



Development of a reverse transcription quantitative real-time PCR-based system for rapid detection and quantitation of hepatitis delta virus in the western Amazon region of Brazil



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A B S T R A C T

Article history:

Received 11 July 2013

Received in revised form

19 November 2013

Accepted 25 November 2013

Available online 4 December 2013

Keywords:

Hepatitis delta virus

HDV RNA

Quantitative real-time PCR

Hydrolysis probe

Viral load

The hepatitis delta virus (HDV) is a pathogen that causes a severe and rapidly progressive disease of hepatocytes. The measurement of viral load in the peripheral blood of patients with HDV infections is important for diagnosis, treatment monitoring, and support for follow-up studies of viral replication during the course of the disease. This study reports the development of an assay capable of detecting and quantifying the abundance of HDV particles in serum samples, based on reverse-transcription quantitative PCR (RT-qPCR). Two standards for calibration were produced for determining the viral load of HDV: a cDNA cloned into a linear plasmid and a transcribed RNA. For validating this assay, 140 clinical samples of sera were used, comprising 100 samples from patients who tested positive for anti-HDV and hepatitis B virus surface antigen (HBsAg) by ELISA; 30 samples from blood donors; 5 samples monoinfected with hepatitis B virus (HBV); and 5 samples monoinfected with hepatitis C virus (HCV). The HDV RT-qPCR assay performed better when calibrated using the standard based on HDV cDNA cloned into a linear plasmid, yielding an efficiency of 99.8% and a specificity of 100% in the *in vitro* assays. This study represents the first HDV RT-qPCR assay developed with clinical samples from Brazil and offers great potential for new clinical efficacy studies of antiviral therapeutics for use in patients with hepatitis delta in the western Amazon region.

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1. Introduction

Hepatitis delta, which is also known as hepatitis D, is an infectious viral illness that causes grave inflammation of hepatocytes (Saracco et al., 1987; Le Gal et al., 2005; Farci and Niro, 2012). Several studies have shown that chronic infection with hepatitis delta virus (HDV) leads to more severe liver disease than mono-infection with chronic hepatitis B virus (HBV), with accelerated progression to fibrosis, cirrhosis, and decompensation of early increased risk of hepatocellular carcinoma (Bensabath et al., 1987; Romeo et al.,

2009; Niro et al., 2010; Wedemeyer, 2011; Buti et al., 2011). HDV infection can therefore occur as either a superinfection or a coinfection. Coinfection is simultaneous infection with both HBV and HDV; it is clinically indistinguishable from acute mono-infection caused by HBV, although it may be more serious. Superinfection is HDV infection in an individual who is chronically infected with HBV (Smedile et al., 1982). Occasionally, HDV superinfection can be self-limiting. In this case, HDV RNA is eliminated in a few years. Antiviral therapy can also hasten HDV RNA elimination, and in some cases, the antibodies against HDV and hepatitis B virus surface antigen (HBsAg) disappear from the sera. In HDV superinfection with persistent viral replication, commonly observed levels of HDV RNA, alanine aminotransferase, and elevated antibodies to HDV are persistently detectable (Hughes et al., 2011). The HDV is a small, spherical particle that is encapsulated by an HBsAg envelope that measures approximately 36 nm. HDV is considered to be a satellite virus of HBV (Bonino et al., 1986). The virion contains a single-strand negative-sense RNA genome of approximately

Abbreviations: HDV, hepatitis delta virus; HBV, hepatitis B virus; RT-qPCR, reverse-transcription quantitative PCR; HBsAg, hepatitis B virus surface antigen.

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1700 nt that is circular and self-complementary (Taylor, 2006; Hughes et al., 2011). Its genetic diversity is related to the geographical origin of the isolate. Thus far, 8 genotypes have been identified and classified as HDV-1 to HDV-8 (Le Gal et al., 2006). HDV-3 is responsible for epidemics of severe hepatitis and is common in northeastern South America (Casey et al., 1993a,b). Studies show that HDV-3 is prevalent in the Brazilian Amazon (Gomes-Gouvea et al., 2009; Alvarado-Mora et al., 2011). This HDV genotype is apparently related to the greater aggressiveness of HDV in this region (Casey et al., 1993b; Viana et al., 2005; Parana et al., 2006).

