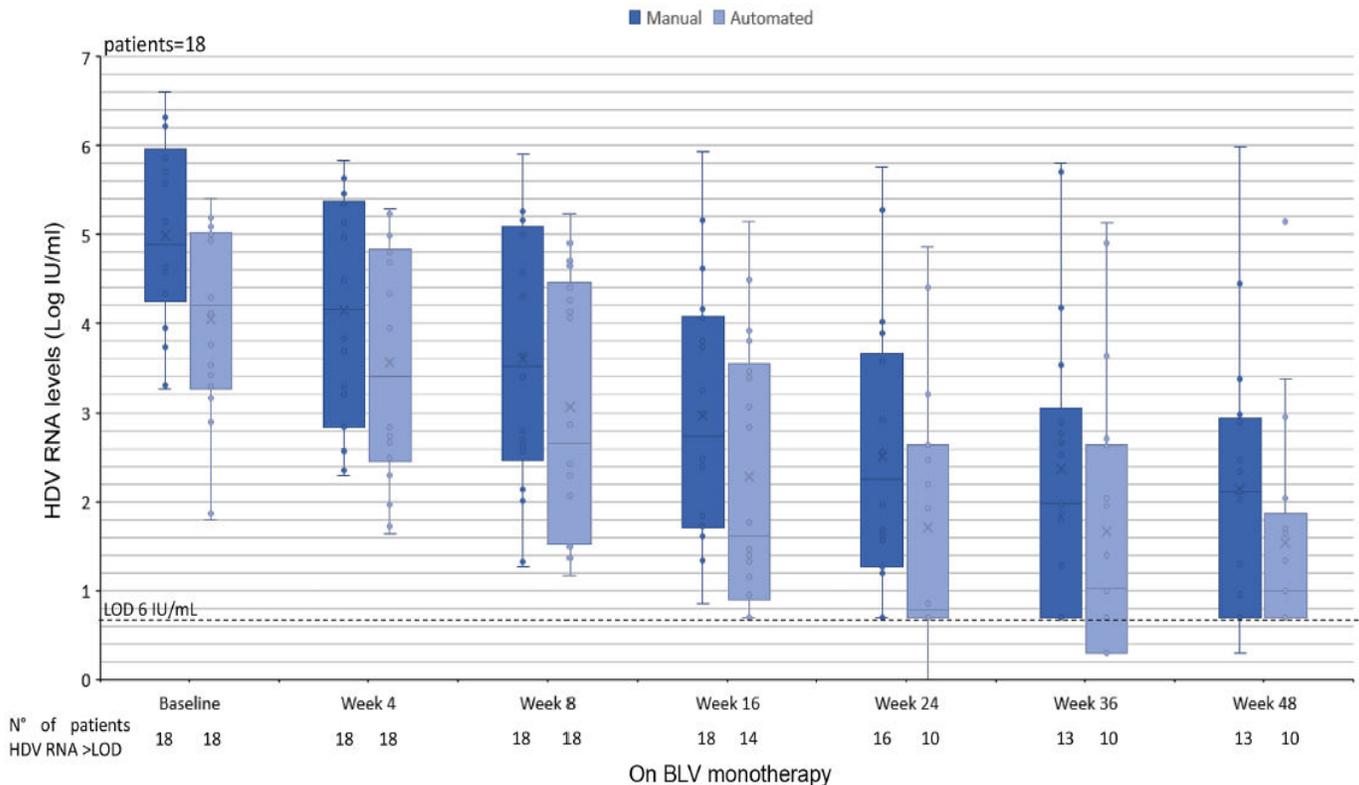
HDV RNA quantification differences during therapy according to extraction methods (I)



Single center Italian study

264 samples from 157 patients (n=18 BLV treated)

Robogene 2.0 Quantification Kit - Manual (INSTANT Virus RNA/DNA kit) vs. Automated (EZ1 DSP Virus Kit) Extraction



- Higher (~1 Log) HDV RNA levels with manual extraction, difference persisting at all time points during BLV
- Virological response* did not differ; patients achieving HDV RNA undetectable differed

