Detection and genotyping of hepatitis D virus from HBsAg positive patients in Iran using RT-PCR

Leila Shahinsaz[1], Farzaneh Sabahi[1*], Mohsen Karimi[2], Farida Behzadian[1], Seyed Moayed Alavian[3], Vahid Zand[4]

[1]Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box 14115-331, Tehran, I.R. Iran [2]Biotechnology Research Center, Pasteur Institute of Iran, Pasteur square, Tehran, I.R. Iran [3]Tehran Hepatitis Center, Vesal Avenue, Tehran, I.R. Iran [4]Hepatitis Clinic, Special Disease Center, Kerman, I.R. Iran

Abstract
Hepatitis Delta virus (HDV) is a degenerate RNA virus or virusoid and a satellite of Hepatitis B virus (HBV). Three distinct genotypes are described for HDV; genotype I is distributed worldwide but other genotypes appear to be more restricted geographically. In the present study, an RT-nested PCR method was set up to detect delta infection from serum samples. Moreover, the target amplified sequences corresponding to the Hepatitis delta antigen (HDAg) C-termini were used for genotyping. The results showed that 63.6% (23 of 36) of (HDAb) positive serum samples (as determined by ELISA) were also positive for HDV-RNA. Sequencing and phylogenic analysis of three Iranian HDV isolates revealed the most homology (93%) with an Italian isolate indicating a close relationship and probably a common origin for these isolates.
Keywords: Hepatitis Delta virus; RT-PCR; phylogenetic tree; sequencing; Iran.

INTRODUCTION

Hepatitis D virus (HDV) is a defective infectious agent that requires helper function from the hepatitis B virus (HBV) to provide envelope proteins, hepatitis B surface antigen (HBs Ag), for assembly and release (Modahal and Lai, 2000). Thus, HDV propagates in human hepatocytes as its natural host only in the presence of HBV, causing fulminant or chronic hepatitis with liver cirrhosis (Modahal and Lai, 2000; Fields *et al*., 2001). The symptoms associated with both chronic and acute HDV infection are frequently more severe than those seen with HBV alone (Modahal and Lai, 2000).

The HDV genome, 1.7 Kb in lengths has extensive intramolecular base pairing that allows it to fold into a non-branched rod-like structure (Wang *et al*., 1986). Like the viroids, replication of HDV genome is carried out by the double rolling circle model during which autocatalytic activity in genomic and antigenomic ribozyme domains is crucial for replication. Unlike the viroids, HDV genome encodes its own nucleocapsid protein named "delta antigen" (Modahal and Lai, 2000; Fields *et al*., 2001) which exists as two species (small and large) (Bonino *et al*., 1986).

HDV infections are found worldwide and cause a wide spectrum of liver disease in humans (Fields *et al*., 2001). Based on genetic sequence analysis, different HDV isolates from different geographic areas are classified into three genotypes (I, II and III) or seven clades as recently proposed (Radjef *et al*., 2004; Watanaba *et al*., 2003). It has been observed that genotype II and III are approximately 75-80% and 60-65% homologous with genotype I, respectively (Theamboonlers *et al*., 2002; Casey *et al*., 1993). Moreover, sequence microheterogeneity has been observed in patients, so HDV can be considered a virus with high rate of diversity rate. The role of HDV genetic variations in the severity of HDV infection is not yet clear, but some studies have suggested a link between these two factors (Shakil *et al*., 1997; Poisson *et al*., 2000).

So far, there has been limited genetic information about HDV isolates from the Middle East, where a high prevalence of HDV infection has been reported (Rizzetto *et al*., 1992; Malekzadeh and Borhanmanesh,

*Correspondence to: **Farzaneh Sabahi, Ph.D.**
Tel/Fax: +98 21 88013030
E-mail: Sabahi_f@modares.ac.ir

1989). Some provinces in Iran, a vast country in the Middle East have reported a 14% prevalence of the delta infection among HBsAg carriers and it is claimed that this infection is endemic in some regions (Rezvan *et al*., 1990; Malekzadeh *et al*., 1989). In the present study we have set a RT-nested PCR procedure as a sensitive and specific molecular tool to detect the HDV genome including an informative region for HDV genotype determination (Shakil *et al*., 1997; Niro *et al*., 1997).

