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ORIGINAL ARTICLE

Phylogenetic Analysis of Twenty-Six Cases of Hepatitis Delta Virus Isolates in Tehran, Iran

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Background and Aims: **Hepatitis delta virus (HDV) is an RNA virus that can lead to severe acute, and chronic forms of liver disease using the helper function of the hepatitis B virus. HDV strains are categorized into three genotypes and eight clades, which distribute geographically. The prevalence rate of HDV infection varies from 2.4 to 10 percent in blood donors for chronic liver disease in Iran. The aim was to find out the phylogenetic background of samples isolated in Tehran.**

Methods: **A molecular phylogenetic analysis in some samples has been conducted in Iran previously. However, the number of cases did not cover the whole country. In addition, based on the restriction in the number of cases, we studied 26 samples.**

Results: **In this study, a phylogenetic distribution of 26 Iranian isolates was determined using a neighbor-joining method. The revealed that all isolates belonged to Genotype I (Clade 1).**

Conclusions: **It is shown that our finding is in concordance with previous studies in Iran. It can be concluded that the strain of HDV being spread in Iran belongs to Genotype 1. This study is in concordance with previous studies in Iran.**

Keywords: **Phylogenetic Analysis, Hepatitis Delta Virus, Iran**

Introduction

Hepatitis delta virus (HDV) is the causative agent of one of the most severe forms of viral hepatitis. HDV co-infects or super-infects hepatocytes already infected with the hepatitis B virus, resulting in an increased risk of cirrhosis or fulminant hepatitis [1, 2]. Hepatitis delta virus is known to be distributed worldwide with enormous geographical variations in its frequency. Hepatitis delta virus (HDV) is a unique human RNA virus that needs the helper function of the hepatitis B virus (HBV) for replication [3]. HDV strains are categorized into at least three genotypes: I, II, and III [4-6], and 8 clades [7] due to HDV's genomic diversity in different regions of the world.

Genotype I has been associated with various types of liver disease, ranging from fulminant hepatitis to asymptomatic chronic [8, 9]. HDV Genotype II shows a less aggressive course [10],

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whereas Genotype III causes a severe clinical course [11]. Delta infection is endemic in Middle Eastern countries but unfortunately there are not enough data on Central Asia. The prevalence rate of HDV infection has been reported to range from 2.4 to 10 percent in blood donors for chronic liver disease patients in Iran [2]. Phylogenetic analyses of HDV isolates in Iranian patients have been conducted previously [12-14]. Because Iran is a large country with different ethnic groups, more data is required to have a better understanding of HDV characteristics. HDV genome is a circular, single-stranded RNA virus that ranges from 1,672 (strain dFr45, accession number *AX741144*) to 1,697 nucleotides (dFr47, *AX741149*) (15). In the present study delta antigen sequences of 26 HDV isolates from Iranian, HDV-infected patients were analyzed to determine HDV genotype distribution.

Materials and Methods

Sera were collected from 26 Iranian patients (19 males and 7 females) with chronic delta hepatitis who were referred to the Tehran Hepatitis Center from 2004 to 2006.

Serological tests

All sera were checked for hepatitis B surface antigens (HBsAg) using an enzyme-linked immunosorbent assay (ELISA) kit (DiaPro Diagnostic BioProbes S.R.L., Milan, Italy). ELISA is also used to test for hepatitis C virus (HCV) and hepatitis A virus (HAV). Anti-HDV antibody was explored using an ELISA kit (Radim SpA, Pomezia, Italy) according to the manufacturer's protocol.

RT-PCR and PCR

Viral RNA was extracted from 200 µl of serum using RNXPlus (CinnaGen, Iran) as mentioned in the manufacturer's protocol. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a random hexamer 0.5 µl (0.2µg/µl), a reverse primer as a GSP 1µl (12.5µM), and an MMULV (Fermentas AB, Vilnius, Lithuania) in a final volume of 20µl. Two µl of cDNA were amplified using 0.3-µM-forward and 0.3-µM reverse primers (F: 5'-TGCCATGCCGACCCGAAGAGGAA-3' R: 5'-G G A G A G A C G G G A T C A C C G AAGAAGGAAGGC-3') by Taq DNA polymerase (CinnaGen, Iran) at 94°C for 5 min and 35 cycles: 94°C for 40 s, 72°C for 1 min (annealing and extension were at 72°C), and ending at 72°C for 5 min. The PCR product, with an expected length of 421bp, was analyzed in 1.5% agar gel electrophoresis.