* HDV RNA decrease ≥ 2 Log from baseline or HDV RNA undetectable

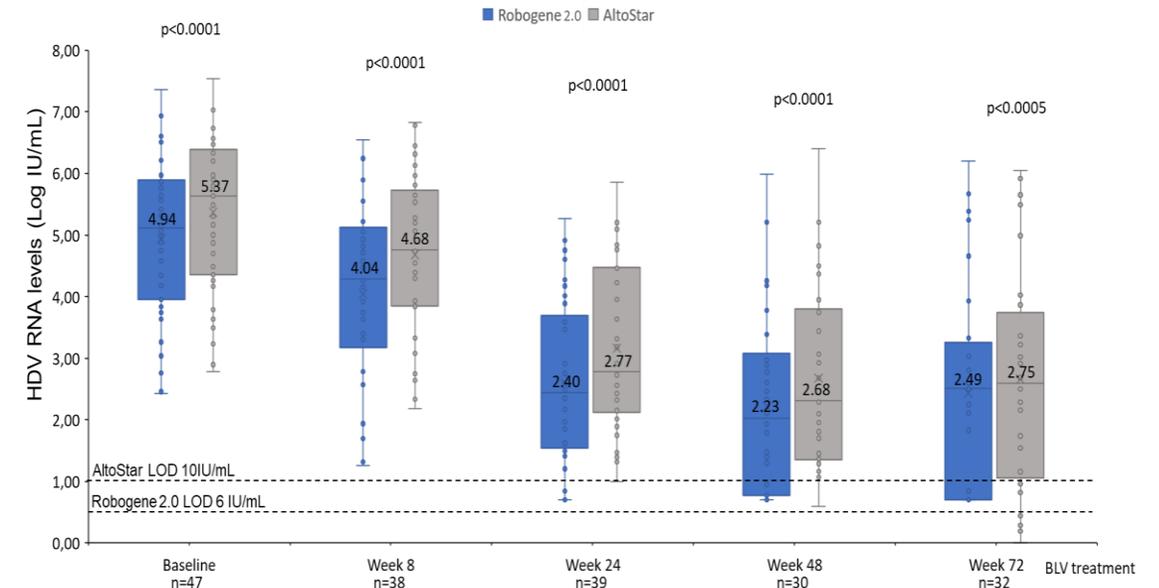
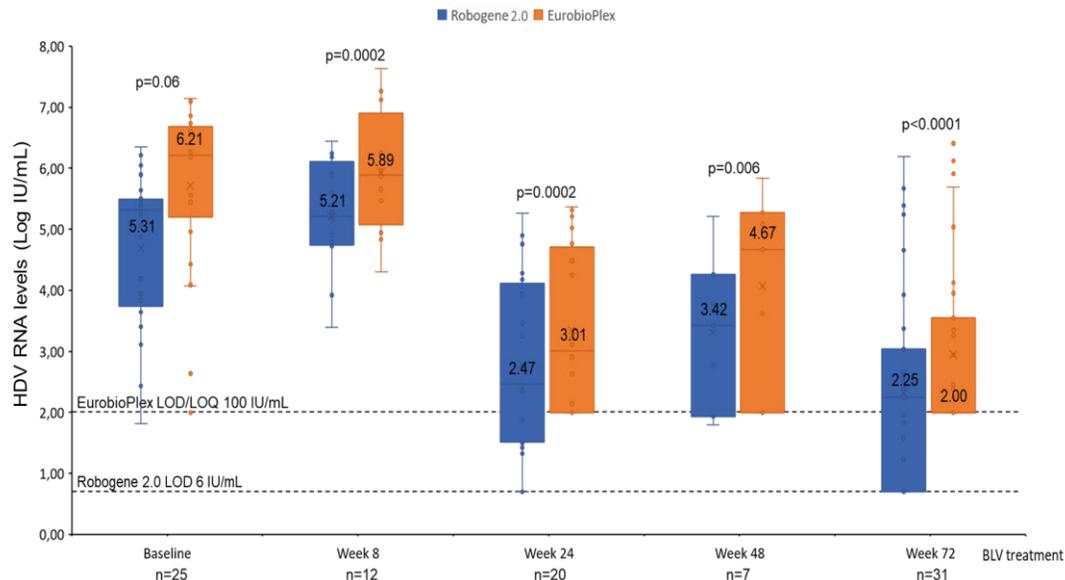
HDV RNA quantification differences during therapy (I)



Single center Italian study

431 samples from 131 patients (n=61 BLV treated)

