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HDV RNA assays: Performance characteristics, clinical utility, and challenges

Heiner Wedemeyer[1,2,3,4], **Mitchell Leus**[5], **Thomas R. Battersby**[6], **Jeffrey Glenn**[7], **Emmanuel Gordien**[8], **Saleem Kamili**[9], **Hema Kapoor**[10], **Harald H. Kessler**[11], **Oliver Lenz**[12], **Marc Lütgehetmann**[13,14], **Tonya Mixson-Hayden**[9], **Christian O. Simon**[15], **Michael Thomson**[16], **Gabriel Westman**[17,18], **Veronica Miller**[5], **Norah Terrault**[19], **Pietro Lampertico**[20,21] **On behalf of the HDV RNA Assays Writing Group at the HBV Forum**

[1]Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover Medical School, Hannover, Germany

[2]Excellence Cluster RESIST, Hannover Medical School, Hannover, Germany

[3]D-SOLVE: EU-funded Network on Individualized Management of Hepatitis D

[4]German Center for Infection Research (DZIF), Partner Site Hannover-Braunschweig, Braunschweig, Germany

[5]Forum for Collaborative Research, School of Public Health, University of California, Berkeley, Washington DC Campus, Washington, District of Columbia, USA

[6]Biomarker-IVD, Gilead Sciences, Inc., Foster City, California, USA

Correspondence: Univ.-Prof. Dr. med. Heiner Wedemeyer Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. wedemeyer.heiner@mh-hannover.de.

AUTHOR CONTRIBUTIONS

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[7]Departments of Medicine (Division of Gastroenterology and Hepatology) and Microbiology & Immunology, Stanford University School of Medicine, Stanford, California, USA

[8]Laboratoire de microbiologie clinique, Centre National de Référence pour les virus des hépatites B, C et Delta, Hôpital Avicenne Assistance Publique – Hôpitaux de Paris, Bobigny, France

[9]Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

[10]Ex Quest Diagnostics, HK Healthcare Consultant LLC, Secaucus, New Jersey, USA

[11]Diagnostic and Research Center for Molecular Biomedicine, Medical University of Graz, Graz, Austria

[12]Clinical Microbiology and Immunology, Janssen Pharmaceutica NV, Beerse, Belgium

[13]Institute for Microbiology, Virology and Hygiene, University Medical Center Hamburg Eppendorf (UKE), Hamburg, Germany

[14]German Center for Infection Research (DZIF), Partner Site Hamburg, Lübeck, Kiel, Germany

[15]Clinical Development and Medical Affairs, Roche Diagnostics Solutions, Rotkreuz, Switzerland

[16]Division of Antivirals, US Food and Drug Administration, Silver Spring, Maryland, USA

[17]Swedish Medical Products Agency, Uppsala, Sweden

[18]Department of Medical Sciences, Uppsala University, Uppsala, Sweden

[19]Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

[20]Division of Gastroenterology and Hepatology, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[21]Department of Pathophysiology and Transplantation, CRC "A. M. and A. Migliavacca" Center for Liver Disease, University of Milan, Milan, Italy

Abstract

Coinfection with HBV and HDV results in hepatitis D, the most severe form of chronic viral hepatitis, frequently leading to liver decompensation and HCC. Pegylated interferon alpha, the only treatment option for chronic hepatitis D for many years, has limited efficacy. New treatments are in advanced clinical development, with one recent approval. Diagnosis and antiviral treatment response monitoring are based on detection and quantification of HDV RNA. However, the development of reliable HDV RNA assays is challenged by viral heterogeneity (at least 8 different genotypes and several subgenotypes), intrahost viral diversity, rapid viral evolution, and distinct secondary structure features of HDV RNA. Different RNA extraction methodologies, primer/probe design for nucleic acid tests, lack of automation, and overall dearth of standardization across testing laboratories contribute to substantial variability in performance characteristics of research-based and commercial HDV RNA assays. A World Health Organization (WHO) standard for HDV RNA, available for about 10 years, has been used by many laboratories to determine the limit of detection of their assays and facilitates comparisons of RNA levels across study centers. Here we review challenges for robust pan genotype HDV RNA quantification, discuss particular clinical needs and the importance of reliable HDV RNA quantification in the context of drug development and patient monitoring. We summarize distinct technical features and performance characteristics of available HDV RNA assays. Finally, we provide considerations for the use of HDV RNA assays in the context of drug development and patient monitoring.

The HDV is a satellite of the HBV, requiring HBsAg provided by HBV for encapsidation, and is responsible for the most severe forms of viral hepatitis.[1] An estimated 9–19 million individuals are infected with HDV worldwide, with prevalence varying substantially between different regions and countries.[2] Pegylated interferon alpha, the only treatment option for chronic hepatitis D (CHD) for many years, has limited efficacy, several side effects, and contraindications. Fortunately, new treatments for CHD are in advanced clinical development. A novel entry inhibitor, bulevirtide, received conditional approval by the European Medical Agency in July 2020,[3,4] and several other drugs are under study in phase 2 or 3 trials.[5] However, the field lacks standardized, reliable, and accurate assays for the diagnosis and monitoring of HDV RNA levels in response to treatment. The HBV Forum convened a working group to assess the current landscape of assays used for HDV RNA quantification, strengthen clarity on the utility of HDV RNA in drug development and patient monitoring, and develop consensus for future needs. Participants included leading experts in academia, clinical practice, pharmaceutical and diagnostic companies, and regulatory agencies.