MATERIALS AND METHODS

Subjects: Thirty six HBsAg positive patients (32 males and 4 females) suffering from liver diseases (3 with acute hepatitis, 23 with chronic hepatitis and 10 HBsAg carriers) were studied. All the patients were positive for HDV anti-HDAg by ELISA (Diasorin, France). Clinical diagnosis was based on liver function tests, hepatitis virus markers, ultrasonography, and liver histopathology. Four patients had history of surgery and three of them were intravenous drug abusers (IVDUs), no one had a history of blood product transfusion or imprisonment. Pearson chi-square test was carried out to detect any meaningful relationship between HDV RNA-positivity and clinical status of patients. Fisher's exact test was performed for groups with small number of patients (Table 1).

RNA extraction and cDNA synthesis: HDV RNA was extracted from 50 µl of serum using the guanidine isothiocyanate-phenol-chloroform method (RNX-plus kit, Cinnagen, Iran), precipitated with isopropanol, and washed with 70% (v/v) cold ethanol. The RNA pellet was resuspended in 25 µl of diethyl pyrocarbonate (DEPC) treated water. Complementary DNA (cDNA) synthesis was performed in a volume of 20 µl containing 5 µl of extracted RNA, 2 units of ribonuclease inhibitor (Fermentas, Lithuania), 10 mM each of four deoxynucleotide triphosphates (dNTPs), 2 units of random hexamer and 200 units of M-Mulv enzyme (Fermentas, Lithuania) in the buffer supplied by the manufacturer, and incubated for 1h at 37ºC. The guidelines of Kwok and Higuchi (1989) were strictly observed to prevent carry-over contamination. Appropriate negative controls for RNA extraction, cDNA synthesis, and PCR were routinely included in each PCR round.

Nested-PCR strategy: A set of outer and inner primers were designed by Gene runner 3.05 (Hastings Software, Inc.) to amplify a 400nt genomic region coding the C-termini of HDAg (nt 880-1280). 5 µl of the cDNA pool was added to the first PCR mixture including outer primers HD1 (5' CCA GGT CGG ACC GCG AGG AGG 3') and HD2 (5' ACA AGG AGA GGC AGG ATC ACC GAC 3') as sense and antisense primers, respectively. The second PCR was carried out by transforming 5 µl of the first PCR product to a new tube with the same PCR mixture including inner primers, HD3 (5' GAT GCC ATG CCG ACC CGA AGA 3') and HD4 (5' GAA GGA AGG CCC TCA AGA ACA AGA 3') as sense and antisense primers (Fig. 1 and Table 2). Both rounds were performed in a total volume of 50 µl PCR mixture containing 10 mM Tris-Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs, 20 pmol of each of the forward and reverse primers, 0.5 U *Taq* DNA polymerase (Cinnagen, Iran) and covered with 50 µl of mineral oil (Sambrook and Russell, 2001). All amplification reactions were performed in a thermocycler (Master Cycler, Eppendorf) under the following profile: 3 min at 95ºC followed by 35 cycles of 1 min at 95ºC for denaturation, 1 min at 65ºC for primer annealing and 1 min at 72°C for extension, concluded by a final extension step at 72°C for 10 min. PCR products were evaluated through 1% (w/v) agarose gel stained with ethidium bromide upon preparation. 200 µl from each sample was then purified from low melting temperature agarose gel and subjected to sequencing using HD3 primer in an automatic cycle-sequencing procedure (Primm, Italy). Annealing temperature of 55ºC was used for the sequencing reactions.

