

Hepatitis B virus genetic heterogeneity and drug resistance among jaundiced patients at Coast General Teaching and Referral Hospital, Mombasa County, Kenya

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ABSTRACT

Objectives: Hepatitis B virus (HBV) infection and emergence of drug resistance have remained one of the major public health puzzles. This study determined circulating HBV genotypes and nucleoside analog resistance to provide information in choosing the best therapy.

Methods: A cross-sectional study was conducted among jaundiced patients visiting Coast General Teaching and Referral Hospital during the period between February and August 2018. A total of 222 patients were recruited and screened for HBsAg following the ethical procedure. Viral DNA was extracted from positive samples, partial HBV-*pol* gene amplified, and directly sequenced and analyzed using web-based software prediction to genotypic resistance mutations.

Results: Forty-seven (21.2%) of the 222 patients tested positive for HBV. Of the 45 samples successfully sequenced, 12 (26.4%) had drug resistance. Six patients (13.3%) had rtV173L, rtL180M, and rtM204V mutations; five subjects (11.1%) with rtL180M and rtM204V while 1 patient (2.2%) had rtM204V mutations. Therefore, all patients had cross-resistance to lamivudine and entecavir. Phylogenetic analysis revealed that HBV genotype A1 35 (74.5%) was predominant. HBV genotypes A3, B, and C2 each occurred once (0.02%). In addition, existence of new HBV genotypes A3, B, and C2 1 (0.02%) in the country was also detected.

Conclusion: Findings suggest that HBV-infected patients should not be put on lamivudine monotherapy. These patients should be on a combination therapy; tenofovir plus lamivudine or emtricitabine to prevent emergence of drug resistance variants. In addition, HBV genotype A1 remains the most predominant genotype in this region. The detected new genotypes variants indicate a possible existence of 0.02% circulation within the population.

Keywords: Drug resistance, genetic heterogeneity, hepatitis B virus, mutations

Introduction

In spite of the existing control and preventive measures, hepatitis B virus (HBV) infections have remained a public health problem worldwide. In the recent past, great advances have been made toward management of these infections. HBV treatment at the onset involved the use of interferon alpha allowing change in natural progression of HBV.^[1] The Kenyan Ministry of Health treatment guidelines for HBV (2014) recommends the use of entecavir and tenofovir as preferred nucleoside analogs treatment with one immune-modulating drug; pegylated interferon alpha 2a. However, long time use of nucleoside analogs can result into emergence of drug-resistant strains.^[2] Therefore, the use of drug combination of entecavir and tenofovir is preferred.^[2-4]

To date, HBV is differentiated into 10 genotypes (A-J) based on the divergence of HBV genomic sequence by either more than 8.0% or greater than 4.0% in the S gene.^[5] The HBV genotypes show specific pathogenesis which result to diverse clinical results among HBV chronic patients world over.^[6] HBV genotype A and B patients respond better to therapeutic interferon based with genotype A having greater tendency of chronicity.^[7] Further, patients infected with genotypes C and D tend to have reduced rate of seroconversion with genotype D having a higher rate of chronicity and mutations.^[6] However, genotypes C and D have higher chances of mutations on core promoter and pre-S genes and disease progression to hepatocellular carcinoma.^[7] HBV genotypes are further segregated into subgenotypes based on the divergence of the genomic sequence between 4.0% and 8.0%.^[5] In Africa, mainly genotype A and to a varying extend

genotypes C, D, and E have been detected.^[5,6] East and Central African countries are invaded predominantly by HBV genotype A of subtype A1 with North Africa countries having D1 and D7 subgenotypes.^[6] In addition, HBV subgenotype A2 has also been detected in South Africa.^[8] In Kenya, HBV genotypes A-E have been detected in some parts of the country.^[9-12] Despite the information on HBV genetic diversity and drug resistance being critical in patients' management, this information is still elusive. Mombasa is populated with high-risk populations to HBV which could drive this infections transmission networks. The information on distribution of HBV genotypes and drug resistance among jaundiced patients in Mombasa County is not clear yet albeit Mombasa being known to be populated with high-risk population to these infections. Therefore, this study was aimed at determining HBV genetic diversity and drug resistance among patients presenting with jaundice and other related symptoms at Coast General Hospital, Mombasa County, Kenya.