MOLECULAR EPIDEMIOLOGY OF HDV

HDV genotypes and subgenotypes

HDV is classified into distinct genotypes and subgenotypes based on phylogenetic data. Two HDV strains are considered to belong to a same genotype if they have > 85% nucleotide sequence similarity over the partial *R0* region or > 80% nucleotide sequence similarity over the full-length genome sequence, together with posterior probability values > 0.99 in phylogenetic trees. Two HDV strains within a given genotype belong to the same subgenotype if they share > 90% of similarity in their full-length genome sequence and with a phylogenetic tree topology supported by posterior probability values close to 1.

Based on extensive molecular characterization of numerous partial (the so-called *R0* region) and complete nucleotide sequences from HDV strains from all continents, by means of phylogenetic trees obtained from various evolutionary models, the HDV genus can be definitively classified into 8 major clades/genotypes (HDV-1 to HDV-8) and several subgenotypes.[6–8] In addition, a high level of spread and diversity of HDV genotypes, with considerable intergenotypic and intragenotypic recombination, drives the emergence of new strains and subgenotypes as reported in recent study in Central Asia.[9]

Worldwide distribution of HDV genotypes and subgenotypes

HDV genotypes and subgenotypes display distinct geographical distribution (Figure 1).[8] HDV-1 genotype is the most prevalent worldwide, is found in nearly all continents, and has a distinct subgenotype distribution. Four HDV-1 subgenotypes, HDV-1a, −1b, −1c, and −1d, characterized by a mean intergroup nucleotide divergence over the whole genome sequence of between 14.6% and 16%, have been clearly identified. Among these subgenotypes, HDV-1a and HDV-1b show the highest intergroup divergence (16%) and are thought to be ancestral HDV-1 strains.[8]

HDV-2 (formerly HDV-IIa) and HDV-4 genotypes localize to Asia, and clearly segregate into 2 distinct subgenotypes. HDV-2a originates from Southeast Asia, whereas HDV-2b circulates in Yakutia, east Siberia. HDV-4a (formerly HDV-IIb) and HDV-4b (formerly HDV-IIb-M) originated in Taiwan and Japan, respectively.

HDV-3 predominates in the north of South America and is the most phylogenetically distant genotype. Two putative subgenotype clusters were identified from sequencing isolates from Brazil, Peru, and Venezuela and Bolivia,[7] but further studies of additional isolates are needed to formally describe HDV-3 subgenotypes.

HDV-5 to HDV-8 have been described only in Africa or in migrant populations in other countries. HDV-5 is the second most frequently reported genotype, with > 90% originating from western and sub-Saharan Africa, mainly in the region between Cameroon and Mauritania. Subgenotypes HDV-7a and HDV-7b have been identified in Cameroonians. HDV-6 and HDV-8 localize in central Africa, in a region spanning Angola and Cameroon. Remarkably, all African HDV genotypes (HDV-5 to HDV-8), as well as HDV-1a and 1b subgenotypes, were found in a central African area around Cameroon, indicating that this region or a neighboring area could have been the main historical source of HDV diversification.[7,8]

There is currently no solid clinical evidence that HDV viral loads vary significantly between HDV genotypes. In one study from the United Kingdom, patients with HDV-5 infection had numerically slightly higher HDV RNA levels than patients infected with HDV-1 without reaching statistical significance.[10] However, differences in viral load may also be biased due to different patient characteristics. *In vitro*, substantial differences between HDV genotypes have been described for HDV replication efficacy, assembly, and secretion.[11] Whether this translates in differences in viral loads and outcomes in patients is unknown.

Clinical and diagnostic aspects of HDV genetic diversity

Data on the association of genotypes or subgenotypes with disease outcomes are scarce. It is generally thought that HDV-1 is associated with a wide range of disease severity, from mild to severe, and often leading to liver cirrhosis and HCC, while HDV-2 causes milder disease, and HDV-3 is often associated with fulminant hepatitis and death.[12–15] However, some studies report different outcomes. In a small cohort from Yakutia, HDV-2-infected individuals were found to have severe disease,[16] and in another cohort from the Miyako Islands in the Far East, HDV-4b infection was also associated with severe disease.[17] Almost all studies have been performed on small cohorts with only 1 or 2 circulating HDV genotypes. A recent retrospective study on a cohort of > 1000 patients reported that cirrhosis occurred twice as often in patients infected with European/Asian HDV-1 strains than those infected with African HDV-1 strains.[18] In addition, within African HDV patients, HDV-1 and HDV-5 were associated with a significantly higher risk of developing fibrosis and cirrhosis, compared with other genotypes.[18] Studies to better characterize the role of HDV genotype in disease severity and the underlying mechanisms are needed.

Very few studies have addressed whether specific genotypes or subgenotypes are more resistant to specific antiviral therapies. Patients infected with HDV-5 appear to have a better response to pegylated interferon alpha treatment than those infected with HDV-1.[10]

Extensive HDV molecular diversity presents a challenge for commercial and research-based assays to properly detect or quantify HDV RNA across all genotypes. Particularly, HDV RNA levels have been dramatically underestimated by most assays in patients infected with strains of African genotypes (HDV-1a, HDV-1b, and HDV-5 to HDV-8).[19–21]

ROLE OF HDV RNA IN PATIENTS WITH CHD

Quantification of HDV RNA levels is critical in diagnosis and monitoring of untreated patients. CHD is defined by the presence of detectable HDV RNA, independent of alanine aminotransferase levels. Natural history studies suggest three patterns of HDV and HBV viremia among persons with CHD: HDV predominant (HDV RNA levels high and HBV DNA levels low or undetectable), HBV predominant (HDV RNA levels low and HBV DNA levels high), nondominance (both HBV DNA and HDV RNA levels high or both low).[22–24] The “HDV predominant” pattern is by far most frequently identified (80% of the patients) but some fluctuations in viral “dominance” over time are well recognized. Some untreated patients do clear viremia over time.[25]

