The challenges for HDV testing

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INSERM
• Assays for HDV RNA monitoring
  ➢ Various assays
  ➢ Extraction methods
  ➢ Amplification methods
  ➢ HDV genotypes

• Screening for HDV antibodies
  ➢ Reflex testing
  ➢ Point of care tests
HDV RNA quantification and HDV genotype heterogeneity

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Commercial Real-Time Reverse Transcriptase PCR Assays Can Underestimate or Fail to Quantify Hepatitis Delta Virus Viremia

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BACKGROUND & AIMS: Hepatitis delta virus (HDV) infection causes fulminant hepatitis and increases the severity of chronic hepatitis B virus infection, leading to cirrhosis, liver failure, or hepatocellular carcinoma. There are 8 HDV genotypes (genotypes 1–8). We previously developed a TaqMan real-time reverse transcriptase (RT)-PCR method that is able to quantify viral load of all HDV genotypes (linear from 2 to 8 log_{10} copies/mL). We compared its results with those from 3 commercial real-time RT-PCR assays: the Lightmix HDV kit (designed to quantify HDV genotype 1 [HDV-1]), and the ReheGene and the DiaPro HDV RNA quantification kits (designed to quantify all genotypes).

METHODS: We selected RNA from 128 clinical samples of all HDV genotypes except HDV-4, with various HDV viral load values. We also analyzed 5 samples, collected over time, from each of 6 patients infected with strains of different genotypes.

RESULTS: Quantification results from the commercial kits for HDV-1 from European or Asian samples were consistent with those from our method, however, they underestimated (0.5–1 log_{10}) with Lightmix and DiaPro) and did not detect (1 and 4 samples with Lightmix and DiaPro, respectively) HDV-1 African samples. Moreover, the commercial kits greatly underestimated HDV viral load of almost all non-genotype-1 strains (about 2–3 log_{10}), and even did not detect HDV-7 or HDV-8 RNA in several samples with high concentrations of virus.

CONCLUSIONS: Commercial kits accurately quantify HDV-1 in samples from European and Asian patients. However, they can dramatically underestimate or fail to quantify HDV viral load from samples from African patients infected with strains of genotypes 1 and 5 to 8.
Figure 1. HDV RNA VL quantification by the NRC-HDVQ technique. Results for (A) all 128 samples according to HDV genotype and (B) the 66 HDV-1 samples according to the European/Asia or African origin of the patients. The box plots display the median values (thick line inside boxes) and the 25th and 75th percentiles (lower and upper limits of boxes).

Figure 3. Performances of the commercial assays for quantification of HDV African strains. Results of quantification of African strains: (A) 15 HDV-5, (B) 16 HDV-6, (C) 17 HDV-7, and (D) 11 HDV-8 by the NRC-HDVQ technique and the 3 commercial assays are presented. The box plots display the median values (thick line inside boxes) and 25th and 75th percentiles (lower and upper limits of boxes).
Original article

Automated nucleic acid isolation methods for HDV viral load quantification can lead to viral load underestimation

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Background: HDV infection is a cause of severe liver disease. Diagnosis and monitoring of HDV RNA are important to patient management. Since 2012, a WHO standard for HDV RNA quantification has been available; however, the impact of RNA extraction methods on HDV viral load quantification has never been evaluated.

Methods: The aim of this study was to compare four commonly used automated nucleic acid (NA) extraction methods (AmplicPrep, MagNA Pure, QiAcube QBK and QiAcube VRK) with a manual NA extraction method (Instant Virus RNA/DNA kit) and evaluate the possible effect of each method on HDV RNA yield with subsequent amplification with the Robogene HDV assay. Serum samples from HDV-positive patients taken before treatment with pegylated interferon-α2a and at treatment weeks 12 and 48 were studied.

Results: The automated extraction methods MagNA Pure, AmplicPrep and QiAcube VRK extraction led to about 10-fold lower HDV RNA values compared with the manual method of NA extraction, while the difference was smaller with QiAcube QBK (about 6-fold lower). The median viral load was 10,665 IU/ml for the manual method, 445 IU/ml for AmplicPrep, 3,209 IU/ml for MagNA Pure, 2,060 IU/ml for QiAcube QBK and 3,508 IU/ml for QiAcube VRK. Use of MagNA Pure led to misclassification of two on-treatment samples with low viral load as being false negative.

