Hepatitis: Global Health Concern

Chapter 1

HBV Genome, HBsAg Isolation and Virotherapy Innovation Using Plant System in the Middle East

Essam M Janahi[1]; Aboul-Ata E Aboul-Ata[2]*; I M El-Kalamawy[3]; Amal Mahmoud[4]; M S El-Ghanam[5]; Kathleen Hefferon[6]

[1]Department of Biology, College of Science, University of Bahrain, PO. Box 32038, Sakhir, Kingdom of Bahrain.

[2]Molecular Biology Lab. II, Plant Virus and Phytoplasma Res. Sec, Plant Path. Res. Inst., ARC, POB 12619, Giza, Egypt.

[3]Department of Genetic Engineering Res, Egyptian Company for Blood Transfusion, VACSERA, Dokki, Giza, Egypt.

[4]Genetic Engineering Inst., Sadat University, Sadat City, Munofia, Egypt

[5]Cairo University, Giza, Egypt.

[6]Department of Cell and Systems Biology, University of Toronto, Canada.

Correspondence to: **Aboul-Ata E Aboul-Ata, Plant Virus and Phytoplasma Res. Sec., Plant Path. Res. Inst., ARC, POB 12619, Giza, Egypt.*

Email: aboulataaboulata@yahoo.com

1. Introduction

HBV is the second member of 5-hepatitis virus (A,B,C,D,E). It belongs to family hepadnaviridae. It has almost dsDNA genome [33]. WHO Have surveyed HBV, D type in different countries in the Middle East. They found that Egypt has 3-11%, Iraq 4-5%, Jourdan has 2.6-10%, Libya has 2-6%, Oman has 2.3-10%, KSA has 7.4-17%, Sudan has 16-20%, Tunisia has 6.5%, UAE has 2-5%, and Yemen has 12.7-18.5%. They used ELISA for virus detection.

The complete infectious hepatitis B virus particle is spherical, with an overall diameter of 42-47 nm. It consists of a 25-27 nm diameter core or nucleocapsid which is mainly comprised

of HBcAg surrounded by a 7 nm lipoprotein bilayer derived from the endoplasmic reticulum (ER) membrane of the host [14, 27, 36, 38]. have described three different glycosylated HBV surface proteins of varying sizes, LHBs (large hepatitis B surface protein), MHBs (middle hepatitis B surface protein), and SHBs (small (major) hepatitis B surface protein), are inserted in lipoprotein bilayer. They added that the viral DNA, a virus-encoded RNA/DNA dependent DNA polymerase, a genome bound protein covalently linked to 5'-end of the negative (-) strand of HBV DNA, and a capped RNA primer covalently linked to the 5'-end of the positive (+) strand of HBV DNA are contained in the nucleocapsid. Purified cores also containing a protein kinase activity detected by its ability to phosphorylate HBc protein *in vitro*; because recombinant C and P proteins do not possess kinase activity, this enzyme is thought to be of host origin. contain viral DNA and are therefore non-infectious. The 20 nm spherical and filamentous structures are found in sera of infected patients, sometimes reaching titres of 10^{12}/ml, and far outnumbering the virion particles (10,000-1000,000:1). The presence of such high concentration of these non-infectious particles acts as an immunological decoy that will protect the infectious viral particles from antibodies neutralization in the blood stream [14,36]. Subviral 20 nm particles are made up largely of SHBs protein, with variable quantities of MHBs polypeptides and few or no LHBs chains. By contrast, Dane particles are substantially enriched for LHBs chains. Because LHBs chains are thought to carry the receptor recognition domain, this enrichment may prevent the more numerous 20 nm particles from competing effectively with virions for cell surface receptors [14].

Electron cryomicroscopy with 3-dimensional computer reconstitution has revealed that the majority of core particles have icosahedral T4 symmetry with 240 protein subunits, and a minor population showed T3 symmetry with 180 subunits [14,27,10]. The nucleocapsid contains holes or channels of up to 10 nm diameter, formerly described in other viruses, which allow the entry of small molecules [10]. The core particle has been shown to have spikes on their surface, which are made up of dimers of core polypeptide in the form of radial bundles of four α-helices [3]. The N-termini of the two core proteins in the dimer are located at the tip of the spike. A more recent study has confirmed these results and showed that the capsid spikes do not penetrate the inner leaflet of the lipid bilayer and that LHBs seems to play an important role in maintaining stable, noncovalent interactions between the capsid and envelope [11].

HBV genotype D is prevalent in the Middle East area. The HBV genome is a partially relaxed-circular dsDNA molecule consisting of a full length strand (minus strand) with a single unique nick and a complementary (positive strand) of variable length. HBV is considered as a para-retrovirus because its replication involves the reverse transcription of an intermediate-RNA function, of pre-genomic RNA (pgRNA). Polymerase and HBVsAg regions have been also isolated from Egyptian patient samples. HBVsAg (S) gene has been identified at the band size 25.42 kDa. Virotherapy, for plant-based vaccine structure, has been suggested for future

work. Cucumber mosaic virus (CMV-HBVsAg chimeric-virus construct). Cucumber mosaic virus (CMV) 26 kDa hybrid coat protein (CP D/S) gene for 2 strains (CMV/S and CMV/D) were isolated and amplified from sgRNA 4 using F and R primers. Replicase gene (RP) and 30 kDa movement protein gene (MP) were used. CaMV promoter (35sP). Nopaline synthase terminator (Nos3T) are constructed between Right and left boarder (RB and LB). One more suggestion is (BeYDV-HBVsAg chimeric virus construct). Long and short intergenic regions (LIR & SIR) of bean yellow dwarf gemini virus as well as capsid protein (CP), movement protein (MP) and replication-associated protein (RepA) genes and as well as 35s P and Nos3T is constructed between Right and left boarder (RB and LB).

2. Genome Organization

2.1. Open Reading Frames

Four scientists [14,27,36,38] have described hepatitis B virus genome which consists of one full length strand of about 3,200 nucleotides in length (negative strand) with a single, unique nick and a complementary incomplete (positive strand) of variable length **(Figure 1)**. One of them [27] has added that lengths of positive strands amongst different molecules range between 50% and 70% of full size. The large [L (-)] strand of the partially double-stranded genome has four overlapping ORFs: preS (surface), preC (core), P (polymerase), and X, that encode at least seven different proteins **(Figure 1)**. Using of ORFs overlapping, the HBV genome can encode one and a half times the information content of an equivalent DNA molecule than non-overlapping ORFs. He added Circularization of the genome is achieved by base pairing of the free 5' end of the S(+) strand with the nicked 5' end of the L(-) strand in a region of approximately 220 nt flanked by two 11-bp direct repeats, DR1 and DR2. The 5′ end of the L(-) strand begins within the direct repeat termed DR1, whereas the S(+) strand begins within DR2. (14, 27, 36 and 38) They have added polypeptide encoded by the amino-terminal region, of the HBV P gene, is covalently linked to the 5' end of the L(-) strand and serves as a primer for L(-) strand. DNA synthesis during replication. They added that 19-nt capped ribonucleotide, covalently attached to the 5' end of the S(+) strand, most likely, serves as a primer for S(+) strand.

2.1.1. The pre-S/S gene

Four scientists [14,27,36,38] have explained that pre-s/s gene is divided into pre-S1, pre-S2 and S domains by three in-frame start codons and thus encodes three different forms of the envelope protein: the small (major) (SHBs), middle (MHBs) and large (LHBs) hepatitis B surface proteins. The SHBs protein is synthesized when a ribosome begins translation at an AUG at position 157 of the preS/S gene. It is comprised of 226 amino acids. They added that MHBs protein is produced from an upstream AUG at position 3174, which adds 55 amino acids onto the small protein, and the LHBs surface protein is generated from the AUG at

position 2850, adding 108 amino acids onto the middle protein. Two more scientists [16 and 42] explained that based on the presence or absence of S-domain glycosylation, there are two isomeric forms for each of the three HBV surface proteins. Furthermore, the MHBs protein carries a further N-linked glycosylation within the pre-S2–specific domain. One more of interested scientist [31] has published that LHBs protein contains a further posttranslational modification that involves a myristic fatty acid group linked by an amide bond to its amino-terminal glycine residue.

