Molecular epidemiology and clinical characteristics of hepatitis delta virus (HDV) infected patients with elevated transaminases in Shanghai, China

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Abstract

**Background:** Patients coinfecte{d} with HBV and hepatitis D virus (HDV) have a greater risk of HCC and cirrhosis. The current study was undertaken to assess HDV genotype distribution and determine clinical characteristics of hepatitis delta virus (HDV) among HBsAg positive individuals in Shanghai.

**Method:**

This retrospective study involved 225 serum samples from HBsAg positive hospitalized patients from October 2010 to April 2013. HDV-specific RT-nested PCR was used to amplify HDV RNA. HDV genotypes were characterized by Next-generation sequencing (NGS), followed by phylogenetic analyses. HDV/HBV co-infected patients and HBV mono-infected patients were compared clinically and virologically.

**Results:** Out of the 225 HBsAg-positive serum samples with elevated transaminases, HDV-RNA was identified in 11 (4.9%) HBsAg positive patients. The HBV loads in the HDV positive group were significantly lower than the HDV negative HBV-infected patients. The aminotransferase enzymes were significantly higher in HDV/HBV co-infected compared to HDV negative patients \( (P<0.05) \). Phylogenetic analyses indicated that HDV-2 genotype being the predominant genotype, other HDV genotypes were not observed. HDV/HBV patients were significantly associated with a rather unfavourable clinical outcome.

**Conclusion**

In summary, our study showed that the prevalence of HDV infection in patients with elevated transaminases is not low and the predominance of HDV genotype 2 infection in Shanghai. This finding helps us to better understand the correlation of HDV/HBV co-infection. Moreover, Next-generation sequencing (NGS) technologies provide a rapid, precise method for generating HDV genomes to define infecting genotypes.

**Background**

More than 240 million people throughout the world are chronically infected with hepatitis B virus (HBV), and approximately 15–25 million are co-infected with hepatitis D virus (HDV), a satellite virus which requires HBV envelope proteins for particle assembly and spread \(^1\). The HDV virions possess an outer layer containing hepatitis B surface antigens (HBsAg) and host lipid surrounding the inner
nucleocapsid that consists of viral RNA. Thus, HDV depends on HBV for its propagation and replication [2]. Moreover, HBV/HDV co-infection leads to the most severe form of viral hepatitis with an accelerated progression to liver fibrosis, cirrhosis and hepatocellular carcinoma [3].

In some regions of the world with vaccination campaigns against HBV, the prevalence of HDV infection has significantly declined. However, prevalence of HDV infection is higher in areas where HBV infection is endemic, as well as the central Europe because of the immigrant population regions [2]. HDV Genotype 1 is present globally, whereas other HDV genotypes occur in specific geographical regions [4–7]. Genotype 2 (previously 2a) prevails in Japan, Taiwan and Russia [8–12]. Genotype 3 which is the most diverged one is exclusively found in Amazon Basin [13–14]. HDV-4 (previously 2b) is found in Japan and Taiwan, whereas genotypes 5–8 are described in individuals of African origin [15–16]. China is one of the countries with the highest number of hepatitis B virus infections in the world, however, no nationwide cohort study has been conducted to assess the prevalence of HDV infection [17]. And yet, local data based on serology and molecular biology of HDV has not done. Usually, the HDV prevalence is commonly described as percentage of anti-HDAg positive individuals in HBsAg-positive patients using commercial ELISA kits. Anti-HDV seropositive specimens still need to be tested for serum HDV RNA [18]. Several commercial anti-HDV tests were used as methods of simultaneous competitive assays. Recently, quantitative microarray antibody capture (Q-MAC) assay was shown closely related with the presence of HDV-RNA (sensitivity, 100%; specificity, 94.3% for Q-MAC assay) [19]. Anti-HDV IgG is the most commonly used tool to screen patients with CHB for concomitant HDV infection or previous infection. Thus HDV RNA is the "gold standard" for the current diagnosis of HDV infection [20–21]. Quantitative determination of HDV RNA can be used to monitor the response of antiviral therapy and to determine HDV genotype by sequencing HDV RNA positive samples. There is no standard commercial PCR method, which brings great challenges to diagnosis of HDV infection.

Cloning of PCR products and subsequent Sanger dideoxy sequencing technique have been widely
used for the genetic analysis, especially in estimating viral populations \[22-23\]. However, the two sequencing methods are time-consuming and laborious. Furthermore, preferential selection of deficient viral genomes can bias the results of molecular cloning. Next-generation sequencing (NGS) method had made it possible to generate hundreds of thousands of clonal sequence reads, which provided the potential to reduce the time and complexity for DNA sequencing without the need for cloning. Furthermore, NGS can characterize genetic diversity with enhanced sensitivity, efficiency, and accuracy \[24\].