Cloning and sequencing

The PCR product was purified by a gel extraction kit (Macherey-Nagel, Düren, Germany) and cloned to the TOPOII.1 T vector (Invitrogen, California, USA) according to the manufacturer's protocol. Plasmid was extracted from positive colonies using a plasmid extraction kit (Core one™, Seoul, South Korea). Confirmation of ligation was performed by restriction enzyme digestion analysis with EcoR1 (Fermentas AB, Vilnius, Lithuania). The inserted genes were sequenced using an M13 forward primer with a Big Dye terminator DNA sequencing kit (Applied Biosystems, Foster City, CA).

Reference sequences and phylogenetic analysis

A total of 55 reference sequences and 26 Iranian sequences were obtained from a Gene Bank and were analyzed along with 26 new, HDV-positive cases in this study.

Phylogenetic analysis

The sequences were edited using BioEdit V.5.0.9 [16]. Alignment, phylogenetic, and molecular-evolutionary analyses were conducted using MEGA version 4 [17] with the neighbor-joining method [18]. A bootstrap test and reconstruction was done 1000 times to confirm the reliability of the phylogenetic tree [19].

Results

Clinical and demographic data

The study group consisted of 26 Iranian, HDV-positive patients. Their mean age was 45.73 ± 9.6, and 19 (73%) were male and 7 (27%) were female. Three patients tested positive for HAV infection. Liver cirrhosis was detected in 12 patients. The mean serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 65.19 ± 39.46 IU/L and 69.09 ± 54.3 IU/L, respectively. According to a questionnaire distributed by the authors, the infected patients had been involved in various high-risk behaviors, as listed in Table 1.

Phylogenetic analysis

The sequences obtained from the 26 Iranian, HDV–positive cases were analyzed and compared with gene-bank sequence data from the 26 previously reported Iranian sequences and 55 reference sequences from Clades 1 to 8. It was found that all the sequences belonged to Genotype I (data not shown) and Clade 1, with a bootstrap value of 99 (Fig.1). The mean distance within Clade 1 for Iranian isolates was 0.091. The mean distance between Clade

Table 1. Potential risk factors for HDV infection in the patients enrolled in the study.

High-risk behavior	Number of patients	Percentage (%)
High-risk sexual contact	2	7.7
High-risk sexual contact and blood cupping	1	3.8
Transfusion	3	11.6
Transfusion	1	3.8
Blood cupping (Hijamat)	5	19.3
Intravenous drug user	1	3.8
Intravenous drug user and High-risk sexual contact	1	3.8
Unknown	12	46.2
Total	26	100

1 and all other clades was 0.089, and the mean distances between Clade 2 and all other clades (as well as Clade 3, etc.) are presented in Table 2.

Discussion

HDV is classified into three genotypes (I, II, III) based on distinct geographical distributions [20]. The sequence analysis shows that, based on the C–terminal sequence of delta antigens, the HDV isolates from the Iranian patients were categorized as Genotype I. HDV is classified into eight clades, and all of the Iranian isolates belonged to Clade 1. This result is in agreement with previous studies in Iran [14] and similar epidemiological studies from other Middle Eastern countries such as Jordan, Kuwait, Turkey, and Tadzhikistan [21-24].

The similarity between Iranian isolates was 9.1%. These isolates were not related closely to any strain from other countries, but all of them were categorized as Clade 1. Nineteen samples from previous studies in Iran [14] showed that 18 out of the 22 Iranian isolates clustered with Egyptian isolates. In this study most of the isolates clustered with previously identified Iranian isolates, with the Egyptian isolate still being the most related one. One Iranian sample was clustered with a Northern South American isolate. Two isolates from Lebanon and Italy are related to our isolates. However there is not enough bootstrap support for the subgroup information. The HBV genotype was not analyzed in the present study. According to previous studies, the HBV Genotype D has predominated in Iran [25]. A phylogenetic analysis of HBV must be performed in order to determine the associations between HDV and HBV in the present study.

Epidemiological studies on HDV in Iran have also been done on the basis of the seroepidemiology of the virus. These studies have shown that the seroprevalence of the anti-HDV antibody is 5.7% in the Golestan province of Iran [26], 2% in Babol [27], and 2.4% in Hamedan [28]. A study from 2005 [2] reported that 5.7% of cases of HDV seropositivity among patients with chronic liver disease were HBsAg positive.

Conclusions

It has been shown that the Iranian HDV genotype is similar to the genotype found previously

Table 2. Mean nucleotide distance between groups of HDAg sequences of Iranian isolates and 55 HDV sequences from Clades 1 to 8. IR indicates Iranian sequences.