HDV viremia is associated with higher rates of liver complications,[18,26,27] indicating that indeed HDV infection is contributing to the disease. For example, in a nucleoside analog-treated cohort from Asia, the 5-year cumulative incidence of HCC was 7% among HDV RNA negative persons compared to 22% with detectable HDV RNA. However, some of the earlier studies must be interpreted with caution due to sensitivity issues of some previous HDV RNA assays and the fact that HDV viremia can be intermittent. Data are lacking on whether a specific threshold of HDV RNA levels is associated with high versus low risk for development of liver complications, in part due to variability in quantitation and limits of detection among HDV RNA assays across different cohort studies.[28] In general, if most recent HDV RNA assays are used, <10% of the patients with CHD may have HDV RNA levels <1000 IU/mL.[29]

Treated patients—definition of response

The goal of HDV therapy is to improve survival by preventing disease progression, particularly development of cirrhosis, decompensation, HCC, and liver-related death. However, these hard clinical endpoints are difficult to measure in clinical trials and real-life studies because of the need for lengthy follow-up. Hence, surrogate endpoints such as HBsAg clearance and HDV RNA levels below lower limit of quantitation (LLoQ) on-therapy or off-therapy have been proposed. In both untreated and IFNα-treated patients, the achievement of these endpoints is associated with a lower risk of disease progression.[30] A sustained suppression of HDV RNA levels (undetectable or below LLoQ [target not detected (TND)] vs. detectable) off-IFNα treatment is associated with a lower incidence of clinical endpoints.[27,31–33]

While the preferred endpoint for HDV therapy is HBsAg loss (± anti-HBs seroconversion) and HDV RNA levels below LLoQ in the absence of HBsAg loss, confirmed HDV RNA below LLoQ during or off-therapy is thought to be the next best alternative endpoint. If this alternative endpoint is not achievable, then an intermediate endpoint of $\geq 2 \log_{10}$ IU/mL HDV RNA decline from baseline combined with normal alanine aminotransferase levels, termed “combined response,” has been used.[30,34] Indeed, this combination endpoint was used in the phase II clinical studies of bulevirtide and in the phase III trials of lonafarnib and bulevirtide.[3,35] The 2-log reduction in HDV RNA levels has not been compared to below LLoQ in terms of liver-related outcomes and has not been validated in natural history studies or trials with noninterferon-based therapies. Moreover, since change in HDV RNA levels over time is expected to vary by length of follow-up, defining a specific threshold (eg, > 1000 IU/mL or > 100 IU/mL HDV RNA) would help identify the populations in need of treatment and define study endpoints.

In addition to defining response, HDV RNA levels can be used to define virological nonresponse and for this, a <1 log decline of HDV RNA levels compared to baseline at week 24 (or week 48) has been used. In bulevirtide-treated patients, 10%–20% of the patients had a virologic nonresponse at week 24.[3,36]

HDV RNA ASSAYS: CHALLENGES FROM A VIROLOGICAL PERSPECTIVE

Variability across assay platforms and laboratories presents significant challenges for standardization of HDV RNA assays. The factors for variability in the performance characteristics of different HDV RNA assays include the RNA extraction methodology, primer/probe design for nucleic acid tests (NAT), lack of automation, and overall dearth of standardization and well-characterized sample evaluation panels across testing laboratories. A World Health Organization (WHO) standard for HDV RNA, available for about 10 years, has been used by many laboratories to determine the limit of detection (LoD) of their assays, and allows for comparison of RNA levels, reported in IU/mL, across study centers. A study utilizing the WHO HDV RNA international standard conducted across 28 different laboratories in 17 countries reported a degree of heterogeneity of performance among the assays, many of which were research based.[21] A multicenter European study compared the sensitivity and accuracy of the conformité européenne *in vitro* diagnostic (CE-IVD)–labeled RoboGene HDV RNA Quantification Kit 2.0 (Roboscreen GmbH) using different extraction methods and platforms.[37] Correction factors based on WHO HDV RNA international standard differed up to $2 \log_{10}$ between protocols, and the LoD varied considerably. Consistent with other studies,[28] protocols using automated extraction platforms had significantly higher LoDs than manual platforms.

Quantitative NAT for HDV RNA design challenges

Primers and probes for quantitative NAT for HDV RNA should target regions with the highest sequence conservation because of the variability in primary sequence and secondary structures, present between and within HDV genotypes. Many research-based and commercial NATs use complementary DNA or transcribed RNA to evaluate assay performance, but such methods do not account for the secondary structure and circular nature of the viral RNA isolated from clinical samples.

Cross-genotype variability has implications for primer/probe design, and the overall sensitivity and specificity of the NAT for HDV RNA. The paucity of sequence data from available clinical isolates, particularly from genotypes other than HDV-1, limiting the feasibility of optimizing primer/probe design and assessing assay performance across genotypes. The estimated evolution rate of HDV genomes within infected individuals ranges from 9.5×10^{-3} to 1.2×10^{-3} substitutions/site/year,[38] so longitudinal studies may be confounded by sequence changes impacting primer/probe binding. For such studies, it would be of value to sequence the primer/probe binding regions to monitor for changes over time.

