

CASE REPORT

An Extremely Aberrant Subtype of Hepatitis B Virus Genotype D in Iran

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Background and Aims: Hepatitis B virus (HBV) infection is a global health problem, with more than 350 million people chronically infected worldwide. Previous studies revealed that *ayw2* is the predominant subtype in Iran. There are also some reports on other HBV subtypes including *ayw1*, *ayw3* and *ayw4* which are widespread subtypes of genotype D. In this study, we reported an exceptional and extremely rare subtype of HBV genotype D in an Iranian patient chronically infected with HBV who was also co-infected with hepatitis delta virus (HDV).

Methods: The HBV and HDV genotypes and sub-genotypes were determined by polymerase chain reaction (PCR) followed by direct sequencing and phylogenetic analysis. The HBV subtype was determined from the amino acid sequence of the region of viral genome encoding the hepatitis B surface antigen (HBsAg).

Results: Phylogenetic analysis based on the HBV *S* gene revealed that the patient was infected with HBV genotype D and sub-genotype D1. Further amino acid mapping on the amino acid of the HBV *S* gene sequence showed that the patient who was chronically infected with HBV was infected by an unusual subtype "ayr" of the virus which is not typical for the genotype D of HBV. HDV phylogenetic analysis also revealed that the patient was co-infected with HDV clade1.

Conclusions: The results indicated the presence of the uncommon subtype "ayr" of HBV genotype D in Iran. This may show that the virus is going further evolution by changing its genome, though the importance of this atypical subtype in genotype D of HBV is still not clear and needs longitudinal studies.

Keywords: Hepatitis B virus, Genotype D, Subtype ayr, Hepatitis D virus, Iran

Hepatitis B is still a significant health concern in the world given the fact that around two billion people have been infected by hepatitis B virus (HBV) and that around 350 millions of them are chronic carriers. HBV infection can lead to chronic carrier state and progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) ⁽¹⁾. HBV belongs to Hepadnaviridae family and has a circular and partially double-stranded DNA genome of almost 3.2 kb containing four overlapping open reading frames that encode the seven viral proteins.

The first classification of HBV isolates was done by serotyping based on reactivity of HBV surface antigen (HBsAg) of the isolate with the standard panel of anti-sera ⁽²⁾. HBsAg epitopes involved in expression of subtype specificities are located in a region that includes the two external loops of the molecule (amino acids 110-180) and that makes the

HBV strains antigenically diverse. The major immunogenic region, the "a determinant" spanning residues 124-147 of HBsAg that consisted of several conformational epitopes, is common to the almost all HBV isolates. The two major subtype epitopes are the *d/y* and *r/w* determinants. Both of these

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determinants are consisted of two epitopes that depend upon the nature of the amino acids at positions 122 and 160 of HBsAg, respectively. If the amino acid at position 122 is Arg (122R), then the subtype is *γ* and if it is Lys (122K), then the subtype is *δ*. Similarly, 160R defines the *r* subtype and 160K defines the *w* subtype ⁽³⁾. The four possible combinations define the major subtypes; additional amino acids contribute to immunogenicity, giving rise to 10 distinct subtypes ⁽⁴⁾.

Furthermore, HBV can be classified into genotypes based on the HBV genome nucleotide sequence divergence exceeding 8%. So far, eight genotypes (A to H) of HBV with distinct geographical distribution have been reported ⁽⁵⁾. Genotypes and serotypes are useful tools in understanding the epidemiology of HBV infection. There is a certain correlation between serotype and genotype worldwide ⁽⁴⁾. For example, strains encoding an HBsAg of serotype *ayr* can be only found in genotype C but *ayw1-4*, *adw1* and *adw3* can be found in genotype D (Table 1) ⁽⁶⁾. Previous studies revealed predominance of HBV genotype D ⁽⁷⁾ and subtype *ayw2* in Iran ^(8, 9).

Table 1. Expected and usual association existed between HBV genotypes and subtypes as reported worldwide.

Genotype	A	B	C	D	E	F	G	H
Subtype	adw2 ayw1	adw2 ayw1 adw3	adrq+ adrq- ayr adw2 ayw1 ayw3	ayw1 ayw2 ayw3 ayw4 adw1 adw3 ayr (the case)*	ayw4 adw3	adw4 ayw4	adw2	adw4

*Our patient. This case interestingly showed an aberrant subtype *ayr* among HBV genotype D which is extremely atypical in this genotype. The *ayr* subtype is frequently reported in HBV genotype C in the world. According to our knowledge it is the first report from Iran and second report in the world.

