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International Journal of Infectious Diseases (2007) **11**, 58—62

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Hepatitis delta virus (HDV) genotypes in patients with chronic hepatitis: molecular epidemiology of HDV in Turkey

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Received 28 June 2005; received in revised form 29 September 2005; accepted 6 October 2005
Corresponding Editor: Jane Zuckerman, London, UK

KEYWORDS
Genotype;
Hepatitis delta virus;
Sequencing

Summary
Objective: Analysis of hepatitis delta virus (HDV) isolates from around the world has indicated that there are at least three phylogenetically distinct genotypes with different geographic distributions. The aim of this study was to determine the distribution of HDV genotypes by direct sequencing in patients with chronic delta hepatitis in Izmir, Turkey.
Design and methods: Serum samples from 32 chronic hepatitis patients (21 males, 11 females; mean age 44.2 years, range 23—70 years) with anti-delta positivity were analyzed for hepatitis B and C serologies. After reverse transcription, cDNA of partial delta antigen was amplified by in-house nested PCR. The products of the HDV PCR were bidirectionally sequenced with internal primers using Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, CA, USA) and ABI Prism 310 Genetic Analyzer (Perkin Elmer, USA). Nucleotide sequences of HDV were compared with previously reported sequences and aligned by using ClustalW (1.82).
Results: HDV-RNA was positive in 26 (81.3%) of 32 anti-delta positive samples. Comparison of the HDV sequences with published sequences of HDV genotypes I, II, and III indicated that all were closely related to HDV genotype I isolates. Similarity among isolated sequences ranged from 84% to 96%.
Conclusion: HDV genotyping was successfully performed by direct sequencing of the amplicons obtained from routine HDV-RNA screening PCR tests. All of the HDV isolates from the chronic delta hepatitis patients included in this study were found to be genotype I.
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Introduction

Hepatitis delta virus (HDV) is a defective RNA virus that requires the helper function of the hepatitis B virus (HBV) for replication.[1] Disease associated with both acute and chronic HDV infection is usually more severe than that due to HBV infection alone.[2]

Recent improvements in molecular techniques have provided increased understanding of the natural course of HDV infection. These advances have enhanced the clinician's ability to evaluate the stage of HDV infection, response to therapy, and occurrence of reinfection after liver transplantation.[3]

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doi:10.1016/j.ijid.2005.10.012

Analysis of HDV isolates from different regions of the world has indicated that there are at least three phylogenetically distinct genotypes with different geographic distributions. HDV genotyping is based on direct sequencing or restriction fragment length polymorphism (RFLP) analysis of reverse transcription (RT) products of the HDV genomes from patients. Genotype I is the most widespread geographically, having been identified in isolates from North America, Europe, and the Middle East. Genotype II has been found in East Asia. Genotype III is found exclusively in South America.[4] Divergence in nucleotide sequences ranges from 27% to 34% and in amino acid sequences is 30% among different genotypes.[5] A new study suggests that the genetic variability of HDV is much more complex than was previously thought and the genus includes at least seven clades.[6] The clinical pattern resulting from infection with HDV genotype I is very variable, ranging from mild to severe disease. Genotype II usually gives rise to a milder hepatitis, while genotype III appears to lead more often to fulminant hepatitis.[7]

Turkey is in the intermediately endemic region and anti-delta seroprevalance was found to be 6.7% in 716 hepatitis B surface antigen (HBsAg) positive samples tested in the Clinical Virology Laboratory of Ege University Hospital, Izmir.[8] The aim of this study was to determine HDV genotypes and analyze the sequences encoding the C-terminal half of the delta antigen by direct sequencing of isolates from patients with chronic delta hepatitis.

Materials and methods

Serum samples from 32 patients (21 males, 11 females; mean age 44.2 years, range 23–70 years) with chronic delta hepatitis were analyzed for hepatitis B, D, and C serological markers including hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBe, anti-HCV (BioMerieux, France), and anti-delta (Diasorin, Italy) by micro-enzyme immunoassay method. HBV-DNA was tested by a quantitative PCR assay according to the manufacturer's instructions (HBV Monitor, Roche Molecular Diagnostics, NJ, USA).