Reference sequences and data analysis: Reference HDV genome sequences were obtained from GenBank and used to compare the sequences of the isolates in this study. The accession number and country of the reported sequences were as follows: U81989 (Ethiopia), M55042, X63373 and X85253 (as Italy1, 2, and 3 references, respectively), M92448 (Taiwan-1), AJ583878 (Egypt), M28267 (USA) and AF098261 (Canada) as genotype I, X60193 (Japan), AB088679 (Japan) as genotype IIa and IIb respectively, L22063 (Peru) and D8349 (Venezuela) as genotype III.

Control of sequencing results, editing of raw data were accomplished by the Choromas (version 1.45-Australia) and Bioedit (version 5.0.9-Tom Hall, Dept. of Microbiology, North Carolina State University) softwares. The 3 new Iranian sequences were aligned with 11 reference sequences from different geographic areas. The alignment of partial genomic nucleotide sequences were carried out with CLUSTAL W program by Bioedit software. Genetic distance was estimated using the Kimura two parameter matrix and based on this phylogenetic trees were constructed by

Table 1. HDV-RNA status among various subgroups.

Situation		HDV-RNA % (count)		Fisher's Exact Test	Pearson Chi-Square
		negative	positive	P value	P value
male female	Gender	84.6% (11) 15.4% (2)	91.3% (21) 8.7% (2)	0.540	0.609
single married	Marital status	7.7% (1) 92.3% 12)	17.4% (4) 82.6% (19)	0.419	0.634
chronic hepatitis acute hepatitis carrier	Diagnosis	46.2% (6) 15.4% (2) 38.5% (5)	73.9% (17) 4.3% (1) 21.7% (5)	0.217	-
positive negative	Anti-H0BcIgM	30.8% (4) 69.2% (9)	4.3% (1) 21.7% (5)	0.028	0.047
positive negative	Anti-HBcIgG	76.9% (10) 23.1% (3)	82.6% (19) 17.4% (4)	0.679	0.686
positive negative	HBeAg	15.4% (2) 84.6% (11)	26.1% (6) 73.9% (17)	0.458	0.682
positive negative	Anti-HBe	53.8% (7) 46.2% (6)	52.2% (12) 47.8% (11)	0.923	1.000
positive negative	HBV-DNA	22.2% (2) 77.8% (7)	30.8% (4) 69.2% (9)	0.658	1.000
yes no	Noninfectious disease	8.3% (1) 91.7% (11)	.0% (0) 100.0% (23)	0.160	0.343
yes no	Addiction	23.1% (3) 76.9% (10)	13.0% (3) 87.0% (20)	0.438	0.645
yes no	Tattoo	7.7% (1) 92.3% (12)	13.0% (3) 87.0% (20)	0.624	1.000
yes no	Transfusion History	16.7% (2) 83.3% (10)	.0% (0) 100.0% (23)	0.044	0.111
yes no	Surgery History	15.4% (2) 84.6% (11)	17.4% (4) 82.6% (19)	0.877	1.000
yes no	Prison History	23.1% (3) 76.9% (10)	.0% (0) 100.0% (23)	0.016	0.040
super infection co infection	HDV infection type	76.9% (10) 23.1% (3)	95.7% (22) 4.3% (1)	0.086	0.124
no yes	family history	38.5% (5) 61.5% (8)	43.5% (10) 56.5% (13)	0.769	1.000

As it can be observed from the table, no significant correlation ($P < 0.05$) between values evaluated and HDV RNA positivity could be observed. The inverse correlation between groups with transfusion history and prison history with RNA positivity is observed that is difficult to interpret.

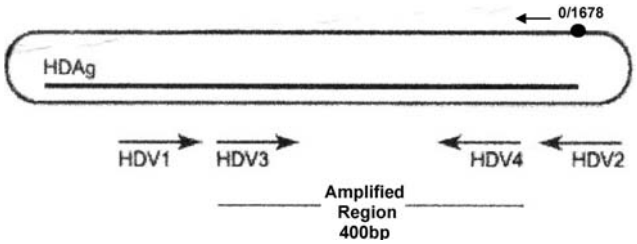

Figure 1. Schematic diagram of HDV RNA and specific primer binding sites. The bold line inside the circle shows the HDAg ORF. The location of the amplified region (880-1280nt) is also demonstrated.

