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One-step real-time PCR assay for detection and quantitation of hepatitis D virus RNA

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A B S T R A C T

Hepatitis D virus (HDV) is a defective virus which requires hepatitis B virus (HBV) surface antigen (HBsAg) for its assembly. Hepatitis B infected individuals co-infected or superinfected with HDV often present with more severe hepatitis, progress faster to liver disease, and have a higher mortality rate than individuals infected with HBV alone. Currently, there are no commercially available clinical tests for the detection and quantitation of HDV RNA in the United States. A one-step TaqMan quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was developed for detection of HDV RNA, designing primers located in the region just downstream from the HDV antigen gene. The assay has the potential to detect all eight HDV genotypes. A quantifiable synthetic RNA control was also developed for use in the determination of HDV RNA titers in clinical samples. The limit of detection of this assay is 7.5×10^2 HDV RNA copies/ml with a dynamic range of six logs. Most clinical specimens tested (40/41) fell within the linear range of the assay. The median HDV RNA titer of the tested specimens was 6.24×10^6 copies/ml, with a range of 8.52×10^3–1.79×10^9 copies/ml. Out of 132 anti-HDV-positive specimens 41 (31.1%) were positive for HDV RNA.

1. Introduction

Hepatitis D virus (HDV), also called hepatitis delta virus, is a small circular negative single-stranded RNA virus of approximately 1700 nucleotides with the ability to replicate only in the presence of hepatitis B virus (HBV) envelope (Rizzetto et al., 1980). Due to many unique features usually found in plant viroids, HDV was assigned its own genus, the *Deltavirus* (Mason et al., 2005). HDV is considered a defective virus because it requires the hepatitis B surface antigen (HBsAg) to assemble (Rizzetto et al., 1980). Due to 74% homology in base pairing, HDV RNA has the ability to fold on itself in an unbranched, double-stranded rod-like structure (Wang et al., 1986). The genome and the antigenome of HDV also contain a ribozyme domain, capable of self-cleavage and self-ligation (Sharmeen et al., 1988; Wu et al., 1989). HDV creates anti-genomic RNA through the double-rolling circle mechanism of replication (Chen et al., 1986), which serves as an mRNA for the hepatitis D antigen (HDAg) (Lai, 2005). There are two forms of the HDAg, both coded by the same open reading frame (ORF). The small hepatitis D antigen (S-HDAg) is 195 amino acids long (24 kDa), while large hepatitis D antigen (L-HDAg) is 19 amino acids longer on the carboxyl-terminal end (27 kDa), consisting of a stretch of variable, yet genotype-specific, membrane-attaching sequence and serves as the virion assembly signal (Lai, 2005).

Like many RNA viruses, HDV exhibits a high level of genetic diversity. Eight genotypes of HDV have been reported (Le Gal et al., 2006; Radjef et al., 2004), with genotype 1 being prevalent worldwide, and other genotypes being endemic to different parts of the world (Hughes et al., 2011). Genotype 2 is found in Southeast Asia, Taiwan, China and Japan, genotype 3 is endemic to the Amazon Basin, genotype 4 is found in Taiwan and Japan, while genotypes 5–8 are prevalent in Africa (Hughes et al., 2011; Pascarella and Negro, 2010).

Individuals with concurrent acute HBV and HDV infection are considered co-infected, whereas patients who are chronically infected with HBV may develop a superinfection with HDV (Pascarella and Negro, 2010). Co-infection or superinfection with HDV can cause more severe hepatitis than HBV infection alone, including more rapid progression to liver disease and higher