HBV infection remains a major public health problem in China, with 10–15% of the population being HBsAg positive \[17\]. Molecular epidemiological studies of HDV prevalence and HDV genotypes in HBV endemic areas in China are needed to assess and improve control measures for HBV/HDV co-infection. Therefore, in the study, we investigated the molecular epidemiology and clinical characteristics of HDV infected patients in Shanghai.

Methods
Patients
A total of 225 serum samples with positive HBsAg were collected from patients in Shanghai Jiao Tong University Affiliated Sixth People’s Hospital from October 2010 to April 2013. All patients were negative for hepatitis C, HIV and other liver diseases, including alcoholic, metabolic or autoimmune hepatitis. Anti-HDV IgG was detected by enzyme-linked immunosorbent assay kit (Beijing Beier Biological Engineering Co., Ltd, China). Serum or plasma samples were collected from those patients’ blood and used for biochemical and other laboratory routine procedures. Samples were stored at -80 °C. Demographic and laboratory data were obtained from the Electronic Medical Record (EMR). All patients were not treated with drugs and did not progress to liver cancer. Ethical approval was obtained from the Human Ethics Committee of Shanghai Sixth People’s Hospital (No.2016-73).

Extraction of HDV RNA and first complementary DNA (cDNA) synthesis
Nucleic acid was extracted from 100 µL serum sample using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa) according to the manufacturer’s instructions and was kept at -80 °C until further use. About 50 ng of the extracted RNA was reverse transcribed into cDNA using Superscript III
reverse transcriptase (Invitrogen) following the manufacturer’s instructions.

**Molecular Detection And Genotyping Of HDV**

The HDV-specific nested polymerase chain reaction (PCR) was carried out with first cDNA samples as template to amplify the HDV delta-gene fragment, which included the HDAg open reading frame (ORF) editing site. Briefly, cDNA was amplified in the first PCR reaction using the HDV-specific primers of PCR forward primer and the reverse primer. The specific primers were synthesized as described in reference \(^{[25]}\) (Table 1). First round PCR amplification was employed in a PCR reaction tube volume of 50 µl containing 10 mM concentration of each of the four deoxynucleotides, 10 uM of each outer sense nucleotides and outer anti-sense primers; and 0.5 ul High-Fidelity DNA Polymerases and 2 ul cDNA. In the thermal cycler, firstly the samples were incubated on 98 °C for 2 min then it was followed by 35 cycles consisting of 98 °C for 10 sec, 65 °C for 30 sec and 72 °C for 15 sec \(^{[10]}\). We used the negative and positive controls along with samples in each run. Finally nested PCR products (10 µl) were electrophoresis on a 1% agarose gel. BLAST database (http://blast.ncbi.nlm.nih.gov) to compare input sequence with related reference sequences in the HDV database from the Gene Bank of the National Center for Biotechnology Information (NCBI). Genotypes were assigned by comparing with reference sequences. The HDV sequences were then referenced to the BLAST database (http://blast.ncbi.nlm.nih.gov) to determine the genotype.

**TABLE 1. Primers used for HDV detection**

| Primer name   | sequence 5’-3’ |
|---------------|---------------|
| HDV-850       | CGG ATG CCC AGG TCG GAC C |
| HDV-1380      | GGA GCW CCC CCG GCG AAG A |
| HDV-887       | GAG ATG CCA TGC CGA CCC GAA GAG |
| HDV-1290      | GAA GGA AGG CCC TCG AGA ACA AGA |

**Table 2**

| Patient | Sex | Age | Cirrhosis | Outcome | Anti HDV | HDV genotype |
|---------|-----|-----|-----------|---------|----------|--------------|
| 1       | Female | 73  | -         | alive   | -        | 2            |
| 2       | Male   | 46  | -         | alive   | -        | 2            |
| 3       | Female | 48  | -         | alive   | -        | 2            |
| 4       | Male   | 26  | -         | alive   | -        | 2            |
| 5       | Female | 53  | -         | alive   | -        | 2            |
| 6       | Female | 43  | -         | alive   | -        | 2            |
| 7       | Male   | 25  | -         | alive   | -        | 2            |
| 8       | Male   | 53  | +         | death   | -        | 2            |
| 9       | Male   | 49  | -         | alive   | -        | 2            |
| 10      | Male   | 26  | -         | alive   | -        | 2            |
| 11      | Female | 58  | +         | alive   | -        | 2            |

**Sequence Alignments And Phylogenetic Analyses**
Positive samples were sequenced using an automatic sequencer. Sequences were compiled using the BioEdit program, MEGA7 (molecular evolutionary genetics analysis, version 7.0). The following eight prototype HDV-sequences retrieved from the NCBI GenBank were used for alignment and HDV genotyping: (HDV-1: AF098261, AJ000558, AY633627, HM046802,NC001653, KF660600, KF660601, KF660602; HDV-2: KF660599, AF104264; HDV-3: AB037947, AB037948, AB037949; HDV-4: AF018077, AF209859; HDV-5: AM183326, AM183331, JA417551; HDV-6: AJ584847, AM183332; HDV-7: AM183333, JA417541; HDV-8: AM183327, AM183330). The phylogenetic tree reconstruction and the mean value of genetic diversity of DNA sequences were carried out using MEGA 7 software.

