Hepatitis B virus subgenotype A1, occurrence of subgenotype D4, and S gene mutations among voluntary blood donors in Kenya

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Abstract Kenya is one of the high endemic zones for hepatitis B virus (HBV) infection. The consensuses on prevalence of the HBV genotypes and the existence of their variants have not been fully established in Kenya. Hence, there is a need to further monitor the diversity of HBV. This study aimed to extend the current molecular and epidemiological information about the geographical distribution of HBV genotypes and subgenotypes, as well as to describe the hepatitis B surface antigen (HBsAg) variants circulating in different Regional Blood Transfusion Centres of Kenya. A total of 32 HBsAg positive blood units from five different blood transfusion centers in Kenya were used in the study. The HBV DNA preS/S-gene was amplified and sequenced. Alignments of S gene were applied using reference sequence from GeneBank. Phylogenetic analysis was performed using the MEGAv4.0 software with the neighbor-joining and maximum composite likelihood methods. Twenty-one plasma samples (65.6 %) were DNA positive and were successfully sequenced. Eighteen out of the twenty-one isolates (85.7 %) belonged to subgenotype A1 Afro-Asian: six were from Nairobi, four from Kisumu, two from Embu, and three each from Eldoret and Mombasa. The other three strains (14.3 %, 3/21) belonged to subgenotype D4 from Mombasa. The HBsAg mutations were detected in nine isolates (42.9 %, 9/21). The HBV/A1 and HBV/D4 are dominant among blood donors in Kenya. This demonstrates that continuous monitoring of the HBV diversity would help reveal circulating genotypes and subgenotypes as well as mutants of clinical significance in Kenya.

Introduction

Chronic infection with hepatitis B virus (HBV) is a common cause of death associated with liver failure, cirrhosis, and

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hepatocellular carcinoma (HCC). Worldwide, approximately 350 million persons have chronic HBV infection, and an estimated 620,000 persons die annually from HBV-related liver disease [1]. Based on genomic nucleotide sequence divergence of greater than 8 %, HBV was initially classified into eight genotypes labeled A through H [2], and the subgenotypes differ by at least 4 % [3, 4]. However, currently, the virus has been classified into nine different genotypes, designated from A to I [5]. The JRB34 strain provisionally designated genotype J was found to be genetically and phylogenetically distinct from any of the previously published strains, including those of genotype I from Vietnam and Laos [6]. The HBV genotypes and their subgenotypes have shown a distinct geographic distribution globally [7].

Africa is one of the highly endemic regions of HBV and three genotypes (A, D, and E) have been identified in different countries [7]. Kenya is one of the high endemic zones for HBV infection and studies in Kenya showed HBsAg carrier rates in the range of 5–30 % [8]. In Kenya, previous reports indicate that the high demand for blood transfusion is exacerbated by the donor seroprevalence of HBV (2.71 %) as the leading Transfusion Transmissible Infection (TTI) followed by HIV (1.27 %) and HCV (1.02 %) [9]. A previous study in Kenya reported the presence of HBV variants with genotypes A (88 %), E (8 %), and D (4 %). In addition, the study demonstrated that there could be a high genetic diversity of HBV in Kenya [10]. This work aimed to extend the current molecular and epidemiological information about the geographical distribution of HBV genotypes and subgenotypes, as well as to describe the hepatitis B surface antigen (HBsAg) variants circulating in different Regional Blood Transfusion Centres (RBTCs) of Kenya.

Materials and methods

This study was carried out at the Kenya Medical Research Institute (KEMRI), Kenya. The study used 32 HBsAg positive rejected blood units collected from five different RBTCs in Kenya from March, 2012 to June, 2012: Mombasa ($n = 7$), Nairobi ($n = 6$), Kisumu ($n = 9$), Eldoret ($n = 5$), and Embu ($n = 5$). The study protocol was approved by KEMRI Ethics Review Committee. The permission to collect the blood samples from the RBTCs was granted by the Kenya National Blood Transfusion Service (KNBTS).

