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Identification and Characterization of Hepatitis B Virus Immune Escape Mutants in Kenya

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Abstract

Hepatitis B Virus (HBV) infections affect about 400 million people globally and cause about 1.4 million deaths annually. The virus displays high levels of genetic variations/mutations, some of which are immune escape mutants. The prevalence of HBV infection in Kenya is high at about 8%. This study aimed at identifying and characterizing HBV immune escape mutants in Kenya. From 547 HBV sequences available in Kenya in NCBI, and HBVdb databases in July 2021, 120 full sequences were retrieved. The *S gene* sequences at position 1-225, which included the "a" determinant region of the gene were analyzed using various bioinformatics tools such as Bioedit software, and Emboss Cons. The clinical significance was flagged from the search of peer-reviewed journals. Forty-six HBV-positive blood donor samples were obtained from the Kenya National Blood Transfusion Services without personal identifiers, DNA extracted, and sequenced targeting positions 1 to 520 of *S genes*. Mutations were similarly identified from seventeen sequences after cleaning and analysis. Out of 120 sequences that were extracted from databases and analyzed, 79 different mutations were identified. Fifteen of them were of clinical importance with an occurrence frequency of at least 5% were obtained. The majority (64.6%, n = 51), with S207N and A194V being most dominant, could result in immune escape and reduced HBsAg detection signals while 24.1% (n = 19) could result in immune escape/reduced HBsAg detection signals and high probability of hepatocellular carcinoma. Most likely to occur on the amino acids Alanine, Lysine, Serine, Asparagine, and Valine in decreasing order. The most dominant genotype was found to be Genotype A (N = 10), while four sequences were Genotype D. In contrast to the *in-silico*

studies, the sequences from HBV samples from blood donors did not demonstrate the presence of S207N and A194V mutations and all the genotypes were type A1. Only two (13.3%) samples showed the same mutations of sK122R and sT143S for both *in-silico* analysis and actual sequenced samples. This study did not identify G145R mutation which is the commonest mutation within the HBsAg immunodominant "a" determinant that is associated with immune escape. The concordance of mutations in "a" determinant region of *HBsAg gene* among various studies is minimal. The study identified new mutations (sA194Y, sS207, sA194S, sS207I, sP46A, sA194T, sS207I, sP46R, and sT143P) that had not been published before. Four (20%) of the mutations were clinically significant. These included sS207R, sT143S, sC76F and sK122R.

Keywords

Hepatitis B Virus, Mutations, Hepatitis B Surface Antigen Gene, Genotype

1. Introduction

Hepatitis B Virus (HBV) infections affect about 400 million people globally about 1.4 million HBV deaths annually with more than 200,000 of these deaths coming from Africa. Kenya has a prevalence rate of >8.0% HBsAg. Several HBV mutants with clinical implications have been identified and documented worldwide, indicating the potential for spreading and developing their own epidemiology [1] [2]. They include the *HBV polymerase gene mutants*, *S genes mutants*, *pre-core/core*, *and X mutants* [1] [2]. However, mutational occurrence within or around the major "antigenic alpha" determinant region (121 - 149 aa) of *HbsAg S gene*, causes conformational changes. This affects HBV antigenicity and induces protective antibodies that make the virus go undetected by the rapid HBsAg test in some patients [3] [4]. Additionally, mutations occur in the S region, which permits the virus to evade/escape immune surveillance such as the body's own immune response, the immunological response caused by vaccination, and anti-HBV immunoglobulin therapy [5] [6]. Thus, these immune escape mutations provide false-negative results by commercial HBsAg assays (occult hepatitis B), failure to vaccine-induced immunity and anti-HBV immunoglobulin therapy which is linked to HBV reactivation, increased mutant viral replication, immunosuppression antiviral treatments failure and hepatic damage [5]. These highlights show the clinical importance of early, cost-effective, accurate, and sensitive detection methods of the viral pathogens, genotypes and mutations that confer resistance to therapy and immune escape profiles [7]. This is key for the effective prevention and clinical management of this disease [2]. The findings can be used to guide effective disease transmission prevention, enables practicing physicians to identify those patients at risk of disease progression and guide the appropriate/suitable first-line anti-viral treatment for pa-

tients. It can also monitor the efficacy and modification of antiviral therapy in patients to avoid the clinical effects of resistance [7] [8] [9] [10] [11]. Thus, ensuring proper clinical management of infected patients with first-line potent antiviral therapy [8] [9]. This can increase a patient's survival, safeguard the public's health, and achieve long-term treatment success [4] [5] [9].

2. Materials and Methods

2.1. Retrieval of Kenyan-Sourced *HBsAg Gene* Sequences from in NCBI and HBV Databases and Their Analysis

From 547 HBV sequences available from Kenya in NCBI and HBV databases, 120 full large Surface (S) gene sequences containing the "a" determinant region of the gene were retrieved. These sequences were translated to the protein sequences using EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/) and aligned with the referenced amino acid sequence from HBV database in Bioedit software version 7.2.5. Mutations or amino acids exchange in each sequence was identified, and recorded in an excel table. The clinical significance of mutations was flagged out from the search of peer-reviewed journals using Pubmed search engine and later submitted to geno2pheno HBV online software (https://hbv.geno2pheno.org/index.php) version 2.0 for further confirmation of drug resistance and immune escape mutation identification.

2.2. Clinical Samples Collection and Processing

A total of forty-six HBV-positive blood donor samples without personal identifiers/information were obtained from the National Blood Transfusion Centers in Kenya. Hepatitis B virus template DNA was extracted from 400 µl positive HbsAg serum samples with an automatic ExiPrep Dx Viral DNA kit (Bioneer, Korea) on an ExiPrep 48 Dx internal system according to the manufacturer's instructions, and 50 µl of elute recovered and stored at −20˚C till use.

