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Betty Chelangat Too, BSc, MSc, National Hospital Insurance Fund, P.O Box 719-00606, Nairobi, Kenya, email: toobettie@gmail.com. Fred Wamunyokoli, BSc, MSc, PhD, Lecturer, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000 – 00200, Nairobi Kenya, email: fwamunyokoli@gmail.com. Javan Okendo, BSc, MSc, Research Assistant, Centre for Research in Therapeutic Sciences, Strathmore University, P.O Box 59857, 00200, Nairobi, Kenya, email: javanokendo@gmail.com. John Oketch, BSc, Research Assistant, Center for Research in Therapeutic Sciences, Strathmore University, P.O Box 59857, 00200, Nairobi, Kenya, email: oketchjohn9@gmail.com. Allan Okwaro, BSc, Research Assistant, Center for Research in Therapeutic Sciences, Strathmore University, P.O Box 59857, 00200, Nairobi, Kenya, email: allanokwaro@gmail.com. Raphael Lihana Wekesa, BSc, MSc, PhD, Lecturer, Center for Clinical Research, Kenya Medical Research Institute, P.O Box 20778-00202, Nairobi, Kenya, email: lihanaraphael@gmail.com. Bernard Ogutu, BSc, MSc, PhD, Lecturer, Center for Research in Therapeutic Sciences, Strathmore University, P.O Box 59857-00200, Nairobi, Kenya, email: ogutu6@gmail.com. Washingtone Ochieng' BSc, MSc, PhD, Lecturer, Department of Immunology and Infectious Diseases, Harvard School of Public Health. 677 Huntington Avenue, Boston, Massachusetts, email: ochiengwashingtone@gmail.com.

Corresponding author: Washingtone Ochieng', BSc, MSc, PhD, Lecturer, Department of Immunology and Infectious Diseases, Harvard School of Public Health. 677 Huntington Avenue- Boston, Massachusetts. Email;ochiengwashingtone@gmail.com

VIRAL HEPATITIS B AND HEPATITIS C CO-INFECTIONS OF HIV-1 INFECTED PATIENTS IN KENYA

B. C. Too, F. Wamunyokoli, J. Okendo, J. Oketch, A. Okwaro, R. W. Lihana, B. Ogutu, and W, Ochieng'

ABSTRACT

Background: **Co-infections with hepatitis B and hepatitis C (HBV/HCV) viruses continue to be of significant concern among HIV-1 patients. In resource limited settings like Kenya where HIV-1 still remains endemic, infections with diverse viral genotypes complicates prognosis and treatment outcomes.**

Objective: **To determine the prevalence and co-prevalence of HCV and HBV among HIV-1 infected antiretroviral-naïve and experienced subjects from selected facilities in Kenya.**

Design: **A cross-sectional study.**

Setting: **Center for Research in Therapeutic Sciences laboratory at Strathmore University, Nairobi, Kenya**

Subjects: **HIV infected patients attending the comprehensive care clinic from six County hospitals in Kenya.**

Results: **Overall, 32 of the 140 HIV infected patients were also infected with either HBV (n=24, 17.1 %) or HCV (n=8, 5.7 %). Five of the 24 HBV positive and**

six of the 8 HCV positive patients were ART experienced, with the rest in these positive categories being ART naïve. All the HBV DNA PCR positive patients were ART experienced. Only 4/24 HIV/HBV+ patients were also co-infected with HCV. Confirmatory genetic testing of the 24 HBV co-infected patients showed that seven were positive by PCR. Genotyping of the seven PCR positive samples revealed 4 of these isolates to be HBV genotype A

Conclusions: **HBV and HCV coinfection among HIV infected patients is higher than previously reported, with majority of ART experienced still at an increased risk for co-infection with both viruses.**

INTRODUCTION

Hepatitis viruses (HBV, HCV) and HIV are among the top 10 leading causes of infectious disease-related deaths worldwide. These viruses share similar routes of transmission which include sexual, blood contact and injecting drug usage (1, 2), which may further potentiate their co-infection burden and risks. It is estimated that over 240 million people are infected chronically with HBV, 130-150 million with HCV and 35 million with HIV/AIDS globally (3-5). The rates of HIV-HBV co-infection are reported as high as 10-20% in countries where HBV is endemic. In most cases high prevalence of these multiple infections leads to increased morbidity and mortality as compared to HIV or HBV mono-infections (6). It has been shown that HIV exacerbates the natural history of HBV by increasing rates of chronic HBV infection, replicative disease, and progression to advanced liver disease among persons with HIV/HBV co-infection (7). On the other hand, HIV also modifies the natural history of HCV infection, with clear evidence of higher HCV viral load and accelerated liver disease progression in persons with HIV/HCV co-infection (8).