Reconfirmation of HBsAg positive samples using ELISA

The HBsAg positive units were all reconfirmed positive using enzyme-linked immunosorbent assay (ELISA) kit,

Fig. 1 A phylogenetic NJ tree constructed using the HBV preS2/S ▶ nucleotide sequences (nt 7–842 according to GenBank accession #AY233274/#AB033559). Twenty-one strains from different geographical regions of Kenya isolated in this study are indicated in *bold* font and *diamonds*. Strain names with abbreviation for Kenya (*KE*), submitted to the GeneBank DDBJ with the accession numbers: AB786643–AB786663. The *numbers* on the nodes are bootstrap values in percentages of occurrences obtained from 1,000 replicates. Reference sequences retrieved from GenBank are indicated by their subgenotype, accession numbers, and origin added within *parentheses* for HBV/A and HBV/D strains

(Hepanostika® HBsAg Ultra, bioMérieux SA). All assays were performed according to the manufacturer's instructions. The blood samples were separated and stored at −80 °C until further use for HBV DNA amplification.

HBV DNA extraction, amplification, and sequencing

HBV DNA was extracted using High Pure Viral Nucleic Acid Kit (Roche Mannheim, Germany) from 200 µl of all the HBsAg positive plasma samples. The HBV DNA preS/S-gene was amplified from the extracted DNA in a nested PCR. This included the primers: S1F—TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC and S1R—TCC AGA CCA GCT GCG AGC AAA ACA for the first round; and S2F—AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG and S2R—AGT TCC GCA GTA TGG ATC GGC AGA GGA for the second round [11]. The PCR amplification profile was hot start of 94 °C for 7 min, followed by 38 cycles of 94 °C for 40 s, 60 °C for 1 min and 72 °C for 2 min and final extension for 72 °C for 15 min in both rounds. Ten microliters of nested PCR product was analyzed on 1.2 % agarose gel in 1× TAE buffer stained with ethidium bromide. PCR products were purified using a MinElute® Gel Extraction Kit (Qiagen, Inc., USA) following the manufacturer's protocol. The purified DNA (50 ng) products were subjected to direct sequencing using a Prism Big Dye v3.0 kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA automated sequencer (Applied Biosystems). The preS2/S sequences were obtained using the same amplification primers.