The HDV genome has a high GC content (60%) and intramolecular base-pairing (74%), conferring extensive secondary structure and an overall rod-like shape.[8] There is a possibility that reverse transcription of the HDV genome and hence assay performance is impacted by the circular genome, causing steric interference of the reverse transcriptase, for example. Also, the robust secondary structure necessitates an initial heating step prior to reverse transcription to improve efficiency.[39] The 85-nucleotide autocatalytic ribozyme region is considered highly conserved, but like the rest of the genome has variable regions, as determined by quasispecies analysis.[40] In the study by Le Gal et al[21] evaluating assay performance across different laboratories, most laboratories used the HDV RNA ribozyme region as the preferred target of real-time NAT for HDV RNA because of its high degree of conservation.

Approaches to standardization

The WHO HDV RNA international standard is an important tool for comparing assay performance across laboratories and clinical trial study sites, enabling data reporting in IU/mL. The use of automation and a separate internal control may help improve reliability and reproducibility. While underestimation of HDV RNA levels has been seen for assays employing automation for viral RNA extraction,[28,37] use of a fully automated platforms provides promise for improved sensitivity and consistency.[41,42] Ideally, the internal control should be extracted and amplified alongside the viral RNA, and be a consistent concentration across tests. Studies using housekeeping genes such as ribosomal RNA and β-actin have shown concentration variability across clinical samples, and freely circulating GAPDH may be in more of a DNA form than RNA.[43] One option to overcome concentration differences is to include unrelated viral RNA, *in vitro* transcribed RNA, or armored/synthetic RNA in the initial extraction step, amplified using primer/probes which do not bind HDV RNA.

HDV RNA limits of detection and quantitation

The lack of consistency with respect to assay sensitivity, and how negative data are documented, confounds the interpretation of HDV RNA data from different laboratories. The standard definition of the LoD of a quantitative assay is the lowest concentration at which 95% of all replicates test positive. Hence, data reported as below the LoD do not necessarily mean an absence of the analyte, because the test result may still be “target detected (TD).” In addition, HDV RNA below LLoQ cannot be reliably quantified and thus it is not possible to determine if HDV RNA levels are greater than, equal to or below the LoD (if LLoQ is, as commonly/frequently the case, greater than LoD). For this reason, HDV RNA levels, which are below the linear range of the assay (LLoQ) should be reported as either <LLoQ (TD) or (TND).[44] An example of interpreting HDV RNA levels according to a lab report is found in Table 1. For the purposes of data analysis, for example, calculation of change from baseline, HDV RNA levels below LLoQ of the assay but “TD” are often “imputed” as half the LLoQ, and “undetectable” test results as 0 or LLoQ/4, for example; however, these imputations are applied for practical purposes and should be interpreted accordingly.

One of the most adequate methods of LoD determination for NATs is the probit regression model when testing serial dilutions close to the expected sensitivity limit, as described in Section 5.5 CLSI guideline EP17-A2.[45] In contrast, the LLoQ is defined as the lowest concentration of analyte that can be quantitatively measured with known confidence (also referred to as Total Analytical Error, see CLSI EP21-A)[46] including potential measurement bias and imprecision. Since the LoD is established at 95% detection probability and up to 5% anticipated negative results, it is standard practice to define the LLoQ at a slightly higher concentration level than the LoD with a detection probability closer to 100%.

TECHNICAL PERFORMANCE SUMMARY OF NATs FOR HDV RNA QUANTITATION

NATs for detection and quantification (quantitative RT-PCR) of HDV RNA were first described in 2004.[47] Since then, many reports of laboratory-developed tests (LDT) have been published (Table 2). [22,39,41,43,52–55,57–60,62,64–67] The methods described generally include a procedure for (1) RNA extraction; (2) reverse transcription and quantitative RT-PCR, that is, specification of primer and probe sequences, reaction (buffers, enzymes, etc.), and thermal cycling conditions; and (3) data analysis to calculate a copy number or RNA levels in the sample. In some cases, digital PCR was used instead of RT-qPCR.[64,65]

Published primer and probe sequences can be compared to newly described HDV RNA sequences for recognizing and correcting potential mismatches. However, assay validation and quality control of reagents such as primers, probes, enzymes, and extraction reagents must be performed by each laboratory, requiring considerable resources. Since the sensitivity and accuracy of the result will depend on the entire process including RNA extraction, reverse transcription conditions, amplification and normalization methods, assays that encompass all steps from sample to result in a defined and controlled workflow are needed to decrease interlab variability in assay performance (especially accuracy and sensitivity). The use of highly automated molecular testing platforms,[41,54] with built-in internal controls, IVD grade reagents and fixed extraction methods have the potential to overcome some of the issues of traditional LDT workflows.

In recent years, several commercial entities have developed products that can be used as part of an assay for quantitation of HDV RNA levels (Table 2). These products are provided as HDV RNA quantitation kits that include RT-qPCR primers and probes, as well as the reagents needed and instructions for use. However, nucleic acid extraction reagents or procedures are usually not included, users may use several instrumentation platforms for nucleic acid extraction, thermal cycling and detection, and post-testing calculations. Since these essential steps can strongly influence assay sensitivity, precision, and accuracy,[28] it should not be surprising that comparative studies of assay performance have revealed unacceptable levels of variability for clinical applications.[19,21,37] This variation can exist even if different labs use the same RNA quantitation kit and results are normalized to the WHO HDV RNA international standard and when some internal controls are included.[37] In contrast to LDTs, manufacturers of commercial kits must monitor reagent quality carefully and provide control materials, but primer and probe sequences are usually proprietary and not disclosed. As a result, users cannot independently monitor emerging inclusivity issues related to rare genotypes or sequence variations. Data on real-world performance of commercial assays are scarce but include the RoboGene HDV RNA Quantification Kit 2.0,[37,49] Diapro HDV Quantitation Real-Time PCR kit,[19] and EurobioPlex HDV kit.[48]

