Results

1. Amplification of extracted DNA samples using PCR:

Strict precautions were followed to avoid cross contamination and appropriate negative and positive controls were included during DNA extraction and PCR amplification steps.

The electrophoresis shows the presence of specific PCR product band visualized by ethidium bromide in agarose gel at the expected molecular weight for surface antigen (HBsAg) ≈1200bp (Fig. 5) and ≈ 800 bp for pc/core region (Fig. 6).

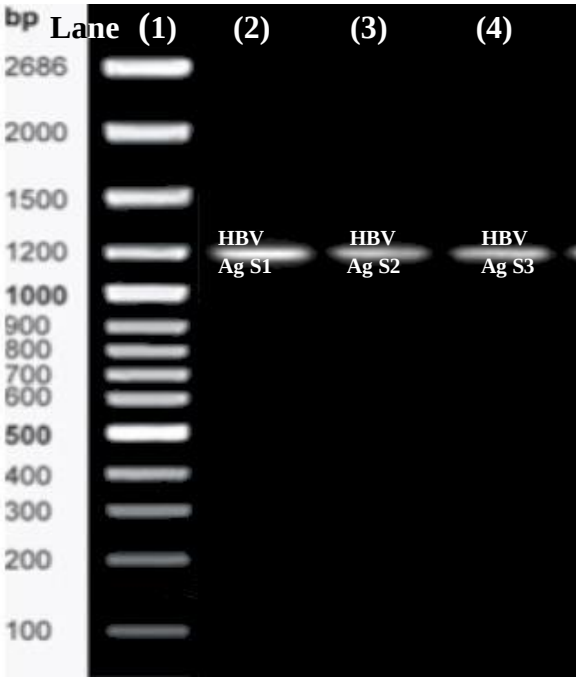


Fig. (5): Agarose gel electrophoresis of surface antigen of HBV showing expected molecular weight. Lane (1): Molecular weight marker. Lane (2, 3, 4): samples of HBsAg.

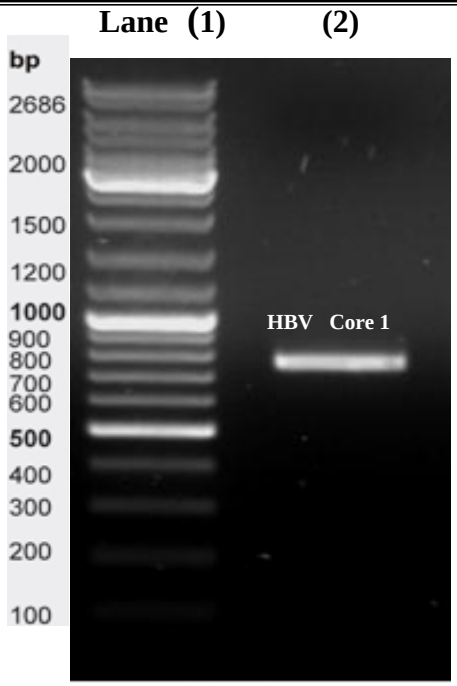


Fig. (6): Agarose gel electrophoresis of HBV Core1 (pc/core.) showing expected molecular weight. Lane (1): Molecular weight marker. Lane (2): HBV sample.

2. Nucleotide sequencing of Surface antigen and Core antigen:

The conditions for performing highly amplified PCRs must include controls to ensure that any sequence changes found are not an artifact of PCR fidelity itself. HBV Surface antigen and Core antigen coding regions sequences were successfully amplified. All isolate in this study submitted to genbank database and accession numbers for each isolate presented in the table (2). Nucleotide sequencing of Surface antigen and Core antigen were shown in figures for HBV Ag S1 Fig. (7), HBVAgS2 Fig. (8), HBV Ag S3 Fig. (9) and HBV core1 (pc/core) Fig. (10).

Isolate Name	Genbank Accession numbers	Isolate Name	Genbank Accession numbers
HBV Ag S1	JF304298	HBV Ag S3	HM014050
HBV Ag S2	HM014050	HBV Cor1	JF304297

Table (2): Genbank Accession numbers of each Isolate

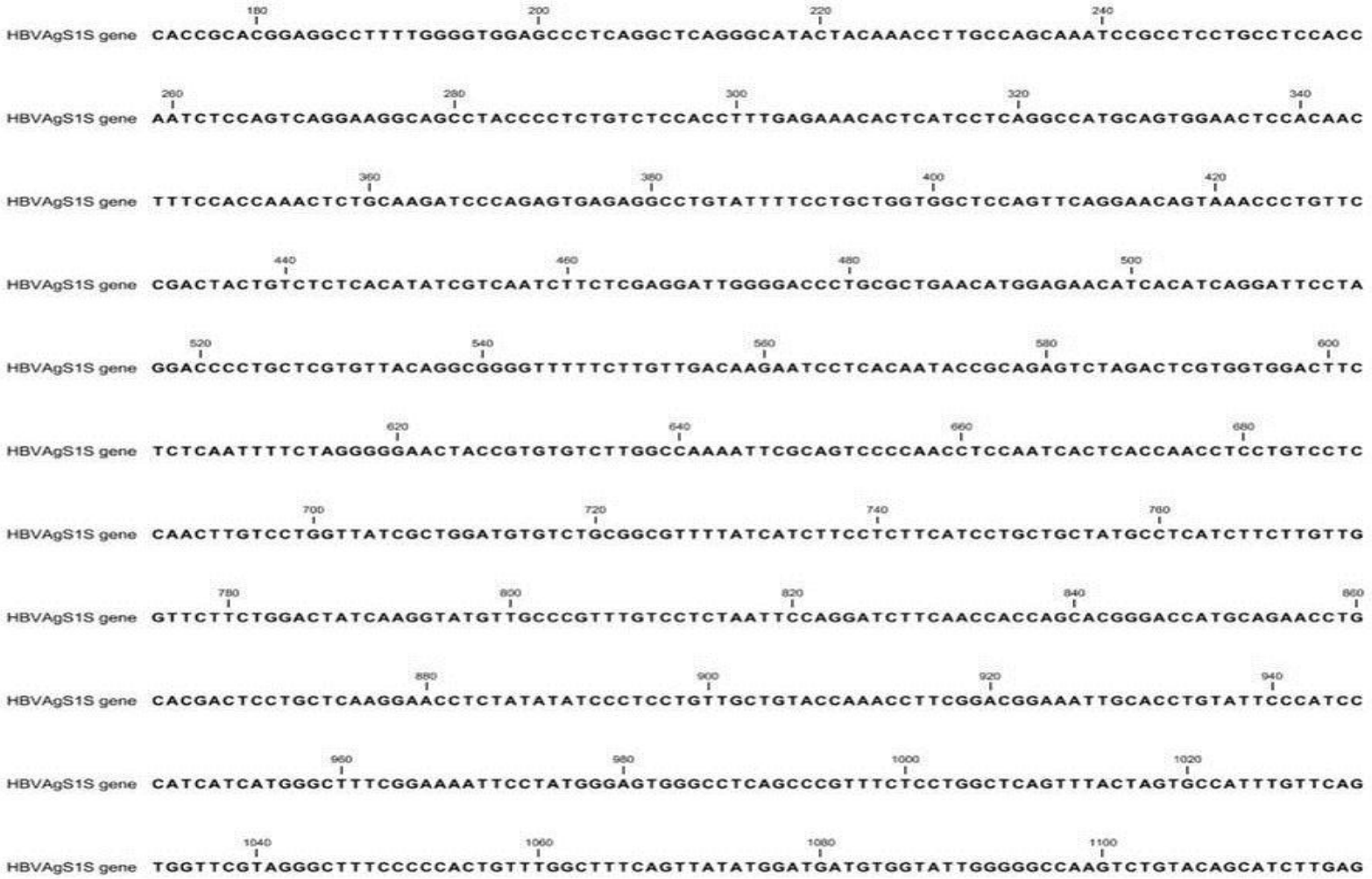


Fig. (7) : Nucleotide sequence of HBV Ag S1.

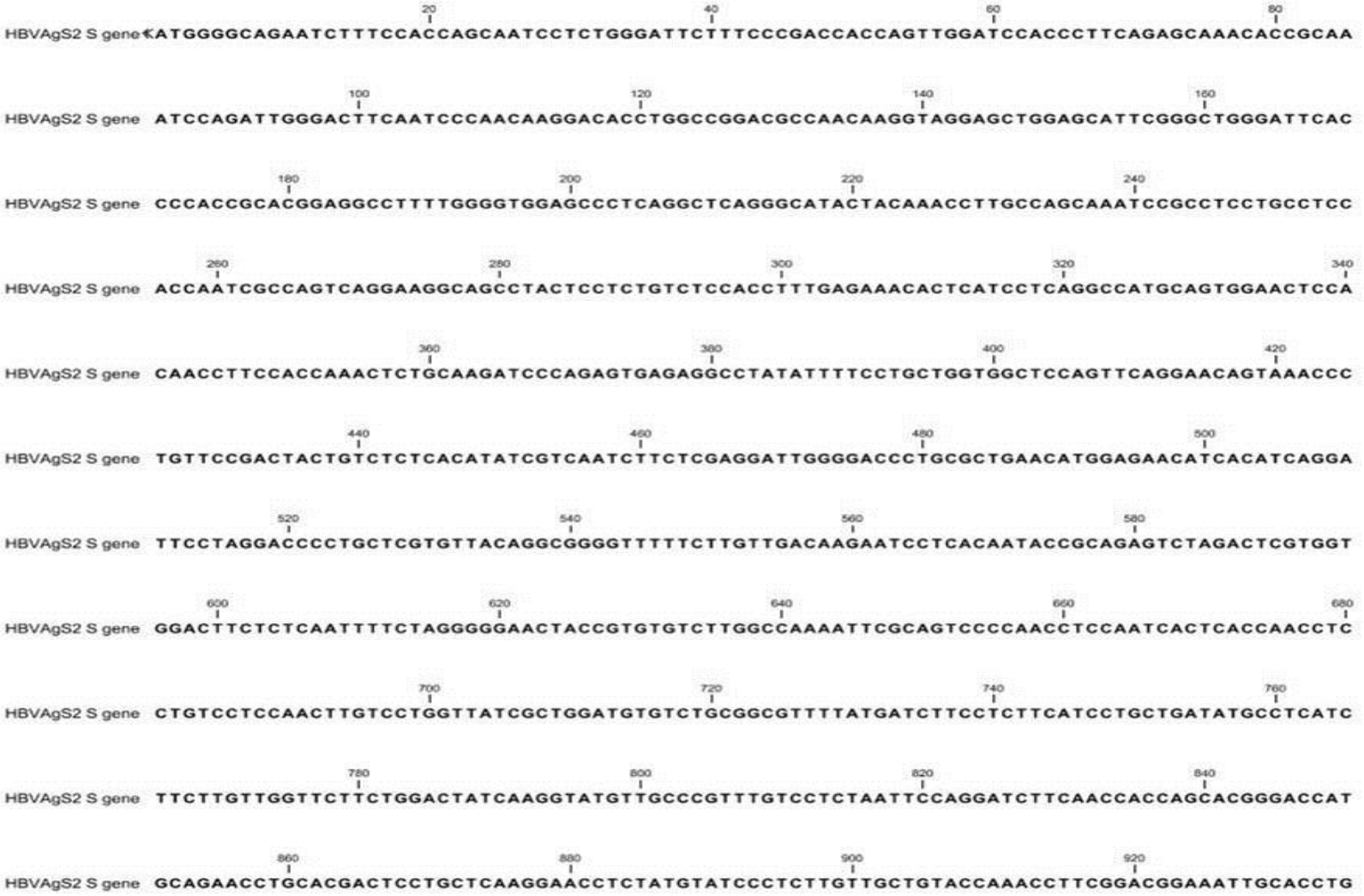


Fig. (8) : Nucleotide sequence of HBV Ag S2.

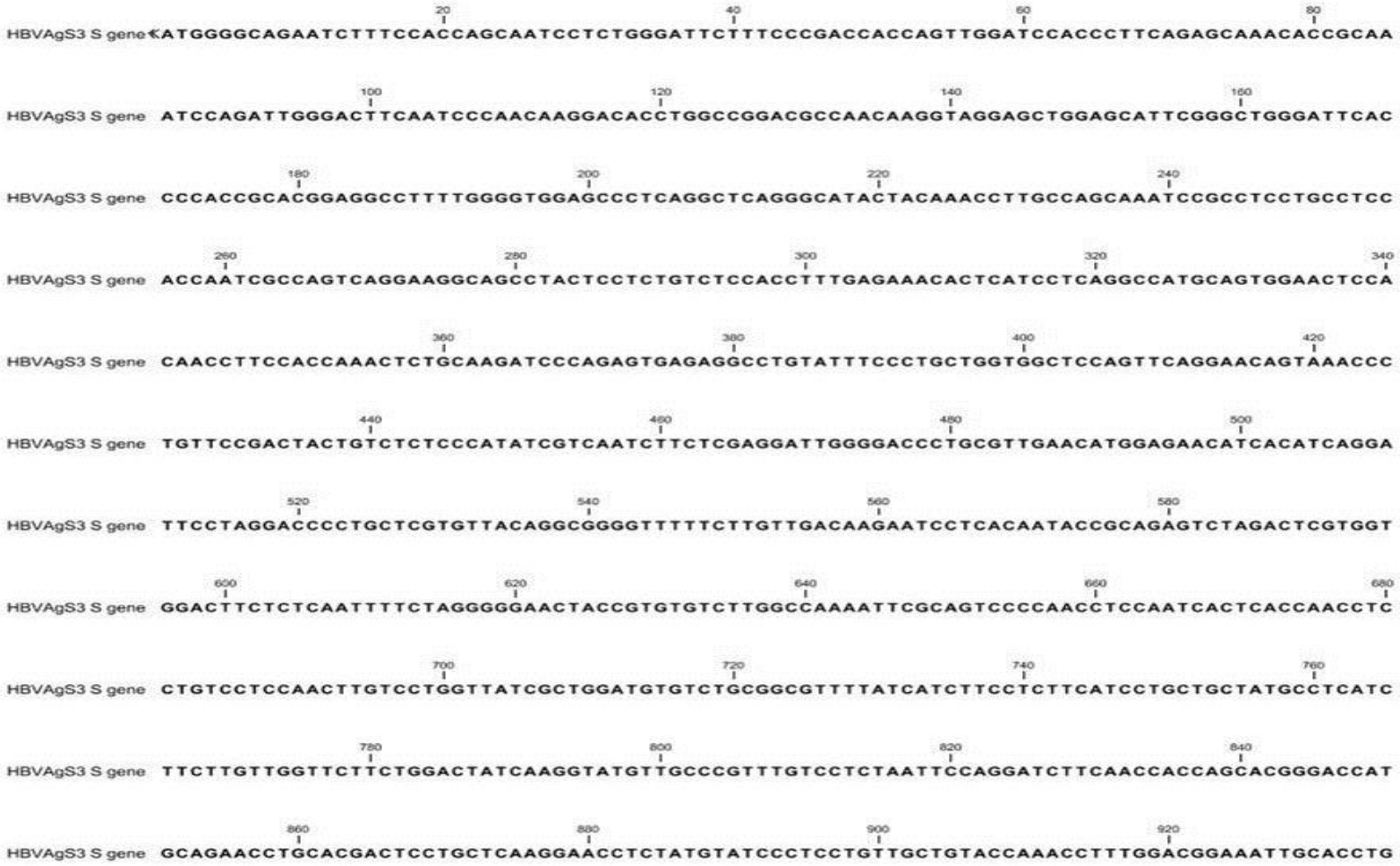


Fig. (9) : Nucleotide sequence of HBV Ag S3.

Fig. (10): Nucleotide sequence of HBV Cor1 (pc/core).

3. Bioinformatics analysis:

Twenty out of hundred HBV DNA samples that were positive by complete HBsAg (pres1/pres2/s) and HBcoreAg PCR, using previously published primers. Three selected isolates were chosen to be amplified for the HBV. The results of sequence of the new isolate samples were compared with 8 reference HBV /A to H genotypes. The percent of divergence and identity between them was reported (all reference sequences were retrieved from Genbank). Phylogentic tree was constructed with 8 references HBV/A-H genotypes to identify the genotype of the isolates. Additional phylogenetic tree was constructed against 87 reference genotyped HBV /D were retrieved from GenBank to confirm that the new isolates are fall under the subgenotype D. Another alignment between the new isolates with circulated HBV/D in Egypt to clear the mutation between the Egyptian isolates retrieved from Genbank with accession number showed in table (3). The percent of divergence and identity between the new isolate samples and circulating HBV/D in Egypt was reported. Phylogenetic tree constructed between references HBV/D, new isolates and circulating HBV/D in Egypt clearing the relation between all isolate. Because of the important of DNA translation sequence to protein to determine the effect of mutation in DNA on protein composition alignment between protein translations of reference HBV/D, new isolate and circulating HBV/D in Egypt was clearing the differences between them. In the following we will present the results of HBsAg (pres1/pres2/s) and HBcAg (pre core /Core) .