Molecular studies based on the quantitation of HDV RNA through real-time reverse transcription polymerase chain reaction (RT-qPCR) with a Light Cycler DNA Master SYBR Green I mix (Boehringer Mannheim, Mannheim, Germany) demonstrated a correlation between levels of RNA HDV circulating in sera and liver damage. In addition, HBV DNA levels showed no correlation among the different clinical stages (Yamashiro et al., 2004). Quantitation of HDV RNA in sera is critical for diagnosing infection and monitoring treatment, because it allows for the identification of profiles of different viral responses to interferon therapy with greater precision than qualitative tests (Le Gal et al., 2005; Mederacke et al., 2010). In 2009, Kiesslich and colleagues performed real-time RT-qPCR analysis to assess the influence of the HBV genotype on the disease course in HBV-HDV-coinfected patients by measuring their viral loads. In 2012, Ferns and collaborators developed a standardized RT-qPCR assay in real time by using a transcript of “full-length genomic RNA” in order to minimize the risk of producing false-negative results and underestimating the viral load. Shang et al. (2012) developed a real-time RT-qPCR assay to determine the viral load using sera or plasma infected with all HDV types. Many studies have used in-house RT-qPCR assays for monitoring treatments (Yamashiro et al., 2004; Le Gal et al., 2005; Castelnuovo et al., 2006; Farci, 2006); thus, many laboratories have developed real-time RT-qPCR assays in-house in order to diagnose and monitor the treatment of HDV infection because there is no commercial test yet available. Currently available methods for serological diagnosis of hepatitis delta have lower specificity than other clinical methods. The method proposed here for molecular HDV RT-qPCR is meant to fulfill the need to increase the clinical specificity of diagnosis and treatment monitoring of hepatitis delta in the Amazon region of Brazil. However, this proposed method is limited by the lack of an international standard to regulate assays for quantification of HDV RNA. The aim of this study was to produce an in-house RT-qPCR test to enable the early diagnosis and quantification of HDV for treatment monitoring, given that the western Amazon region of Brazil is considered to be an area of high endemicity for this virus.

2. Methods

2.1. Clinical samples

The study included 100 serum samples from patients found to be infected with HBV/HDV following outpatient service in viral hepatitis at the Research Center for Tropical Medicine from Rondônia, Porto Velho, Brazil. To test specificity, a control group of 30 blood donors was included, all of whom were negative for human immunodeficiency virus (HIV)-1 and HIV2, HBsAg, anti-HBc, and anti-hepatitis C virus (HCV). We also tested 5 serum samples from individuals monoinfected with HCV and 5 samples from individuals who were HBV monoinfected. This study was approved by the ethics committee of the Research Center for Tropical Medicine (approval 33/10 CEP/Research Center Tropical Medicine and Registration No. 110/2010).

2.2. HDV RNA extraction

HDV RNA extraction was performed with the QIAamp Viral RNA Mini Kit (Qiagen, Germany) using 200 μ L sera, according to the manufacturer's instructions. The RNA precipitate was re-suspended in 50 μ L of elution buffer. To prevent false-positive results, strict procedures and proposed techniques for nucleic acid amplification diagnostics were followed (Kwok and Higuchi, 1989).

2.3. Reverse transcription

HDV RNA was denatured at 95 °C for 5 min and 15 μ L of the RNA extraction was converted into cDNA using 200 U of M-MLV enzyme (Sigma-Aldrich®, Saint Louis, USA), 1 μ L of 10 \times M-MLV reverse transcriptase buffer, 1 μ L of random primer, and 1 μ L of 10 mM dNTP mix. The thermocycling conditions for cDNA synthesis were as follows: 70 °C for 10 min, 37 °C for 50 min, and 94 °C for 10 min.