Hepatitis is an infectious inflammatory condition of the liver and remains an important public health issue in developing countries, including Kenya (9). The proportion of deaths attributable to liver disease has increased despite the introduction of highly active antiretroviral therapy (HAART) (10, 11). As majority of HIV-1 infected individuals continue to gain increasing access to antiretroviral treatment (ART), selective pressure of treatment and mixed adherence issues help generate new profiles of ART resistant viral strains which are readily transmitted to untreated persons, (12). It is not uncommon to find patients progressing adversely in spite of HAART, and those with hepatitis infections developing even more compounding prognostic indicators like liver-related diseases (13, 14). There remains increasing need for new and robust cumulative data on the burden of HBV and HCV among patients with HIV in resource-limited settings where ART compliance, access and adherence are suboptimal.

MATERIALS AND METHODS

Study design, population and samples: This was a cross-sectional study of HIV-1 positive subjects from six health facilities distributed across different, mostly rural regions of Kenya where HIV prevalence rates have been high. These were: Ndhiwa hospital (Homabay county), Ahero hospital (Kisumu county), Naivasha hospital (Nakuru county), Kitengela health centre (Kajiado county), Kiambu

hospital (Kiambu county), and Omari rehabilitation center (Malindi County). Screening for HCV was done on plasma using DiaSpot one step HCV test strip according to the manufacturer's instructions (15). HBV screening was done on plasma using DiaSpot one step HBsAg test strip as previously described (16).

Extraction and amplification of HBV DNA: HBV DNA was extracted from HBsAg positive samples using QiaAmp DNA Mini kit as per the manufactures protocol (QIAGEN, 2013). The extracted DNA was amplified by nested PCR using gene-specific primers (16). Each PCR reaction contained, 25 µl final individual reaction volume: 2.5 µl of 1x PCR buffer, 0.125 µl of 5 u/ml Taq Polymerase, 0.5 µl of 10 mM dNTPs, 2.0 µl of 25 mM $MgCl_2$, 1.25 µl of 10 µM of each of the primers. After aliquoting 22 µl into individual tubes, 3 µl of DNA template was added. The primer pairs HBV-Z (Reverse; 5'AGC CCT CAG GCT CAG GGC ATA3´) and HBV-3(Forward; 5´-CGT TGC CKD GCA ACS GGG TAA AGG -3´) were used in the first round, and primers HBV-P (Reverse; 5'-TCA TCC TCA GGC CAT GCA GT-3') and HBV-M (Forward; 5'-GAC ACA CTT TCC AAT CAA TNG G-3') in the second round. The amplification conditions for the first and second PCR were as follows: Initial denaturation at 94˚C for 3 minutes; then 40 cycles of denaturation at 94˚C for 30 seconds; annealing at 53˚C for 30 seconds and extension at 72˚C for one minute. Final extension at 72˚C for 5 minutes was done then 4˚C for infinity.

