Standardized One-Step Real-Time Reverse Transcription-PCR Assay for Universal Detection and Quantification of Hepatitis Delta Virus from Clinical Samples in the Presence of a Heterologous Internal-Control RNA

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As for other chronic viral diseases, quantification of hepatitis delta virus (HDV) loads may be useful for patient management. We describe a one-step quantitative reverse transcription-PCR assay that is reliable and automatable and meets the regulatory authorities’ standards for accurate quantification of the major HDV genotypes. It includes an internal control and uses in vitro-transcribed RNAs as standards. Its linearity range is 500 to 1.7 × 10^11 copies/ml, its sensitivity is around 150 copies/ml, its repeatability is around 15%, and its reproducibility is below 0.25 log10 copies/ml.

TABLE 1 Sequences of the primers and probes used in this study

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgD-F1</td>
<td>5’TGGGTCTGTCCTCCGGCTGA3’</td>
<td>1158–1175</td>
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<td>AgD-F2</td>
<td>5’TGGGTCTGTCCTCCGGCTGA3’</td>
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<tr>
<td>AgD-R</td>
<td>5’AAGGAGGCGCTTCCGAGAA3’</td>
<td>1287–1268</td>
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<tr>
<td>Probes</td>
<td></td>
<td></td>
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<tr>
<td>TaqMan-MGB</td>
<td>5’CTTTCTTCTTCTCCCTGCTGA3’</td>
<td>1215–1234</td>
</tr>
<tr>
<td>LNA-BHQ</td>
<td>5’CTTCTTCTTCTCCCTGCTGA3’</td>
<td>1217–1234</td>
</tr>
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<tr>
<td>dLy131</td>
<td>5’CTTCTTCTTCTTCTCTGCTGA3’</td>
<td>1215–1234</td>
</tr>
</tbody>
</table>

a TaqMan-MGB and LNA-BHQ probe sequences were aligned with patients’ sequences correctly detected by the LNA-BHQ probe but not by the TaqMan-MGB probe. Boldface letters in the two forward primer sequences indicate differences. Boldface letters in the probe sequences show the differences between the probe and patient sequences.

b Numbering refers to the HDV-1 genome (accession number M21012).

c LNA nucleotides are underlined.

For other chronic viral infections, quantification of HDV loads may improve patient management and help clarify the pathophysiology of HDV infection. Commercial kits accurately quantify HDV-1 genotypes and primers provided in the kit (Simplexa Extraction and Amplification Control RNA; Focus Diagnostics, Cypress, CA, and Centre de Recherches en Cancérologie de Lyon, Department Immunity, Microenvironment Virus, UMR INSERM, U1052 UMR CNRS 5286, Lyon, France) by following the Generic 2.0.1 protocol. A one-step qRT-PCR was performed with the Quantitect Virus kit (Qiagen, Courtaboeuf, France) as described in the supplemental material on a Rotor-Gene 6000 device (Qiagen). As for other chronic viral diseases, quantification of HDV loads may be useful for patient management. We describe a one-step quantitative reverse transcription-PCR assay that is reliable and automatable and meets the regulatory authorities’ standards for accurate quantification of the major HDV genotypes. It includes an internal control and uses in vitro-transcribed RNAs as standards. Its linearity range is 500 to 1.7 × 10^11 copies/ml, its sensitivity is around 150 copies/ml, its repeatability is around 15%, and its reproducibility is below 0.25 log10 copies/ml.
conserved positions of the HDV target, further allowed some polymorphism in less conserved positions (Table 1; see Fig. S1 in the supplemental material). The linearity threshold values for the IC were very stable, at around 26.2 ± 1.7, even for high viral loads. Its presence did not significantly affect HDV quantification compared to that with the assay without an IC (difference of 0.34 ± 0.23 log10 copies/ml). The majority of the previously published qRT-PCR assays for HDV RNA quantification did not include an IC and employed plasmids or cDNAs as calibrators (7, 9, 10, 11, 14). Plasmid-based standard curves usually underrate RNA samples (16). In vitro-transcribed RNAs (HDV-1, -5, -6, -7, and -8) were thus serially diluted in QuantiTect nucleic acid dilution buffer (Qiagen, Courtaboeuf, France) and used as standards. The standard curve generated with HDV-1 standards is shown in Fig. 1. We found a 1-log difference when plasmid standards were used instead of RNA standards (data not shown), a difference also described by Terlizzi et al. (16). Indeed, the use of in vitro-synthesized RNA standards provides the control for both the RT and PCR steps and is thus more reliable than other methods using DNA-based standards.