2.4. Design and optimization of primers and fluorescence resonance energy transfer probes

The primers and hydrolysis probe (TaqMan Probes, Applied Biosystems, Foster City, CA, USA) were designed based on known and complete HDV sequences that are deposited in GenBank and were obtained from the National Center for Biotechnology Information (NCBI) using CLUSTAL W 2.1 software (European Bioinformatics Institute). Nucleotide sequences of the 8 genotypes that are described in the database were aligned and mapped in terms of their conserved regions. To analyze the *in silico* specificity of the primers, we used the Basic Local Alignment Search Tool (BLAST) at the NCBI. Primer concentration was optimized using a concentration gradient from 100 nM to 900 nM. Optimization of the probe concentration was conducted using a concentration gradient from 50 nM to 300 nM using the TaqMan® PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

2.5. HDV-qPCR

HDV-qPCR was performed on the ABI 7500 platform (Applied Biosystems, Foster City, CA, USA) with a 25- μ L reaction volume containing 12.5 μ L TaqMan Universal Master Mix 2 \times (Applied Biosystems, Foster City, CA, USA), 5 μ L of cDNA, 100 nM of the forward primer HDVq (nt) 5'-TGGCTCTCCCTTAGCCATCCGA-3', 100 nM primer Reverse HDVqb (nt) 5'-GGGTTTCCACTCACAGGTTTGC-3', and 100 nM of the probe 5'-FAM-CCGCGAGGAGGTGGAGATGCCAT-TAMRA-3', which amplify a fragment of approximately 135 nt production corresponding to HDAg-L.

2.6. Generation of a standard curve

2.6.1. RT-PCR

For of standardized controls, HDV RNA was extracted and reverse transcribed (as described in Sections 2.2 and 2.3); we used a sample of serum found to be positive for anti-HgsAg and HDV, as determined by ELISA (DiaSorin, Saluggia, VC, Italy). For the RT-PCR, we used 10 μ L of extracted RNA and added it to 40 μ L of PCR mix (20 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each of the 4 dNTPs, 10 pmol of each primer, and 1.25 U of Taq DNA polymerase [Invitrogen™ Life Technologies, Carlsbad, CA, USA]). PCR was performed under the following conditions: 2 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 50 s at 60 °C, and 45 s at 72 °C, with a final extension step of 5 min at 72 °C.