2.3. Amplification of the HBV *S Gene*

Amplifications of the HBV *S gene* were run by nested PCR using Veriti PCR System. First round of the PCR was performed using specific outer primer pair 230F (5'-TCACAATACCGCAGAGTCT-3') and 800R primers (5'AACAGCGGTATAAAGGGACT-3') targeting position 1 to 571 bp of *S gene*. The PCR was performed at 40 cycles using the following thermocycling parameters, 94˚C for 5 min; followed by 45 cycles at 94˚C for 4 min; 94˚C for 45 seconds, annealing at 55˚C for 1 min, extension at 72˚C for 2 min and final extension at 72˚C for 7 minutes. The Second round nested PCR was conducted using specific Inner primers pair P7(+) (5'-GTGGTGGACTTCTCTCAATTTTC-3') and P8(−) primers (5'-CGGTATAAAGGGACTCAAGAT-3') targeting position 1 to 541 bp of *S gene*. Under the same thermocycling conditions as of the 1st PCR except that annealing at 50˚C with 20 µl master mix and 5 µl of the first-round product as per the method of Lindh *et al*. (2007). All amplified PCR

products were examined on a 2% agarose gels stained with Gel Red and viewed using a UV transilluminator. A digital image of the gel was taken using a gel documentation system. The products were then purified, prepared for sequencing and directly sequenced targeting position 1 to 571 bp of *S gene*. Using the same PCR primer pair and an automated sanger technic Applied Biosystems ABI3500xl sequencer (Macrogen, Netherlands).

2.4. Sequence, Genotyping, and Mutations Analysis of *S Gene* Sequences

All Forward and reverse sequences obtained by the sanger technique were analyzed, edited and assembled using Finch TV (http://www.geospiza.com/Products/finchtv.shtml), and Bioedit sequence alignment editor software version 7.2.5 [12] and then subjected to NCBI nucleotide BLAST for quality check. Of the 46 sequences, only 17 were of good quality, qualified for genotyping and mutation analysis. Genotypes and subgenotypes of the 120 retrieved sequences from the database and 17 assembled HBV sequences obtained from blood donors were determined using geno2pheno HBV online software (https://hbv.geno2pheno.org/index.php) version 2.0 [13]. Mutation analysis of sequences from donors was carried our as described previously at the beginning of this section.

2.5. Phylogenetic Analysis

A phylogenetic analysis was performed using MEGA version 11 [14]. The analysis was done using the Maximum-likelihood statistical method, Tamura-Nei model, and the bootstrap method of 1000 replicates.

2.6. Ethical Approval

Jomo Kenyatta (JKUAT) Institutional Ethical Review Committee approved this study protocol (Ref. No. (IERC) (JKU/IERC/02316/0508)).

2.7. Data Analysis

All the statistical analyses were performed using SPSS version 19.0 and GraphPad software for P-Value.

3. Results

3.1. *In-Silico* Identification of the Predominant and Clinically Significant Mutations of *HBsAg Gene* in Kenya from NCBI/HBVdb Databases

A total of one hundred (120) sequences containing complete *Hepatitis B Surface Antigen gene sequences* from Kenya were retrieved from NCBI database/HBVdb databases and analyzed. Out of these sequences, 79 different types of mutations were identified of which 15 mutations were of clinical importance with the occurrence frequency of at least 5% were identified (**Table 1**).

Table 1. Predominant and clinically important mutations of *HBsAg gene* in Kenya as retrieved from NCBI database/HBVdb databases.

Mutation	Genotype	Frequency	Clinical importance	References for associated clinical significance
S207N	A	53	Immune escape, HbsAg detection failure, Increased HBsAg reactivity in immunological diagnostic assays, causes hepatocellular carcinoma/cirrhosis and is an asymptomatic carrier	[13] [15]
A194V	A	36	Immune escape, Drug (tenofovir) resistance and others	[2] [16]
A194V	D	4	Immune escape, Drug (tenofovir) resistance and others	[2] [16]
K122R	A, D	14	Immune escape and is an occult infection related mutation	[15] [17]
T114S	A, D	12	Causes immunosuppression and HBV reactivation	[18]
V168A	A, D	11	*Detection failure and is a* occult infection related mutations	[17]
N131T	A, D	7	Immune escape	[15] [19]
Y161F[7]	A	7	Immune escape	[15]
N40S	A	6	Reduced HBsAg detection signals	[15]
I68T	A	6	Hepatocellular carcinoma (HCC)	[15] [19]
T143S	A	6	Immune escape, Vaccine escape, HBIg therapy escape, detection failure of HbsAg and alter the antigenic properties of variant HBsAg	[15] [20] [21]
A159G	A	6	Immune escape	[15] [19]
S45T[5]/A[5]	A	5.5	Reduced HBsAg detection signals	[15] [19]
P46T[5]	A		Reduced HBsAg detection signals	[15]
C76Y[5]	A		Immune escape (detection failure) and causes Hepatocellular carcinoma/cirrhosis and is an asymptomatic carrier	[15] [21]

Majority (97.5%, n = 77) of the identified mutations were immune escape mutants and they could reduce HBsAg detection with the serological assays. Out 51 (66.2%) were reported as immune escape mutants and they could reduce HBsAg detection with the serological assays alone. Further, 24.6% (n = 19) of mutations were associated with immune escape/reduced HBsAg detection signals and high probability of hepatocellular carcinoma (HCC). Five (6.3%) of mutations were associated hepatocellular carcinoma, two were associated with immune escape/Drug (tenofovir) resistance while one (1.3%) each were associated with immunosuppression/HBV reactivation and Low serum HBV DNA respectively (**Figure 1**).

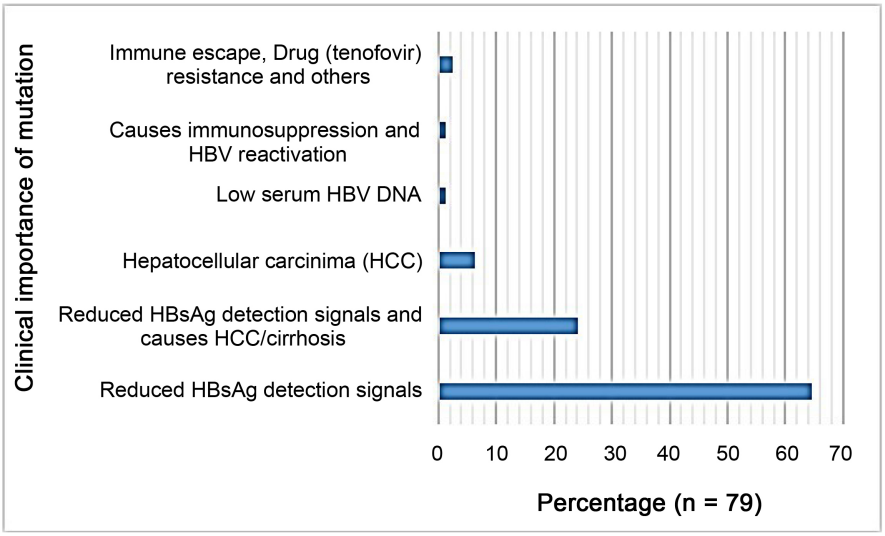

Figure 1. Frequency of clinical significance associated with mutations on *HBsAg gene* (overall).