HDV-RNA was extracted from 150 μL of serum samples by Nucleospin RNA virus extraction kit (Macherey-Nagel, Germany) and 50-μL eluate was prepared. The RNA pellet was reverse transcribed to complementary DNA (cDNA) using random hexanucleotide mix and M-MuVL in a final volume of 20 μL. The mixture was incubated in a thermal cycler at 42 °C for one hour. Ten microliters of the resultant cDNA preparation were added to 40 μL of a PCR solution containing 25 pmol of external sense primer (nucleotides (nt) 858–881) 5′ GCC CAG GTC GGA CCG CGA GGA GGT 3′ and external antisense primer (nt 1289–1312) 5′ ACA AGG AGA GGC AGG ATC ACC GAC 3′, and 2U Taq polymerase. The mixture was amplified by 35 cycles of PCR (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) followed by a 7-min extension at 72 °C. Five microliters of the product were used for the second round PCR using 25 pmol internal sense primer (nt 883–906) 5′ GAG ATG CCA TGC CGA CCC GAA GAG 3′ and internal antisense primer (nt 1265–1288) 5′ GAA GGA AGG CCC TCG AGA ACA AGA 3′ set with the same conditions as the first PCR. PCR working precautions were strictly followed and negative controls were added between samples. Products were analyzed by electrophoresis on 1% agarose gel and the expected 359 base pair length was confirmed.[9]

The products of the HDV PCR were sequenced bidirectionally with internal primers using Big Dye Terminator DNA Sequencing Kit (Applied Biosystems CA, USA) and ABI Prism 310 genetic analyzer (Perkin Elmer, USA). Sequences obtained from the sera of the 26 patients with HDV infection and 17 sequences representing all types of HDV from the database of GenBank/EMBL/DDBJ, were aligned by ClustalW (1.82). Phylogenetic comparison was done by TREECON version 1.3b after bootstrapping 100 replicates.[10]

Nucleotide sequence accession numbers of the reference HDV sequences

The accession numbers of the reference sequences used were as follows: Tw-2b, AF018077; dFr-48, AJ583871 (Cameroon); DFr-644, AJ583882 (Congo), dFr-47, AJ583870 (Guinea); L22063 (Peru); AF251367 (Venezuela); X60193 (Japan); AY261457 (Tw-2a); AF008372 (France); AF008371 (USA); AF008417 (Italy); U81989 (Ethiopia); U81988 (Somalia IC); AF008875 (Egypt); AF008320 (Greece); AF008309 (Albania); AF008319 (Romania).

The sequences of 26 patients in this study are available at GenBank with accession numbers from DQ223507 to DQ223532.

Results

HDV-RNA was positive in 26 (81.3%) of 32 anti-delta positive samples. All the samples were HBsAg positive and anti-HCV negative. The HBV-DNA levels of HDV-RNA positive patients, and HBeAg and anti-HBe status of the patients are summarized in Table 1. Since clinical information and data about therapy and duration of therapy could not be obtained from

Table 1 HBeAg/anti-HBe status and HBV-DNA levels of HDV-RNA positive patients (HBV-DNA levels were determined for 22 of 26 HDV-RNA positive patients)

	Total	HBV-DNA level (copy/mL)			
		<200	200–200 000	>200 000	Unknown
HBeAg+, anti-HBe−	5	–	1	2	2
HBeAg−, anti-HBe+	18	8	7	1	2
HBeAg−, anti-HBe−	2	2	–	–	–
HBeAg+, anti-HBe+	1	–	–	1	–
Total	26	10	8	4	4

the patients, the correlation of HDV-RNA and HBV-DNA levels was not evaluated. Comparison of the HDV sequences with the reported sequences from HDV genotypes I, II, and III indicated that all were closely related to HDV genotype I isolates. The phylogenetic tree of 26 sequences with 17 references by TREECON version 1.3b is shown in Figure 1.[10]

Nucleotide similarity among the 26 isolated sequences ranged from 84% to 97% (mean 89.8%). Amino acid sequence similarity was found to be in the range of 75—95% (mean 85.8%). By pair-wise comparison, nucleotide similarity ranged from 82% to 96% (mean 86.9%) in the hepatitis delta antigen (HDAg) segment with the GenBank database type I sequences (AF008309, AF008320, AF008319, AF008375, AF008372, AF008371, AF008417, U81989, U81988), the U81988 Somalia IC isolate being the least, and Albania AF008309 isolate the most, similar. When compared with previously reported sequences from Turkey (accession numbers AY148019—AY148027) nucleotide divergence was found to be 9.1% (3.9—14.5). Suggested representatives of the new clades, Tw-2b, dFr-47, dFr-48, and dFr-644, were

