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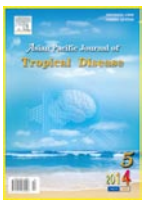

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Phylogenetic analysis of hepatitis delta virus isolated from HBsAg positive patients in Shahrekord, Iran

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PEER REVIEW

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Comments

This is an interesting study in which authors determined delta antigen sequences of 5 HDV isolated from HBsAg positive patients. The study is useful to the researchers in future study. Further study is needed.
Details on Page 396

ABSTRACT

Objective: To find out the phylogenetic background of hepatitis delta virus (HDV) samples isolated in Shahrekord, Iran.

Methods: A total of 350 hepatitis B surface antigen (HBsAg) positive sera samples were found from blood donors and HBsAg positive patients in blood transfusion center and clinical laboratory in Shahrekord, Iran. HDV RNA was extracted using RNXPlus (CinnaGen, Iran). A total of 421 bp corresponding to hepatitis delta antigen have been isolated from HDV in Shahrekord, then were amplified in polymerase chain reaction system, sequenced for determining nucleotide sequence and compared with identified nucleotide sequences of these genes in other countries.

Results: Among 350 HBsAg positive samples, we could detect HCV RNA in only two samples. After sequencing, the nucleotide sequences had a variability of 1/7-3/0 for *HD Ag* gene. The greatest sequence similarity existed between Iranian *HD Ag* sequence and JF694493-Iran, U25667-China with a sequence similarity of 99.7% and the least relationship between Iranian *HD Ag* sequence and AF008420-USA with a similarity of 92.9%.

Conclusions: It is suggested that precise genotype of HDV circulating in the region can be determined by more expansive sampling from different parts of Chahar mahal and Bakhtiari province and neigh bouring provinces (Esfehan and Khoozestan).

KEYWORDS

Hepatitis delta virus, Phylogenetic analysis, Sequencing, Shahrekord

1. Introduction

The hepatitis delta virus (HDV) (Delta) is a defective RNA pathogen which was first discovered in 1977. Around 1986, several other labs began to work on the molecular virology of this agent and over and over, HDV has provided us with intriguing and unique phenomena in molecular virology[1-3].

HDV requires helper functions from the hepatitis B virus (HBV) for virion assembly and propagation. Thus, HDV infection is necessarily associated with HBV infection because HDV ribonucleoprotein buds through the hepatitis B surface antigen (HBsAg) excretory pathway[4-6].

The HDV genome is a circular single-stranded RNA

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genome of approximately 1 680 bases with extensive intramolecular complementarity. Part of the HDV genome might have historical homology to viroids or plant virus satellite RNA sequences, and a rolling-circle model has been developed for viral RNA replication[5,7,8].

HDV is now well known to induce a spectrum of both acute and chronic liver diseases[9]. Super infection with HDV in HBV carriers leads to more progressive chronic liver disease (80%), with higher incidence of cirrhosis and hepatocellular carcinoma[10-13].

More than 350 million individuals worldwide are HBV carriers and over 15 million people are infected with HDV worldwide and its prevalence in Italy, Eastern Europe and western region of Asia is higher than in the rest of world and appears to be endemic in the Middle East[8,14-16].

The genetic diversity of HDV is related to the geographic origin of the isolates. Apart from HDV-1, which is ubiquitous, each virus clade is geographically localized: HDV-2 (previously labeled HDV-IIa) is found in Japan, Taiwan, and Yakoutia, Russia, HDV-4 (previously labeled HDV-IIb) in Taiwan and Japan, HDV-3 in the Amazonian region, and HDV-5, HDV-6, and HDV-7 in Africa[8,17].

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

HDV genotyping has been performed by various methods such as hybridization, direct sequencing, and restriction fragment length polymorphism analysis of reverse transcription (RT) product of the HDV genome[8,18].

Phylogenetic analyses of HDV isolated in Iranian patients have been conducted previously. Because Iran is a large country with different ethnic groups, more data are required to have a better understanding of HDV characteristics. In the present study, delta antigen sequences of 5 HDV isolated from HBsAg positive patients, were analyzed to determine HDV genotype distribution[9].

