

## MOLECULAR VARIABILITY OF THE S GENE OF *HEPATITIS B VIRUS* IN EGYPT

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### ABSTRACT

*Two* novel mutants of the *hepatitis B virus* surface antigen (HBsAg) were characterized. The mutants were isolated from asymptomatic patients who were found to be persistently positive for both HBsAg and anti-hepatitis B surface antibody (anti-HBs) and a long-lasting anti-HBc (core) IgM. Polymerase chain reaction (PCR) and sequencing were performed to surface antigen region and revealed two genotype D mutants HBV. Aligned with known HBsAg sequences from GenBank, the mutant variants matched to consensus with eight distinct genotypes (A to H) of hepatitis B virus. Multiple sequence alignment provided a more sensible way of detecting sequence homology and identifying the HBV isolates. From this alignment, certain positions were highly conserved with the previously identified and recorded Egyptian isolates, while other positions were not. The importance of the two variants lies in the observation that mutations in the surface antigen coding region can change the immunodominant region structure and therefore alter the group specific determinant antigenicity. Phylogenetic tree based on HBsAg partial nucleotide sequence was displayed. In conclusion, by understanding the HBsAg major immunodominant region nucleotide sequence, highly sensitive diagnostic assays can be achieved for detection of HBV in clinical specimens. Control of these HBV mutants, which will require new drugs, vaccines, and treatment strategies, will become the next major challenge on the path to eventual elimination of HBV infection.

**Keywords:** HBsAg, PCR, Sequencing, Phylogenetic.

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## INTRODUCTION

*Hepatitis B virus* (HBV) infection is a global health problem with more than 2 billion infected individuals. HBV infection leads to diverse outcomes ranging from acute to fatal fulminant hepatitis, and chronic hepatitis (CHB), which may result in severe complications as liver cirrhosis (LC) and hepatocellular carcinoma (HCC). HBV is one of the important human DNA viruses having strong oncogenic potential (De Oliveira, 2007). *Hepatitis B virus* is the prototype member of the family *Hepadnaviridae*. It has a compact, circular DNA genome of about 3.2 kb in length with four overlapping open reading frames (ORFs) encoding the polymerase (P) core (C), surface antigen (S), and X protein (Yang *et al.*, 1995).

Genetic and phylogenetic analysis of HBV DNA sequences has led to the definition of eight genotypes designated A through H, based on more than an 8% diversity in the whole genome, and further into their subgenotypes (more than 4%, but less than 8% intragenotype diversity), having distinct ethnogeographical distribution. Some studies have clearly demonstrated that

genotype variability of different HBV genetic regions can influence the clinical manifestation, and even response to therapy (Schaefer, 2005). In the past few years, there is growing evidence suggesting that HBV genotypes influence clinical outcomes, HBeAg seroconversion rates, mutational patterns in the precore and core promoter regions, and response to antiviral therapy. (Alexander *et al.*, 2008)

Few reports described the frequency of HBV genotypes in Egypt. In one study, the genotypes of HBV isolated from 105 serum samples from Egyptian carriers were determined by sequencing and found that HBV genotype D are the most prevalent in Egypt (Saady *et al.*, 2003). Based on partial-length of genome (HBsAg) analysis, we have revealed that two new subtype according to the comparison with registered subtype in GenBank (Manuilav *et al.*, 2010).

The main aim of this study was to investigate the genetic heterogeneity of HBsAg sequences in our population. We analyzed and compared the HBsAg sequence from our study population with related GenBank sequences. In this manuscript we discuss the genetic variability of HBsAg.

## MATERIALS & METHODS

**Samples collection,** one hundred sera samples were collected from Egyptian patients suffering from chronic HBV infection. All patients, Blood Donors, were diagnosed HBV infection with no co-infected with other hepatitis viruses such as HCV, HDV, or HIV were excluded. All sera Samples were divided to 200  $\mu$ l and stored in -20 OC until were used.

