Arch Iranian Med 2009; 12 (3): 238 – 243

Original Article

Genotype Analysis of Hepatitis Delta Virus from Hepatitis B Surface Antigen-Positive Patients Using PCR-RFLP in Tehran, Iran

Hamideh Mirshafiee MSc**, Mahmood Mahmoodian-Shooshtari PhD•*, Zohreh Sharifi PhD*, Seyed-Masoud Hosseini PhD**

Background: Hepatitis delta virus is a unique human pathogen responsible for some 20 million infections globally. This virus is dependent on hepatitis B virus for transmission and propagation. Currently, at least three genotypes of hepatitis delta virus with different geographic distribution and clinical manifestations are described.

Methods: In this study, hepatitis delta virus RNA of 35 patients' sera were analyzed by RT-semi-nested polymerase chain reaction. Based on genomic differences of hepatitis delta antigen coding region of hepatitis delta virus RNA among hepatitis delta virus RNA-positive sera, the polymerase chain reaction products were digested with restriction enzymes and studied by restriction fragment length polymorphism.

Results: Out of 35 samples, 13 (38.46%) were positive for hepatitis delta virus RNA by RT-semi-nested polymerase chain reaction. All polymorphisms were shown to be genotype I. Out of 13 hepatitis delta virus RNA-positive (13/35), eight were HBeAg negative.

Conclusion: Our data indicated that hepatitis delta virus isolates in Tehran are exclusively genotype I.

Archives of Iranian Medicine, Volume 12, Number 3, 2009: 238 – 243.

Keywords: Genotyping • hepatitis delta virus • RFLP • semi-nested PCR • Tehran

Introduction

Hepatitis delta virus (HDV) was first discovered by Rizzetto et al.[1] in a patient with chronic hepatitis B virus (HBV) infection. In 1980, it was shown that HDV was an infectious agent responsible for exacerbation of liver disease in these patients.[2] HDV is a subviral agent that can lead to severe acute and chronic forms of liver disease in association with HBV.[3] HDV is a 36-nm viral particle which depends on the HBV to provide hepatitis B surface antigen (HBsAg) for virion assembly and propagation.[4] HDV particles consist of a negative sense, circular single-strand RNA genome, approximately 1700 nucleotides (nt) in length[5] which assembles with two viral proteins; HD Ag-S, HDAg-L (short and long forms) to form a ribonucleoprotein.[6] Two HDAg protein forms are translated from viral mRNA through a process known as RNA editing.[8]

Both HBV and HDV are blood-borne infections.[9] HDV infection can occur either as a coinfection with HBV or as a superinfection in patients with chronic HBV infection.[7] Super infection with HDV in HBV carriers leads to more progressive chronic liver disease (80%), with higher incidence of cirrhosis and hepatocellular carcinoma.[7] In contrast, individuals with HBV-HDV coinfection have more severe acute disease and a higher risk of fulminant hepatitis compared to those infected with HBV alone[7]; only 2% of these patients resulted in chronic infection.[4]

HDV genotyping has been performed by various methods such as hybridization, direct sequencing. or restriction fragment length

Authors' affiliations: *Virology Laboratory, Research Center, Iranian Blood Transfusion Organization, Tehran, Iran, **Department of Microbiology, Faculty of Biological Sciences, Shaheed Beheshti University, Tehran, Iran.
•Corresponding author and reprints: Mahmood Mahmoodian-Shooshtari PhD, Virology Laboratory, Research Center, Iranian Blood Transfusion Organization, Tehran, Iran.
Tel: +98-218-860-1501-30, Fax: +98-218-860-1555
E-mail: m.shooshtari@ibto.ir
Accepted for publication: 25 December 2008

polymorphism (RFLP) analysis of reverse transcription (RT) product of the HDV genome.[10] Since 1993, it has been known that based on HDAg coding region, at least three major HDV genotypes exist (I, II, and III)[12] with different geographic distribution.[12] The sequence differences among these genotypes are significant; there is advergence of 40% in nucleotide sequence and 35% in amino acid sequence of HDAg.[12] Genotype I is more widespread geographically (North America, Asia, Africa, Middle-East, and Europe). Genotype II has been found in Japan, Taiwan, and Yakutia[11]; and genotype III has identified only in northern part of South America.[11,12]

