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Virology

Quantitative detection and typing of hepatitis D virus in human serum by real-time polymerase chain reaction and melting curve analysis

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Abstract

Hepatitis D virus (HDV) infection is an important etiologic agent of fulminant hepatitis and may aggravate the clinical course of chronic hepatitis B infection resulting in cirrhosis and liver failure. This report describes the establishment of a real-time reverse transcriptase polymerase chain reaction method that allows the quantitative detection of HDV-1 and HDV-3 with a sensitivity in a linear range of 2×10^3 to 10^8 copies/mL. Additionally, the new assay provides the opportunity to distinguish HDV-1 from HDV-3 by a subsequent melting curve analysis, an important option because these HDV types are highly associated with severe clinical outcome. The results of the melting curve analysis of 42 HDV sequences obtained in this study and the phylogenetic analysis based on 139 full-length sequences from GenBank were consistent and showed that all sequences described here cluster within the HDV-1 clade. Therefore, this assay is useful for monitoring of antiviral treatment and molecular epidemiologic studies of HDV distribution.

Keywords: Hepatitis delta virus; Genotyping

1. Introduction

Hepatitis D virus (HDV) is a subviral satellite that requires the presence of hepatitis B surface antigen (HBsAg) for its assembly and transmission (Rizzetto et al., 1980). The virus is endemic worldwide, with prevalence and patterns of infection varying in different regions. It is speculated that around 20 million individuals suffering from hepatitis B virus (HBV) infection are also infected with HDV (Radjef et al., 2004). Coinfection of HBV and HDV (simultaneous infection) results in both acute type B and acute type D hepatitis, which usually exhibits a self-limited course. The chronic form of hepatitis D is seen in less than 5% of HBV/HDV coinfected patients (Hadziyannis, 1997). In contrast to the coinfection, the superinfection of chronic HBV carriers with HDV is frequently associated with fulminant acute hepatitis and higher progression rates to severe chronic active hepatitis and cirrhosis (Farci, 2003). The circulation of HDV has declined significantly down to approximately 8% to 10% in HBsAg carriers in Europe within the last 2 decades (Gaeta et al., 2007), thus reducing the number of hepatocellular carcinoma (HCC) and mortality because of cirrhosis. It has been shown that HDV infection increases the risk for HCC 3-fold and the mortality 2-fold in patients with cirrhosis (Fattovich et al., 2000). However, persistent reservoirs of HDV in Europe still remain, composed of people who became infected with HDV during the last epidemics in the 1970s and 1980s and of immigrants from endemic areas (Erhardt et al., 2003).

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HDV is the only animal virus with a small circular, single-strand minus RNA genome. HDV utilizes the host RNA polymerase II, which is redirected to carry out HDV RNA-directed transcription (Taylor, 2006). The mutation rate was determined to vary between 3×10^{-2} and 3×10^{-3} base substitutions per nucleotide per year (Lee et al., 1992), thus leading to an overall nucleotide sequence variability of about 40% (Deny, 2006). Based on the complex genetic variability, HDV isolates collected worldwide have been classified into 8 groups, HDV-1 (former genotype I), HDV-2 (former genotype IIa), HDV-3 (former genotype III), HDV-4 (former genotype IIb), and HDV-5 to HDV-8 (Radjef et al., 2004). These HDV types show different geographic distribution and are associated with different disease patterns. HDV-1 has a worldwide distribution with a broad spectrum of associated diseases, ranging from asymptomatic to fulminant hepatitis. In contrast, HDV-2 and HDV-4 are mainly prevalent in Asia, HDV-3 so far is prevalent in the northern part of Latin America, and HDV-5 to HDV-8 are common in Africa (for review, see Chao, 2007). HDV-3 was found to be often associated with severe acute hepatitis especially in association with HBV genotype F, whereas HDV-2 and HDV-4 generally lead to a less severe outcome of acute and chronic HDV infection.

The aim of this study was to establish a reliable polymerase chain reaction (PCR) assay for the quantitative detection of all HDV types and subsequent identification of the HDV-1 and HDV-3 types. The nucleic acid amplification testing (NAT) might be helpful in the improved monitoring of antiviral treatment of the hepatitis D and for molecular epidemiologic studies. Furthermore, the first HDV sequences were obtained from 42 patients living in Germany.