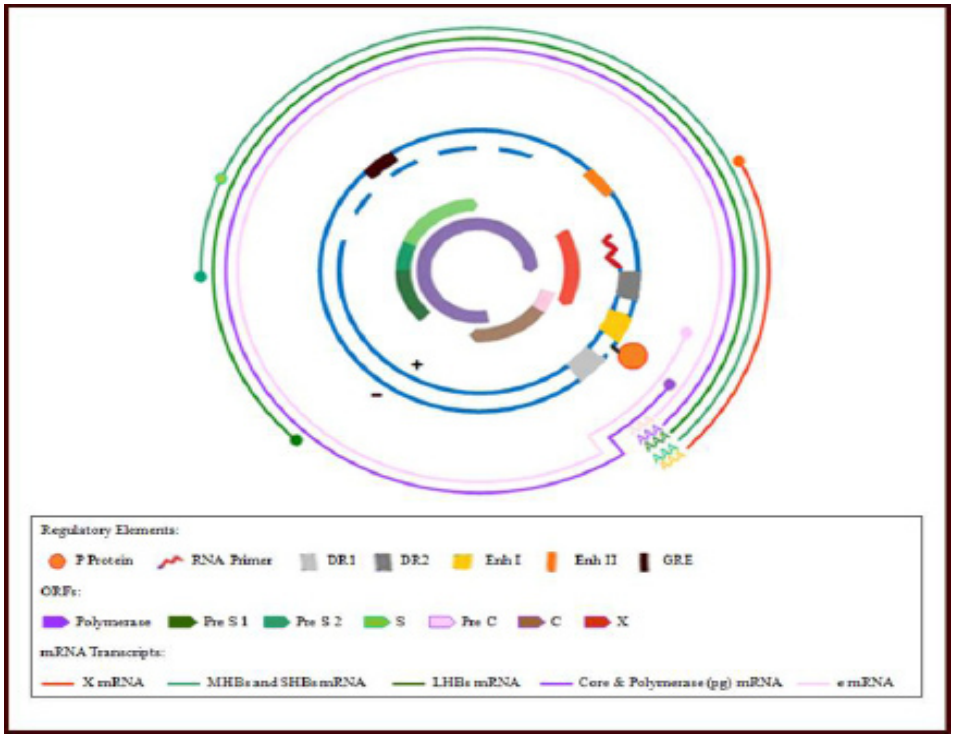

Figure 1: Organization of HBV Genome. The partially double-stranded circle is comprised of L(-) and S(+) strands with cohesive complementary 5' ends. Locations of the direct repeats 1 and 2 (DR1 and DR2), the enhancers (EnhI and EnhII) as well as glucocorticoid-responsive element (GRE) is indicated. The genome bound protein and the oligoribonucleotide at the 5' end of the L(-) strand and at the 5' end of the S(+) strand, respectively are also indicated. The inner arrows denote viral ORFs (preS1/S2/S, preC/C, P, and X). The outer circle depicts unspliced viral transcripts with heterologous initiation sites and the common polyadenylation site, (adapted from [6].

2.1.2. PreC/C ORF

The scientist [32] has stated that Initiation codes, of the core protein (HBcAg, the structural protein of the nucleocapsid), initiates by AUG at position 1903 and terminates at position 2451. An upstream 29 amino acid precore sequence may precede it in the same reading frame starting from AUG at position 1816 to form the 'e' antigen (HBeAg). HBc encapsulates the HBV nucleic acid and it has nucleic acid binding capacity as well. Two of scientists [30 and 41] have added more information on pre-C polypeptide (HBeAg precursor) which has a signal peptide in its first 19 N-terminus amino acids. This signal directs the pre-C polypeptide to the endoplasmic reticulum where the N- and C-terminus amino acids are cleaved by host proteases. After further processing by the Golgi apparatus. Some scientists [41,8,36] have explained how secretory protein is released in to extracellular medium (or serum) as the 17-kDa protein known serologically as 'e' antigen (HBeAg). The physiological function of HBeAg

is less clear than that of the structural proteins, as it is not required for viral replication, nor acute infection *in vivo*. Suggestion has been made by three scientists [41,30,26] that HBeAg which is conveniently used clinically as an index of viral replication, infectivity, and severity of disease, it may have an immunomodulating in the course of infection, playing an important role in the natural history of disease. One scientist [29] has proposed that one function of HBeAg is to maintain chronicity suggesting the induction of fulminant hepatitis following primary infection in animal models with HBeAg negative variants of hepadnaviruses. Group of scientists [4,6,9,29] stated that the precore region at the position 1896, a mutation is found at guanosine (G) into adenosine (A), resulting in a stop codon (TGG to TAG). These two mutations together stabilize the stem loop structure, which is a secondary structure with a pre-genome encapsidation sequence of the precore/core region. Moreover two scientists [6,29] stated that those mutations are relatively common in patients in Mediterranean countries, particularly in Greece and Italy. Such mutated viruses continue to replicate, but do not secrete HBeAg. In a clinical setting, these patients are anti-HBe positive with high HBV DNA levels. The mutations are believed to occur during seroconversion to anti-HBe under immunological pressure during the immune clearance phase.

2.1.3. The P ORF

One reference [37] wase being clear for P ORF which extends over more than 75% of the genome from the AUG at position 2309 to the termination codon at position 1622 (equivalent to 832 amino acids). Four domains are found in the P ORF. The amino-terminal domain encodes a terminal protein (primase), required for priming (-) strand synthesis. The next domain is not known its function yet. The third domain encodes an RNA/DNA-dependent DNA polymerase, which serves as a reverse transcriptase and DNA polymerase. The fourth domain in the carboxyl terminal, encodes an RNase H, and the RNA template has to be removed in order to allow second-strand DNA synthesis of the (+) strand. Finally, the X ORF which initiates at position 1374 and terminates at position 1836, encodes a complex trans-activating protein (HBx) of 154 amino acids. Two more scientists [14 and 38] have explained that this protein is conserved among all mammalian hepadnaviruses but not in avian hepadnaviruses and it is essential for the life cycle of HBV *in vivo* but not *in vitro*.

3. Regulatory Elements

Regulatory sequences act as binding sites for various proteins and are important for viral replication and gene regulation. Several regulatory sequences have been identified in different regions of the HBV genome. These include 4 promoters, 2 enhancers, the glucocorticoid-responsive element (GRE), 2 short direct repeats (DR1 and DR2), the Epsilon-Stem loop, the polyadenylation signal sequences and a posttranscriptional regulatory element.

3.1. Promoters

Two scientists [11,14] have discussed that promoter functions are binding site for RNA polymerase and the initiation of RNA transcription. Four promoters have been identified in the HBV genome. The three different hepatitis B surface proteins are expressed from three different in-frame start sites within the S ORF. Two promoters (preS1 and preS2) control the expression of those proteins. The preS1 promoter gives rise to a single 2.4kb transcript, which contains the full S ORF. While the preS2 promoter gives rise to various transcripts of 2.1kb in length. Two references [6,7] have added that preS2 promoter is located within the coding region of the pre-S1 domain, and lacks a classic TATA box sequence. They also added preS2 promoter, is stronger than preS1 promoter, consequently more MHBs and SHBs are expressed, compared to LHBs protein. The preS1 promoter can be down-regulated by a poorly understood mechanism which is dependent on the 3160 to 3221 nucleotide sequence within the preS2 promoter.