Infection with HDV is widespread and some endemic areas have been reported, such as southern Italy, parts of Africa, South Asia, and the Middle-East.[4] Although Iran is located in the Middle-East, unfortunately there are limited genetic and epidemiologic data from Middle-East. The prevalence of HBV in Iran is 1.7%[13] and that of HDV in subjects with hepatitis B infection is 5.7 %.[14] In addition, results showed that the prevalence rate of HDV infection has been declined from 18.03% in 1990 [15] to 5.7% in 2005 among patients with hepatitis B.[14]

The aim of this study was to determine the prevalence of HDV genotypes in Iran in patients who were HBsAg and anti-HDV positive. We have performed semi-nested PCR-RFLP method as a sensitive and specific molecular tool to detect the HDV genotypes.

Materials and Methods

Samples

Sera were obtained from 35 patients, HBsAg and anti-HDV positive, by ELISA method. All patients (eight females and 27 males, with the mean age of 34 years, ranging from 14 – 78 years) were referred to the Iranian Blood Transfusion Organization in Tehran, Iran. All of them were analyzed for HDV RNA. Genotyping was determined by RFLP. As a control, four patients of HBsAg positive and anti-HDV negative were tested for HDV infection.

HDV RNA extraction and cDNA synthesis

HDV RNA was extracted from 200 µL serum using the High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's instructions and RNA was suspended in 30 µL of elution buffer. Subsequently, cDNA was synthesized in total volume of 20 µL containing 5 µL of eluted RNA with 24U of avian myeloblastosis virus (AMV) reverse transcriptase, 10 pM of each primers (D120, DH1), 10 mM of the dNTPs, 10 X reaction buffer, 25 mM of $MgCl_2$, and 50U RNas inhibitor (Roche, Germany). The amplification procedure involved reverse transcription at 42°C for 60 minutes and AMV reverse transcriptase inactivation at 99°C for five minutes.

Primers

The most widely used region for genotyping is spanning nt 908 – 1265 which encodes the second half of HDAg protein. This region comprises highly conserved domains with a 14 – 19% divergence among genotypes but also the carboxyl-terminal domain with a 56% divergence among different genotype isolates.[16] In this study, for RT-PCR and semi-nested PCR, three oligonucleotide primers[17] were used that synthesized at the Metabion International AG Company (Germany) (Table 1). Also, HDV genotyping was investigated using RFLP analysis of the amplified region of nucleotides 907 – 1308, which is generally accepted to be ideal for the genotyping.

Semi-nested PCR

For cDNA amplification, in the first round of semi-nested PCR, 5µL of cDNA was mixed with 10 pM of each primer (D120, DH1), 10X PCR buffer+Mg^{2+}, 10 mM dNTP, and 5U Taq DNA polymerase (Roche, Germany). In the second round, PCR reagent II, composed of 10 pM of each primers (D120, D118), 10X PCR buffer+Mg^{2+}, 10 mM dNTP, and 5U Taq DNA polymerase (Roche, Germany) with 2 µL of the first PCR product. The first and second PCRs were carried out under the following conditions: four minutes at 95°C for predenaturation, followed by 35 cycles of two minutes at 95°C, annealing at 60°C for one minute

Table 1. HDV primer sequences used for RT-PCR.

Name	Position	Sequence
D120	884-907	5'-ATGCCATGCCGACCCGAAGAGGAA-3'
DH1	1334-1313	5'-GGCCTCTCAGGGGAGGATTCAC-3'
D118	1329-1308	5'-CTCAGGGGAGGATTCACCGACA-3'

and extension at 72°C for one minute, with a final extension step of 10 minutes at 72°C. PCR products were analyzed by electrophoresis in 2% agarose gel stained with ethidiume bromide and the expected 441 bp length was confirmed (Figure 1).[17]

HDV genotype analysis

For genotype determination, RFLP analysis was applied,[17] digesting 15 µL each of the PCR product with 10U of Sma I and Xho I (Fermentas, Switzerland) in a total volume of 20 µL each, for overnight at 30°C and 37°C, respectively. The resulting restriction fragments were analyzed by electrophoresis on a 3% agarose gel (Table 2). Molecular weight marker (Roche, Germany) and undigested PCR product were included in each analysis.