3.2. Frequency of Substitution at *HBsAg Gene* and Its Correlation with Amino Acid Usage in Reference HBV Virus [NC_00397] *HBsAg Gene*

The frequency of substitutions in *HBsAg gene* at the specific loci from 79 samples and their correlation with amino acid preferential usage in Reference HBV virus [NC_00397] *HBsAg gene* was determined (**Figure 2**). The ratio of amino acid usage in Reference HBV virus [NC_00397] *HBsAg gene* and the Frequency of substitution at *HBsAg gene* (Z/B) was highest for Alanine (Z/B = 11.00), followed by Lysine (Z/B = 7.00), Serine (Z/B = 3.52), Asparagine (Z/B = 3.40) and Valine (Z/B = 2.60) (**Figure 2**). Although technically a positive correlation, the relationship between amino acid preferential usage and the frequencies of mutations was weak (R = 0.4136, P-Value = 0.000151, at $p < 0.05$). This implies that the mutations at this amino acid positions were highly biased in *HBsAg gene* (**Table 2**).

Mapping of the locations of Alanine, Lysine, Serine, Asparagine, and Valine on the Reference HBV virus [NC_00397] *HBsAg gene* showed that there some large loci where these amino acids were not clustered (**Figure 3**).

The most predominant mutations of clinical importance in the *in-silico* analysis were S207N (n = 53), followed by mutation A194V (n = 40). The two mutations are associated with immune escape while the latter is also associated with Drug resistance especially that of tenofovir.

Fifteen mutations in the *HBsAg gene* from donors' samples were of clinical significance with 13 (86.7%) of them being associated with immune escape and reduced chances of being detected by the diagnostics kits that function by detection of HBsAg such as ELISA and immunochromatographic assays. One mutation, T114S, was associated with immunosuppression and HBV reactivation while another, I68T, was associated with Hepatocellular carcinoma (HCC) (**Figure 4**).

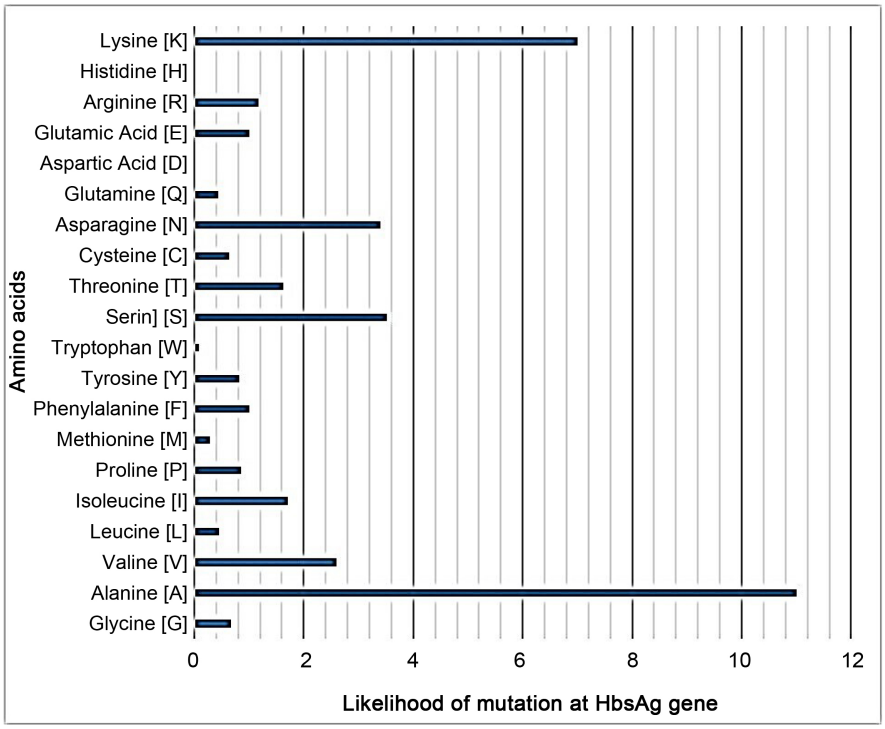

Figure 2. Frequency of substitution at *HBsAg gene* and its correlation with amino acid usage in Reference HBV virus [NC_00397] *HBsAg gene.*

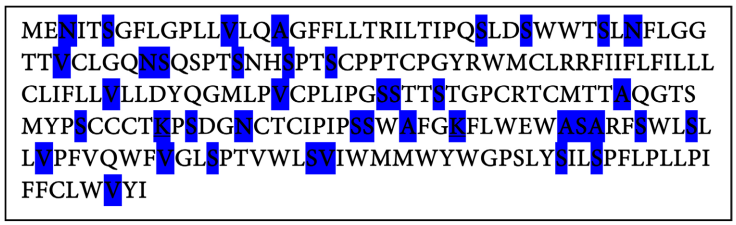

Figure 3. Frequency of substitution at *HBsAg gene* and its correlation with amino acid usage in Reference HBV virus [NC_00397] *HBsAg gene.*

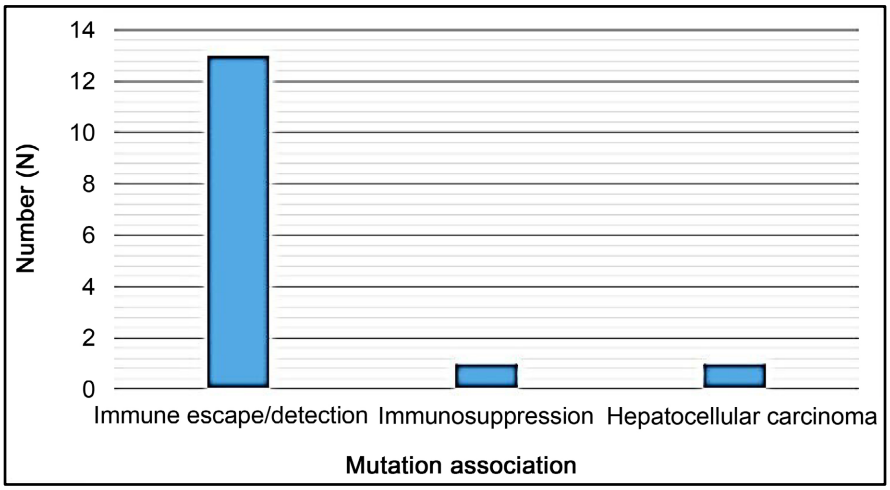

Figure 4. Frequencies of clinical significance associated with mutations on the *HBsAg gene*.