One more reference [7] has thought that core promoter gives rise to multiple RNAs, with different sequences at the 5' end. These transcripts are the core antigen, 'e' antigen, polymerase, and pre-genomic RNA (pgRNA) transcripts. The core promoter is regulated by both HBV enhancer elements and a negative regulatory element (NRE). However, its location has to be accurately defined, as it has been roughly mapped in the 1591 to 1851 nucleotide region. A reference [34] has stated that Transcription by C promoter is tightly restricted to liver. They added that liver-specific expression of C gene transcription is thought as result from the requirement of several liver-enriched transcription factors to bind to the C promoter. Two scientists [34 and 46] have published about C promoter is also susceptible to both positive and negative regulation. They also added that part of the hepatotropism of HBV is likely to be attributable to liver-specific transcription of the C promoter, which is required for synthesis of pre-genomic RNA

3.2. Pregenomic RNA

Two publishing [14 and 27] discovered that polymerase gene does not have an upstream promoter element. Its expression seems to have ribosome scanning of the pgRNA for transcript and initiation of translation at the P ORF start codon. Two more scientists [14,60] stated that ORF X has its own promoter that gives rise to the 0.9 kb RNA transcript which is in turn translated to a 17 kDa protein (the hepatitis B X Protein (HBx)). The promoter is thought to belocated within the 1230 to 1376 nucleotide sequence.

4. Virotherapy Strategies and Recombinant Plant-Protein Vaccine

An estimated 2 billion people have been infected with HBV. Moreover, 350 million people across the world continue to carry chronic (long-term) infection. It is most commonly

found in South East Asia, the Middle and Far East, Southern Europe and Africa. It is highly infectious, 50-100 times more than HIV and causing death worldwide [20]. Between 500,000 and 700,000 HBV-diseased people die each year [45]. Different HBV genotypes have distinct geographical distributions i.e. Genotype A is found mainly in Northwest Europe, the United States, India, and Sub-Saharan Africa. Genotypes B and C prevail in East Asia, while genotype D is common in the Mediterranean countries. Genotype E is only found in Africa and genotype F is found mainly in Central and South America [2].

Many interested scientists [1,19,50] in plant vaccine of HBV strategies are studied. HBV has different immunogenic genes i.e. longest open-reading frame encodes the viral polymerase (Pol open-reading frame). The envelope open-reading frame, which is located in a frame-shifted manner, overlaps with the core (C) and the X open-reading frames. The covalently closed circular DNA (ccc DNA) is the template that is transcribed to generate four major RNA species: the 3.5-, 2.4-, 2.1-, and 0.7-kb **(Figure 1)**. Expression of these four transcripts is directed by the enhancer (enh 1 and enh2) and (DR1 and DR2) as promoter. II/basal core, large surface antigen (L), major surface antigen (S), and enhancer I/ X gene promoters, respectively. One reference [17] has added that HBV has different immunogenic genes as target for getting vaccine prepared i.e. surface Antigen (HBVsAg), Core Antigen (HBVcAg), Short and Long intergenic Region (HBVSiR) and (HBVLiR), Polymerase, and x genesVirotherapy strategies for HBV-vaccine production are different i.e. a). Protein vaccine through Plant-virus genomic structure as vector, b). DNA vaccine and c) Exomes for vaccine production. Following is some details on the first items (Plant-viral vector). One more reference [46] has explained that many research groups have different ideas.

4.1. Isolation and identification of HBsAg and polymerase as immunogenic-gene target

4.1.1. DNA Extraction

In 2012 at Cairo Univ. (15), have isolated HBVsAg (S). They have determined the protein of surface antigen (S) as 226 aa and 25.42 kDa. In a trial that was done by [14], they have collected 100 blood serum samples. They tested all samples against HBV, HCV, HDV and HIV by serological assay. They were positively reacted against HBV where they were negative against HCV, HDV and HIV. This test was done to be sure that they are working HBN-diseased patients. In another trial done by [26], DNA was extracted from HBV-infected blood serum by using QIAamp DNA extraction kit (QIAGEN GmbH, Hilden Germany). Serum samples of eight HBV positive patients from Sohag governorate-Egypt were used where isolates Sohage 1 and 7 were obtained from HBV positive patients with hepatocellular carcinoma (HCC).

4.1.2. PCR procedure and DNA sequencing

Sense 5’-TCA CCA TAT TCT TGG GAA CAA-3’ and antisense 5’-CGA ACC ACT

GAA CAA ATG GC-3' primers were used to amplify 1110 bp of open reading frames of polymerase and S genes of HBV genome. An Egyptian scientist [14 has isolated and amplified HBsAg immunogenic target using specific primers (GAACAAGAGCTACAGCATGGG) and (CTTTTGTCTTTGGGTATACAT) in HBsAg domain between positions 2850-2871 for the 1st primer and 810-831 for the 2nd one under band size 681 bp. Briefly, 100 µl of reaction mixture containing 10 µl of extracted

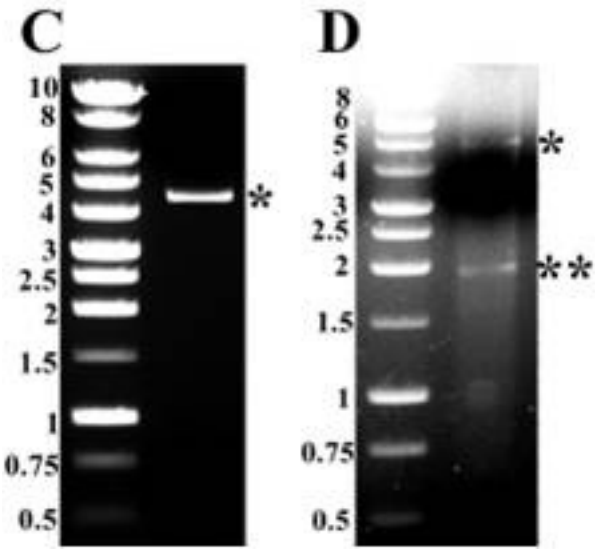

Figure 2: (C) Image of an agarose gel showing the supercoiled pRP-HBsAg(S)-GST and the * indicates the position of the plasmid. (D) Image of an agarose gel showing the KpnI/ EcoRI digestion products of pRP-HBsAg(S)-GST. The * indicates the position of the linearized plasmid and the ** indicates the position of the dropped insert. The left lane in each gel contains 1 kb DNA ladder (Promega, USA); the sizes of the respective bands is indicated on the left side of each panel. After [14]. BMC Research Notes 2012, 5:125 http://www.biomedcentral.com/1756-0500/5/125

DNA, 50 mM potassium chloride, 10 mM Tris hydrochloric acid (pH 8.3), 2 mM magnesium chloride, 200 µM deoxyribonucleosides, 2 U of *AmpliTaq Gold DNA* polymerase (perkin-Elmer, Norwalk, Conn), and 20 pmol each of the oligonucleotide primers. Amplification was performed for one cycle at 95°C for 10 min followed by 35 cycles, each consisting of denaturing for 20 sec. at 94°C, annealing for 20 sec at 55°C, and extension for 1 minute at 72°C. DNA sequencing was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 373xl DNA Analyzer.

