



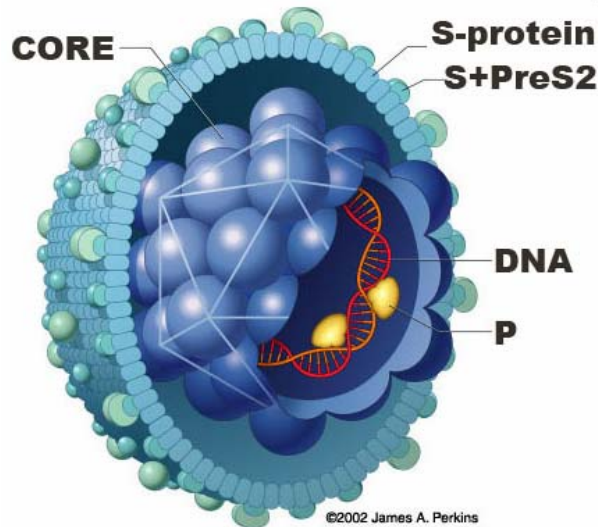
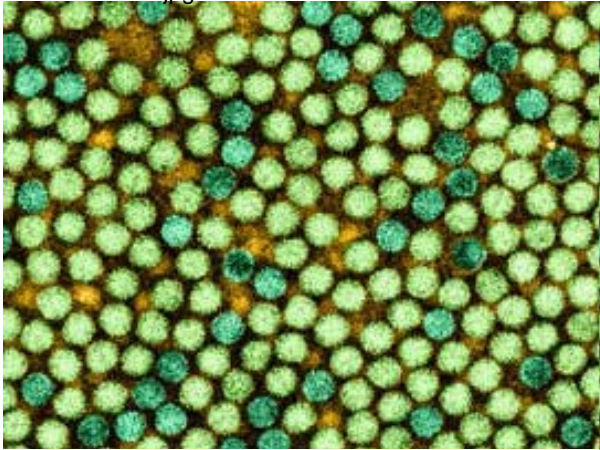
SINGLE BASE MUTATIONS IN THE S-GENE OF PHILIPPINE HEPATITIS B VIRUS (HBV) ISOLATES MAY GIVE RISE TO “ESCAPE MUTANTS”

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HBV

<http://www.foundation.org.cn/images/UpPic/2007720134439684.jpg>

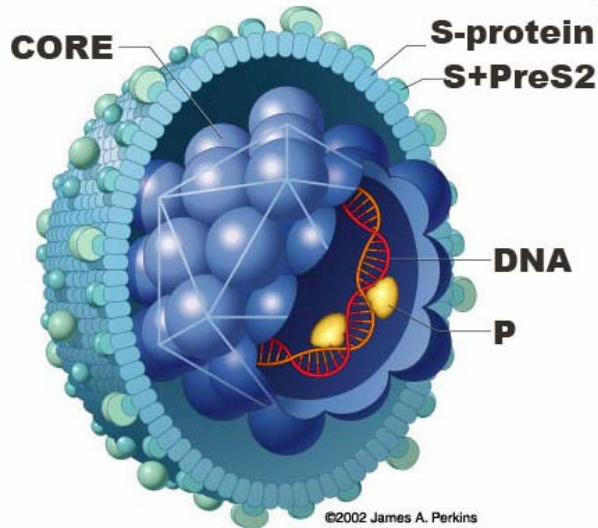
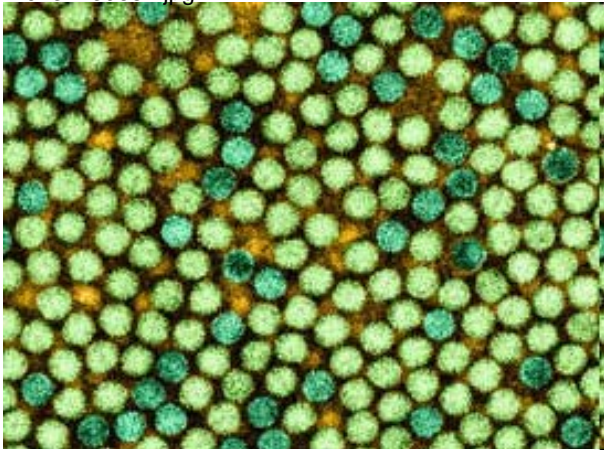


<http://www.foundation.org.cn/en/images/UpPic/2007822155857549.jpg>

- Genus *Orthohepadnavirus*, Family *Hepadnaviridae*
- Small, circular, partially dsDNA ~3.2 kb in size
- It has 4 genes with partially overlapping ORFs: pre-S/S, C, P and X genes
- The overlapping ORFs generate 7 proteins: polymerase, core antigen, e antigen, large, medium and small surface antigen and X protein

HBV

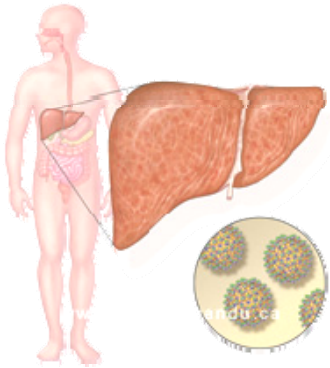
<http://www.foundation.org.cn/images/UpPic/2007720134439684.jpg>



<http://www.foundation.org.cn/en/images/UpPic/2007822155857549.jpg>

- HBV has a mutation rate $\sim 10X$ greater than that of other DNA viruses
- The overlapping ORFs may constrain some viral variability but **mutant or variant** forms have been identified for all 4 genes.
- It has 8 major genotypes (A-H) on the basis of **>8% intergenotypic difference** in the entire nucleotide sequence
- HBV genotypes demonstrate geographic diversity

Mechanism of HBV Mutant Generation



http://www.sexualityandu.ca/images/sti/hep_e.jpg

~400M HBV infected individuals

Replication via a **RNA reverse transcriptase** intermediary step

HBV quasispecies pool

HBV MUTANTS

Selective pressures
immune response &
treatment options

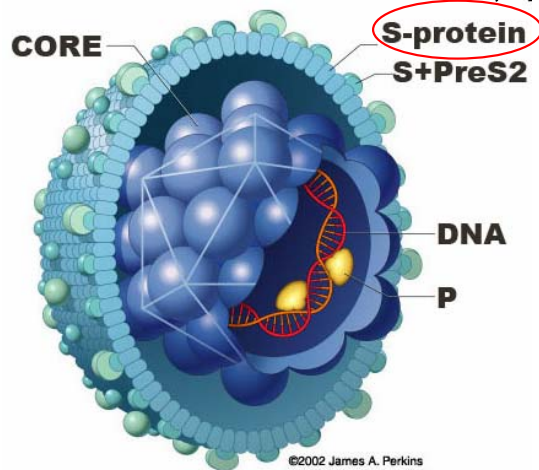
May alter current diagnostic & treatment algorithms





HBV forms currently relevant in clinical practice

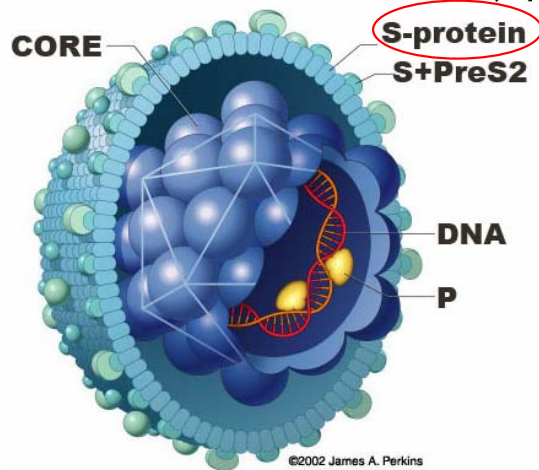
1. Wild type
2. Precore mutants
3. Core mutants
4. YMDD mutants
5. Surface antigen mutants
 - escape or immune escape mutations



<http://www.foundation.org.cn/en/images/UpPic/2007822155857549.jpg>

small HBsAg

- The first serological marker to appear after infection.
- Protein encoded by the S gene
- carries a group-specific “a” determinant common to all subtypes of the HBV
- The “a” determinant is located on the major hydrophilic region (MHR) of the S gene, which covers amino acids 120 to 160 and a highly immunogenic area that forms the basis for anti-HBs response