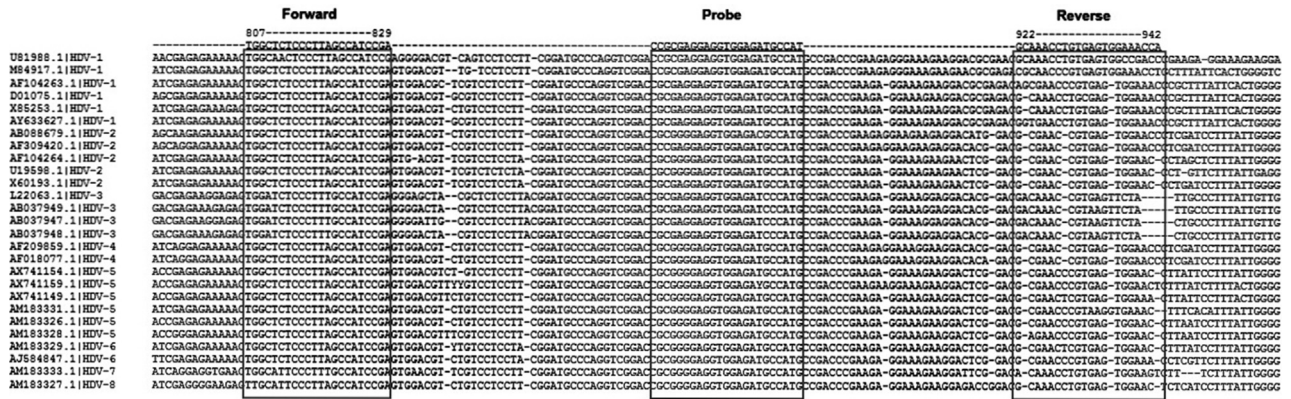


Fig. 1. Multiple alignment using CLUSTAL W 2.1 for HDV RNA sequences that were drawn from GenBank and corresponded to 8 genotypes (HDV-1 through HDV-8). The forward primer demonstrated a score from 90 to 100, the reverse primer demonstrated a score from 77 to 95, and the probe demonstrated a score from 95 to 100 between the aligned sequences of HDV RNA (GenBank IDs: U81988, M84917, AF104263, D01075, X85253, AY633627, AB088679, AF30942, AF104264, U19598, X60193, L22063, AB037949, AB037947, AB037948, AF209859, AF018077, AX741154, AX741159, AX741149, AM183331, AM183326, AM183328, AM183329, AJ584847, AM183333, and AM183327).

2.6.2. Cloning of cDNA into plasmids and RNA transcription *in vitro*

The PCR product was purified using an ExoSAP-IT® enzyme (USB, Cleveland, OH, USA) and the fragment was inserted into a cloning plasmid pTZ57R/T (Fermentas®, Hanover, MD, USA) connected with T4 DNA Ligase (Promega, Madison, USA), and cloned into a prokaryotic system. The cloned cDNA was digested with *EcoRI* (Invitrogen™ Life Technologies, Carlsbad, CA, USA) and *BamHI* (Invitrogen™ Life Technologies, Carlsbad, CA, USA) and the presence of the insert was confirmed by electrophoresis on an agarose gel, followed by subsequent linearization with *EcoRI*, resulting in calibration standard HDV cDNA. Thereafter, 10 µL of cDNA was inserted into the linearized plasmid, which was then transcribed *in vitro* by using T7 RNA polymerase (Promega®, Madison, WI, USA) with a 100 µL reaction volume containing transcription-optimized 1× buffer, 100 mM DTT, ribonuclease inhibitor 40 U/µL, 2 nM of each of the 4 rNTPs, and 19 U/µL phage RNA polymerase. The mixture was incubated at 37 °C for 2 h to obtain calibration standard HDV RNA.

2.6.3. Quantitation and control of calibrators

The integrity calibrators were confirmed by performing agarose gel electrophoresis. The concentration was measured spectrophotometrically using a NanoDrop® ND-1000 (Thermo Scientific NanoDrop Products, Wilmington, Delaware), and the measurements were recorded in units of nanograms per microliter, which was converted into copies per microliter using the following equation: $([\times \text{ ng}/\mu\text{L} \times 10^{-9}] / [\text{DNA bps} \times 660]) \times 6.022e^{23} = y \text{ copies}/\mu\text{L}$ $e([\times \text{ ng}/\mu\text{L} \times 10^{-9}] / [\text{RNA bps} \times 350]) \times 6.022e^{23} = y \text{ copies}/\mu\text{L}$ (Qiagen, West Sussex, UK). Both solutions were diluted to obtain the linear dynamic range of the calibrators. Subsequently, the calibration dilutions of standard RNA were converted into cDNA.

2.7. Analytical performance

2.7.1. Analytical sensitivity and linear dynamic range

The limit of detection and the linear dynamic range were established by linear regression production of HDV-qPCR assays *in vitro*, using 3 repetitions of a serial dilution of 6-log₁₀ (10²–10⁷ copies/reaction) of both standard controls (Bustin, 2010).

2.7.2. Analytical specificity

To demonstrate the analytical specificity of HDV RT-qPCR, we tested 100 serum samples from patients infected with HDV/HBV (HBsAg, anti-HBc, which were found to be anti-HDV positive by ELISA). We also used 30 samples from blood donors, 5 serum

samples from patients chronically infected with HCV, and 5 serum samples from HBV monoinfected patients. All samples were subjected to HDV-qPCR to determine their viral load.

