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Mutations in the "a" Determinant Region of Hepatitis B Virus Genotype A among Voluntary Kenyan Blood Donors

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Abstract
Background: Occurrence of mutations within the major antigenic alpha determinant region of hepatitis B surface antigen (HBsAg can alter HBV antigenicity resulting in failures in diagnosis, vaccine and hepatitis B immunoglobulin therapy.
Objective: This study aimed at detection of mutations in the "a" determinant region of HBV surface antigen among voluntary blood donors in Kenya.
Design: A cross sectional study involving serology and molecular techniques
Settings: This study involved analysis of samples from blood transfusion centers
Subjects: A total of 301 blood samples from donor blood were collected for the study.
Methods: Sero-status for HBsAg was determined using Enzyme-Linked Immunosorbent Assay (ELISA). A fragment of the *S* gene including the "a" determinant was amplified by PCR from the HBsAg positive samples and sequenced for mutation analysis. Mutations and phylogenetic analyses were performed using Mega 6 software, Bioedit software and GENETYX® software version 9.1.0.
Results: Out of the 301 samples tested 69/301 (22.9%) were Polymerase Chain Reaction (PCR) positive including 2/69(2.9%) were sero-negative for HBsAg. All isolates were genotype A, sub-genotype A1. A total of 29 mutations were observed of which 37.9% were located within the "a" determinant. Mutations T143M and K122R were the most frequent in this study. Escape mutations associated with diagnostic failure, vaccine and immunoglobulin therapy escape were also identified.
Conclusions: These findings are important for policies related to vaccine implementation and therapeutic and diagnostic guidelines.
Keywords: Escape mutants, genotype, hepatitis B virus, antigenic determinant, surface antigen.

1 Introduction

Hepatitis B virus infection (HBV) is a major public health concern especially in Africa, Middle East and Asia (Akbar *et al*., 2004, McMahon, 2005). More than 4 billion people have had contact with HBV worldwide (Ying-Hui, 2012). Of these, about 360 million people are chronic carriers of HBV that can cause death from liver cirrhosis and hepatocellular carcinoma (HCC) (Lavanchy, 2004; WHO, 2015).

Though HBV is a DNA virus, it replicates via reverse transcriptase (polymerase enzyme) which has no proof-reading activity leading to the generation of mutations occurring naturally during infections (Kramvis 2014).

Hepatitis B surface antigen (HBsAg) is a primary target for diagnosis and immunoprophylaxis of HBV infection (El-sherif *et al.,* 2007). The HBsAg which is a 226 amino acid sequence contains a highly conformational, hydrophilic domain known as the "a" determinant located at positions between aa124-aa147 within the Major Hydrophilic Region (MHR; aa110-aa165 (Hu *et al*., 2009). The dominant epitopes of "a" determinant are the targets of neutralizing antibodies used in active, passive immunization and in diagnostic assays. (Kay *et al.,* 2007). Mutations in this region can reduce the binding affinity between the HBsAg and antibody to the HBsAg resulting in weak reactivity of serological assays. Although, mutations have been described in all the four open reading frames (ORFs) of the HBV, S escape mutants is the most worrying since it may be spread by blood transfusion. HBV DNA detection by nucleic acid amplification technology (NAT) is not mandatory for blood donor screening in many countries, especially in those areas where the prevalence of S escape mutants is projected to be high (Fischinger *et al.,* 2010). It is imperative that the prevalence and types of variants of the "a" determinant found in the population be monitored, because this will affect policy decisions relating to vaccine and diagnostic reagents design (Zuckerman, 2000; Francois *et al.,* 2001).To date cases of blood donations with diagnostic escape mutations not detected by HBsAg screening assay have been reported (Levicnic, 2004). Therefore, we aimed at detection of mutations in the "a" determinant region within the S gene among voluntary blood donors in Kenya.

2. Methods

This study was cross-sectional where 301 plasma samples were collected from blood transfusion centers in Kenya. The samples were then transported to Kenya Medical Research Institute (KEMRI) HBV laboratory through cold chain and stored at -80°C till use. The presence of HBsAg was tested using Hepanostika *HBsAg Ultra* (BioMérieux, Lyon, France) kit as per the manufacturer's instructions.