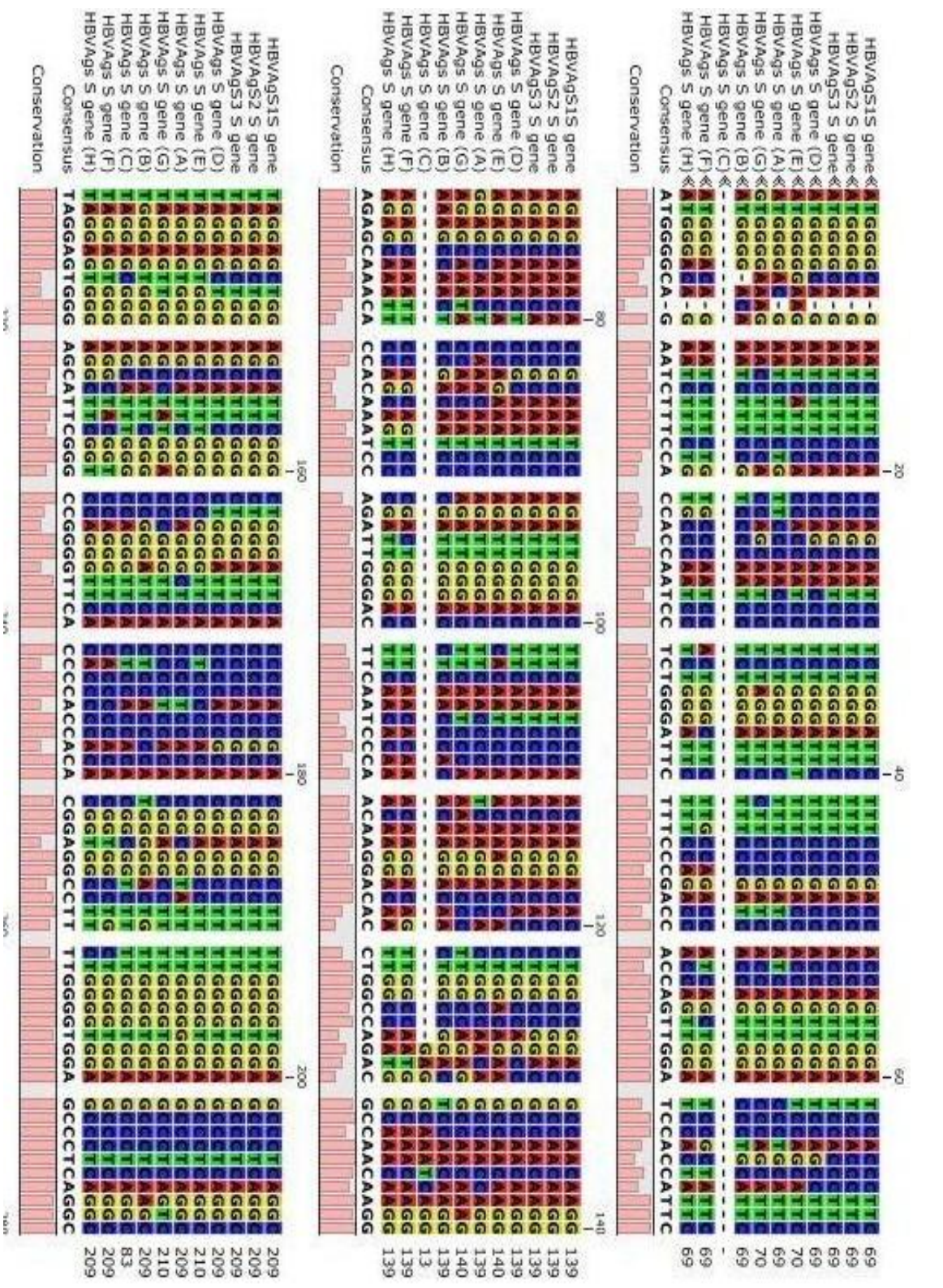
3.1. Bioinformatics analysis of Surface antigen:

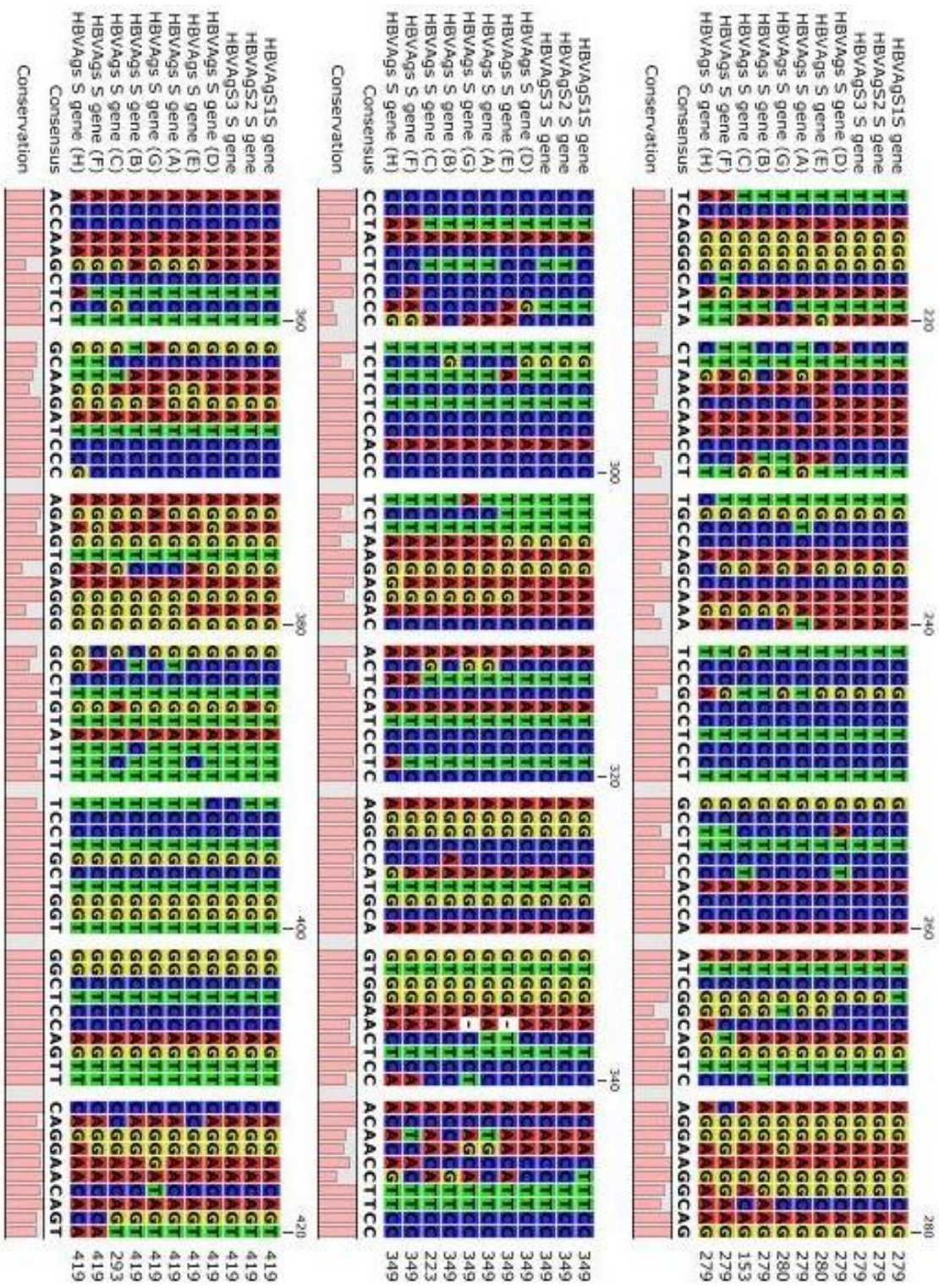
3.1.1. Alignment Surface antigen with HBV/A-H reference:

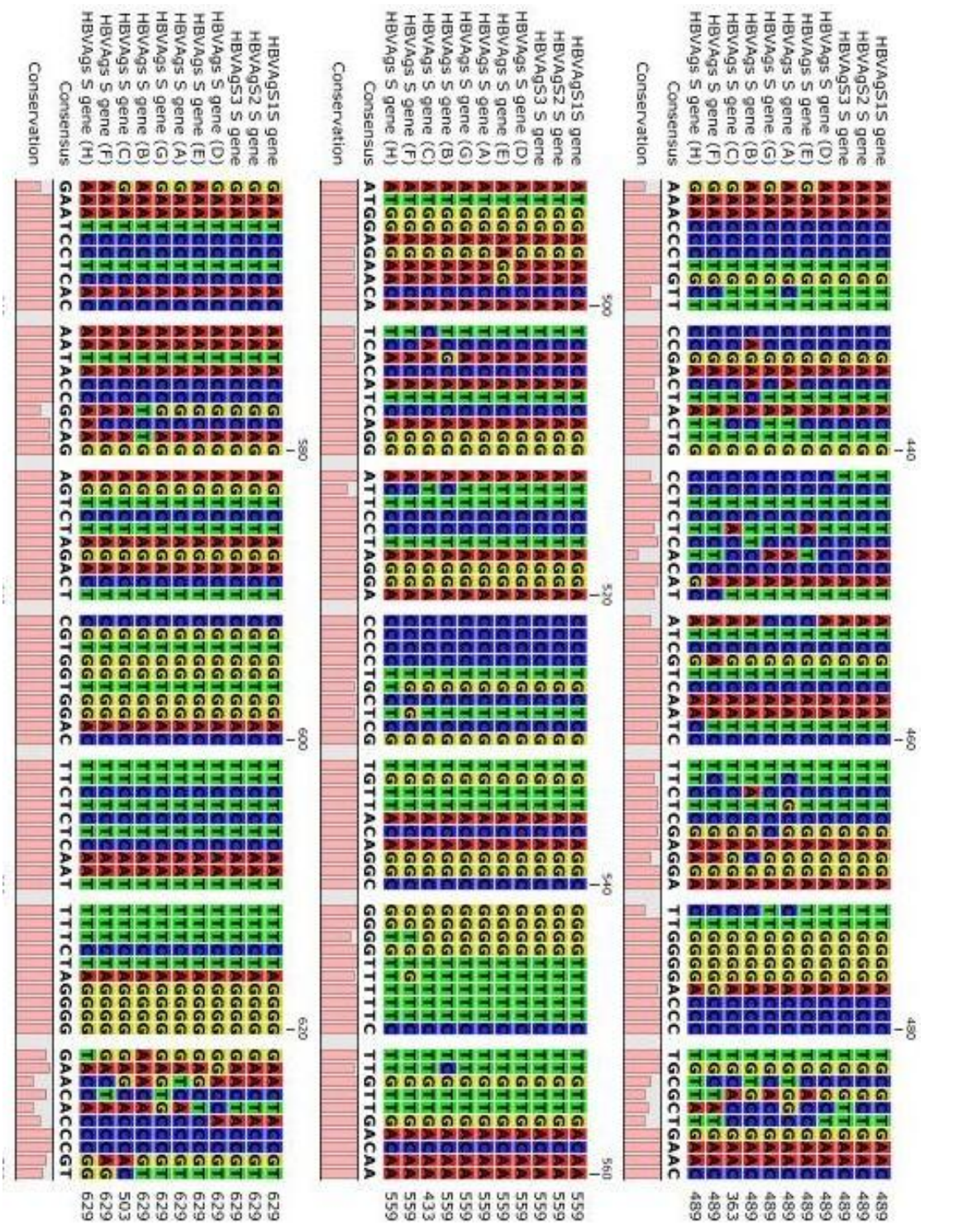
A genetic classification of HBV strains based on nucleotide divergences of 8% or more between the strains, enabled the identification of eight groups of clones and designated A to H genotypes. Using the nucleotide sequence of the partial genome, surface antigen was performed on three HBV isolates from Egyptian patient. In this study, the Surface antigens of representatives of HBV Ag S1 to HBV Ag S3 were sequenced to establish their phylogenetic relationship to the genomic groups.

To genotype HBV accurately and robustly, sequence comparisons of the partial genomic sequences of the HBsAg isolates, sequence alignment was performed for surface antigen using Megalign (DNASTAR, Window version 3.12e).

Multiple-alignment algorithm in Megalign (DNASTAR, Window version 3.12e) populated with sequence, surface antigen sequence of the HBV, obtained from Egyptian patient with sequences of eight reference genotypes (A to H), sequences retrieved from the GenBank, (Fig. 11). Our present isolates showed variations among all published sequences with close related to genotype D.


HBVAgS1S gene
HBVAgS2 S gene
HBVAgS3 S gene
HBVAgs S gene (D)
HBVAgs S gene (E)
HBVAgs S gene (A)
HBVAgs S gene (G)
HBVAgs S gene (B)
HBVAgs S gene (C)
HBVAgs S gene (F)
HBVAgs S gene (H)
Consensus
Conservation


HBVAgS1S gene
HBVAgS2 S gene
HBVAgS3 S gene
HBVAgs S gene (D)
HBVAgs S gene (E)
HBVAgs S gene (A)
HBVAgs S gene (G)
HBVAgs S gene (B)
HBVAgs S gene (C)
HBVAgs S gene (F)
HBVAgs S gene (H)
Consensus
Conservation
220
240
260
280
300
320
340
360
380
400
420