2. Materials and methods

2.1. Patient samples

A total of 50 serum samples of patients with an acute or chronic HDV infection were selected in the routine diagnostic laboratories of the Institutes of Virology in Berlin and Essen. All patients lived in Germany but almost half of them had a migration background; thus, they possibly became infected in their home countries: 18 German, 10 Russian, 13 Turkish (7 of them born in Germany), 3 from other European, and 6 from other Asian countries.

All patient samples were positive for HDV RNA as determined by an established method (Hopf et al., 1991) or by an in-house assay in the Institute of Virology (Essen). HBV viral loads were between 20 and 70 Mio IU/mL as determined by commercial assays (COBAS® AmpliPrep/ COBAS® TaqMan® HBV Test [Roche Diagnostics, Mannheim, Germany] and VERSANT Hepatitis B Virus DNA 3.0 Assay [Siemens Healthcare Diagnostics, Eschborn, Germany]). The limits of detection are 54 IU/mL for the COBAS assay and 20 IU/mL for the VERSANT assay. All samples were HBV genotyped by conventional sequencing. HBsAg was determined by the ARCHITECT HBsAg assay (ABBOTT, Illinois, USA). Antibodies to HDV were detectable in all samples by ETI-AB-DELTAK-2 anti-HD (DiaSorin Deutschland, Dietzenbach, Germany).

2.2. Quantitative determination of HDV RNA concentration

Viral RNA was isolated from serum samples applying the QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Reverse transcriptase (RT) reaction with SuperScript™ III RT (Invitrogen, Karlsruhe, Germany) and Primer "Random ($p(dN)_6$)" (Roche Applied Science, Mannheim, Germany) was followed by real-time PCR. The RT reaction was performed in 20-μL total volume containing 4 μL of 5× PCR buffer, 0.1 μL of dithiothreitol (0.1 mol/L), 3 μL of desoxynucleotid-triphosphate (dNTP)s (2.5 mmol/L), 1 μL of protector RNase inhibitor (40 U/μL), 0.5 μL of Super Script III (200 U/μL), 0.5 μL of $p(dN)_6$ primer (600 ng/μL), and 10 μL of heat-treated (5 min/70 °C) RNA. The transcription was set up on ice and performed as follows: 5 min/25 °C to 35 min/50 °C to 2 min/95 °C. Two microliters of the RT reaction were amplified in a LightCycler 2.0 instrument (Roche, Basel) in 20-μL reaction volume containing the following: 1 μL of primers HD10 and HD11 (each 10 pmol/μL), 3 μL of donor probe Hepatitis D locked nucleic acid (HD LNA) (4 pmol/μL), 1 μL of acceptor probe HD13R (4 pmol/μL), and 4 μL of

Table 1
Primers and probes used for HDV RNA detection and site-directed mutagenesis

Primer/probe	5'-sequence-3'	nt position
Screening and genotyping		
HD10	AGTGAGGCTTATCCCGG	469–485
HD11	CTCGGATGGCTAAGGGAG	836–819
HD LNA	AG+CCT+CCT+CG+CTGGCGCC-FL	703–720
HD13R	LCRed640-GCTGGGCAACGATCCGAGG-ph	722–740
Cloning and site-directed mutagenesis		
pSC-A fwd	GACCATGATTACGCCAAGCG	Vector
pSC-A rev	TCGCTATTACGCCAGCTGG	Vector
HDV fwd	GCTGGGCAACGATCCGAG	722–739
HDV rev	CTCGGATCGTTGCCCAGC	739–722

fwd = forward, rev = reverse.

Table 2
Protocol for PCR and melting curve analysis

	T (°C)	T (s)
Activation	95	600
Touchdown	95	5
	62–48	15
	72	10
Amplification (35 repeats)	95	5
	48	15
	72	10
Melting curve	95	0
	45	0
	85[a]	0

[a] Transition rate, 0.2 °C/s.