Table 2. HDV primer sequences used in this study.

Primers	sequences	Location*
HDV1	5'-CCA ggT Cgg ACC gCg Agg Agg-3'	855-872
HDV2	5'-ACA Agg AgA ggC Agg ATC ACC Gac-3'	1284-1307
HDV3	5'-gAT gCC ATg CCg ACC CgA AgA-3'	880-901
HDV4	5'-gAA ggA Agg CCC TCg AgA AcA AgA-3'	1260-1280

* Numbering according to Casey *et al*. (1993).

the neighbor-joining (NJ) method. The stability of nodes and three branching was determined by bootstrapping using 1,000 repeats of drawing and cut off value of 70% was accepted to indicate stable nodes.

RESULTS

Sera from 36 patients that had been tested positive for HBsAg and anti-HDVAg using ELISA were also analyzed for HDV-RNA by RT-nested PCR amplification. Target region corresponded to the C-terminal portion of the HDAg coding region and included the RNA editing site (Lee *et al*., 1993) and the polyadenylation signal (Shakil *et al*., 1997). As demonstrated in Figure 2, samples from different patients have shown the formation of 400 bp amplicons compared to the DNA molecular weight marker and no signal was observed in the negative control sample, indicating the absence of contamination and a successful RT-PCR procedure.

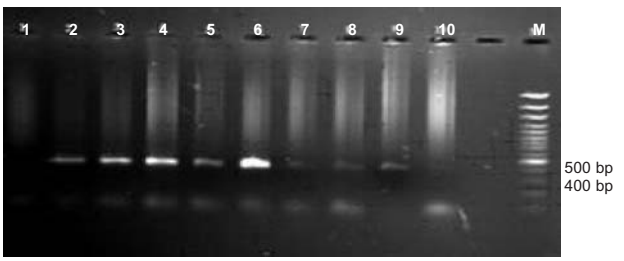

Figure 2. An example of gel electrophoresis for detection of HDV RNA by nested RT-PCR demonstrating 405 Kb band amplified in positive specimens. Lanes 2-9 positive specimens. Lanes 1 and 10 negative specimens. The last 2 lanes indicate negative contral and molecular weight marker, respectively.

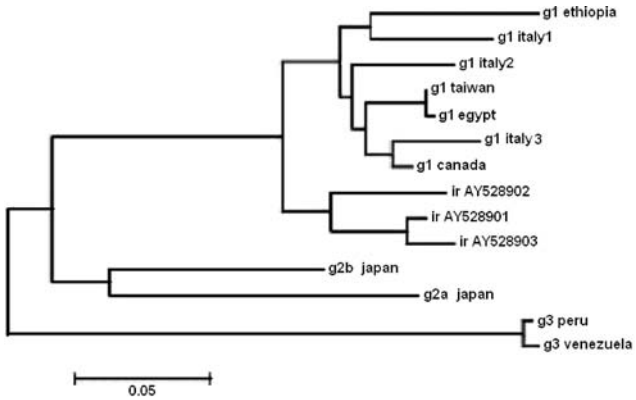

Figure 3. Tree created by phylogenetic analysis based on Kimura two-parameter distance method followed by ClustalW-Neighbour Joining for nucleotides corresponding to Hepatitis D virus HDV antigen gene partial cds880-1280.

The rate of positivity for HDV-RNA was 63.8% (23 of 36) and we could not show any statistical association between HDV-RNA positivity and clinical characteristics of patients among this small group.

As demonstrated in Figure 3, phylogenetic analysis was carried out following sequence analysis on three samples showing that Iranian isolates clustered with genotype I isolates and were clearly distant from the isolates reported as genotypes II and III.

The genetic distances computed by Kimura two parameter models showed 92-93%, 86%, and 82% homology between Iranian isolates and prototypes from genotype I, II and III respectively in the subject region (nt880-1280).