Table 2. Frequency of substitution at *HBsAg gene* and its correlation with amino acid usage in Reference HBV virus [NC_00397] *HBsAg gene.*

Amino Acid Type	Amino Acid	Amino acid usage in Reference HBV virus [NC_00397] *HBsAg gene* (B)	*Frequency of substitution at HBsAg gene* (Z)	(Z/B)
Hydrophobic: Aliphatic	Glycine [G]	15	10	0.67
	Alanine [A]	5	55	11.00
	Valine [V]	10	26	2.60
	Leucine [L]	35	16	0.46
	Isoleucine [I]	14	24	1.71
	Proline [P]	21	18	0.86
	Methionine [M]	7	2	0.29
Hydrophobic: Aromatic	Phenylalanine [F]	16	16	1.00
	Tyrosine [Y]	6	5	0.83
	Tryptophan [W]	13	1	0.08
Hydrophilic: Polar Uncharged	Serine [S]	25	88	3.52
	Threonine [T]	19	31	1.63
	Cysteine [C]	14	9	0.64
	Asparagine [N]	5	17	3.40
	Glutamine [Q]	7	3	0.43
Hydrophilic: Acidic	Aspartic Acid [D]	3	0	0.00
	Glutamic Acid [E]	2	14	7.00
Hydrophilic: Basic	Arginine [R]	6	7	1.17
	Histidine [H]	1	0	0.00
	Lysine [K]	2	14	7.00

The most dominant genotype was found to be Genotype A in both *in-silico* analysis and blood donors' sequences (Figure 5).

3.3. Phylogenetic Analysis of Sequenced Samples from Blood Donors in Kenya

Phylogenetic analysis of sequenced portion of *HbsAg gene* (Figure 6) showed that HBV PGENE 10 and PGENE2F clustered with the standard Genotype A NC_003977.1 references while HBV10, HBV7 and HBV13 clustered with the sub-genotype A1 AY128092.1, X51970.1 reference sequences from Africa. Sequences from samples HBV26, HBV46, HBV48, HBV38, HBV39, HBV27,

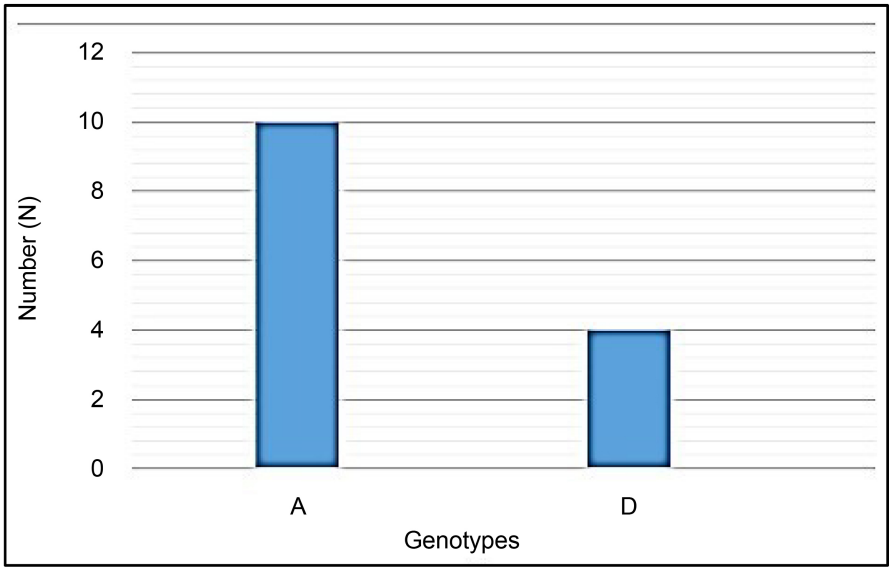

Figure 5. Frequencies of various genotypes HBV from analysis of *HBsAg gene*.