2. Materials and methods

2.1. Serological tests

In this study, a total of 300 HBsAg positive sera samples that were found from blood donors and HBsAg positive patients in blood transfusion center and clinical laboratory in Shahrekord, Iran. All sera were checked for HBsAg using an ELISA kit (Enzygnost HBsAg 5.0 DADE BEHRINC). Anti-HDV antibody was explored using an ELISA kit (DIA-PRO, Italy) according to the manufacturer's protocol.

2.2. RT-polymerase chain reaction (PCR) and PCR

HDV RNA was extracted from 200 μL 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 (0.2 μg/μL), a reverse primer as a GSP 1 μL (12.5 μmol/L), and an MMULV (Fermentas AB, Vilnius, Lithuania) in a final volume of 20 μL. About 2 μL of cDNA were amplified using 0.3 μmol/L forward and 0.3 μmol/L reverse primers:

F: 5′-TGC CAT GCC GAC CCG AAG AGG AA-3′

R: 5′- GGA GAG ACG GGA TCA CCG AAG AAG GAA GGC-3′) by Taq DNA polymerase (Fermentase) at 94 °C for 5 min and 35 cycles: 94 °C for 40 seconds, 72 °C for 1 min (annealing and extension were at 72 °C), and ending at 72 °C for 5 min[9]. Finally, the PCR products were electrophoresed on a 2% agarose gel prepared in 1× Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide, and evaluated under UV transilluminator. The PCR product with an expected length of 421 bp was analyzed in 1.5% agar gel electrophoresis.

2.3. Sequence analysis

The nucleotide sequences were edited using Edit View v.1.0.1 (Applied Biosci-ence, Australia). The 14 sequences regis-tered in GenBank (accession numbers JF694493-Iran, AF425644-Taiwan, U25667-China, AF309420-Japan, AJ309880-Russia, U81989-Ethiopia, U81988-Somalia, JX888135-Luxembourg, AB037949-Venezuela, KC590319-Brazil, L22065-South America, AF247965-Sweden, HF679406-France, M55042-Netherland) were aligned separately using the Clustal W v1.81 in order to obtain a consensus sequence. Subsequently, the sequences were analysed using the BioEdit package v.7.0.4.1 to compare the nucleotide sequences.

The Two nucleotide sequences of the Iranian IBR *gB* and *gD* genes were compared with the corresponding sequences from other regions of the world. Unrooted dendrogrammes were constructed using the Njplot software. Statistical support for dendrogrammes was obtained by boot-strapping using 1 000 replicates.

3. Results

Among 350 HBsAg positive samples, we could only detect HCV RNA in two samples. The results are shown in Figure 1.

The nucleotide sequences of the 421 bp fragments of the *HD Ag* gene from Iranian isolates were compared with the sequences of the *HD Ag* gene from the known reference sequences obtained from the GenBank nucleotide sequence

Table 1

Sequence identity matrix of partial *HD Ag* gene of Iranian HDV isolates in comparison with other sequences.

