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 log10 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 RoboGene 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 log10 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 log10), 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.

Keywords: Diagnosis; Test; Screen; Virology.

Hepatitis delta virus (HDV) is a 36-nm viral particle that depends on the hepatitis B virus (HBV) envelope for virion assembly and propagation. The HDV particle consists of the delta ribonucleoprotein, comprising a circular negative single-stranded RNA genome of 1672 to 1697 nucleotides, closely linked to the 2 viral proteins, the small and the large delta antigen, and an envelope comprising HBV proteins.

Molecular analyses of numerous isolates have led to the classification of the HDV genus into 8 distinct genotypes, with intergenotype divergence ranging from 20% over the whole genome nucleotide sequence, to as much as 40% for, in particular, HDV-3 compared with the other genotypes.

Genotypes have a specific geographic distribution: HDV genotype 1 (HDV-1) is ubiquitous; HDV-2 and HDV-4 are found in Far Eastern Asia; HDV-3 is found in northern South America, and HDV-5 to HDV-8 is found in western and central Africa. However, because of migration from endemic countries, all genotypes except HDV-4 are present and spreading in France and several other European countries.

HDV infection can cause severe liver disease, with fulminant hepatitis occurring at least 100 times more frequently than in HBV monoinfection. Chronicity rates reach 70% to 90% in superinfected patients. In many cases, chronic delta hepatitis evolves to cirrhosis (60%–70%) and hepatocellular carcinoma. In addition, the efficacy of the reference treatment, currently based on 12 (preferably 18) months of weekly pegylated interferon, is unsatisfactory.

The diagnosis of HDV infection relies on the detection of specific anti-HDV antibodies. All individuals exposed to HDV will be positive for IgG antibodies, which persist after viral

Abbreviations used in this paper: HBV, hepatitis B virus; HDV, hepatitis delta virus; HDV-1, hepatitis delta virus genotype 1; HDVQ, hepatitis delta virus quantification; LOQ, limit of quantification; NRC, National Reference Center; RT, reverse transcriptase; VL, viral load.
clearance. IgM antibodies persist in a large proportion of patients with chronic infection, and are considered by some investigators as a surrogate marker for HDV replication.\textsuperscript{15,16}

Currently, the detection or quantification of HDV RNA is the only reliable marker of HDV replication, which is positive in all patients with acute or chronic infection and negative in spontaneous or treatment-induced viral clearance.

We previously developed an in-house consensus technique for HDV RNA quantification in blood samples using reverse-transcriptase (RT) and TaqMan real-time PCR technologies that can quantify all HDV genotypes (HDV-1 to HDV-8) with wide range linearity (from $10^2$ to $10^8$ copies/mL).\textsuperscript{17}

Very recently, several manufacturers have developed commercial blood HDV RNA quantification assays using real-time RT-PCR technology.

The aim of the present work was to evaluate the performances of 3 commercially available kits: the Lightmix kit HDV (Roche, Meylan, France), designed for HDV genotype 1; the RoboGene HDV RNA quantification kit (Aj-Roboscreen, Leipzig, Germany); and the HDV RNA Quantification (QT) (DiaPro, Milano, Italy). Each theoretically designed to quantify all HDV genotypes.

**Materials and Methods**

**Clinical Samples**

A total of 128 clinical samples sent to the virology unit of Avicenne Hospital, the laboratory associated with the French National Reference Center (NRC) for hepatitis B, C, and delta, were selected for this study. Samples with various genotypes and viral load (VL) values were chosen and coded by the scientific coordinator. The panel of samples was composed of 33 HDV-1 samples from European or Asian patients (HDV-1Eu/As), 33 HDV-1 samples from African patients (HDV-1Afr), 2 HDV-2 samples, 1 HDV-3 sample, 15 HDV-5 samples, 15 HDV-6 samples, 17 HDV-7 samples, 11 HDV-8 samples, and 1 negative control (negative hepatitis B surface antigen).