<http://www.foundation.org.cn/en/images/UpPic/2007822155857549.jpg>

HBV S gene Mutants

- Escape or immune escape mutants
- Mutations in the MHR can disrupt the antigenicity of HBsAg
- Results to a condition known as **occult hepatitis B infection**
- Responsible for reactivation of hepatitis B, **diagnostic assay failure** and reinfection in HBV-infected recipients of orthotopic liver transplantations
- These mutations are **stable** and can **transmitted horizontally and vertically.**

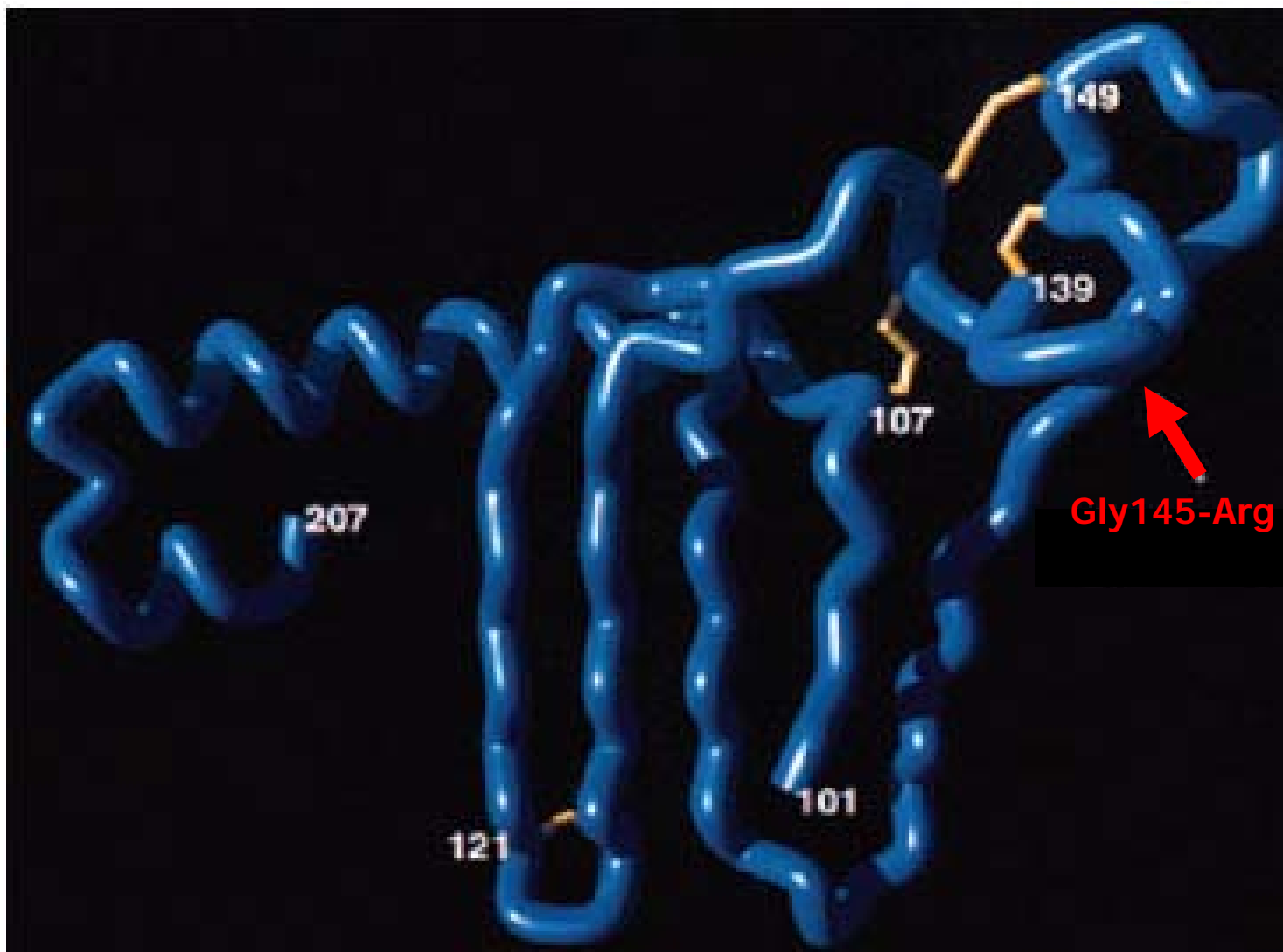
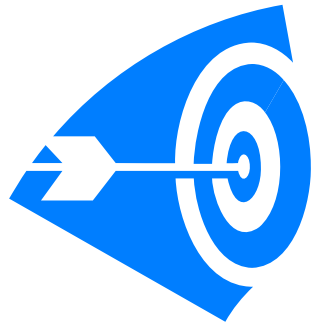


Figure. **Gly145Arg** mutant in the projecting amino acid 139-147 antigenic loop of the "a" determinant. This mutant produces **false-negative results** in some **commercial assays**.



OBJECTIVES

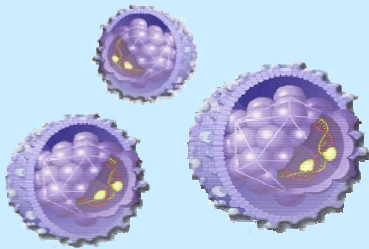
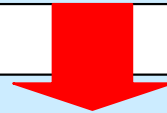
- To determine genotypes of Philippine HBV isolates not typable by PCR-RFLP
- To identify by sequence analysis single base mutations in the S gene that may give rise to “escape mutants”

METHODOLOGY



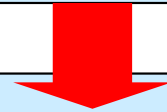
Samples

1,183 sera/plasma received for HBV DNA quantitation from 2002-2007



Sample Selection

436 frozen samples with HBV DNA > 1000 copies/ml



DNA Extraction

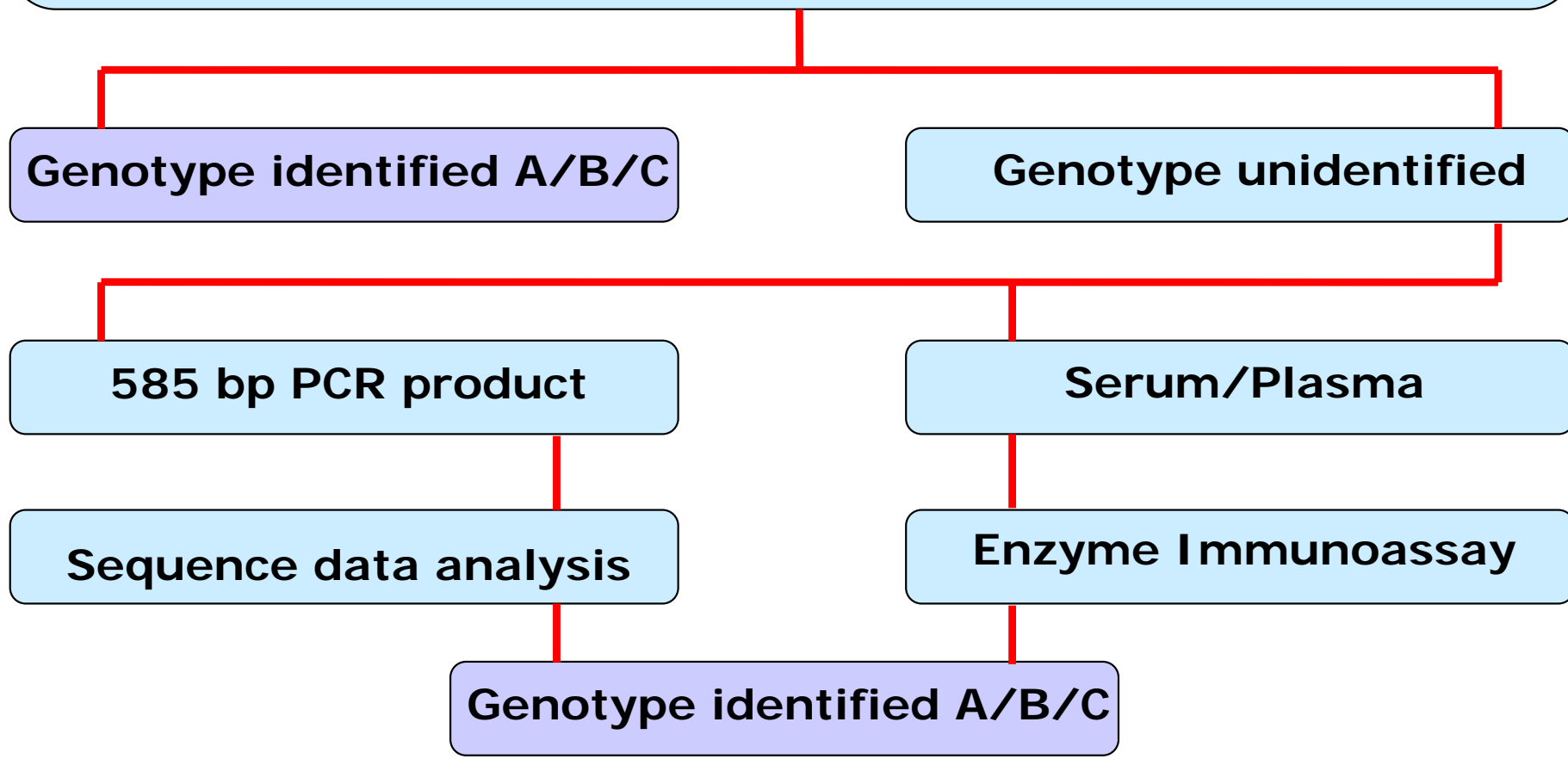
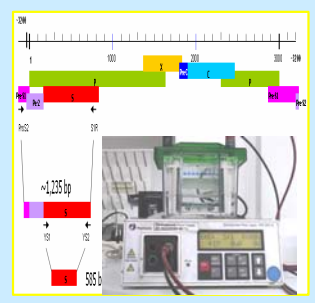
From 200 μ l serum/plasma of 436 frozen samples