Conclusions: The NA extraction method had a significant impact on the measured HDV viral loads determined by the commonly used Robogene assay, with the manual RNA method yielding consistently higher values of viral load. ClinicalTrials.gov Identifier: NCT00932971.

Figure 1. HDV quantification results of automatically extracted NA significantly underestimated the viral load, when compared with the results of the manual extraction.

Nucleic acid (NA) extraction was performed using the following methods: AmplicPrep, MagNA Pure, QiAcube QBK, QiAcube VRK, Instant Virus RNA/DNA kit. The viral loads were compared using the Wilcoxon matched-pair signed-rank test. HDV, hepatitis D viral load.
Reliable quantification of plasma HDV RNA is of paramount importance for treatment monitoring: A European multicenter study

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A R T I C L E   I N F O

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A B S T R A C T

Objective: Quantification of plasma hepatitis D virus (HDV) RNA is the essential tool for patient management under antiviral therapy. The aim of this European multicenter study was to improve the comparability of quantitative results reported by different laboratories using the CE/IVD-labeled RoboGene HDV RNA Quantification Kit 2.0 (Robocore GmbH) with different manual or automated nucleic acid extraction protocols/platforms and amplification/detection devices.

Methods: For harmonization of HDV RNA concentrations obtained by different protocols, correction factors (CF) were determined using the 1st WHO International Standard for HDV RNA. The limit of detection (LOD) and accuracy were determined for each protocol by using reference material. Furthermore, clinical samples were analyzed and results compared.

Results: The CF ranged from 20 to 1,870 depending on the protocol used. The LOD was found between 4 and 450 IU/ml. When accuracy was tested, external quality control (EQC) samples containing low HDV RNA concentrations were not detected by those protocols with higher LODs. For EQC samples, the maximum standard deviation of HDV RNA concentration was found to be 0.53 log10 IU/ml for clinical samples 0.87 log10 IU/ml.

Conclusions: To ensure reliability in quantification of HDV RNA, any modification of the extraction and amplification/detection protocol validated by the manufacturer requires revalidation. With the 1st WHO International Standard for HDV RNA, the CF could easily be calculated leading to harmonization of quantitative results. This warrants both accurate monitoring of response to existing anti-HDV treatment and comparability of study results investigating novel anti-HDV drugs.

Fig. 1. Quantitative results of accuracy testing utilizing the Quality Control for Molecular Diagnostics 2013 Hepatitis D Virus EQA Program 2013 (designation of panel members according to the EQA program). Correction factors were applied for all quantifications.

Fig. 2. Quantitative results of patient sample testing. Correction factors were applied for all quantifications.
Comparisons of three HDV-RNA quantitative commercially available tests in untreated and in mycophenolate-M treated patients with HDV related chronic hepatitis in a real-life setting

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Background and Aim: As new antiviral (HCV) therapies are being developed, highly sensitive and reliable quantitative tests are needed. Aim of the study was to compare three commercially available HDV RNA kits. Methods: 52 serum samples from 4 categories of patients were tested for HDV RNA by assays: Robogene (HDV-RNA quantification. 2.0, Aj-Robocare, Germany, LLOQ 6 IU/ml); Eurobioflex (HDV-qRT-PCR, Eurobio, France, LLOQ 100 IU/ml) and DiaPro (HDV-RNA Quantifications, Dia.Pro Diagnostic Bioprobes, Italy, 50 IU/ml). Total RNA was extracted by QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany).

Results: Group 1: 116 Caucasian with known active HDV-hepatitis (50 years, 56% males, 73% cirrhosis, 69% on tenofovir/entecavir, 50% previously interferon-exposed). ALT 66 (24-304) IU/L had a median HDV-RNA of 5.5 (1.1-7.1), 5.9 (0.8-4), 3.9 (0.6-6) log IU/ml by Robogene, Eurobioflex and DiaPro, respectively. Group 2: 15 HBsAg-positive patients (age 42, 27% cirrhosis, 7% HBVAg positive, 40% on tenofovir/entecavir, 76% undetectable HDV-DNA, 3 with abnormal ALT) had HDV-RNA undetectable with all 3 assays except for one subject who had HDV-RNA 697 IU/ml with DiaPro. Group 3: 15 with the same 3 unselected patients. The only true negative sample tested negative by all the 3 assays, while the 8 positive controls were correctly identified in 100%, 87.5% and 25% of the cases by different assays. Group 4: 21 sera collected during Mycophenolate-B treatment. First patient: baseline HDV-RNA was 29,000, 1,400,000, 12,283 IU/mL during therapy, RNA progressively declined with both Robogene and Eurobioflex, till undetectability at week 36; DiaPro gave 5 false-negative results. Second patient: baseline HDV-RNA 392,000, 4,248,000, 1,140 IU/ml; RNA declined with the first two assays but not with the last one (2 false-negative results). Overall, the HDV-RNA undetectable was 100%, 65% and 86%, respectively. Conclusions: Robogene is the most sensitive and reliable test for HDV-RNA quantification.