Phylogenetic and mutation analysis

The reference HBV DNA sequences of genotypes of A–H and A and D subtypes from GenBank (Fig. 1) together with obtained ($n = 21$) Kenyan sequences obtained in the study were aligned using CLUSTAL W software program in MEGAv4.0 software, with subsequent confirmation by visual inspection and manual modification. The obtained nucleotide sequences were aligned with standard hepatitis B sequence for subgenotype A1 Accession number;
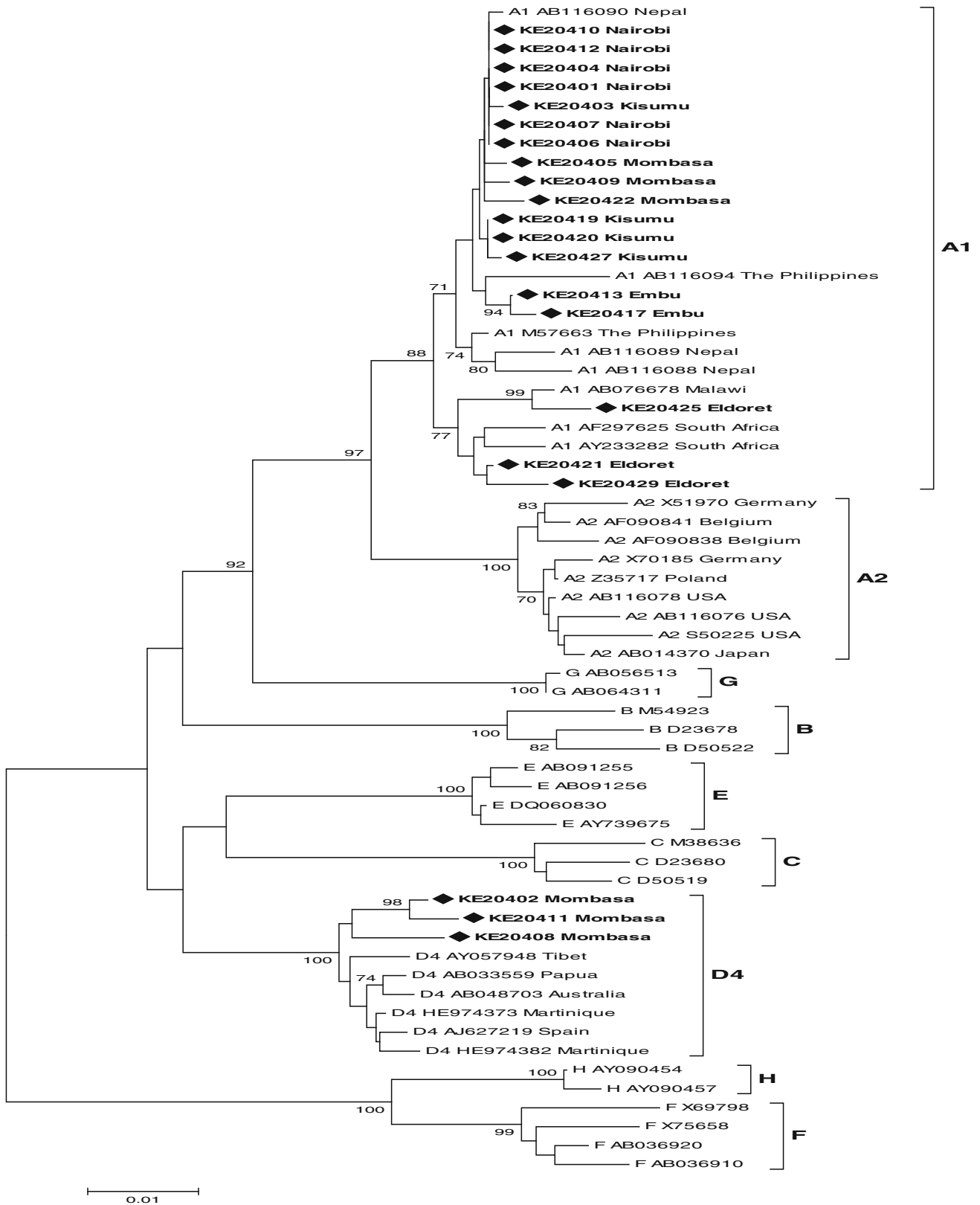
AY233274 [12] from Gene Bank database and published consensus subgenotype D4 sequence [13] to detect mutations. Phylogenetic analysis was performed using neighbor-joining method and maximum composite likelihood (MCL) methods [14] of MEGAv4.0 software, with 1,000 bootstrap replicates.

Results

All the 32 HBsAg positive blood units were reconfirmed as HBsAg positive in the two successive rounds of the tests using ELISA. In these units, HBV S gene was successfully amplified from 21 HBsAg plasma samples (65.6 %). The rest of the samples could not amplify specifically; these were considered either to have very low DNA that could not be detected by the nested PCR or false-positives. The HBV large S coding region sequences were successfully amplified from the 21 DNA positive samples.

The sequences were subjected to a BLAST similarity search throughout GenBank, and the most similar strains were used for phylogenetic analysis together with the reference sequences of human HBV genotypes. Phylogenetic relationship based on approximately 836 nucleotides of preS2/S sequences of the HBV strains is represented in Fig. 1. The 27 sequences of all other reported human HBV genotypes were selected for phylogenetic tree. The phylogenetic tree revealed 18 HBV Kenyans strains (85.7 %, 18/21) grouping together in the subgenotype HBV/A1 (Africa-Asian) cluster with bootstrap value 88 %. Of the 18 strains, six were from Nairobi, four from Kisumu, two from Embu, and three each from Mombasa and Eldoret. The HBV/A1 grouped distinctively from HBV/A2 (European) clusters within the HBV/A cluster (bootstrap index 97 %). The other three Kenyan strains from Mombasa (14.3 %, 3/21) belonged to subgenotype D4 cluster, supported by 100 % bootstrap value (Fig. 1).