2.7.3. Amplification efficiency and linearity of HDV-qPCR

The linearity of HDV-qPCR was evaluated by means of 4 rounds of a serial dilution of 6-log₁₀ of both standard controls (Bustin, 2010). The efficiency was calculated using the equation: Amplification Efficiency = $[10^{(-1/\text{slope})} - 1] \times 100$ (Le Gal et al., 2005).

2.7.4. Reproducibility and repeatability

Intra-assay reproducibility was evaluated by means of 4 rounds of a serial dilution of 6-log₁₀ of both standard controls. To evaluate inter-assay repeatability, we performed the assay with 6 sera samples in 4 rounds on consecutive days (Bustin, 2010).

2.8. Statistical analysis

Statistical analyses of both standard controls were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA). The non-parametric data were compared using the Mann–Whitney 2-tailed *U*-test, and a *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. In silico analysis of primers and FRET probes

In silico analysis of the primers and FRET probes demonstrated that these were highly specific for the HDV genome. The primers and FRET probes were capable of binding to the conserved regions among the 8 presently known genotypes of HDV RNA with high specificity (Fig. 1).

3.2. Analytical performance

3.2.1. Analytical sensitivity and linear dynamic range

The linear dynamic ranges that were successfully tested for HDV-qPCR were 1.3 × 10²–1.3 × 10⁷ and 8.4 × 10¹–8.4 × 10⁶ copies/mL for the calibration standard HDV cDNA and RNA, respectively (Figs. 2 and 3). The limits of detection were 1.3 × 10² and 8.4 × 10¹ copies/mL for the calibration standard HDV cDNA and RNA, respectively (Figs. 2 and 3).

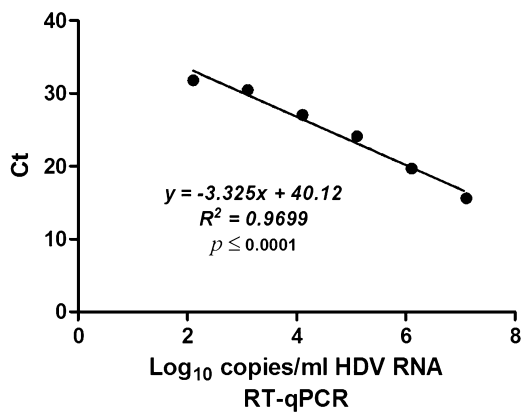


Fig. 2. Linear regression curve of the calibrator standard HDV cDNA. The linear regression curve was shown to be significant ($p \leq 0.0001$) with a slope of -3.325 , linear correlation coefficient $R^2 = 0.96$, generated using the following formula to determine the viral load: $y = -3.325x + 40.12$.

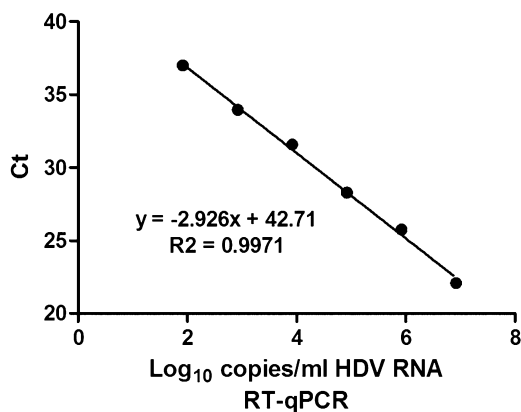


Fig. 3. Linear regression curve of the calibrator standard HDV RNA. The linear regression curve was shown to be significant ($p \leq 0.0001$) with a slope of -2.926 , linear correlation coefficient $R^2 = 0.99$, generated using the following formula to determine the viral load: $y = -2.926x + 42.71$.