**Amplification of HBV DNA and nucleotide sequencing,** DNA was extracted from 100 serum samples; Total DNA was extracted from 200  $\mu$ l serum using a QIAamp DNA Blood Mini Kit (*Qiagen, Cat. No. 5114*) (*Goodarzi et al., 2007*). The S gene region was amplified in 20 out of 100 serum samples by

polymerase chain reaction (PCR) using two primers designed in Metabion Company, Germany (**Table 1**), PCR amplification was done according to *Stuyver et al. (2000)*. using Taq PCR Master Mix Kit. (*Qiagen, Cat. No. 201443*) PCR reactions were done in 100  $\mu$ l mixture reaction, according to the instruction of the manufacturer. PCR was performed with the following parameters: preheat-ing at 95°C for 1 min, 35 cycles of 95°C for 1 min, 48.3°C for 1 min, and 72°C for 1 min; finally at 72°C for 10 min as a final extension step. The PCR products were separated using 1% agarose gel electrophoresis with ethidium bromide stain (0.5  $\mu$ g/ml) and purified using QIA-quick Gel Extraction Kit. (*Qiagen, Cat. No. 28704*).

**Table 1.** Oligonucleotide primers for amplification and their positions

Primer Sequence 5'-3'	Polarity	Length	Domain	Position
GAACAAGAGCTACAGCATGGG	S	21	HBsAg	2850-2868
CTTTTGTCTTTGGGTATACAT	As	21	HBsAg	810-831



Purified PCR products were sequenced directly using a Prism Big Dye v3.1 kit (Applied Biosystems, Cat. No. 4336917) on an ABI 310 DNA automated sequencer (Applied Biosystems) (Nguyen *et al.*, 2009). All samples were analyzed in both forward and reverse directions. For sequence comparisons of S gene sequences of the eight reference HBV genotypes (A, B, C, D, E, F, G and H), sequence alignment was performed using the multiple-alignment algorithm in Meg align (DNASTAR, Window version 3.12e).

**Phylogenetic analysis:** Two phylogenetic trees created the first one based on the S gene nucleotide sequences, phylogenetic analysis was performed on our eight HBV reference genotypes HBV (A to H) retrieved from Genbank and the second compared with 87 reference HBV/D strains retrieved from Genbank. Phylogenetic trees were inferred using the neighbor joining method, which was carried out using MEGA version 2.1 (Kumar *et al.*, 2001). The resultant neighbor-joining tree and its topology were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings.

## RESULTS

### **Amplification of S gene and alignment**

HBV S gene region sequences were successfully amplified from 20 out of 100 serum samples. The sequence data was aligned using dnastar software with eight reference genotypes (A, B, C, D, E, F, G and H) sequences of HBV S gene retrieved from GenBank/ DDBJ (Figure 1).

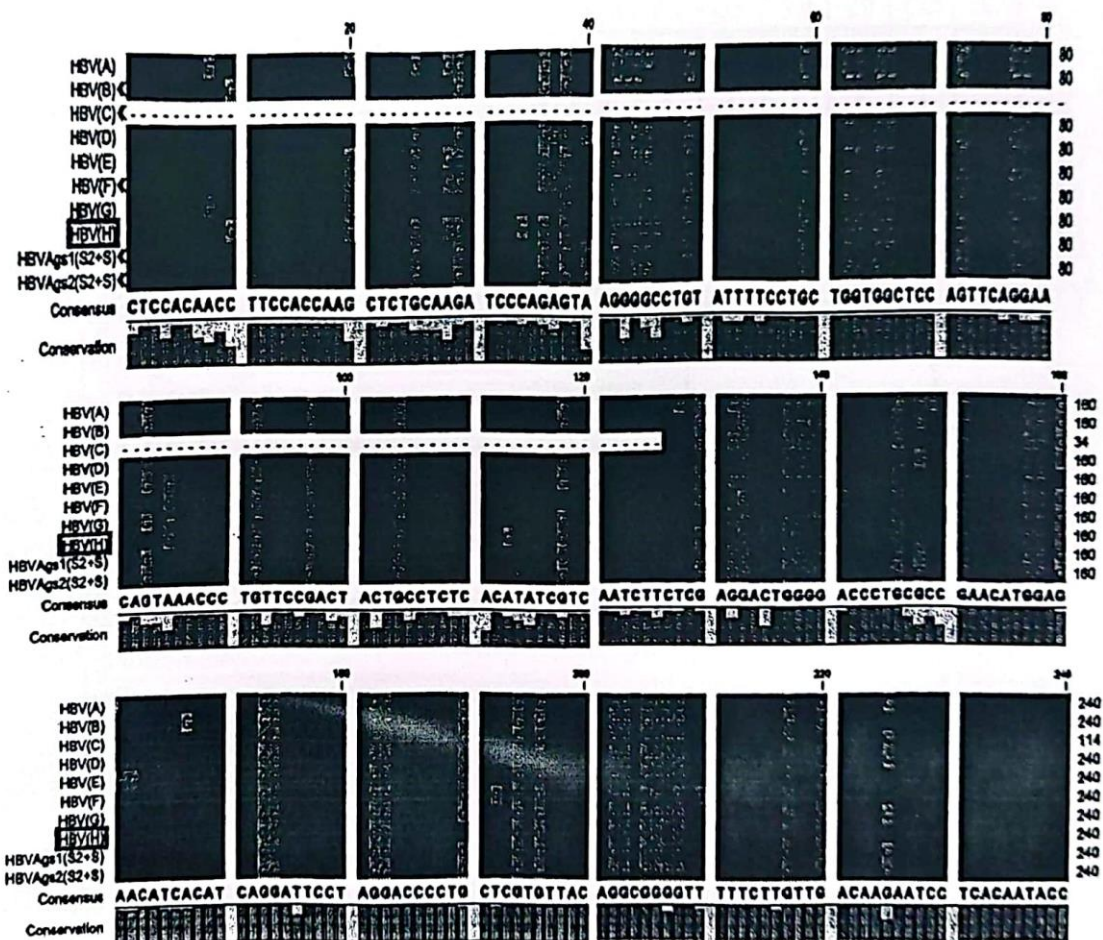
### **Divergences percentage and phylogenetic tree creation**