LightCycler FastStart DNA Master plus Kit (Roche Diagnostics). All primers and probes used are listed in Table 1. The touchdown protocol and the conditions for the melting curve analysis are shown in Table 2. The fluorescence was measured during the annealing phase starting with cycle 16 (no measurement during the touchdown cycles). Two dilutions of the plasmid pSC-A HDV-1, equivalent to 10^4 and 10^6 copies/mL, serve as recalibrators for the quantification. The control for the melting curve analysis consisted of a mix of pSC-A HDV-1/pSC-A HDV-3 (see below) in equal parts.

2.3. Site-directed mutagenesis to generate an HDV-3–like target

The PCR product derived from an HDV-1–positive sample was inserted into the cloning vector pSC-A-amp/kan (StrataClone PCR Cloning Kit, Invitrogen) following the instructions of the manufacturer. One clone (referred to as pSC-A HDV-1) was sequenced by the method of Sanger et al. (1977), applying the 3130 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany), and used as target for site-directed mutagenesis. In order to synthesize an HDV-3–like target, 2 PCR products were generated with primers pSC-A fwd/HDV rev and HDV fwd/pSC-A rev (Table 1) in a 50-μL reaction volume containing 5 μL of 10× PCR buffer, 4 μL of $MgCl_2$ (2 mmol/L), 4 μL of dNTPs (each 200 μmol/L), 1 μL of primers (0.2 μmol/L), 0.25 μL of AmpliTag Gold (Applied Biosystems), and 5 μL of DNA (approximately 2 ng/μL). Both PCRs were run following the same protocol: 10 min/95 °C, 35 repeats of 15 s/95 °C to 15 s/50 °C to 20 s/72 °C, and a final extension of 7 min/72 °C. After a gel purification step of the resulting amplicons, a primer extension (14 repeats for 45 s/41 °C to 45 s/72 °C) was performed. The resulting product was amplified as described above using the vector-specific primers pSC-A fwd and pSC-A rev in a final concentration of 0.5 μmol/L. PCR protocol was 10 min/95 °C, 35 repeats of 15 s/95 °C to 45 s/52 °C to 45 s/72 °C, and a final extension of 7 min/72 °C. The type 3-like fragment was cloned as described above and its correctness confirmed by Sanger sequencing. The plasmid is referred to as pSC-A HDV-3. Primers and probes were purchased from BioTeZ and TIB MOLBIOL (Berlin, Germany), respectively.

2.4. Sequence retrieval and alignment, tree reconstruction

One hundred thirty-nine different complete HDV genome sequences were downloaded from National Center for Biotechnology Information (NCBI) GenBank under the taxonomy ID 12475. The genomes were aligned with M-Coffee (Wallace et al., 2006) that combines the alignments from 4 different methods: T-Coffee (Notredame et al., 2000), Mafft (Katoh et al., 2002), ProbCons (Do et al., 2005), and Muscle (Edgar, 2004). The sequence alignment was done separately for the coding region (the gene for the hepatitis delta antigen) to account for coding sequence (CDS) and for noncoding parts. The 42 HDV sequences of the German patients (accession numbers GQ423683-GQ423724) were aligned to the full genome alignment using ClustalW profile alignment (Thompson et al., 1994). The NCBI accession numbers of the retrieved sequences are available in the supplementary material.

HDV maximum likelihood trees were reconstructed using IQPNNI (Vinh and von Haeseler, 2004) and RAxML (Stamatakis et al., 2008). The reliability of the tree were assessed by nonparametric bootstrap with 1000 replicates for Important Quartet Puzzling and Nearest Neighbor Interchange (IQPNNI) and with the rapid bootstrap for Randomized Axelerated Maximum Likelihood (RAxML) (Stamatakis, 2006). For both methods, the GTR+Γ model of substitution was used (Tavare, 1986). A majority-rule consensus tree was constructed from the 1000 RAxML bootstrap trees using TREE-PUZZLE (Schmidt et al., 2002). Finally, the consensus tree was drawn using the iTOL software (Letunic and Bork, 2007).

The testing of the samples was allowed by the institutional ethics committee without an additional approval.

