MOLECULAR GENETICS MUTATIONS OF THE S GENE OF HEPATITIS B HBV VIRUS IN EGYPTIAN PATIENTS

M. EL-KALAMAWY[1], S. EL-HADDAD[1], S. H. ABDEL-AZIZ[2] AND HANAN A. FAHMY[3]

1. Genetic Engineering Research Dept. Egyptian Company for Blood Transfusion Services Vaccine Seraum (VACSERA)

2. Botany Department, Faculty of Science, Benha University

3. Biotech. Dept., Animal Health Research Institute

Hepatitis B virus (HBV) infection is a global health problem with more than 2 billion infected individuals. HBV infection from Egyptian Company for Blood Transfusion Services 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 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 (more than 4%, but less than 8% intragenotype diversity), having distinct ethno geographical 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 last 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 (Saudy *et al.,* 2003). Based on partial-length of genome (HBsAg) analysis, we have revealed that two new subtypes 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 AND METHODS

Samples collection, one hundred sera samples were collected from Egyptian patients suffering from chronic HBV infection from Egyptian Company for Blood Transfusion Services. All patients, blood donors, were diagnosed HBV infection with no co-infected with other hepatitis viruses such as HCV, HDV, or HIV was excluded. All sera samples were divided to 200 µl and stored in -20°C until were used.

Amplification of HBV DNA and nucleotide sequencing, DNA was extracted from 100 serum samples, total DNA was extracted from 200 µl 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 µl mixture reaction according to the instruction of the manufacturer. PCR was performed with the following parameters: preheating 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 µg/ml) and purified using QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28704).

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 1000 resamplings.

RESULTS AND DISCUSSION

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 alignmented 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 (Fig.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 Fig. (2). This tree was established that the new isolates are relatively to subtype HBV/D.

Additional phylogenetic tree was constructed against 87 reference genotypes 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. (Fig. 3)

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 (Fig. 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 has 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 (Fig. 2) constructed with eight reference HBV genotypes 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 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 has 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 recommend that HBV genotyping becomes 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 nucleotides sequence, highly sensitive diagnostic assays can be achieved for detection of HBV in clinical specimens to be conducted in new drugs, vaccines, and treatment strategies.

SUMMARY

Two novel mutants of the hepatitis B virus surface antigen (HBsAg) were characterized. The mutants that were isolated from asymptomatic patients 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 coding 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 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.

REFERENCES

Abdel-Rahman, N. Z., M. H. Mohamed, Nahed I. Mohamed, Zeinab K. Hassan, Manal, H. El-Sayed, M. K. Mohsen and M. Tarek (2007). Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virology Journal 4: 74 doi: 10.1186/1743-422X-4-74.

Alexander, T., Y. Shiou-Hwie, L. Chun-Jen, C. Claudia and C. Pei-Jer (2008). Viral hepato-carcinogenesis: from infection to cancer. Liver International, 28: 175-188.

De Oliveira, D. E. (2007). DNA viruses in human cancer: an integrated overview on fundamental mechanisms of viral carcinogenesis. Cancer Lett., 247: 182-196.

Ding, X., M. Mizokami, X. Ge, E. Orito, S. Iino, R. Ueda and M. Nakanishi (2002). Different hepatitis B virus genotype distributions among asymptomatic carriers and patients with liver diseases in Nanning, Southern China. Hepatol. Res., 22: 37-44.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791.

Goodarzi, Z., R. Malekzadeh, G. Montazeri, S. M. Alavian, M. Qurbanalizadgan, M. Daram and S. M. Jazayeri (2007). Phylogenetic analysis of HBV based on PreS region in Iranian hepatocellular carcinoma patients. Hepatitis, 7: 201-205.

Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei (2001). Molecular evolutionary genetics analysis software. Tempe, Arizona: Arizona State University.

Manuilov, V. A., L. P. Osipova, I. G. Netesova, E. V. Chub, L. V. Tsoy, R. V. Dul'beev, L. R. Alekseeva, H. Norder, L. O. Magnius and S. V. Netesov (2010). Incidence of genotype of hepatitis B subvirus and HBsAg subtypes in native people of Northern and South Eastern Siberia. Molecular Genetics, Microbiology and Virology, 25: 172-177.

Naito, H., S. Havashi and K. Abe (2001). Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J. Clin. Microbiol., 39: 1686.