Patients and Methods

A hospital-based cross-sectional study was done and a total of 222 samples collected among jaundice patients at the casualty, pediatric clinic, hepatic clinic of outpatient departments, and inpatients seeking medical services at Coast General Teaching and Referral Hospital, Mombasa County, Kenya. The study participants of which were both in and outpatients were recruited during the period between February 2018 and August 2018. Ethical approval was obtained from Kenyatta University Ethical Review Committee; Ref. KU/ERC/APPROVAL/VOL.I (104) and National Ethical Review Committee (NACOSTI/P/18/46294/25080) before execution. Structured questionnaire was administered to patients who consented to obtain demographic data, clinical history of jaundiced patients due to HBV infection, and clinical symptoms. Venous blood samples were obtained and screened for HBV infections.

Serological analysis

HBV infections were confirmed using HBsAg by Heparostika® HBsAg Ultra ELISA kit (Biomereux, Netherlands).^[10,12,13] The viral DNA was extracted from confirmed positive samples.

HBV amplifications

Approximately 5 ml blood samples were used and viral DNA extracted using QIAamp DNA Blood Mini Viral Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.^[4,10] HBV *pol* gene (HBV-*pol*) was amplified by nested polymerase chain reaction (PCR) using primers; F₁ 5'-CCTGCTGGTGGCTCCAGTTC-3' and R₁ 5'-CGTCCCGCG (AC)AGGATCCAGTT-3' in the first PCR and the second PCR primers were; F₂ 5'-CYTGGCCWAAATTCGCAGTCCC-3' and R₂ 5'-GCAAANCCCAAAGACCACAAT-3'.^[9] This was performed in 50 µL reaction volume constituting; 20 µL genomic DNA, 10 µL 10× PCR buffer (Qiagen), 4 µL dNTPs (Thermo Fisher Scientific), 2.5 µL of each primer sequence, 0.5 µL Hot Start Taq DNA Polymerase, (Qiagen GmbH, Hilden, Germany), and 10.5 µL of 2 mM MgCl₂. The amplifications conditions were similar Hot Start; initial activation at 95°C for 15 min in 35 cycles with each cycle 94°C for 45 s denaturation, annealing at 60°C for 45 s, extension at 72°C for 60 s, and final extension at 72°C for 10 min were used in both the first and second round PCRs.^[10,12] The PCR amplification was confirmed by visualization with ethidium bromide staining of the gel. The confirmed products from second round PCR were then purified using QIAquick kit (Qiagen Inc., Valencia, CA).^[10,12,13] followed by bidirectional population sequencing using an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA) [Figure 1].^[10] The generated sequences were phylogenetically analyzed using MEGA X version 10.0.4.^[10,12,13] [Figure 2] Genotypic drug resistance in the HBV *pol* region was defined as the presence of one or more resistance mutations as specified by the consensus mutations in the HBV <http://hbv.geno2pheno.org/index.php>.^[4,10] The HBV isolate sequences were submitted to a web-based software prediction to genotypic resistance for mutations which are genotype-specific and polymerase gene (RT mutation) (Max-Planck-Institute for Informatik, Germany) at <https://hivdb.stanford.edu/HBV/HBVseq/development/hbvseq>.^[10]

Statistical analysis

Statistical analyses were performed using the software SPSS 24.0 (IBM Corporation, Armonk, NY, USA). Age, gender,