3. Results

3.1. Quantitative detection and genotyping of HDV RNA by real-time RT-PCR

The quantitative real-time RT-PCR is established on a LightCycler device using 2 hybridization probes for the fluorescence resonance energy transfer. In comparison to TaqMan probe-based assays, this approach facilitates a subsequent typing based on melting curve analysis.

The primer binding sites are highly conserved within the HDV genomes of all 8 classified types and are located upstream of the region coding for the HD antigen. Additionally, the amplified target includes conserved regions that can be used as target for the hybridization probes (Fig. 1 and Table 2). Because of the extensive intramolecular complementary of the HDV genome, it became necessary to carry out a touchdown real-time RT-PCR. The binding region of the donor probe constitutes a very stable stem loop;

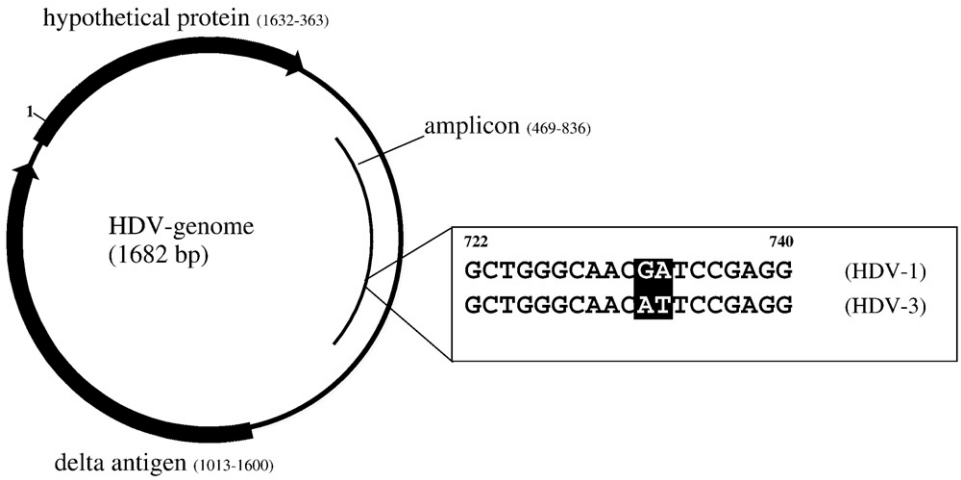

Fig. 1. Schema of the HDV genome (NC_001653, HDV-1 type) with the position of the PCR product. Nucleotides from 722 to 740 serve as target for acceptor probe binding. The 2 highlighted adjacent mismatches are used to differentiate between HDV-3 and the other types by a melting curve analysis after the RT-PCR.

approximately 80 °C being required to melt off the loop. To enable binding of the donor probe, we supplied this oligonucleotide with locked nucleic acids (designated as +C) and in a 3-fold excess compared to the acceptor probe. An amplicon of an HDV-1 RNA-positive patient sample was cloned and used for determination of the sensitivity of the assay. A 10-fold serial plasmid dilution equivalent to 10^6 to 1 copies/reaction was used to plot a standard curve (Fig. 2A, error of 7.6% and an *r* value of -1). A detection limit of the assay of 10 plasmid copies/reaction with 95% probability of detection was determined. Ten samples were additionally tested by the method of Erhardt et al. (2006). The obtained similar results confirmed the reliability of our method (data not shown).

Fifty nonselected HDV RNA and HBs antigen-positive serum samples of patients from the university hospitals Charité Berlin and Essen were quantified by the new assay. The HDV viral load ranged from 883 to 6.9 Mio copies/mL