HBVAgS1S gene
HBVAgS2 S gene
HBVAgS3 S gene
HBVAgs S gene (D)
HBVAgs S gene (E)
HBVAgs S gene (A)
HBVAgs S gene (G)
HBVAgs S gene (B)
HBVAgs S gene (C)
HBVAgs S gene (F)
HBVAgs S gene (H)
Consensus
Conservation
AAACCCTGTT CCGACTACTG CCTCTCACAT ATCGTCAATC TTCTCGAGGA TTGGGGGACCC TGCCGCTGAAC
ATGGAGAACA TCACATCAGG ATTCCTAGGA CCCCTGCTCG TGTTACAGGC GGGGGTTTTTC TTGTTGACAA
GAATCCTCAC AATACCGCAG AGTCTAGACT CGTGGTGGAC TTCTCTCAAT TTTCTAGGGG GAACACCCGT
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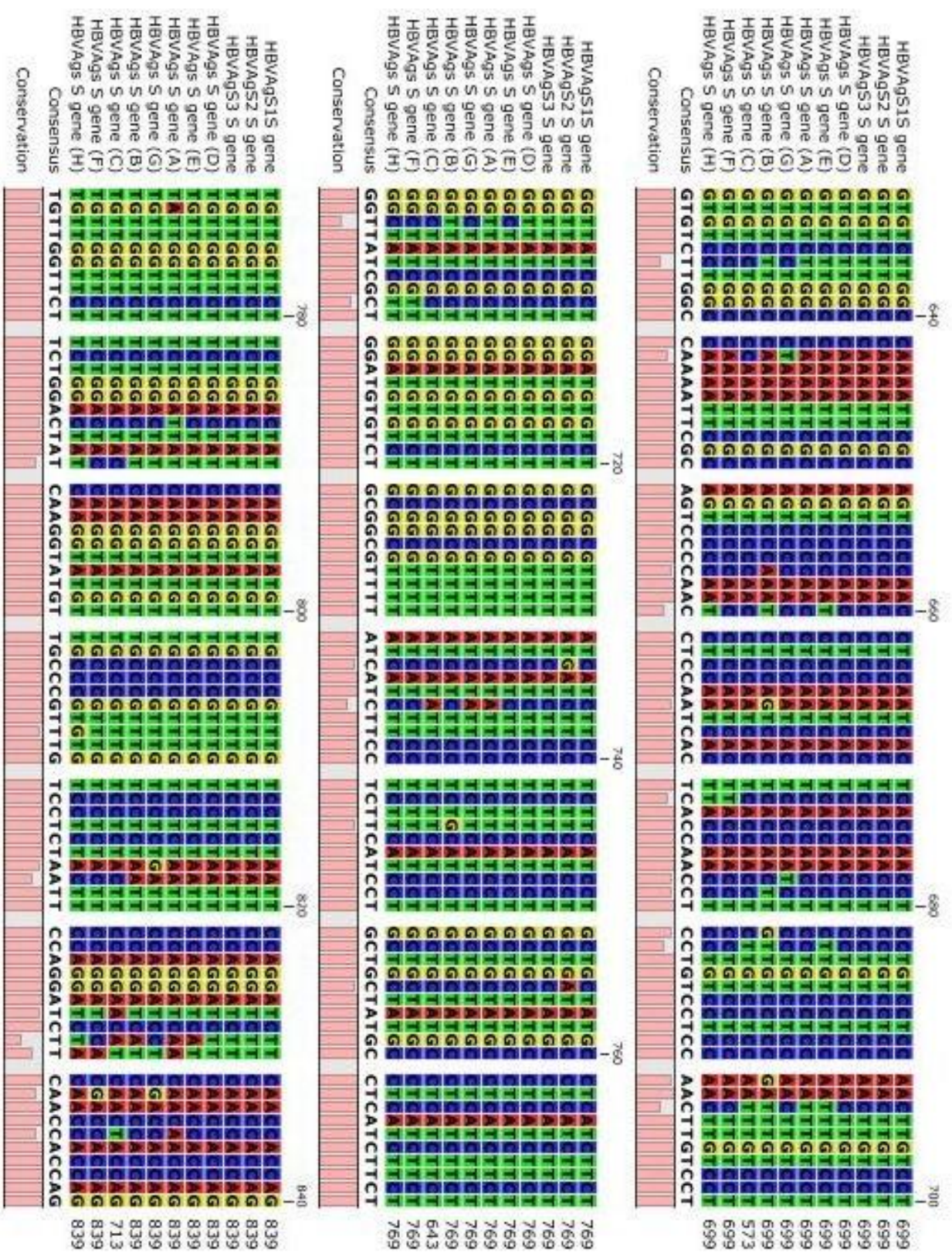
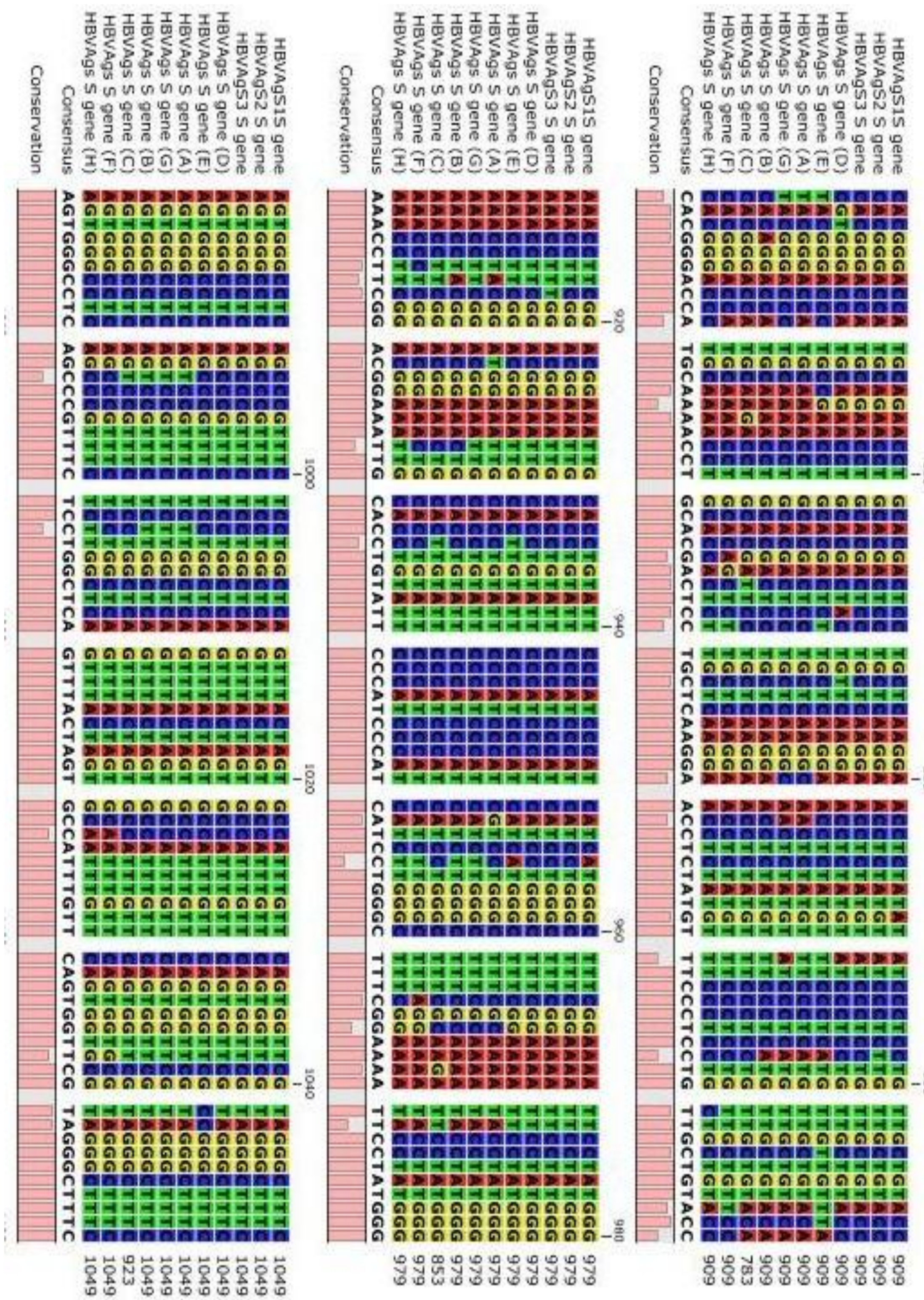
559
629
503
363
433


HBVAgS1S gene
HBVAgS2 S gene
HBVAgS3 S gene
HBVAgs S gene (D)
HBVAgs S gene (E)
HBVAgs S gene (A)
HBVAgs S gene (G)
HBVAgs S gene (B)
HBVAgs S gene (C)
HBVAgs S gene (F)
HBVAgs S gene (H)
Consensus
Conservation
GTGTCTTGGC CAAAATTCGC AGTCCCCAAC CTCCAATCAC TCACCAACCT CCTGTCCTCC AACTTGTCCT
GGTTATCGCT GGATGTGTCT GCGGCGTTTT ATCATCTTCC TCTTCATCCT GCTGCTATGC CTCATCTTCT
TGTTGGTTCT TCTGGACTAT CAAGGTATGT TGCCCGTTTG TCCTCTAATT CCAGGATCTT CAACCACCAG


HBVAgS1S gene
HBVAgS2 S gene
HBVAgS3 S gene
HBVAgs S gene (D)
HBVAgs S gene (E)
HBVAgs S gene (A)
HBVAgs S gene (G)
HBVAgs S gene (B)
HBVAgs S gene (C)
HBVAgs S gene (F)
HBVAgs S gene (H)
Consensus
Conservation
CACGGGACCA TGCAAAACCT GCACGACTCC TGCTCAAGGA ACCTCTATGT TTCCCTCCTG TTGCTGTACC
AAACCTTCGG ACGGAAATTG CACCTGTATT CCCATCCCAT CATCCTGGGC TTTCGGAAAA TTCCTATGGG
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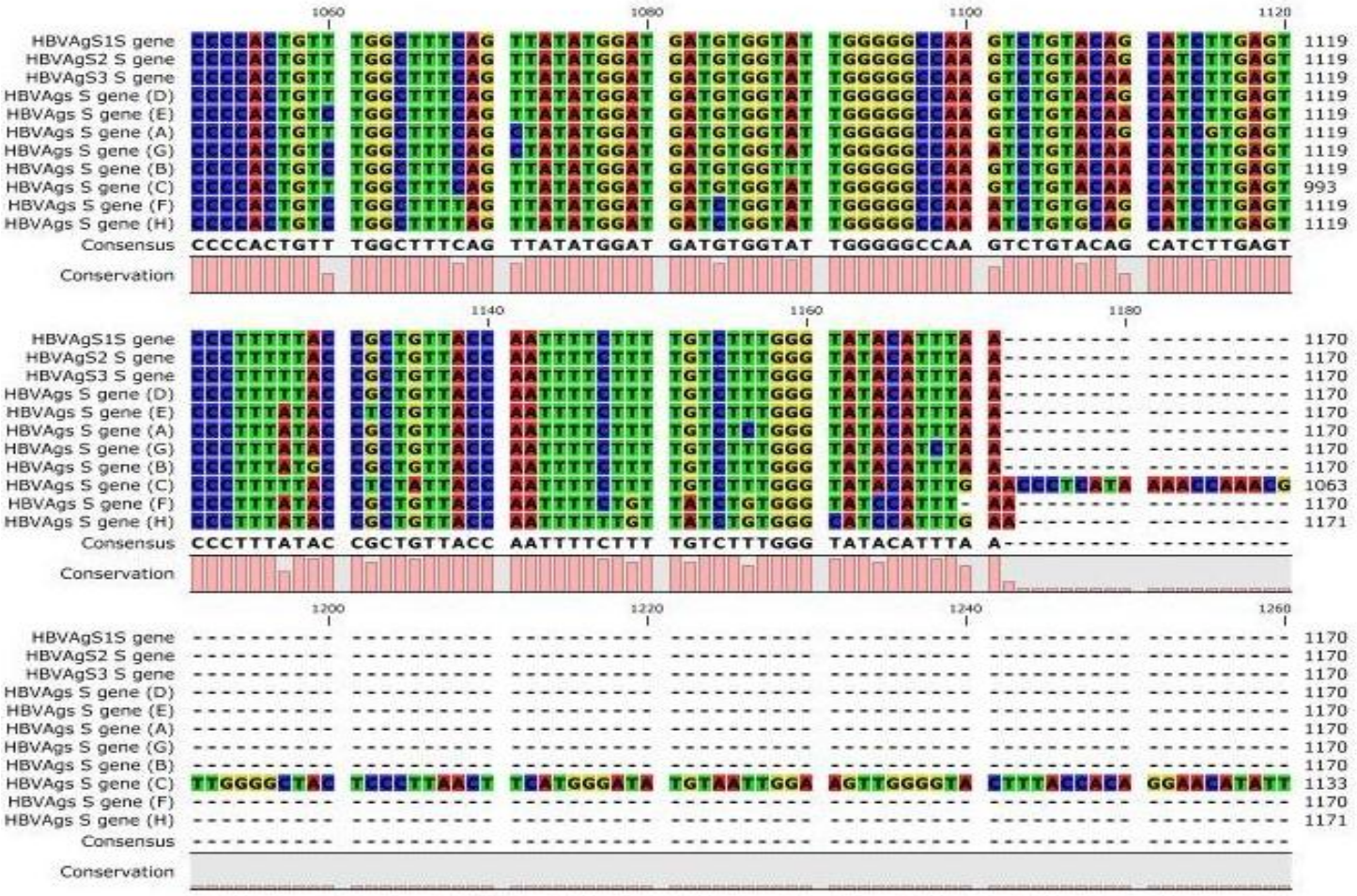


Fig. (11): Alignment of HBVsAg(pres1/pres2/s) gene sequences of the new isolates and eight reference genotypes (A, B, C, D, E, F, G and H) retrieved from GenBank.

Nucleotide identity and divergence between the three isolates (HBV Ag S1, HBV Ag S2 and HBV AgS3) region sequence corresponding to eight human HBV strains belonging to genotypes A to H were determined in Table (3). The percent nucleotide identity between the Egyptian strains and genotype D was ranged from 97.9 to 98.2% indicating how closely related with divergence rang from 1.8 to 2.1 %. According to divergence and identity percent of the three isolates, it showed the close relatedness to each other by recording 0.9% divergence between each other and identity reached 99.2%.

Table (3): Mean percent nucleotide identity and divergence between the surface antigen sequence of the new isolates and eight human HBV strains belonging to genotypes A to H retrieved from GenBank.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		99.2	99.1	98.0	76.4	88.0	90.3	90.6	26.0	77.0	87.4	1	HBVAgS1S gene-1.seq
2	0.8		99.2	97.9	76.2	87.8	90.4	90.6	25.7	76.7	87.3	2	HBVAgS2 S gene-1.seq
3	0.9	0.8		98.2	76.2	87.9	90.3	90.8	25.6	76.8	87.4	3	HBVAgS3 S gene-1.seq
4	2.0	2.1	1.8		75.9	87.5	90.3	90.0	25.6	76.2	87.3	4	HBVAgs S gene (D)-1.seq
5	28.5	28.9	28.9	29.3		72.8	76.6	75.1	26.9	91.5	72.8	5	HBVAgs S gene (G).seq
6	13.1	13.4	13.3	13.7	33.9		87.5	86.8	26.1	73.1	94.5	6	HBVAgs S gene (H).seq
7	10.5	10.3	10.4	10.5	28.2	13.7		91.0	25.9	75.5	87.1	7	HBVAgs S gene (A).seq
8	10.1	10.1	9.9	10.8	30.4	14.5	9.6		25.3	75.2	86.0	8	HBVAgs S gene (B).seq
9	350.0	350.0	350.0	350.0	364.2	350.0	350.0	350.0		27.9	26.3	9	HBVAgs S gene (C).seq
10	27.6	28.0	27.9	28.7	9.0	33.5	29.9	30.3	268.5		72.8	10	HBVAgs S gene (E).seq
11	13.8	14.0	13.9	14.0	33.9	5.7	14.2	15.6	374.3	33.9		11	HBVAgs S gene (F).seq
	1	2	3	4	5	6	7	8	9	10	11		

The phylogenetic analysis of the surface antigen of the three isolated sequences with eight GenBank HBV genotypes (A to H) references sequences (Fig 12) showed eight distinct clusters corresponding to the HBV genotypes (A to H), however genotype D, clustered with the three isolated

sequences. Phylogenetic tree of the three sequences generated in this study with this tree, established that the new isolates are relatively tight to subtype HBV/D.

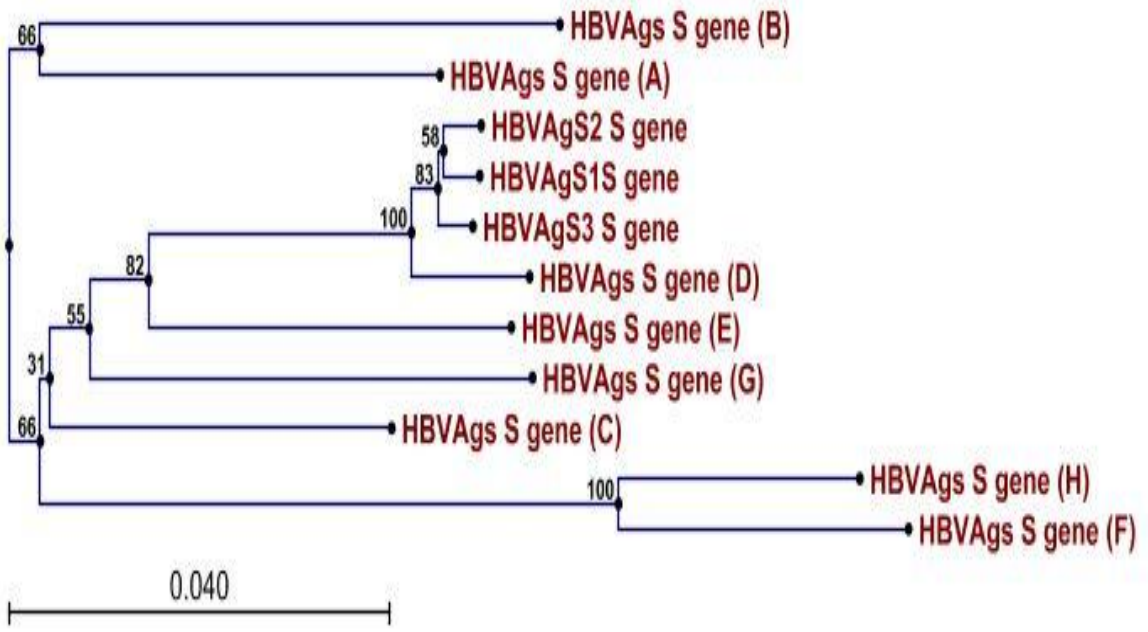


Fig. (12): Phylogenetic tree constructed of the surface antigen of HBV AgS1, HBV AgS2 and HBV AgS3 using the entire nucleotide sequences (A to H) genotypes retrieved from GenBank. Genetic distance is indicated below the tree.

Additional phylogenetic tree was constructed using the entire nucleotide sequences against 87 reference sequence genotyped HBV/D from different countries, were retrieved from GenBank. Reference sequences are denoted by accession numbers and the country of origin. The HBV isolates are illustrated in Fig. (13).The new tree confirmed the previous one and indicates that the new isolates are fall under the subgenotype D.

