

*Original Research Article*

# Frequency and genotype distribution of hepatitis delta virus in HBsAg-positive patients, Kerman, Southeast of IRAN

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**Abstract**

Hepatitis Delta Virus (HDV) and Hepatitis B Virus (HBV) have been identified as a unique human pathogen responsible for some 20 million infections globally. HDV virus is dependent on hepatitis B virus for transmission and propagation. In this study, 400 HBsAg-positive patients referred to the Kerman Besat clinic were examined to investigate the HDV and HBV infections; initially Enzyme Linked Immuno Assay (ELISA) test was done on plasma samples for HBsAg and HDVAg. The Real-time PCR test performed on positive samples and for positive cases, Reverse Transcriptase -nested PCR (RT-nested PCR) was performed. HDV genotype was determined according to electrophoresis results and Restriction Fragment Length Polymorphism (RFLP) tests. To confirm the genotype, the PCR products were sequenced. Out of 400 positive HBsAg patients, 67 samples were positive for HDV Ag. After more confirmation tests by Real-time PCR, seven patients have positive HDV RNA. After PCR-RFLP for detection genotype of HDV, all polymorphisms were shown to be genotype I. HDV genotyping was successfully performed by direct sequencing and our results point to a low prevalence of HDV in the Kerman population which is in sharp contrast to data reported from Iranian scientists done years ago. HDV genotype I is the main genotype in Kerman province, Iran and the middle East.

**Keywords:** Hepatitis Delta virus, HBs-Ag, HDV genotype, PCR-RFLP, Kerman

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## INTRODUCTION

Hepatitis D virus has circular single-stranded RNA with negative polarity. The genome contains several Open Reading frame (ORF) coding region with the length of 1700 nucleotides. Each virus contains about 70 molecules of HDVAg. HDVAg consists of lipids, proteins S, M and L derived from HBsAg (Mohebbi et al., 2008). The small form of HDVAg is 24-kDa (S-HDVAg), which is essential for viral replication and the large form of HDVAg is 27-kDa (L-HDVAg), which is necessary for viral assembly and prevents viral replication. HDV virus is classified in 8 clades and the nucleotide diversity of the isolated strains

within a genotype is 10-15 percent, while the rate is 40% for the different isolates (Gondert et al., 2006). HDV requires the help of HBV for viral assembly and transmission. HDV clinical signs are varied from a mild to chronic hepatitis or acute hepatitis. Hepatitis D infection may occur in two forms: The co-infection with hepatitis B and D and acute hepatitis D infection in chronic carriers of hepatitis B (super-infection) (Braga et al., 2014; Liao et al., 2014). In co-infection, the HDV acquires the helper function of HBV surface proteins (pre-s1) in order to mature and achieve further penetration into hepatocytes

and replication. In general, super-infection usually leads acute hepatitis and liver damage (Lusida et al., 2003). The mortality rate of co-infection is 1-10% and in super-infection is 5-20%. Acute infection is more likely than chronic in patients with co-infection with hepatitis B and D infection (Qureshi et al., 2009). These patients have higher risk of fulminate hepatitis, and only 2% of these patients will progress to chronic disease. In super-infection, 80% of cases progresses to chronic liver disease and cirrhosis, in which the cancer risk is an important issue (Liao et al., 2014). Among genotypes of the virus, genotype I is more prevalent. This genotype is common in North America, Asia, Africa, the Middle East and Europe. Genotype II in Japan, Taiwan, Russia, and genotype III was found only in the northern part of South America. Genotype I have several virulence, but most cases leads to acute HDV infection (Servant-Delmas et al., 2014; Villiers et al., 2015). Genotype II causes milder disease and genotype III mainly will lead to fulminate hepatitis. Worldwide epidemiological studies show that the prevalence of hepatitis D infection among hepatitis B carriers is about 5% (Lusida et al., 2003). It is estimated that approximately 15 million of HBs Ag carriers in the world are infected with hepatitis D virus. 1.7% of the Iranian population are carriers of hepatitis B which is estimated nearly 2 million people. The prevalence of hepatitis D in HBsAg carriers without symptoms in Jordan, Kuwait, Saudi Arabia and Turkey is 2%, 31%, 3.3%, 5.2% respectively. The incidence in acute patients in Egypt, Jordan, Kuwait, Tajikistan, is 16.94%, 16%, 4%, 9.2%, respectively. In chronic liver disease in Yemen, Turkey, Jordan and Egypt is 2%, 32.7%, 23%, 23.53% respectively (Mohebbi et al., 2008; Amini et al., 2011). Based on limited studies in Iran, the prevalence of HDV infection in blood donors is 2.4% and in chronic liver disease is 10%. Hepatitis D virus is a blood-borne virus may be transmitted through contact with contaminated blood products and other common ways of transmission are the use of shared needles or blood transfusions, injection for drug users and blood transfusions, sexual contact and infection in the hospital (De Paschale et al., 2012; De Paschale et al., 2014). The fulminate hepatitis with HDV in tropical areas of the Amazon (South America) was reported HDV genotype III in carriers with the genotype of HBV F. Recently Fulminate hepatitis with genotype I HDV reported in Russia (Ivaniushina et al., 2001; Tallo et al., 2008). Worldwide epidemiological studies show that the prevalence of hepatitis D infection among hepatitis B carriers is about 5%. It is estimated that approximately 15 million people of HBs Ag carriers in worldwide have been infected with the hepatitis D (Flodgren et al., 2000). In Turkey, the prevalence of anti-HDV cases in HBsAg carriers with liver disease and cirrhosis, decrease from 43.3% to 24% in 1980 and 31% to 11% in 2005 (Altuglu et al., 2007). However, after the implementation of vaccination programs against HBV, the prevalence of HBV and HDV in Iran has been decreased.