4.1.3. Sequence analysis

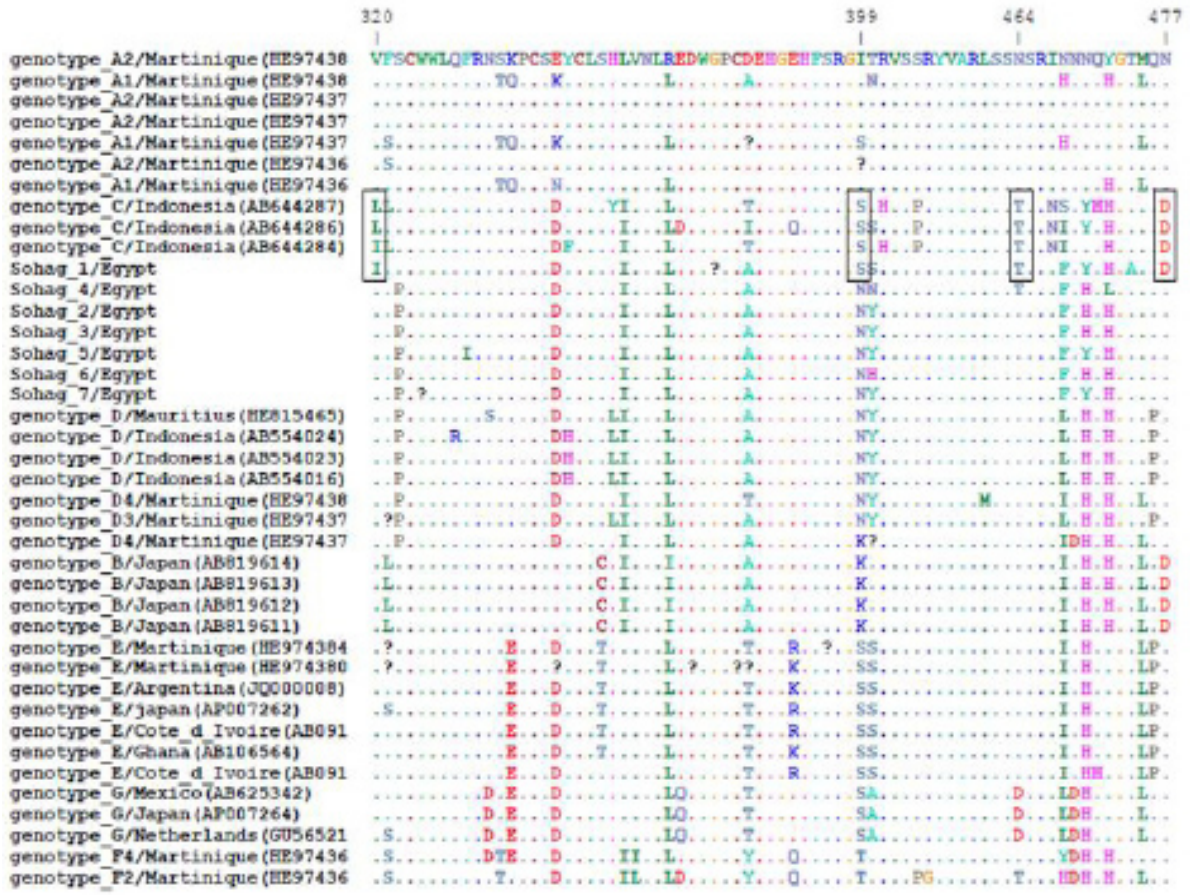

Figure 3: Multiple amino acids sequence alignment of HBV polymerase gene. Amino acids positions correspond to complete genome sequence of genotype C (accession number AB644287). After [25]

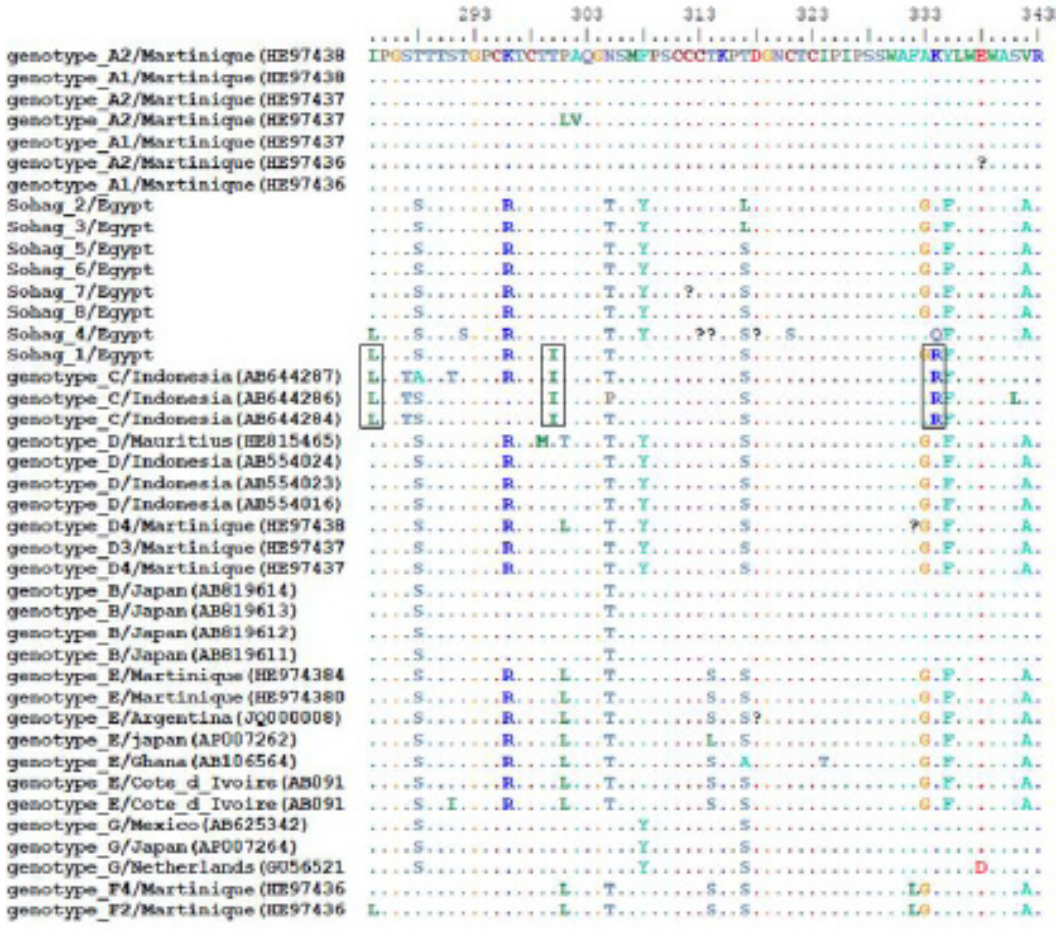

Figure 4: Multiple amino acid sequence alignment of HBV envelope gene. Amino acids positions correspond to complete genome sequence of genotype C (accession number AB644287). After [25].

Table 1: Mean percent nucleotide identity and divergence between S gene sequences of new isolates and eight human HBV strains belonging to genotypes (A-H)

	1	2	3	4	5	6	7	8	9	10	HBV Strains
1		90.3	81.7	89.6	88.7	86.2	89.7	88.6	89.3	89.7	**HBV/sAg S gene (A) Seq.**
2	9.6		80.2	89.1	87.9	84.8	87.2	86.0	89.8	89.9	**HBV/sAg S gene (B) Seq.**
3	7.4	9.3		80.9	81.5	78.0	80.2	78.1	81,3	81.5	**HBV/sAg S gene (C) Seq.**
4	10.3	10.6	8.4		91.4	86.4	89.5	86.6	79.9	97.9	**HBV/sAg S gene (D) Seq.**
5	10.5	11.4	7.5	8.0		85.6	91.0	86.2	92.1	92.0	**HBV/sAg S gene (E) Seq.**
6	14.0	15.4	12.3	14.0	14.3		85.7	94.3	86.4	86.2	**HBV/sAg S gene (F) Seq.**
7	9.4	11.9	8.8	9.6	9.0	14.0		85.6	90.3	90.3	**HBV/sAg S gene (G) Seq.**
8	13.4	14.2	12.6	13.6	13.3	5.6	12.3		87.0	68.8	**HBV/sAg S gene (H) Seq.**
9	10.3	9.9	8.2	2.0	7.0	13.8	8.7	13.0		99.2	**HBV/sAg S gene Seq.**
10	10.1	9.9	8.0	2.1	7.3	14.0	8.7	13.3	0.8		**HBV/sAg S gene Seq.**