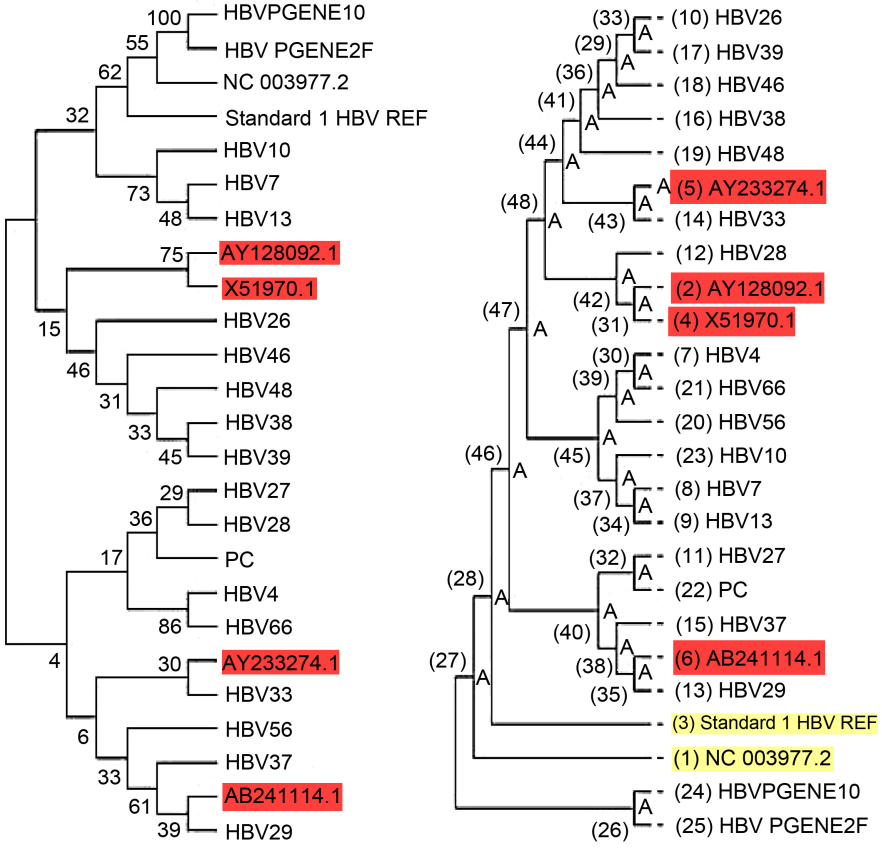

Figure 6. A phylogenetic tree constructed from HBV 17 sequences obtained from the 46 HBV blood donors samples obtained from KEMRI with 6 HBV reference sequences.

HBV28, PC, HBV66 clustered with sub-Genotype A1 AY233274.1 from Africa. This shows the close relationships of the above donor sequences between refer-

ences AY233274.1 and AY128092.1, X51970.1. Nevertheless, HBV33, HBV56, HBV37, and HBV29, clustered with clustered with sub-Genotype A1 AY233274.1 from Africa and subgenotype A1 AB241114.1. This show that all these sequences clade for HBsAg region analysis. Further genetic analysis of the A genotype showed that HBV subgenotype A1 (94.20%) predominated Phylogenetic analysis.

The phylogenetic analysis shows that the evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates) are shown next to the branches [3]. Initial tree(s) for the heuristic or experimental search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 19 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 1203 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

3.4. Analysis of Mutations/Substitutions on the HBV S-Region of the Sequenced Samples at the Same Amino Acid Loci of the Most Two Clinically Important Dominant Mutants amongst HBV Sequences in Kenya

Analysis of HBsAg of amino acid loci of the most common clinically important mutations amongst HBV sequences in Kenya demonstrated twenty (22) types of mutations/substitutions (**Table 3**). Ten (45.5%) mutations (sA194Y, sS207, sA194S, sS207I, sA159I, sP46A, sA194T, sS207I, sP46R, and sT143P] according to current knowledge, had not been published before. Four (20%) of the mutations were clinically significant. These included sS207R, sT143S, sC76F and sK122R.

Out of ten samples that showed amino acid substitutions, six had more than two substitutions. Comparison of mutations of the HBsAg from the blood donors' samples with those that were determined *in silico* from the Hepatitis B virus database from Kenya show a marked difference between them (**Table 3**).

Only two (13.3%) samples showed the same mutations of sK122R and sT143S for both *in-silico* analysis and actual sequenced samples (**Figure 7**). The two mutations were of clinical importance as they are associated with immune escape and occult infection as they are within the "a determinant" region of HBsAg.

Comparison of mutations on 14 most common mutations of clinical importance that were identified in the *in-silico* analysis of mutations from HBsAg Analysis in Kenya, those identified in the sequenced samples and the mutations in same loci of *HBsAg gene* in the past studies as identified by [22] showed

Table 3. Analysis of mutations/substitutions on the HBV S-region of the sequenced samples at the same amino acid loci of the most two clinically important dominant mutants amongst HBV sequences in Kenya.

	Mutations determined *in silico*	*Mutations in the samples sequenced* on same Loci
1.	S207N	sS207L, sS207R
2.	sA194V	sA194Y, sA194S, sA194T
3.	K122R	sK122R*, sK122V, sK122T
4.	sT114S	None observed
5.	sV168A	None observed
6.	N131T	None observed
7.	sY161F[7]	None observed
8.	sN40S	sN40I, sN40K
9.	sI68T	None observed
10.	sT143S*	sT143S* [the same], sT143T, sT143P
11.	sA159G	sA159I
12.	sS45T[5]/A[5]	None observed
13.	sP46T[5]	sP46A, sP46R
14.	sC76Y[5]	sC76F, sC76G

NB: *Common substitutions.

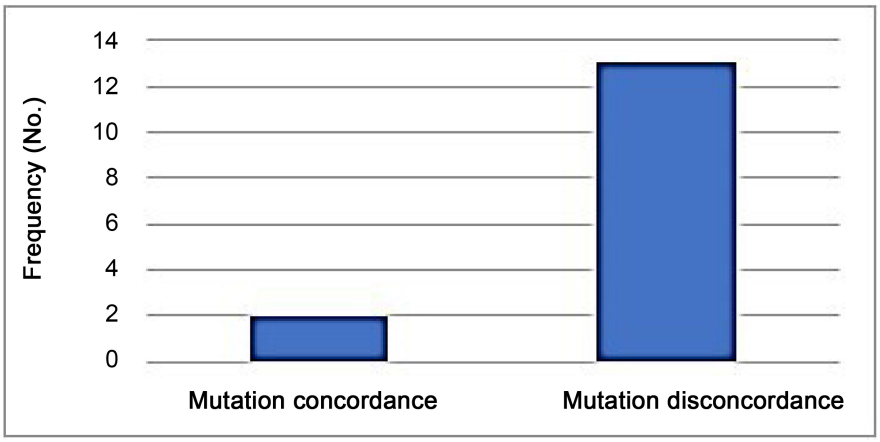

Figure 7. Concordance between HBsAg analyses *in silico* on blood donor sequenced samples.

general lack of consistency of mutations in a given loci (**Table 4**). Mutation sK122R was the most common mutation (n = 4) in these specific loci followed by mutation sT143S (n = 3), and sA194V, sT114S, sY161F, and sA159V (n = 2 each). The frequency (n = 2) of occurrence of mutation sK122R and sT143S for the HBsAg sequenced in this study was the same with that noted by Kiptoon [24].

Table 4. Comparison of *HBsAg gene* mutations from various studies in Kenya.