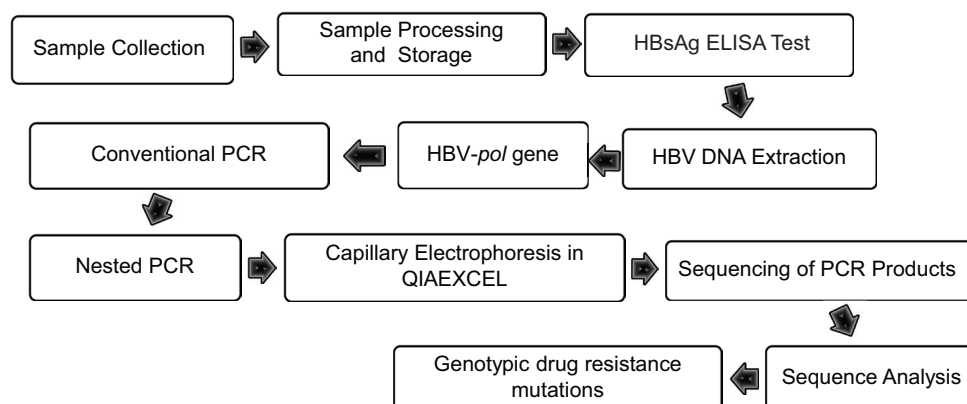


Figure 1: Flowchart of protocol used in the study

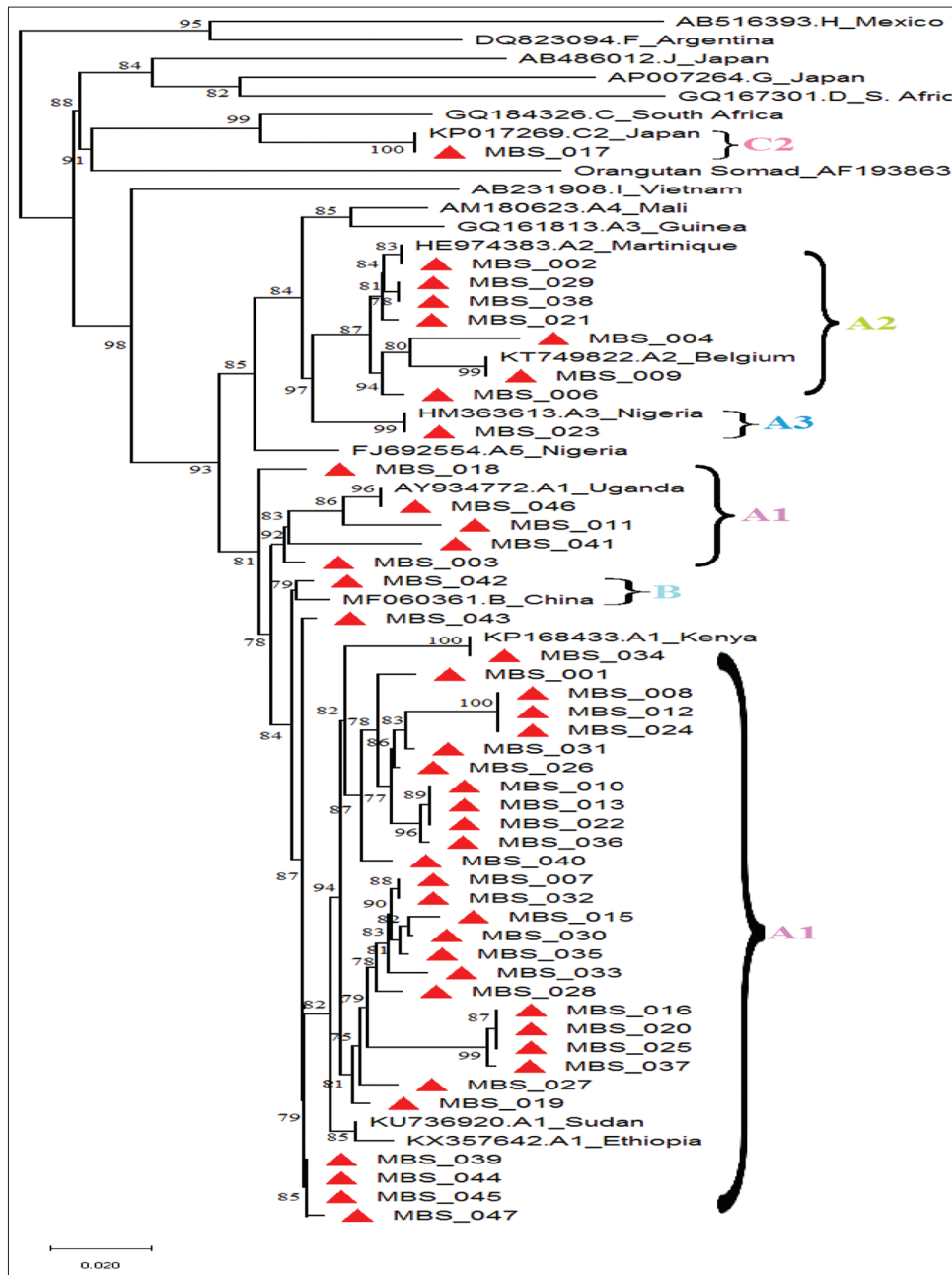


Figure 2: Phylogenetic tree of hepatitis B virus (HBV) *pol* gene sequences from Coast General Teaching and Referral Hospital, Mombasa, Kenya. Neighbor-joining method at 1000 bootstrap replicates was used. Chimpanzee HBV (Orangutan Samad – AF193863) was the outgroup used. Bootstrap values >70% are shown and the HBV isolates from participants of the study are specified in ▲

marital status, occupation, and area of residence were compared in relation to HBV infection using Chi-square test. $P < 0.05$ was considered statistically significant.

Results

Demographic characteristics of the jaundiced patients

A total of 222 participants were recruited into the study. Of these 124 (55.9%) were female and 98 (44.1%) were male.

Their ages ranged between 5 years and 75 years with the mean of 23.6 ± 17.2 years. For females, their mean age was 22.4 ± 15.5 years while that of men was 25.1 ± 19.2 years old. More than half of the study participants were not married 142 (64.0%) who mostly resided in Likoni 92 (41.4%) followed by Kisauni with 54 (24.3%). Most of the study participants were also unemployed 151 (68.0%) constituting mostly housewives 31 (14.0%). $P < 0.05$ was considered statistically significant [Table 1]. Serological analysis revealed that 47 (21.2%) were HBV positive and the positive samples were molecularly analyzed.

Table 1: Demographic characteristics of jaundiced patients visiting Coast General Teaching and Referral Hospital

Gender	All, n=222	Females, n (124)	Males, n (98)	P-value
Mean age	23.6	22.4	25.1	
Median age	29	23.0	26.0	
SD		15.5	19.2	
Age groups				
≤10.0	68	37 (54.4)	31 (45.6)	P=0.001