PCR-RFLP Genotyping

Digestion of PCR-amplified fragment of the S gene (585bp) by Restriction Enzymes *Bsrl*, *Styl*, *HpaII*, *EaeI* and *DpnI*



RESULTS

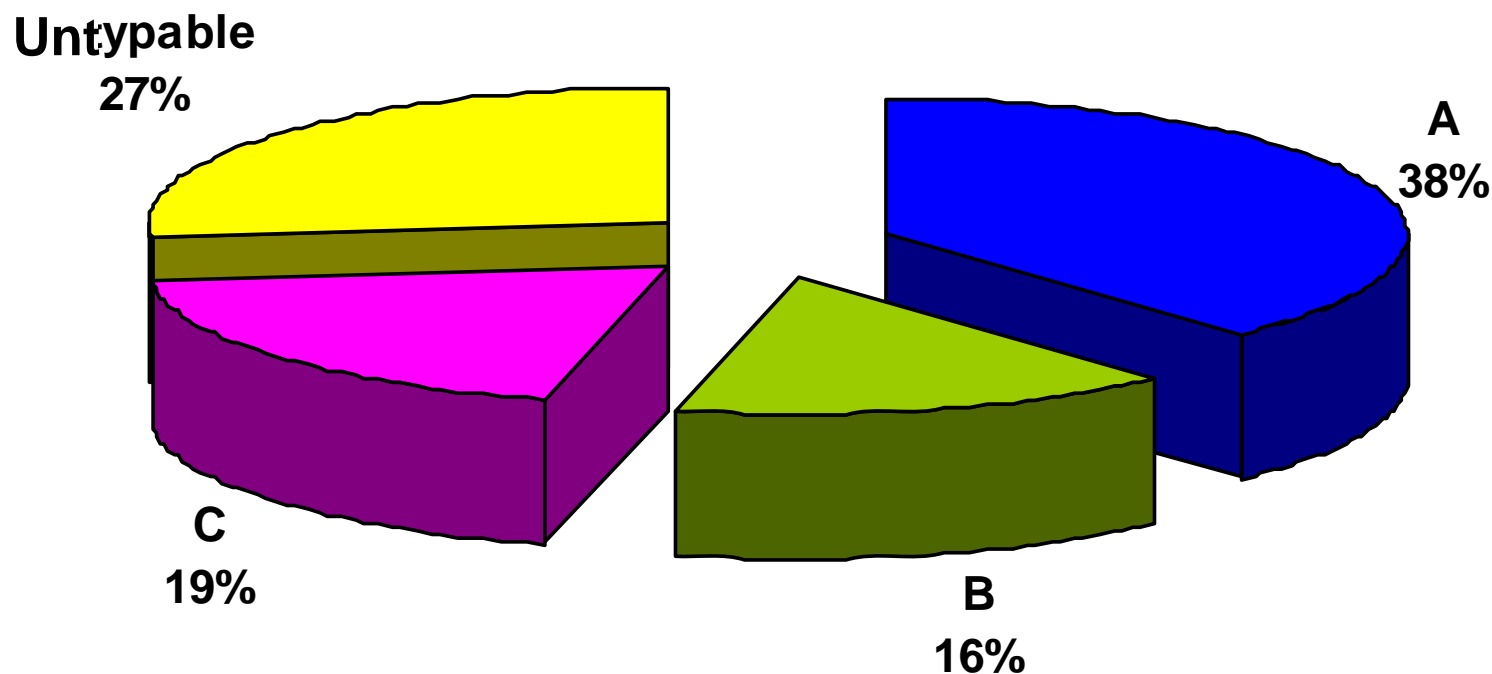


Fig. 1 Distribution of **Philippine HBV genotype** identified by PCR-RFLP (**N=436**) among patients seen at St. Luke's Medical Center from 2002 to 2007.

Fig. 2. PCR-RFLP untypable isolates genotyped using EIA (n=35).

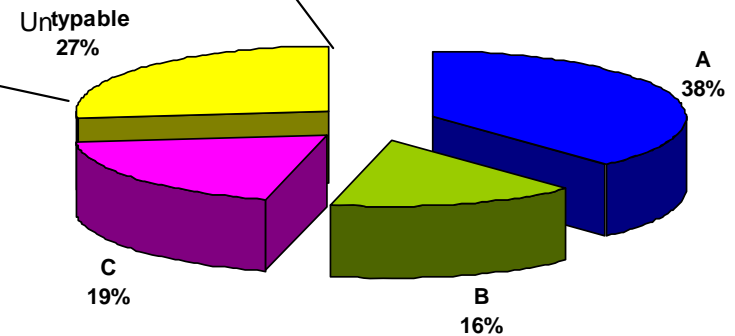
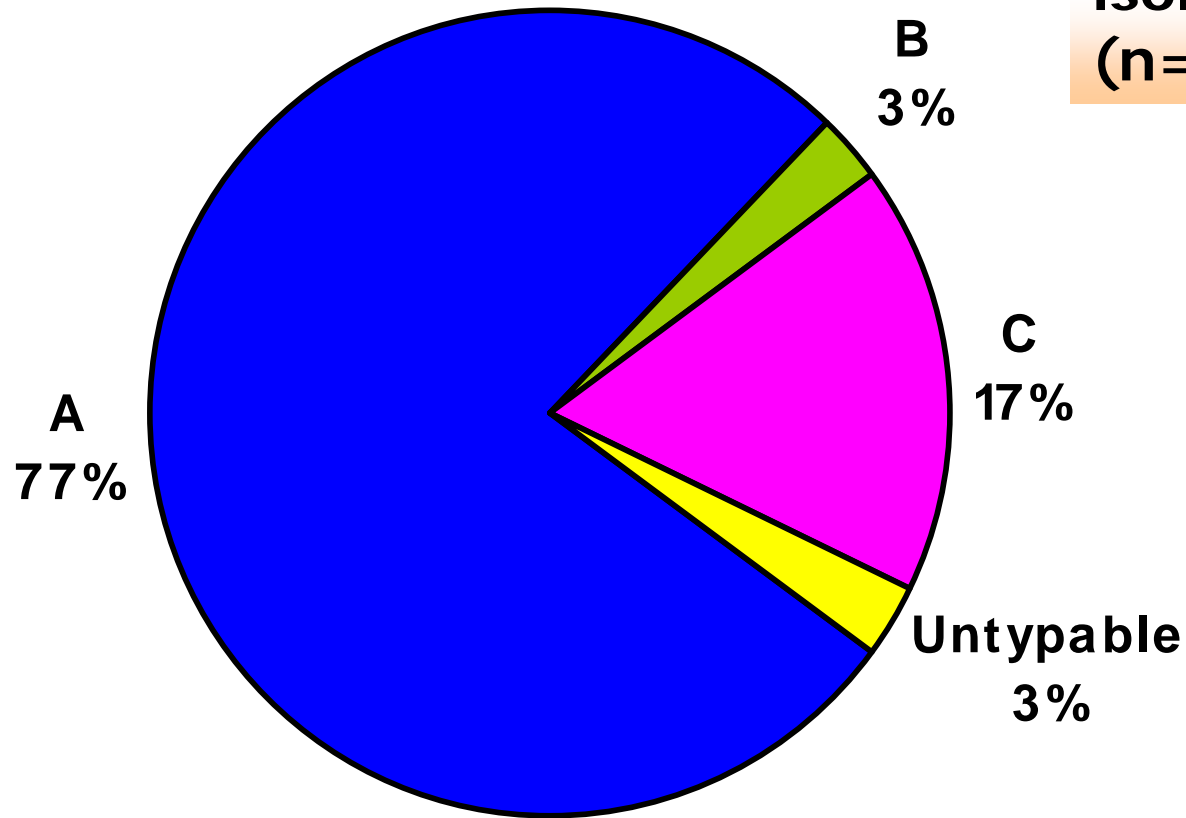


Fig. 1. HBV genotyping by PCR-RFLP(N=436)

Table 1. Sequence analysis confirmed the genotype of 21 Philippine HBV isolates as determined by EIA.