Viral DNA was extracted from 200µl aliquoted plasma using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was eluted in 60µl DNAse free water and stored at -20C till use. A 780 base pair (bp) fragment of HBV was amplified by nested PCR using Kemtaq DNA polymerase kit from Kenya and HBV specific primer pair S1(LLf) 5'-TCCTGCTGGTGGCTCCAG-3'as sense primer and S1(LLr) CGTTGACATACTTTCCAATCAA)-3' as anti-sense primer (Osilowy *et al.,* 2009). Nested PCR was performed by an ABI Thermocycler 9600 system (Applied Biosystems, USA). The conditions for first round PCR consisted of 94 °C for 5 min; followed by 40 cycles at 94 °C for 4 min; 94 °C for 45 seconds min; and 46 °C for 30 seconds; and final extension at 72 °C for 10 min. A total volume of 25µL was amplified in first-round, containing QIAGEN PCR buffer, 0.5µM of each primer, 0.2 mM dNTP mix, 2.5 U *Taq* polymerase (KEMRI Taq) and 10µL of extracted DNA. The second PCR round was done under the same conditions except that annealing was done at 50°C with 10µl of the first-round product using the primer S2nLLf 5'-ACCCTGYRCCGAACATGGA-3'as sense primer and S2 nLLr) 5'- CAACTCCCAATTACATARCCCA -3' as anti-sense primer (Osilowy *et al.,* 2009). PCR-amplified products (10 µl) were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and viewed under ultraviolet light.

HBV DNA positive samples were sequenced using a big dye terminator and the obtained nucleotide sequences edited and aligned using BioEdit sequence alignment editor software version 5.0.9 (Hall, 1999). The newly sequenced (query sequences) were aligned along with reference sequences available in the gene bank. This was followed by CLUSTAL W multiple sequence alignment (Thompson, 1994). After some manual editing by stripping the ends the phylogenetic and molecular evolutionary analyses were conducted using MEGA6 (Tamura *et al.,* 2013). Maximum parsimony test was used for phylogenetic reconstructions. The phylogenetic tree was constructed using MEGA 6. Bootstrapping was performed using 1000 replicates. The nucleotide sequences detected in this study were deposited in the GenBank and these are their accession numbers (KR816101-KR816152). The aligned nucleotide sequences were translated into amino acid sequences using GENETYX® software version 9.1.0. to allow for determination of any mutations within the S protein.

Approval to conduct the study was granted by Kenya Medical Research Institute (Kemri) Scientific and Ethics Review Unit (SERU) (SSC NO. 2443) and Regional Blood Transfusion Centre.

RESULTS

Hepatitis B Virus DNA PCR was done in 301 samples; PCR amplified 69(22.9%) samples. Of these 2 (2.9%) were sero-negative for HBsAg. Table 1summarizes the ELISA and PCR results.

The PCR products obtained were sequenced and 52 (75 %) successfully genotyped. A phylogenetic tree was generated for all 52 nucleotide sequences as shown in Figure 1. The predominant genotype was A.

Prevalence of HBV escape mutations

A total 29 mutations were observed in the present study. Eleven of the 29 mutations (37.9%) were located within the "a" determinant (124-147), whereas 63.6% (7/11) were in the first loop (positions 120-137) and 36.4% (4/11) in the second loop (positions 139-147). The amino acid substitutions detected in the "a" determinant of HBsAg included mutations at positions M133I, K133E, M134S, and F137L and T143M in 5 sequences .The most frequent mutations observed were T143M and K122R detected each in (3/52) 5.8% of sequences .

Twenty sequences (38.5%) had amino acid changes outside the 'a' determinant identified among them; Y161F, E164G W165L, S167L, V184A, T189I, S193L and A194V.In addition 13 had a single mutation (65.0%), 7(35%) had double to triple mutations.