All 3 sequences of Iranian isolates were reported to Genbank for the first time with following accession numbers: AY528901.0, AY528902.1 and AY528903.1.

DISCUSSION

HDV can exclusively infect and propagate in individuals infected by HBV, whose liver cells produce HBsAg. In both patterns, co-amplification and superinfection, the morbidity and mortality rate is high compared to HBV infection alone. The other factor which has been identified to be related to the length and severity of liver disease is the genotype of HDV infecting HBV positive patients (Casey *et al*., 1993; Wu *et al*., 1995; Hadler *et al*., 1984).

Today, the diagnosis of active HDV infection is usually dependent on measurement of antibody response against HDVAg (Modahal and Lai, 2000). However, the anti-HDVAg antibody is usually detectable around one month after infection and its level may be low and undetectable in certain patients. The more direct assessment of HDV infection depends

on the presence of HDVAg using ELISA that does not have a desired sensitivity (Di Bisceglie, 2001). Immunohistochemistry is the gold standard for HDV diagnosis; however, the need for liver biopsy has made it difficult to be used as a routine procedure in clinical practice (Popper *et al*., 1983). Several studies have shown that serum HDV RNA detection is a sensitive and reliable method for diagnosis of HDV infection which can be detectable from the first week post-infection (Jardi and Rodriguez, 1994). Almost similar results obtained by others (Jardi and Rodriguez, 1994).

In the present study, we showed that 63% of HDVAb ELISA positive cases are positive for HDV RNA by RT-nested PCR. The following factors have to be considered when explaining differences between results obtained by ELISA and those of RT-PCR: i) lower viral load in some samples, ii) high secondary structure naturally present in HDV RNA genome that can affect priming stage iii) cases with past acute HDV disease who retain anti-HDAg but have resolved viremia, may have been included among study subjects, iv) stability of anti-HDAg IgG for several years after active self-limited infections and iiv) low rate of viral replication in chronic infection cases. Because previous studies have shown that the HDAg open reading frame specific region corresponding to the C-terminus of the long form of HDAg is one of the most distinctive sequences useful for genotyping, the primers used here were designed to target the mentioned region (nt883-1288) (Shakil *et al*., 1997; Nitro *et al*., 1997).

In order to assess the situation of the HDV genotype of Iranians, three isolates from patients with different clinical histories were subjected to molecular phylogenetic analysis along with strains isolated from other regions. The emerged phylogenetic tree revealed close branching of Iranian isolates clustered with genotype I isolates which has been identified in most areas of the world and is represented by many different isolates (Radjef *et al*., 2004; Shakil *et al*., 1997). Sequence of these three isolates showed the highest homology (92-93%) with an Italian reference isolate type I. This is in agreement with our previous study (Behzadian *et al*., 2005), indicating possible evolutionary relationship between Iranian and Italian HDV strains.

Although previous local studies indicate that tattooing, dentistry procedures and endoscopy are risk factors of HDV infection in Iran (Alavian S.M., unpublished data); we could not show any link between these factors in this small subjected group. Molecular phylogenetic analysis of more isolates from Iran along with epidemiologic studies can provide more precise information about the Delta infection status in this geographic area.

References

Behzadian F, Sabahi F, Karimi M, Sadeghi Zadeh M, Maghsoudi N, Sarrami Forooshani R, Shahinsaz L (2005). Molecular phylogenetic analysis of Iranian HDV complete genome. *Virus Genes*. 30: 383-393.

Bonino F, Heermann KH, Rizzetto M (1986). Hepatitis delta virus: protein composition of delta antigen and its hepatitis B virus-derived envelope. *J Virol*. 58: 945-950.

Casey JL, Brown TL, Colan EJ (1993). A genotype of hepatitis D virus that occurs in northern South America. *Pro Natl Acad Sci USA*. 90: 9016-20.

Zuckerman AJ, Thomas HC (2001). *Viral Hepatitis*. Second edition. Churchill-Livingstone.

