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Quantitative longitudinal evaluations of hepatitis delta virus RNA and hepatitis B virus DNA shows a dynamic, complex replicative profile in chronic hepatitis B and D

Melanie Schaper[1], Francisco Rodriguez-Frias[1,3,*], Rosendo Jardi[1,3], David Tabernero[3], Maria Homs[3], Gerardo Ruiz[1], Josep Quer[2,3], Rafael Esteban[2,3], Maria Buti[2,3]

[1]*Department of Biochemistry, Hospital Universitario Vall d'Hebron, Paseo Valle Hebrón s/n, Barcelona 08035, Spain;* [2]*Department of Hepatology, Hospital Universitario Vall d'Hebron, Barcelona, Spain;* [3]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain*

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Background & Aims: This study presents a real-time reverse-transcription PCR (rt-RT-PCR) assay for hepatitis delta virus (HDV) RNA quantification, designed to clarify the interplay between HDV and hepatitis B virus (HBV) in chronic infection.

Methods: Serum HDV-RNA and HBV-DNA were analysed by rt-RT-PCR in a cross-sectional study of 37 untreated chronic HDV patients, 25 of whom were also longitudinally studied.

Results: In the cross-sectional study, both viruses were active in 15 (40.5%) patients and inactive in 4 (10.8%); HDV alone was active in 12 (32.4%) and HBV in 6 (16.2%). The longitudinal study showed seven replication profiles, with considerable fluctuating activity of one or both viruses, including alternating predominance. In 20% of cases, longitudinal HBV/HDV viral loads differed from cross-sectional results, indicating a risk of misinterpreting HBV/HDV interactions when assessing a single determination. Fluctuating HBV replication only increased in the presence of fluctuating HDV activity. HBsAg levels, stable in HBV single infection, fluctuated in HDV chronic infection. The results of both the cross-sectional and longitudinal study call into question the major suppressor effect of HDV over HBV, revealing an important role of HBV.

Conclusions: Longitudinal evaluation of viremia shows a complex interaction between HBV and HDV and is essential to understand the pathophysiology of chronic HDV infection.

Keywords: Hepatitis delta virus; Hepatitis B virus; Chronic delta infection; HDV-RNA quantification; Real-time PCR; Dynamic replicative profiles.

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* Corresponding author. Fax: +34 93 2746831.

E-mail address: frarodri@vhebron.net (F. Rodriguez-Frias).

Abbreviations: HDV, hepatitis delta virus; HBV, hepatitis B virus; rt-RT-PCR, real-time reverse-transcription PCR; PCR, polymerase chain reaction; ALT, alanine aminotransferase; HBsAg, HBV surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody against HBeAg; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; CV, coefficient of variation; KW, Kruskal–Wallis test; CHD, chronic hepatitis D.

Introduction

Hepatitis delta virus (HDV) is a satellite RNA virus that depends on the envelope protein of hepatitis B virus (HBV) to enter hepatocytes and assemble new HDV particles [1–3]. Dual HBV/HDV infection is associated with fulminant acute hepatitis and rapid progression to liver cirrhosis and hepatocellular carcinoma [4–6]. The mechanisms underlying this dismal clinical progression are uncertain.

Contrasting results have been obtained on the role of each virus in the pathogenicity of HBV/HDV infection. Some authors suggest that liver disease activity is related to HDV [7–11], whereas others have found that HBV replication, regardless of HBV-DNA levels, is associated with adverse outcome and lower survival, suggesting that HBV replication may modulate the pathogenesis of HDV in chronic delta hepatitis [12–14].

These inconsistent results are from cross-sectional studies. Longitudinal evaluation of HBV/HDV activities has not yet been performed in these patients. In addition, the viral relationships have been determined with qualitative methods or quantitative methods with low sensitivity [7,12,15]. In a previous report, we investigated the role of HBV/HDV in chronic dual infection and found that HBV replication was associated with lower viremia than was detected in patients with HBV infection alone. However, in that previous study, a qualitative method was used to detect HDV-RNA [16].

The purpose of this study was to develop a quantitative real-time, reverse-transcription PCR assay (rt-RT-PCR) to measure HDV-RNA levels, and to apply it in a cross-sectional and longitudinal study of serum samples from patients with chronic HBV/HDV infection, to clarify the interplay between these viruses and better understand the biological and clinical features of this disease.