Comparison between Robogene 2.0, EurobioPlex EBX004 and AltoStar HDV RT-PCR Kit 1.5



n° of patients with HDV RNA TND/<LOD	0 (0%) 1 (4%)	0 (0%) 0 (0%)	2 (10%) 7 (35%)	0 (0%) 2 (29%)	11 (36%) 16 (52%)	n° of patients with HDV RNA TND/<LOD	0 (0%) 0 (0%)	0 (0%) 0 (0%)	4 (10%) 1 (3%)	5 (17%) 1 (3%)	10 (31%) 7 (22%)
n° of patients with HDV RNA TND	0 (0%) 1 (4%)	0 (0%) 0 (0%)	1 (5%) 4 (25%)	0 (0%) 1 (14%)	4 (13%) 10 (32%)	n° of patients with HDV RNA TND	0 (0%) 0 (0%)	0 (0%) 0 (0%)	1 (3%) 0 (0%)	2 (7%) 0 (0%)	5 (16%) 1 (3%)

- EurobioPlex and Altostar reported significantly higher HDV RNA than Robogene at all timepoints during BLV treatment
- Rates of virological response* did not differ significantly, HDV RNA undetectability rates differed across assays during BLV therapy

HDV RNA quantification differences during therapy (II)

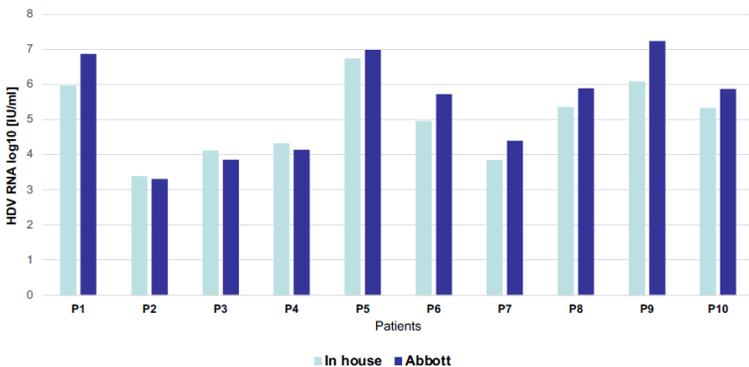


Single center UK study

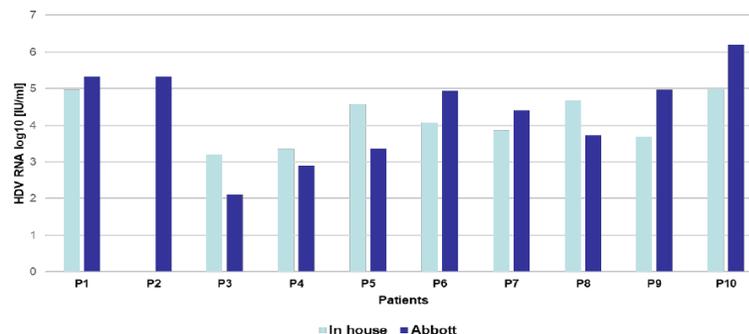
30 samples from 10 BLV-treated patients

In-house real-time PCR assay (LLOQ 677 IU/ml) and HDV RNA test mRealTime by Abbott Diagnostics (RUO test, LLOQ 5 IU/ml)