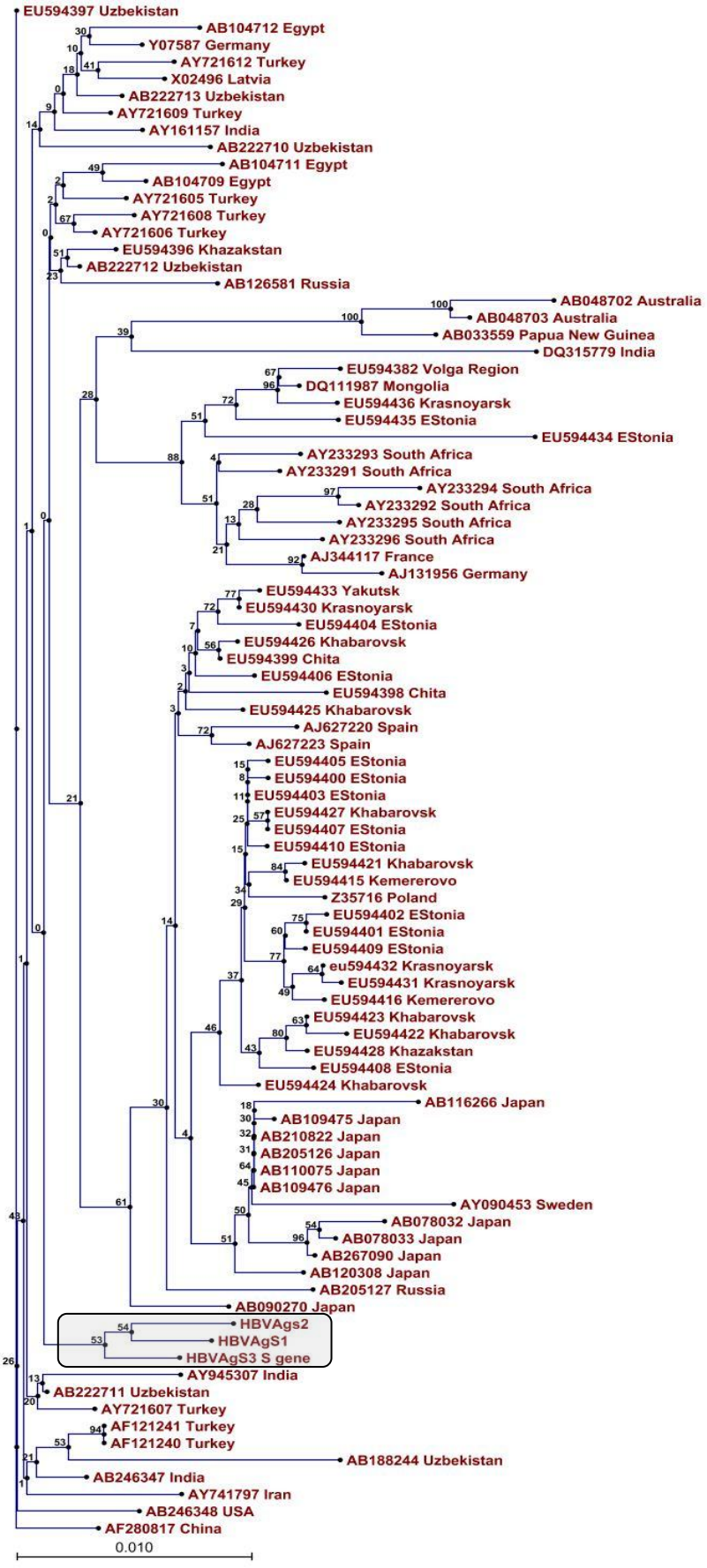


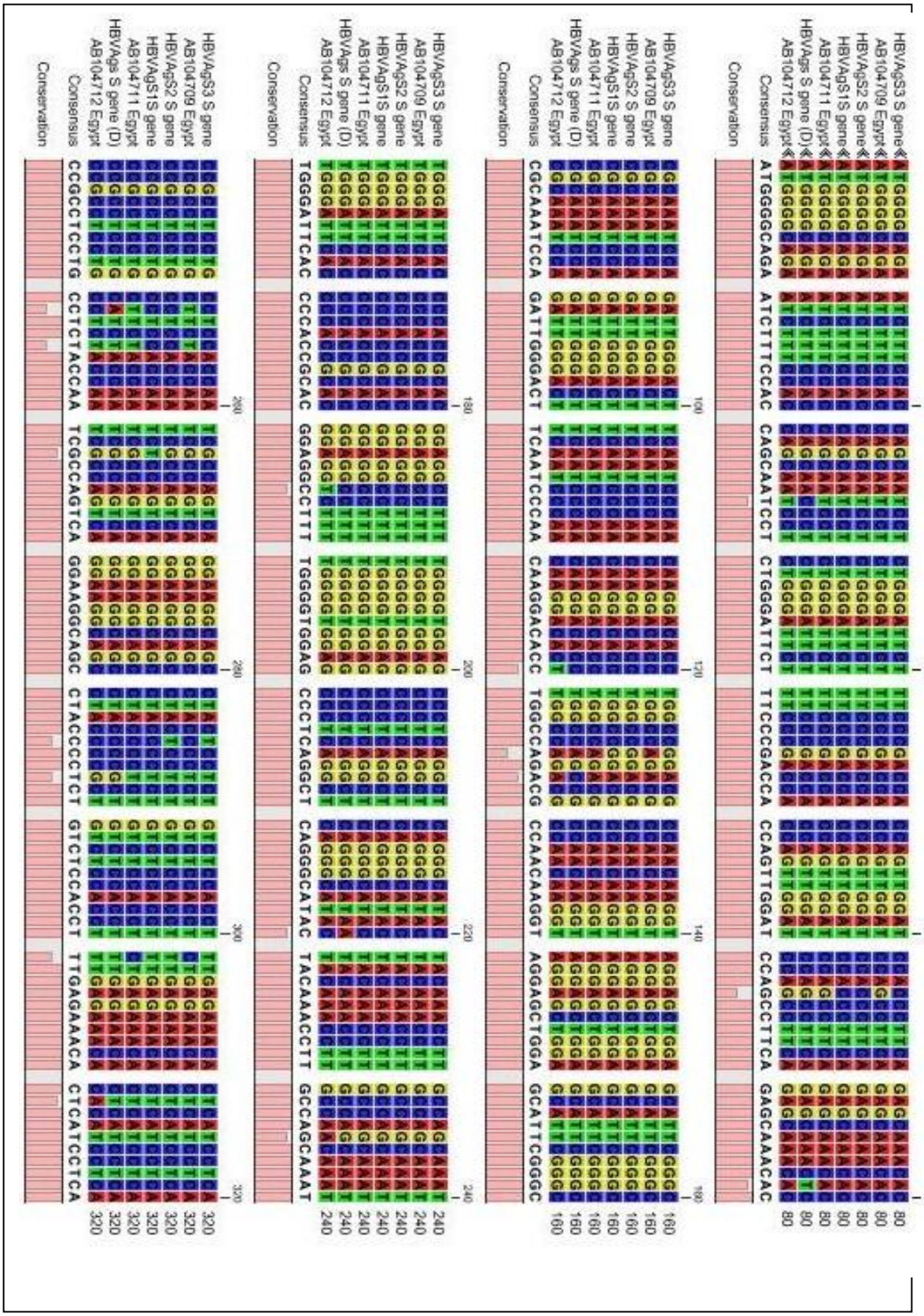
Fig. (13): Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present three isolates and 87 HBV/D isolates retrieved from DDBJ/GenBank. Reference sequences are denoted by accession numbers and the country of origin. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

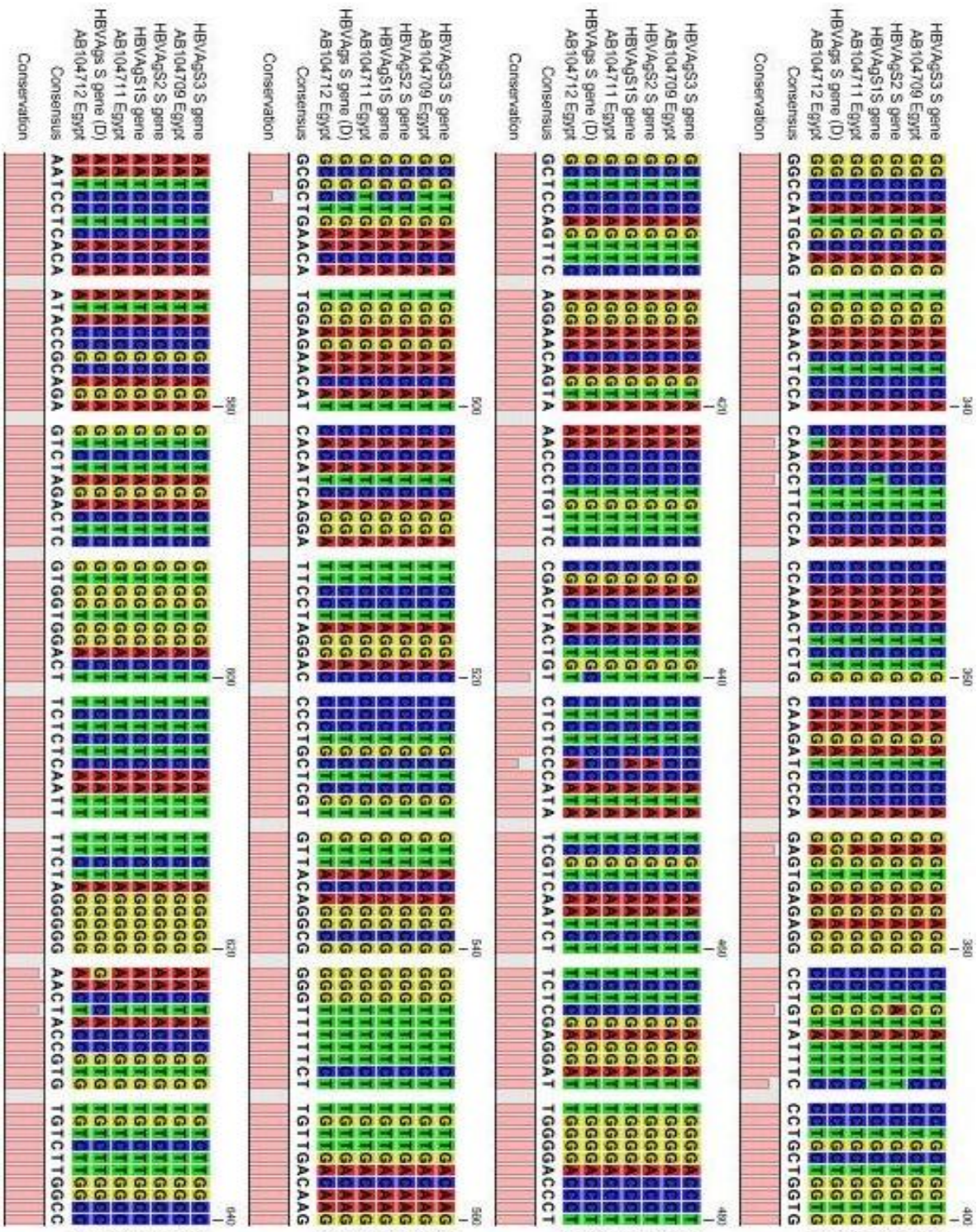
3.1.2. Alignment of isolated Egyptian HBV 2010 with currently circulated HBV/D in Egypt of Surface antigen:

To determine whether the isolated Egyptian HBV is related to the currently circulating strains, alignment of nucleotide sequences of the surface antigen of the isolated Egyptian 2010 with comparison to previously isolated Egyptian reference sequences and their accession numbers that submitted to GenBank database were shown in table (4) with comparison to the related genotype D (Fig. 14). The isolated Egyptian HBV 2010 is much more related to the isolated Egyptian HBV strains that submitted to GenBank database with the accession numbers at table (4) with some mutation which reported in table (5) for HBVAgS1 at nt. 204, 873, 889 and share HBV AgS2 at nt. 391. HBVAgS2 characterized by mutation at nt. 385, 733, 873 and 898 and share HBVAg3 at nt. 286. HBVAgS3 share isolate Egy.104709 at nt 918, 1110.finally all Egyptian isolate shred mutation at nt. 80, 126, 156, 289, 373, 485, 622, 625,and 842 but Egy.104711 defer from them at nt. 65and 441.

Table (4): The accession numbers of the Egyptian HBV strains that submitted to GenBank database.

Reference Isolated Egyptian strain	Gene sequenced	Accession numbers
Egy. 104709	**HBsAg(Pres1/Pres2/s) genes and HBcAg (pc /core) genes.**	**AB104709**
Egy 104711	**HBsAg(Pres1/Pres2/s) genes and HBcAg (pc /core) genes.**	**AB104711**
Egy 104712	**HBsAg(Pres1/Pres2/s) genes and HBcAg (pc /core) genes.**	**AB104712**


HBVAgS3 S gene
AB104709 Egypt
HBVAgS2 S gene
HBVAgS1S gene
AB104711 Egypt
HBVAgs S gene (D)
AB104712 Egypt
Consensus
Conservation


HBVAgS3 S gene
AB104709 Egypt
HBVAgS2 S gene
HBVAgS1S gene
AB104711 Egypt
HBVAgs S gene (D)
AB104712 Egypt
Consensus
Conservation
340
360
380
400
420
440
460
480
500
520
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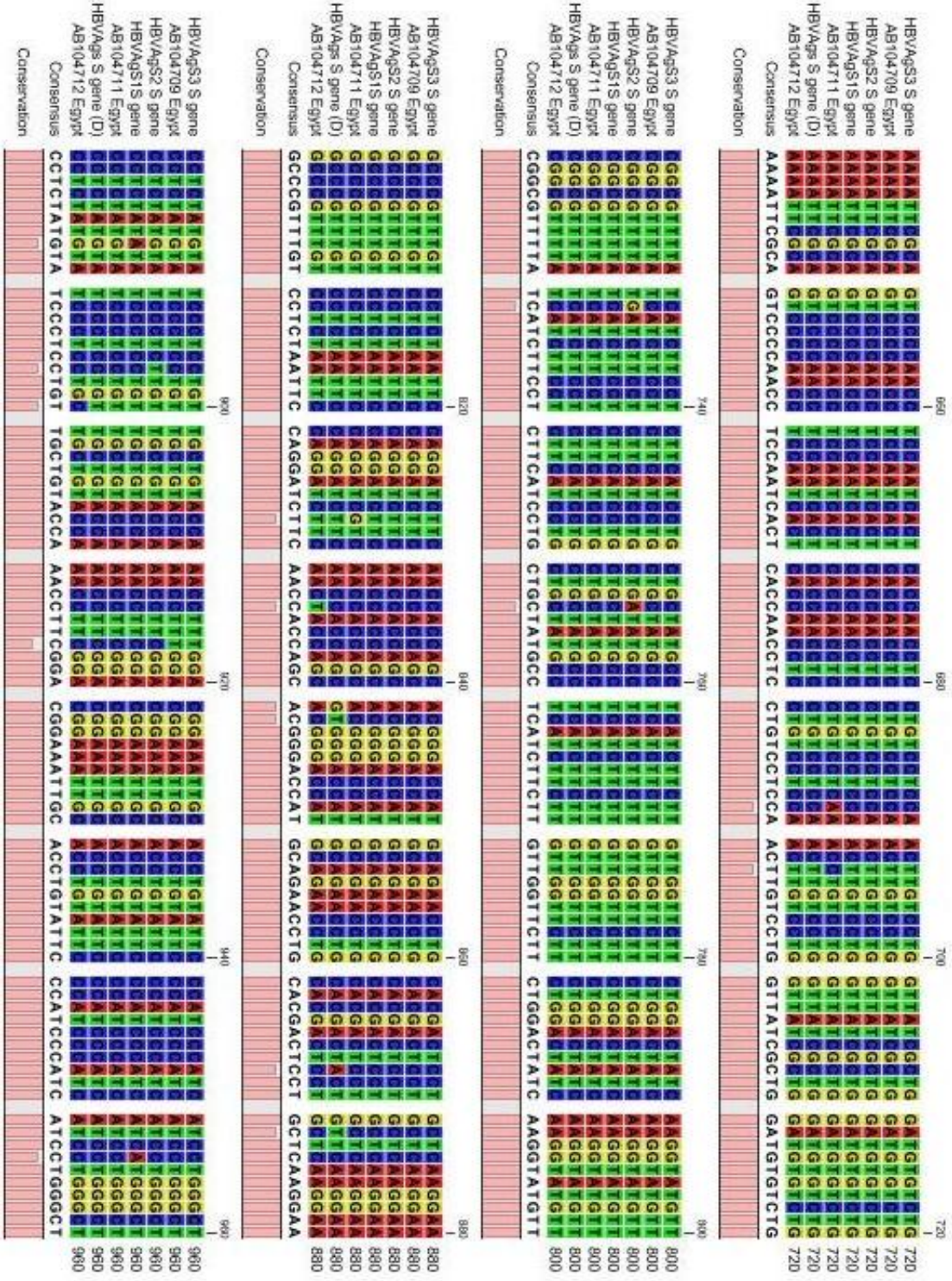
560
580
600
620
640