Patients and methods

From January 2006 to April 2007, 37 consecutive Spanish Caucasian patients with chronic HBV/HDV infection admitted to our hospital were included in this study. All patients had histological evidence of chronic hepatitis, serum alanine amino-

transferase (ALT) levels >1.3-fold above the normal upper limit, detectable HBV surface antigen (HBsAg), and positive status for IgG antibody to HDV. Exclusion criteria were positive testing for hepatitis C virus antibody or human immunodeficiency virus antibody, current antiviral therapy, or specific treatment in the 2 years before study initiation. The patients' main demographic and serological characteristics were a mean age of 42 years (range 11–59), 73% males, and 81% positive for hepatitis e antigen (HBeAg) antibody (anti-HBe). Liver histology was available in 35 patients: 14 had liver cirrhosis and 21 had chronic active hepatitis.

This study contained two parts, a cross-sectional analysis and a longitudinal analysis. The cross-sectional study used serum samples from all 37 subjects obtained at the time they were enrolled. The longitudinal analysis included 25 of the 37 patients for whom sequential serum samples from the last 4 to 8 years (collected every 6 months) had been stored at −80 °C. Six patients (24%) were HBeAg-positive; two of them seroconverted to anti-HBe during follow-up. Regarding disease evolution, 21 (84%) patients remained stable, whereas 4 progressed to hepatic decompensation (1 received a liver transplant and another died of liver-related complications). Hepatocellular carcinoma (HCC) did not develop in any patient during follow-up. The serological, virological, and histological data of patients in the longitudinal analysis are shown in Table 1.

To analyse the effect of HDV activity on HBV parameters (HBV-DNA and HBsAg), a control group of 10 patients (80% men; median age 44 years, range 25–59), with chronic hepatitis B (CHB) and no delta infection were longitudinally tested during a period without antiviral treatment (2–4.5 years). The disease remained stable in all cases. Mean ALT at baseline was 49 IU/L (17–198). Three of these patients (33%) were HBeAg-positive; no seroconversion to anti-HBe was observed during follow-up. Five patients (50%) were infected by HBV genotype A and 5 by genotype D (50%). ALT flares were observed in four patients (three had one, and one had two flares). Biopsies demonstrated chronic active hepatitis in seven cases and cirrhosis in three. To compare HDV/HBV activities, a virus was considered predominant when its viral load was $\geqslant 1$ $\log_{10}$ greater than the viral load of the other virus.

Serological testing

HBsAg, HbeAg, and anti-HBe were determined with the Vitros System (Ortho Clinical Diagnostics, Amersham, Bucks, UK) and anti-HD with the Liasson System (Dia-Sorin, Milano, Italy). For HBsAg determination, a calibration curve was used (range, 2–750 IU/ml) prepared from highly positive pooled serum quantified according to the WHO International HBsAg Standard (NIBSC code: 00/588). Results are expressed as $\log_{10}$ IU/ml. Coefficients of variation (CVs) of HBsAg values, assessed at three levels, were 5.89% for a sample of 2.1 IU/ml (0.324 $\log_{10}$ IU/ml), 0.98% for a sample of 24.4 IU/ml (0.681 $\log_{10}$ IU/ml), and 0.3% for a sample of 750 IU/ml ($2.875 \times \log_{10}$(IU/ml)).

Quantification of HDV-RNA by real-time reverse-transcription PCR

HDV-RNA levels were determined by rt-RT-PCR in the LightCycler™ system (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, HDV-RNA was extracted from 200 µl of serum and resuspended in 50 µl of water. Twenty microlitres of extracted RNA were denatured by intense thermal shock (heating at 100 °C for 5 min and immediate freezing at −70 °C in ethanol) to relax the robust secondary structure of the HDV genome. Ten microlitres of denatured RNA were mixed with 10 µl of Tth DNA polymerase reaction mix (LightCycler RNA Master HybProbe™, Roche Diagnostics), containing 10 pmol of the specific primers DP1 (position 887): 5′-TGGCTCTCCCTTAGCCATCCGA-3′ and DP2 (position 993): 5′-TCCTTCTTTCCTCTTCGGGT-3′ and 4 pmol of each fluorescent probe, HDV-FL (position 921) 5′-TCCTCCTTCGGATGCCCAGGTCG-3′-fluorescein and HDV-LC (position 947) 5′-LC640-CCGCGAGGAGGTGGAGATGCCATp-3′. PCR consisted of an initial RT step at 61 °C for 20 min, followed by a denaturing step at 94 °C for 30 s, and 45 cycles consisting of denaturation at 95 °C for 4 s, annealing at 55 °C for 15 s, and extension at 72 °C for 15 s. Primers and probes defined a 107-bp fragment located in a highly conserved region of HDV-RNA, between the autocatalytic cleavage sites. The mean nucleotide homologies for primers and probes within the eight HDV clades were 96% for DP1, 97% for DP2, 98% for HDV-FL, and 96% for HDV-LC (Fig. 1). A cDNA clone containing three copies of the complete HDV-RNA sequence (plasmid psVL-D3) was used as the primary standard [17].