3.2.2. Analytical specificity in vitro

Of 100 sera samples of HBsAg- and anti-HDV-positive patients who were tested by performing HDV-qPCR, 54% were positive, with a viral load greater than or equal to the detection limit and 46% were considered to be negative because they were below the detection limit of the assay, as determined by the standard calibration HDV

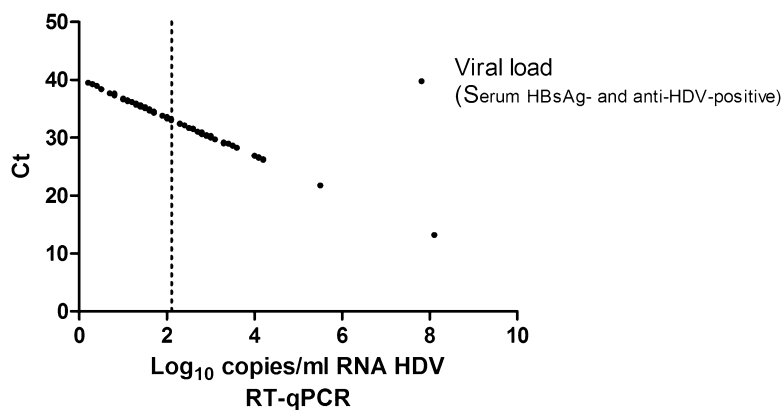


Fig. 4. Viral load (\log_{10} copies/mL) of serum HBsAg- and anti-HDV-positive samples as determined from ELISA. The dashed line represents the detection limit of the HDV-qPCR assay ($2.11 \log_{10}$ or 1.3×10^2 copies/mL). At the left side of the dashed line are the 46% of the samples that were considered negative as determined from the calibrator standard HDV cDNA, and to the right side of the dashed line are the 54% of the samples that were considered positive with viral loads greater than or equal to the detection limit of the HDV-qPCR assay. The viral load of the samples was determined using the equation presented in Fig. 2.

Table 1
Intra-run repeatability of HDV cDNA standards evaluated with 3 replicates of 6-fold standard dilutions.

| cDNA (copies/mL) | 1st run (Ct) | 2nd run (Ct) | 3rd run (Ct) | Mean | SD(\pm) | CV (%) |
|-----------------------------------|--------------|--------------|--------------|--------|-------------|--------|
| 1.3×10^7 | 14.95 | 15.71 | 16.16 | 15.61 | 0.61 | 0.04 |
| 1.3×10^6 | 18.92 | 19.81 | 20.32 | 19.68 | 0.71 | 0.04 |
| 1.3×10^5 | 22.92 | 24.35 | 25.12 | 24.13 | 1.12 | 0.05 |
| 1.3×10^4 | 26.55 | 27.09 | 27.47 | 27.04 | 0.46 | 0.02 |
| 1.3×10^3 | 30.07 | 30.60 | 30.82 | 30.50 | 0.38 | 0.01 |
| 1.3×10^2 | 31.52 | 32.04 | 31.87 | 31.81 | 0.27 | 0.01 |
| Slope | -3.427 | -3.336 | -3.211 | -3.325 | 0.07 | |
| Intercept | 39.95 | 40.31 | 40.10 | 40.12 | 0.27 | |
| Correlation coefficient (R^2) | 0.98 | 0.97 | 0.96 | 0.97 | | |

Table 2
Intra-run repeatability of HDV cDNA standards evaluated with 3 replicates of 6-fold standard dilutions.

| cDNA (copies/mL) | 1st run (Ct) | 2nd run (Ct) | 3rd run (Ct) | Mean | SD(\pm) | CV (%) |
|-----------------------------------|--------------|--------------|--------------|---------|-------------|--------|
| 8.4×10^6 | 22.09 | 22.10 | 22.07 | 22.09 | 0.02 | 0.00 |
| 8.4×10^5 | 25.76 | 25.51 | 26.00 | 25.76 | 0.25 | 0.01 |
| 8.4×10^4 | 28.29 | 28.19 | 28.38 | 28.29 | 0.10 | 0.00 |
| 8.4×10^3 | 31.57 | 31.50 | 31.63 | 31.57 | 0.07 | 0.00 |
| 8.4×10^2 | 33.95 | 33.83 | 34.07 | 33.95 | 0.12 | 0.00 |
| 8.4×10^1 | 37.00 | 37.35 | 36.65 | 37.00 | 0.35 | 0.01 |
| Slope | -3.146 | -29.860 | -2.867 | -29.260 | 0.14 | |
| Intercept | 41.66 | 42.95 | 42.47 | 42.36 | 0.65 | |
| Correlation coefficient (R^2) | 0.99 | 0.99 | 0.99 | 0.99 | | |