Seq	Sample 1	Sample 2	JF694493-Iran	AF425644-Taiwan	U25667-China	AF309420-Japan	AJ309880-Russia	U81989-Ethiopia	U81988-Somalia	JX888135-Luxembourg	AB037949-Venezuela	KC590319-Brazil	L22065-South America	AF247965-Sweden	HF679406-France	M55042-Netherland	AF008420-USA
Sample 1	ID	0.99	0.991	0.971	0.975	0.969	0.964	0.952	0.950	0.948	0.942	0.94	0.939	0.931	0.931	0.930	0.929
Sample 2	0.990	ID	0.997	0.900	0.991	0.97	0.965	0.956	0.951	0.949	0.945	0.941	0.940	0.935	0.936	0.931	0.930
JF694493-Iran	0.991	0.997	ID	0.981	0.982	0.968	0.963	0.953	0.951	0.950	0.940	0.940	0.938	0.930	0.930	0.930	0.927
AF425644-Taiwan	0.971	0.900	0.981	ID	1.000	0.998	0.972	0.972	0.970	0.962	0.958	0.945	0.960	0.938	0.929	0.926	0.924
U25667-China	0.975	0.991	0.982	1.000	ID	0.996	0.970	0.971	0.972	0.965	0.956	0.946	0.962	0.939	0.927	0.925	0.922
AF309420-Japan	0.969	0.970	0.968	0.998	0.996	ID	0.961	0.964	0.968	0.96	0.950	0.948	0.961	0.940	0.928	0.928	0.924
AJ309880-Russia	0.964	0.965	0.963	0.972	0.970	0.961	ID	0.958	0.948	0.952	0.948	0.945	0.958	0.938	0.926	0.927	0.936
U81989-Ethiopia	0.952	0.956	0.953	0.972	0.971	0.964	0.958	ID	0.940	0.951	0.944	0.944	0.954	0.936	0.921	0.921	0.932
U81988-Somalia	0.95	0.951	0.951	0.970	0.972	0.968	0.948	0.94	ID	0.948	0.942	0.938	0.950	0.932	0.920	0.920	0.936
JX888135-Luxembourg	0.948	0.949	0.950	0.962	0.965	0.960	0.952	0.951	0.948	ID	0.938	0.932	0.948	0.940	0.912	0.919	0.928
AB037949-Venezuela	0.942	0.945	0.940	0.958	0.956	0.950	0.948	0.944	0.942	0.938	ID	0.929	0.940	0.928	0.908	0.901	0.921
KC590319-Brazil	0.940	0.941	0.940	0.945	0.946	0.948	0.945	0.944	0.938	0.932	0.929	ID	0.935	0.920	0.899	0.897	0.920
L22065-South America	0.939	0.940	0.938	0.960	0.962	0.961	0.958	0.954	0.950	0.948	0.940	0.935	ID	0.924	0.910	0.908	0.904
AF247965-Sweden	0.931	0.935	0.930	0.938	0.939	0.940	0.938	0.930	0.932	0.940	0.928	0.920	0.924	ID	0.921	0.913	0.921
HF679406-France	0.931	0.936	0.930	0.929	0.927	0.928	0.926	0.921	0.920	0.912	0.908	0.899	0.910	0.921	ID	0.921	0.928
M55042-Netherland	0.930	0.931	0.930	0.926	0.925	0.928	0.927	0.921	0.920	0.919	0.901	0.897	0.908	0.913	0.921	ID	0.914
AF008420-USA	0.929	0.930	0.927	0.924	0.922	0.924	0.936	0.932	0.936	0.928	0.921	0.920	0.904	0.921	0.928	0.914	ID

database (14 sequences). The nucleotide sequences had a variability of 1/7-3/0 for *HD Ag* gene (Table 1). The greatest sequence similarity existed between Iranian *HD Ag* sequence and JF694493-Iran, U25667-China with a sequence similarity of 99.7% and the least relationship between Iranian *HD Ag* sequence and AF008420-USA with a similarity of 92.9%. The results are shown in Table 1 and Figure 2.

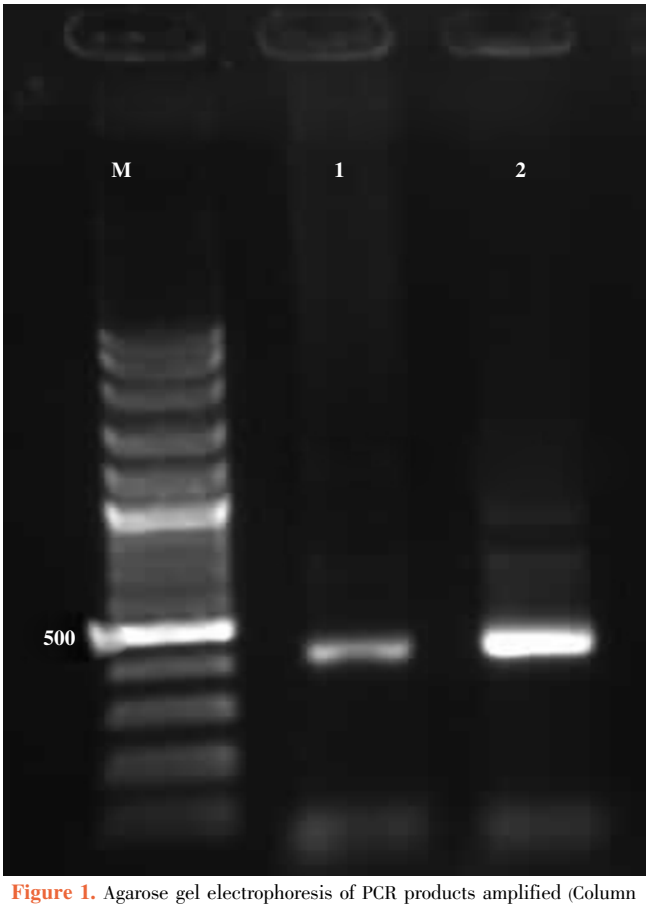

Figure 1. Agarose gel electrophoresis of PCR products amplified (Column M=100 bp DNA Ladder, Columns 1 and 2 are positive samples).