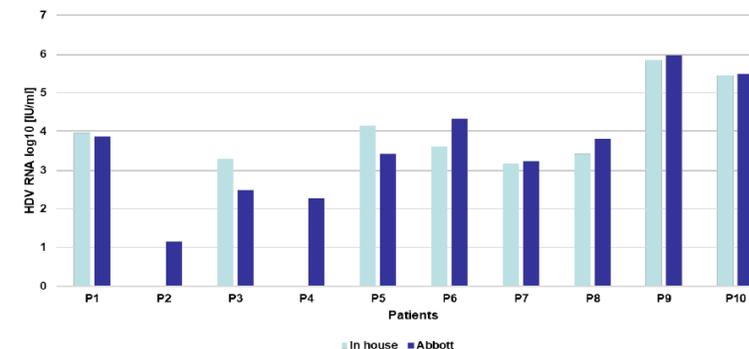
At therapy start



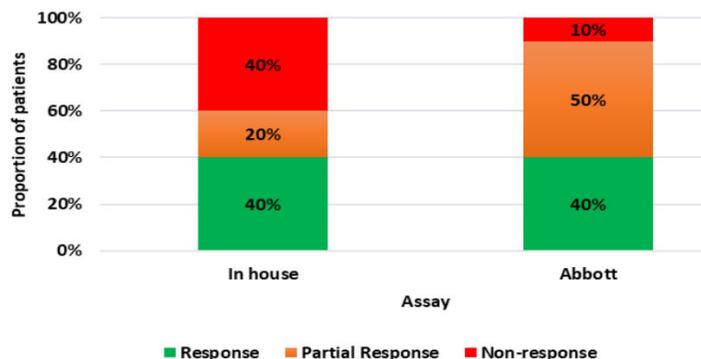
Week 12



Week 24



Week 24 Response



- Median HDV RNA at baseline, week 12 and week 24 did not differ significantly ($p=0.76$, $p=0.86$, and $p=0.89$)
- Number of patients categorized as virological responders* were similar by both assays. 2/10 samples negative at week 24 by inhouse assay, positive by Abbott assay

LLOQ, lower limit of quantification; RUO, research use only

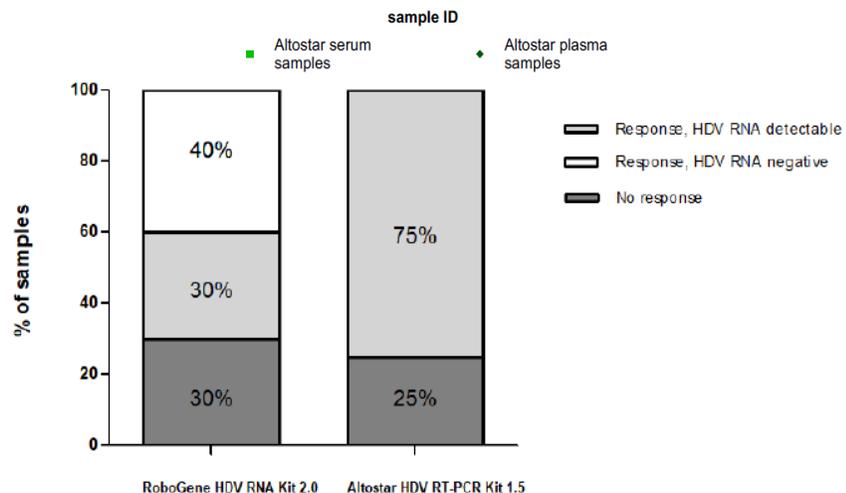
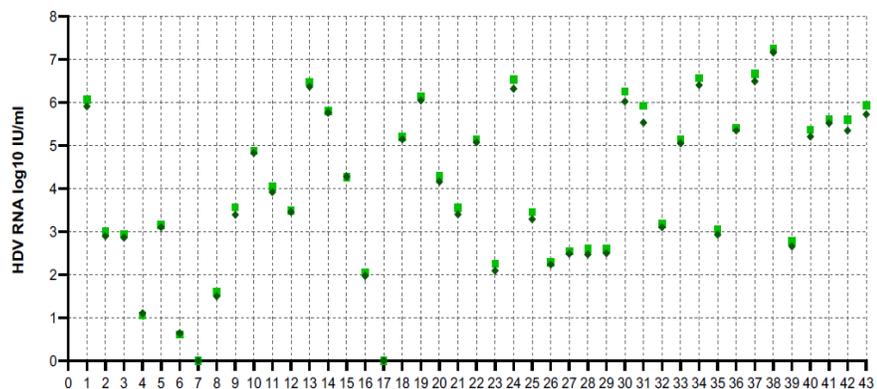
HDV RNA quantification differences during therapy (III)



Single center German study

43 samples from 40 BLV-treated patients

Comparison between Robogene 2.0 (QIAamp DNA Blood Mini Kit) and AltoStar HDV RT-PCR Kit 1.5



- No significant difference at baseline (mean difference 0.11 log IU/ml)
- Mean HDV RNA levels at BL and during BLV were comparable across assays ($p=0.72$, $p=0.66$)
- No significant differences in virological response rates* (up to 65 weeks), but 8/20 samples undetectable with Robogene 2.0 yet quantifiable by AltoStar HDV RT-PCR Kit 1.5