Nine Kenyan isolates (42.9 %, 9/21) showed amino acid substitution in the S region that codes amino acids 1–226. Twelve of the isolates (57.1 %) did not show any mutations in the S region. These included; subgenotype A1 of all the six isolates from Nairobi, three from Kisumu, and one from Eldoret, as well as two isolates of subgenotype D4 from Mombasa. The *a* determinant region (amino acids 124–147) of HBsAg did not show any mutation. However,

substitution K122R occurred within the HBsAg major hydrophilic region (MHR) of the amino acids in positions 99–169. The alignment of amino acid sequences are shown in Figs. 2 and 3. The nine isolates in this study demonstrated different amino acid substitution based on amino acids of the HBsAg (Table 1). The amino acid substitution S45P was common in isolates KE20425 and KE20429 of subgenotype A1 from Eldoret. In the same region, amino acid substitutions F20S, P46A, K122R, and T189I occurred in Isolate KE20425 as well as S55F, Y200F, and S204N in isolate KE20429.The amino acid substitutions, S193L and S204N, were common in all the two isolates of subgenotype A1 from Embu. Additional substitution S34L was also observed in isolate KE20417 from Embu. The isolate KE20427 of subgenotype A1 from Kisumu showed amino acid substitution G44E. The substitutions P203R was observed in KE20422 of subgenotype A1 from Mombasa. In addition, P67Q amino acid substitution was found in isolate KE20405 as well as I226F in isolate KE20409. In the other three isolates of subgenotype D4 from Mombasa only, S210R was observed in isolate KE20408.

Discussion

This is the second study on the distribution of HBV genotypes among the blood donors in Kenya. In the study, the HBsAg positive plasma samples were obtained from the five major RBTCs in different geographical regions of Kenya. The results of the present study indicated that HBV/A1 is a major subgenotype among the blood donors. The subgenotype A1 was found to be a predominant (85.7 %) in all the five Regions of Kenya. However, the lower predominance of subgenotype D in smaller proportion (14.3 %) appears to occur only in Mombasa region with transition to subgenotype D4.
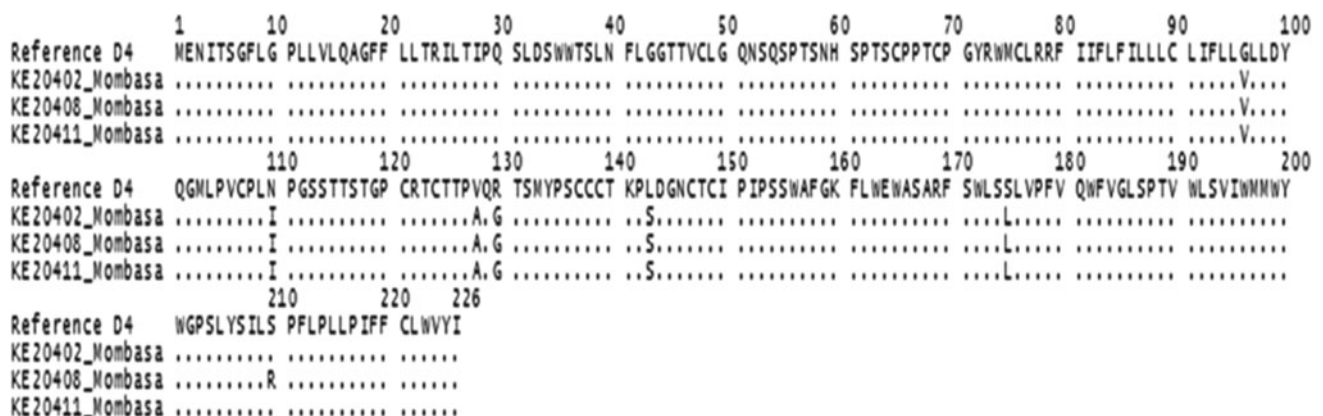
Fig. 2 Alignment of amino acid sequences encoded by the S gene from 1 to 226 amino acids with the consensus sequence of subgenotype D4 [13]. Kenyan isolate KE20408 from Mombasa showed S210R mutation