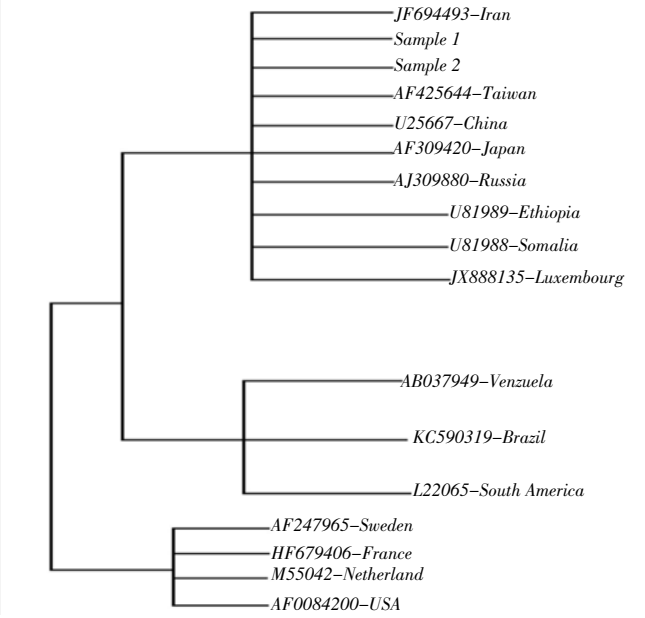

Figure 2. Dendrogramme based on sequence alignment analysis of two Iranian isolates and 15 of the reference isolates from other regions of the world for Delta gene of HDV.

4. Discussion

For the first time, HDV was known as a new nuclear antigen in hepatocytes patients infected with HBV. It was in conjunction with acute or chronic hepatitis. Then this antigen was called Delta antigen and was considered as gene products of HBV. Later, it was understood that Delta antigen is along with a transferable factor of disease and it is not from products of HBV genome. In patients liver or serum, *HD Ag* is found as two kinds based on number of amino acid or its weight. Small Delta antigen with weight of 24 000 KD and big Delta antigen with weight of 27 000 KD differ from each other in terms of 19 amino acids and

the sum of these two kinds is mRNA. *HD Ag* is important in forming structure of virus and enters nucleus of liver cellsn.

In this study, a piece with length of 412 open pairs from Delta antigen was used for philo genetic analysis of Delta virus. Infection with Delta virus is an infection which is seen throughout the world, but its prevalence is different in various geographical regions. Among 350 million people who are infected with HBV around the world, 5% suffers from both HBV and HDV. Geographical regions of the world has been divided to three groups (based on geographic spread): some regions with high epidemic such as Amazon in Venezuela, some African countries, regions such as south of Italy, Greece, Mediterranean countries, Bangladesh and some regions with low prevalence like developed countries which is seen among people with high risk like addicts injective drugs. RNA virus almost contains 1 700 nucleotides. It is a single ring-shaped filament which is not structurally similar to HBV genome. Heterogenecity of virus is high and RNA virus in each person differs the other person in terms of sequence of nucleic acids. *HD Ag* is the only protein which is made by HDV and considered as internal part of virus structure.

Delta virus has one serotype and eight genotypes. Genotypes I, II, III are the most abundant ones based on geographical distribution. Therefore, genotype III is common in Southern America or among Yoopka natives in Venezuela country and causes acute diseases of liver. This genotype can cause Amazon black fever in lower region of Amazon river which is a kind of heavy hepatitis of this virus. Genotype II of Delta virus produces a milder disease in Eastern Asia (with a slower trend). Genotype I is common in Europe and Northern America so that produced disease is often fast or leads to progressive cirrhosis (with fast trend)[19].