Recent studies show the increase in HDV prevalence among HBsAg-positive patients (Amini et al., 2011). A study published the incidence of HDV is 9.7% in chronic HBV patients in Shiraz. In the central area of Iran, the HDV incidence reported 2.4% in HBsAg carriers (Tajbakhsh et al., 2011). In other studies for anti-HDVAg on high risk cases, 2.5% in chronic asymptomatic HBsAg carriers, 33.33% in hemophilia patients, 44.5% in Hemodialysis patients with HBsAg were reported in Iran (Amini et al., 2011). Seroprevalence studies of HDV in Iran showed 9.7% in Shiraz in 2011, 9.3% in Tabriz and Tehran in 2009 and 17.3% in Hamadan in 2010 (Seifi and Ghannad, 2010). The only effective way to prevent infection with HDV is vaccination against hepatitis B, which has dramatically reduced the incidence of hepatitis D. 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 (Gust and Dimitrikakis, 1987). 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. The purpose of this study was to investigate the molecular features of HDV in this region. These features can contribute to a better understanding of the epidemiologic and clinical aspects of these viruses in this region.

## MATERIALS AND METHODS

### Patients

In a retrospective study, 400 HBsAg positive patients 39 cases Male (78%) and 11 cases Female (22%) were collected from Jun 2013 to Jun 2014 in our Laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, IRAN). All patients were tested for Anti HCV, Anti HIV, and excluded from this study if were positive. Mean±SD male patients were 54±4.5 and for female were 48±3.2.

### Serological Tests

All samples were tested with HDV Ag, HBs Ag, ELISA kit (Diapro, Italy). SGOT, SGPT and ALkPh were tested for HDV PCR Positive samples.

### DNA and RNA Extraction

HBV Viral DNA was isolated from 200 µl serum, using a QIAamp Viral DNA Mini kit (Qiagen, Germany) and HDV Viral RNA was extracted using Viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA and RNA pellets were re-

**Table 1.** Primer sequences for HDV nested PCR

<b>Primers</b>			
<b>Sequence</b>	<b>Name</b>	<b>Polarity</b>	<b>Use</b>
5'-ATGCCATGCCGACCCGAAGAGGAA-3'	D120	Sense	1 <sup>st</sup> PCR
5'-GGCCTCTCAGGGGAGGATTCAC-3'	DH1	Antisense	
5'-ATGCCATGCCGACCCGAAGAGGAA-3'	D120	Sense	2 <sup>nd</sup> PCR
5'-CTCAGGGGAGGATTCACCGACA-3'	D118	Antisense	

**Table 2.** RFLP enzymes pattern for different genotypes of HDV virus

<b>Sample</b>	<b>Size after SmaI digestion (bp)</b>	<b>Sample</b>	<b>Size after XhoI digestion (bp)</b>
Genotype I	179 ,226	Genotype I	382 ,59
Genotype II	405	Genotype II	159 ,81 ,30
Genotype III	103 ,104	Genotype III	358 ,83
Iranian samples	179 ,226	Iranian samples	382 ,59

suspended in 100µl of pre-warmed Elution buffer and stored at -70°C until use.