S/No.	Studies of specific locations of HBV S-regions in Kenya				
	Mutations from *in silico* study	**Mutations from 16 sequenced sample**	**Mutations identified by Nyairo [23]**	**Mutations identified by Kiptoon [24]**	**Mutations identified by Aluora [25]**
1.	sS207N	sS207L sS207R			S207K
2.	sA194V	sA194Y, sA194S, sA194T	sA194V		sA194V
3.	sK122R	sK122R, sK122V, sK122T,	sK122R (3)	sK122R	
4.	sT114S	None observed	sT114S sT114K		sT114P
5.	sV168A	None observed			
6.	sN131T	***None*** observed		sT131N	
7.	sY161F7	***None*** observed	sY161F, sW165L		
8.	sN40S	sN40I, sN40K			
9.	sI68T	***None*** observed			
10.	sT143S	sT143S sT143T, sT143P	**sT143M**	sT143S	**sT143M**
11.	sA159G	sA159I	sA159V		sA159V
12	sS45T[5]/A[5]	None observed			
13	sP46T[5]	sP46A, sP46R			
14.	sC76Y[5]	sC76F, sC76G			

When mutations on *HBsAg gene* position 1 - 225 were mapped for sequences that were obtained in the *in-silico* analysis and compared with a comparable mapping that was done by [26] on the same gene and region it was found that the regions of mutations clustering and conserved regions were in concordance (**Figure 8**).

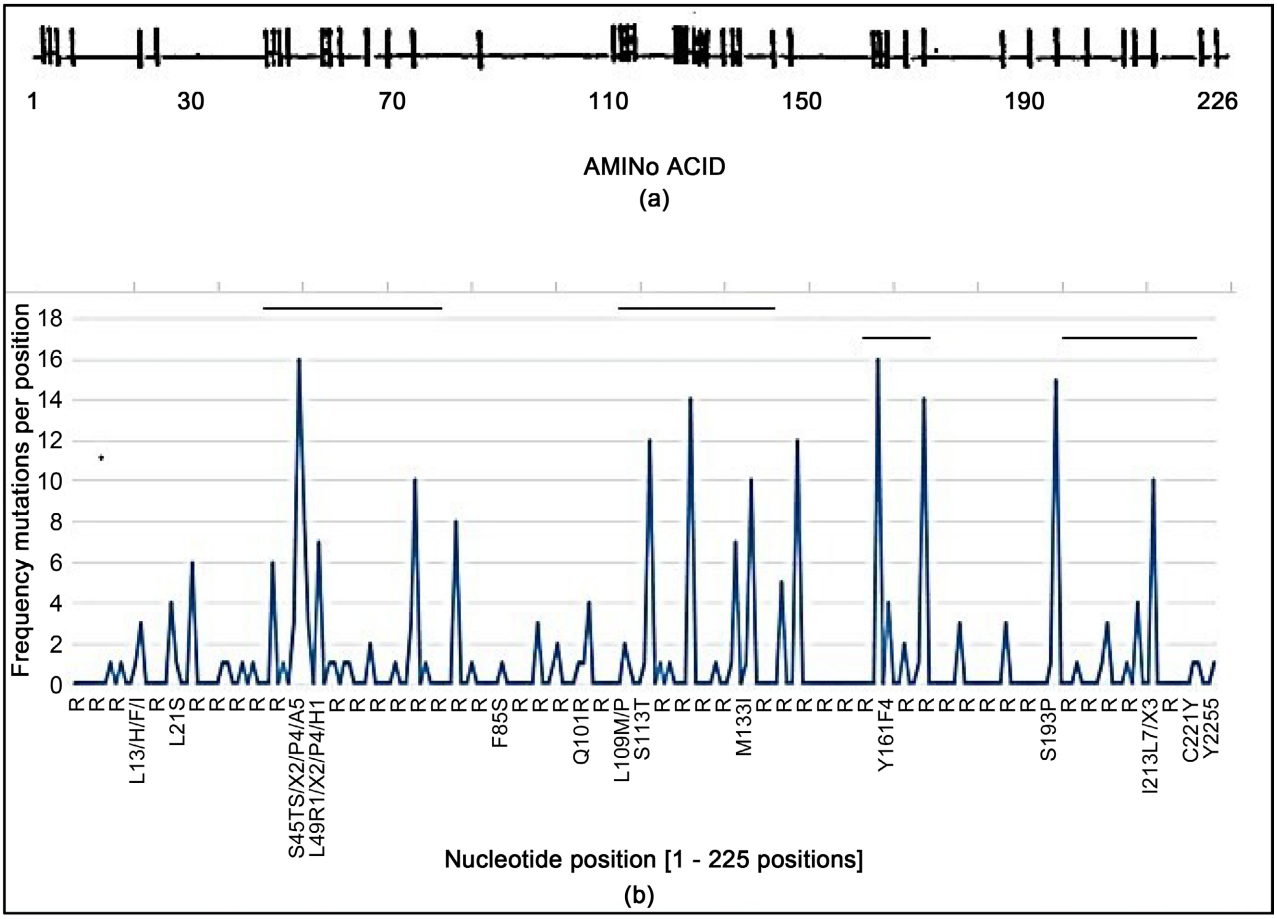

Figure 8. Frequency of mutations at specific loci of *HBsAg gene* [1 - 226 positions] from Lauder *et al.* (1993) study [A] and the *in-silico* analysis of HBV sequences in Kenya [B] comparing the mutation clustering between the two studies. The regions of clustering are comparable.

4. Discussion

In-Silico Identification of the Predominant and Clinically Significant Mutations of *HBsAg Gene* in Kenya from NCBI/HBVdb Databases

From 100 *HBsAg gene* sequences extracted from the NCBI, and HBV database from Kenya, 79 different types of mutations were identified after *in-silico* analysis. The most common mutations of clinical significance (with occurrence frequency > 5%) were: S207N, A194V, A194V, K122R, T114S, V168A, N131T, Y161F7, N40S, I68T, T143S, A159G, S45T5/A5, P46T5, and C76Y5 starting from the highest to the lowest [15] [17]. Majority (64.6%, n = 51) of the mutation were associated with immune escape and reduced HBsAg detection signals. This was followed by 24.1% (n = 19) of mutations that were both associated with immune escape/reduced HBsAg detection signals and high probability of hepatocellular carcinoma (HCC). Five (6.3%) of mutations were associated hepatocellular carcinoma, with one being associated with immune escape/drug (Tenofovir) resistance while one (1.3%) each were associated with immunosuppression/HBV reactivation and Low serum HBV DNA respectively [15]. Mutations

on the "a" determinant region of *HBsAg gene* are associated with escape from vaccine-induced immunity have been reported by various studies and they include T116N, P120S/E, I/T126A/N/I/S, Q129H/R, M133L, K141E, P142S, D144A/E, and G145R/A S143L. [15] [17] [19]. Of these mutations only T116N was identified from the *in-silico* analysis of the 100 sequences in this study. Although studies in Sub-Saharan Africa, have identified G145R as the commonest mutation within the HBsAg immunodorminant "a" determinant the *in-silico* and sample analysis in this study together with other studies [23] [24] [25] carried out in Kenya have not found this mutation.