Numerous researches have been done about genotyping of HDV which we will describe some of them. In research done by Behzadian *et al.*[20], for the first time, sequence of genome of HDV was specified in Iran completely. In Iran, isolates of HDV has a length with 1 676 nucleotides. Results obtained from this research showed that HDV in Iran belongs to genotype I. In this research, complete sequence of these isolates showed that there is the most similarity with Italian isolates (type I) (92.6%) and it indicates that there are evolutionary relations between these two isolates. While there was the least similarity with Peruvian isolates (type III)[20].

In research done by Esmaeeli *et al.* on 26 patients infected with hepatitis delta in Tehran in 2009, all samples belonged to genotype I[9].

Mohebbi *et al.* conducted a study on 25 patients infected with hepatitis delta in 2008. And after philogenetic study, they specified that all samples belonged to genotype I of virus[21].

For determining frequency and genotype of HDV in patients infected with HIV and patients who were under dialysis, a study was done by Aghasadeghi *et al.* in 2013[22]. Among 70 people under study (120 patients under dialysis and 600 patients infected with HIV), all cases of positive HBsAg were reported. Nested PCR method was used for approving positive HDV-RNA in the samples and also anti-HDV. According to the result among 120 patients under dialysis about nine people (7.5%) and among 600 patients suffering from HIV about nine people (1.5%) showed positive HBsAg. According to nested PCR, three patients (33.3%) under dialysis and five patients (55.5%) suffering from HIV were positive in terms of anti-HDV. Totally, RNA of Delta virus was extracted from samples (37.5%), (2.5% patients under dialysis and 0.8% patients suffering from HIV). Positive samples were specified after philogenetic study and the sample belongs to clade 1[22].

For studying outbreak of hepatitis delta and hepatitis B in blood donors without sign in Iran, research was conducted by Attaran *et al.* in 2013[23]. This research showed that among 854 people under study, positive HBsAg of 18 people (2%) were as positive anti-HDV and 154 people (18%) were HDV-DNA and 6% from whole samples was reported positive HDV-RNA. These results were approved by two methods: real-time PCR and seminested PCR. After philogenetic analysis of positive samples, it was specified that samples belong to HDV 1 group[23].

A research was done by Lee *et al.* in south of Taiwan in 2013[24]. Generally, 64 patients suffering from serum outbreak of anti-HDV Abs, HDV genotype, clinical demonstrations among patients with and without HDV infection. The result obtained from this study was that among 64 patients suffering from HIV, while about seven patients (10.9%) had HDV genome. After philogenetic analysis, it was specified that positive cases belonged to HDV 11[24].

In 2012, a research was done Mansour *et al.* in Mauritania for studying molecular epidemiology of hepatitis B and HBV in pregnant women and patients suffering from hepatitis[25]. In this study, 1 200 pregnant

women and 946 hepatitis patients were considered. Among pregnant women and hepatitis patients, 10.6% and 18.3% had positive HBsAg, respectively, Also, anti-HBc Ab was reported (66.3%), and (76.5%) HDV-RNA was found in 10.1% of pregnant women and 17.3% of patients. After philogenetic analysis of positive samples, it was specified that common was 9.7%. This study approved high outbreak of HBV and HDV in Mauritania and genetic variety of virus in this region[25].

In addition, Mansour *et al*. conducted a research in 2012 for epidemiological study of hepatitis delta among blood donors of Nouakchott, Mauritania[26]. This study was done among 1 700 patients suffering from hepatitis with positive HBsAg. After studying and doing experiments, it was specified that 19.78% of donors were positive HDV Ab and 56 people (62.2%) were positive HDV-RNA. Philogenetic study showed that genotype 5 with 10.7% and genotype 1 with 89.3% had high outbreak in this region. It means that HDV in blood donors of Mauritania was one of the reasons of chronic hepatitis, liver cirrhosis or hepato cellular cancer of liver[26].

Le Gal *et al*. conducted a research in center and east of Turkey in 2012 for studying genetic variety on 34 patients of HBV/HDV[27]. Philogenetic study of piece 900-1 280 of genome HDV showed that all sides belonged to genotype 1 HDV. According to the results, two infections of HDV and HBV are very native in Turkey and both of them are repeated very much. Also, it has a wide genetic variety which may reflect evolution or outbreak of successive diseases[27] .