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Clinical Application of Droplet Digital PCR for Hepatitis Delta Virus Quantification

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Abstract: Droplet digital PCR (ddPCR) is a novel developed PCR technology providing the absolute quantification of target nucleic acid molecules without the need for a standard curve and regardless PCR amplification efficiency. Our aim was to develop a ddPCR assay for Hepatitis Delta virus (HDV)-RNA viremia quantification and then evaluate its performance in relation to real-time PCR methods. Primers and probe were designed from conserved regions of HDV genome to detect all the 8 HDV genotypes; the World Health Organization (WHO)-HDV international standard was used to calculate the conversion factor transforming results from copies/mL to IU/mL. To evaluate the clinical performance of ddPCR assay, plasma specimens of HDV-infected patients were tested and results were compared with data obtained with two real-time quantitative PCR (RT-qPCR) assays (i.e., in-house assay and commercial RoboGene assay). Analyzing by linear regression a series of 10-fold dilutions of the WHO-HDV International Standard, ddPCR assay showed good linearity with a slope coefficient of 0.966 and R2 value of 0.980. The conversion factor from copies to international units was 0.97 and the quantitative linear dynamic range was from 10 to 1 × 108 IU/mL. Probit analysis estimated at 95% an LOD of 9.2 IU/mL. Data from the evaluation of HDV-RNA in routine clinical specimens of HDV patients exhibited strong agreement with results obtained by RT-qPCR showing a concordance correlation coefficient of 0.95. Overall ddPCR and RT-qPCR showed highly comparable technical performance. Moreover, ddPCR providing an absolute quantification method may allow the standardization of HDV-RNA measurement thus improving the clinical and diagnostic management of delta hepatitis.

Keywords: chronic hepatitis D; ddPCR; HDV

Figure 3. Scatter plot with regression lines between nominal and measured concentration of a 10-fold dilution series of WHO-HDV international standard using real-time qPCR (A) and ddPCR (B). Three replicates were performed per dilution. Abbreviations: hepatitis D virus (HDV); World Health Organization (WHO).

Figure 5. Probit analysis of HDV-RNA amplification data obtained by RT-qPCR assay (A) and ddPCR assay (B) for the estimation of the 95% and 50% limit of detection (LOD). Probit curves are depicted in blue; the two additional red dashed curves represent the 95% confidence interval for each probit curve. Abbreviations: hepatitis D virus (HDV); World Health Organization (WHO).
Figure 8. Deming regression analysis of HDV-RNA viral loads of 20 clinical specimens determined by ddPCR and RoboGene HDV RNA Quantification Kit 2.0.

Figure 9. Histogram showing the comparison of HDV-RNA values obtained by RT-qPCR, ddPCR and RoboGene HDV RNA Quantification Kit 2.0. Blue bars represent values obtained by RT-qPCR. Data obtained by ddPCR and RoboGene HDV RNA assay are represented by red and gray bars, respectively.