cDNA (Fig. 4). All 30 sera samples from the blood donors, the 5 sera samples from the patients chronically infected with HCV, and the 5 sera samples from the patients monoinfected with HBV were negative.

3.2.3. Amplification efficiency and linearity of HDV-qPCR

The linearity of HDV-qPCR was evaluated by constructing a linear regression curve for each run of serial dilutions of 6- \log_{10} calibration standard HDV cDNA and RNA, and we found a strong correlation between the dilutions (correlation coefficient $R^2 = 0.97$ and 0.99) for both standards (Tables 1 and 2). The amplification efficiency was calculated from the slope, and was -3325 (99.8%) and -2.92 (119%) for calibration standard HDV cDNA and RNA, respectively.

Table 3
Inter-run reproducibility of HDV cDNA standards evaluated with 3 replicates of 6 samples.

| Sample | 1st run (copies/mL) | 2nd run (copies/mL) | 3rd run (copies/mL) | Mean | SD(±) | CV (%) |
|--------|---------------------|---------------------|---------------------|-------------------|-------------------|--------|
| 1 | 9.1×10^2 | 8.3×10^2 | 8.1×10^2 | 8.5×10^2 | 5.3×10^1 | 0.06 |
| 2 | 1.3×10^3 | 1.2×10^3 | 1.2×10^3 | 1.2×10^3 | 6.2×10^1 | 0.05 |
| 3 | 8.6×10^2 | 7.6×10^2 | 5.9×10^2 | 7.4×10^2 | 1.3×10^2 | 0.18 |
| 4 | 2.5×10^2 | 2×10^2 | 2.5×10^2 | 2.3×10^2 | 2.9×10^1 | 0.13 |
| 5 | 1.3×10^2 | 1.5×10^2 | 1.3×10^2 | 1.3×10^2 | 1.1×10^1 | 0.08 |
| 6 | 1.9×10^3 | 1.7×10^3 | 1.3×10^3 | 1.6×10^3 | 2.8×10^2 | 0.17 |

3.2.4. Reproducibility and repeatability

Repeatability was measured by determining the intra-assay standard deviation (SD), where the SD varied from 0.27 to 0.71 and from 0.02 to 0.35 for calibration standard HDV cDNA and RNA, respectively (Tables 1 and 2). Reproducibility of the HDV-qPCR assay was confirmed by inter-assay analyses, where the HDV RNA was quantified in sera samples using 6×3 runs on consecutive days (Table 3). The HDV-qPCR assay is highly capable of determining the viral load (using the equation shown in Fig. 2) of sera samples with low variability between runs. Tests showed that the SD was 1.1×10^1 – 2.8×10^2 copies/mL and that the coefficient of variation (CV) was 0.05–0.18.

4. Discussion

The high genetic diversity of HDV has been problematic for the standardization of in-house real-time PCR assays, especially when using probe-based techniques, because HDV's small genome combined with its high genetic diversity hamper design of HDV hydrolysis probes that bind efficiently to all viral genotypes (Yamashiro et al., 2004; Shang et al., 2012). In this study, we designed primers and hydrolyzable probes that are capable of detecting all genotypes (Fig. 1) without the need for additional primers as described in other studies (Le Gal et al., 2005; Schaper et al., 2010). The primers aligned with HDV RNA sequences of all genotypes, and the isolates that are deposited in GenBank scored 90–100, 95–97, and 95–100 for the forward primer, probe, and reverse primer, respectively, which corresponded to optimal specificity *in silico*. Analytical specificity was confirmed with *in vitro* tests where blood samples from HBV-negative, HCV-monoinfected individuals were subjected to RT-qPCR HDV, thereby demonstrating that the test did not produce false-positive results (*in vitro* specificity = 100%).