We compared S gene sequence determined in our present study with eight reported S gene sequence and confirmed their genotype to be D genotype by phylogenetic analysis. Divergences in the entire genome sequences of the present two isolates with HBV/D subgenotype isolates registered with GenBank are shown in Table (2). The two isolates in subgenotype D showed 0.8% divergence from each other. Among the sequences registered with GenBank (A, B, C, D, E, F, G and H) showed divergences with the two isolates up to 14% and identity up to 97.9% were shown in Table(2).



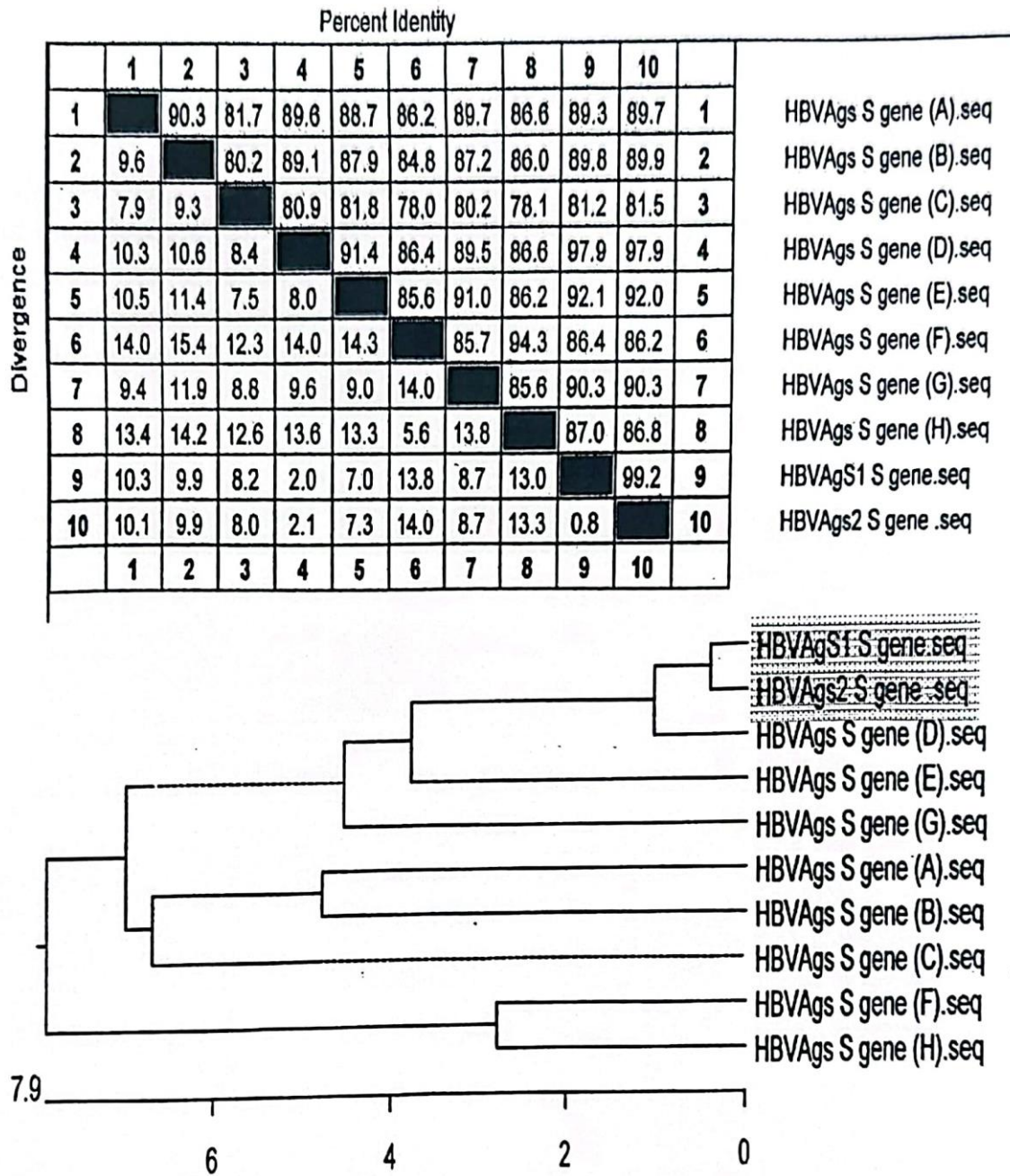
We constructed a phylogenetic tree by using the entire nucleotide sequences determined from the two isolates and representative genotypes (A, B, C, D, E, F, G and H) from DDBJ/GenBank. The HBV isolates are illustrated in **Figure (2)**. This tree was established that the new isolates are relatively to subtype HBV/D.