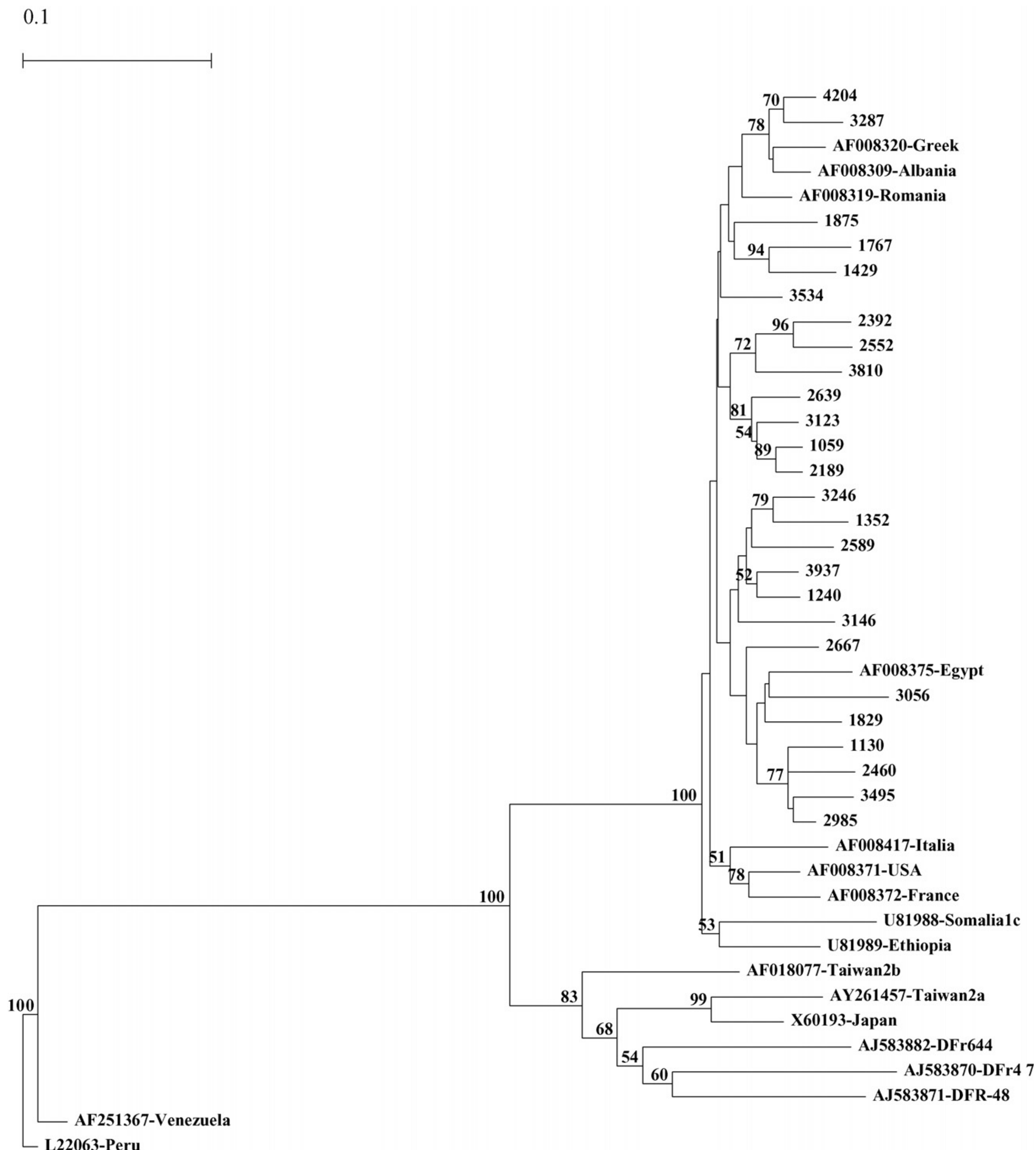

Figure 1 Phylogenetic tree of 26 sequences with 17 references by TREECON version 1.3b.[10]

also included in our study. By pair-wise comparison, nucleotide similarity of our isolates with Tw-2b, dFr-47, dFr-48, and dFr-644 ranged between 75% and 81% (mean 75.4%), 71% and 78% (mean 74.9%), 71% and 79% (mean 75.5%), and 70% and 76% (mean 74.1%), respectively. Nucleotide similarity among the isolated sequences and GenBank sequences is shown in Supplementary data in Appendix A.