Isolate Code	GENOTYPE	
	EIA	Sequencing
02-157	B	B
02-171	C	C
02-211	C	C
03-227	C	C
05-960	A	A
06-1164	A	A
06-1249	A	A
06-1252	A	A
06-1320	A	A
06-1322	A	A
07-1402	A	A
05-954	A	A
04-584	A	A
05-957	C	C
06-1248	A	A
06-1296	A	A
06-1293	A	A
04-407	A	A
06-1048	A	A
06-1292	A	A
06-1281	C	C

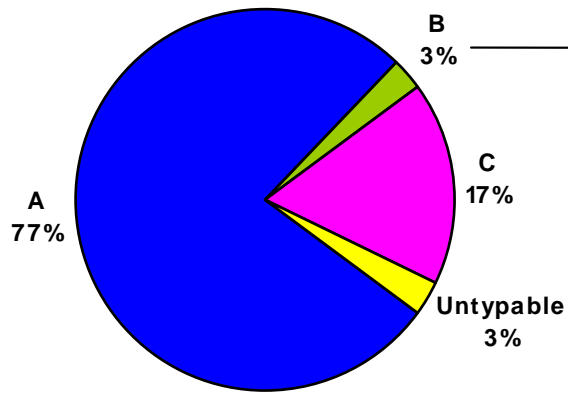


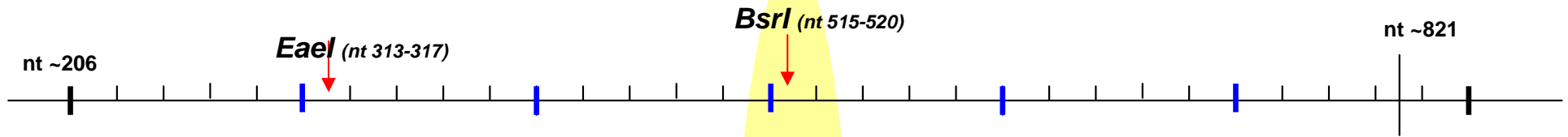
Fig. 2.
PCR-RFLP
untypable
isolates
genotyped
by EIA
(n=35)

HBV Genotypes PCR-RFLP profile

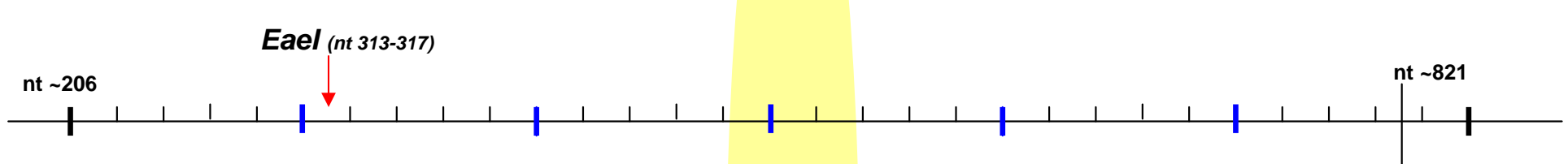
GEN	Bsrl	Styl	Hpall	Eael	Dpnl	Hpall
A	+	-	-	+		
	nt 515-520			nt 313-317		
B	+	-				
	nt 339-343					
C	-	+				
		nt 468-473				
D	-	-			+	-
					nt 503-506	
E	-	-	+	+		
			nt 724-727	nt 313-317		
F	-	-			+	+
					nt 503-506 Nt 768-771	nt 302-305
G	-	-	-	-		
H	-	-			+	+
					nt 503-506	nt 302-305

HBV Genotype A Reference Sequences

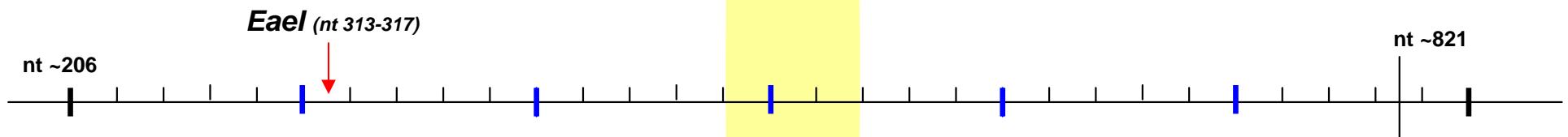
HBV/A (AM295800), S-gene (585 bp)



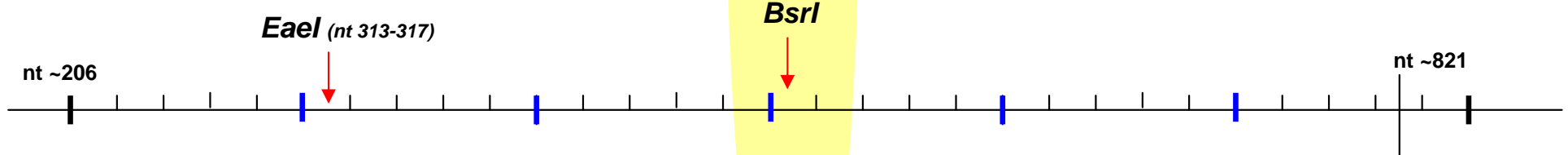
HBV/A (AB246317_Tanzania), S-gene (585 bp)



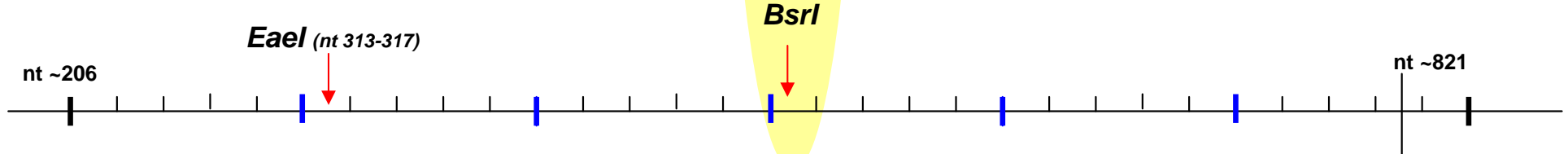
HBV/Aa (DQ315786_India), S-gene (585 bp)