Ideally any NAT for HDV RNA should detect and accurately quantify all eight HDV genotypes and therefore should target highly conserved portions of the genome. All of the molecular assays published or on the market target either the ribozyme or the delta antigen —85% of the positions within the ribozyme exhibit 100% conservation.[40]

Analytical sensitivity of commercial and research-based quantitative HDV RNA assays reporting results in IU/mL varies widely (Table 2). Three published research-based assays with input volumes of serum or plasma ranging from 0.4 mL to 1 mL have well-described analytical sensitivity studies and associated assay LoDs in the range 3.9–9.2 IU/mL. Among the 4 CE-IVD HDV RNA assays in Table 2, only the Robogene HDV kit reports an assay LoD in the range of these research assays (6–14 IU/mL, depending on instrument). The Robogene assay is the only assay of the CE-IVD and research-based assays in Table 3 reporting LoD from a probit analysis of a HDV dilution series in serum or plasma. The EurobioPlex HDV kit EBX-004 instructions for use describes a dilution series study with limited replicates that can be used to roughly estimate the LoD of the assay at around 100 IU/mL. A new version, EBX-071, has recently been developed and is in the final validation phase. In addition to major improvements for routine use, the kit’s standard range has been recalibrated in line with the WHO standard. This is an essential step towards universal reporting of viral load results (E. Gordien, personal communication, abstract submitted to the American Association of the Study of the Liver, AASLD 2023). The remaining CE-IVD and research use only assays do not describe studies from which LoDs were determined or do not even report LoD. As discussed above, LoD and the LLoQ are important analytical performance characteristics needed to understand an assay’s capabilities and limitations and to ensure “fit for purpose.”[68]

A comprehensive list of HDV RNA quantitative assays both commercial or LDT is presented in Table 2. While the characteristics of an “ideal” assay will depend on available equipment, human resources, local regulation, and numbers of samples to be processed at a given time, we recommend that only assays or products that include the following components be considered for drug development and/or clinical applications:

- Quantification methods normalized (calibrated) to the WHO HDV RNA international standard for viral load monitoring purposes.[69]
- An internal control that can be used to monitor the entire process including RNA extraction and quantitation.
- Cover all 8 known HDV genotypes with similar analytic sensitivity and quantitative accuracy.

Detailed performance characteristics for assays and products fulfilling these criteria, based on publicly available manufacturer data or publications, are summarized in Table 3.

FUTURE STUDIES, PERSPECTIVES, AND RECOMMENDATIONS

For many chronic viral infections, fully automated quantitative NATs which are Food and Drug Administration (FDA) approved or CE marked or WHO-prequalified, are commercially available. However, for HDV RNA detection and quantification, different laboratory-developed assays are typically used and no FDA-approved assay is available. In recent years, more quantitative assays have been developed and implemented at specialized laboratories and are being used to monitor treatment effects and to perform studies to assess the correlation of HDV RNA levels/declines with clinical outcome.

Most data available from validation studies and the use of HDV RNA assays are for HDV-1, which also appears to be the most prominent genotype in clinical studies to date. The lack of sufficient and accessible non-HDV-1 samples limits the evaluation of performance characterization of the HDV RNA assays to a few non-HDV-1 patient samples and *in vitro* transcribed non-HDV-1. A widely accessible repository of non-HDV-1 HDV samples would facilitate the comprehensive cross-genotype characterization of the available and new HDV RNA assays, which could become key for use in countries with a high prevalence of non-HDV-1.

The achievement of suppressed HDV RNA levels, ideally “undetectable” or TND, is considered a desirable endpoint, especially for off-treatment responses.[30,34] However, it remains unclear if an undetectable HDV RNA level, as determined with the most sensitive assay, is needed for improved clinical outcome, or if suppressing to a low but not undetectable level, preferably in combination with normalization of hepatic transaminase levels, can provide similar benefit. Of note, in other chronic viral infections, such as HIV and HBV infections, achieving viral suppression below specific thresholds (rather than undetectable) is sufficient for clinical benefits.[70,71] Future studies may assess if achieving undetectable HDV RNA levels with the most sensitive assays has clinical benefit compared with HDV RNA levels below a certain threshold (eg, LLoQ or 100 IU/mL) for long-term suppressive treatment and/or predicting off-treatment sustained response versus HDV RNA relapse.

To facilitate the comparison of clinical data from different studies, the LLoQ and LoD of the assay used should be described with consistent nomenclature applied. In addition, for the main analyses describing the proportion of patients with HDV RNA levels below LLoQ, subanalyses may explore proportion of patients with HDV RNA levels below LLoQ (TD) or

HDV RNA levels below LLoQ (TND). Because multiple different HDV RNA assays with different performance characteristics are being used, studies comparing these assays will be instructive.

No rapid HDV RNA test is currently available as is the case for several other viruses. A rapid HDV RNA assay would be very useful to guide patient management and treatment in particular in resource-limited settings. A rapid point-of-care test for the serodiagnosis of HDV infection has recently been developed.[72] However, for a rapid HDV RNA test similar challenges and performance requirements as for "regular" assays would apply.

Lastly, with novel HDV drugs being approved or in clinical studies, recommendations will be needed to guide the efficient use of HDV RNA assays for patient monitoring during treatment with these drugs (Table 4).