Additional phylogenetic tree was constructed against 87 reference genotyp HBV /D which were retrieved from DDBJ/GenBank. The HBV isolates are illustrated in The new tree confirmed the last one and indicates that the new isolates were grouped to subgenotype D **Figure (3)**.



**Figure 1.** Alignment of partial S gene of the new isolates and eight reference genotypes (A, B, C, D, E, F, G and H).

**Table 2.** Mean percent nucleotide identity and divergence between the S gene sequence of the new isolates and eight human HBV strains belonging to genotypes A to H.



**Figure 2.** Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present two isolates and eight HBV subtypes (A to H) retrieved from DDBJ/GenBank . Bootstrap values are indicated



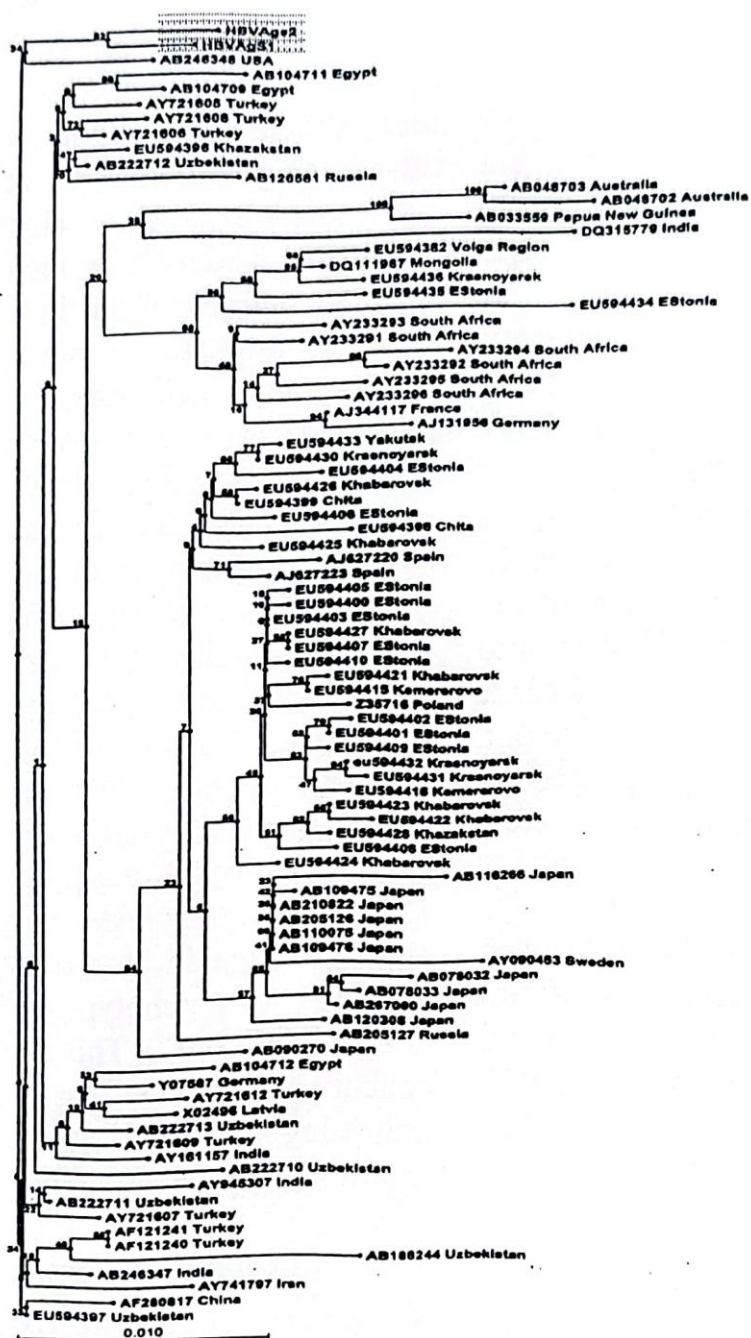


Figure 3. Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present two isolates and 87 HBV/D isolates retrieved from DDBJ/GenBank.

## DISCUSSION

To our knowledge, this is the first investigation to characterize HBV in Egypt using S gene sequencing data. HBV/D was the most prevalent HBV genotype. Two new subgenotypes are characterized by clear phylogenetic separation (**Figure 2**), Genetic and phylogenetic analysis of HBV DNA sequences has lead to the definition of eight genotypes designated A through H, based on more than an 8% diversity in the whole genome, and further into their subgenotypes having distinct ethno-geographical distribution or a 4 % divergence or greater in surface (S) antigen (**Schaefer, 2005**) (**Table 2**).

Our study have clearly demonstrated the divergence (**Table 2**) between reference Genotype HBV/D and HBV AgS1(2%) and HBV AgS2 (2.1%) this divergence ratio has been established that the new isolates are tight to subtype HBV/D. HBV Ag S1and 2 clustered to HBV/D when phylogenetic tree (**Figure 2**) constructed with eight reference HBV genotype A-H retrieved from Gene Bank. This finding was demonstrated by **Saudy et al. (2003)** who studied the