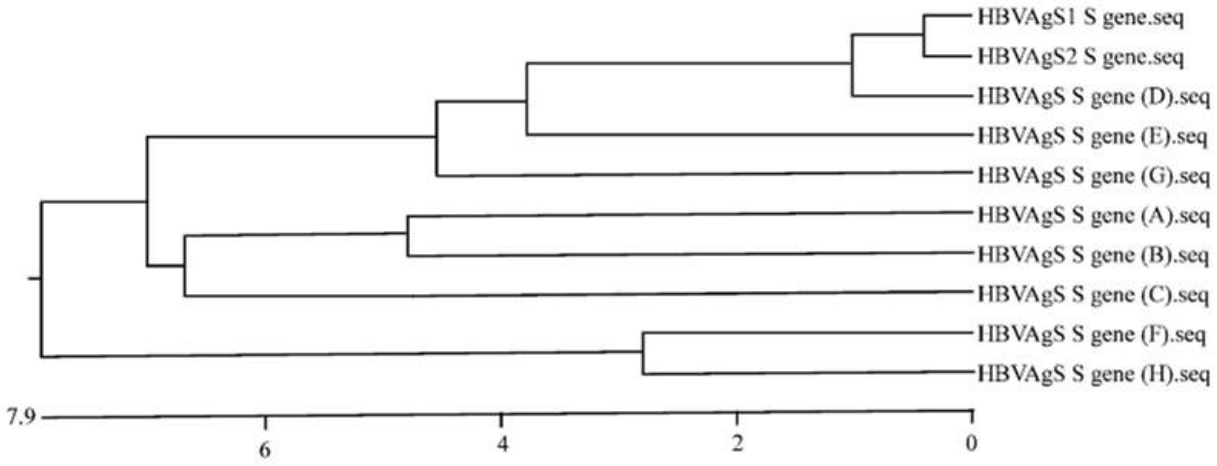

Figure 5: Phylogenetic tree of entire nucleotid sequences constructed by neighbor joining method using the two isolates, isolated in this work with eight HBV using joining method after [14] e

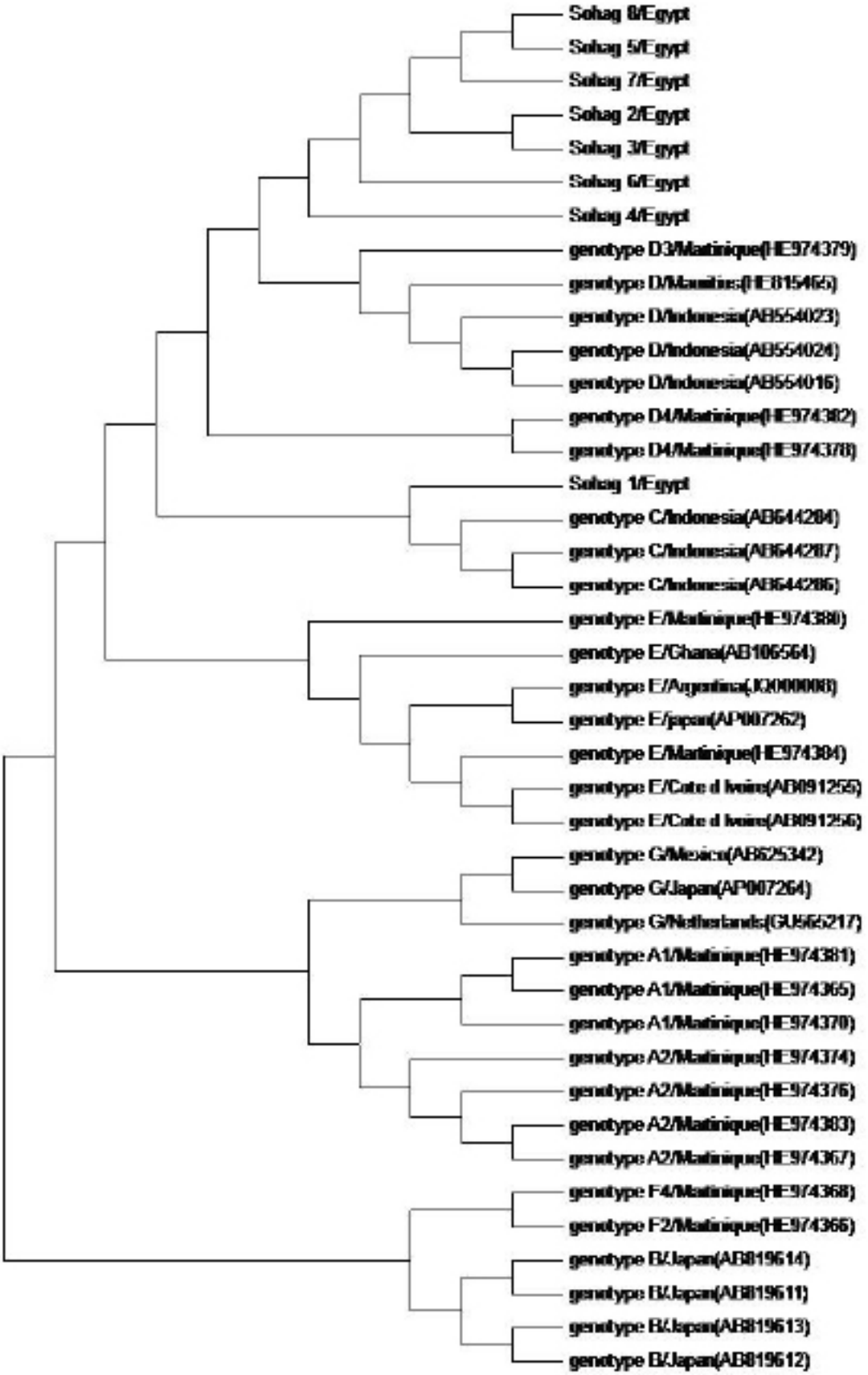

Figure 6: Phylogenetic analysis of HBV nucleotide sequences. DDBJ GeneBank. Bootstrap values are indicated.