10.1–19.0	25	19 (76.0)	6 (24.0)	
19.1–28.0	41	22 (53.7)	19 (46.3)	
28.1–37.0	41	26 (63.4)	15 (36.6)	
37.1–46.0	23	12 (52.2)	11 (47.8)	
46.1–55.0	16	5 (31.3)	11 (68.7)	
55.1+	8	4 (50.0)	4 (50.0)	
Marital status				
Single	142 (64.0)	78 (54.9)	64 (45.1)	
Married	80 (36.0)	47 (58.8)	33 (41.2)	
Area of residence				
Likoni	92 (41.4)	50 (54.3)	42 (45.7)	P=0.067
Kisauni	54 (24.3)	26 (48.1)	28 (51.9)	
Mvita	41 (18.5)	24 (58.5)	17 (41.5)	
Mwishomoroni	34 (15.3)	13 (38.2)	21 (61.8)	
Nyali	1 (0.005)	1 (100)	0 (100)	
Occupation				
Unemployed	182 (82.0)	85 (46.7)	96 (52.7)	P=0.002
Government employed	21 (9.5)	12 (57.1)	9 (42.9)	
Self-employed	19 (8.6)	8 (42.1)	11 (57.9)	

"N" represents total number of study subjects while "n" total number by gender.

HBV drug resistance

This study also analyzed HBV polymerase drug resistance among 45 samples of participants. In overall, 12 (26.7%) successfully sequenced chronic HBV patients' samples showed HBV drug resistance mutations causing entecavir and lamivudine resistance albeit data on their prior exposure to these treatment therapies were not captured. These mutations were all identified in HBV genotype A and showed resistance to 3TC, ETV, and LdT. Mutations presenting resistance to tenofovir and adefovir were not detected in the study. Mutation rtM204V conferring resistance to 3CT occurred predominantly at 26.7% in all the patients followed by rtL180M (20.0%) and rtV173L at 13.3%. Triple nucleoside analog linked to mutation existed at rtM204V with secondary mutation at rtL180M and mutation rtV173L. Six patients (13.3%) had rtV173L multiple mutations at rtM204V with rtL180M secondary mutation. On the other hand, 5 patients (11.1%) had rtM204V mutations with rtV173L and rtL180M secondary mutations [Table 2].