Moreover, we found that HBV subtype *ayw2* is the predominant subtype (94.4%) in Iran followed by subtypes *ayw1* (2.8%), *ayw3* (2%) and *ayw4* (0.4%) ⁽¹⁰⁾. Herein, we reported on a very aberrant subtype of HBV genotype D in an Iranian chronically-infected patient who was also co-infected with hepatitis delta virus (HDV).

During our study on the molecular epidemiology of HBV in Iran from 2004 to 2008, we found an interesting case regarding its virological feature. The

case was a 64-year-old Iranian man diagnosed with HBV and HDV co-infection. His known high risk behaviors included shared shaving razors, blood splashing and high risk sexual contacts. He had no intravenous drug abuse, alcohol intake nor transfusion. He did not receive any antiviral drug medications before; he had no trip abroad. The patient had liver cirrhosis (CHILD C). His abdomen has distended for last three months and was found to have tense ascites. Serologic tests for HBsAg, hepatitis B e antigen (HBeAg), antibody to hepatitis B e antigen (anti-HBe), antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis C virus (anti-HCV) and antibody to hepatitis delta virus (anti-HDV) antibodies were carried out by commercially available enzyme-linked immunosorbent assay (ELISA) procedures (DIA PRO Diagnostic Bioprobes, Srl., Italy). Laboratory findings are shown in Table 2.

HBV DNA was extracted from serum using QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany). HBV subtypes were determined using nested-PCR, followed by sequencing of the HBV S ORF as previously described ⁽¹¹⁾. HDV RNA was extracted from 140 µL of serum with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was conducted using primers selected from the well conserved regions of HDV which were capable of amplifying all genotypes of HDV ⁽¹²⁾. Product was analyzed bi-directionally using internal primers. The nucleotide sequences of HBV and HDV isolates obtained in this study were compared to the previously reported HBV and HDV reference gene sequences retrieved from GenBank. Subsequently, phylogenetic analysis was carried out by MEGA software version 3.1.

Phylogenetic analysis of the HBV DNA from the patient revealed that the patient was infected with genotype D, sub-genotype D1 (data not shown). The result showed that he was also infected with HDV clade 1 (data not shown). The nucleotide sequence was translated into amino acid sequence according to the ORF of the HBV S gene. The

Table 2. Laboratory findings in our patient.

Laboratory tests	ALT (IU/L)	AST (IU/L)	Total protein (g/dL)	Serum Albumin (g/dL)	PT (sec)	PTT (sec)	HBsAg	HBeAg	Anti-HBe	Anti-HBc	Anti-HCV	Anti-HDV
Results	31	77	6.9	3.8	18	49	+ve	-ve	+ve	+ve	-ve	+ve

ALT: alanine aminotransferase; AST: aspartate aminotransferase; PT: prothrombin time; PTT: partial thromboplastin time

HBsAg subtype was carefully deduced from the amino acids at specified positions. Surprisingly, HBV isolate of this patient belonged to *ayr* subtype of HBV genotype D based on the presence of Arg at positions 122 and 160. The results were reconfirmed by nested-PCR and sequence re-analysis.

The *ayr* subtype is widespread in genotype C of HBV and it is uncommon in other genotypes (Table 1). According to our search in the related databases, only one study reported this strange subtype in genotype D in a Spanish patient⁽¹³⁾. Therefore, the patient is the second report of this bizarre *ayr* subtype belonging to HBV genotype D isolate. From the virological point of view, this report is very interesting, because it may show that the virus are going to possess different genetically features in future by evolution which may affect the diagnostic assays and probably the outcome of disease, though its clinical consequences are still not clear. The importance of subtypes in disease progression and drug resistance is still ambiguous, nonetheless, Zolner and coworkers reported that HBV subtype *ayw* respond better to lamivudine monotherapy than HBV subtype *adw*^(14, 15). In another study, the seroconversion rate was different and higher in *adw* than *adr* subtype of HBV⁽¹⁶⁾.

Taken all together, we report an extremely rare subtype *ayr* in genotype D of HBV from Iran, while this subtype totally belongs to genotype C of HBV. Following this patient may shed light over some important issues of presence of this atypical subtype in comparison with the common *ayw2* subtype in Iran.

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