4. Discussion

In this study we report on HBsAg mutations and HBV genotypes among voluntary blood donor population in Kenya . Genotype A sub-genotype A1 was observed in all the HBV DNA positive plasma samples tested. This was comparable with other published data in Kenya (Kwange *et al.,* 2013). Genotype A was found to be predominant in Kenya followed by genotypes E and D (Mwangi *et al.,* 2008; Ochwoto *et al.,* 2013). This probably suggest the trends in HBV genotypes has remained the same especially in this study population

This study also identified mutations of interest in HBV genome. Vaccine escape, immunotherapy and diagnostic escape mutants within and outside the "a" determinant region of HBsAg were both detected among the volunteer blood donor population. Since the basis for effective prevention of HBV infections lies in the use of effective vaccination and diagnostic programming, results of this study therefore provides relevant and valuable information on genetic variability of HBV.

This study found different mutations in the S region of HBV genomes, where the percentage of samples with these mutations was 29/52(42.3%).

In comparison to studies that have been conducted in Kenya Kwange *et al.,* 2013 reported S gene mutations including K122R, T189I and S210R in samples collected from various parts of the country. However, they did not report any amino acid substitution in "a" determinant. Our finding showed that 37.9% mutations were located within the "a" determinant 124-147.

In a study carried out in Iran (Moradi *et al.,* 2012) the following amino acid changes were reported in genotype D: Y100F, L109R, I110L, G112R, S117I, P120S/T, R122K/T, M133I, Y134H, T140I, S143L, G145R, G159R, and E164V. Five of 40 mutations (12.5%) occurred in ''a'' determinant region and they included; M133I, Y134H in the first loop of ''a'' determinant and the rest of substitutions including T140I, S143L and G145R occurred in the second loop of ''a'' determinant. In this study 11 of the 29 mutations (37.9%) were located within the "a" determinant, 63.6% (7/11) in the first loop (amino acid positions 120-137) and 36.4% (4/11) in the second loop (amino acid positions 139-147).The only similarity that is seen between the two studies is amino acid substitutions at M133I which is associated with vaccine escape. The differences might be attributed to the difference in genetic variability of HBV.

Our study reported a pattern of more frequent mutations including K122R and T143M. A mutation K122R substitution was detected in three samples within the MHR (aa110-aa165) this is a polymorphism typically linked with determining *d/y* sub-serotype specificity (Martin *et al*., 2010 ;Amponsah *et al.,* 2013). Two previous studies in Kenyan; one by Ochwoto *et al*., (2013), conducted among liver disease patients and that of Kwange *et al*, 2013 among a blood donor population reported mutations in HBV genome. The current study, however reports diversity of mutations within a larger study population and diverse geographical localities in Kenya.

Moreover, Yong *et al.,* 2012 reported mutations at positions D99N, Y100F, Q101R/H, L110I, T113S, S114T, S117T, T118M, I126T/V, W165L, and V168A. Our findings showed different feature of mutations with four similar mutations at positions Q101H, K122R, T143M, and W165L. This difference could be due to high variability of geographical location. A known mutation T143M frequent in this study was reported in Indonesian blood donors and has been associated with altering antigenic properties of variant HBsAg, this mutant also has been associated with problems in diagnostic assays and escape to vaccine and HBIg therapy as reported by Le *et al*., 2010. Here, T143M was found in 3 out of 52 samples. Among these samples, two samples were sero-negative for HBsAg but positive for the corresponding HBV- DNA. Repeat testing of these samples consistently gave negative results despite using different test kits including ELISA and rapid tests. In addition these samples were observed to have mutations; T143M, M133I respectively. Since these substitutions has been associated with failure of HBIg therapy and problems in detection assays (Ren *et al.,* 2006). It is likely that failure to detect positivity by serology on the samples could be due to mutations. Such reports of false-negative diagnostic results due to HBsAg mutants have been described previously (Coleman, 2006; Rebecca, 2011).This could also be due to low concentration of analyte.We however did not carry out viral load testing due to unavailability of resources.