In a study done by Barros *et al.* in 2011 on 133 Brazilian vectors of chronic HBsAg, after determining sequence, it was specified that genotype 2, 3 and 8 are the most common genotypes in this region[28].

In 2011, Kim *et al*. conducted a research among patients suffering from chronic hepatitis B to study synchronous infection outbreak in South Korea[29]. This research was conducted on 940 patients that three of them just had positive anti-HDV. After philogenetic study, all positive cases belonged to group 1 of HDV. This study showed that synchronous infection of HDV can not have significant clinical effect on Korean patients suffering from chronic infection of HBV[29].

In study which has been done by Foupouapouognigni *et al.* on 233 HBsAg Cameroonians in 2011, RNA of HBV was extracted from serum of HDV-Ab people[30]. In this study, among 233 patients with positive HBsAg, about 41 cases were diagnosed as positive HDV-Ab and RNA of Delta virus was indentified among 25 cases of them. philogenetic analysis done on 25 samples showed that 22 samples (88%) of sterines belonged to genotype I[30].

In a study done by Gomes-Gouvêa *et al.* on 14 Brazilion suffering from sudden hepatitis in 2009, it was specified that all patients were positive in terms of having HBV DNA and HDV RNA[31]. Philogenetic analysis of HDV sequence showed that all positive cases belong to genotype III[31].

A study was done by Moriyama *et al*. on three Japanese patients suffering from chronic hepatitis B in 2005[32]. RNA of HDV was separated from serum of these people. After comparing HDV genomic regions, it was specified that 80% of Miyako samples in Japan belong to genotype II b[32].

Saudy *et al*. conducted a research on 105 patients suffering from positive HBsAg in Greek in 2003[33]. According to this study, nine patients had positive HDV-Ab. And after extracting RNA, nucleotides within 853-1 265 were sequenced. Philogenetic analysis showed that 9 isolates of HDV belong to genotype I[33].

According to a study done by Ivaniushina *et al*. on 29 Russian patients with positive HBsAg in 2001, 14 patients were diagnosed positive in terms of HDV-Ag[34]. After philogenetic analysis, it was specified that 14 isolates of HDV belong to genotypes I and II[34].

In a study which was done by Sakugawa *et al*. on six Japanase patients suffering from chronic hepatitis in 1999, RNA HDV was separated from six people. Philogenetic study showed that genotype II b of this virus is common in Okinato, Japan[35].

In the present study, nucleotide sequence of coding-gene of Delta antigen from two positive serum samples was determined in Shahrekord and Philogenic proximity (similarity) of determined sequence was compared with positive sequence of this virus in gene bank. According to the results, samples under study had 99.1%-99.7% similarity to sequence of this virus from South Eastern Asian countries (China and Taiwan). We can put these viruses in phylum of genotype II of HDV. Perhaps, this genetic variety can be justified based on geographic spread of this virus. Because the amount of travels to American and European countries has been more limited in Iran, especially after victory of Islamic Revolution, so it seems that infection with this virus in the region under study (Shahrekord) has been brought from Southeast Asian countries (men go to these countries especially Japan and Korea for working).

Generally, it is suggested that precise genotype of HDV circulating in the region can be determined by more expansive sampling from different parts of Chahar mahal and Bakhtiari province and neigh bouring provinces (Esfehan and Khoozestan) and determining sequence of more positive samples and doing methods of molecular biology like RFLP and the other genotyping methods.

Acknowledgements

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Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

HDV virus is a RNA pathogen associated with HBV. HDV is known to induce a spectrum of both acute and chronic liver diseases. The genetic diversity of HDV is related to the geographic origin of the isolates.

Research frontiers

The present study was carried to determine delta antigen sequences of 5 HDV isolated from HBsAg positive patients.

Related reports

Many authors reported the prevalence of HDV around the world. In research done by Esmaeeli *et al.* on 26 patients infected with hepatitis delta in Tehran in 2009, all samples belonged to genotype 1.

Applications

It is significant to know HDV genotype distribution in the world, which will be useful to researchers in their future study.

Peer review

This is an interesting study in which authors determined delta antigen sequences of 5 HDV isolated from HBsAg positive patients. The study is useful to the researchers in future study. Further study is needed.

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