HCV RNA Isolation and Amplification: Total RNA was extracted from plasma using Qiagen viral RNA mini kit according to the manufacturer's instructions (15). The RNA samples were then subjected to one-step reverse transcriptase polymerase chain reaction (RT-PCR) protocol (Qiagen) followed by nested PCR as reported in one of our publications (16). Primers NS5BF1 5'-TGATACCCGCTGYTTTGACTC-3' and NS5BR1 5'-GTACCTCATAGCCTCCGTG-3' were used in the forward and reverse RT-PCR reactions. Briefly, the reaction comprised 5µl of RNA added to a final volume of 25µl PCR reaction mixture that contained 0.5µm of each primer, 2µl of dNTP mix, 2.5µl of 10x PCR buffer, 11.5µl of RNase -free water, 1µl Qiagen One-step RT-PCR enzyme mix (Invitrogen, Carlsbad, CA, USA). The RT-PCR was performed under the following thermos-cycling conditions; 50˚C for 30 min, 95˚C for 15 min, 45 cycles at 94˚C for 30 sec, 58˚C for 30 sec, 72˚C for 1 min and a final extension at 72˚C for 5 minutes. This was then put on hold at 4˚C. Second round PCR reaction was done using HotStart taq polymerase (New England Biolabs) in a 25µl reaction volume comprising of 10x PCR buffer, 0.5Mm dNTPs, 2Mm $MgCl_2$, 1.25 µM forward and reverse primers, 0.125 units of the enzyme and 5µl of first PCR product. PCR was done using the following amplification conditions; denaturation at 94°C for 3 min followed by 40 cycles of 94°C denaturation for 30 sec, annealing at 55°C for 4o sec, extension at 72°C for 1 minute followed by a final extension at 72°C for 5 min. Primers NS5BF2 5'-ATACCCGCTGYTTTGACTCAN-3' and NS5BR2 5'-ACCTGGTCATAGCCTCCGTGA-3' were used in the nested PCR.

Nucleotide sequencing and genotyping of HBV: The PCR products were purified with QIAquick purification columns and prepared on-site for sequencing at an outside facility. Sequencing was completed using standard Big-Dye chemistry (Macrogen, Netherlands) Approximately 2µl of primers HBV-P (Reverse; 5'-TCA TCC TCA GGC CAT GCA GT-3') and HBV-M (Forward; 5'-GAC ACA

CTT TCC AAT CAA TNG G-3') were used for the sequencing reactions. Sequences were manually inspected, joined in pairwise alignment (BioEdit ver. 7.2.5), and subjected to multiple sequence alignment using Clustal W (www.liebertpub.com/aid). Two reference sequences were included in the alignment for each subtype query sequence.

RESULTS

A total of 140 samples from HIV infected ART-naive and ART-experienced patients were analyzed. Overall, 111 (79%) were receiving ART while 29 (21%) were not receiving ART. Majority of the patients, 107 (76%) were female. When grouped by age, 56 (40%) patients were aged 18 to 35 years, 67 (47.9%) patients were aged 36 to 50 years while 17 (12%) were above 50 years. Distribution the 140 samples by each clinical site was as follows; 5 (3.6%) were from Kiambu, 41 (29.3%) were from Kisumu, 9 (6.4%) were from Kajiado, 43 (30.7%) were from Malindi, 8 (5.7%) from Nakuru and 34 (24.3%) were from Ndhiwa. Out of the 140 patients, 61 (43.6%) had CD4 counts of <=350 cells/mm^3, 27 (19.3%) had CD4 counts of >350 to <=700 cells/mm^3 while 9 (6.40%) had CD4 counts of > 700cells/mm^3. Of the ART experienced, 36 were on first-line AZT+3TC+NVP, 25 on D4T+3TC+NVP while 31 were on TDF+3TC+NVP at enrollment. These data are detailed in Table 1.

Table 1
Demographic/Clinical and Infection Characteristics of HIV Patients

Demographic/Clinical and Infection Characteristics of Patients	Grouping Variable and indicators	Number of patients enrolled (%)	Number ART experienced (%)
Region's clinical site	Kiambu	3.6	3.60
	Kisumu-Ahero	29.3	29.3
	Kitengela	6.4	6.40
	Malindi	30.7	30.7
	Naivasha	5.7	5.70
	Ndhiwa	24.3	24.3
Gender	Male	23.6	23.6
	Female	76.4	76.4
Age bracket	18-35years	56	40.0
	36-50years	67	47.9
	Above 50years	17	12.1
CD4 T-cell levels of Patients	Unknown	43	30.7
	<=350 cells/mm^3	61	43.6
	>350 to <=700 cells/mm^3	27	19.3
	> 700cells/mm^3	9	6.40
ART Experience	Naive	29	20.7
	Experienced/Initiated	111	79.3
	Total	100	100

Prevalence and mono-infection of HBV and HCV among HIV patients: Overall, out of the 140 HIV patients, 27 (19%) tested positive for HBV by serology. Of the HBV serology positive subjects, 26 (96%) were receiving ART, one patient had not been initiated ART. In terms of HCV status, 8 of the 140 patients, (5.7%) tested positive for HCV by serology. Of the 8 HCV-serology positive subjects, 6 (75%) were receiving ART while 2 (25%) were not ART initiated. PCR conducted to confirm presence of viral genomes revealed that 7 of the 27 (26.6%) HBV seropositive specimens contained HBV genomic material while none of the HCV seropositives were confirmed positive by genomic (PCR). We suspect that degradation of the more labile HCV RNA may have made it difficult to recover and amplify HCV genomic material.