In addition, we performed a longitudinal study in 6 patients infected with strains of different genotypes (1 HDV-1Eu/As, 2 HDV-1Afr, 2 HDV-5, and 1 HDV-8) who had at least 5 successive samples available.

**Methods**

**RNA Extraction**

Total RNA extraction was performed on 250 µL of patient serum, using the QIAamp MinElute Virus Vacuum kit (Qiagen, Courtaboeuf, France). RNA was eluted in 100 µL of RNase-free water, aliquoted in several tubes, and stored at $-80^\circ$C until use.

**Hepatitis D Virus Genotyping**

HDV genotyping was performed by sequencing and phylogenetic analyses of the R0 region of the HDV genome exactly as previously described.\textsuperscript{2}

**Hepatitis D Virus Viral Load Quantification Techniques**

The main characteristics of the different assays were as follows: (1) the laboratory associated with the NRC in-house consensus HDV real-time RT-PCR method (NRC-hepatitis delta virus quantification [HDVQ]), (2) the Roche Lightmix HDV kit (Lightmix Roche), (3) the Robogene HDV RNA quantification kit (Aj-Roboscreen), and (4) the DiaPro HDV RNA Quantification (QT) (DiaPro) are summarized in Table 1.

**Study Design**

The quantification of the coded samples was performed blindly by 2 technicians, strictly according to the manufacturers’ protocols (except for the RNA extraction step) from stored

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**Table 1. Main Characteristics of the HDV RNA Quantification Assays**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Instrument</th>
<th>IC</th>
<th>Cycling and RT-PCR program</th>
<th>Probe characteristics</th>
<th>Probe detection</th>
<th>Probe/primer genome localization</th>
<th>Standard (genotype)</th>
<th>Quantification range, log copies/mL</th>
<th>Quantification slope</th>
<th>Genotype detection</th>
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<tr>
<td>NRC-HDVQ</td>
<td>ABI 7000</td>
<td></td>
<td>RT 95°C, 5 min</td>
<td>Hydrolyze TaqMan</td>
<td>HDV: 6'-FAM</td>
<td>Ribozymes (DNA (HDV-5))</td>
<td>2–8</td>
<td>−3.68</td>
<td>1–8</td>
<td></td>
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<tr>
<td></td>
<td>(In progress)</td>
<td></td>
<td>PCR 42°C, 45 min</td>
<td>Hydrolyze TripleHyb</td>
<td>HDV: 6'-FAM</td>
<td>RNA (HDV-1)</td>
<td>2.7–10</td>
<td>−3.47</td>
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<td>Hybridization</td>
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<td>2 probes</td>
<td>Antigen</td>
<td>DNA (HDV-1)</td>
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<td>−3.34</td>
<td>1–8</td>
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<td>DiaPro</td>
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<td>Hybridization</td>
<td>HDV: 6'-FAM</td>
<td>Ribozymes (DNA (HDV-5))</td>
<td>2–8</td>
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IC, internal control.
aliquots of RNA samples extracted as described earlier. Results that showed a discordance greater than 1 log_{10} copies/mL between 2 techniques or invalid internal controls were re-tested with each assay. Similarly, all samples with VL values greater than the linearity range were re-tested after dilution.

**Statistical Analyses**

HDV VL values were converted into log_{10} copies/mL for analysis. To estimate the cumulative distribution of left-censored data, the Turnbull method was used, assuming normal distribution for each technique. In further analyses, these non-detected values were replaced by sampling in the fitted distribution. Bland–Altman plots were drawn to assess the agreement level between each assay and the NRC-HDVQ. Predictive analysis of the tests according to sex, age, geographic origin, or to HDV genotype was assessed by Tobit regression. Statistical analyses were performed with R (http://www.R-project.org/), using fitdistr and censReg packages. All tests were 2-sided, with a P value less than .05 considered statistically significant.