Nguyen, T., J. V. Alexander, T. S. Bowden1, C. Croagh, S. Bell, P. V.

Desmond, M. Levy and Locarnini S. A. (2009). Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: A perspective on Asia. J. of Hepatology, 52: 508-513.

Ogawa, M., K. Hasegawa, T. Naritomi, N. Torii and N. Hayashi (2002). Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol. Res., 23: 167-177.

Saudy, N., F. Sugauchi, Y. Tanaka, S. Suzuki, A. Aal, M. A. Zaid, S. Agha and M. Mizokami (2003). Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. J. Med. Viol., 70: 529-536.

Schaefer, S. (2005). Hepatitis B virus significance of genotypes. J. Viral. Hepat., 12: 111-124.

Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi and R. Rossau (2000). A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. Journal of General Virology, 81: 67-74.

Yalcin, K., H. Degertekin, I. H. Bahcecioglu, A. Demir, M. Aladag, B. Yildirim, S. Horasanli, S. Ciftci and S. Badur (2004). Hepatitis B virus genotype D prevails in patients with persistently elevated or normal ALT levels in Turkey. Infection, 32: 24-29.

Yang, Z., I. J. Lauder and H. J. Lin (1995). Molecular evolution of the hepatitis B virus genome. Journal of Molecular Evolution, 41: 587-596.

Zekri, A. R. N., M. M. Hafez, N. I. Mohamed, Z. K. Hassan, M. H. El-Sayed, M. M. Khaled and T. Mansour (2007). Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virology Journal, 4: 74.

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

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.

Percent Identity

|	1	2	3	4	5	6	7	8	9	10		
1		90.3	81.7	89.6	88.7	86.2	89.7	86.6	89.3	89.7	1	HBVAgs S gene (A).seq
2	9.6		80.2	89.1	87.9	84.8	87.2	86.0	89.8	89.9	2	HBVAgs S gene (B).seq
3	7.9	9.3		80.9	81.8	78.0	80.2	78.1	81.2	81.5	3	HBVAgs S gene (C).seq
4	10.3	10.6	8.4		91.4	86.4	89.5	86.6	97.9	97.9	4	HBVAgs S gene (D).seq
5	10.5	11.4	7.5	8.0		85.6	91.0	86.2	92.1	92.0	5	HBVAgs S gene (E).seq
6	14.0	15.4	12.3	14.0	14.3		85.7	94.3	86.4	86.2	6	HBVAgs S gene (F).seq
7	9.4	11.9	8.8	9.6	9.0	14.0		85.6	90.3	90.3	7	HBVAgs S gene (G).seq
8	13.4	14.2	12.6	13.6	13.3	5.6	13.8		87.0	86.8	8	HBVAgs S gene (H).seq
9	10.3	9.9	8.2	2.0	7.0	13.8	8.7	13.0		99.2	9	HBVAgS1 S gene.seq
10	10.1	9.9	8.0	2.1	7.3	14.0	8.7	13.3	0.8		10	HBVAgs2 S gene .seq
	1	2	3	4	5	6	7	8	9	10		