BL, baseline; BLV, Bulevirtide

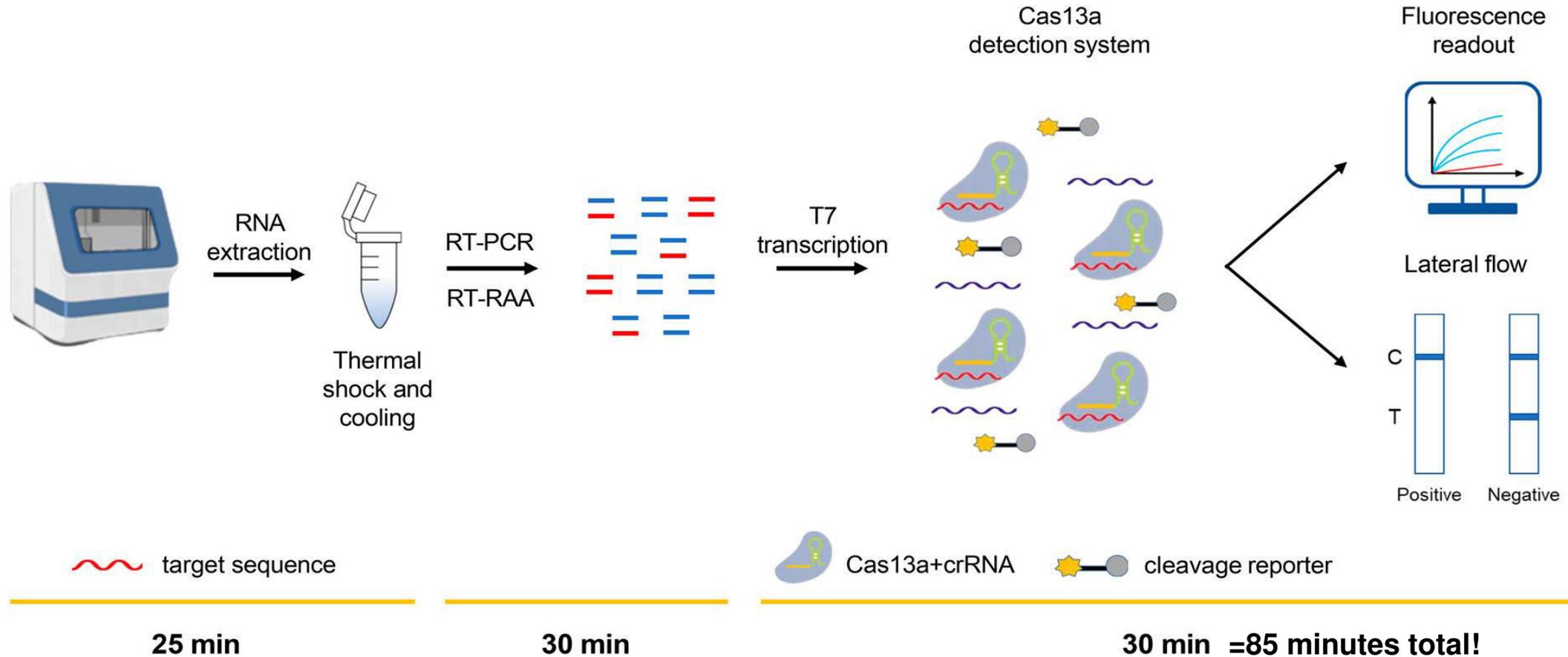
Outline

- HDV RNA quantification significantly differs according to extraction methods in untreated patients
 - HDV RNA quantification significantly differs according to assays in untreated patients
 - HDV RNA quantification differences during therapy
 - **Beyond RT-qPCR: new approaches**
-

Beyond RT-qPCR: new approaches (I)



Development of a HDV RNA assay using the CRISPR–Cas13a system combined with RT–PCR and RT RAA