HBV genotypes analysis

Nucleotide sequence identity analysis among HBV genotype A showed 92–96% similarity with an average identity of 96.8%.

Table 2: HBV drug resistance pattern among jaundiced patients at Coast General Teaching and Referral Hospital

Mutations	n=45 f %	Parameters
rtM204V	12 (26.7)	
rtV173L	6 (13.3)	
rtL180M	9 (20.0)	
Combined mutations pattern		Associated drugs
rtL180M, rtM204V	5 (11.1)	ETV, 3CT, and LdT
rtV173L, rtL180M, and rtM204V	6 (13.3)	ETV, 3CT, and LdT
rtM204V	1 (2.2)	LdT
No major mutations	33 (73.3)	Susceptible
Genotypes	% circulation	% resistance
A1	35 (74.5)	12 (26.7)
A2	7 (14.9)	0 (0.0)
A3	1 (2.1)	0 (0.0)
B	1 (2.1)	0 (0.0)
C2	1 (2.1)	0 (0.0)

ETV: Entecavir, 3CT: Lamivudine, LdT: Telbivudine. HBV: Hepatitis B virus

In genotypes B and C2, sequence similarities indicated 95–98% and an average identity of 98.9%. Out of the 47 sera samples that were analyzed, 45 were successfully amplified and sequenced. Generated sequences were phylogenetic analyzed using MGEA X version 10.0.^[10,12] Phylogenetic analysis revealed that HBV genotype A1 was 35 (74.5%), followed by A2 7 (14.9%) and 1 (0.02%) occurrence each for A3, B, and C2 [Figure 2].

Discussion

The HBV overall infection level was higher than those previously detected in Kenya; 2.36%,^[14] 3%,^[10] 3.8%,^[14] 6.0%,^[15] 7.25%,^[10] 13.3%,^[16] and 14.6%,^[17,18] Uganda 14.9%,^[19] and in other African countries such as Zambia 9.9%, Malawi 6.7%, Uganda 4.9%, Ethiopia 4.7%, and Rwanda 2.4%.^[15] This high HBV infection rate in this study is attributed to high likelihood of study participants being of HBV high-risk groups, especially intravenous drug users, therefore, treatment and management among this group are of necessity.^[20] In comparison, the finding was lower than some studies conducted in Ghana 54.2%^[21] and Kenya 50.6%.^[11] The variations depicted in the prevalence rates were related to the sample size and the study populations.

Kenyan Ministry of Health directs the use of entecavir and tenofovir for the treatment of HBV through the clinical protocol for HBV therapeutic guidelines for hepatitis B (2014). On the other hand, long duration use of these drugs could be a factor to emergence of HBV-resistant strains. HBV drug resistance mutations have been highlighted in the treatment and management of chronic HBV patients,^[3] therefore, tracking of primary and secondary resistance mutations among patients is of great value in monitoring, optimizing, and choosing the suitable treatment in achieving sustainable virological response, thus reducing disease progression to hepatocellular carcinoma.

This study found high overall drug resistance prevalence of 12 (26.7%) [Table 2] which could be linked to very low genetic barrier to lamivudine. This prevalence was higher compared to the previous studies.^[10,22,23] This disparity could be due to different therapeutic management periods of patients and low genetic barrier to LAM.^[10] Mutation rtM204V occurred predominantly followed by rtL180M; rtM204V [Table 2] mutation known to be primary mutation since it is susceptible to HBV and lamivudine while rtL180M mutation is taken to be secondary mutation due to its ability to enhance viral replication.^[3] This finding concurs with observations in the previous studies in Kenya and other countries.^[3,10,13,24] This is in contrary to a study in Ethiopia on HIV/HBV patients on second-line therapy.^[25] In addition, there was no rtM204I mutation detected in the study; absence of this mutation with detection of rtM204V mutation confirms the predominance of genotype A in drug resistance mutations.^[22] This finding is similar to the previous studies.^[10,22]