Results

In this study, there was a male predominance, in which 27 (77%) out of 35 patients were males. We used a 441-nt HDV cDNA fragment, encompassing the HDAg region. HDV RNA was positive in 13 (37%) of 35 anti-HDV-positive samples and the mean age of HDV RNA-positive patients was 38.46 years. All of sera were HBsAg and anti-HDV positive (Table 3).

Thirteen samples of HDV RNA positive showed Sma I and Xho I restriction patterns advocating a HDV genotype I profile (Figure 2). Viral marker of HBV replication, serum HBeAg was absent in a majority of the HDV RNA-positive subjects (eight cases), and only one case of positive HDV RNA had HBeAg positivity.

Table 2. Sma I and Xho I digestion patterns of HDAg coding fragment (441bp) (17).

	Restriction enzyme fragments	
	Sma I (bp)	**Xho I (bp)**
Genotype I	219+222	382+59
Genotype II	441 (undigested)	81+301+59
Genotype III	306+135	83+358
Iranian samples	219+222	382+59

Discussion

Based on sequence comparison, HDV is classified into three genotype (I, II, and III)[18] with different geographic distribution and clinical manifestations. Recent extensive analysis of HDV sequences from strains isolated from patients of African origin, has indicated that the various HDV genotypes fall into at least seven genotype [5] and in a study [3] proposed an extended classification of the delta virus genus to eight clades, which is very similar to the human HBV genetic variability[19] (A to H).[5] Although it is suggested that HDV genotype III is linked to HBV genotype F[20] in Peruvian Amazon basin, no particular HDV genotype (I, II, or I+II) has been linked to HBV genotype B or C infection in Taiwan.[21] In the Middle-East, predominant genotypes of HBV and HDV are D and I, respectively. The association of HDV genotype I with HBV genotype D has been reported from other countries where HBV

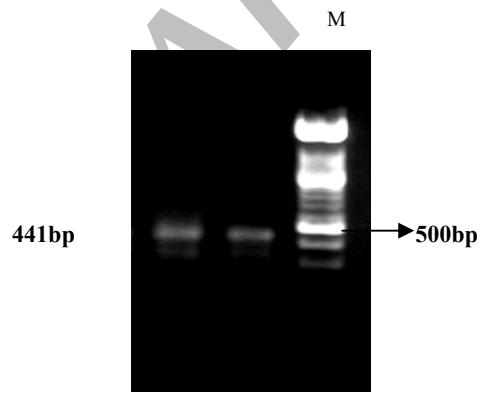

Figure 1. Detection of HDV RNA by semi-nested PCR demonstrating 441 bp band amplified in positive samples, followed by 2% LE agarose gel electrophoresis. (M) 100 bp ladder molecular size marker.

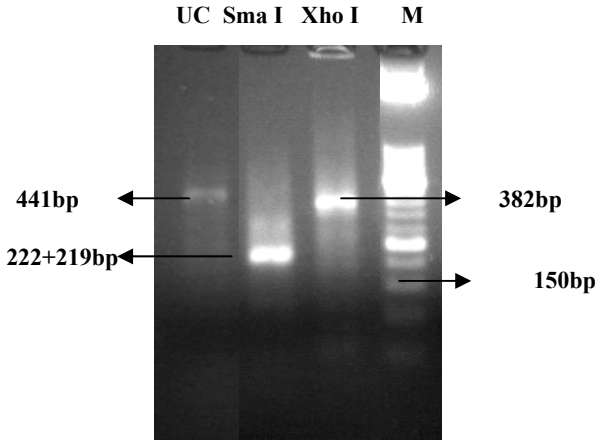

Figure 2. The Xho I and Sma I cleaved PCR products of HDV genomes were electrophoresed in a 3% LE agarose gel. Lanes, (M) molecular size marker (50 bp), (UC) undigested PCR product.

Table 3. Characteristics of anti-HDV-positive patients.