### Virus Detection using Real time PCR

Real time PCR was carried out using HBV and HDV Real Time PCR kit (Inter Lab Service, Russia) following instruction manual.

### cDNA synthesis for Semi-Nested PCR

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<sub>2</sub>, and 50U RNase 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 HDVAg 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. In this study, for RTPCR and semi-nested PCR, three oligonucleotide primers 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 (Theamboonlers et al., 2002).

### Semi-nested PCR

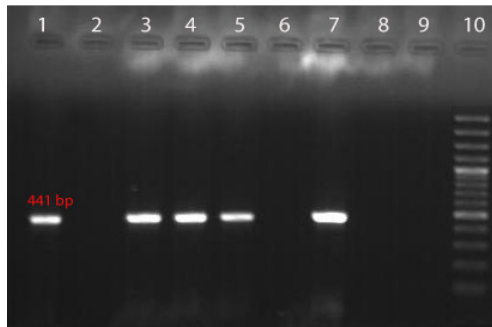
For cDNA amplification, in the first round of semi-nested PCR, 5µL of cDNA was mixed with 10 pM of each primer (D120, DH1), 10X PCR buffer, 10 mM dNTP, and 5U Taq DNA polymerase (Roche, Germany). In the second round, PCR reagent II, composed of 10 pM of each primers (D120, D118), 10X PCR buffer, 10 mM 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 pre-denaturation, followed by 35 cycles of two minutes at 95°C, annealing at 60°C for one minute 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 ethidium bromide and the expected 441 bp length was confirmed.

### HDV genotype analysis

For genotype determination, RFLP analysis was applied, 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 100bp (Roche, Germany) and undigested PCR product were included in each analysis.

### RESULTS

The ELISA test was performed on positive HBsAg patients to determine HDVAg. From 400 positive HBsAg samples, 67 samples were positive for HDVAg which is about 16.75% of positive HBsAg cases. For more



**Figure 1.** The results of gel electrophoresis of RT-nested PCR test to detect the presence of the genome of the hepatitis D. Number 1,3,4 and 5 are positive HDV samples with 441 bp. Number 2 and 6 are negative control. Number 7 is positive HDV viral genome. Number 10 is size marker 100bp.

**Table 3.** Mean of Viral characteristic the seven Positive patients for HDV and Negative patients

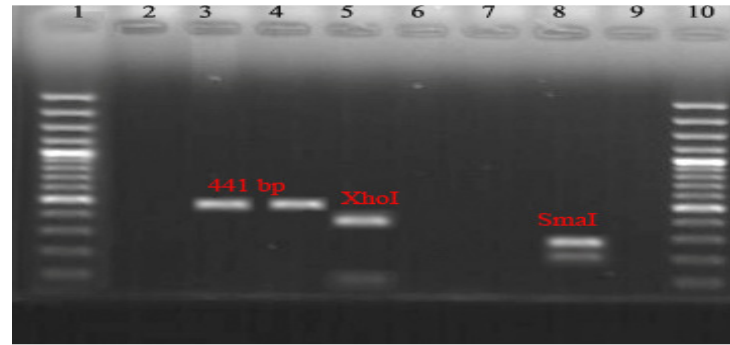
HBV Load	HDV Load	SGOT	SGPT	AlkPh	Age
$8.5 \times 10^5 \pm 1.3 \times 10^5$	$6.8 \times 10^4 \pm 1.1 \times 10^4$	931.1 $\pm$ 100.8	377.1 $\pm$ 95.1	1942 $\pm$ 607	48 $\pm$ 5
$3.5 \times 10^3 \pm 1.5 \times 10^3$	-----	450 $\pm$ 120	210 $\pm$ 100	650 $\pm$ 56	55 $\pm$ 10

detailed confirmation of positive samples, Real-time PCR method was used. Due to the sensitivity and precision of the method, from 67 positive samples, 7 samples were determined conclusively as positive. To avoid any confusion, the experiment was repeated two times. The 1.75% of positive HBsAg patients have hepatitis D virus RNA. After genome extraction the cDNA was made and the nested PCR was done by using specific primers for the C-terminal of HDVAg Hepatitis D (D120 and D120 primers for first PCR and D118 and DH1 primers for second PCR). Figure 1 illustrates RT-nested PCR test to detect the presence of the genome of the hepatitis D. The final PCR product was analyzed by electrophoresis on 2% agarose gel. The size of 441 bp band indicates Hepatitis D virus genome in our sample.