Discussion

It is known that HDV infection is highly prevalent in patients with chronic HBV infection in Turkey. Overall anti-HDV seropositivity in Turkey ranges between 2.9% and 33%; in asymptomatic HBsAg carriers it is 0–11.2% and in patients with chronic hepatitis it is 6.8–53.4%.[11–13]

In a study in which the epidemiology and clinical presentation of HDV in Turkey were studied, the prevalence of HDV infection was significantly higher in patients with chronic liver disease than in asymptomatic carriers of HBsAg. The frequency of 'severe' or fulminant hepatitis was similar in HBV infected and HBV/HDV coinfected individuals. The frequency of biphasic hepatitis was significantly higher in the latter group than in those with classical hepatitis B.[14]

Since genotypes may influence the clinical outcome of the disease and display geographic dissimilarities, it seems that it is important to determine the occurrence of diverse genotypes in a population, which may contribute to the clinical setting. Reports on HDV genotypes in Turkey are limited. In a study performed on different isolates from various countries, five isolates from Turkey were found to be genotype I.[15] In a recent study from Turkey, a typing method of HDV, based on RFLP patterns of amplified HDAg of HDV genome was used for HDV-RNA typing. RFLP analysis revealed an HDV genotype I pattern in all patients. In the aforementioned report, for 13 patients, direct sequencing was also performed, and these data were used for phylogenetic analysis.[16]

In our study, the genotype assignment was based on the analysis of the sequences that corresponded to nucleotides between 906 and 1265, and included sequences encoding the C-terminal half of HDAg. Sequences encoding the C-terminal half of the HDAg were up to 15% variable among genotype I isolates.[9] In another study on geographic distribution and genetic variability of hepatitis delta virus genotype I, sequence divergence among genotype I isolates from different countries ranged from 3.1% to 13.4%.[15] In a study from Turkey, by pair-wise comparison, isolates differed in the HDAg from other GenBank type I sequences by $6 \pm 1.0\%$ (range 1–7.1%) in the HDAg segment (nucleotides between 904 and 1210). The mean nucleotide divergence of HDV RNA sequences from 13 patients was reported as $2.5 \pm 0.6\%$.[16] In our isolates, a longer HDAg segment (nucleotides between 923 and 1262) was analyzed and by pair-wise comparison, nucleotide similarity among isolated sequences ranged from 84% to 97% (mean 89.8%). The number of analyzed sequences is greater than the previous report, which may account for the greater nucleotide divergence in the study. Greater nucleotide difference may also be due to the length of the analyzed segment which is 33 base pairs longer than the previous one with different starting and ending points (nt 904–1210 vs. nt 923–1262).

In a study on molecular phylogenetic analysis of delta virus, HDV genetic variability was extended to at least seven major clades, bringing the variability of the virus closer to that of HBV. In this study, the sequences from the serotype II B complex form a distinct clade (proposed as HDV 4), and dFr-47, dFr-48, and dFr-644 are considered as the representatives of the new African clades.[6] Suggested representatives of the new clades were also included in our phylogenetic analysis. By pair-wise comparison, nucleotide similarity of our isolates with Tw-2b, dFr-47, dFr48, and dFr-644 ranged between 75% and 81% (mean 75.4%), 71% and 78% (mean 74.9%), 71% and 79% (mean 75.5%), and 70% and 76% (mean 74.1%), respectively, showing that they are not related to these isolates that represent the new clades.

In this study HDV genotyping was successfully performed by direct sequencing of the amplicons obtained from routine HDV-RNA screening PCR tests. All of the HDV isolates from the chronic delta hepatitis patients included in this study were found to be genotype I. HDV type I seems to be the unique genotype found in the Turkish population, which is an expected finding in agreement with similar epidemiological studies from the Mediterranean region and confirms previously published data.

Conflict of interest: No conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j. ijid.2005.10.012.

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