HBVAgS3 S gene
AB104709 Egypt
HBVAgS2 S gene
HBVAgS1S gene
AB104711 Egypt
HBVAgs S gene (D)
AB104712 Egypt
Consensus
Conservation
720
740
760
780
800
820
840
860
880
900
920
940
960

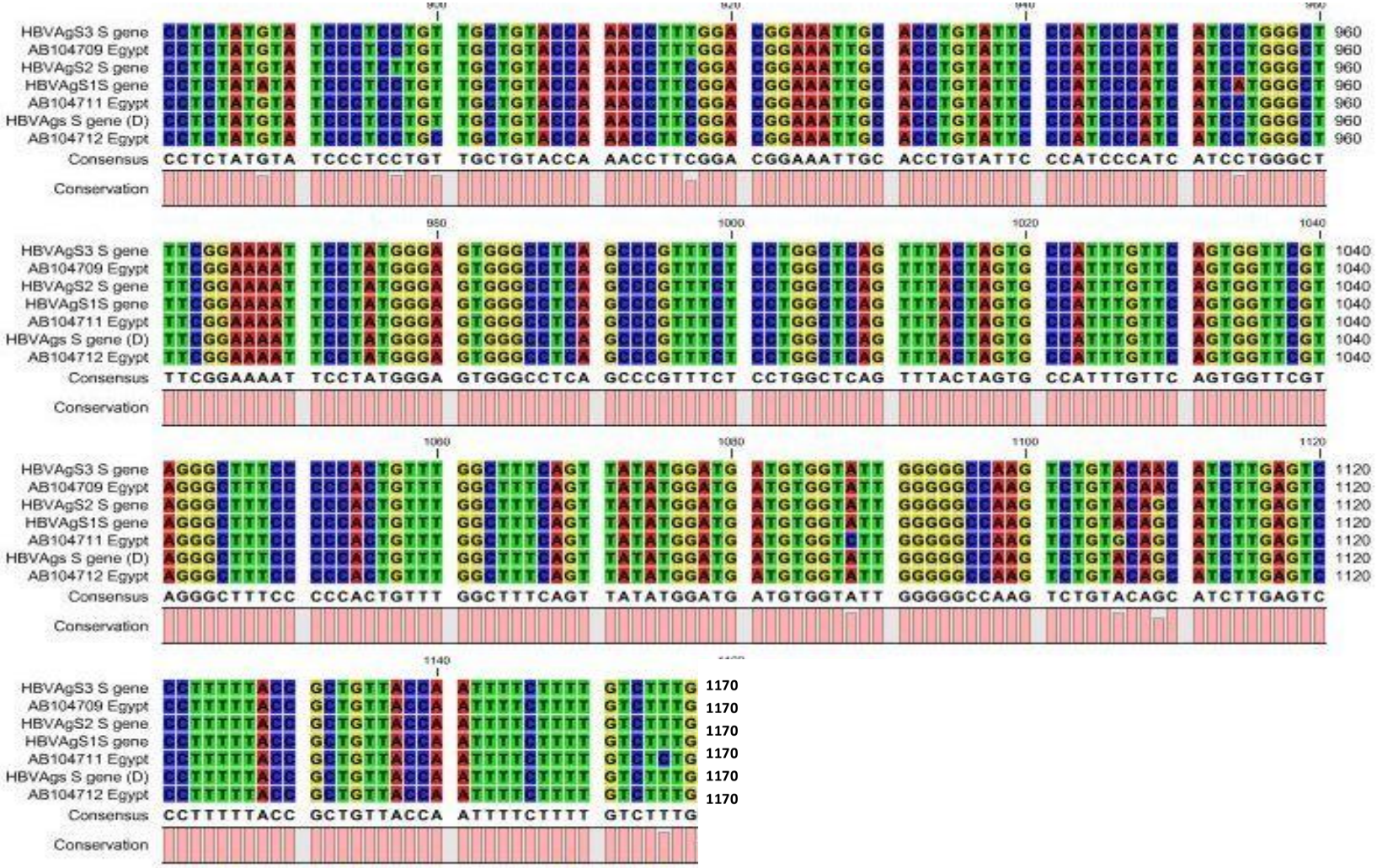


Fig. (14): Alignment of nucleotide sequences of HBsAg region of the isolated Egyptian HBV with comparison to the isolated Egyptian HBV strains that submitted to GenBank database with the accession numbers (Egy. 104709, Egy 104711 and Egy 104712) and genotype B and D.

Table (5) : Positions of mutations at Nucleotide level between the circulating HBV/D in Egypt and reference HBV/D with HBV AgS1, HBV Ag S2 and HBV AgS3 isolates.

Genotype / Nucleotide Position	HBV/D	HBV AgS1	HBV AgS2	HBV AgS3	Egy.104709.	Egy 104711	Egy 104712
65	G	C	C	C	C	G	C
80	T	A	A	A	A	A	A
126	A	G	G	G	G	A	G
156	T	C	C	C	C	T	C
204	G	T	G	G	G	G	G
286	C	C	T	T	T	C	T
289	G	T	T	T	T	T	T
302	T	T	T	T	T	C	T
343	A	A	A	A	A	A	T
373	G	A	A	A	A	A	A
385	G	G	A	G	G	G	G
391	C	T	T	C	C	C	C
441	C	T	T	T	T	T	C
485	T	C	C	C	C	T	C
622	G	A	A	A	A	A	A

Genotype / Nucleotide Position	HBV/D	HBV AgS1	HBV AgS2	HBV AgS3	Egy.104709.	Egy 104711	Egy 104712
625	C	T	T	T	T	T	T
694	T	T	T	T	T	C	T
733	C	C	G	C	C	C	C
829	T	T	T	T	T	G	T
835	C	C	C	C	C	C	T
842	G	A	A	A	A	A	A
873	T	G	C	C	C	C	C
889	G	A	G	G	G	G	G
898	C	C	T	C	C	C	C
901	T	T	T	T	T	T	C
918	C	C	C	T	T	C	C
1117	A	A	A	A	A	G	A
1110	G	G	G	A	A	G	G
1156	T	T	T	T	T	C	T

Nucleotide identity between the new isolate samples and circulating HBV/D in Egypt was averaged from 98.4 to 99.5 % indicating how closely related, while divergence was ranged between 0.5 - 1.7%. The percent nucleotide identity between as shown in (Table 6). Nucleotide identity and divergence percentage between the isolated Egyptian S gene with genotype D were ranged from 97.9% to 98.1% and from 1.8% to 2.1% respectively.

Table (6): Nucleotide identity and divergence percent between surface antigen sequence of the new isolates HBV AgS1 , HBV AgS2 and HBV AgS3 with genotype D and the three circulating Egyptian HBV strains with the accession numbers (Egy. 104709, Egy 104711 and Egy. 104712).

Percent Identity

	1	2	3	4	5	6	7		
1		99.3	98.8	98.8	98.7	99.5	98.4	1	AB104709 Egypt-1.seq
2	0.7		98.4	98.4	98.4	98.8	98.1	2	AB104711 Egypt-1.seq
3	1.2	1.6		98.6	98.5	98.6	98.2	3	AB104712 Egypt-1.seq
4	1.2	1.6	1.4		99.2	99.1	97.9	4	HBVAgS1S gene-1.seq
5	1.3	1.7	1.5	0.8		99.2	97.9	5	HBVAgS2 S gene-1.seq
6	0.5	1.2	1.4	0.9	0.8		98.1	6	HBVAgS3 S gene-1.seq
7	1.6	1.9	1.8	2.0	2.1	1.8		7	HBVAgs S gene (D)-1.seq
	1	2	3	4	5	6	7		

Divergence

Phylogenetic analysis was performed on surface antigen sequences representing genotype D with three circulating Egyptian HBV strains with the accession numbers (Egy. 104709, Egy 104711 and Egy. 104712).

The genotypes D determined sequences in this study of the surface antigen region matched to great extend with three patterns (HBV AgS1. HBV AgS2 and HBV Ag S3) obtained with surface gene region sequences.

The three strains were matched exactly with each other and come from the same point. Despite this similarity, genotype D sequences clustered with the all Egyptian HBV strains (Fig. 15). Genetic distance is indicated below the tree. HBV AgS3 was most frequently detected followed by HBV AgS1 and HBV AgS2. All Egyptian clustered with genotype D (Fig. 15).

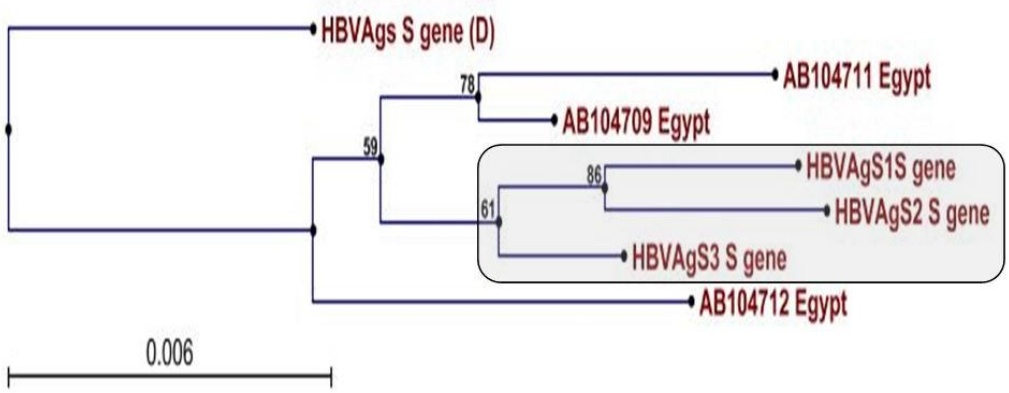
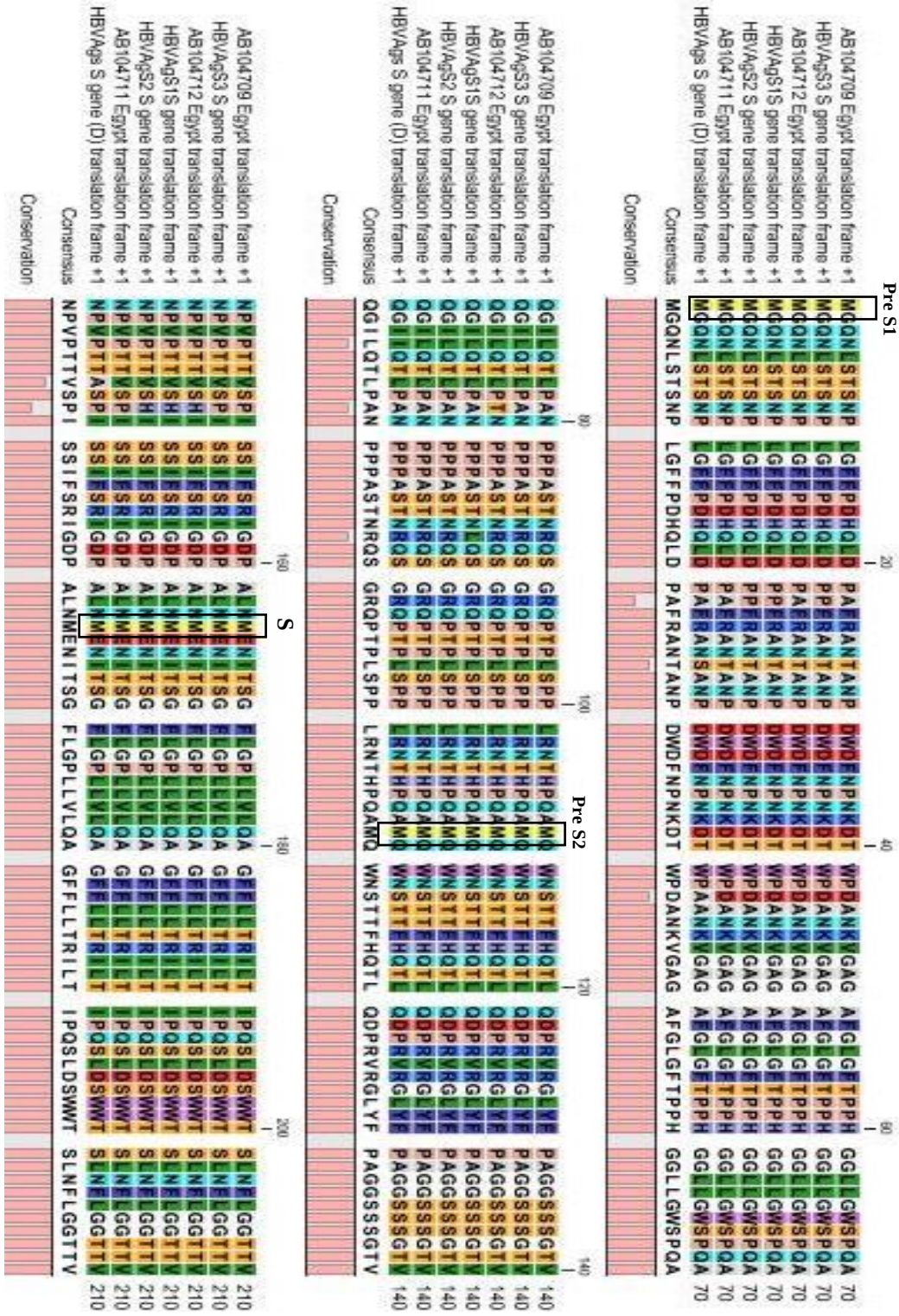


Fig. (15): Phylogenetic tree constructed of the S gene region of the three HBV AgS 2010 representing genotype D with three circulating Egyptian HBV strains with the accession numbers (Egy. 104709, Egy 104711 and Egy. 104712).

At the level of AA, the two Egyptian isolates 2010 HBV (AgS1AgS2 and AgS3) are related to each other with some mutation reported in table (7). It is clear to note that there are six mutations at the same positions recorded in the three isolates and circulating HBV/D in Egypt compared with reference D. HBV AgS1 characterized with the mutation at AA. 88 and 296, HBV AgS2 also characterized by mutation at position 244. On the other hand HBV AgS3 show identical with isolate with accession number (Egy. 104709) at position 306 and 370 except at AA 22 it share the other two new isolates.


Pre S1
Pre S2
S
AB104709 Egypt translation frame +1
HBVAgS3 S gene translation frame +1
AB104712 Egypt translation frame +1
HBVAgS1S gene translation frame +1
HBVAgS2 S gene translation frame +1
AB104711 Egypt translation frame +1
HBVAgs S gene (D) translation frame +1
Consensus
Conservation

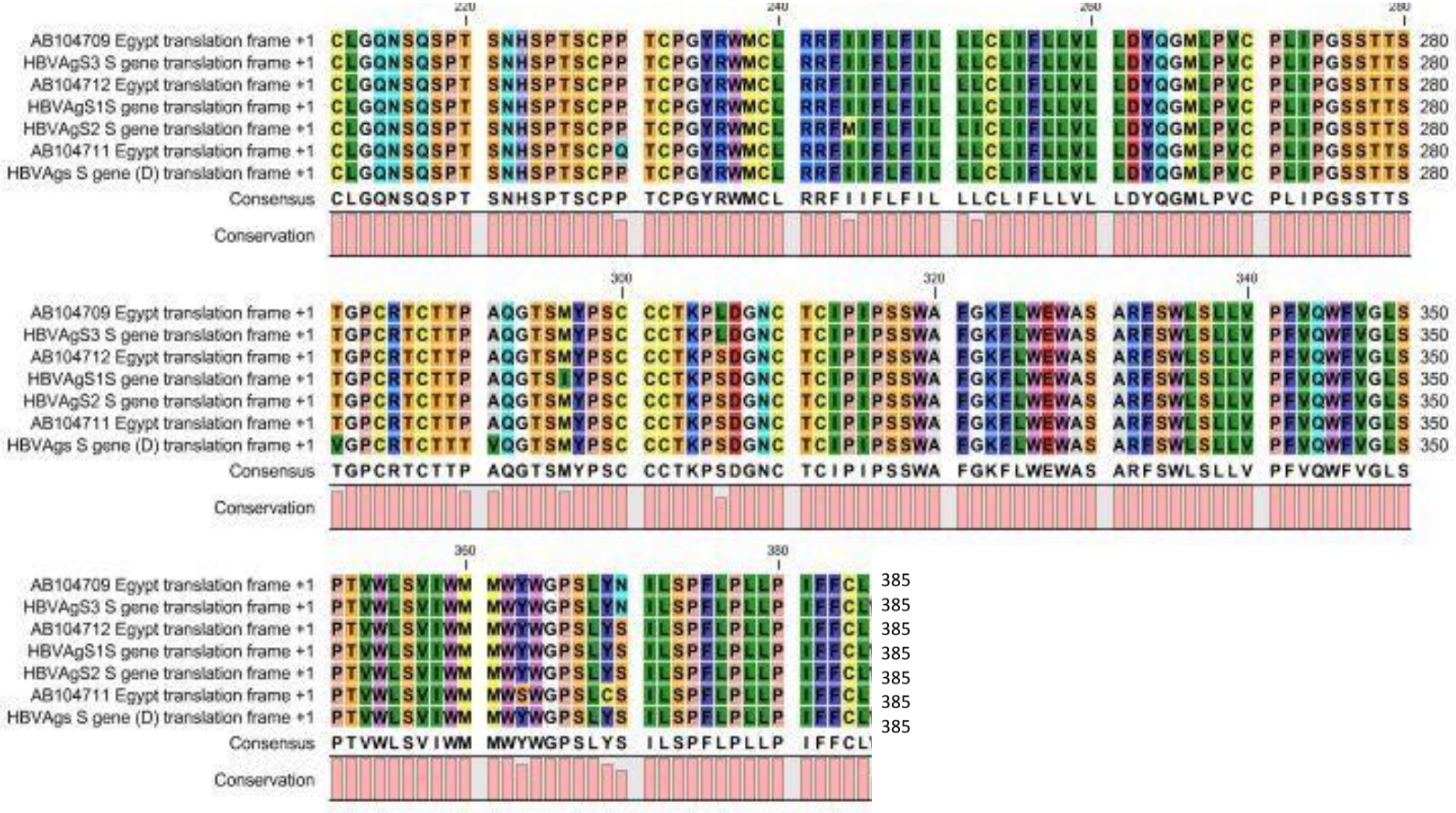


Fig (16): Alignment of deduced amino acid sequences in the HBsAg (pres1/pres2/S) gene of HBV isolates obtained in this study from Egyptian (HBV AgS1, HBV AgS2and HBV AgS3) corresponding to the isolated Egyptian HBV strains with the accession numbers of that submitted to Genbank database with reference genotypes D.