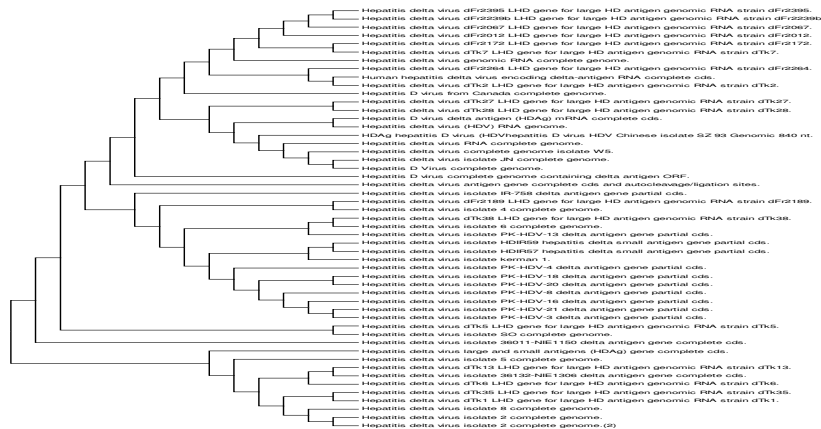
In our study, from 400 positive HBsAg patients, 67 samples have HDV-Ag. After more confirmation tests by Real-time PCR, 7 patients have positive HDV RNA. All seven positive samples were tested by HBV viral Load, HDV viral Load, liver function tests and RT-nested PCR for hepatitis D virus genome detection and RFLP analysis was done to determine HDV genotypes in positive samples (Table3). After testing samples by RT-nested PCR, 7 positive PCR were carried on by appropriate restriction enzyme respecting digestion pattern. According to the location and size of the products the type of hepatitis D virus genotype was determined. As it is shown in table 2, the product size after cutting by SmaI enzyme are 226bp and 179bp for genotype one, 405bp for genotype 2 and 130bp, 104bp for HDV genotype 3. The product size after cutting by XhoI are 382bp and

59bp for genotype one, 159bp, 81bp and 30bp for genotype 2 and 358bp, 83bp for HDV genotype 3.

Based on information in Table 2, the size of bands after RFLP by XhoI and SmaI enzymes were illustrated in Figure 2. In all 7 Iranian samples, 226bp and 179bp were for SmaI enzyme and 382bp and 59bp were reported for XhoI which approved HDV genotype I for all Iranian samples. In order to confirm the genotyping results obtained by RFLP, PCR product was sequenced by MacroGen Company (Korea) and 441 nucleotides were analyzed by BioEdit software. The BLASTN program was used and one of sequences was used to blast and phylogenetic analysis. After blasting, 50 sequences with the highest sequence similarity with the pattern were chosen and according to rank points, were used to draw a phylogenetic analysis. The analysis of the nucleotide and amino acid sequence to specify the variable and instant regions, the number of differences in nucleotides between varieties, determination of the types (Sequence type) and drawing a phylogenetic tree were performed by using MEGA5 software. The mean of nucleotide differences between patients 1 and clade 1 is 0.08, and the low difference represents the placement of patients in clade 1. All the sequences with high simulated ranking were analyzed by MEGA5 software and all of them were categorized by Clustal W and the Alignment. Using the method Kimura's2-parameter genetic distance estimated and the phylogenetic tree was drawn based on Neighbor-Joining method and the mean of the nucleotide differences was calculated 0.37. To ensure the accuracy of the drawn tree, the statistical (Bootstrap method 1000



**Figure 2:** The results of RFLP in gel electrophoresis for Hepatitis D and restriction enzyme cut sites. Number 3, 4 are with 441bp size before enzymatic reaction. Number 5 is under XhoI reaction with 59bp, 382bp size. Number 8 is under SmaI reaction with 226bp, 179bp size. Number 2 is negative control. Number 1, 10 are size markers 100bp.