Divergence
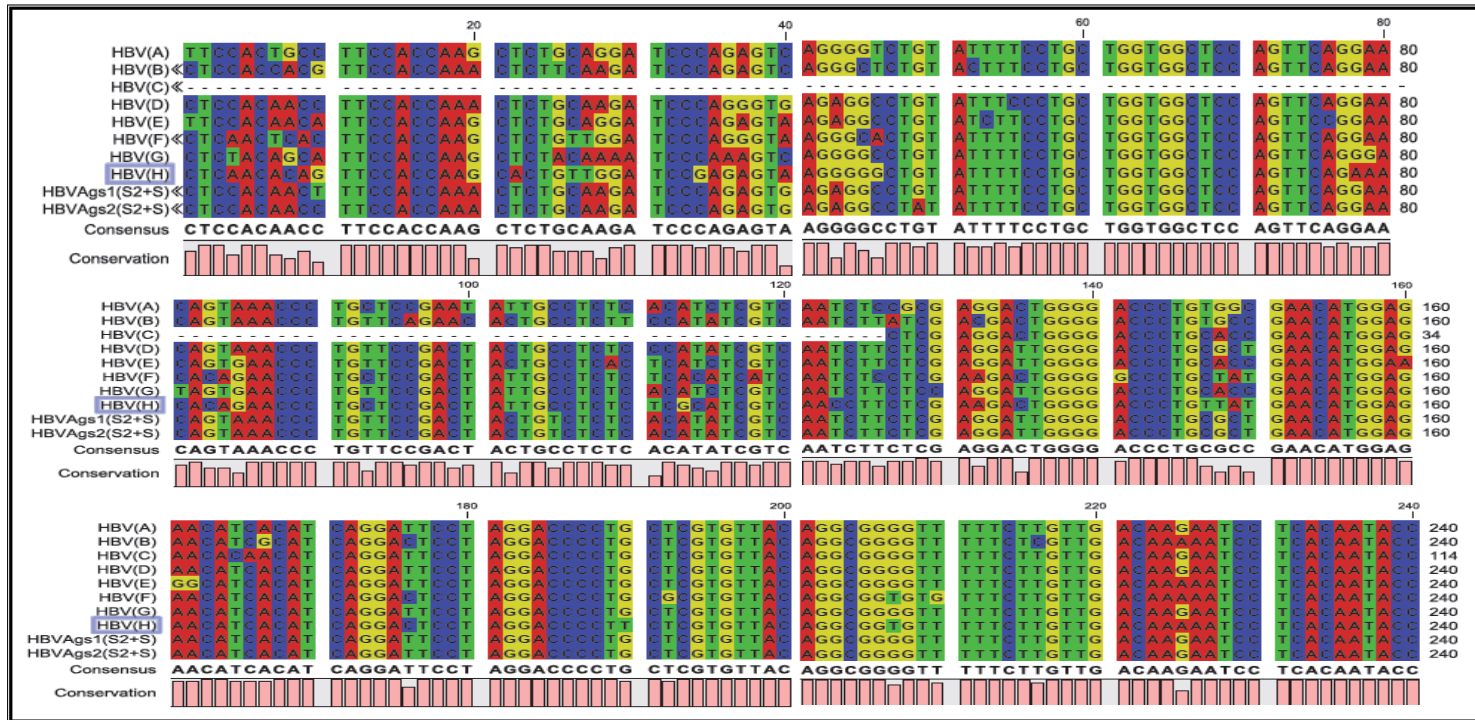
Fig. (1): Alignment of partial S gene of the new isolates and eight reference genotypes (A, B, C, D, E, F, G and H).
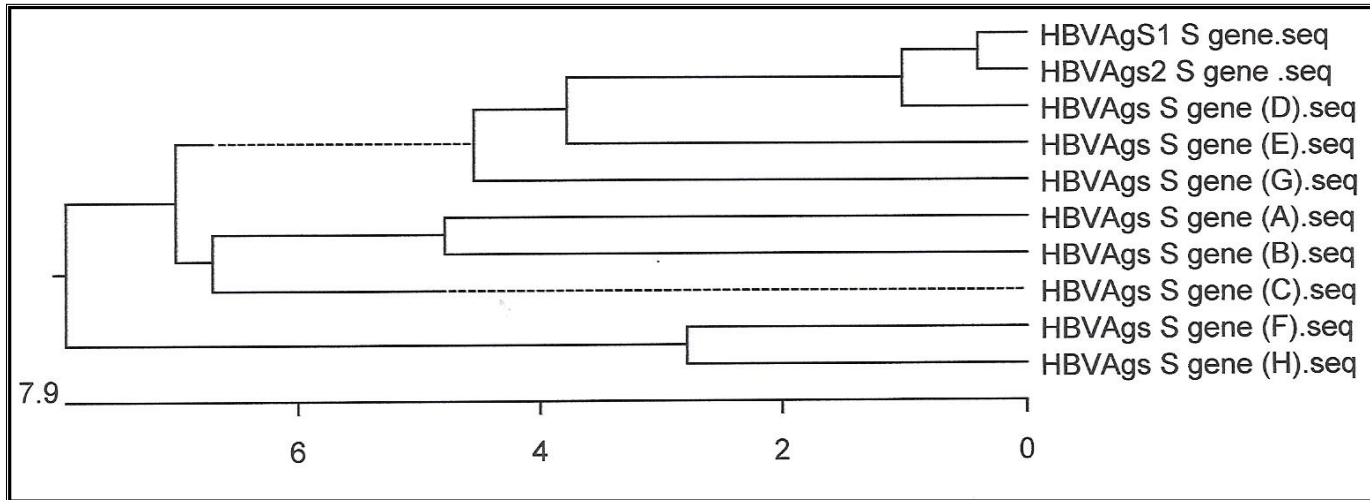
Fig. (2): Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present