In conclusion, a wide range of quantitative HDV RNA assays is currently in use and improved versions are expected to become available in the future. Understanding the performance and the comparability of these assays will be crucial for the development and use of novel anti-HDV drugs.

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Abbreviations:

CE-IVD	conformité européenne *in vitro* diagnostic
CHD	chronic hepatitis D
ddPCR	droplet digital PCR
FDA	Food and Drug Administration
GT	genotype
HDAg	hepatitis D antigen
IC	internal control
IFU	instructions for use
IVDR	in vitro diagnostic regulation
IVT	*in vitro* transcription
LDT	laboratory-developed test
LLoQ	lower limit of quantitation

LoD	limit of detection
NAT	nucleic acid test
qPCR	quantitative PCR
RT-PCR	real-time PCR
RUO	research use only
TD	target detected
TND	target not detected
ULoQ	upper limit of quantitation
WHO	World Health Organization
WHO IS	World Health Organization International Standard

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KEY POINTS

- Clinical development of treatments for chronic hepatitis D will rely on standardized, reliable, and accurate assays for the diagnosis and monitoring of HDV RNA levels in response to treatment.
- Development of reliable HDV RNA assays is challenging due to distinct virologic characteristics of HDV, resulting in substantial variability in performance characteristics of existing research-based and commercial HDV RNA assays.
- We summarize available research-based and commercial HDV RNA assays, outline their performance characteristics, and discuss technical features that are sources of assay variability.
- Finally, we provide considerations for the use of HDV RNA assays in the context of drug development and patient monitoring of HDV RNA levels in response to treatment.

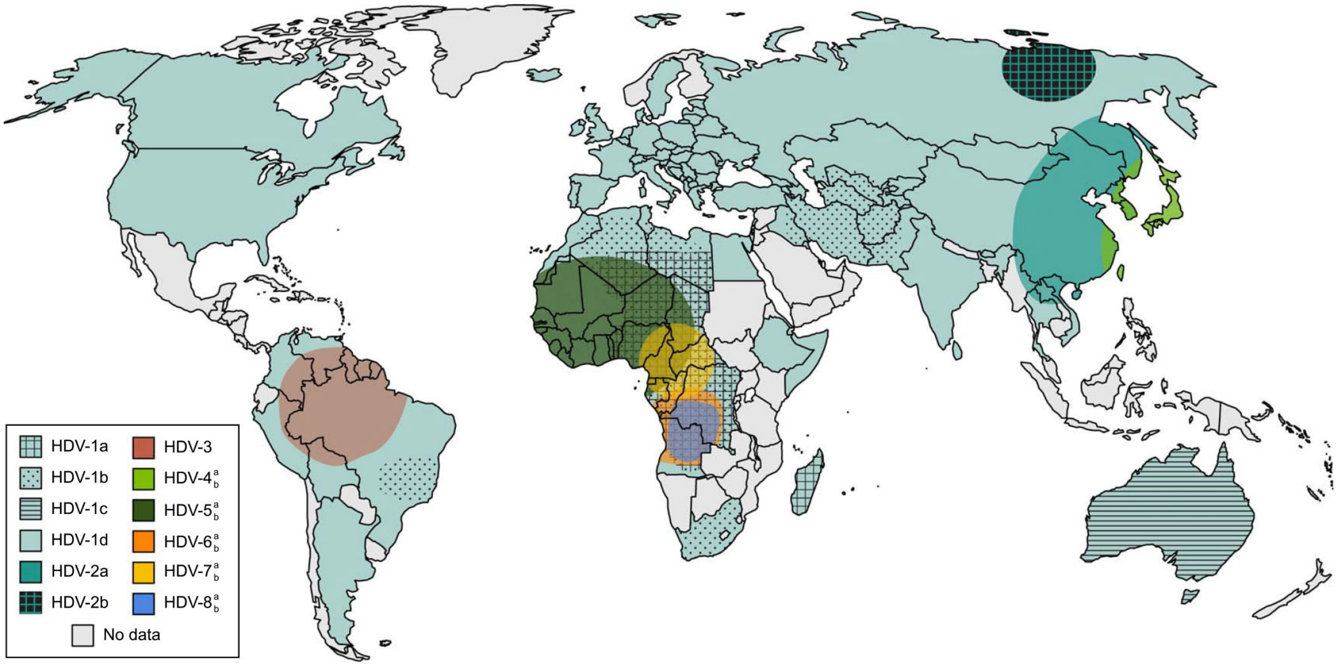

FIGURE 1.

Worldwide distribution of HDV genotypes and subgenotypes. Countries harboring HDV-1 are in pale teal. HDV-1a and HDV-1b subgenotypes are localized to Africa and Madagascar, and HDV-1c to the Oceania islands, whereas HDV-1d is found in the Middle East, eastern and western Europe, Asia, and North America. HDV-2 (teal) is found in Asia, including in Taiwan and Japan for HDV-2a and Siberia for HDV-2b. HDV-3 (red) is present in the north of South America. HDV-4a and HDV-4b (light green) circulate in the Far East. HDV-5, HDV-6, HDV-7, and HDV-8 (dark green, orange, yellow, and blue, respectively) are present in western, sub-Saharan, and central Africa. Grey areas indicate regions where no data are yet available.