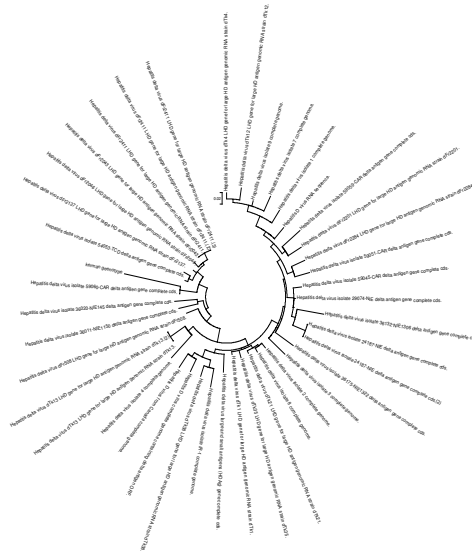


**Figure 3.** Neighbor-joining phylogenetic tree based on the sequences obtained using the C-terminal region of HDAG of hepatitis D viruses. Comparison with 50 full genetic sequence of the hepatitis D virus with the eight clades derived from Worldwide gene databank. Average rate is above 70% based on Bootstrap value 1000 replicate method

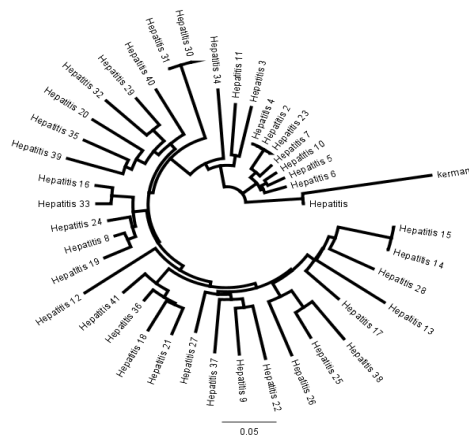
replicate) was used and shown in Figure 3 based on Neighbor-joining method. The average above 70 percent is reliable. As it is shown in Figure 4 and 5, The phylogenetic tree (Unrooted) using Maximum likelihood based on sequences obtained from amplification of the C-terminal HDVAg hepatitis D virus was drawn and the sample from Kerman is categorized in genotype I for HDV virus. Comparing the results of genotyping by RFLP and sequencing of the C-terminal HDVAg hepatitis D and Alignment results with the phylogenetic analysis, Clustering of different isolates of genotypes correctly reported and HDV genotype I in our sample was confirmed by sequencing.

In our study all information was analyzed by SPSS Version19. Based on our data, the average age of patients is 38 and minimum age is 12 years and maximum is 72 years. The mean of age for patients was 38 years and 64.9% of HDV-positive patients were male

and 35.1% were women. In Table 4, different risk factors which are effective for infection were mentioned. As it is clear, from 400 patients, 258 (64.6%) people had the history of dental surgery which is a shining reason of infection in our research. 182 (45.5%) of cases had the family history for HBV infection and 174 (43.4%) patients had the history of surgery in their life. 85(21.2%) patients had done ear piercing and 32(8%) cases had got tattoo which can be reasons for hepatitis infection. From 400 patients, 4(1%) samples had got contaminated needles, 4(1%) had sexually transmitted diseases and 8(2%) patients were drug injecting users. According to our data, some activity such as endoscopy, cupping and unprotected sex can be concerned for HBV and HDV infection in Iranian population and significant association between HDV infection and blood transfusion, surgical and tattoos were shown by reporting the P.value <0.001.



**Figure 4.** Unrooted phylogenetic tree using Maximum likelihood based on C-terminal sequences of HDAg obtained by replication of hepatitis D virus.



**Figure 5.** Unrooted phylogenetic tree using Maximum likelihood method based on C-terminal sequences of HDAg hepatitis D virus.

**Table 4.** The frequency of different risk factors in HBsAg positive patients.

Risk factors	Num.(%)
Injection drug Use	8 (2)
Sex outside marriage	4 (1)
Family history of hepatitis B	182 (45.5)
Ear piercing	85 (21.2)
Tattoo	32 (8)
History of surgery	174 (43.4)
History of dental surgery	258 (64.6)
Endoscopy	56 (14)
Cupping	20 (5)
Sexually transmitted disease	4 (1)
Needle stick	4 (1)
Exposure of hepatitis	109 (27.3)