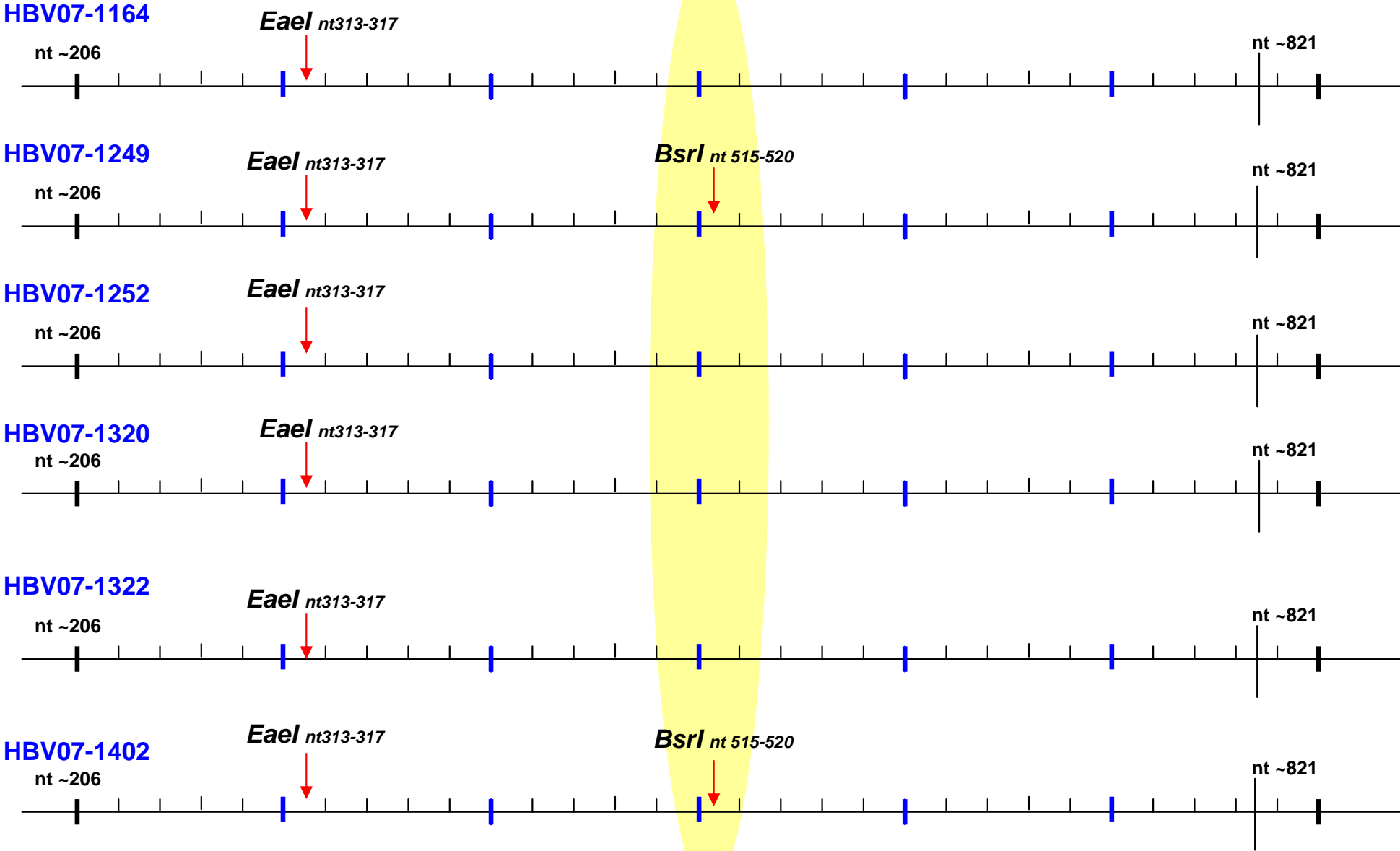
HBV/A (AM184126_Gabon), S-gene (585 bp)



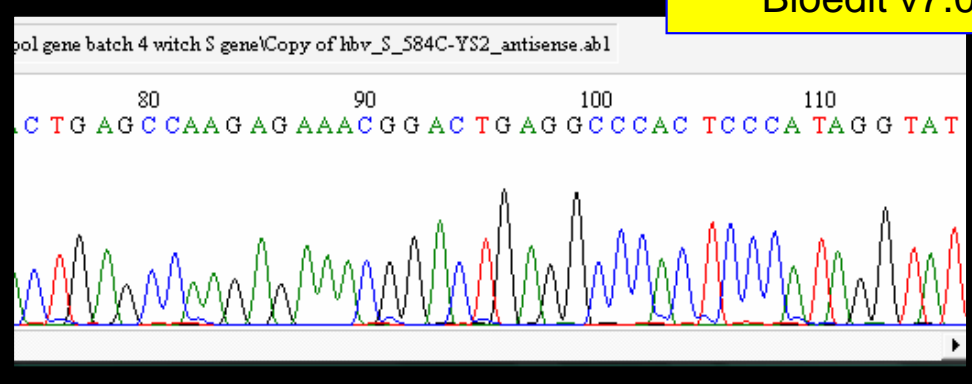
HBV/A (AY934774_Philippines), S-gene (585 bp)



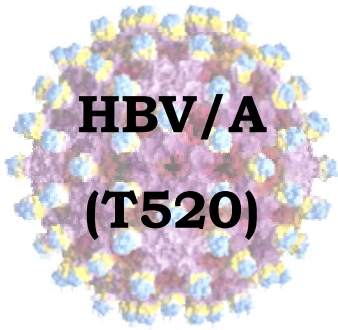
RBD non-A/B/C Isolates by PCR-RFLP



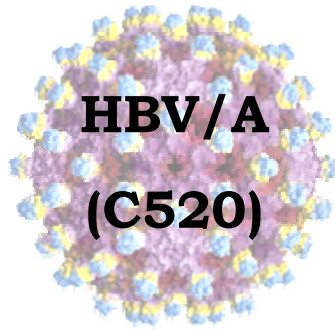
Bioedit v7.0



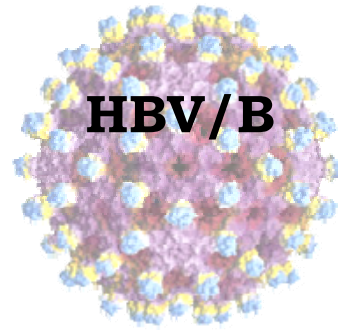
(n=21)



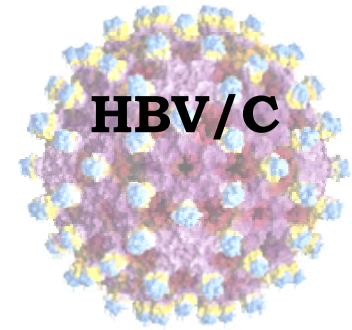
Group I



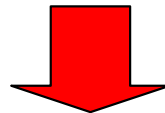
Group II



Group III

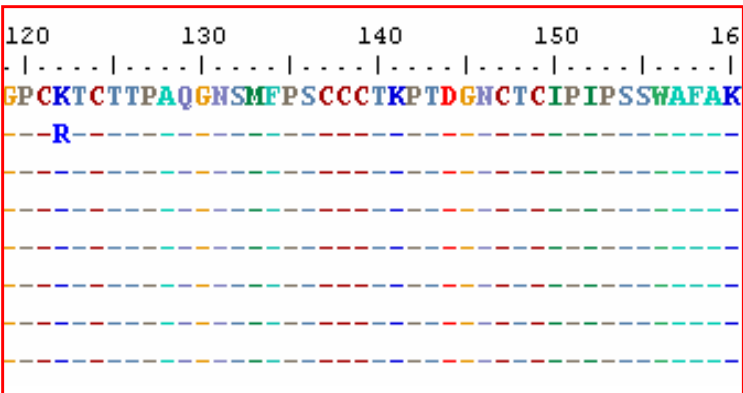


Group IV



S gene nucleotide sequence analysis

	20	30	40	50	60	70	80	90	100																																																																															
HBV/A AY934774 (Philippines)	F	L	L	T	R	I	L	T	I	P	Q	S	L	D	S	W	T	S	L	N	F	L	G	G	S	P	V	C	L	G	Q	N	S	Q	S	P	T	S	N	H	S	P	T	S	C	P	P	I	C	P	G	Y	R	W	M	C	L	R	R	F	I	F	L	F	I	L	L	C	L	I	F	L	L	V	L	L	D	Y										
HBV/A AM184126 (Gabon)																																																																															
HBV/A AY934774 (Philippines)																																																																															
hbv_S 960 consensus																																																																															
hbv_S 1048 consensus																																																																															
hbv_S 1249 consensus																																																																															
hbv_S 1402 consensus																																																																															
hbv_S 1296 consensus																																																																															
	110	120	130	140	150	160	170	180	190																																																																															
HBV/A AY934774 (Philippines)	Q	G	M	L	P	V	C	P	L	I	P	G	S	T	T	T	S	T	G	P	C	K	T	C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	T	K	P	T	D	G	N	C	T	C	I	P	I	P	S	S	W	A	F	A	K	E	L	W	E	A	S	V	R	F	S	W	L	S	L	L	V	P	F	V	Q	W	F	V	G	L	S	P	T	X
HBV/A AM184126 (Gabon)																																																																															
HBV/A AY934774 (Philippines)																																																																															
hbv_S 960 consensus																																																																															
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hbv_S 1402 consensus																																																																															
hbv_S 1296 consensus																																																																															
	190	200																																																																																						
HBV/A AY934774 (Philippines)	X	G	F	Q	L	Y	G	*	C	G																																																																														
HBV/A AM184126 (Gabon)																																																																															
HBV/A AY934774 (Philippines)																																																																															
hbv_S 960 consensus																																																																															
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hbv_S 1402 consensus																																																																															
hbv_S 1296 consensus																																																																															