Figure 10. Comparison between kinetic patterns of HDV-RNA viral load measured by RT-qPCR (blue line) and ddPCR (red line) in longitudinal plasma samples from four patients (from A-D) treated with PEG-IFNα. HDV-RNA was evaluated at baseline and at months 6, 12 and 18 during treatment.
Development and performance of prototype serologic and molecular tests for hepatitis delta infection

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Worldwide, an estimated 5% of hepatitis B virus (HBV) infected people are co-infected with hepatitis delta virus (HDV). HDV infection leads to increased mortality over HBV mono-infection, yet HDV diagnostics are not widely available. Prototype molecular (RNA) and serologic (IgG) assays were developed for high-throughput testing on the Abbott m2000 and ARCHITECT systems, respectively. RNA detection was achieved through amplification of a ribozyme region target, with a limit of detection of 5 IU/ml. The prototype serology assay (IgG) was developed using peptides derived from HDV large antigen (HDAg), and linear epitopes were further identified by peptide scan. Specificity of an HBV negative population was 100% for both assays. A panel of 145 HBSAg positive samples from Cameroon with unknown HDV status was tested using both assays: 16 (11.0%) had detectable HDV RNA, and 23 (15.7%) were sero-positive including the 16 HDV RNA positive samples. Additionally, an archival serial bleed panel from an HDV superinfected chimpanzee was tested with both prototypes; data was consistent with historic testing data using a commercial total anti-Delta test. Overall, the two prototype assays provide sensitive and specific methods for HDV detection using high throughput automated platforms, allowing opportunity for improved diagnosis of HDV infected patients.
Clinical establishment of a laboratory developed quantitative HDV PCR assay on the cobs6800 high-throughput system

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Background & Aims: Currently available HDV PCR assays are characterized by considerable run-to-run and inter-laboratory variability. Hence, we established a quantitative reverse transcription real-time PCR (RT-qPCR) assay on the open channel of a fully automated PCR platform (cobs6800, Roche) offering improved consistency and reliability.

Methods: A primer/probe-set targeting a highly conserved region upstream of the HDV antigen was adapted for use on the cobs6800. The lower limit of detection (LLOD) was determined using a dilution panel of the HDV WHO standard (n = 21 dilution). Linearity and inclusivity were tested by preparing 10-fold dilution series of cell culture-derived virus (genotype GT 1-8; n = 5/dilution). Patient samples containing a variety of bloodborne viral pathogens were tested to confirm exclusivity (n = 60).

Results: The LLOD of the HDV utility-channel (HDV_UtC) assay was determined as 3.86 IU/ml (95% CI 2.95-5.05 IU/ml) with a linear range from 10-10.8 IU/ml (GT1). Linear relationships were observed for all HDV GTs with slopes ranging from -3.481 to -4.134 cycles/log and R² from 0.918 to 0.994. Inter-run and intra-run variability were 0.3 and 0.6 Ct (3xLLOD), respectively. No false-positive results were observed. To evaluate clinical performance, 110 serum samples of anti-HDV-Ab+ patients were analyzed using the HDV_UtC and CE-IVD RoboGene assays. 58/110 and 49/110 samples were concordant positive or negative, respectively (overall agreement 97.3%). Quantitative comparison demonstrated a strong correlation [R² 0.8733; 95% CI 0.8514-0.9695; p value <0.0001].

Conclusion: The use of highly automated, sample-to-result solutions for molecular diagnostics holds many inherent benefits over manual workflows, including improved reliability, reproducibility and dynamic scaling of testing capacity. The assay we established showed excellent analytical and clinical performance, with inclusivity for all HDV GTs and a limit of quantification of 10 IU/ml, making it a sensitive new tool for HDV screening and viral load monitoring.

Lay summary: The hepatitis delta virus (HDV) causes a severe form of inflammation in the liver. We developed a tool for molecular diagnostics, a polymerase chain reaction HDV assay that showed great performance. It can be used to improve diagnosis of HDV, as well as for monitoring treatment responses. The assay allows for quantification of the virus in the tested samples and is performed on a fully automated platform (cobs6800), which provides various benefits including less hands-on time and excellent comparability of test results.