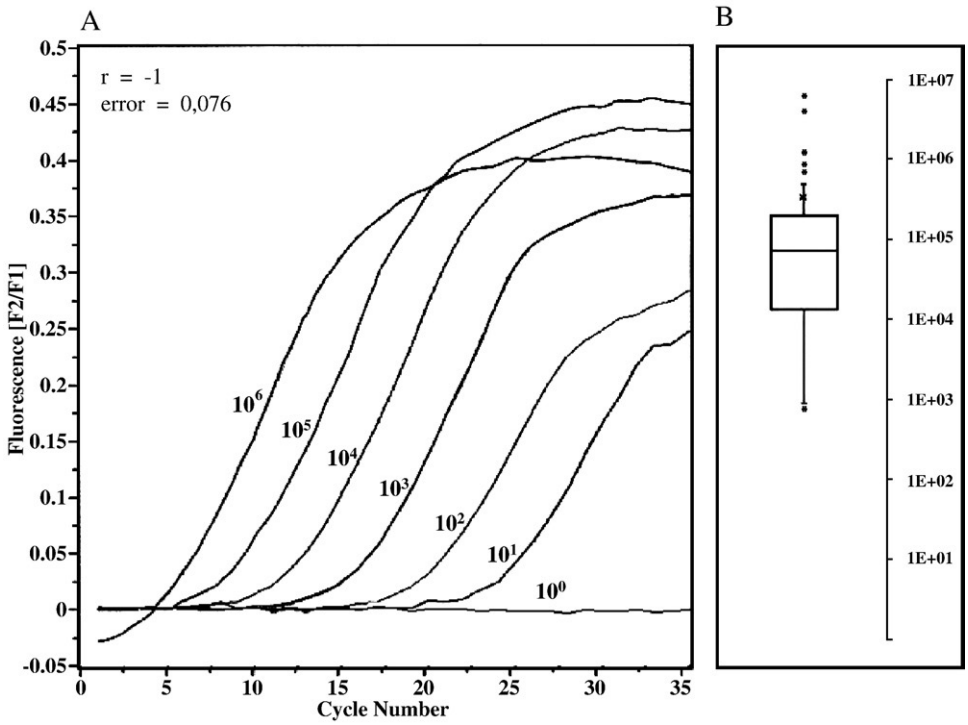

Fig. 2. Determination of the assay sensitivity. (A) HDV-PCR was performed with plasmid DNA in a dilution series from 10^6 to 1 copies/reaction, which was then used for plotting the standard curve. Because of the touchdown protocol and associated settings, the initial 15 steps are not recorded; thus, cycle 5 in the graph reflects PCR cycle 20. (B) Box plot graph of 50 HDV viral loads of German patient samples determined by the new NAT assay with an average of approximately 73 000 HDV RNA copies/mL.

(median, 73 000 copies/mL, and mean, 443 000 copies/mL, Fig. 2B). HBV-DNA was also detected in approximately 70% of the HDV RNA-positive samples. All DNA-positive samples belong to HBV genotypes A or D, which are predominant in Germany.

The typing approach was focused on the detection of HDV-1 and HDV-3 types, which can be associated with severe clinical outcome. Because of the nonavailability of HDV-3 isolates, it was necessary to generate an HDV-3–like target by site-directed mutagenesis of an HDV-1 sequence. Two adjacent nucleotide mismatches at position 732/733 are used for differentiation by a melting curve analysis. The acceptor probe is complementary to the HDV-3 sequence. This probe melts off from an HDV-3–like target at approximately 68 °C and from an HDV-1 target at approximately 54 °C (Fig. 3). The difference of 12 to 14 K allows a rapid identification of HDV-3 strains. The other HDV types (HDV-2, 4–8) are not distinguishable from HDV-1 by this approach but are not considered as causative agents for severe clinical cases. The HDV-4 sequences contain mutations in the probe binding sites, which could have an influence on the sensitivity of the assay and the melting point for those targets. However, the strains used in this study were all from the Miyako Islands in Japan and can be reduced to 2 different clades (Watanabe et al., 2003).

In order to confirm the typing results, all amplicons derived from the patient samples were sequenced bidirectionally.

3.2. Sequencing and phylogenetic analysis of the PCR products

For 42 out of the 50 patient samples, sequence data were obtained. Two samples had a viral load of 883 and 1000 copies/mL, respectively. These small loads might be below the detection limit of the PCR in the settings of the sequencing reaction. The sequence data of the remaining 6 samples could not be interpreted because of a massive overlay of signals, perhaps caused by mixed viral variants in the sample. In order to assess the proportion of virus variants in samples with heterogenous sequences, 1 amplicon from a patient exhibiting 150 400 cop/mL (accession number GQ423697) was cloned as described. The sequences of 2/10 clones were identical, and the remaining 8/10 clones revealed 3 to 9 nucleotide exchanges (Fig. 4). The phylogenetic tree shows that all clone sequences cluster together with the sequence of the amplicon. This finding is indicative for the presence of quasispecies and does not provide evidence for mixed HDV types in this patient sample.