Co-prevalence of HBV and HCV among HIV patients: Out of the 8 HIV patients who had tested positive for HCV by serology, 4 (50%) also tested positive for HBV. All these dual positive subjects were actively receiving ART. Similarly, for the 27 HIV patients who had tested positive for HBV by serology, 4 (14.8%) were also HCV seropositive and ART initiated. Out of the 27 HIV patients who tested positive for HBV by serology, 22 (82%) were female while 5 (19%) were male. Among the 8 HIV patients who were HCV positive, 5 (63%) were female while 3 (38%) were male. All the 4 patients who had tested positive for HBV and were infected with HCV were female.

More than half of the patients who tested positive for HBV by serology were aged 36 to 50 years while 11% were over 50 years of age. Similarly, for the 8 HIV patients who tested positive for HCV by serology, 7 were aged over 35 years. Likewise, 75% of all the HCV seropositives who were infected with HBV were aged 36 or older. The same trend was true for patients who had tested positive for HBV and were also HCV seropositive, with 75% aged over 35 years.

In terms of statistical associations, seroprevalence of HBV among HCV seropositive subjects was significantly associated with the gender (χ^2=4.800, p= 0.028), while seroprevalence of HCV was significantly associated with the age (χ^2=6.105, p= 0.047). Chi-square test did not show any significant association of these co-infections with ART status (experienced).

HBV genotypes: Of the 24 HbsAg positive samples, seven were positive for HBV-DNA by PCR. Gene-specific sequencing of HBV pol region was successfully completed for the seven HBV-DNA positive samples. Phylogenetic analysis of the sequences showed all the isolates to cluster with HBV genotypes A1, with the isolates clustering closely with A1 reference sequences from East African countries of Kenya and Ethiopia (figure 1).