**Results**

The VL values of the 128 positive samples ranged from 2 to 9.2 log_{10} copies/mL as determined by the NRC-HDVQ assay and covered all HDV genotypes except HDV-4 (Figure 1A). In the HDV-1 sample group, VL values depended on the geographic origin of the patients, being significantly higher in non-African vs African patients (by about 1 log_{10} copies/mL; P = .003) (Figure 1B). In all techniques, the VL values depended only on the HDV genotype; they were not affected by either age or sex.

**Analytical Performances of the Commercial Assays**

**Overall quantification performances.** Analysis of HDV RNA quantification results revealed that, compared with NRC-HDVQ, many samples were under the limit of quantification (LOQ) of the commercial techniques: 15 (11.7%), 24 (18.8%), and 61 (47.7%) for the Lightmix Roche, Aj-Roboscreen, and DiaPro assays, respectively.

Results from each commercial assay then were compared with those of the NRC-HDVQ by plotting differences against the mean of the obtained measures, according to the Bland–Altman method (Figure 2A). As shown, Lightmix Roche, Aj-Roboscreen, and DiaPro techniques led to lower HDV VL values on average: 0.77 log_{10}, 0.56 log_{10}, and 1.68 log_{10}, respectively. It should be noted that the Roche test is designed for HDV-1 quantification only. All assays identified the negative control correctly.

**Hepatitis D virus-1 sample quantification.** Because HDV-1 is ubiquitous and found in 80% of infected patients in France, we evaluated the performances of the 3 assays on the 66 HDV-1 samples. As shown in Figure 2B, all samples were very well quantified with the Aj-Roboscreen technique compared with NRC-HDVQ with an HDV VL mean difference of less than 0.1 log_{10} copies/mL. For the Lightmix Roche and DiaPro assays this mean difference was 0.29 log_{10} and 0.35 log_{10} copies/mL, respectively (P < .01).

We further evaluated the performances of the commercial assays among patients infected with HDV-1 genotype strains according to their geographic origin (33 HDV-1Eu/As and 33 HDV-1Afr patients).

We found excellent concordance for HDV-1Eu/As samples: VL value differences were <0.3 log_{10} copies/mL (Figure 2C).

For HDV-1Afr samples, Aj-Roboscreen results showed high concordance with NRC-HDVQ (Figure 2C). However, the Roche assay underestimated VL values by 0.5 to 1 log_{10} copies/mL and failed to detect 1 sample whose VL was 5 log_{10} copies/mL with the NRC-HDVQ. Similarly, DiaPro failed to amplify 4 samples with VLs ranging from 2 to 8 log_{10} copies/mL and underestimated 4 others by more than 2 log_{10}.

**Hepatitis D virus–non-1 sample quantification.** Fifty-eight samples belonging to HDV-5 to HDV-8 genotypes, found only in patients originating from sub-Saharan Africa, were tested with the 3 commercial assays, even though the Roche technique was not designed for HDV–non-1 samples (Figure 3). As expected for the Roche kit, most HDV-5, HDV-6, and HDV-8 samples were strongly underestimated, whereas only 2 samples with low VL (<3 log_{10} copies/mL) were under the LOQ of this
assay. Conversely, for HDV-7 strains, only samples with high VL ($>6 \text{ log}_{10}$) could be detected.

For the DiaPro assay, all HDV-5 and HDV-6 samples were under the LOQ of the technique. Only 4 HDV-7 and 1 HDV-8 sample with very high VL ($>7 \text{ log}_{10}$) were detected, but the results were underestimated dramatically.

For Aj-Roboscreen, almost all HDV-5 to HDV-8 samples also were underestimated. Samples with VL values less than $5 \text{ log}_{10}$ copies/mL were usually under the LOQ of the technique. For HDV-8, only samples with more than $6 \text{ log}_{10}$ copies/mL were detected by this assay.