TABLE 1

Interpretation of HDV RNA levels according to lab report

As example, we considered a putative assay with LoD = 10 and LLoQ = 100 IU/mL			
HDV RNA levels	**HDV RNA limits**	**HDV RNA interpretation**	**Comments**
Below LLoQ	< 100 IU/mL	Low positive viremia [below 100 IU/mL but not quantifiable, ie, HDV RNA target detected (TD)], or negative for viremia [ie, HDV RNA target not detected (TND)]	The LLoQ depends on the assay’s performance characteristics. A result of below LLoQ includes both low level viremia and negative viremia test results
Below LoD	Interpreted as <10 IU/mL	Low positive viremia (not quantifiable) or negative for viremia	Below LoD is not recognized by regulatory agencies as the assay cannot determine the concentration for samples <100 IU/mL, ie, the concentration could be <10 IU/mL or between 10 and 100 IU/mL
TND	Undetectable	Undetectable	No virus (HDV RNA) detectable in sample (ie, “negative”). This result is frequently/ sometimes referred to HDV RNA <LLoQ TND

Abbreviations: LLoQ, lower limit of quantitation; LoD, limit of detection; TD, target detected; TND, target not detected.

TABLE 2

Comprehensive list of published methods (LDTs) and RNA quantitation kits

Assay name	Manufacturer/provider	Type	Source of data	Reference(s)	Technique	Calibrated to WHO international standard
EurobioPlex HDV Kit EBX 004	Eurobio Scientific	Commercial kit	Le Gal (2017)	[48]	qPCR	Yes
RoboGene HDV RNA Quantification Kit 2.0	Roboscreen GmbH	Commercial kit	Wang (2018); IFU/website (2020)	[37,49]	qPCR	Yes
AltoStar HDV RT-PCR Kit 1.5	altona Diagnostics GmbH	Commercial kit	Supplier		qPCR	Yes
RealStar® HDV RT-PCR Kit 1.0 RUO	altona Diagnostics GmbH	Commercial kit	Supplier		qPCR	Yes
AmpliSens HDV-FRT	Federal Budget Institute of Science	Commercial kit	IFU/website		qPCR	Yes
Fluorion HDV QNP 2.1 Real-Time PCR Kit	Iontec	Commercial kit	IFU/website		qPCR	Yes
SYSTAAQ HDV Real-Time PCR Kit	SYSTAAQ	Commercial kit	IFU/website		qPCR	Yes
LIPSGENE HDV Kit	VL-Diagnostics GmbH	commercial kit	IFU/website		qPCR	Yes
Bosphore HDV Quantification-Detection Kit v1	Anatolia Geneworks	commercial kit	IFU/website		qPCR	No
genesig Real-time PCR Detection Kitfor HDV	Primerdesign	Commercial kit	Supplier		qPCR	No
HDV Real-TM Qual Real-time PCR Test	Sacace Biotechnologies	Commercial kit	IFU/website		qPCR	No
LightMix Kit HDV[a]	TibMolBiol/Roche	commercial kit	IFU/website		qPCR	No
HDV Real-time RT-PCR Kit	Creative Biogene	commercial kit	IFU/website		qPCR	Unknown
HDV Quantitation Real-Time PCR kit	Dia.Pro Diagnostic Bioprobes s.r.l	Commercial kit	IFU/website		qPCR	Unknown
ViroReal Kit HDV[a]	Ingenetix	Commercial kit	IFU/website		qPCR	Unknown
HDV Real-Time RT-PCR Kit	Liferiver	Commercial kit	IFU/website		qPCR	Unknown
Hepatitis Delta virus One-Step RT-qPCR Kit[a]	NZYTech	Commercial kit	IFU/website		qPCR	Unknown
PCRmax LtdTM qPCR test Hepatitis Delta	PCRmax	Commercial kit	IFU/website		qPCR	Unknown
Hepatitis Delta Virus by Quantitative PCR	ARUP	LDT	Website		qPCR	Yes
HDV RNA, Quantitative Real-Time PCR	Quest Diagnostics	LDT	Website/coauthor		qPCR	Yes
NA (LDT)		LDT	LeGal (2005)	[50,51]	qPCR	No
NA (LDT)		LDT	Tseng (2008)	[52]	qPCR	No
NA (LDT)		LDT	Hofmann (2010)	[53]	qPCR	No
NA (LDT)		LDT	Mederacke (2010)	[54]	qPCR	No
NA (LDT)		LDT	Schaper (2010)	[22]	qPCR	No
NA (LDT)		LDT	Ferns (2012)	[55]	qPCR	No

Assay name	Manufacturer/provider	Type	Source of data	Reference(s)	Technique	Calibrated to WHO international standard
NA (LDT)		LDT	Scholtes (2012)	[56]	qPCR	No
NA (LDT)		LDT	Shang (2012)	[57]	qPCR	No
NA (LDT)		LDT	Katsoulidou (2013)	[58]	qPCR	No
NA (LDT)		LDT	Kodani (2013)	[59]	qPCR	No
NA (LDT)		LDT	Botelho-Souza (2014)	[60]	qPCR	No
NA (LDT)		LDT	Karataylı (2014)	[61]	qPCR	No
NA (LDT)		LDT	Coller (2018)	[62]	qPCR	Yes
NA (LDT)		LDT	Pflüger (2021)	[63]	qPCR	Yes
NA (LDT)		LDT	Olivero (2022)	[64]	ddPCR/qPCR	Yes
NA (LDT)		LDT	Xu (2022)	[65]	ddPCR	

[a] No longer available.

Abbreviations: ddPCR, droplet digital PCR; IFU, instructions for use; LDT, laboratory-developed test; qPCR, quantitative PCR; RT-PCR, real-time PCR; RUO, research use only; WHO, World Health Organization.