The concordance of mutations in "a" determinant region of *HBsAg gene* among various studies is minimal. In a study done in Central Africa Republic seven mutations (sL127I, sY100C, sA128V, sG130S, sM133T, sF134I, and S140T) within the "a" determinant region were identified of only one mutation (sG130S) was similarly identified in this study [27]. Avellón and Echevarria analyzed amino-acid positions 112 - 157 in the HBV "a" determinant region and established the following mutations: M133T (2.2%); Q129H, M133I, F/Y134N (1.8%); F/Y134L, G145A (1.5%), and P120T (1.1%) and G145R (0.4%) [28]. None of these mutations were captured in the current study. Following these findings, it is very important to continuously monitor the mutations of HBV in each specific geographical region as the general findings from other geographical regions may not be fully relied on to inform a virus mutation states. The demonstrated evidence of mutations on the "a" determinant region of *HBsAg gene* calls for the need for identification and development of other biomarkers to produce better serology-dependent tests like Enzyme-Immunosorbents Assays (ELISA) and immunochromatographic tests. Otherwise, the future could be in development of multiplex PCR tests that will detect various loci of HBV gene [29] Similarly, the indication of development of vaccine escape HBV mutant points to the future trend of increased use of multivalent HBV vaccine that will be able to capture escape mutants. Already, a recombinant HBsAg of the Wild-Type and the G145R Escape Mutant vaccine is in the development pipeline [30] though it could end up not being useful in countries like Kenya where this study has demonstrated the low prevalence of G145R Escape Mutant.

The *in-silico* analysis identified the following mutations that were associated with hepatocellular carcinoma (HCC): I68T, Q30R, G44E2/X1, L49R1/X2/P4/H1, Q54H, S64C, P67Q2/L1, Q101R, L109M/P, and F134S1/Y4/L1. The analysis also identified mutations A194V40/X1 with potential to lead to drug resistance for drugs like Tenofovir. The presence of these mutations points to the need of carrying out HBV sequence analysis regularly for immune escape, drug resistance, and detection of diagnostic markers. The frequency of specific amino acid mutations at *HBsAg gene* from the 79 sequences demonstrated significant bias ($R = 0.4136$, P-Value = 0.000151, at $p < 0.05$). The preference of substitution at *HBsAg gene* was highest for Alanine, followed by Lysine, Serine, Asparagine and Valine. When mutations on *HBsAg gene* position 1 - 225 in the *in-silico* analysis were compared with a similar mapping that was done by Lauder *et al*. [26] on

the same gene and region it was found that the regions of mutations clustering and conserved regions were in concordance. It is noteworthy that the mutation clusters at these positions of *HBsAg gene* have not been over a period of 30 years.

Analysis of mutations on the *HBsAg gene* of 17 sequenced samples in this study at the same amino acid loci as those determined by the *in-silico* study demonstrated twenty (22) types of mutations/substitutions. Ten (45.5%) mutations (sA194Y, sS207, sA194S, sS207I, sA159I, sP46A, sA194T, sS207I, sP46R, and sT143P) had not been published before, according to current knowledge. Four (20%) of the mutations were clinically significant. These included sS207R, sT143S, sC76F and sK122R.

5. Conclusion

This study, like other related studies in Kenya, did not identify G145R which is the commonest mutation within the HBsAg immunodorminant "a" determinant that has been identified in other regions of the world with closely associated with immune escape. The concordance of mutations in "a" determinant region of *HBsAg gene* among various studies is minimal. The study identified new mutations (sA194Y, sS207, sA194S, sS207I, sP46A, sA194T, sS207I, sP46R, and sT143P) that had not been published before, according to current knowledge. Four (20%) of the mutations were clinically significant. These included sS207R, sT143S, sC76F and sK122R.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Nyairo, E.K., *et al*. (2016) Mutations in the "a" Determinant Region of Hepatitis B Virus Genotype A among Voluntary Kenyan Blood Donors. *Journal of Biology, Agriculture and Healthcare*, **6**, 89-97.

[2] Horvat, R.T. (2011) Diagnostic and Clinical Relevance of HBV Mutations. *Laboratory Medicine*, **42**, 488-496. https://doi.org/10.1309/LM7SF4QZMEG5LVPN

[3] Poortahmasebi, V., *et al*. (2020) Conformational Analysis of Hepatitis B Virus Surface Antigen Mutations among HIV-Positive Patients Diagnosed with Occult Hepatitis B Virus. *Future Virology*, **14**, 813-827. https://doi.org/10.2217/fvl-2019-0096

[4] Samal, J., Kandpal, M. and Vivekanandan, P. (2012) Molecular Mechanisms Underlying Occult Hepatitis B Virus Infection. *Clinical Microbiology Reviews*, **25**, 142-163.

https://doi.org/10.1128/CMR.00018-11

[5] Coleman, P.F. (2006) Detecting Hepatitis B Surface Antigen Mutants. *NIH Public Access*, **12**, 198-203. https://doi.org/10.3201/eid1203.050038

[6] Zuckerman, J.N. and Zuckerman, A.J. (2003) Mutations of the Surface Protein of Hepatitis B Virus. *Antiviral Research*, **60**, 75-78. https://doi.org/10.1016/j.antiviral.2003.08.013

[7] Kim, Y.J. (2019) Evaluation of Dual Priming Oligonucleotide (DPO)-Based Multiplex PCR for Detection of HBV YMDD Mutants. *Archives of Virology*, **153**, 2019-2025. https://doi.org/10.1007/s00705-008-0218-3

[8] Kim, J., *et al*. (2008) Direct Detection of Lamivudine-Resistant Hepatitis B Virus Mutants by a Multiplex PCR Using Dual-Priming Oligonucleotide Primers. *Journal of Virological Methods*, **149**, 76-84. https://doi.org/10.1016/j.jviromet.2008.01.003

[9] Zoulim, F. (2010) Hepatitis B Virus Resistance to Antiviral Drugs: Where Are We Going? *Liver International*, **31**, 111-116. https://doi.org/10.1111/j.1478-3231.2010.02399.x

[10] Cooksley, W.G.E. (2010) Do We Need to Determine Viral Genotype in Treating Chronic Hepatitis B? *Journal of Viral Hepatitis*, **17**, 601-610. https://doi.org/10.1111/j.1365-2893.2010.01326.x

[11] Chan, H.L., *et al*. (2003) Hepatitis B Virus Genotype Has No Impact on Hepatitis B e Antigen Seroconversion after Lamivudine Treatment. *World Journal of Gastroenterology*, **9**, 2695-2697. https://doi.org/10.3748/wjg.v9.i12.2695

[12] Alzohairy, A.M. (2011) Mfold©: RNA Modeling Program.