The other HDV genotypes are rarely found in patients living in France. Two HDV-2 samples (from 2 patients born in Vietnam and China), and 1 HDV-3 sample (patient from Bolivia) were included in this study. The 2 HDV-2 samples were underestimated by 1.5, 3, and $5 \text{ log}_{10}$ copies/mL with Aj-Roboscreen, Lightmix Roche, and DiaPro, respectively. The HDV-3 sample, whose VL value was estimated at $5 \text{ log}_{10}$ by the NRC-HDVQ, weakly was detected, only by the Aj-Roboscreen (<LOQ of the assay).

**Longitudinal Study**

We thus conducted a longitudinal study to investigate the performances of the commercial assays in patient follow-up evaluation. Six patients infected by different HDV genotype strains and who had at least 5 available consecutive samples were selected. For this part of the study, positive VL values under the LOQ of the techniques were recorded as unquantifiable positive values. The results are shown in Figure 4.

As expected, no significant difference was observed for patient 1 (HDV-1Eu/As) (Figure 4A), whose VL ranged between 5 and $8 \text{ log}_{10}$ copies/mL. The evolution pattern was identical across the 3 commercial assays and similar to that obtained with the NRC-HDVQ assay.

Patients 2 and 3 were infected with an HDV-1Afr strain (Figure 4B and C). As shown, the Aj-Roboscreen assay showed the same pattern and the same VL values as NRC-HDVQ for all samples, whereas the Lightmix Roche assay slightly underestimated VL values for patient 2 (<1 log$_{10}$). For patient 3, however, the VL was underestimated dramatically (>3 log$_{10}$), and 4 of 5 values were under the LOQ of the assay. Conversely, the DiaPro assay quantified the different samples of patient 3 quite well, whereas it significantly underestimated those of patient 2. Thus, mixed results were obtained with the Lightmix Roche and DiaPro in 2 patients infected with an HDV-1Afr strain.

Patient 4, who was infected with an HDV-5 strain, displayed high VL on the first 2 samples (samples 1 and 2, approximately $6 \text{ log}_{10}$ copies/mL) (Figure 4D). After treatment, a complete virologic response (samples 3 and 4) was obtained, followed by a relapse at the end of the treatment (sample 5, VL = $2 \text{ log}_{10}$). Aj-Roboscreen and Lightmix Roche assays were able to well-
quantify samples 1 and 2, although with lower values (−1.5 to −2.5 log10 copies/mL). However, both failed to detect the viral rebound at the end of the treatment.

Similarly, for patients 5 and 6, infected with HDV-5 and HDV-8 strains, the VLs were underquantified dramatically by the Lightmix Roche and the Aj-Roboscreen assays, respectively, and some HDV-8 samples were missed entirely by the latter test (Figure 4E and F). Furthermore, and as expected from the results of the retrospective study, the DiaPro assay failed to quantify patients 4, 5, and 6 strains (Figure 4D–F).

Discussion

HDV is thought to co-infect 5% to 20% of HBV-infected patients and is responsible for more aggressive hepatic disease.10,11 In this study, we evaluated the performances of 3 commercially available assays for quantification of HDV RNA VL in routine use, compared with the in-house TaqMan real-time RT-PCR technique we developed previously.17 By using this technique, in a study including 14 HDV-infected patients treated by pegylated interferon α2b, Castelnau et al12 identified 4 response patterns: responder, incomplete responder, nonresponder, and responder-relapser; showed that HDV kinetics at month 3 were predictive of response; and showed that after 6 months of therapy a negative HDV RNA was predictive of sustained virologic response. Consequently, as it is for HBV or HCV, the quantification of the VL by real-time PCR methods constitutes the main tool for correctly classifying patients, detecting early relapse, and defining clear therapeutic end points.