A DNA alignment comprising 139 different full HDV genomes contains 1890 sites including gaps; the 42 German sequences of this study were aligned to the genome alignment.

The majority-rule consensus tree from the bootstrap analysis with RAxML is displayed in Fig. 4. Overall, this tree corroborates previous studies. Moreover, the important groups exhibit high bootstrap supports. The tree clearly separates the 3 former genotypes I, II, and III. It is also congruent with the new assignment of HDV-1 to HDV-8 groups. Moreover, our sequences and the previously known genotype I sequences clearly form a monophyletic group (RAxML-BP: 100, IQPNNI-BP: 98%). Computations of the pairwise distances within the sequences of our patients, within the non-German sequences, and the in-between distances of these 2 groups revealed no distinct cluster for our sequences within the HDV-1 group (data not shown).

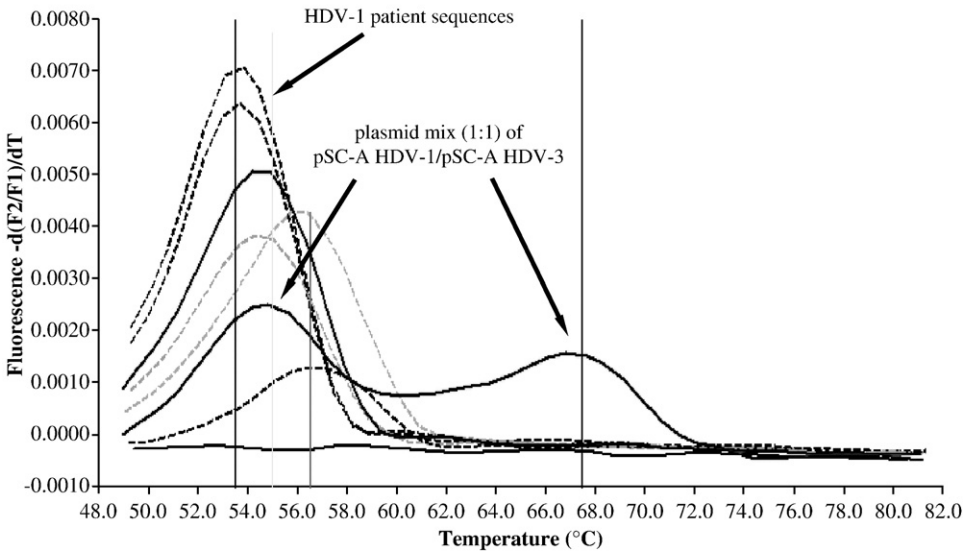

Fig. 3. Melting curve analysis of plasmid controls and of various patient sequences (dotted lines). pSC-A HDV-1 contains a cloned PCR product of an HDV-1 strain; pSC-A HDV-3 comprises an HDV-3–like sequence obtained by site-directed mutagenesis. The acceptor probe is complementary to HDV-3 and melts off from an HDV-3 target at approximately 68 °C and from an HDV-1 strain at approximately 54 °C. The control used for the melting curve analysis consists of a mix of both plasmids (each 5000 copies).