	HDV RNA positive (*n*=13)	HDV RNA negative (*n*=22)
Mean age	38.46 years	30 years
Sex		
Female	3	5
Male	10	17
HBeAg*		
Positive	1	4
Negative	8	4

*HBeAg was not tested in 18 cases.

genotype D is predominant such as in Italy,[16] Turkey,[22] Egypt,[23] and Pakistan.[24] Apart from HDV-1 and HDV-3 which are distinct geographically, each virus clade is geographically localized: HDV-2 (genotype IIa) is found in Japan, Taiwan, and Yakutia, Russia; HDV-4 (genotype IIb) in Taiwan and Japan; HDV-5, -6, -7, -8 in Africa.[3]

Clinically, the disease pattern resulting from infection with HDV genotypes is different; genotype I associated with a broad spectrum of pathogenicity. Recently, it has been associated with fulminant hepatitis in Samara, Russia.[25] Genotype II usually gives rise to a milder hepatitis, while genotype III appears to lead more often to fulminant hepatitis.[20,26] Expect genetic variability of HDV, other different factors may influence the clinical outcomes of the disease such as co-infection or superinfection of HDV with HBV. Co-infection has been associated with a higher rate of fulminant hepatitis than HBV alone but also higher rate of self-limited infection.[27] HDV superinfection has been associated with acute exacerbation of chronic HBV infection which may result in chronic HDV infection.[28]

Another study shows that HDV genotype I was detected using RT-PCR and sequencing.[29] In the present study, we determined HDV genotype using RFLP patterns of amplified HDAg region of HDV genome. RFLP analysis, a simple and reliable method for HDV genotype characterization,[17] revealed an HDV genotype I pattern in all HDV RNA- positive patients. This result is agreed with predominant genotype of HDV in Iran's neighboring countries and in the Middle-East region such as Lebanon,[30] Egypt,[23] Turkey,[10] and Pakistan.[24] Another study,[31] based on molecular phylogenetic analysis of the Iranian HDV complete genome revealed that at amino acid level, predicted HDAg sequence of HDV have the most homology with those of the Italian and Lebanese isolates.

In most cases of HDV infection, HBV replication is suppressed to very low levels by HDV[32] which leads to subsequent clearance of HBeAg and even HBsAg.[33] In our study, most of HDV RNA-positive cases were negative for HBeAg except one. In a study,[34] it was suggested that the level of HDV RNA in serum by real-time RT-PCR, in the presence of active HDV replication, was associated with the severity of liver disease, whereas the level of HBV DNA did not.

The HDV RNA negativity among the anti-HDV-positive patients can be attributed to: 1) previous HDV infection and stability of anti-HDAg IgG for several years after active self-limited infections, 2) level of HDV RNA under the threshold of detection, as determined using the semi-nested PCR, because of lower viral load in some samples or low rate of viral replication in chronic infection cases, and 3) high secondary structure naturally present in HDV RNA genome that can affect priming stage,[35,29] while HDV RNA positivity reflecting active ongoing delta infection.

Due to lack of effective therapy for chronic HDV infection and high treatment expenses,[36] the only prevention and effective approach for delta hepatitis is anti-HBV vaccination. Because of introducing mandatory HBV vaccination to all newborns in Iran in 1993, along with other preventive measures such as wide use of disposable needles for blood sampling and other hygienic purposes in high-risk populations, it is anticipated that a decrease in the prevalence of HBV infection may deplete the carrier reservoir of HBV and may lead to decrease the number of subjects susceptible to HDV infection. Collectively, the declining prevalence of both HBV and HDV infection may herald complete control of HDV infection in Iran in the near future.

In conclusion, our findings indicated that the predominant genotype of HDV in Iran's neighboring countries and in the Middle-East region was HDV genotype I. HBeAg was absent in a majority of the HDV RNA-positive patients (eight cases) that could be due to inhibition of HBV replication by HDV.

Acknowledgment

We gratefully acknowledge the virology laboratory staff of the Iranian Blood Transfusion Organization, for their advising and technical

support. The authors specially thank Dr. H. Keivani (Keivan Virology Laboratory) for some sample collections.

References

1 Rizzetto M, Canese MG, Arico S, Crivelli O, Bonini F, Verme G. Immunofluorescence detection of new antigen-antibody system (delta/antidelta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut*. 1977; **18:** 997 – 1003.