Ideally, the tool must be able to quantify with high sensitivity and reproducibility all circulating HDV strains, according to the high genetic variability of HDV. In particular, we have characterized 4 African genotypes, HDV-5, HDV-6, HDV-7, and HDV-8, and a putative African HDV-1 subgenotype.2,3 Because of migration, all of these African strains are spreading in France (and in several European countries), where they account for 20% (HDV-5 to HDV-8) of HDV cases. This number increases to 60% when HDV-1Afr is included. Therefore, quantification of HDV VL must take this wide genetic diversity into account to detect all circulating strains.

Although the variability of the NRC-HDVQ technique may appear high relative to that of the 3 commercial assays (as depicted in Figure 3), the HDV–non-1 samples were underestimated dramatically, and sometimes undetected, by the 3 commercial assays in our tests. We thus highly suggest that these tests be used in the management of HDV-1–infected patients only.

There are several challenges in developing accurate, sensitive, and consensus tests for HDV RNA VL quantification in blood samples, in particular the HDV genetic diversity. The most conserved regions of the genome are located in genomic and antigenomic ribozymes. In our assay, primers and probes are designed in these regions and permit detection/quantification of all HDV genotypes.

The hepatitis D antigen coding region also can be used, but discrepancies at the nucleotide level between genotypes require using degenerate nucleotides in primers/probes, thus reducing PCR efficiency.
Commercial assays have several advantages: most steps are automated (including nucleic acid extraction), and the more recent assays include an internal control. Moreover, they are ready to use and are thought to be robust, with high repeatability and reproducibility, and to provide comparable results among different laboratories.

Our study design did not permit an evaluation of the extraction step. Indeed, this will need further evaluation because the volume of serum or plasma may influence the sensitivity of the technique. This step should not be a critical point because the use of automatic extraction methods can be validated very easily.23

Recently, several laboratories have developed in-house assays with various protocols.23–27 Primers and probes are located either in the hepatitis D antigen or in the ribozyme region. Volumes of extracted serum/plasma vary between 140 and 500 μL, and extractions are performed by either manual or automated devices. HDV RNA amplifications are performed with 1 or 2 distinct steps and use classic hybridization or modified TaqMan probes. Quantification standards consist of DNA or RNA molecules, and results are expressed in complementary DNA copies/mL or in RNA copies/mL.24 An internal control, either RNA or DNA, is included in some assays. However, no international calibration standard currently is available to standardize the VL results, and, unfortunately, not all of these assays have been subjected to study with clinical samples, or, when performed, the tests did not always include HDV–non-1 samples.

Therefore, the development of international quality controls for the various in-house or commercial assays should be organized and implemented, to assess the specificity and sensitivity of the techniques and their ability to quantify all circulating strains.

In conclusion, this study describes the performances of 3 real-time RT-PCR systems and compares their results with those obtained via the NRC-HDVQ technique. Correlation and agreement with this assay were excellent for HDV-1Eu/As strains (accounting for approximately 40% of spreading strains in France). However, these commercial kits significantly underestimate or fail to quantify HDV VL for all other HDV genotypes, the only exception being Aj-Roboscreen for HDV-1Afr. Therefore, overall improvement of in-house and commercial assays is needed urgently, especially for the quantification of non–HDV-1 genotype strains to propose a fully adequate tool for therapeutic management of HDV infection.

Figure 4. Longitudinal study. Six patients (patients 1–6, panels A–F, respectively) with at least 5 consecutive samples were included in a longitudinal study. The genotype and the origin of the infecting strain are specified for each patient. Serums were quantified with each assay as indicated. Results are expressed in log copies/mL. The top and bottom shaded areas correspond to the LOQ of the Aj-Roboscreen (2.7 log_{10} copies/mL) and of the NRC, Lightmix Roche, and DiaPro assays (2 log_{10} copies/mL), respectively.
References


Reprint requests

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S.B., F.L.G., and A.B. contributed equally to this work.

Conflicts of interest

The authors disclose no conflicts.