Le Gal et al. (2005) used a specificity assay with 26 negative control samples, of which 5 were positive for HBsAg and HBV DNA, 6 were positive for HCV RNA, reagents 04 anti-HAV-IgM 05 HIV-RNA 01–05 HEV-RNA and negative for HBV, HCV, HAV, HEV, and HIV. Other studies have also used samples containing viruses other than HDV to assay specificity, and have reported *in vitro* specificity of 100% using negative control samples; these results are corroborated by the present study (Kiesslich et al., 2009; Mederacke et al., 2010; Ferns et al., 2012; Shang et al., 2012).

Of the 100 clinical samples that were HBsAg- and anti-HDV positive in the study, 54% were positive as determined using HDV-qPCR. Serological diagnosis of HDV infection is complex due to the natural history of the infection itself. It is important to consider that the serological ELISA that is used in the routine evaluation of patients detects IgM or IgG antibodies and may not reflect the presence of viral RNA in the sera of these individuals, since we know that each patient can be at a different stage of infection (Jardi et al., 1994; Wedemeyer, 2011). A study by Jardi et al. (1994) demonstrated that sera anti-HDV IgM is the least specific marker for HDV chronic infection that is diagnosed by the detection of intra hepatic HDAG or HDV RNA. In the present study, we developed a method that can be used to detect HDV in patient sera directly, because it is based on detection of HDV RNA, which may explain why 46% of the samples

were negative for the presence of RNA HDV when screened with anti-HDV reagent (Le Gal et al., 2005). These patients may have had super-infection with viral clearance via their immune response or been HDV patients with viral loads below the limit of detection. Regardless, the test offers guidelines to exclude antiviral therapy in this group of patients. Currently, routine laboratory diagnosis of HDV is accomplished by serologic methods. However, in recent years molecular methods for nucleic acid amplification-based techniques in-house for real-time qPCR has been shown to diagnose a variety of diseases with greater specificity.

The detection limit and linear dynamic range of our test (1.3×10^2 – 1.3×10^7 and 8.4×10^1 – 8.4×10^6 copies/mL) proved to be capable of detecting up to 130 and 84 copies/mL of standard HDV cDNA and pattern RNA, respectively. The correlation coefficients (Pearson) of our RT-qPCR were $R^2 = 0.97$ and 0.99 , demonstrating excellent linearity of the linear regression curves that were produced. The efficiency of the HDV RT-qPCR assay using HDV standard cDNA was greater (average of 99.8% and slope -3.325) than that using HDV standard RNA (average of 119% and slope -2.926); this result may be explained by the natural instability of RNA.

The intra-assay repeatability test showed that the HDV-qPCR assay developed in this study is accurate and reliable (0.38 and $0.35 \pm$ SD) for both standards (Tables 1 and 2), and that the inter-assay reproducibility (CV% 0.18–0.05) showed low variation in terms of quantifying the viral load in sera samples (Table 3). Several in-house RT-qPCR HDV assays have been developed with linear dynamic ranges, detection limits, efficiency FRET probes, and primers; the use of different standards has resulted in the absence of an international consensus for quantitation of viral load (Yamashiro et al., 2004; Le Gal et al., 2005; Kiesslich et al., 2009; Mederacke et al., 2010; Schaper et al., 2010; Ferns et al., 2012; Shang et al., 2012).

We have developed an HDV-qPCR assay that can be used to quantify HDV RNA in sera of patients with HDV that will serve as tool for rapid, accurate, and reliable diagnosis in the western Amazon region of Brazil.

5. Conclusions

These results open up possibilities for new clinical studies of the efficacy of anti-viral therapies used on patients with hepatitis delta. They will also improve our understanding of the viral kinetics of HBV and allow determination of how these viruses interact at different time points throughout the infection, especially during treatment.

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