Statistical Analysis
All the data was analyzed and the summary statistic was carried out by SPSS version 19.0 (a statistical package). T-test was used to determine the risk factors for acquiring HDV infection. Levels of HBV DNA were expressed as log10 copies/mL. A P value < 0.05 were considered significant.

Results
Sample characteristics and HDV prevalence
Overall, we analyzed clinical characteristics of 225 cases screened from October 2010 to April 2013, 159 (70.7%) were females and 66 (29.3%) were males. Results indicated that median age of mono-HBsAg positive patients and HDV-RNA/HBsAg-positive patients were 41.4 ± 13 years and 45.5 ± 14.9 years, respectively (P < 0.05). Among these HDV-RNA positive samples, there were not positive for anti-HDV antibodies (Fig. 1 and Table.2). However, 11 of the 225 HBsAg-positive serum samples demonstrated detectable HDV-RNA given a HDV prevalence of 4.9% in our study, which may contribute to underestimating the burden of HDV infection.

Detection of HDV RNA in HBsAg Positive Patients by agarose gel electrophoresis
In 225 patients with HBsAg positive HBV, HDV RNA target gene amplification was detected, and agar sugar electrophoresis detected 11 cases of HDV RNA positive specimens. Electrophoresis display of agarose gel from partial nested PCR products (Fig. 1).

Distribution Of HDV Genotypes
To determine HDV genotype in Shanghai, we obtained sequence data of HDV RNA-positive samples via amplifying the HDV delta-gene fragment (L-HDAG region) using automatic sequencer. All of the sequences obtained were compiled using BioEdit, CLUSTX. Distribution of HDV genotypes based on
phylogenetic analysis is shown in Fig. 2 and Fig. 3. Phylogenetic analysis was performed using sequences of the HDV amplicons and analysis showed that all analyzed HDV genotypes belong to HDV-2 strain. Other HBV genotypes were not detected in this study population. The HDV strains also clustered in the Asian clade.

HDV And Chronic HBV Infection Clinical Outcomes

The clinical and subclinical characteristics of the patients with or without HDV infection were presented in Table 3. The HBV DNA loads in the HBV/HDV coinfected group were significantly lower in comparison to the HDV negative group (3.85 log10 copies/mL vs. 5.16 log10 copies/mL; P = 0.038). And the aspartate aminotransferase was significantly higher in HDV positive compared to HDV negative HBV-infected patients (P < 0.05).

| characteristics                  | HDV/HBV (n = 11) | HBV (n = 214) | P value |
|----------------------------------|------------------|---------------|---------|
| Age (yr)                         | 45.45 ± 14.94    | 41.39 ± 13.01 | .026    |
| Male:Female                      | 6/5              | 60/154        | .885    |
| ALT (IU/l)                       | 1485.65 ± 935.53 | 768.58 ± 824.03 | .006    |
| AST (IU/l)                       | 977.56 ± 939.71  | 424.29 ± 474.41 | .000    |
| HBV-DNA (log10 copies/mL)        | 3.85 ± 2.11      | 5.16 ± 2.00   | .038    |
| TBIL (µmol/L)                    | 80.71 ± 68.13    | 64.36 ± 87.19 | .942    |
| DBIL (µmol/L)                    | 73.07 ± 71.03    | 43.52 ± 63.15 | .446    |
| AKP (U/L)                        | 151.30 ± 66.58   | 114.01 ± 63.52 | .442    |
| γ-GT (U/L)                       | 196.34 ± 119.76  | 144.93 ± 122.32 | .064    |

IU: international unit; data are given as median with range; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin; DBIL: direct bilirubin; AKP: alkaline phosphatase; γ-GT: γ-glutamyltransferase. P values are presented for comparisons between HBV-HDV positivity vs. HBV monopositivity.

Discussion

HDV, the defective satellite RNA virus, was first discovered 41 years ago by Mario Rizzetto that can only assemble and propagate in patients with hepatitis B virus (HBV) [26]. Most countries of the Asian-Pacific region are known to be endemic for HBV [27–28]. Understanding the prevalence of HDV and its genotypes, which are now identified into eight major genotypes, is very important as part of a molecular clue for distribution of HDV.