Table (7): Positions of mutations at protein level between HBV AgS1, HBV Ag S2 and HBV AgS3 isolates with the circulating HBV/D in Egypt and reference HBV/D.

Genotype / PROTIEN position		HBV/D	HBV AgS1	HBV AgS2	HBV AgS3	Egy. 104709.	Egy 104711	Egy 104712
Pre S1	22	A	P	P	P	A	A	A
	27	S	T	T	T	T	T	T
	43	A	D	D	D	D	D	D
	79	A	A	A	A	A	A	T
	88	R	L	R	R	R	R	R
Pre S2	147	A	V	V	V	V	V	V
	149	P	H	H	P	P	P	H
S	230	P	P	P	P	P	Q	P
	244	I	I	M	I	I	I	I
	281	V	T	T	T	T	T	T
	290	T	P	P	P	P	P	P
	291	V	A	A	A	A	A	A
	296	M	I	M	M	M	M	M
	306	S	S	S	L	L	S	S
	363	Y	Y	Y	Y	Y	S	Y
	369	Y	Y	Y	Y	Y	C	Y
	370	S	S	S	N	N	S	S

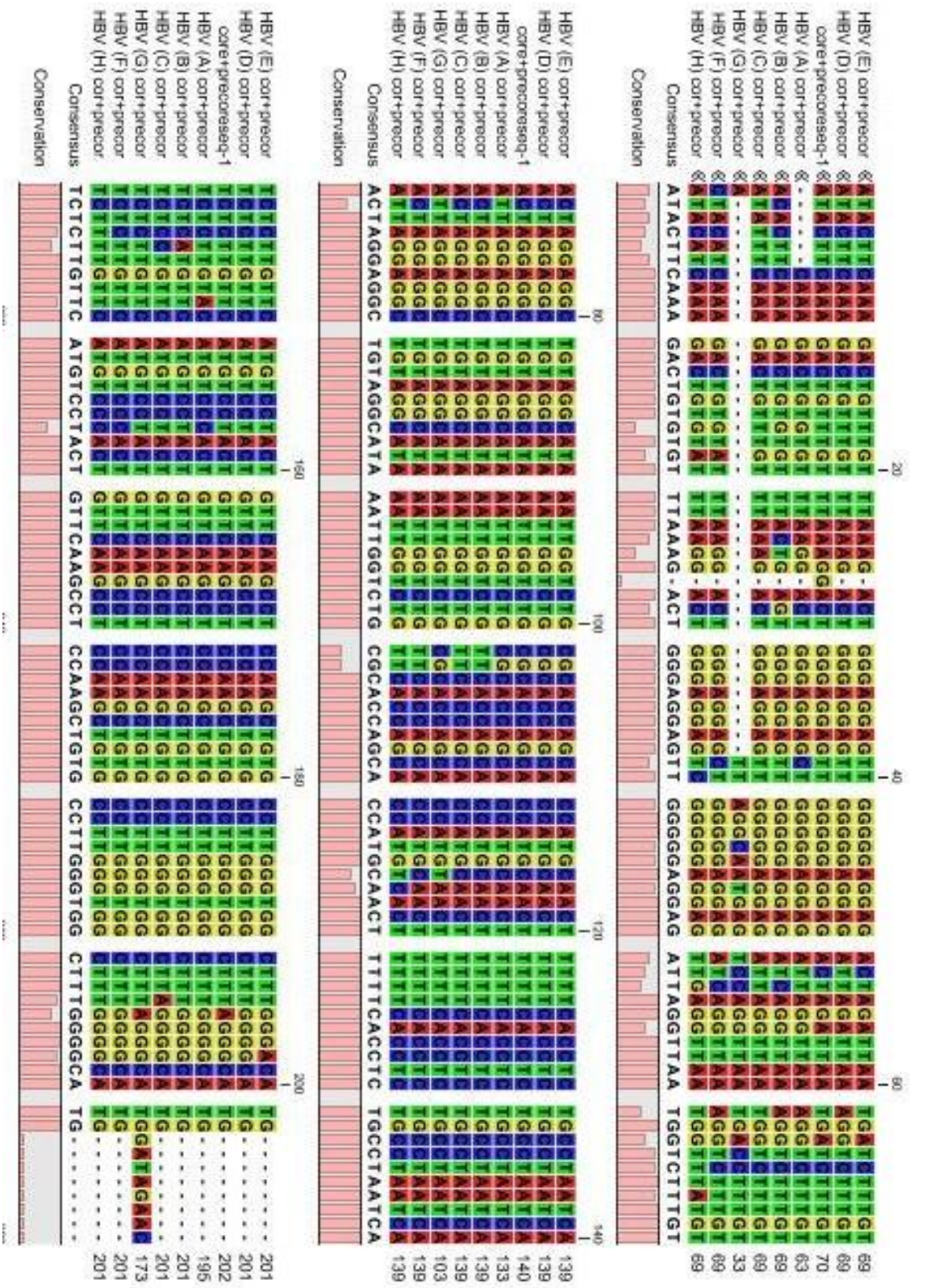
3.2. Bioinformatics analysis of Core antigen:

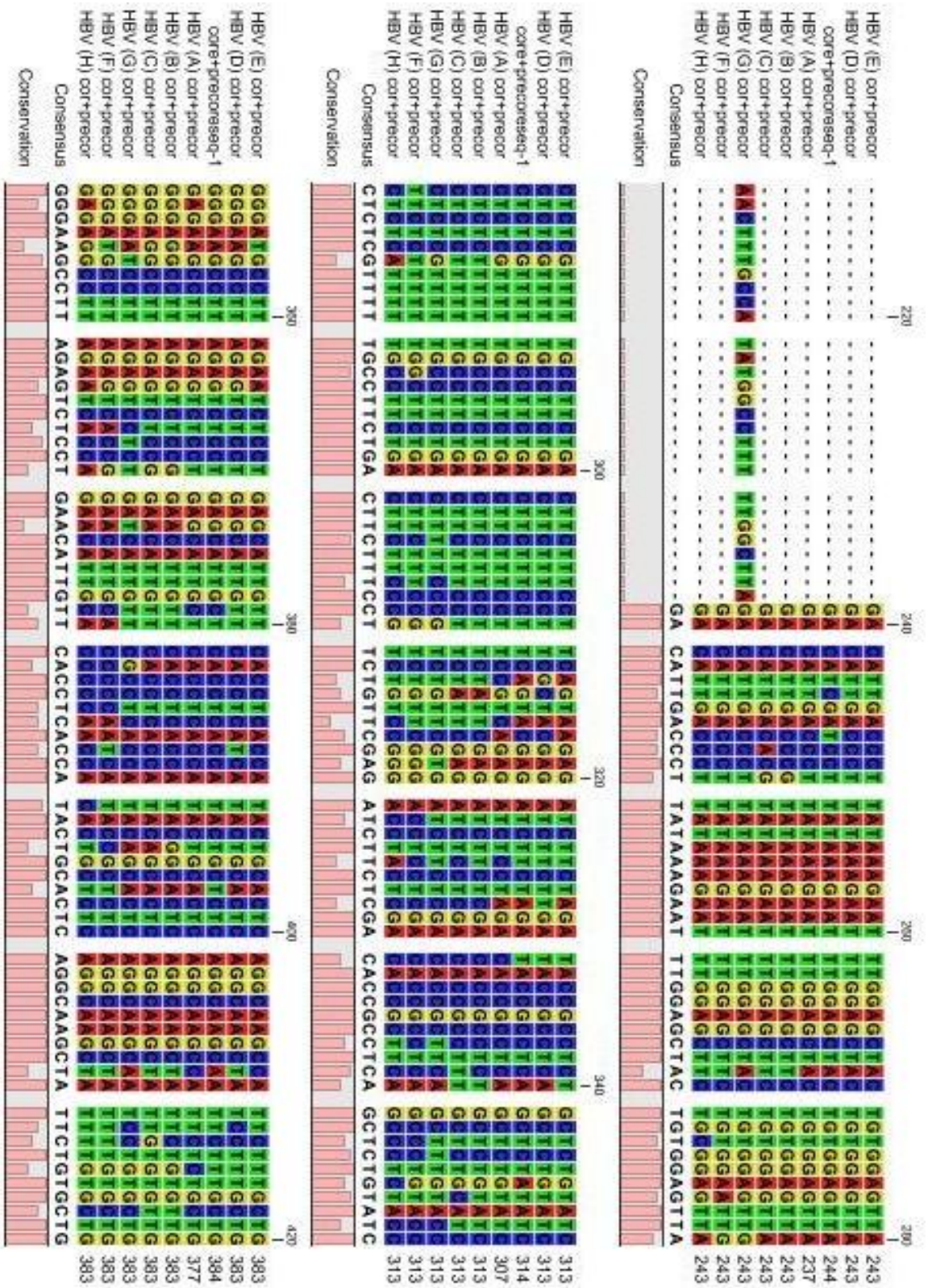
3.2.1. Alignment of Core antigen with HBV/A-H reference genotypes:

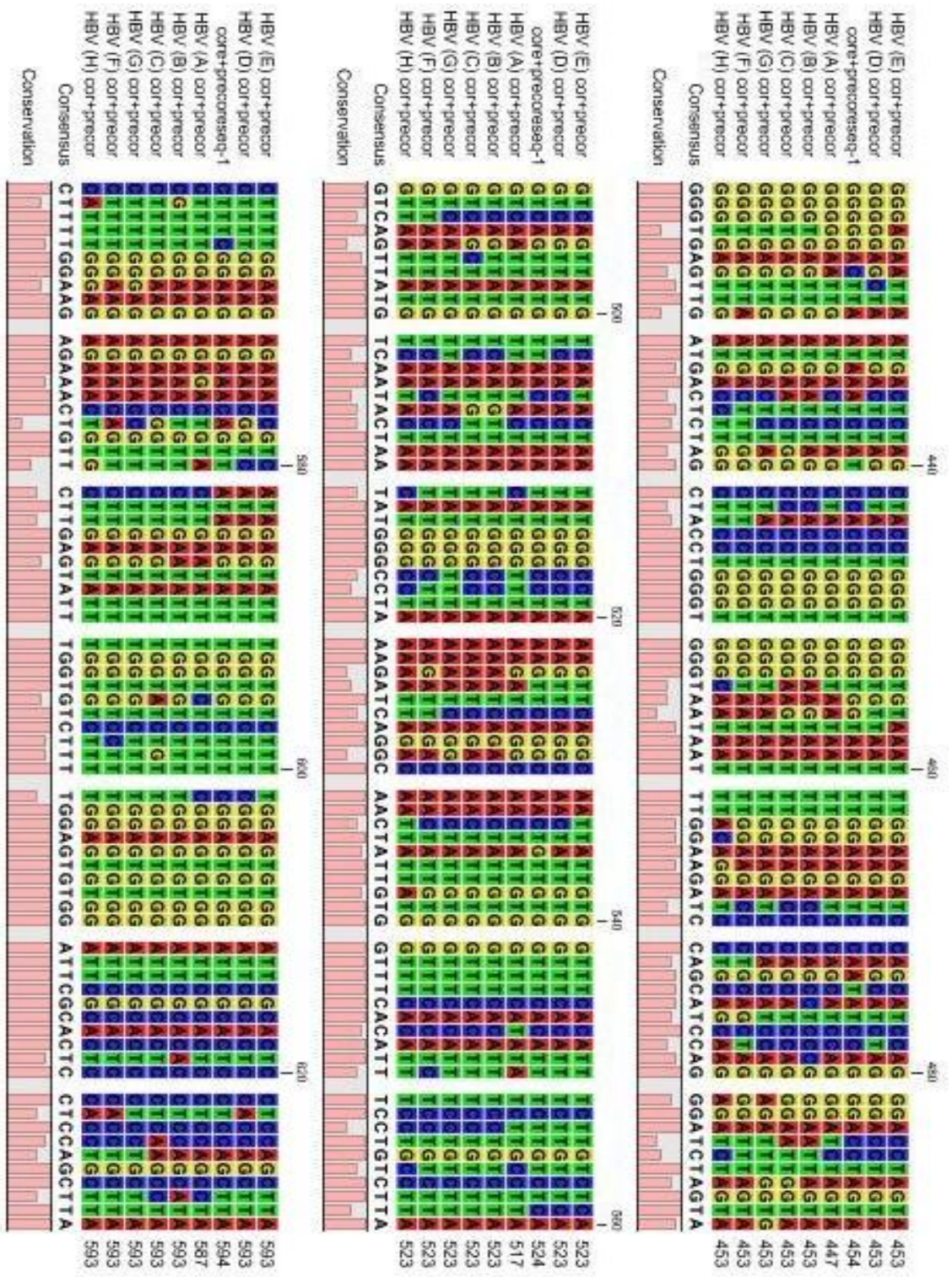
For sequence comparisons of the partial genomic sequences of the HBcAg isolates, sequence alignment was performed for Core antigen (pc/core region) using the multiple-alignment algorithm in Megalign (DNASTAR, Window version 3.12e).

Comparison of the consensus of HBcAg sequences to be amplified for the HBV genome region corresponding to nts 1 to 770 with reference eight genotypes (A to H) sequences retrieved from the GenBank. The partial genome for Core region sequence of HBV was obtained from our Egyptian patient with chronic hepatitis. When compared with previously reported major genotypes HBV A to H using Megalign (DNASTAR, Window version 3.12e) revealed that these strains were very closely related to genotype D (Fig. 17).

Nucleotide identity and divergence between the Core antigens sequence from the same Egyptian (HBV AgS1) corresponding to eight human HBV strains belonging to genotypes A to H were determined in Table (8). It showed high overall identity (93.6%) with genotype D and divergence (4.7%). The results of identity and divergence improved that this isolate related to genotype D.