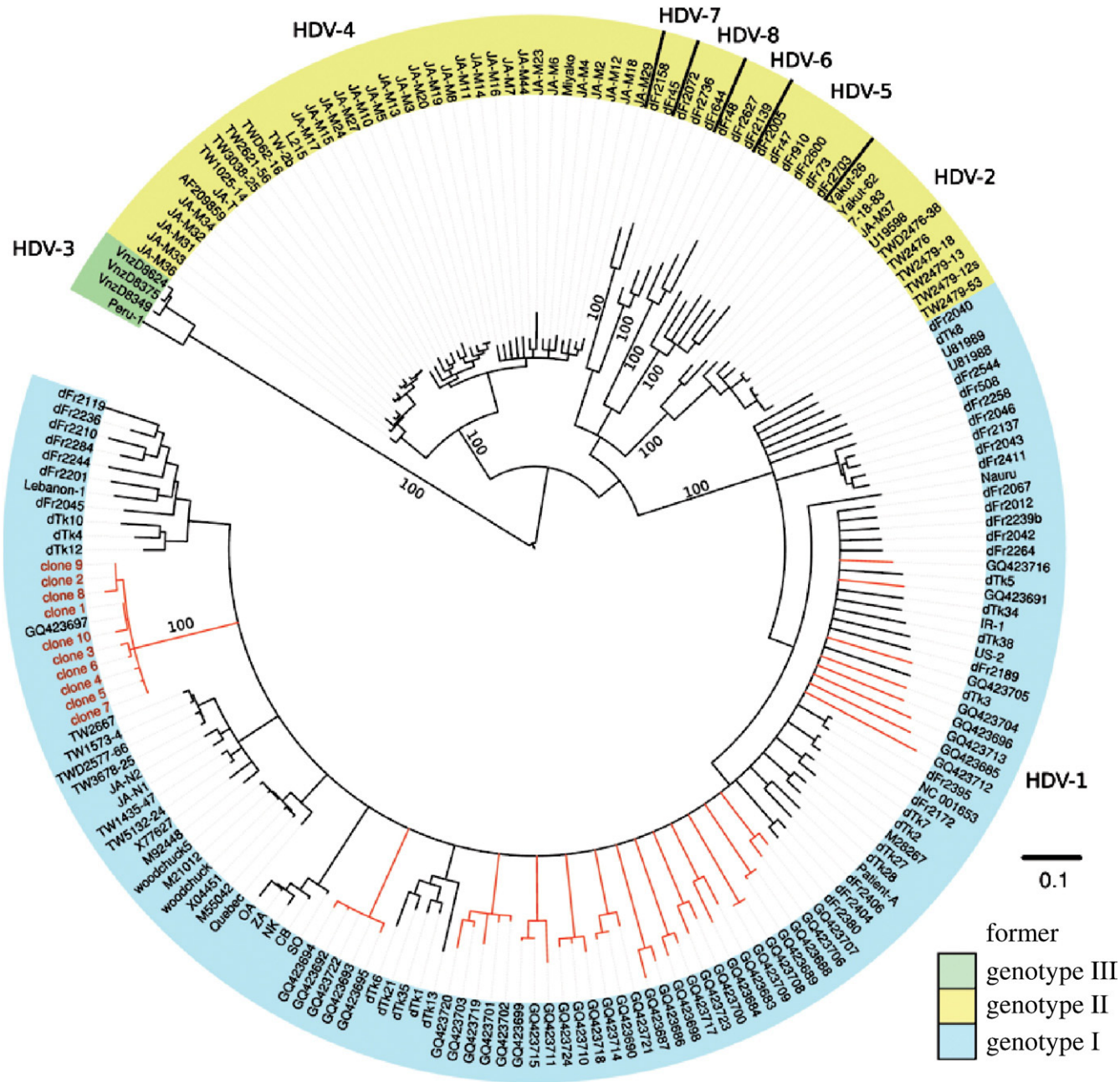

Fig. 4. Classification of the patient sequences into the phylogenetic tree consisting of all published HDV full-length sequences. The tree is rooted at the branch separating HDV-3 from all other HDV clades. Taxa of the former genotype I, II, and III are colored in cyan, yellow, and light green, respectively. The branches leading to the German taxa are highlighted in red. Numbers associated with each deep branch are the bootstrap support values by RAxML. The bootstrap values determined by IQPNNI are 100% except 98% for HDV-1. The 10 clone sequences obtained from 1 patient sample (accession number GQ423697) cluster together with the original sequence.