2 Rizzetto M, Canese MG, Gerin JL, London WT, Sly DL. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J Infect Dis*. 1980a; **141:** 590 – 602.

3 Gal FL, Gault E, Rapault MP, Serpaggi J, Trinchet JC, Gordien E, et al. Eight clade for hepatitis delta virus. *Emerging Infect Dis*. 2006; **12:** 1447 – 1450.

4 Gerin JL, Cassey JL, Purcell RH. Hepatitis delta virus. In: Hollinger FB, Purcell RH, Gerin JL, eds. *Viral Hepatitis*. Philadelphia: Lippincott Williams and Wilkins; 2002: 169 – 182.

5 Radjef N, Gordien E, Ivaniushina V, Gault E, Anais P, Drugan T, et al. Molecular phylogenetic analyses indicate a wide and ancient radiation African hepatitis delta virus, suggesting a deltavirus genus of at least seven major clades. *J Virol*. 2004; **78:** 2537 – 2544.

6 Gal FL, Gordien E, Affolabi D, Hanslik T, Alloui C, Dény P, et al. Quantification of hepatitis delta virus RNA in serum by consensus real-time PCR indicates different patterns of virological response to interferon therapy in chronically infected patients. *J Clin Micro*. 2005; **43:** 2363 – 2369.

7 Yamaguchi Y, Deléhouzée S, Handa H. HIV and hepatitis delta virus: evolution takes different paths to relieve blocks in transcriptional elongation. *Microb Infect*. 2002; **4:** 1169-1175.

8 Polson AG, Bass BL, Casey JL. RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature*. 1996; **380:** 454 – 456.

9 Taylor JM. Hepatitis delta virus. *Virol*. 2006; **344:** 71 – 76.

10 Altuğlu I, Özacar T, Sertoz RY, Erensoy S. Hepatitis delta virus (HDV) genotypes in patients with chronic hepatitis: molecular epidemiology of HDV in Turkey. *Inter J Infec Dis*. 2005; **11:** 58 – 62.

11 Ivaniushina V, Radjef N, Alexeeva M, Gault E, Semenov S, Salhi M, et al. Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia. *J Gener Virol*. 2001; **82:** 2709 – 2718.

12 Casey JL, Polson AG, Bass BL, Gerin JL. Molecular biology of HDV: analysis of RNA editing and genotype variation. In: Rizzetto M, Purcell RH, Gerin JL, eds. *Viral Hepatitis and Liver Disease*. Turin, Italy: Edizioni Minerva Medica; 1997: 290 – 294.

13 Alavian SM. Ministry of Health in Iran is serious about controlling hepatitis B. *Hep Monthly*. 2007; **7:** 3 – 5.

14 Alavian SM, Assari SM, Joybari HM, Lankarani M, Doroudi T, Haji-Beigi B, et al. Frequency and risk factors of hepatits D virus in hepatitis B patients [in Persian]. *Govaresh*. 2005; **10:** 21 – 26.

15 Rezvan H, Forouzandeh B, Taroyan S, Fadaiee S, Azordegan F. A study on delta virus infection and its clinical impact in Iran. *Infect*. 1990; **18:** 26 – 28.

16 Niro GA, Smedile A, Andriulli A, Rizzetto M, Gerin JL, Casey JL. The predominance of hepatitis delta virus genotype I among chronically infected Italian patients. *Hepatology*. 1997; **25:** 728 – 734.

17 Heamboonlerns A, Hasurabhanon T, Verachai V, Chonsrisawat V, Poovorawan Y. Hepatitis D virus infection in Thailand: HDV genotyping by RT-PCR, RFLP, and direct sequencing. *Infect*. 2002; **30:** 140 – 144.

18 Zhang YY, Tsega E, Hansson BG. Phylogenetic analysis of hepatitis D virus indicating a new genotype I subgroup among African isolates. *J Clin Microbiol*. 1996; **34:** 3023 – 3030.