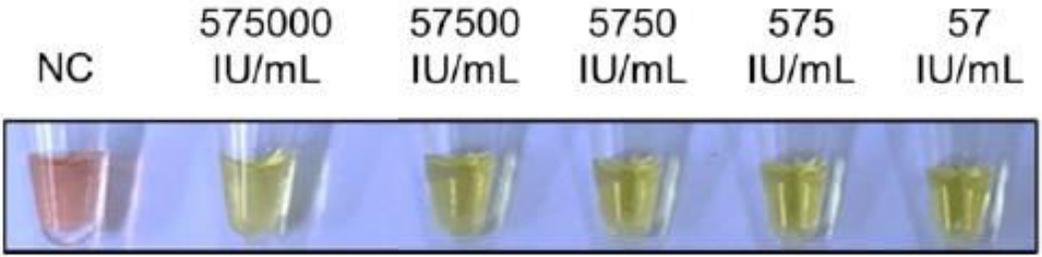
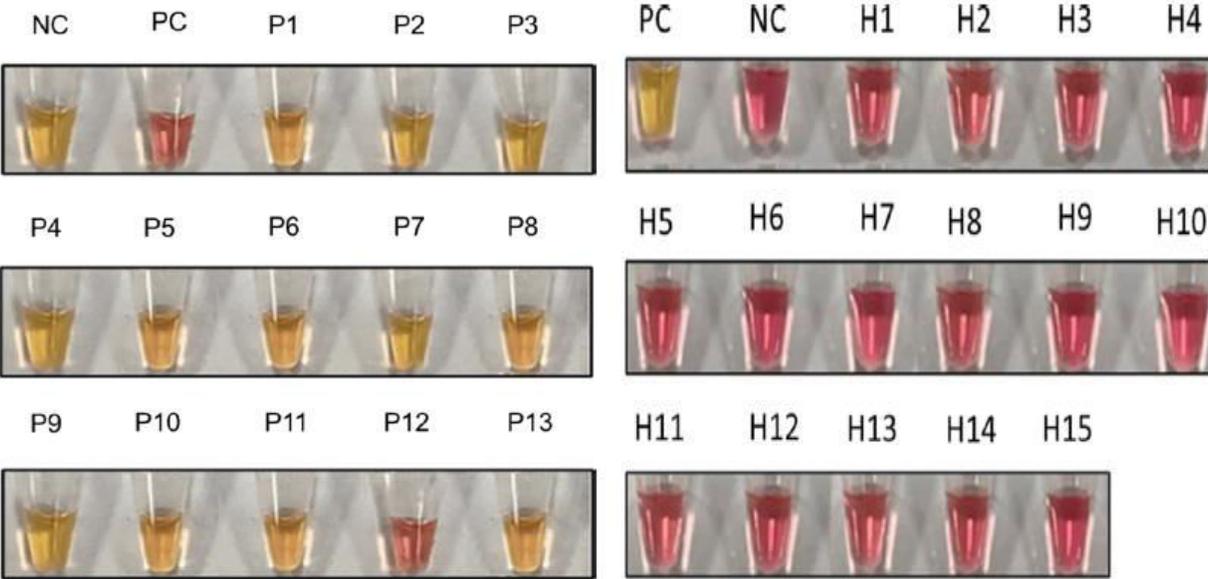


RT-qPCR, Real-time quantitative PCR; RT-RAA, Real-time recombinase aided amplification technology; CRISPR, clustered regularly interspaced short palindromic repeats, WHO-IS, World Health Organisation International Standard

Beyond RT-qPCR: new approaches (II)



LAMP-PCR is a low-cost alternative to RT-qPCR, potential simple screening assay



- Positive RT-PCR samples tested with LAMP PCR returned one false negative; Negative RT-PCR samples tested with LAMP PCR were all negative
- LAMP-PCR LOD 57 IU/mL

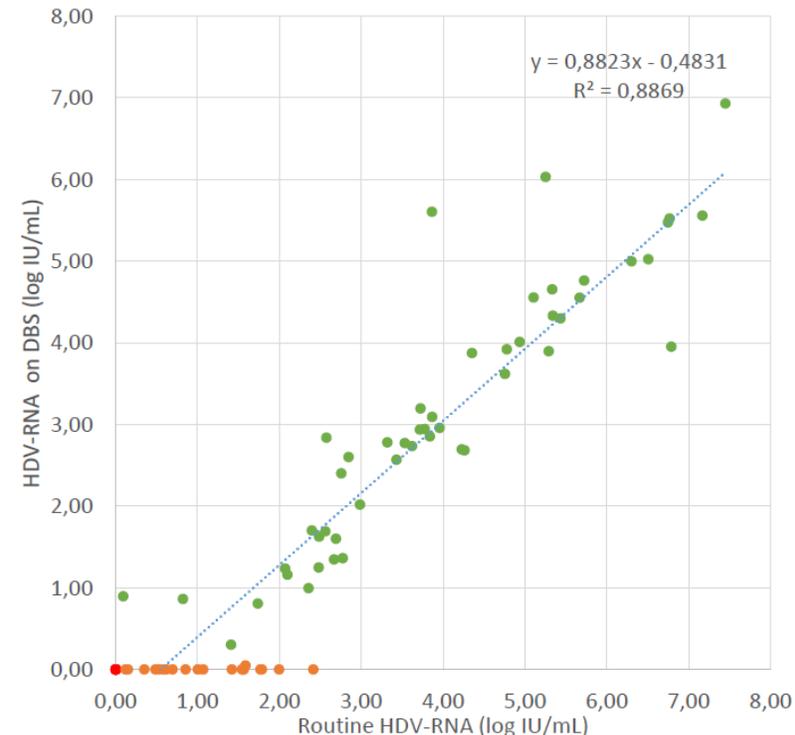
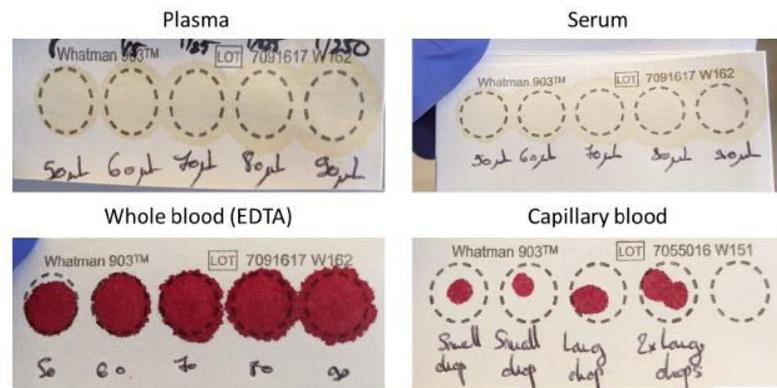
RT-qPCR, Real-time quantitative PCR; LAMP-PCR, Loop-mediated isothermal amplification PCR