4. Discussion

Many factors such as the time and duration of infection, the viral load, the appearance of defective HDV RNAs (Wu et al., 2005), the origin of the HBV helper strain, and the genetic background of the patient (Dieye et al., 1999) contribute to the pathogenicity of HDV. Additionally, the HDV genotype has an influence on the clinical outcome of an infection (Rizzetto, 2009). Therefore, a NAT assay that provides the possibility to type HDV isolates is required, preferentially in a quantitative format. Commercial assays are not available, and only a few methods for quantification of HDV RNA have been published yet. Furthermore, they are limited to distinct HDV types (Yamashiro et al., 2004) or represent consensus PCRs but without a typing option (Le Gal et al., 2005). The method presented here is suitable for a rapid quantification of HDV RNA with a linear range from 2×10^3 to 10^8 HDV RNA copies/mL. The quantification of

50 HDV RNA-positive samples revealed viral loads that fall within the linear range. The viral loads are also comparable with those reported in other studies (Castelnau et al., 2006; Le Gal et al., 2005).

It has been demonstrated that the quantitative detection of HDV RNA in serum is the most reliable biologic marker for treatment monitoring (Castelnau et al., 2006). Treatment of an HDV infection is usually performed with pegylated interferon (PEG-IFN) alpha. Depending on the duration of therapy, only 15% to 50% of the patients have a sustained virologic response (Castelnau et al., 2006; Erhardt et al., 2006). To distinguish between responders and nonresponders, the decrease of HDV RNA count provides predictive information. This information may lead to, for instance, an extension in the therapy schedule of treated patients with an insignificant viral load decrease or of slow responders.

Among the patient samples, no correlation between the viral loads of HDV RNA and HBV DNA was seen. This was expected because the majority of the patients experience an antiviral therapy with nucleoside reverse transcriptase inhibitors (NRTIs) to HBV, which, in turn, has little or no effect on HDV replication (Lau et al., 1999; Niro et al., 2005). To demonstrate the suitability of the new quantitative HDV-PCR, a study with PEG-IFN alpha-treated patients has recently been started. Preliminary data obtained from the first patients over a period of 1 year demonstrate that the HDV-PCR is a useful tool to monitor the viral load (Fülöp et al, in preparation).

Besides the quantitative detection of HDV RNA, the described PCR method provides the opportunity to identify HDV-3 by a subsequent melting analysis. Two adjacent nucleotide mismatches in the acceptor probe binding site (Fig. 1) correspond exclusively with HDV-3 sequences and were used for rapid type identification. Because HDV typing is not routinely performed in HDV-3 endemic areas, relevant samples to confirm this hypothesis were not available. Therefore, an artificial HDV-3–like target was generated by site-directed mutagenesis and used as a positive control (Fig. 3). Considering global tourism and human migration, the method is suitable for epidemiologic studies addressed to the type distribution of HDV strains. The opportunity to quantitate and to genotype HDV in a single run without additional efforts should be of benefit even in developing countries.

Although our patient samples were only collected from 2 geographic regions in Germany, the results are indicative for a predominance of HDV-1 strains. In rare cases, where the differentiation into HDV-1 and HDV-4 to HDV-8 is necessary, sequencing of the PCR fragment is sufficient for determination of the HDV type.

Reliable interpretation of the sequence data of the German patients could be obtained for approximately 90% of samples. In 6 cases, heterogenous sequences were found suggesting the presence of HDV RNAs of different origins. This can be caused by several factors including the presence of quasispecies, defective RNAs (Wu et al., 2005), or intra- and intergenotypic mixed infections. The analysis of a certain number of clones of 1 sample revealed the presence of quasispecies of the HDV-1 type resulting from errors in the transcription process of the viral genome. Defective RNAs have been found in serum of almost 80% of patients with chronic hepatitis D, however, in proportions of less than 4% compared to full-length RNA (Wu et al., 2005). Those species might not be detectable by testing of only 10 clones as done in this approach. The clinical significance of defective RNAs needs further investigation. Mixed intergenotypic infections seem to be very rare events but have been described (Wang and Chao, 2005).

Taken together, this newly established NAT method allows a simple and rapid quantitative detection of HDV RNA in serum samples and subsequent typing of the amplicon. This assay can be used for both monitoring of antiviral treatment and molecular epidemiologic studies.

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