Fig. 3 Alignment of amino acid sequences encoded by the S gene from 1 to 226 amino acids with the consensus of subgenotype A1 (accession number: AY233274). Eight Kenyan isolates of subgenotype A1 showed mutations

The first study conducted among the blood donors in Kenya [10] demonstrated that HBV genotype A is the most predominant genotype in Kenya with both subgenotype A1 and A2 present as well as genotype D and E. The study presented a phylogenetic analysis based on pre-S1 region of subgenotype A1 and A2 HBV isolates from a population of donors in Kenya [10]. Additional sequences obtained in the present study of the pre-S/S region of HBV isolates from population of blood donors of different geographical regions are an extension from the previous study [10] in which only the pre-S1 genes were sequenced. However, in our study, 18 isolates clustered with A1 subgenotypes from Nepal, The Philippines, Malawi, and South Africa, suggesting that subgenotype A1 could be circulating in the five regions of Kenya. The presence of A1 in Kenya population suggests significant role of population movements from Asian and other African countries to Kenya introducing this HBV strain in Kenya. Based on the sequences of the pre-S2/S region genotype A of subgroup (A1 Afro-Asian) was first recognized in 60 % of HBV isolates from South

Table 1 Distribution of amino acid substitutions in nine HBV sequences from four Regional Blood Transfusion Centres in Kenya with previously recognized S gene mutations in boldface

No.	Isolates	Genotype	S gene mutations
1.	KE20413Embu	A1	**S193L**, **S204N**
2.	KE20417Embu	A1	**S193L**, **S204N**, S34L
3.	KE20422Mombasa	A1	**P203R**
4.	KE20425Eldoret	A1	**F20S**, S45P, P46A, **K122R**, **T189I**
5.	KE20427Kisumu	A1	**G44E**,
6.	KE20429Eldoret	A1	S45P, S55F, **Y200F**, **S204N**
7.	KE20405Mombasa	A1	P67Q
8.	KE20409Mombasa	A1	I226F
9.	KE20408Mombasa	D4	**S210R**

Africa [15]. Moreover, in Malawi, 65 % of the HBV genotype A isolates clustered with subgroup A1 previously reported in carriers from South Africa and Zimbabwe [16].

In this study, genotype D was less predominant in smaller proportion (14.3 %) with its subgenotype D4 in all the three strains from Mombasa. This suggests that this genotype and its subgenotypes could be circulating in Mombasa. However, these findings may not be conclusive in Mombasa since the number of sequences obtained for this subgenotype in the region was small. The data presented were descriptive but revealed the presence of subgenotype D4 in the region. The subgenotype D4 isolates from Kenya are different from the consensus D4 sequence which was derived from various regions outside Africa. The isolates are like other D4 strains which also have the amino acid exchanges; 128A, 130G, and 143S [13]. Thus, the D4 strains from Kenya were probably not imported from Asia. However, Mombasa occupies a geographical area which is the main sea gate to East Africa where there are significant population movements of different ethnic groups which may have introduced the subgenotype D4. The presence of subgenotype D4 has been reported in the neighboring countries such as Somali and Rwanda, as well as in South Africa [4, 17, 18].