HBV (E) cor+precor
HBV (D) cor+precor
core+precoreseq-1
HBV (A) cor+precor
HBV (B) cor+precor
HBV (C) cor+precor
HBV (G) cor+precor
HBV (F) cor+precor
HBV (H) cor+precor
Consensus
Conservation


HBV (E) cor+precor
HBV (D) cor+precor
core+preconseq-1
HBV (A) cor+precor
HBV (B) cor+precor
HBV (C) cor+precor
HBV (G) cor+precor
HBV (F) cor+precor
HBV (H) cor+precor
Consensus
Conservation
220
240
260
280
300
320
340
360
380
400
420


HBV (E) cor+precor
HBV (D) cor+precor
core+precoreseq-1
HBV (A) cor+precor
HBV (B) cor+precor
HBV (C) cor+precor
HBV (G) cor+precor
HBV (F) cor+precor
HBV (H) cor+precor
Consensus
Conservation
440
460
480
500
520
540
560
580
600
620
640
660
453
523
587
593
594

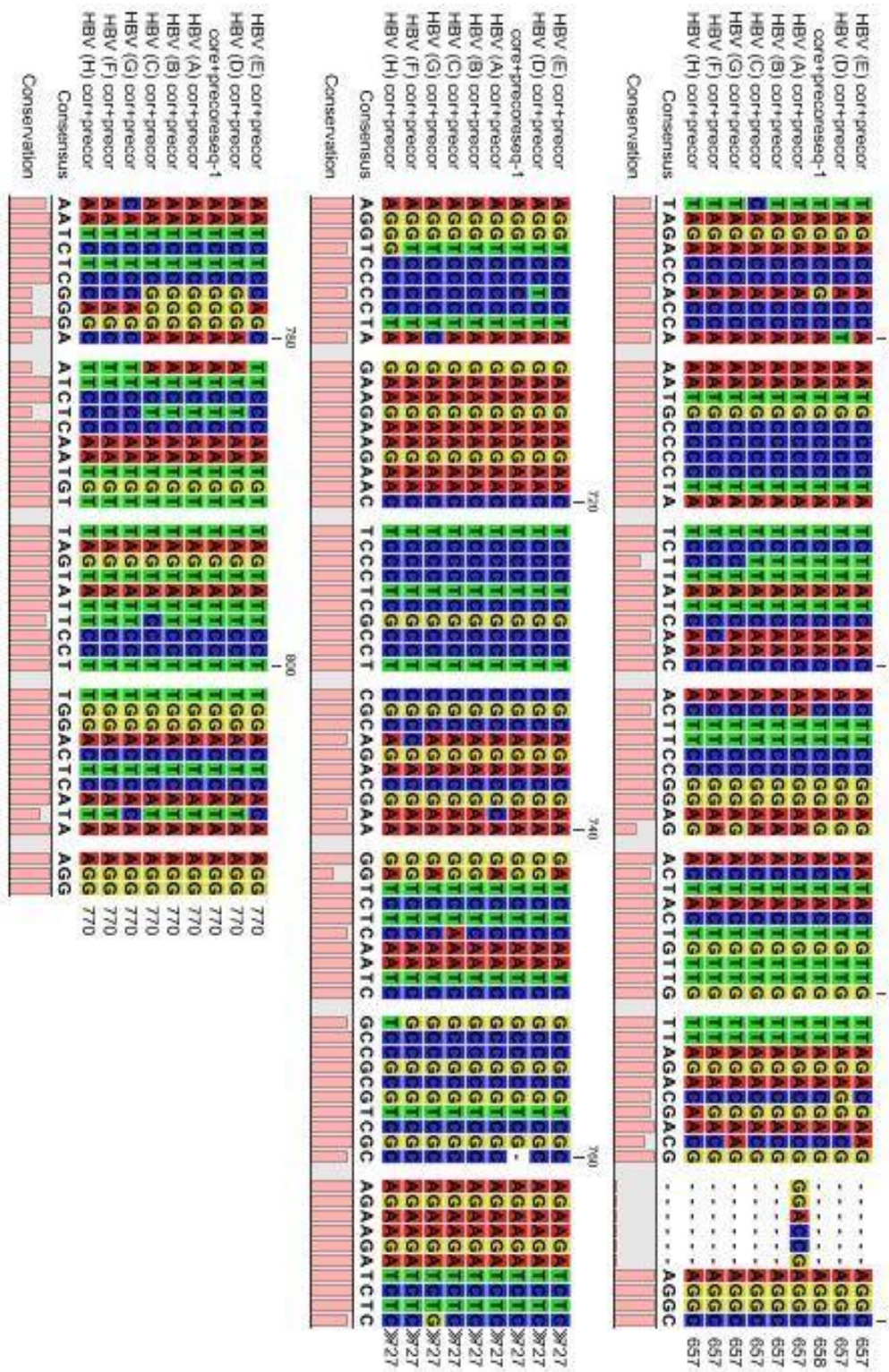


Fig. (17): Alingnment of partial Core antigen sequences of the new isolate and eight reference genotypes (A, B, C, D, E, F, G and H)

Table (8): Mean percent nucleotide identity and divergence between the pc/core gene region sequence of the Egyptian isolates 2010 and eight human HBV strains belonging to genotypes A to H.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	
1		89.1	90.0	88.4	91.2	90.5	87.4	83.5	85.6	1
2	8.1		89.0	88.2	93.6	92.9	84.5	82.3	82.1	2
3	8.8	9.8		95.6	91.3	90.0	89.4	83.8	86.1	3
4	10.0	10.4	4.3		90.1	89.4	88.2	82.7	84.9	4
5	7.3	4.7	8.7	9.5		95.1	88.2	83.4	84.5	5
6	7.9	5.4	9.7	10.4	4.8		87.0	85.2	85.6	6
7	11.2	13.3	10.2	11.1	11.4	12.2		83.4	91.8	7
8	10.8	11.9	11.4	12.4	11.7	9.8	11.4		82.2	8
9	13.1	16.3	13.4	14.4	14.9	13.8	8.3	12.4		9
	1	2	3	4	5	6	7	8	9	

1 HBV (A) cor+precor .seq
2 HBV Ag1 cor+precor .seq
3 HBV (B) cor+precor .seq
4 HBV (C) cor+precor .seq
5 HBV (D) cor+precor .seq
6 HBV (E) cor+precor .seq
7 HBV (F) cor+precor .seq
8 HBV (G) cor+precor .seq
9 HBV (H) cor+precor ..seq

A phylogenetic tree based on the Core antigen sequence, compared with HBV genotypes (A-H) retrieved from GenBank, showed that HBV Cor1 clustered within genotype D, as illustrated in Fig. (18) by using Megalign (DNASTAR, Window version3.12e).

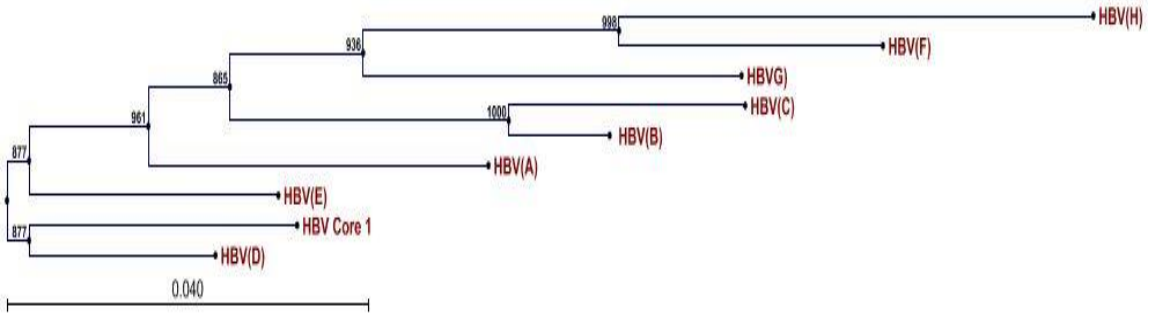


Fig. (18): Phylogenetic tree constructed of the Core antigen of HBV Ag1 using the entire nucleotide sequences (A to H) retrieved from GenBank.

Phylogenetic tree was constructed for HBV isolate from Egyptian patient using the entire nucleotide sequences against 87 reference genotyped HBV/D were retrieved from GenBank. Reference sequences are denoted by accession numbers and the country of origin (Fig. 19).

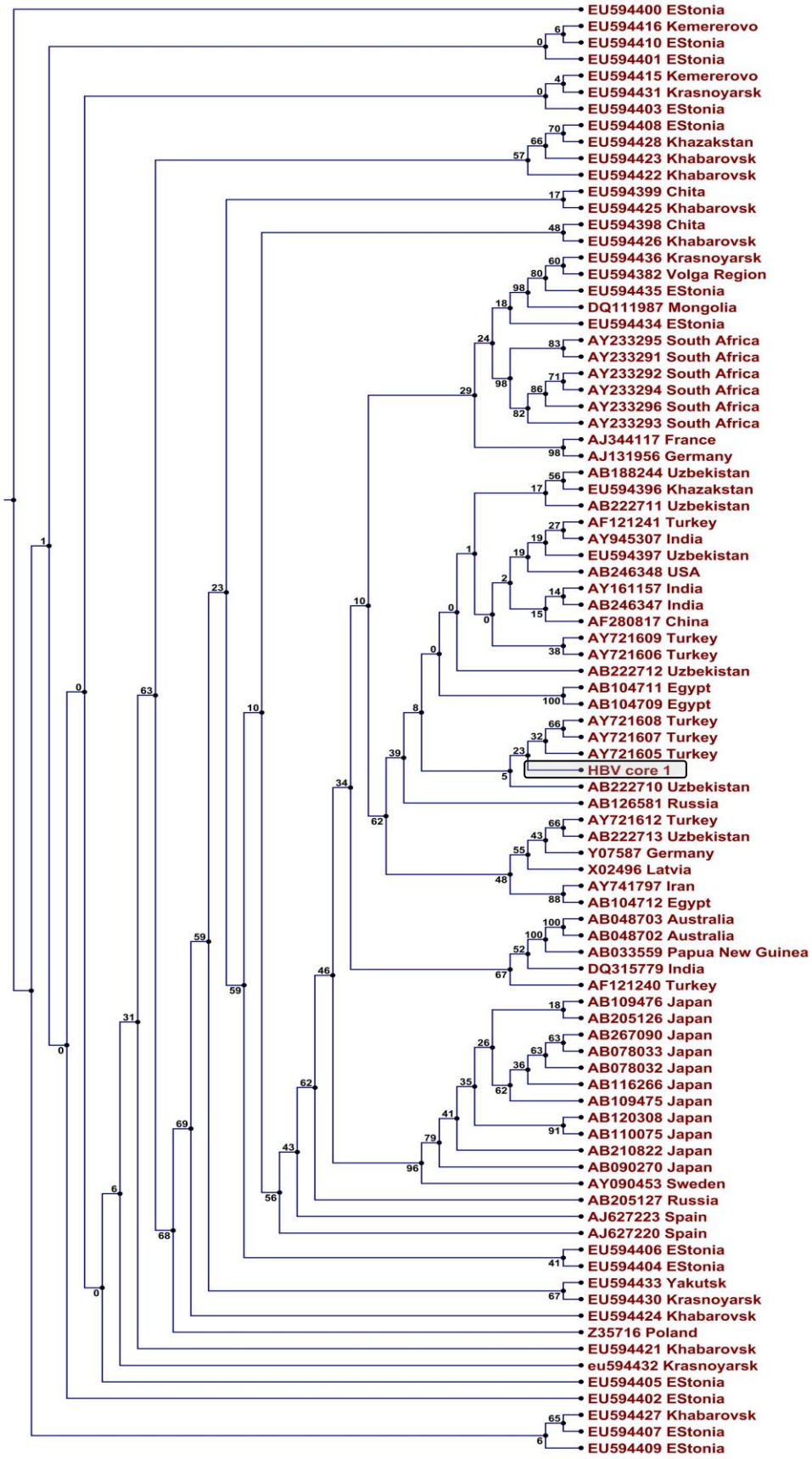


Fig. (19): Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present pc/core gene isolates and 87 HBV/D isolates retrieved from GenBank. Reference sequences are denoted by accession numbers and the country of origin. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

3.2.2. Alignment with currently circulating HBV/D in Egypt at Core antigen:

Alignment of nucleotide sequences of the Core antigen region of the isolated Egyptian 2010 with comparison to previously isolate Egyptian reference sequences and their accession numbers that submitted to GenBank database were shown in table (3) with comparison to the related genotype D (Fig. 20). The isolated Egyptian HBV 2010 is much more related to the circulating Egyptian HBV strains with identity percent ranged from 94.7 to 95.3% and diverge of 2.9 to 3.6% were reported in table (9). Nucleotide identity and diverge with genotype D were 93.6% and 4.7% respectively.

Table (9): Nucleotide identity and divergence between Core antigen sequence of HBV 2010 corresponding to genotype D and circulating Egyptian HBV strains.

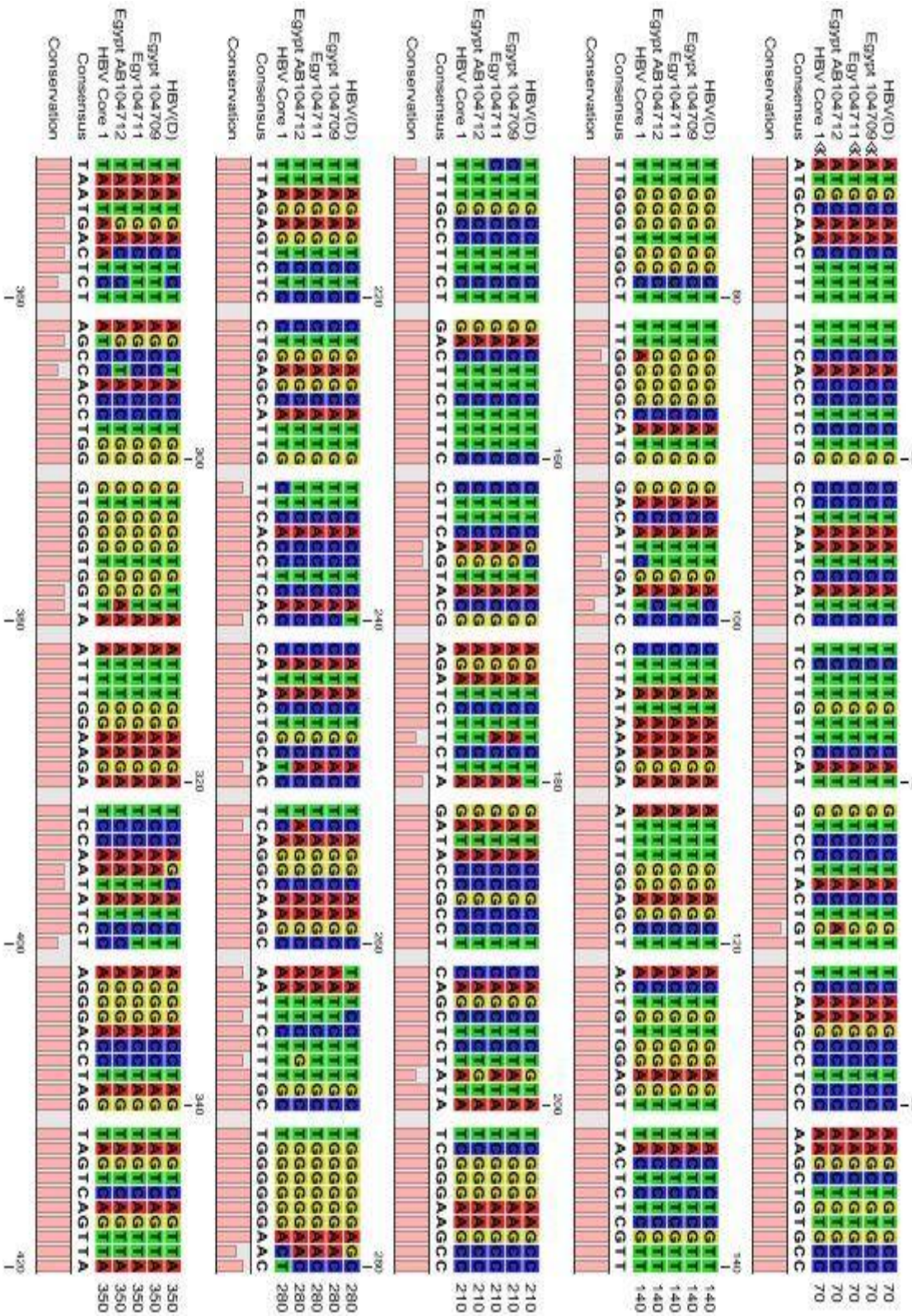
Percent Identity

Divergence

	1	2	3	4	5	
1		93.6	94.7	95.3	95.3	1
2	4.7		97.0	97.1	97.1	2
3	3.6	3.1		98.1	98.1	3
4	2.9	2.9	2.0		100.0	4
5	2.9	2.9	2.0	0.0		5
	1	2	3	4	5	

1 HBV Ag1 cor+precor .seq
2 HBV (D) cor+precor .seq
3 Egypt AB104712 cor+precor .SEQ
4 Egy104709 cor+precor .SEQ
5 EGY104711 cor+precor .SEQ

HBV isolate was grouped into genotype D by phylogenetic analysis based on partial core region (Fig. 18).Changing of nucleotides from genotype D at nt. positions summarized in table (10) at nt. 83, 96, 231, 249, 279, 280, 285,287, 292, 356, 417, 432 and 489. These substitutions may affect structures of AA or not according to at which places they happened.