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Fig. 1. Probit analysis and linearity for HDV genotype 1 and test results of the new HDV_UCT assay compared to the CE-trademarked IVD BoboCene assay. (A) A Probit analysis was computed based on the results of a 2-fold dilution panel of the WHO standard (n = 21 repeats/dilution). The plot is displaying the calculated LOD of 3.86 IU/ml (dotted green line) and the corresponding 95% CI 2.95–5.05 (dotted light green line) as well as the probit curve (green dashed line; 95% CI: light dashed green lines). The observed hit rates are marked by violet dots. (B) Displayed are the results (violet dots) of a 10-fold dilution panel of the WHO standard (GT1; n = 5 repeats/dilution). The dashed green line shows the linear regression line with a slope of -3.42 (R² 0.998). (C) Comparison between the test results (violet dots) of the HDV_UCT and the CE-IVD assays demonstrated a strong correlation (R² 0.8733; 95% CI 0.8014–0.9609; p value <0.0001). The linear regression line is plotted as a dashed green line (95% CI: light green dashed lines). Values are log-transformed. (D) Bland-Altman analysis based on the log-transformed test result (violet dots) in IU/ml of the HDV_UCT and the CE-IVD assays. The mean of the bias was calculated as -0.2997 (95% CI 0.014 to -1.291). Only 3 data points were detected lying outside of the margins of ± 1.96 SD. CE-IVD, CE-marked in vitro diagnostics assay; HDV_UCT, HDV utility-channel; LOD, lower limit of detection; WHO, world health organization.

Fig. 2. Linearity and Incluability of cell culture-derived HDV genotypes 1-6. A linear relationship was observed for all 8 genotypes with slopes ranging from -3.491 to -4.134. The linear regression line is plotted (dashed green line). Pearson’s correlation coefficients (R²) range from 0.994 to 0.998. HDV concentration in IU/ml were calculated based on the linearity experiments using the WHO standard 10-fold dilution series. (H) GT9; log transformed. Gray dots mark the values considered outside of the linearity range that were not included in the calculation of the slope. GT, genotype; HDV_UCT, HDV utility-channel; WHO, world health organization.
A Rapid Point-of-Care Test for the Serodiagnosis of Hepatitis Delta Virus Infection

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Abstract: Hepatitis Delta virus (HDV) is a satellite of the Hepatitis B virus (HBV) and causes severe liver disease. The estimated prevalence of 15–20 million infected people worldwide may be underestimated as international diagnostic guidelines are not routinely followed. Possible reasons for this include the limited awareness among healthcare providers, the requirement for costly equipment and specialized training, and a lack of access to reliable tests in regions with poor medical infrastructure. In this study, we developed an HDV rapid test for the detection of antibodies against the hepatitis delta antigen (anti-HDV) in serum and plasma. The test is based on a novel recombinant large hepatitis delta antigen that can detect anti-HDV in a concentration-dependent manner with pan-genotypic activity across all known HDV genotypes. We evaluated the performance of this test on a cohort of 474 patient samples and found that it has a sensitivity of 94.6% (314/332) and a specificity of 100% (142/142) when compared to a diagnostic gold-standard ELISA. It also works robustly for a broad range of anti-HDV titers. We anticipate this novel HDV rapid test to be an important tool for epidemiological studies and clinical diagnostics, especially in regions that currently lack access to reliable HDV testing.
Figure 2. Principle of the HDV rapid test. (A) Schematic of the lateral flow assay for the detection of anti-HDV in patient serum. (B) Proof-of-principle of the HDV rapid test using anti-HDV-positive and -negative patient sera.

Figure 3. Test validation of the HDV rapid test. (A) Characterization of test validation sera or plasma according to their pre-known anti-HDV status. (B) Test validation for anti-HDV ELISA. The sensitivity of the HDV rapid test was compared to the gold-standard ELISA. (C) Sensitivity and specificity of the HDV rapid test compared to the gold-standard ELISA. 

Figure 4. Pan-genotypic activity of the HDV rapid test and HBsAg multiplexing. (A) Pan-genotypic activity of the HDV rapid test. Anti-HDV-positive samples of patients that were infected with specific HDV genotypes were run on the HDV rapid test. Anti-HDV-negative samples of HBsAg-positive and -negative patients were used as controls. (B) Multiplexing of the HDV rapid test with the detection of HBsAg. Anti-HBsAg was spotted as a third line on the HDV rapid test for the detection of HBsAg in patient serum or plasma. The conjugate pad was treated with a mixture of gold-conjugated goat anti-human IgG and gold-conjugated mouse anti-HBsAg. The result of a single proof-of-principle experiment is shown.
HDV tests - Conclusions

• Monitoring of HDV viremia
  - Diagnosis of CHD
  - Monitoring of antiviral therapy
  - Need for robust quantitative assays

• Anti-HDV Ab testing
  - Reflex testing of HBsAg+ carriers
  - Point of care test for HBsAg and HDV

Better assays are needed for an optimal diagnosis and treatment management