**Genotype A
Group I**

Kyte & Doolittle Scale Mean Hydrophobicity Profile
Scan-window size = 13

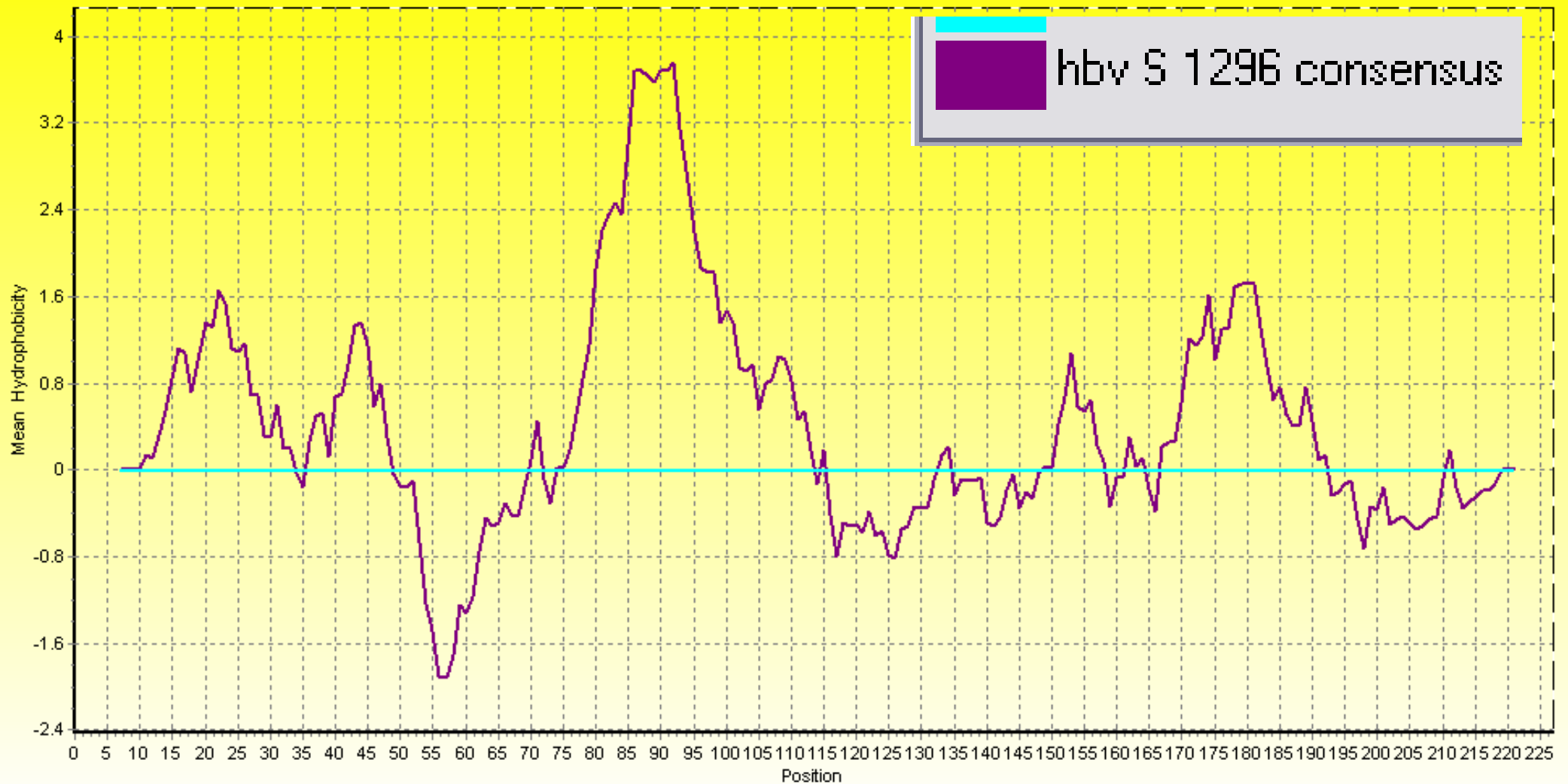





Fig. 3. **Group I** consists of HBV **genotype A** isolates whose sequence mutations in the S antigen outside the “a” determinat region do not affect hydrophobicity as compared to those of the reference sequences.

Plot Legend	
	HBV/A AY934774 (Philippines) 155...835 S protein nt263(G),nt289(T),nt359(T),nt459(A)
	HBV/A AM184126 (Gabon)
	HBV/A AY934774 (Philippines)