two isolates and eight HBV subtypes (A to H) retrieved from DDBJ/GenBank. Bootstrap values are indicated.
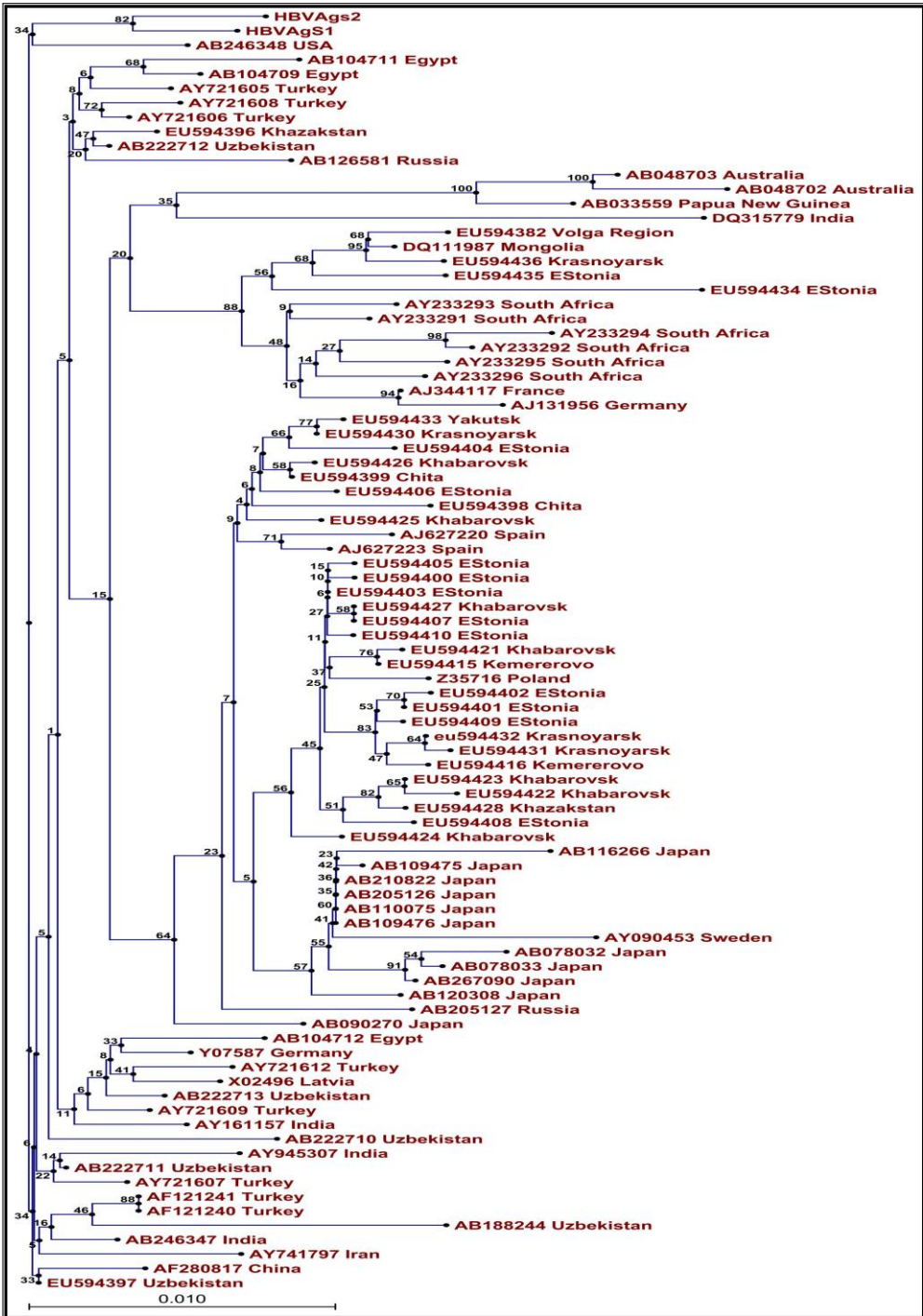
Fig. (3): Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present two isolates and 87 HBV/D isolates retrieved from DDBJ/GenBank.