genotypes of HBV isolated from 100 serum samples of Egyptian carriers by sequencing and he found that HBV genotype D was the most prevalent type in Egypt. This result has been confirmed later on by **Abdel-Rahman et al. (2007)**, who reported that HBV/D have the highest infection ratio in Egypt Followed by mixed infection with HBV/A+D. On the other hand **Naito et al. (2001)** examined 2 serum samples positive for HBV DNA by primer specific to be of genotype D but they didn't find other genotypes as they only examined 2 serum samples. A third study was done on 70 pediatric cancer patients suffering from hepatitis and they were diagnosed as HBV infection, where genotype D was reported as the predominant HBV genotype in form of two new subtype of genotype HBV/D (**Zekri et al., 2007**). This study also concurs with previous studies, indicating that HBV genotype D prevails in the Mediterranean area, near and Middle East (**Ogawa et al., 2002; Ding et al., 2002; Yalcin et al., 2004**).

We recommended that HBV genotyping become a routine work in clinical medicine and molecular epidemiology. As genotypes have



different biological and epidemiological behavior, medically significant. Although a small number of samples were employed in our investigation, a novel two HBV/D subgenotype were reported according to the entire nucleotide sequences compared with reference HBV/D from the DDBJ/GenBank, where there have been scant data on HBV. It shows that they belong to subgenotype D. Further studies, including other genotypes and their clinical relevance, should be conducted. In conclusion, by understanding the HBsAg major immunodominant region nucleotide sequence, highly sensitive diagnostic assays can be achieved for detection of HBV in clinical specimens to be conduct in new drugs, vaccines, and treatment strategies.

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**This sequence will appear in Genbank with accession No. JF304298 & JF304299.**