Qualitative detection of HDV-RNA and HBV-DNA, and HDV and HBV genotyping

Qualitative detection of HDV-RNA and quantitation of HBV-DNA were performed as previously reported [18–19]. HDV-RNA was genotyped by direct sequencing after RT-PCR, using a reported method [20], and nucleotides were numbered according to Makino et al. [21]. Consensus nucleotide sequences were compared to GenBank pattern sequences (accession numbers in Fig. 1), corresponding to the eight HDV clades [22]. HBV genotyping was performed by INNO-LIPA assay, as described [23].

Statistical analysis

The Mann–Whitney *U* test, one-way ANOVA, and the Kruskal–Wallis (KW) test were applied to compare quantitative data. The CV was used to evaluate fluctuations in quantitative parameters (HDV-RNA, HBV-DNA, HBsAg) in longitudinal study profiles. SPSS, version 15.0, was used for the statistical analyses. Significance was set at a p value of less than 0.05.

Results

Sensitivity, specificity, and reproducibility of rt-RT-PCR for HDV-RNA quantification

A linear relationship from 10^3 to 10^8 copies/ml was observed between cycle threshold values and number of HDV cDNA copies used as standard ($r > 0.99$). To obtain a standard as similar as possible to human samples, a serum sample from a chronic hepatitis D (CHD) patient highly positive for qualitative HDV-RNA was quantified in triplicate using the HDV cDNA standard curve, showing a level of 10^9 HDV cDNA copies/ml (HDV-RNA equivalent/ml). Sensitivity and linearity of the method were assessed by serial dilutions of CHD standard (10–10^9 HDV-RNA equivalent/ml). Sensitivity was 10^3 HDV-RNA equivalent/ml, and linearity was from 10^3 to 10^7 (correlation coefficient, 0.99) (Fig. 2). To evaluate the specificity of the method, 20 serum samples qualitatively positive for HDV-RNA and 20 anti-HD-negative/HBsAg-positive were quantified in duplicate with rt-RT-PCR. Of the 20 qualitatively positive samples, 19 were positive by quantitative methods, with a range from 3.3 to 5.1 $\log_{10}$ HDV-RNA equivalent/ml. The 20 anti-HD-negative/HBsAg-positive patients had an HDV-RNA value lower than 10^3 HDV-RNA equivalent/ml.

Intra-assay variability was determined by analysing 10 times in a single day two dilutions of CHD standard (10^4 and 10^7 HDV-RNA equivalent/ml), which yielded CVs of 5.2% and 2.7%, respectively. Inter-assay CVs, calculated by analysing the same dilutions once a day for 10 days, were 7.3% and 4.8%, respectively. In all HDV-RNA quantitation experiments, serial dilutions (10^3–10^7 HDV-RNA equivalent/ml) of CHD sample were processed as standard.

Cross-sectional study: HDV-RNA quantitation and relationship with HBV-DNA levels; HBV/HDV genotypes

All 37 patients were enrolled in the cross-sectional study. The rt-RT-PCR technique detected HDV-RNA (>10^3 equivalent/ml) in 27 (73%) cases, whereas HBV-DNA ($>3 \times 10^2$ copies/ml) was observed in 21 (56.8%). Active dual infection was detected in 15 patients (40.5%), inactive HBV/active HDV in 12 (32.4%), active HBV/inactive HDV in 6 (16.2%), and no viral activity in 4 (10.8%). Median HBV-DNA was 3.4 $\log_{10}$ (<2.5–8.6), and median HDV-RNA was 5.3 $\log_{10}$ (<3.0–7.9) ($p = 0.02$).

An analysis of replicative activity by HBV-DNA and HDV-RNA levels when both viruses were active showed that when HBV was predominant, median HBV-DNA was $\log_{10}$ 6.4 (4.1–8.6) and HDV-RNA $\log_{10}$ 4.3 (3–5.1), whereas when HDV was predominant, median HBV-DNA was $\log_{10}$ 3.8 (3.1–4.9) and HDV-RNA