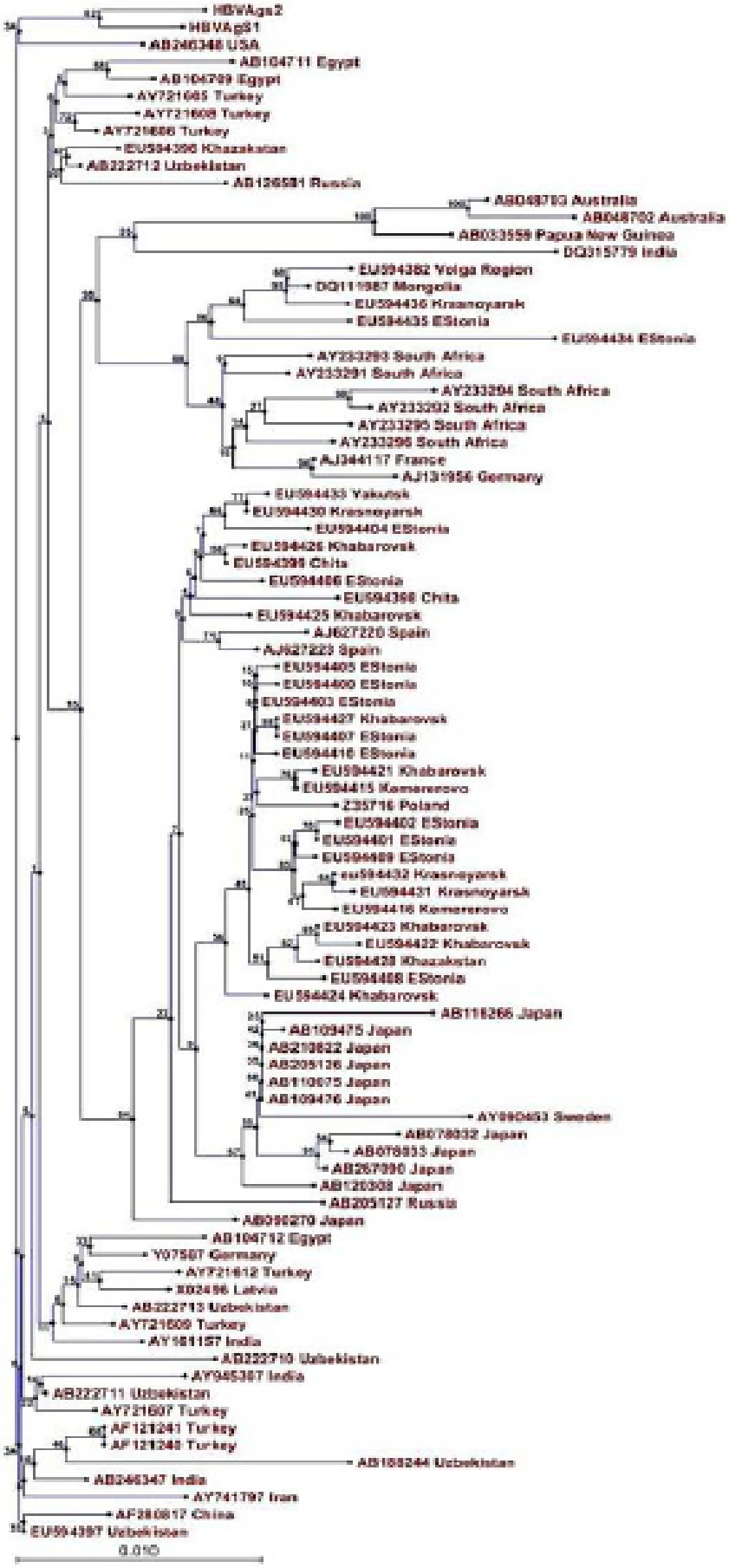

Figure 7: Phylogenetic tree of entire nucleotide sequences constructed by the neighbor-joining method using the present two isolates and 87 HBV/D isolates retrieved .from DDBJ/GenBank After [14].

Sequence analysis was done using Blast programs from National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov/Blast). One hundred sequences of

HBV isolates from different types were obtained from GenBank and were used to construct Neighbor-Joining tree between Sohag and GenBank isolates.

4.1.4. Plasmid vectors for DNA fragments and chimeric virus construction

An Egyptian scientist [15], at Cairo University, has used DH5α bacterial cells for cloning and expression systems. The plasmid vector pRc/CMV-HBs(S) (Aldevron, USA) was used as a source for the S gene encoding the HBsAg(S). This vector is 5618 bp andcarries the HBsAg(s) gene. In addition the plasmid carries the HBV-3'-UTR which was found essential for the production of protein in *E. coli* cells. The expression vector pRP265 (NCCB, Netherlands) was used to express encoding the S gene as a fusion protein in *E. coli*. This vector which is 4979 bp for cloning and for recombinant protein production. PCR amplification of S gene and the 3'-UTR were designed to clone the desired fragment Primer MS-1 (5'tgGGTACCacATGGAGAACA TCACATCAGGAT-3' and primer MS-2 (5'-gGAATTCCGAGATCCTCGCCGTCGGGCAT-3'; Previous Egyptian scientist [15] has used the following primers for cloning i.e. primers pair AA437 (5'-AACAGCTATGACCATG-3') and AA438 (5'-GTAAAACGACGGCCAGT-3') that bind in the pCR2.1 vector plasmid was designated pCR2.1-HBsAg(S). He [15 has looked for effect of temperature and timing for protein induction and it was 7.9 µg/50 ml culture at 3.0 hr.) .He has used the same previous system of [15] in cloning and amplification of HBVsAg target gene. They used the following primers for amplification from the plasmid pTBIk2 (5' GATCGTCGACCATGGATCTGCAGACTCATG-3' and 5'-TCTAGAAGATCTGTTTAAA TGTATACCCAAAGACAGAA-3'). Resulting amplicon was digested with NcoI and BglII restriction endonucleases and inserted into the plasmid pRT104, which was transformed into E. coli XL1-Blue cells. The DNA construct contained the strong constitutive transcriptional p35S promoter It was excised from plasmid pRTTBI-HBS-6, which is 4478 bp, using HindIII. This fragment was inserted into the binary vector pBINPLUS/ARS and resulting construct, pBINp35STBI- HBS#15, was confirmed by restriction analysis and nucleotide sequencing. It was inserted into *Agrobacterium tumefaciens* LBA4404 was transformed with the plasmid pBINp35STBI-HBS#15

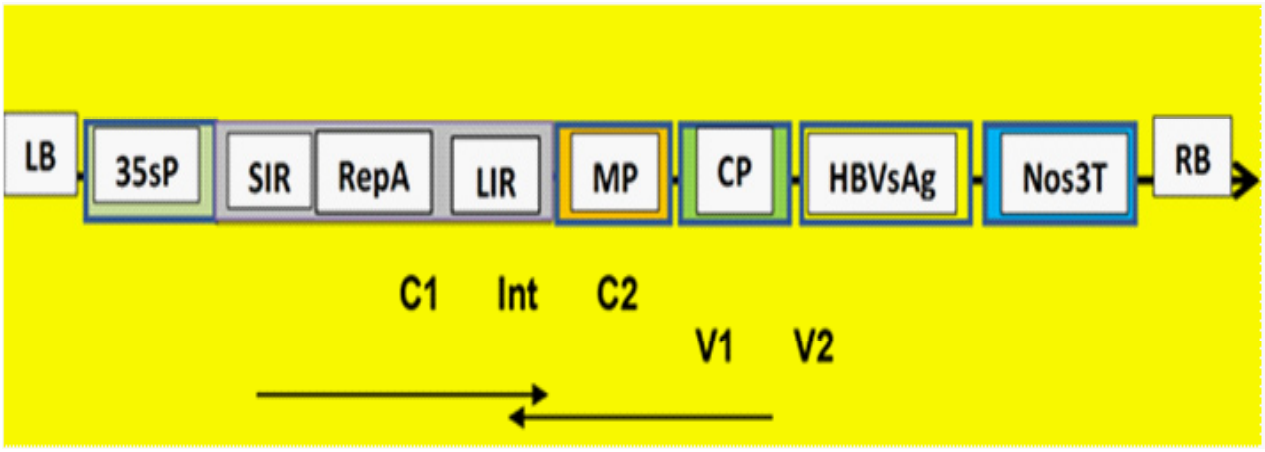

Figure 8: Suggested BeYDV-HBVsAg chimeric virus construct, 25.42 kDa HBVsAg (S) protein that early isolated (Elghanam, et al., 2012) is constructed with Bean Yellow Dwarf Begomovirus. Long and short intergenic regions (LIR & SIR) of bean yellow dwarf gemini virus as well as capsid protein (CP), movement protein (MP) and replication-associated protein (RepA) genes with HBVsAg (S) target gene as well as CaMv 35s P and Nos3T are experimented. CaMV 35s promoter (35sP). Nopaline synthase terminator (Nos3T) are constructed between Right and left boarder (RB and LB). Arrow directions are the orientation activities for C1, C2 and V1, V2 with intron (Int.). The construct is inserted in pBY002 binary vector and transferred onto H5α *E. coli* cell. The chimeric virus is transfected into suitable bioreactors