The distribution of HDV is still present worldwide, and with a higher incidence in Amazonas, Mongolia, Kiribati, and in Asian countries [29]. In China, a large reservoir of HBV infection, testing for HDV is limited and the burden of HDV is likely underestimated [17]. In a study from Taiwan, high-risk populations like human immunodeficiency virus (HIV) infection and injection drug users (IDUs) had
higher prevalence of HDV infection, contrasting with the HBsAg positive subjects \[^{30}\]. Our research is the first one to describe the molecular epidemiology of HDV in mainland China, which is a very important study as it describes the HBV/HDV co-infection using molecular methods. Our study showed that the epidemiology of HDV infection among the HBsAg positive subjects from Shanghai area remained low in this study (4.9%). HDV-1, HDV-2 and HDV-4 are found in China, surprisingly, our current study showed HDV-2 is the predominant distribution of HDV genotype. However, further longitudinal studies are needed to confirm this distribution of HDV genotype in China.

Next generation sequencing (NGS) methods are expected to be complementary to conventional diagnostic methods, providing a powerful tool to face the challenges of old and emerging viral infections \[^{31}\]. The nest-PCR assay had higher sensitivity comparing to the multiplex quantitative PCR. The primers were specific to L-HDAg (nucleotides 888 to 1122 as referred to) region. PCR was considered as a golden standard contrary to other techniques, due to its high specificity and sensitivity. The specificity and sensitivity of nest-PCR is higher comparing to the normal PCR. Several studies had shown that HBV/HDV co-infection could suppress HBV replication with lower levels of HBV viraemia seen in patients positive with HBsAg \[^{32}\]. And HDV genotypes may also affect the natural history of HDV and influence the outcome of liver disease. For example, HBV/HDV co-infection led to exacerbation and rapid progression of chronic liver disease, which further enhances liver cirrhosis and hepatocellular carcinoma (HCC) \[^{33}\]. In line with mentioned studies, our results indicated that the levels of HBV-DNA were suppressed in patients with HBV/HDV co-infection, suggesting inhibitory effects of HDV on HBV. The potential virological mechanism of inhibition HBV by HDV may be HDV proteins p24 and p27 inhibitor HBV enhancer \[^{34}\]. Importantly, higher ALT/AST levels in HDV/HDV co-infection patients were detected in our study.

A limitation of our study is that we don’t have sufficient patients. We collected only 225 serum samples for HDV RNA detection and all samples were tested for anti-HDV antibodies. However, none of the HDV-RNA positive samples was anti-HDV antibodies positive. It can be hypothesized that the current commercial ELISA kits have insufficient sensitivity. Meanwhile, our present study of the
prevalence of HDV infection is mainly focused on Shanghai area. Our results might not be representative for whole China. However, slight variations can significantly modify the national data. Our study provides new insights into the prevalence and genotype distribution in Shanghai. Moreover, further studies are needed to understand the molecular epidemiology of HDV in different areas in China.

Conclusions

In conclusion, our study showed that the prevalence of HDV infection in patients with elevated transaminases is not low and the predominance of HDV genotype 2 infection in Shanghai. This finding also helps us to better understand the correlation of HDV/HBV co-infection.

Abbreviations

HDV
Hepatitis D virus
HBV
Hepatitis B virus
ALT
Alanine aminotransfase
AST
Aspartate transaminase
PCR
Polymerase chain reaction
HIV
Human immunodeficiency virus
HCC
Hepatocellular carcinoma
NGS
Next generation sequencing
IUD
Injection drug users
HBsAg
Hepatitis B surface antigen
DBIL
Direct bilirubin
AKP
Alkaline phosphatase
γ-GT
γ-glutamyl-transferase

Declarations

**Ethics approval and consent to participate**

This study has been ethically approved by the Human Ethics Committee of Shanghai Sixth People’s Hospital (No.2016-73). The given ethics committee waived the need for written consent since all the samples used were collected during the course of routine medical care based on clinicians’ request.

**Consent to publish**

Not applicable.

**Availability of data and materials**

The datasets generated and/or analyzed during the current study may be made available from the corresponding authors on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' Contributions**

SW, YZ, YY, and XC designed the study. SW and YZ collected and analyzed data. YT, TY, ML, ZT, and GZ interpreted the results. SW primarily wrote the manuscript. YY, XC, ZT, and GZ provided valuable insight for revising the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Representative agarose gel electrophoreses of amplified HDV-products. Representative agarose gel electrophoresis of amplified HDV nested-PCR products using primers indicated in Table.1. The final PCR product length is 403bp. P, positive control was amplified from a confirmed HDV patient. N, negative control. M, marker.
Figure 2

Nucleotide alignment of genomic sequences of HDV isolates
Figure 3

Phylogenetic analysis based on HDV delta-gene sequences. The horizontal branch was drawn in accord with the relative genetic distance. A number of commonly used reference delta-gene sequences for classifying HDV genotypes were also included and are indicated by accession numbers.