Mutation M133I observed in the present study in one sample was reported in a Spanish study and is associated with the failure of HBIg therapy since it causes a reduced HBsAg affinity to anti-HBs and reduced HBsAg detection in commercial diagnostic assays (Le *et al.*2010). Other different amino acid substitutions G130S, F137L, T114S, T114K and M134S within MHR were also found in the samples from HBV subtype A1. The effects of these variations on the HBsAg are unknown. The other important finding in the current study is the magnitude of mutations. In total, 29 mutations were detected in 20 of the 52 samples analyzed. These mutations were distributed across the sequenced genome fragment including 11 of 29 in 'a 'determinant, 7 and 4 of 11 in the first and second loop respectively. Since these samples were collected from across different sites in Kenya, this probably demonstrates an underlying genotypic mutation dynamics of HBV occurrence in the population. The major concern arising from HBsAg mutations is the 3 clinical related outcomes which includes; diagnostic escape mutants affecting reliability of results of various diagnostic assays, escape from vaccine-induced immunity, antiviral resistance and passively transferred neutralizing responses in newborn babies or liver-

transplant recipients (Sayiner *et al*.,2007;Ma, and Wang, 2012). Considerably, these events could be taking place in our population undetected, except for blood transfusion, work place, and infants' vaccination; there is no universal policy on HBV prevention in Kenya. This situation could be similar in most countries across Africa.
Both genotype variability and mutations are important consideration in development of effective vaccines. Hepatitis B virus has been shown to display a certain geographical distribution. Our study however indicates a single genotype on all the samples collected from different regions in Kenya. This is however understandable since genotype A has been shown to be the most prevalent. Whereas genotypes have been shown to influence the disease progression and treatment responses (Bartholomeusz and Schaefer, 2004; Chotiyaputta and Lok, 2009). A strong link between HBV genotype and the natural history of disease as well as the better response to interferon treatment in genotype A is encouraging. Given the high prevalence of HBV in Kenya, and in Africa, where blood transfusion is a critical intervention monitoring and prevention of infections is crucial. While mutations in the major hydrophilic region of HBV Surface antigen have been reported in developing countries, largely, there is limited data on Hepatitis B in Kenya partly due to lack of official programming, universal testing strategy and prioritized research. Currently, the public health impact of Hepatitis is drawing attention and the future is likely to see more efforts and focused momentum towards hepatitis. Availability of information on mutations and genetic variants of HBV in this region is required to inform development of clinical and surveillance mechanisms.

5. Conclusions

Genotypes A, sub-genotype A1 was found to be prevalent. Escape mutations which have been associated with diagnostic failure, vaccine escape and immunoglobulin therapy are present within the population. Other than T143M, different aa substitutions G130S, F137L, T114S, T114K and M134S within MHR were also found in the samples from HBV subtype A1. The effects of these variations on the HBsAg are unknown and there is need for further studies. This finding is an important monitoring tool in guiding future policies related to laboratory diagnostic procedures.

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Table 1: Prevalence of HBV-DNA in Donor Blood

Variable	HBsAg status	HBV DNA status
Positive	67(22.2%)	69(22.9%)
Negative	234(77.8%)	232(77.1%)
Total	301	301