[13] Hermans, L.E., *et al*. (2016) Combined Analysis of the Prevalence of Drug-Resistant Hepatitis B Virus in Antiviral Therapy—Experienced Patients in Europe (CAPRE). *The Journal of Infectious Diseases*, **213**, 39-48. https://doi.org/10.1093/infdis/jiv363

[14] Tamura, K., Stecher, G. and Kumar, S. (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, **38**, 3022-3027. https://doi.org/10.1093/molbev/msab120

[15] Coppola, N., *et al*. (2015) Clinical Significance of Hepatitis B Surface Antigen Mutants. *World Journal of Hepatology*, **7**, 2729-2739. https://doi.org/10.4254/wjh.v7.i27.2729

[16] Cuestas, L., *et al*. (2006) Unusual Naturally Occurring Humoral and Cellular Mutated Epitopes of Hepatitis B Virus in a Chronically Infected Argentine Patient with Anti-HBs Antibodies. *Journal of Clinical Microbiology*, **44**, 2191-2198. https://doi.org/10.1128/JCM.00057-06

[17] Wang, Q., *et al*. (2020) A Combination of Human Broadly Neutralizing Antibodies against Hepatitis B Virus HBsAg with Distinct Epitopes Suppresses Escape Mutations. *Cell Host Microbe*, **28**, 335-349.E6. https://doi.org/10.1016/j.chom.2020.05.010

[18] Rajput, M.K. (2020) Mutations and Methods of Analysis of Mutations in Hepatitis B Virus. *AIMS Microbiology*, **6**, 401-421. https://doi.org/10.3934/microbiol.2020024

[19] Araujo, N.M., Teles, S.A. and Spitz, N. (2020) Comprehensive Analysis of Clinically Significant Hepatitis B Virus Mutations in Relation to Genotype, Subgenotype and Geographic Region. *Frontiers in Microbiology*, **11**, Article ID: 616023. https://doi.org/10.3389/fmicb.2020.616023

[20] Chaouch, H., Villano, U., *et al*. (2016) Naturally Occurring Surface Antigen Variants of Hepatitis B Virus in Tunisian Patients. *Intervirology*, **59**, 36-47. https://doi.org/10.1159/000445894

[21] Olinger, C.M., Weber, B., Otegbayo, J.A., Ammerlaan, W. and Muller, N. (2007) Hepatitis B Virus Genotype E Surface Antigen Detection with Different Immunoassays and Diagnostic Impact of Mutations in the preS/*S Gene*. *Medical Microbiology and Immunology*, **196**, 247-252. https://doi.org/10.1007/s00430-007-0050-5

[22] Downs, L.O., Campbell, C., Yonga, P. and Ansari, M.A. (2022) A Systematic Review of Hepatitis B Virus (HBV) Prevalence and Genotypes in Kenya: Data to Inform Clinical Care and Health Policy. https://doi.org/10.1101/2022.04.08.22273611

[23] Nyairo, E.K. (2018) Hepatitis B Virus *Gene*tic Diversity and Surface Antigen Mutations among Voluntary Blood Donors in Kenya. Jomo Kenyatta University of Agriculture and Technology, Juja. http://ir.jkuat.ac.ke/bitstream/handle/123456789/4564/NYAIRO3REVISED17.5.18bb.pdf?sequence=1&isAllowed=y

[24] Beatrice, K., *et al*. (2020) Characterization of Occult Hepatitis B in High-Risk Populations in Kenya. *PLOS ONE*, **15**, e0233727.

[25] Aluora, P.O., Muturi, M.W. and Gachara, G. (2020) Seroprevalence and Genotypic Characterization of HBV among Low-Risk Voluntary Blood Donors in Nairobi, Kenya. *Virology Journal*, **17**, Article No. 176. https://doi.org/10.1186/s12985-020-01447-2

[26] Proutski, V. and Holmes, E.C. (1998) SWAN: Sliding Window Analysis of Nucleotide Sequence Variability. *Bioinformatics*, **14**, 467-468. https://doi.org/10.1093/bioinformatics/14.5.467

[27] Wilfried, G., *et al*. (2020) Detection of Circulating Hepatitis B Virus Immune Escape and Polymerase Mutants among HBV-Positive Patients Attending Institute Pasteur de Bangui, Central African Republic. *International Journal of Infectious Diseases*, **90**, 138-144. https://doi.org/10.1016/j.ijid.2019.10.039

[28] Avellón, A. and Echevarria, J.M. (2006) Frequency of Hepatitis B Virus "a" Determinant Variants in Unselected Spanish Chronic Carriers. *Journal of Medical Virology*, **78**, 24-36.

[29] Gani, A.W., *et al*. (2019) An Automated, Quantitative, and Multiplexed Assay Suitable for Point-of-Care Hepatitis B Virus Diagnostics. *Scientific Reports*, **9**, Article No. 15615. https://doi.org/10.1038/s41598-019-52147-z

[30] Konopleva, M.V., Borisova, V.N., Sokolova, M.V., Semenenko, T.A. and Suslov, A.P. (2022) Recombinant HBsAg of the Wild-Type and the G145R Escape Mutant, Included in the New Multivalent Vaccine against Hepatitis B Virus, Dramatically Differ in Their Effects on Leukocytes from Healthy Donors *in Vitro*. *Vaccines*, **10**, Article No. 235. https://doi.org/10.3390/vaccines10020235