Cross-resistance to 3CT, ETV, and LdT was detected in 11 samples in this study [Table 2], however, data on prior exposure to therapies were not captured. Therefore, there could be long-term 3CT therapy failure and exposure to viral strains harboring resistance mutational patterns associated with cross-resistance.^[2] In addition, resistance mutation rtV173L, rtL180M, and rtM204V can cause alterations in the S gene resulting to immune escape variants hence greater number of viral strains with cross-resistance.^[3,26] In addition, the presence of secondary mutation in amino acid position 180 (rtL180M) in *pol* gene can reinstate hepatitis B capacity to replicate.^[2] Further, hepatitis B has the ability to stay for long as covalently closed circular DNA even under seroclearance or treatment making the covalently closed circular HBV DNA to act as a store for drug resistance strains.^[2] This finding is similar to observations in the previous studies.^[2,10]

There was no detected resistance mutation to adefovir and tenofovir in this study. This concurs with finding in the previous studies.^[10,13,25] This is linked to their high anti-HBV potency with high genetic barrier to drug resistance.^[26] Therefore, this supports the reference by the World Health Organization that tenofovir be used in treatment patients with HIV/HBV coinfection.^[13] However, this was in contrary to an observation from the previous study.^[22] This discrepancy in observations could be associated to diverse treatment periods.

The detected genotypes concur with the previous studies that have also shown the same distribution.^[10-12] The phylogenetic analysis revealed that genotype A1 was the most predominantly circulating among the studied subjects [Figure 2]. These findings agree with the previous studies that have been conducted in the country and other surrounding countries.^[6,9-12] HBV genotype A1 sequences were clustered with reference sequences from Kenya and Uganda.^[6] This trend indicates its possible East African origin with an implication of these strains circulating locally among Kenyan

residents. By virtue of population, migration across East African countries could explain this alignment. Subgenotype A2 sequences were aligned with those from Martinique and Belgium as an indication of its possible origins [Figure 2].^[1,6] In addition, sequences of subgenotypes A3, B, and genotype C2 clustered with reference sequences from Nigeria, China, and Japan.^[6,8] The detected viral genotypes confirm the persistent and stability of this viral strains circulating within the country. In contrary to the previous studies conducted in the country, this study confirmed possible existing of other HBV genotypes circulating among Kenyan population. HBV genotypes A3, B, and C2 were detected even though at low proportion. Despite low proportion of the sample used, there are newly detected genotypes in this study. As a result, there is a likelihood of existence of possible other genotypes in this region.^[9,10,12] The genotypes D and E in the previous studies were detected among patients who had a history of intravenous drug use.^[9,10,12]

Conclusion

The high HBV drug resistance was detected among hepatitis B patients, high resistance to LAM and cross-resistance to entecavir. As a result, we recommend a combination of tenofovir and lamivudine for the treatment and management of HBV patients. HBV genotype A1 remains the most predominant genotype 35 (74.5%) with new genotypes A3, B and C2 1(0.02%) being detected in this region. Detection of new HBV genotypes in Kenya calls for continuous surveillance of HBV infections and circulating trends of HBV genotypes.

Authors' Declaration Statements

Ethical approval and consent to participate

The study was approved before execution by Kenyatta University Scientific (Ref. KU/ERC/APPROVAL/VOL.I (104)) and Ethical Review Committee and Coast General Hospital Ethics Review Committee (Ref ERC-CGH/MSc/VOL.I/38). The study was conducted as per the Helsinki Declaration criteria. Each participant signed consent form before collection of demographic data and blood.

Availability of data and materials

The sequences in this study have been deposited in the GenBank under the accession numbers MK127847-MK127860 and MK834343-MK834372.

Authors' Contributions

G.O.K. designed the study, collected the samples, and analyzed and drafted the article. K.O.O helped in data interpretation and analysis while J.M.M. and A.K.N. helped in designing the experiment, sequence analysis, supervised analysis of the

samples, and interpretation of the data and review of the article. All authors approved the final article on reading.

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