A specificity of 100% was achieved (verified with plasma from HDV-negative patients replicating hepatitis B virus [HBV], HIV, hepatitis C virus [HCV], cytomegalovirus, or hepatitis E virus and apparently uninfected patients).

HDV-1 standard RNA at extreme concentrations (3.38 × 109 and 3.38 × 1010 copies/μl) were detected but out of the linear range. Taking into account the dilution factor inherent to the extraction technique (1/50), the dynamic range of HDV RNA quantification (linearity) determined with both RNA transcripts and serum samples extended from 500 to 1.7 × 1011 copies/ml of serum for clinical samples. Standard curves generated within the dynamic range in 15 independent experiments showed a mean R2 value of 0.999 and a PCR efficiency of 97%.

The lower limit of detection (LOD) was established from triplicate analyses of 2 10-fold dilutions of the standard RNA (27 and 2.7 copies/reaction mixture) and determined to be 168 copies/ml. Dilutions of clinical samples confirmed the LOD to be around 150 copies/ml (see Fig. S2 in the supplemental material).

The linearity and accuracy of the assay were verified by using dilutions of patient plasma (see Fig. S2 in the supplemental material) and of HDV transcripts of the HDV-5, -6, -7, and -8 genotypes (see Fig. S3 in the supplemental material). Their theoretical concentrations were compared with those measured based on the HDV-1 standard curve. Measured and expected HDV RNA levels were well correlated (R2 values of 0.999, 0.999, 0.988, and 0.998, respectively) with differences of 0.00 to 0.46 log10 copies/ml. Due to the influx of migrants, more than 30% of the HDV carriers in France were born in Africa and are likely infected with an African strain (HDV-5, -6, -7, or -8) or an African HDV-1 variant. Accurate detection and quantification of all genotypes are mandatory for a gold standard qRT-PCR assay.

Repeatability, evaluated with 20 extractions of serum samples from two patients, yielded coefficients of variation (CVs) of 15 and 15.3% (7.41 ± 0.07 and 5.63 ± 0.07 log10 copies/ml), respectively, with a variation of only 0.07 log10. Results from 15 independent assays of the low-, medium-, and high-level RNA transcripts and one patient’s serum varied from 0.03 log for the high-level standard to 0.11 log for the low-level standard (CVs of 21.9, 15.5, 7.4, and 14.7, respectively). This is in the range of commercially available assays for HBV, HCV, or HIV and below the maximum allowed analytical variability of 0.25 log (3).

Samples from 80 patients were quantified using the qRT-PCR with the LNA probe. Viral loads ranged from 2.7 to 10.4 log10 copies/ml with a median viral load of 6.0 log10 copies/ml. The four highest viral loads corresponded to sera from two young patients and two HIV-positive patients, in accordance with previous observations (15). Two samples had 150 and 500 copies/ml. Twenty-nine patient samples (28.6%) were found to be negative (below the LOD). Among these samples, 25 were also found to be negative with the qualitative assay and were HDV IgM negative. The other four samples were also found to be negative with the qualitative assay but were still positive for anti-HDV-IgM antibodies; two of them had very low hepatitis B surface antigen levels.

In the context of new emerging antiviral molecules, it is crucial to achieve the most sensitive results. Here we found 18 samples out of 76 with quantitative results between the quantification limit (5 × 102 copies/ml) and 105 copies/ml, while the method of Ferns et al., the other in-house assay that includes an IC, found only 1 sample out of 44 below 105 copies/ml (5), placing its sensitivity in question. The target amplified, the antigen delta gene in the present assay versus the highly structured ribozyme in the other one, might explain this discrepancy.

The duplex one-step qRT-PCR assay presented here allows accurate quantification of the major HDV genotypes with a single primer-probe mixture and provides essential features mandatory for diagnostic use (12). These characteristics are wide dynamic range encompassing the viral range of HDV-infected patients, good sensitivity and variability, inclusion of an IC, and a reduced contamination risk due to closed-tube procedures and a one-step reaction. Hepatitis delta is still an orphan hepatitis in terms of knowledge of its pathogenicity, diagnostic tools, and antiviral treatment. The present method could help improve understanding of the pathophysiology and clinical evolution of HDV infection.

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FIG 1 Typical real-time amplification plot generated from a 10-fold dilution series of the short RNA HDV-1 standard. From left to right, the curves represent 2.7 × 1011 to 2.7 × 102 HDV RNA copies/reaction. The curve that stayed below the threshold corresponds to 2.7 copies/reaction. Norm. Fluor., normalized fluorescence.
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REFERENCES