TABLE 3

Detailed performance characteristics for assays and products calibrated to WHO IS, detect GT1–8, and used IC for RNA extraction

Assay name	Manufacturer/ provider	Target site	LoD (IU/mL)	LLoQ (IU/mL)	ULoQ (IU/mL)	Regulation	RNA extraction method	Detection equipment (cycler)	Comments
EurobioPlex HDV	Eurobio Scientific	HDAg	10	562	3.16E+08	CE-IVD	m2000sp	CFX96	
RoboGene HDV RNA Quantification Kit 2.0	Roboscreen GmbH	HDAg	6	60	1.00E+08	CE-IVD	Instand Virus RNA/DNA Kit	Several options	
HDVQNP2.1 Real-Time PCR Kit	Iontec	Proprietary	400	1000	1.00E+10	CE-IVD	Fluorion i12, i24/i12 Kit	Several options	*no information on GT8
AltoStar HDV RT-PCR Kit 1.5	altona Diagnostics	Proprietary	< 10*	100*	1.00E+06	RUO**	AltoStar AM16r	CFX96	*Still under verification. **The kit is CE-IVDR ready
RealStar HDV RT-PCR Kit 1.0	altona Diagnostics	Proprietary				RUO	Several options	Several options	LoD depend on extraction/detection method
SYSTAAQ HDV Realtime PCR Kit	SYSTAAQ	Proprietary	10	10	8.00E+06	RUO	Several options	Not specified	LoD depend on extraction/detection method
HDV RNA, Quantitative Real-Time PCR	Quest Diagnostics	Proprietary	5	40	1.00E+07	LDT	MagNA Pure 96	ABI 7500	
Pflüger (LDT)		Ribozyme	3.9	10	1.00E+08	LDT	cobas 6800	cobas 6800	
Olivero (LDT)		Ribozyme	9.2	10	1.00E+06	LDT	EZ1 Advanced XL	CFX96 QX200	

Abbreviations: CE-IVD, conformite europeenne *in vitro* diagnostic; GT, genotype; HDAg, hepatitis D antigen; IC, internal control; IVDR, in vitro diagnostic regulation; LDT, laboratory-developed test; LLoQ, lower limit of quantitation; LoD, limit of detection; RT-PCR, real-time PCR; RUO, research use only; ULoQ, upper limit of quantitation; WHO IS, World Health Organization International Standard.

TABLE 4

Challenges and recommendations for the development of quantitative HDV RNA assays

Challenges	Recommendations
HDV RNA sequence variability	
Primary sequence and secondary structures vary considerably between and within genotypes. There are replicative and defective quasispecies in clinical samples	Primer/probe design for RT-PCR assays should focus on highly conserved regions, and assay validation should assess geographically and temporally diverse clinical isolates (eg, at least 10–20 isolates), rather than cDNA or IVT RNA which do not have the same degree of secondary structure
Sequence data of HDV are limited (especially non-HDV-1), complicating the primer/probe design for RT-PCR assays	HDV sequencing should be performed consistently in clinical trials and epidemiological studies to increase the available sequence data
Long-term studies may be confounded by natural sequence variation, potentially impacting primer/probe binding affinity	Longitudinal studies should assess the primer/probe binding regions over time to monitor for sequence changes
Assay platforms and validation	
Different assays with different performance characteristics are used across laboratories and trials	Assays should use the WHO international standard for validation, and an internal RNA control of known concentration at the RNA extraction stage, with primers/probe distinct from those used for HDV RNA. Additional cross trial/lab studies should be performed to allow comparison of results from clinical trials and to provide guidance for the use of these assays for patient management. Clinical trials and patient management should use a central laboratory with a validated assay (FDA approved/CE marked if available)
Assay performance data in non-HDV-1 are limited and difficult to generate given scarcity of non-HDV-1 samples	Non-HDV-1 *in vitro* transcribed RNA can be used for assay characterization, with the caveat that it lacks the secondary structures associated with viral RNA and therefore may have limited accuracy with respect to assay sensitivity and linearity. A repository of non-HDV-1 samples for assay characterization and validation would be valuable to the scientific community
Manual RNA extraction has been reported to be more sensitive than automated procedures but is prone to higher variability and is more labor intensive	Automated assays, ideally on standard platforms, should be developed/ used as much as possible
Data interpretation	
Use of assays with different performance characteristics complicates data interpretation	Data should be reported in IU/mL. When selecting an assay platform, the sensitivity and specificity should be reported, and preferably these should be comparable to the best performing assays available
Clinical relevance of undetectable vs. detectable HDV RNA is uncertain	Clinical studies are needed to assess threshold of HDV RNA for longterm clinical outcomes (suppressive therapy) and/or viral relapse (finite therapy)
Different ways to report HDV RNA values below LLoQ are used	Use consistent nomenclature to report HDV RNA values below the quantitative range (below LLoQ): ie, data should be reported as either below LLoQ, target detected or below LLoQ, target not detected. Use of reporting below LoD[a] should be avoided because it incorrectly implies virus absence
For novel treatments, there is no clear guidance/consensus on frequency of HDV RNA testing during treatment and during follow-up	Guidelines will need to be developed considering resource-limited regions

[a] Limit of detection (LoD) is defined as the lowest concentration at which ≥ 95% of replicates test positive.

Abbreviations: CE, *conformite europeenne;* FDA, Food and Drug Administration; IVT, *in vitro* transcription; LLoQ, lower limit ofquantitation; LoD, limit ofdetection; RT- PCR, real-time PCR; WHO, World Health Organization.