Clade	IR	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8
IR	0.091								
Clade 1	0.089	0.078							
Clade 2	0.428	0.420	0.043						
Clade 3	0.276	0.276	0.473	0.068					
Clade 4	0.279	0.274	0.421	0.176	0.107				
Clade 5	0.375	0.360	0.397	0.244	0.259	0.120			
Clade 6	0.333	0.326	0.459	0.179	0.197	0.213	0.086		
Clade 7	0.338	0.344	0.404	0.224	0.212	0.234	0.200	0.083	
Clade 8	0.332	0.319	0.429	0.200	0.214	0.182	0.211	0.220	0.045

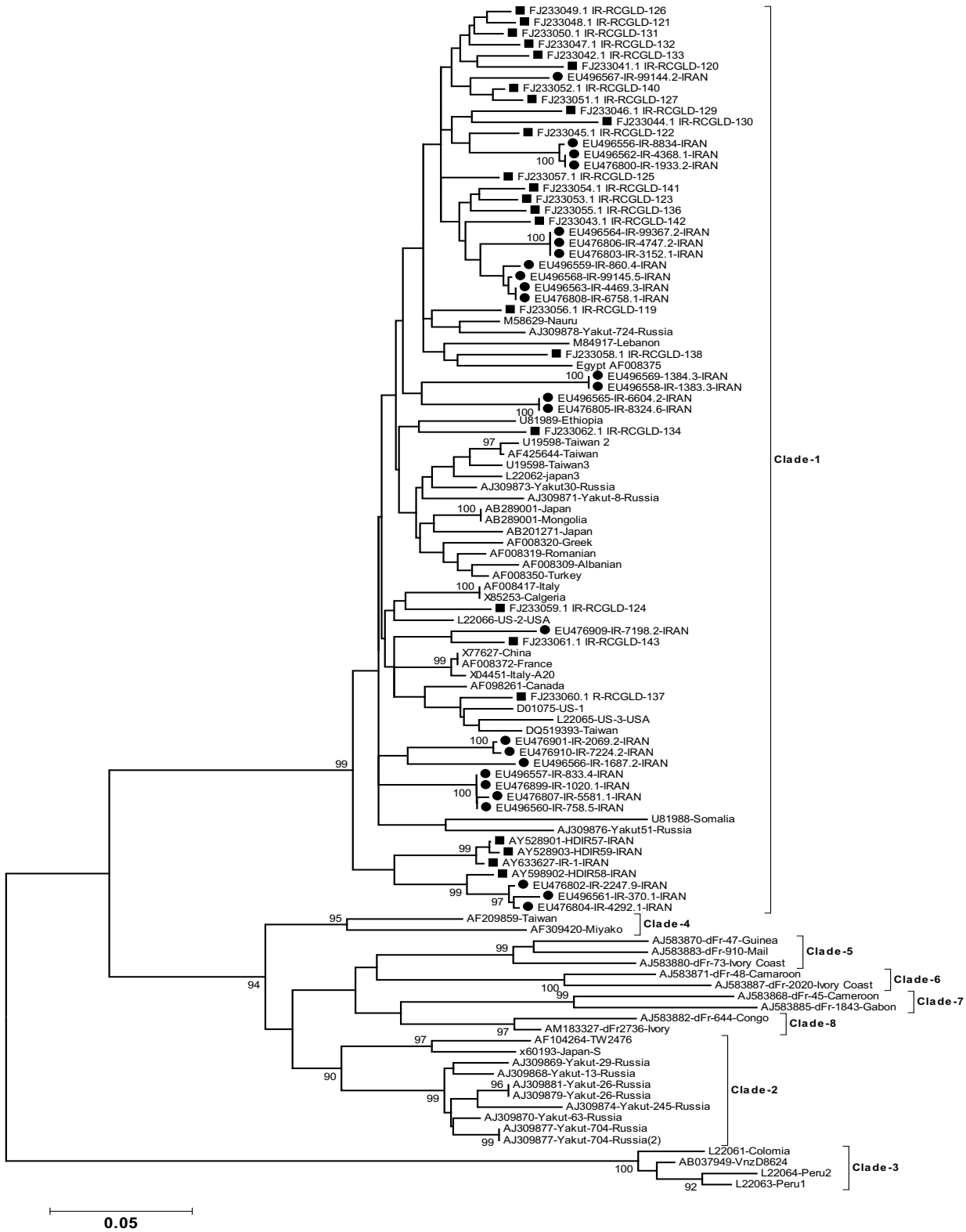

Figure 1. The figure shows a phylogenetic tree of 26 Iranian patients, 26 Iranian isolates that have been reported previously in the literature, and 54 reference isolates based on a neighbor-joining method. Iranian patients from other studies are marked with black squares and patients' samples from the present data are marked by black circles. The numbers show the bootstrap percentage values.

by Behzadian *et al.* [12] and Mohebi *et al.* [14] and belong to Genotype 1. In addition, it seems that Genotype I does not only belong to Iranian territory but may also be common in the Middle Eastern countries, including Jordan and Kuwait.

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