Kyte & Doolittle Scale Mean Hydrophobicity Profile
Scan-window size = 13

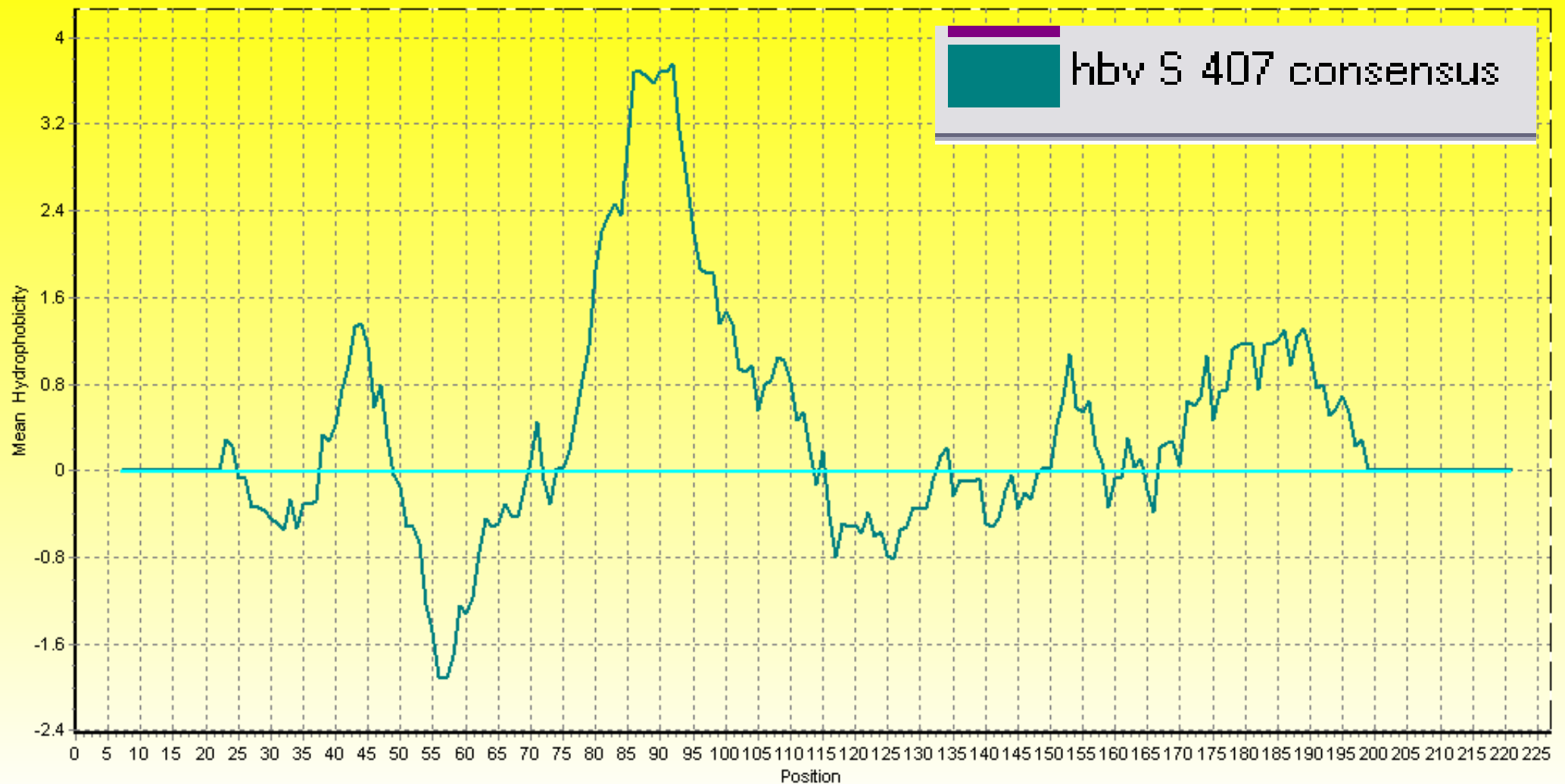


Fig. 4. **Group II** consists of HBV **genotype A** isolates whose sequence mutations in the S antigen showed an increase in hydrophobicity only in 1292 (a.a. 130-140 of “a” determinant) as compared to those of the reference sequences.



```

      20      30      40      50      60      70      80      90      100
      |.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
HBV/B DQ993711 (Taiwan) S prot FSLTKILTIPQSLDSWWTSLNFLGXTPVCLGQNSQSQISSHSPTCCPPICPGYRWMCLRRFIIFLCILLL*LIFLLVLLDY
HBV/B EU306681 (China)      -L-----G-----C-----C-----
HBV_B EU350409 (China)     -L-----G-----C-----C-----
HBV/B EU305548 (China)     -L-----G-----C-----C-----
HBV/B AB368295 (Vietnam)   -L-----S-E-----C-----C-----
HBV/B DQ993711 (Taiwan)    -----*-----
HBV/B AB241117 (Philippines) -L-----S-G-----C-----C-----
HBV/B AB287329 (Japan)    -L-----S-G-----C-----C-----
hbv_S 157 consensus       -L-----S-G-----Y-----C-----

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           110      120      130      140      150      160      170      18
      |.....|.....|.....|.....|.....|.....|.....|.....|
HBV/B DQ993711 (Taiwan) S prot QGMLPVCPLIPGSSTTSTGPCKTCTTPAQGTSMFPSCCCTKPTDGNCTCIPIPSSWAFAYLWEWASVRFSWLSLLVPPFV
HBV/B EU306681 (China)      -----S-----
HBV_B EU350409 (China)     -----I-----
HBV/B EU305548 (China)     -----I-----
HBV/B AB368295 (Vietnam)   -----R-----
HBV/B DQ993711 (Taiwan)    -----R-----T-----
HBV/B AB241117 (Philippines) -L-----R-----T-----
HBV/B AB287329 (Japan)    -----L-----R-----T-----
hbv_S 157 consensus       -----R-----T-----

```

```

           0      170      180      190      200
      |.....|.....|.....|.....|
HBV/B DQ993711 (Taiwan) S prot YLWEWASVRFSWLSLLVPPFVQWFVGLSPTVWLSVIWMMWY
HBV/B EU306681 (China)      -----F-----
HBV_B EU350409 (China)     -----F-----
HBV/B EU305548 (China)     -----I-F-----
HBV/B AB368295 (Vietnam)   -----I-----
HBV/B DQ993711 (Taiwan)    -----
HBV/B AB241117 (Philippines) -----R-----F-----
HBV/B AB287329 (Japan)    -----F-----
hbv_S 157 consensus       -----V-G-----

```

**Genotype
B**

Kyte & Doolittle Scale Mean Hydrophobicity Profile
Scan-window size = 13

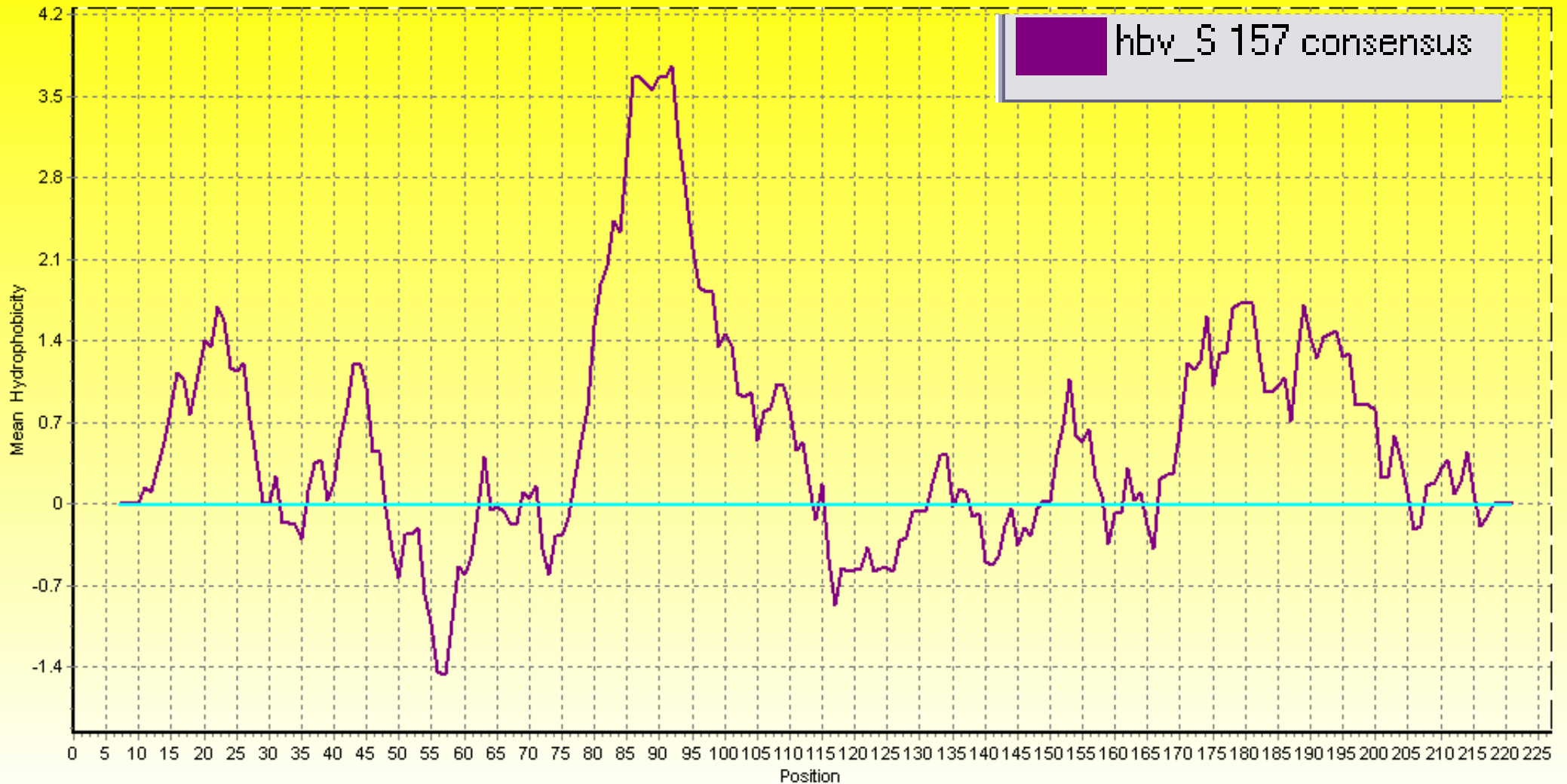


Fig. 3. **Group III** represents the HBV **genotype B** isolate whose sequence mutations in the S antigen do not affect hydrophobicity as compared to those of the reference sequences.