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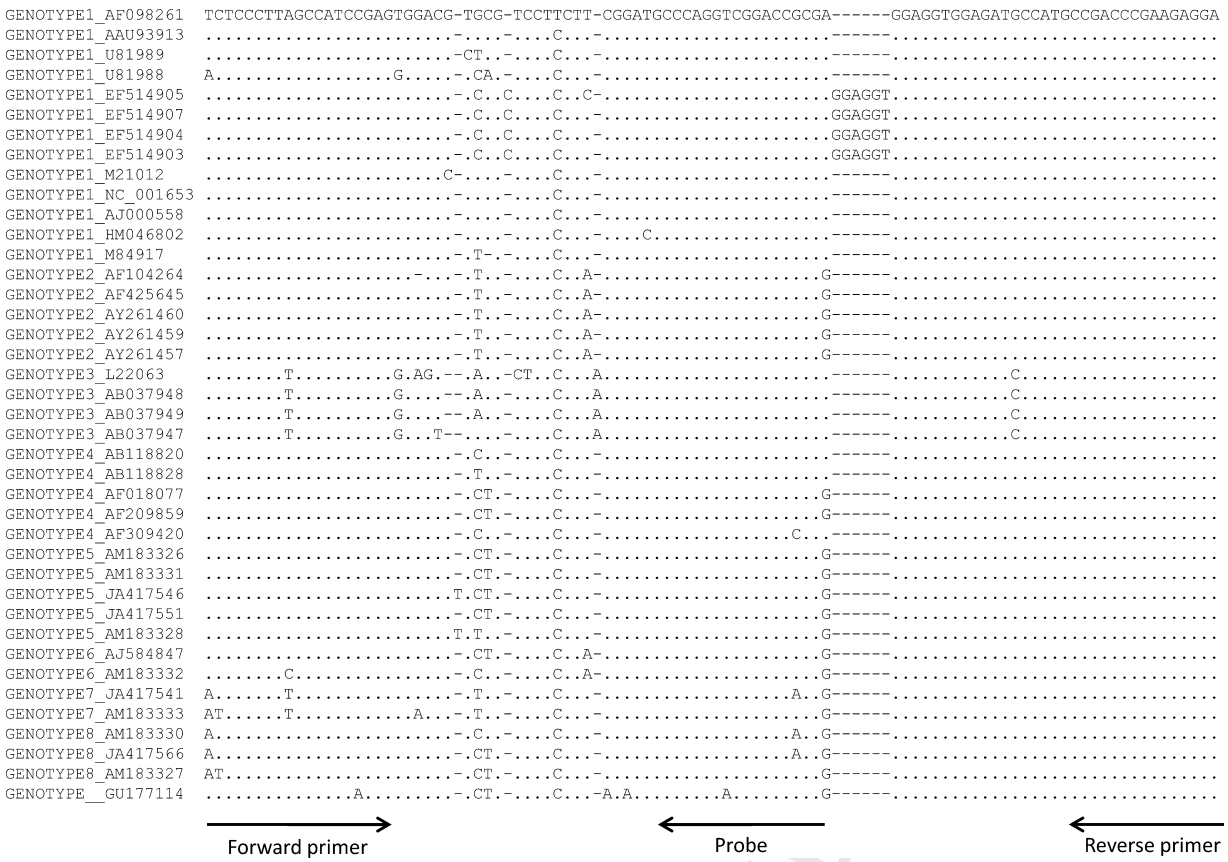

Fig. 1. Alignment of the qRT-PCR amplicon region using sequences representing all HDV genotypes. Assignments on left indicate genotype and GenBank accession number. Positions of qRT-PCR primers and probe are also indicated.

mortality rates (Hadler et al., 1984). Due to this risk, it is important for patients who are HBsAg positive to be tested for HDV infection (Hughes et al., 2011; Wedemeyer and Manns, 2010).

Although anti-HDV antibody status can assess whether an individual has been exposed to HDV, it cannot determine if the infection is active or resolved. The aim of this study was to establish an easy-to-use, one-step quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay for detection and quantitation of HDV RNA in human serum.

2. Materials and methods

2.1. Primer and probe design

Sequence alignment of full-length HDV genomes was used to locate conserved regions of the genome to identify potential regions suitable for HDV RNA amplification (Fig. 1). Primers PAN-HDVF and PAN-HDVR and probe PAN-HDVP had the best analytical performance (Table 1). These primers amplify a sequence located upstream of the L-HDAg ORF (Fig. 1).

2.2. Analytical validation of the qRT-PCR assay

An HDV RNA standard for evaluation of HDV RNA PCR is not currently available. Because of lack of a standard dilution panel, an anti-HDV positive human serum specimen was used to make 10-fold serial dilutions in normal human serum for nucleic acid extraction. This material was used for initial analytical validation in which the best performance conditions for amplification of HDV RNA were determined. Various concentrations and ratios of primers and probes were tested to optimize the assay for fluorescence intensity level, efficiency, and specificity. In addition, HBV DNA Genotype Performance Panel PHD201(M) (SeraCare, Milford, MA) was tested to verify that there was no cross reactivity with HBV using this HDV qRT-PCR assay. Specificity of the assay was determined using 48 anti-HDV-negative human sera.

2.3. Clinical specimens

The evaluation panel included 132 HBsAg positive sera previously referred to our laboratory that had been stored at −70 °C. These sera originated from the United States, Moldova, Romania and Venezuela. The HBsAg positive sera from this cohort were screened for anti-HDV positivity. All HBaAg/anti-HDV positive sera were screened by the newly designed assay for HDV RNA positivity. Qualitative confirmation of the HBsAg-positive status was determined using the VITROS ECi Immunodiagnostic System (Ortho-Clinical Diagnostics, Inc., Rochester, NY). Total anti-HDV was detected using the EIA-AB DELTAK-2 kit (DiaSorin, Stillwater, MN); positive samples were tested for HDV RNA in two independent runs by the qRT-PCR assay.

2.4. Extraction of viral nucleic acids and one-step qRT-PCR

Total nucleic acid was extracted from 200 μl of each serum specimen using the MagNA Pure LC Instrument and the MagNA Pure Total Nucleic Acid Isolation kit (Roche Diagnostics, Pleasanton, CA), eluted in 50 μl of elution buffer and stored at −70 °C until further use.

qRT-PCRs were performed on the ABI 7500 real-time PCR instrument using the AgPath-ID One-Step kit (Applied Biosystems, Foster City, CA) following the manufacturer-recommended protocols. All

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