## DISCUSSION

Like many RNA viruses, Hepatitis D virus shows genetic changes. However, based on the sequence similarity between isolates, eight clades were classified, which are associated with different geographical distribution and clinical signs (Villiers et al., 2015). HDV genotype I is very common and leads serious infection in North America, Asia, Africa and Europe. Genotype II exists in Japan, Taiwan, and Russia causes milder disease (Liao et al., 2014). While genotype III is just in South America and makes fulminate hepatitis. Definitive diagnosis (golden standard) is a phylogenetic analysis of HDV genotype but a simple method for the detection and genotyping is (Restriction fragment length polymorphism (RFLP) (Ghamari et al., 2013). In this method, the RT-PCR products are broken with the restriction enzyme XhoI, SmaI for genotyping. Approximately 400 million people in worldwide are chronically infected with hepatitis B virus infection and 15 million of them have hepatitis D (Wang et al., 2013). Although hepatitis D infection has been reported in many countries, but in the Middle East, the Mediterranean, Central and northern South America is more significant (Tajbakhsh et al., 2011). Also HDV is a major public health issue especially in endemic areas of Iran. In this study From 400 positive HBs Ag patients, 67 cases have positive Anti-HDV and among them, seven samples were positive by Real-time PCR test.

In our study, the mean of age for patients was 38 years and 64.9% of HDV-positive patients were male and 35.1% were women. Comparing with a gender distribution in other countries such as Romania and Iraq our data is similar. The sero-positivity rate among infected patients with HDV in positive HBV cases is 16.75%. Due to possible cross-reactivity between antigens of HDV and HBV, a highly accurate and sensitive test was done by Real-time PCR and shows that only 7 samples had HDV-RNA and the prevalence 1.75% was reported. This prevalence is sharply less than other results (9.7% in Shiraz, 9.3% in Tehran, 17.3% in Hamedan) which had been done in Iran (Behzadian et al., 2005; Mohebbi et al., 2008; Amini et al., 2011). The main reason of this reduction is the huge vaccination campaign which has been doing in Iran. In 2012, Seroprevalence of HDV among HBsAg-positive patients in Pakistan was 26.8% while this rate in India was 14.2%, in Turkey was 6% and in Saudi Arabia was reported 13.6% (Perveen et al., 2012). Based on our study, compared with the mentioned countries, HDV is endemic and serological prevalence is fewer. In our study based on risk factors, a significant association between HDV infection and blood transfusion, surgical and tattoos were shown by reporting the P.value <0.001.

27.3% of the positive HDV patients had the same family history for both HDV and HBV infections. According to the same methods of transmission, it seems family roots is one of HDV acquisition in patients.

Previous studies suggest that HBV and HDV co-infection increases the severity of hepatitis B disease and leads cirrhosis (Abbasi et al., 2014). In our study, for 7 patients with positive RT-nested PCR to detect the presence of hepatitis D virus genome, RFLP analysis was performed to determine the genotype. Enzymatic digestion of the 7 patients (1.75%) shows genotype I in each case. The phylogenetic analysis of nucleotide differences between groups by using Kimura 2-Parameter and MEGA5 software, and the mean of the nucleotide differences was calculated 0.37 (Behzadian et al., 2005).

The mean of nucleotide differences between patients 1 and clade 1 is 0.08, and the low difference represents the placement of patients in clade 1. A study was conducted in 2005 by a Behzadian and colleagues in Iran, after cloning and sequencing the complete genome of HDV and genotype phylogeny survey, type one was determined (Behzadian et al., 2005). In another study by Mohebbi and colleagues in 2008 in Iran, 22 positive HDV RNA patients was tested by direct sequencing and showed the genotype I in all patients (Mohebbi et al., 2008). In 2009 in Iran, Mirshafiee et al studied on 13 positive HDV RNA patients by RFLP and genotype I was determined in all patients (Mirshafiee et al., 2009). Our data shows the same results which had been done previously in Iran conducted on the genotyping of hepatitis D virus. Similar studies in other countries such as Afghanistan, Turkey, Pakistan, Lebanon and Egypt reported genotype I in their countries, Where the clade I is the most probable source of HDV in these countries and Iran (Altuglu et al., 2007; Khan et al., 2011; Gomaa et al., 2013). In conclusion viral hepatitis is a common disease in Iran and few studies have been done in the Middle East and Iran on hepatitis D, but so far no comprehensive study has been done to indicate the epidemic data in Iran. Also, because of the different genotypes of the virus causing different clinical protests, broader investigation seems necessary in Iran.

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