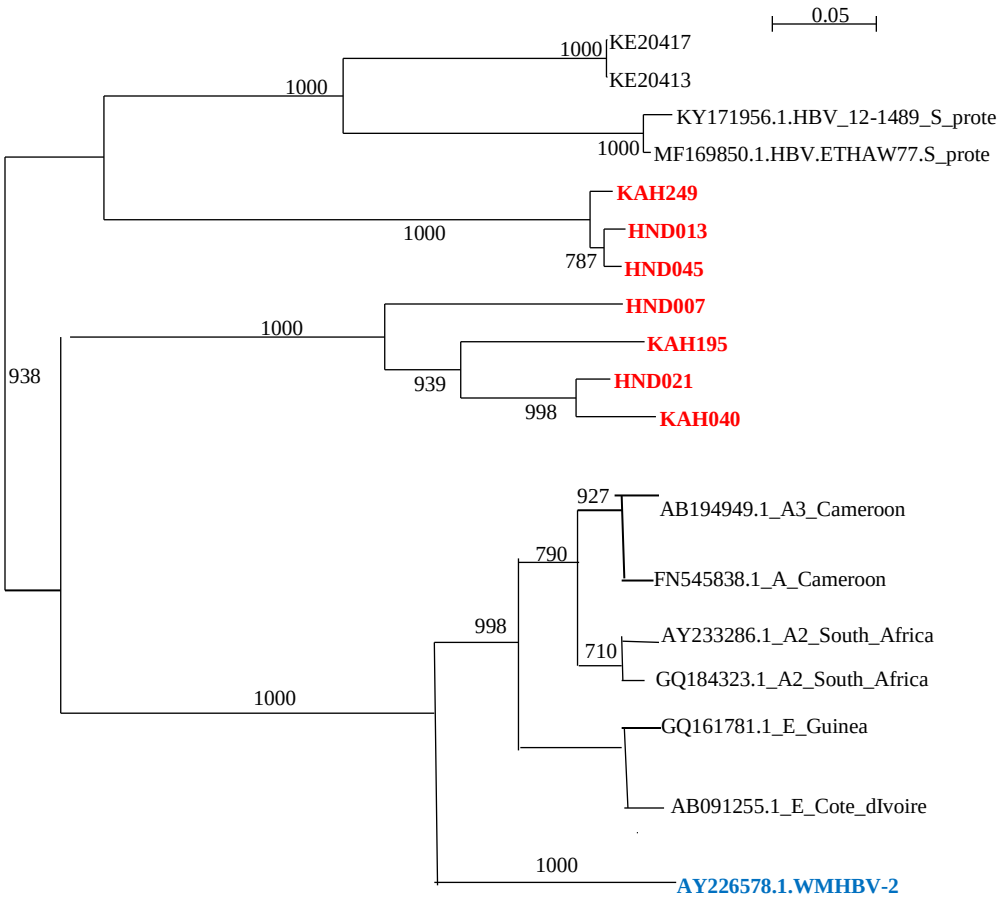

Figure 1: phylogenetic tree of the seven HBV isolate. Neighbour-joining method based on 1000 bootstrap replicates and p-distances were used for generating the tree. References from Genbank together with their country of origin are represented as accession numbers. Woolly monkey HBV (AY226578-WMHBV) was used as the out-group. HBV isolates from the study participants are in red. Bootstraps values above 70% are indicated.

DISCUSSION

In this study, we report the infection and co-infection pattern and prevalence of both Hepatitis B and C among HIV-1 Infected Patients. We observed HCV co –infection rates of about 6% and HBV co-infection of about 20% of the total HIV positive population studied. These data were comparable to that we reported in a related but more homogenous population of injecting drug users (16). However, the rates are still substantially higher than what we could expect in the general population (17, 18), or those reported by others among population settings in Africa (19-23).

Out of the 8 HIV patients who had tested positive for HCV by serology, 4 (50%) also tested positive for HBV. Despite the limited sample size, this observed proportion of dual HCV/HBV co-infection of HIV patients may be considered to be high for a sample population that was regularly seeking CD4 and viral load testing to monitor their immunological and virological status. It is reasonable to assume that in this and similar population setting, risk and prevalence of hepatitis virus infection among HIV-infected persons may be higher than that of the

general population. The fact that these viruses share a mode of transmission, and the continuing stigma associated with HIV infection from this region could both converge to limit uptake of screening, diagnostic and treatment services that would traditionally help mitigate infection risk.

Similarly, out of the 140 HIV patients, 27 (19%) tested positive for HBV by serology, a proportion that we find to be higher than those obtained in Nigeria (24), South Africa (25), Uganda and Rwanda (26). The observed disparity in prevalence rates across different countries have been shown to be associated with the diversity of patient behaviors and risk factors from the different population groups, sample size, and study methodologies (27).

Four patients from this study were found to be co-infected with all the three viruses. Previous studies from Kenya have reported lower co-infection rates, (19, 29). While it is plausible that co-infection rates are on the rise, this cannot be deduced with veracity based on the current limited study population.

All the HBV samples tested were of genotype A1, results that were consistent with the findings from a previous study of HIV infected patients and commercial sex workers in (30 - 32), and among Intravenous Drug Users (IDUs) and non-IDUs at the coastal region of Kenya (33) that showed predominance of genotype A1. Whereas we expect that other HBV genotypes would be circulating consistent with previously published data from Kenya and other African countries (34, 35, 36), the limited sample population precludes generalization of these data. None of the eight samples that tested positive for Anti-HCV IgG turned positive for HCV PCR (37), which we could attribute to RNA sample integrity under the prevailing storage condition, as well as repeated freeze-thaw cycles for use in related studies.

CONCLUSION

Based on the current study findings which are supported by our previously published data, the burden of HBV and HCV as either mono or dual infections still remains high in this setting of HIV positive patients, regardless of ART-experience. We suggest that guidelines for screening, monitoring, treatment and management of HIV patients be refined to allow for early detection and targeted intervention if the burden is to be minimized

GenBank Accession Numbers

The seven sequences reported in this study have been deposited in GenBank and assigned the following Accession numbers: MH347485 - MH347491.

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