Beyond RT-qPCR: new approaches (III)



Single center French study to validate threshold for HDV testing on DBS

Retrospective testing of serum and plasma samples + prospective validation of whole blood samples using DBS (anti-HDV & HDV RNA)



- HDV RNA quantification: 98% Sensitivity, samples with low viral load not detected
- Whole blood on DBS test ongoing

DBS, dry blood spots

Beyond RT-qPCR: new approaches (IV)

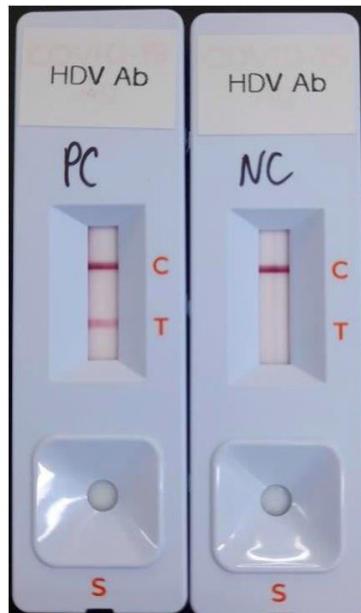


Multicenter study (Mongolia, Korea, Singapore)

LUCA AICell anti-HDV RDT by lateral flow assay technique

200 samples (122 HDV RNA positive, 49 HDV RNA negative HBSAg+, 29 healthy individuals)

Reference standard ELISA test & in house HDV RNA PCR assay



15 min incubation

Group	n	HBsAg ELISA +	anti-HDV ELISA +	HDV-RNA +	anti-HDV RDT +
anti-HDV +	122	122	122	122	122
HBsAg +, anti-HDV -	49	49	0	N/A	0
Healthy	29	0	N/A	N/A	0

100% agreement between RDT and ELISA tests

RDT, rapid diagnostic test; ELISA, enzyme-linked immunosorbent assay

Summary

- Lack of standardized PCR techniques for HDV RNA used in different laboratories because of heterogeneity in technical tools (extraction methods, RT–qPCR devices, internal controls).
- Quantification of HDV RNA levels may be significantly influenced by the assays as well as the extraction methods.
- Discrepancies could be due to the genetic diversity of HDV and to primer-probe mismatches.
- Since discontinuation of BLV monotherapy could be considered only in patients who achieve and maintain undetectable viremia long-term, the use of low-sensitive HDV RNA quantification assays may lead to discontinue BLV in patients who are still HDV RNA positive at low levels.
- Further studies are needed to assess the real impact of the assay on defining virological response to BLV therapy.
- Rapid, user-friendly HDV RNA assays are in development and could be useful tools in resource-limited settings.

Thank You for Your Attention!



FONDAZIONE IRCCS CA' GRANDA
OSPEDALE MAGGIORE POLICLINICO



UNIVERSITÀ
DEGLI STUDI
DI MILANO