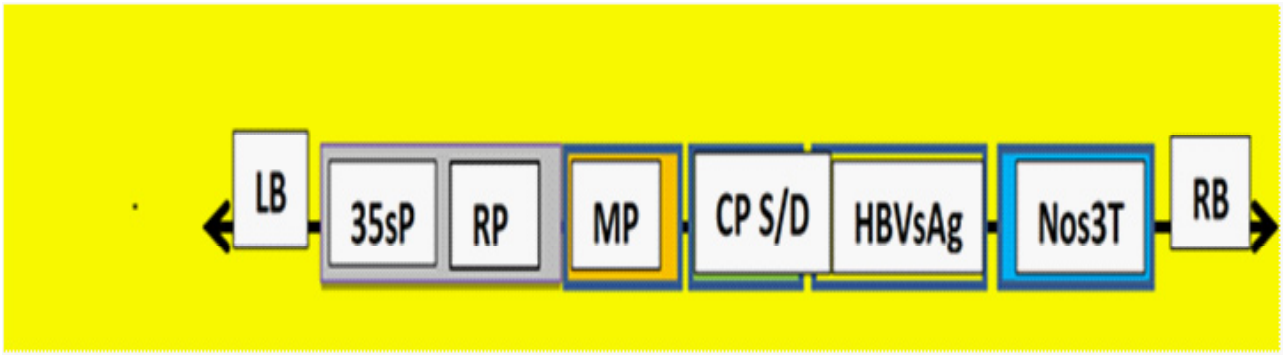

Figure 9: Suggested CMV-HBVsAg chimeric virus construct, 25.42 kDa HBVsAg (S) has been isolated and identified S gene by (15) as target gene from Egyptian patients samples. Cucumber mosaic virus (CMV) 26 kDa hybrid coat protein (CP D/S) gene from 2 strains (CMV/S and CMV/D) are isolated and amplified from sgRNA 4 using specific primers. Replicase gene (RP) and 30 kDa movement protein gene (MP) is helping for target gene replication. CaMV 35s promoter (35sP) and Nopaline synthase terminator (Nos3T) are also constructed between Right and left boarder (RB and LB). They are inserted into a binary vector. The construct is transfected into a bioreactor host.

5. Evolutionary Relationships

The evolutionary history was inferred using the Neighbor-Joining method [20]. Phylogenetic analyses were conducted in MEGA4. Eight HBV isolates from Sohag governorate, Egypt were confirmed by polymerase chain reaction (PCR), resulted in 1110 nt correspond to partial sequence of polymerase (pol) and envelop (env) genes Phylogenetic analysis of pol and env genes nucleotide sequence (Figure 1) revealed that 7 out of the 8 isolates (Sohag 2, 3, 4, 5, 6, 7-HCC and 8) were closely related to genotype D. However, isolate Sohag 1-HCC was closely related to genotype C. Similar data was obtained when amino acid of pol and env genes were used (supplementary data). Multiple amino acid sequence alignment showed specific substitutions distinguished genotype C and Sohag 1-HCC from the other genotypes (Figure 3,4). Thus amino acid substitutions (V_{320}→L and N_{464}→T) and (I_{284}→L, T_{300}→I and K_{334}→R) were detected in pol and env genes respectively. HBV is a leading risk factor for hepatocellular

carcinoma (HCC), with over eighty percent of HCC cases occurring in the regions where HBV is endemic**.** In this study**,** multiple sequence alignment showed unique amino acid substitutions in the envelope genes of Sohag1-HCC and genotype C. These substitutions were V_{320}→L and N_{464}→T for pol gene and I_{284}→L, T_{300}→I and K_{334}→R for env gene. Upon Phylogenetic analysis of the nucleotide sequence and the amino acid of pol or env genes, 7 isolates from patients without HCC (Sohag 2,3,4,5,6) and 1 isolate from patient with HCC (Sohag 7-HCC) were closely related to genotype D. Only one isolate (Sohag 1-HCC) of the 2 HCC isolates was clustered with genotype C which is known as risk factor of HCC. Association between HCC and HCV was previously studied (Mahmoud and Hashem 2012). Genotype D is the most widely distributed genotype and has been found universally, with its highest prevalence in a belt stretching from Southern Europe and North Africa**.** Genotypes B and C prevail in East Asia [29]. The same author [29] reported that genotype C hepatitis B virus is associated with a higher risk of HCC as compared to other major HBV genotypes (A, B and D). As well as, mutations in S gene sequence were observed in this study that may change the antiviral target site and virus replication. The results of this study indicate that further prospective studies are needed to confirm the role of the mutations in polymerase and envelope genes in the development of HCC.

6. Conclusion

HBV has been methodically examined during the last couple of decades, and the major principles of HBV structure, genome and replication cycle have been resolved. Additionally, the nature of viral infections in animal models and, to some extent, in infected patients has been also described. Despite of these successes, many gaps in our understanding of the viral life cycle still exist. Two of the major unresolved issues are related to the identity of HBV receptor and the role of the X gene product (HBx) in the replication cycle. Furthermore, the current combination therapy is hurdled with the severe side effects and the development of resistant mutants; hence it is necessary to pursue the development of better and cheaper antiviral therapies for the enormous number of HBV chronic patients. Therefore, it is still crucial to continue studying HBV gene products as well as it replication cycle in order to design and develop effective antivirals that can inhibit different stages of the viral life cycle such as attachment and penetration, conversion from rcDNA to cccDNA, assembly, budding and release of viral particles.

HBV has different immunogenic antigens i.e. HBVsAg,(Pre S 1, 106 aa, Pre S 2 55 aa and S 226 aa) HBVcAg, (pre C 29 aa and Core Antigen, 185 aa) HBVpAg (Polymerase Antigine, 832 aa) and X Antigen, 154 aa [20]. Those previously mentioned antigens could support give a high possibility for using some of them as immunogenic genes. Bioreactor and adjuvant are affecting expression for protein vaccine. Selection of plant viral vectors for engineering is the good tool for expressed-recombinant protein against target sequence (Figure 10, 11). In this

text we proposed two types of vectors i.e. CMV as RNA virus and BeYDV as DNA Gemini virus. Hybrid S/D CMV has given valuable amount of expressed protein vaccine. Moreover Long and short intergenic regions (LIR and SIR) has been used extensively for replication by [18].

Hybrid plasmid role in Edible oral vaccine production has been discussed by [20] as follow: The plasmid No. 1, pRTTBI-HBS-6, can carry the TBI-HBS-coding sequence, p35S, polyA and polyadenylation signal, respectively. The plasmid No. 2, pBINPLUS/ARS, has BR and BL and in-between, T-DNA (HindIII, P-ubi3, T-ubi3, promoter and terminator, and NPTII), moreover, ori RP4, ori ColE1 replication origins, NPTIII gene rendring bacterial cells resistant to kanamycin XhoI and NcoI restriction endonucleases, The hybrid plasmid has TBI-HBS vaccine antigen. The location of T- and B-cell epitopes of the HIV-1 ENV and GAG proteins TBI protein, 226 aa HBsAg is fused to the 3' terminus of the TBI protein Having plasmid-hybrid technology can help in getting chimeric virus constructs created for different target seuences i.e. target gene sequence, promoters and terminators could be inserted into first plasmid vector. Plant viral vector constructs (CP, MP and polymerase) could be inserted into the second plasmid vector (Figure 10,11).

The hybrid vector could play a role in getting all previous fragments together. It also can be supporting in getting more than one expressed-protein sequences of two immunogenic genes of two different infectious viruses. More than that doubling the immunogenic epitopes can produce double amount of expressed protein vaccine [47]. Bioreactor and adjuvant are affecting expression for protein vaccine.

7. Acknowledgment

We would like to expressour thanks and sincere appreciation for Ms. Sakina Mustafa for designing and drawing all the figures in this manuscript. Very special thanks are also extended to Dr. Michael J. McGarvey, Department of Medicine - Imperial College of London, for critically reviewing the draft of the manuscript.

8. Author Contributions

This study has been done in collaboration with co-authors from Egypt, Bahrain and Canada. Each co-author has submitted part in this work i.e.

1. E. Janahi, Bahrain has submitted genome organization of HBV as part of his Ph.D. in England,

2. A. E. Aboul-Ata, M. El-Kalamawy, Amal Mahmoud, M. El-Ghanam, Egypt has submitted isolation of HBsAg as well as identification.

3. Kathleen Efferon, Canada has submitted her experience in HBsAg-BeyDV chimeric virus

as well as revising the scientific material.