	20	30	40	50	60	70	80	90	100
HBV/C DQ683578 S gene									
HBV/C AB368297 S gene	FL	L	T	R	I	L	T	I	P
HBV/C DQ683578 (S. Korea)S gen	Q	S	L	D	S	W	T	S	L
HBV/C AB368297 (Japan)S gene	N	F	L	G	G	A	P	T	C
HBV/C EU306729 (China)S gene	P	G	Q	N	S	Q	S	P	T
hbv_S_957C_consensus	S	N	H	S	P	T	S	C	P
hbv_S_1281_consensus	P	P	I	C	P	G	Y	R	W
hbv_S_171_consensus	M	C	L	R	R	F	I	I	F
hbv_S_211_consensus	L	L	L	L	L	L	L	L	L
hbv_S_227_consensus	C	L	I	F	L	L	V	L	L

	110	120	130	140	150	160	170	180
HBV/C DQ683578 S gene	Q	G	M	L	P	V	C	P
HBV/C AB368297 S gene	L	P	G	T	S	T	T	S
HBV/C DQ683578 (S. Korea)S gen	T	T	T	T	T	T	T	T
HBV/C AB368297 (Japan)S gene	T	T	T	T	T	T	T	T
HBV/C EU306729 (China)S gene	T	T	T	T	T	T	T	T
hbv_S_957C_consensus	T	T	T	T	T	T	T	T
hbv_S_1281_consensus	T	T	T	T	T	T	T	T
hbv_S_171_consensus	T	T	T	T	T	T	T	T
hbv_S_211_consensus	T	T	T	T	T	T	T	T
hbv_S_227_consensus	T	T	T	T	T	T	T	T

	170	180	190	200
HBV/C DQ683578 S gene	F	L	W	E
HBV/C AB368297 S gene	W	A	S	V
HBV/C DQ683578 (S. Korea)S gen	R	S	W	L
HBV/C AB368297 (Japan)S gene	S	L	L	V
HBV/C EU306729 (China)S gene	P	F	V	Q
hbv_S_957C_consensus	W	F	A	G
hbv_S_1281_consensus	L	S	P	T
hbv_S_171_consensus	W	L	S	V
hbv_S_211_consensus	I	T	W	M
hbv_S_227_consensus	M	W	M	W

**Genotype
C**

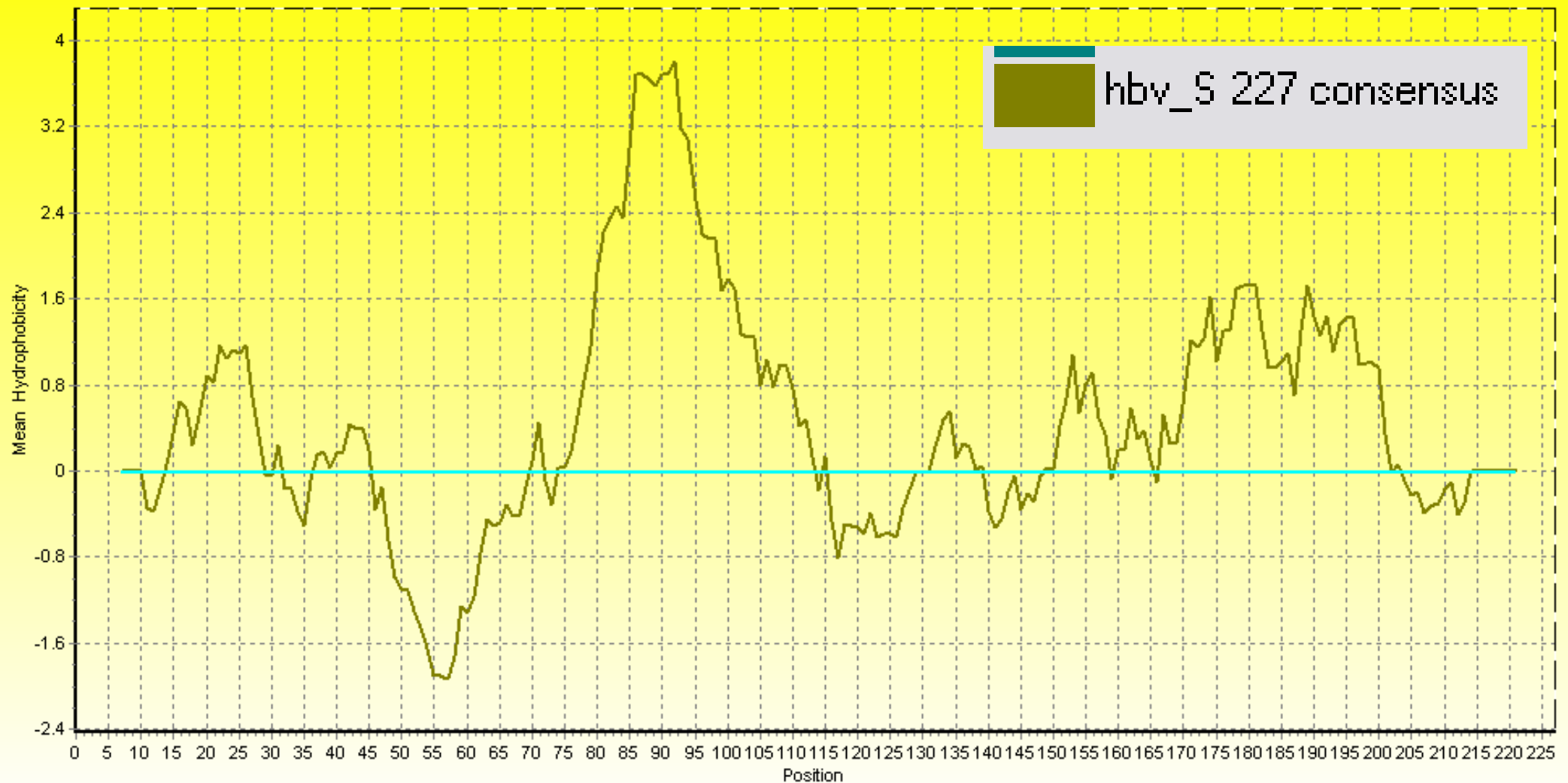


Fig. 3. **Group IV** represents the HBV **genotype C** isolates whose sequence mutations in the S antigen are found in the “a” determinant region and alter its hydrophobicity as compared to those of the reference sequences.

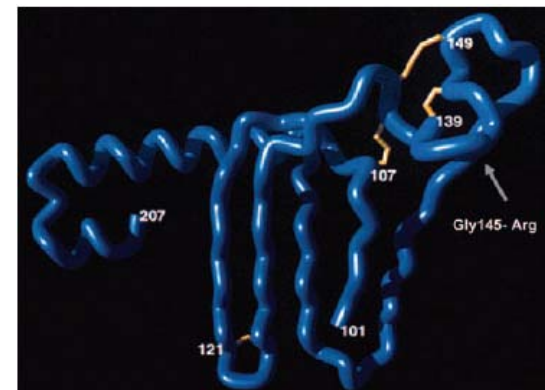


DISCUSSION

- The samples included in the study represent those with >1000 copies/ml (Amplicor HBV Monitor, Roche) collection of the Research and Biotechnology Division, St. Luke's Medical Center.
- The study revealed genotype A (181, 42%) as the major Philippine HBV genotype among the samples collected followed by C (90, 21%) and then B (70, 16%) only after using several molecular techniques.
- The genotyping results were comparable to the data reported by Usuda, S. *et al.* in 1999 also on Philippine (N=68) by ELISA.

- The EIA method used proved to be useful in the identification of PCR-RFLP untypable isolates.
- Sequence data obtained from the untypable isolates could be used to re-design the RFLP protocol used to appropriately genotype Philippine isolates.

- Mutations in the MHR can disrupt the antigenicity of HBsAg in a number of ways.
 - Amino acid modification directly involved in expression of the antigen
 - Alterations to the structure of HBsAg can also disrupt binding of polyclonal antibodies to it
 - Modifications to protein **hydrophobicity**, electric charge and acidity
 - Amino acid insertions or creation of stop codons



- “While most research into HBsAg escape mutants have been directed specifically at the MHR, additional research has shown that mutations outside the MHR can lead to the creation of escape mutants”.

Kohno, H. et al., Mutations in the envelope gene of hepatitis B virus variants co-occurring with antibody to surface antigen in sera from patients with chronic hepatitis B. J Gen Virol 1996;77:1825-31.

CONCLUSION

- The use of three genotyping methods were required to identify the genotypes of Philippine HBV isolates.
- HBV genotype A group I (T520) isolates revealed sequence mutations in the S antigen outside the “a” determinant region but have no effect on hydrophobicity as compared to those of the reference sequences.
- Only isolate 1292 of the HBV genotype A group II (C520) altered the hydrophobicity in a region inside the “a” determinant of the S antigen when compared to those of the reference sequences.

- HBV **genotype B** isolate whose sequence mutations in the S antigen do not affect hydrophobicity as compared to those of the reference sequences.
- HBV **genotype C** isolates revealed the most number of sequence mutations in the S antigen found in the “a” determinant region and altered hydrophobicity as compared to those of the reference sequences.

FUTURE STUDIES

- **Use sequence data obtained to evaluate which genotyping method would best identify genotypes of Philippine HBV isolates**
- Include in the sample population HBsAg negative samples to compare results and associate molecular and clinical data

Life changing consequences



- employability
- vertical transmission
- high cost of treatment and disease monitoring
- disease progression or liver failure

Thank you

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Mabuhay!