19 Arauz-Ruiz P, Norder E, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol*. 2002; **83:** 2059 – 2073.

20 Casey JL, Niro GA, Engle RE, Vega A, Gomez H, McCarthy M, et al. Hepatitis B virus (HBV)/hepatitis D virus (HDV) coinfection in outbreaks of acute hepatitis in the Peruvian Amazon basin: the roles of HDV genotype III and HBV genotype F. *J Infect Dis*. 1996; **174:** 920 – 926.

21 Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis D virus genotypes in intravenous drug users in Taiwan: decreasing prevalence and lack of correlation with hepatitis B virus genotypes. *J Clin Microbiol*. 2002; **40:** 3047 – 3049.

22 Bozdayi AM, Aslan N, Bozdayi G, Türkyilmaz AR, Sengezer T, Wend U, et al. Molecular epidemiology of hepatitis B, C, and D viruses in Turkish patients. *Arch Virol*. 2004; **149:** 2115 – 2119.

23 Saudy N, Sugauchi F, Tanaka Y, Suzuki S, Aal AA, Zaid MA, et al. Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. *J Med Virol*. 2003; **70:** 529 – 536.

24 Moatter T, Abbas Z, Shabir S, Jafri W. Clinical presentation and genotype of hepatitis delta in Karachi. *World J Gastro*. 2007; **13:** 2604 – 2607.

25 Flodgren E, Bengtsson S, Knutsson M, Strebkova EA, Kidd AH, Alexeyev OA, et al. Recent high incidence of fulminant hepatitis in Samara, Russia: molecular analysis of prevailing hepatitis B and D virus strains. *J Clin Micro*. 2000; **38:** 3311 – 3316.

26 Nakano T, Shapiro CN, Hadler SC, Casey JL, Mizokami M, Orito E, et al. Characterization of hepatitis D virus genotype III among Yucpa Indians in Venezuela. *J Gen Virol*. 2001; **82:** 2183 – 2189.

27 Rizzetto M. The delta agent. *Hepatology*. 1983; **3:** 729 – 737.

28 Alexopoulou A, Dourakis SP. Genetic heterogeneity of hepatitis virus and its clinical significance. *Curr Drug Targets Inflamm Allergy*. 2005; **4:** 47 – 55.

29 Shahinsaz L, Sabahi F, Karimi M, Behzadian F, Alavian SM, Zand V. Detection and genotyping of hepatitis D virus from HBsAg positive patients in Iran using RT-PCR. *Iranian J Biotecho*. 2006; **4:** 174 – 179.

30 Ramia S, El-Zaatari M, Sharara AI, Ramlawi F, Farhat B. Current prevalence of hepatitis delta virus (HDV) infection and the range of HDV genotypes in Lebanon. *Epidemiol Infect*. 2007; **135:** 959 – 962.

31 Behzadian F, Sabahi F, Karimi M, Sadeghizadeh M, Maghsoudi N, Forooshani RS, et al. Molecular

phylogenetic analysis of Iranian HDV complete genome. *Virus Genes*. 2005; **30:** 383 – 393.

32 Farci P, Karayiannis P, Lai ME, Marongiu F, Orgiana G, Balestrieri A, et al. Acute and chronic hepatitis delta virus infection: direct or indirect effect on hepatitis B virus replication? *J Med Virol*. 1988; **26:** 279 – 288.

33 Liaw YF, Dong JT, Chiu KW, Sheen IS, Chu CM. Why most patients with hepatitis delta virus infection are seronegative for hepatitis B e antigen. A prospective controlled study. *J Hepatol*. 1991; **12:** 106 – 109.

34 Yamashiro T, Nagayama K, Enomoto N, Watanabe H, Miyagi T, Nakasone H, et al. Quantitation of the level of hepatitis delta virus RNA in serum, by real-time polymerase chain reaction and its possible correlation with the clinical stage of liver disease. *J Infect Dis*. 2004; **189:** 1151 – 1157.

35 Arakawa Y, Moriyama M, Taira M, Hayashi N, Tanaka N, Okubo H, et al. Molecular analysis of hepatitis D virus infection in Miyako Island, a small Japanese island. *J Virol Hepatitis*. 2000; **7:** 375 – 381.

36 Hoofnagle JH, Bisceglic AMD. The treatment of chronic viral hepatitis. *New Engl J Med*. 1997; **336:** 347 – 356.