HBV(D)
Egypt 104709
Egy104711
Egypt AB104712
HBV Core 1
Consensus
Conservation

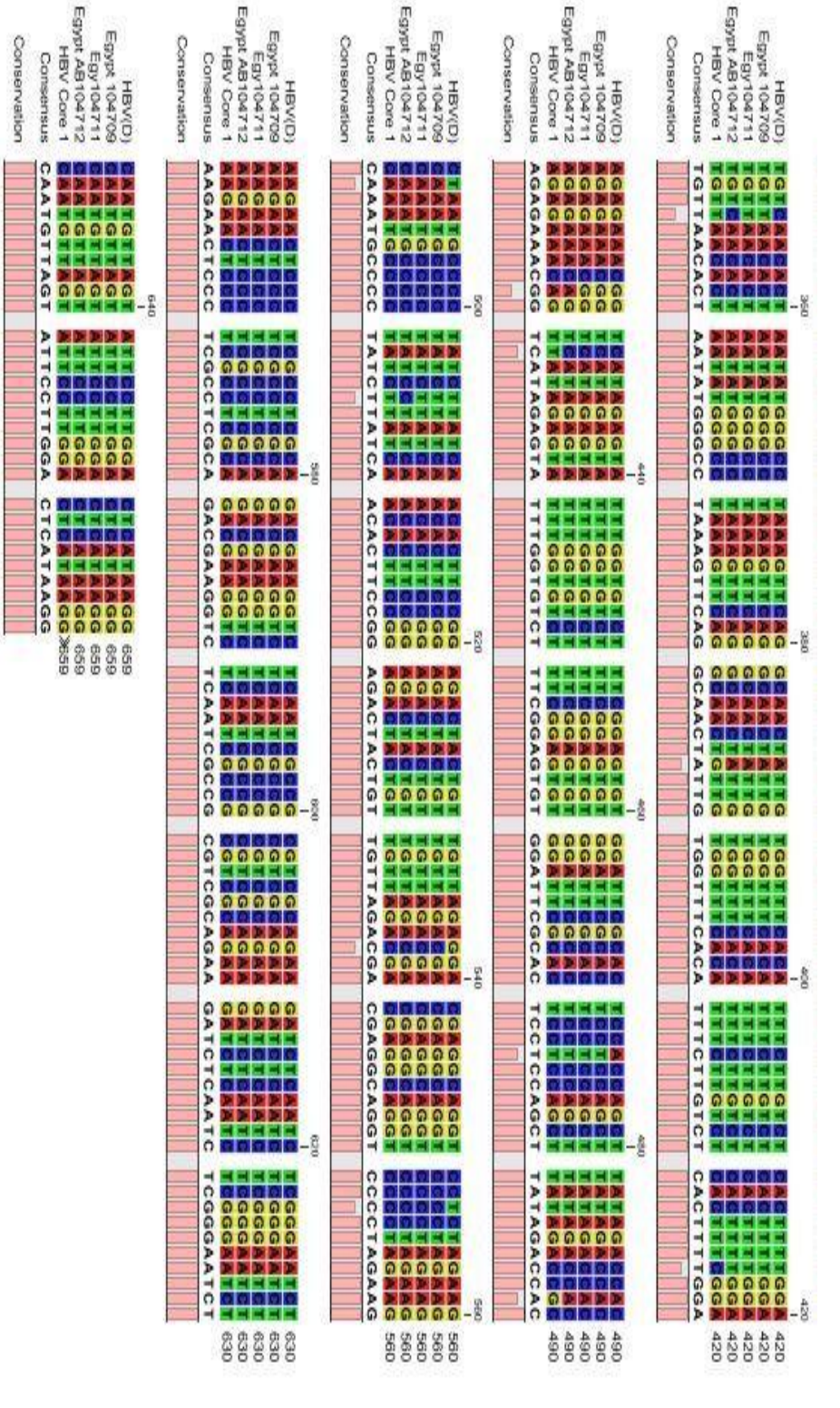


Fig. (20): Alignment of nucleotide sequences of Core antigen region of the isolated Egyptian HBV with comparison to the isolated Egyptian HBV strains that submitted to Genbank database with the accession numbers (Egy. 104709, Egy 104711 and Egy 104712) and genotype B and D.

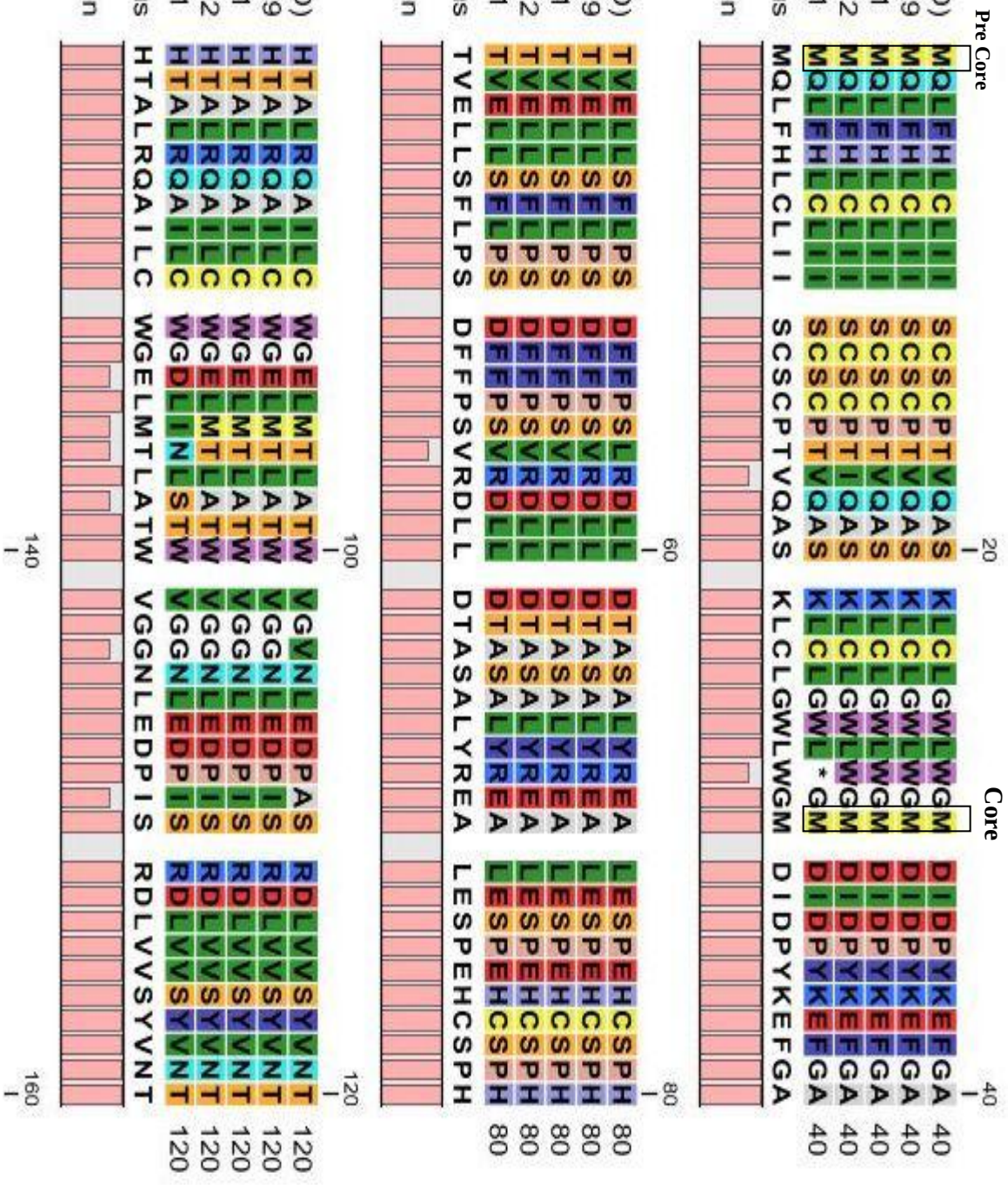
Table (10): Positions of mutations at Nucleotide level between HBV Core 1 isolate with the circulating HBV/D in Egypt and reference HBV/D.

Genotype / Nucleotide position	HBV/D	HBV Cor1	Egy. 104709.	Egy 104711	Egy 104712	Genotype / Nucleotide position	HBV/D	HBV Cor1	Egy. 104709.	Egy 104711	Egy 104712
49	G	G	G	G	A	285	G	A	G	G	G
83	G	A	G	G	G	287	C	A	C	C	C
96	T	C	T	T	T	289	C	C	T	T	C
99	C	T	T	T	C	292	G	T	G	G	G
141	T	T	C	C	T	294	T	C	C	C	T
165	G	A	A	A	A	309	T	T	T	T	A
166	C	G	G	G	G	325	G	A	A	A	A
177	T	T	A	A	T	326	C	T	T	T	T
180	T	A	A	A	A	330	T	C	T	T	C
198	G	A	A	A	G	354	C	T	T	T	C
231	T	C	T	T	T	356	A	G	A	A	A
240	T	C	C	C	C	417	T	C	T	T	T
249	A	T	A	A	A	429	C	A	C	C	A
252	C	C	C	C	A	432	C	T	C	C	C
261	T	A	A	A	A	474	A	T	T	T	T
264	C	T	T	T	T	489	A	G	A	A	A
279	G	C	A	A	A	492	T	A	A	A	A
280	C	T	C	C	C	538	G	C	C	C	C
						553	T	C	C	C	C

The genotypes determined of pc/core region sequence of HBV Core1, the Egyptian isolates 2010 with reference sequences representing genotype D retrieved from the GenBank and circulating Egyptian HBV strains with the accession numbers (Egy. 104709, Egy 104711 and Egy. 104712). Genotype D generated with the same clustered with Egy. 104709, Egy. 104711 and HBV Core1 (Fig.21).

Fig. (21): Phylogenetic tree constructed of the Core antigen of HBV Core1 representing genotype D with three circulating Egyptian HBV strains with the accession numbers (Egy. 104709, Egy 104711 and Egy. 104712).

Amino acid alignment of nucleotide sequences of the Egyptian HBV Core antigen were 219 amino acids long corresponding to the isolated Egyptian HBV strains with the accession numbers of that submitted to Genbank database **(**Egy. 104709, Egy 104711 and Egy. 104712) with reference genotypes D (Fig.22). Changing the amino acid in Egy Core1 at position 28, 93, 95, 96 as well as 98 than genotype D and other Egyptian circulating strains (Fig.22). Compared with reference genotype D, amino acids substitution of HBV Core 1, 2010 had been detected with the location of mutations at AA103, 109, 180 and 185 (Table, 11).


Pre Core
Core
HBV(D)
Egypt 104709
Egypt 104711
Egypt 104712
HBV core 1
Consensus
Conservation
MQLFHLCLII SCSCPTVQAS KLCLGWLWGM DIDPYKEFGA
MQLFHLCLII SCSCPTVQAS KLCLGWLWGM DIDPYKEFGA
MQLFHLCLII SCSCPTVQAS KLCLGWLWGM DIDPYKEFGA
MQLFHLCLII SCSCPTIQAS KLCLGWLWGM DIDPYKEFGA
MQLFHLCLII SCSCPTVQAS KLCLGWL*GM DIDPYKEFGA
MQLFHLCLII SCSCPTVQAS KLCLGWLWGM DIDPYKEFGA
20
40
TVELLSFLPS DFFPSLRDLL DTASALYREA LESPEHCSPH
TVELLSFLPS DFFPSVRDLL DTASALYREA LESPEHCSPH
TVELLSFLPS DFFPSVRDLL DTASALYREA LESPEHCSPH
TVELLSFLPS DFFPSVRDLL DTASALYREA LESPEHCSPH
TVELLSFLPS DFFPSVRDLL DTASALYREA LESPEHCSPH
TVELLSFLPS DFFPSVRDLL DTASALYREA LESPEHCSPH
60
80
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
HTALRQAILC WGELMTLATW VGGNLEDPAS RDLVVSYVNT
100
120
HTALRQAILC WGELMTLATW VGGNLEDPIS RDLVVSYVNT
140
160

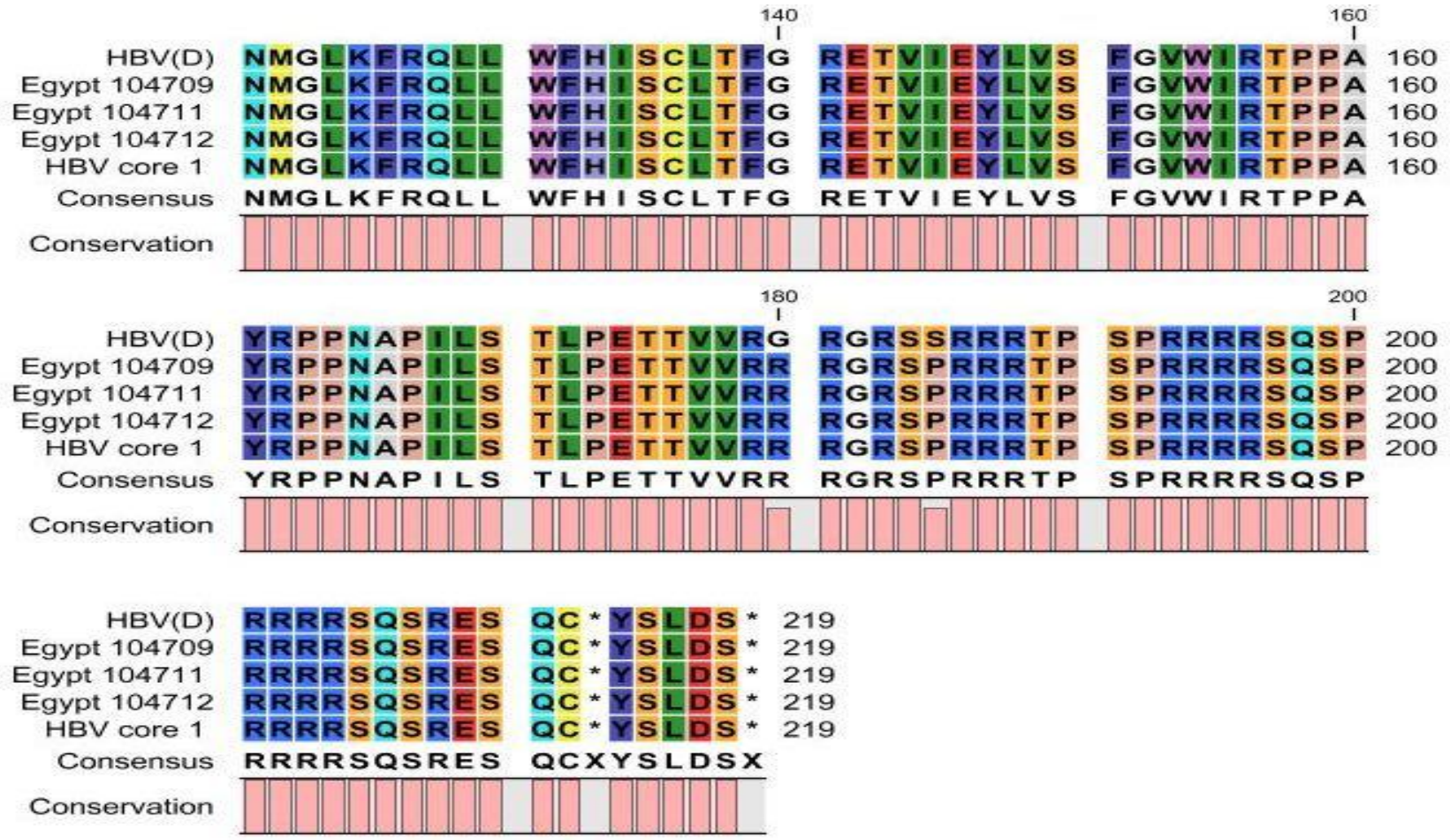


Fig (22): Amino acid alignment of nucleotide sequences of the pc/core region of the isolated Egyptian HBV corresponding to the eight human HBV strains belonging to genotypes A to H submitted to Genbank

Table (11): Positions of mutations at protein level of Core antigen between the HBV Core1 and-circulating HBV/D in Egypt and reference HBV/D with HBV Core 1 isolate.

Genotype / PROTIEN position		HBV/D	HBV Cor1	Egy. 104709.	Egy 104711	Egy 104712	Genotype / PROTIEN position		HBV/D	HBV Cor1	Egy. 104709.	Egy 104711	Egy 104712
Pre Core	17	V	V	V	V	I	Core	98	A	S	A	A	A
	28	W	-	W	W	W		103	V	G	G	G	G
Core	93	E	D	E	E	E		109	A	I	I	I	I
	95	M	I	M	M	M		180	G	R	R	R	R
	96	T	N	T	T	T		185	S	P	P	P	P