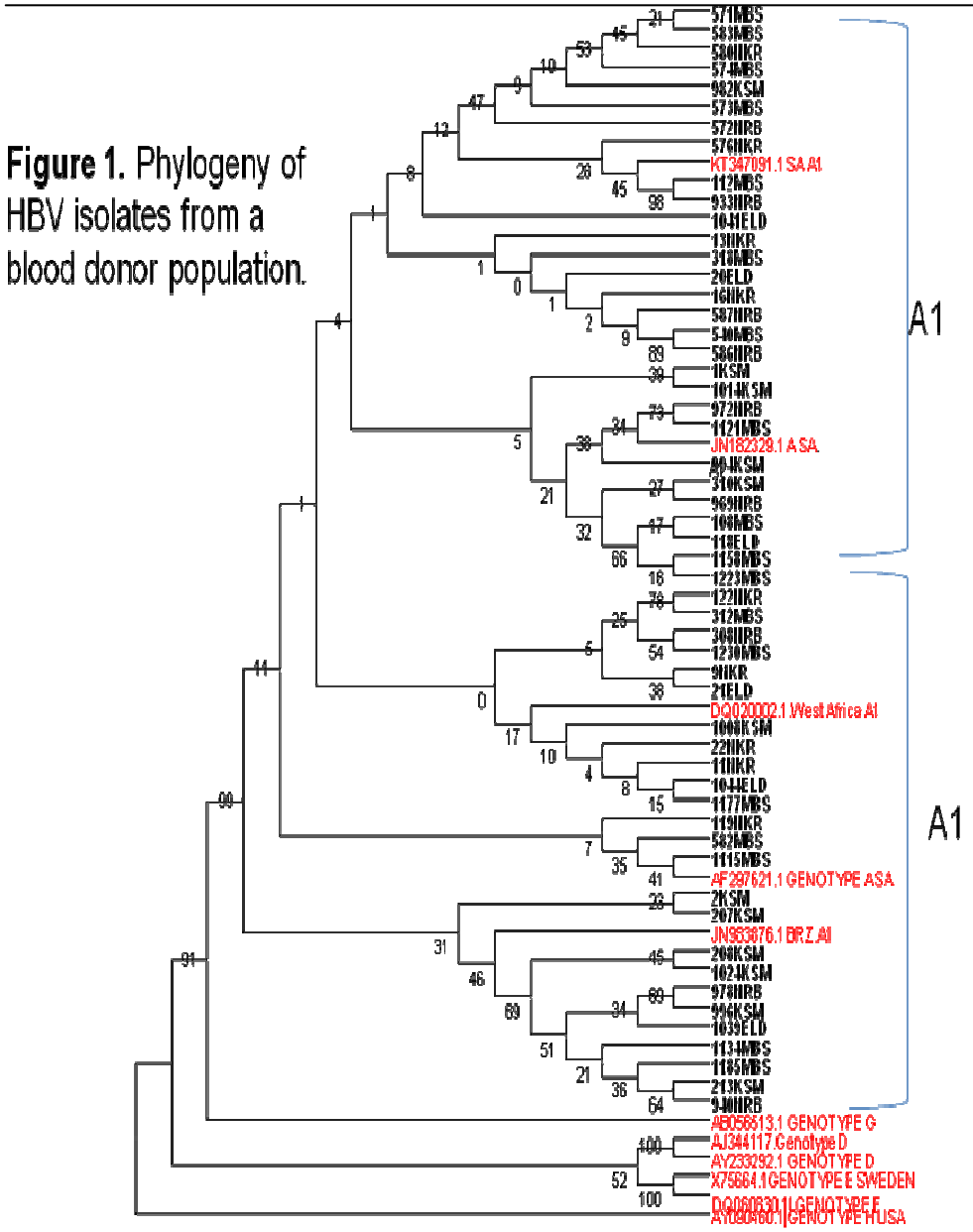

Figure 1: Phylogenetic analysis based on nucleotide sequencing of the S region. The tree contains sequences from 52 samples and a set of representative sequences belonging to genotypes and subgenotypes retrieved from Genebank. The phylogenetic tree was constructed using the neighbor-joining method in Clustal W

Mutations in the ‘‘a’’ determinant domain of HBV isolates.

Figure 2: Multiple sequence alignment with hierarchical clustering based on the protein sequences of the MHR of HBsAg from genotype A. Dots indicate identity to the genotype A consensus.

and

Figure 3: Multiple sequence alignment with hierarchical clustering based on the protein sequences of the MHR of HBsAg from genotype A. Dots indicate identity to the genotype A consensus.

Table 2: Mutation analysis of the ‘‘a’’ determinant_ in HBV donors

Amino acid position	wildtype	mutation	Frequency
101	Q	H	1
114	T	K	1
114	T	S	1
118	T	A	1
122	K	R	3
130	G	S	1
133	M	I	1
133	K	E	1
134	M	S	1
137	F	L	1
143	T	M	3
146	N	K	1
149	C	R	1
159	A	V	1
161	Y	F	1
164	E	G	1
165	W	L	1
167	S	L	2
184	V	A	2
189	T	I	1
193	S	L	1
A194	A	V	1