4. AE Aboul-Ata, The author was leading a Swedish-funded project on HSV-2 plant vaccine and he has published a review on edible vaccine strategies at Advances in Virus Research.

9. References

1. Aboul-Ata E. Aboul-Ata, Antonella Vitti, Maria Nuzzaci, Ahmad K. El-Attar, Giuseppina Piazzolla, Cosimo Tortorella, et al. Plant-Based Vaccines: Novel and Low-Cost Possible Route. for Mediterranean Innovative Vaccination Strategies. Advances in Virus Research. 2014; 89: 1-35.

2. Antonucci TK, Rutter WJ. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J Virol. 1989; 63: 579-583.

3. Arauz-Ruiz, Norder PH, Robertson BH, Magnius LO. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol. 2002; 83: 2059-2073.

4. Bottcher B, Wynne SA, Crowther RA. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. Nature. 1997; 386: 88-91.

5. Brunetto MR, Giarin, M, Saracco G, Oliveri F, Calvo P, Capra G, et al. Hepatitis B virus unable to secrete e antigen and response to interferon in chronic hepatitis B. Gastroenterology. 1993; 105: 845-850.

6. Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet. 1989; 2: 588-591.

7. Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, Williams R, et al. Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology. 1991; 14: 219-222.

8. Chang HK, Ting LP. The surface gene promoter of the human hepatitis B virus displays a preference for differentiated hepatocytes. Virology. 1989; 170: 176-183.

9. Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J Viro. 1992; l66: 5682-5684.

10. Cote PJ, Korba BE, Miller RH, Jacob JR, Baldwin BH, Hornbuckle WE, et al. Effects of age and viral determinants on chronicity as an outcome of experimental woodchuck hepatitis virus infection. Hepatology. 2000; 31: 190-200.

11. Crowther RA, Kiselev NA, Bottcher B, Berriman JA, Borisova GP, Ose V, et al. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell. 1994; 77: 943-950.

12. Dryden KA, Wieland SF, Whitten-Bauer C, Gerin JL, Chisari FV, Yeager M. Native hepatitis B virions and capsids visualized by electron cryomicroscopy. Mol Cell. 2006; 22: 843-850.

13. Eble BE, Lingappa VR, Ganem D. Hepatitis B surface antigen: an unusual secreted protein initially synthesized as a transmembrane polypeptide. Mol Cell Bio. 1986; l6: 1454-1463.

14. El-Kalamawy IM, El-Haddad S, Swelim MA, Hamdy SM, Fahmy HA. Molecular variability of S gene of Hepatitis B virus in Egypt. International Journal of Virology. 2011; 3: 109-115.

15. Elghanam MS, Attia AS, Shoeb HA, Hashem AM. Expression and purification of hepatitis B surface antigen S from Escherichia coli; a new simple method. BMC Research Notes. 2012; 5: 125-134.

16. Fields, Bernard N, Knipe David M, Howley, Peter M, Griffin, et al. Fields virology.5th ed. Lippincott Williams & Wilkins, Philadelphia. 2007.

17. Ganem D, Schneider R. Hepadnaviridae: The viruses and their replication. In: Knipe DM, Howley PM, editors.

Fields Virology. Philadelphia: Lippincott-Raven. 2001; 2923–2970.

18. Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. Large surface proteins of hepatitis B virus containing the pre-s sequence. J Virol. 1984; 52: 396-340.

19. Hefferon KL. DNA Virus Vectors for Vaccine Production in Plants: Spotlight on Geminiviruses. Vaccines. 2014; 2: 642-653.

20. Jaccard P. Nouvelle recherches sur la distribution florale. Bulletin de la Société Vaudoise des Sciences Naturelles. 1908; 44: 223-270.

21. Kuttikan M, kshmi J, Kalyanaraman N, Pitchappan N. Hepatitis B Virus Genetic Diversity: Disease Pathogenesis. INTECH. Chapter. 2013; 3: 69-82.

22. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004; 11: 97–107.

23. LondonShafritz DA, Lieberman HM. The molecular biology of hepatitis B virus. Annu Rev Med. 1984; 35: 219-232.

24. Lu CC, Yen TS. Activation of the hepatitis B virus S promoter by transcription factor NF-Y via a CCAAT element. Virology. 1996; 225: 387-39.

25. Mahmoud A, Hashem HM, Phylogenetic subtyping of hepatitis C virus 5' UTR isolated in Egypt and the effect of 2 transitions in subdomain IIId on the apical loop structure. Life Science J. 2012; 9.

26. Mahmoud A, Hashem HM. Bioinformatics analysis of polymerase and envelope genes of hepatitis B virus in Egypt. Egyptian J Virol. 2014; 9: 22-30.

27. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B. surface antigen in transgenic plants. Proc. Natl Acad. Sci. USA. 1992; 89: 745–749.

28. Milich, D, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. Hepatology. 2003; 38: 1075-1086.

29. Monjardino J. Molecular biology of human hepatitis viruses. Imperial College Press. 1998.

30. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenic relatedness and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology. 1994; 198: 489-503.

31. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology. 1993; 194: 263-276.

32. Ou JH, Laub O, Rutter WJ. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc Natl Acad Sci USA. 1986; 83: 1578-1582.

33. Persing DH, Varmus HE, Ganem D. The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J Virol. 1987; 61: 1672-1677.

34. Petit MA, Pillot J. HBc and HBe antigenicity and DNA-binding activity of major core protein P22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. J Virol. 1985; 53: 543-551.

35. Qirbi N, Hall AJ. Epidemiology of Hepatitis B Virus Infection in the Middle East. La revue sante de la Mediterrranee orientale. 2001; 7: 1034-1046.

36. Raney AK, Johnson JL, Palmer CN, McLachlan A. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J Virol. 1997; 71: 1058-1071.

37. Richter LJ, Thanavala Y, Arntzen CJ, Mason HS. Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat Biotechnol. 2000; 18: 1167–1171.

38. Shafritz DA, Lieberman HM. The molecular biology of hepatitis B virus. Annu Rev Med. 1984; 35: 219-232.

39. Schlicht HJ, Salfeld J, Schaller H. The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core proteins but not for virus formation. J Virol. 1987; 61: 3701-3737.

40. Schlicht HJ, Schaller H. Analysis of hepatitis B virus gene functions in tissue culture and in vivo.Curr Top Microbiol Immunol. 1989; 144: 253-263.

41. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev. 2000; 64: 51-68.

42. Shchelkunov SN, Salyaev RK, Pozdnyakov SG, Rekoslavskaya NI, Nesterov AE, Ryzhova TS, et al. Immunogenicity of a novel, bivalent, plant-based oral vaccine against hepatitis B and human immunodeficiency viruses. Biotechnol Lett. 2006; 28: 959–967.

43. Siddiqui A, Jameel S, Mapoles J. Expression of the hepatitis B virus X gene in mammalian cells.Proc Natl Acad Sci USA. 1987; 84: 2513-2517.

44. Standring DN, Ou JH, Masiarz FR, Rutter WJ. A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigens in Xenopus oocytes. Proc Natl Acad Sci U S A. 1988; 85: 8405-8409.

45. Stibbe W, Gerlich WH. Structural relationships between minor and major proteins of hepatitis B surface antigen. J Virol. 1983; 46: 626-628.

46. Streatfield. Oral hepatitis B vaccine candidates produced and delivered in plant material. Immunology and Cell Biology. 2005; 83: 257-226.

47. Su H, Yee JK. Regulation of hepatitis B virus gene expression by its two enhancers. Proc Natl Acad Sci U S A. 1992; 89: 2708-2712.

48. World Health Organization. Viral hepatitis: Report by the Secretariat. 2009.

49. Yu X, Mertz JE. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. J Virol. 1997; 71: 9366-9374.

50. Yusibov V, Rabindran S. Recent progress in the development of plant-derived vaccine. Vaccines. 2008; 7: 1173-1183.