Fields B, Knipe DM, Howley PM (2001). *Fields' Virology*. 4th ed. USA Lippincott Williams and Wilkins. P: 3037-3050.

Hadler S, Monzon A, Ponzeto E (1984). Delta virus infection and sever Hepatitis an epidemic in the Yupaindians of Venezuela. *Ann Inten Med*. 100: 339-344.

Jardi R, But M, Rodriguez F (1994). Comparative analysis of serological markers of chronic delta infection: HDV RNA, Serum HDAg and anti-HDVIgM. *J Virol Methods*. 50: 59-66.

Kwok S, Higuchi R (1989). Avoiding false positive with PCR. *Nature* 339: 237-238.

Lee CZ, Lin JH, Chao M (1993). RNA-binding activity of hepatitis delta antigen involves two arginin-rich motifs and is required for hepatitis delta virus RNA replication. *J Virol*. 67: 2221-7.

Malekzadeh R, Borhanmanesh F (1989). Privalence and prognostic implications of hepatitis delta Infection in asymptomatic hepatitis B surface antigen carriers in Iran. *Ir J Med Sci.*14: 33-37.

Modahal L, Lai M (2000). Hepatitis delta virus: The Molecular Basis of Laboratory diagnosis. *Crit Rev Clin Lab Sci*. 37: 45-92.

Niro GA, Smedile A, Andriulli A, Rizzetto M, Gerin JL, Casey JL (1997). The predominance of hepatitis delta virus genotype I among chronically infected Italian patients. *Hepathology* 25: 728-734.

Poisson V, Menard DB, Frost E, Perreault JP (2000). A Canadian isolate of hepatitis delta virus. *Can J Gastroenterol*. 14: 36-40.

Popper H, Thung SN, Gerber MA, Hadler SC, de Monzon M, Ponzetto A, Anzola E, Rivera D, Mondolfi A, Bracho A (1983). Histological studies of severe delta antigen infection in Venezuelan Indians. *Hepatology* 3: 906-912.

Radjef N, Gordien E, Ivaniushina V, Gault E, Deny P (2004). Molecular phylogenetic analyses indicate a wide and ancient radiation of African HDV, suggesting a Delta virus genus of at least seven major clades. *J Virol*. 78: 2537-44.

Rezvan H, Forouzandeh B, Taroyan S, Fadaiee S, Azordegan F (1990). A study on delta virus infection and its clinical impact in Iran. *Infection* 18: 26-28.

Rizzetto M, Haddzyannis S, Hansson BG, Toukan A, Gust I (1992). hepatitis delta virus infection in the world: epidemiological patterns and clinical expression. *Gastroenterol Int*. 5: 18-32.

Sambrook J, Russell D (2001). *Molecular cloning: A laboratory manual*. 3rd ed. USA. Cold spring Harbor.

Shakil AO, Hadziyannis S, Hoofnagle JH, Di Bisceglie AM, Gerin JL, Casy JL (1997). Geographic distribution and genetic vari-

ability of hepatitis delta virus genotype I. *Virology* 234: 160-167.

Theamboonlers A, Hansurabanon T, Veranchi V, Chongsrisawat V, Poovorawan Y (2002). Hepatitis D virus infection in Thailand: HDV genotyping by RT-PCR, RFLP and direct sequencing. *infection* 3: 140-144.

Wang KS, Choo QL, Weiner AJ (1986). Structure, sequence and expression the hepatitis delta viral genome. *Nature* 323: 508-14.

Watanaba H, Nagayama K, Enomoto N, Chinzei R, Yamashiro T, Izumi N (2003). Chronic hepatitis delta virus infection with genotype IIb variant is correlated with progressive liver disease. *J Gen Virol*. 84: 3275-89.

Wu JC, Choo KB, Chen CM (1995). Genotyping of hepatitis delta virus by restriction-fragment length polymorphism and relation o outcome of hepatitis D. *Lancet* 346: 939-41.