A previous study indicated that the increased risk of HCC and an earlier age at cancer diagnosis among HBV carriers with genotype A was entirely attributable to the effect of subgenotype A1 [19]. In another study in India, genotypes A and D were found in patients with chronic HBV infection and showed that genotype D is associated with more severe diseases, and may predict the occurrence of HCC in young patients as well [20]. In the present study, genotypes A and D with their respective subtypes A1 and D4 were revealed. Voluntary blood donors found to be HBsAg positive during blood-donation screening are not always followed up in Kenya. Hence, the progression of

HBV infection among the blood donors is unknown. Therefore, active infection could become HBsAg-negative or possibly develop to cirrhosis as well as HCC.

Since the HBsAg positive blood samples were obtained from a population of self-selected "healthy" individuals, the prevalence of HBsAg variants in the blood donor setting was expected to remain low. The HBsAg mutations present in 42.9 % of the 21 isolates indicate that the HBV circulating in Kenya could exhibit significant proportion of mutations. Thus, different and common mutants specific to different regions occurred suggesting that the main epidemiological linkage to be the isolates belonging to the same geographical area. However, the blood donors could not be traced. A large number of MHR mutants have been reported to be in association with failure of HBsAg detection, vaccine, and immunotherapy escape [21]. The known substitution K122R occurred within the HBsAg MHR region in one isolate. However, this substitution has been previously reported to be associated with lower reactivity in HBsAg assays [22]. It is worthy to note that the substitution K122R was detected in HBsAg positive sample. Nevertheless, mutations: I226F, P67Q, S34L, S55F, S45P, and P46A occurred outside the MHR in HBV/A1 isolates. This may suggest the possible existence of new mutants. These mutants have not been previously reported as being associated to immunological evasion and/or to diagnostic failure. The uniquely present amino acid change V194A observed in subgenotype A1 may possibly be typical for Kenya. None of the samples had failed to be detected by the ELISA-based HBsAg screening, thus suggesting that the mutations observed have a minimal influence on the performance of diagnostic tests.

The following amino acid substitution in the S gene were reported in the present study: G44E, T189I, S204N, S210R, and S193L. Hence, it was interesting to note that these mutations have been reported among chronic carries and HBsAg negative samples [23, 24]. The mutations described occurred in HBsAg positive blood donors screened for HBV at a given point of time with no or unknown history of HBV vaccination. Kenya is one of the high endemic zones for HBV infection and the most widely used strategy of HBsAg screening test may not be sufficient. Hence, different serologic "markers" or combinations of markers to identify different phases of HBV infection were not used. This realistically depicts a situation where there was lack of follow-up confirmatory tests to ascertain true infection status and avoid the downstream consequences of false positives. However, the present study confirmed the presence of HBV infection among the blood donors and revealed natural HBsAg mutants: F20S, Y200F, and P203R, known to cause diagnostic difficulties in HBsAg negative or low-reactivity samples which are HBeAg- and HBV-DNA positive [23, 24]. A previous

study has reported viral mutants naturally circulating among patients because none of them was vaccinated against HBV infection or had immunoglobulin therapy [21]. Moreover, naturally occurring HBsAg variants have been reported worldwide in persons who have not been immunized [25].

Conclusion

This study revealed that HBV subgenotype A1 was the most predominant, followed by subgenotype D4 among the studied blood donor population. This is the first study in which the presence of circulating subgenotype D4 and mutants were detected among the blood donors in Kenya. Therefore, continuous monitoring of the HBV diversity among voluntary "healthy" blood donors would help reveal circulating genotypes and subgenotypes as well as mutants of clinical significance in Kenya. Since they are the volunteers who freely donate blood, specific policies, plans, and protocols should be put in place to ensure clear links between various public health intervention programs which can help promote health in the blood donor setting. Moreover, transfusion guidelines should be formulated, HBsAg positive blood donors should be promptly followed up, for medical evaluation, even if they do not feel sick; and for receiving additional advanced molecular diagnosis, follow-up testing, additional counseling information, and advice on treatment considerations.

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