Epidemiology of HBV infection in a cohort of Ugandan HIV-infected patients and rate and pattern of lamivudine-resistant HBV infection in patients receiving antiretroviral therapy

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Background: Many HIV-infected patients in sub-Saharan Africa are not routinely screened for hepatitis B virus (HBV) infection and are on antiretroviral therapy (ART) regimens containing only lamivudine as anti-HBV active drug.

Methods: In 2009-2011, we screened for hepatitis B surface antigen (HBsAg) in 2820 HIV-infected adults patients at the Mbarara Hospital Uganda and investigated risk factors for HBV infection. Using samples of dried plasma or blood spots, we tested for HBV viral load and HBV drug resistance mutations in all HBsAg-positive patients on ART for ≥12 months.

Results: In this study, 109 patients tested HBsAg positive (3.9%; 109/2820). HBsAg-positive patients were more likely to have had >4 lifetime sexual partners (p=0.01). Of the 55 HBsAg-positive patients on ART for ≥12 months, 53 were only on lamivudine as anti-HBV active drug and two were on tenofovir and lamivudine. HBV-DNA was detected in 30 patients (54.5%; 30/55), all on lamivudine-monotherapy. Of the 23 patients in whom HBV-DNA sequencing was successful, 17 had lamivudine-resistant HBV strains harbouring rtM204V/I mutations accompanied by secondary/compensatory mutations.

Conclusions: Our study suggests that sexual transmission may represent a major mode of spread of HBV in southwest Uganda and confirms the importance of screening for HBV and of using ART regimens containing tenofovir in HIV/HBV co-infected patients.

Keywords: Epidemiology, HBV, HIV, Lamivudine, Resistance, Sub-Saharan Africa

Introduction

Co-infection with hepatitis B virus (HBV) and HIV is common in sub-Saharan Africa, where both viruses are highly endemic. With a hepatitis B surface antigen (HBsAg) prevalence rate of 10.3% in the general population, in Uganda there are approximately 1.8 million adults infected with HBV, while the number of HIV-infected adults is around 1.4 million. In Uganda, there are limited data on the prevalence of HBV co-infection among people living with HIV/AIDS. In the national hepatitis B sero-survey incorporated into the 2005 Uganda HIV/AIDS sero-behavioural survey (UHSBS), prevalence of HBsAg-positivity among adults living with HIV/AIDS was 8.3%, which was not significantly different from the prevalence rate in the HIV-negative population. This study also found marked variations in the rates of current and past hepatitis B infections in the different regions of Uganda, with the northeastern region having a six-fold higher prevalence of HBsAg-positivity compared to the southwestern region (23.9% vs 3.8%).

HIV infection exerts a negative impact on the course of HBV infection at various levels. In HIV-infected individuals, hepatitis B is more likely to progress to chronicity after an acute infection, HBV DNA blood levels tend to be higher, rates of hepatitis B e antigen (HBeAg) loss are lower, progression to end-stage liver disease is accelerated and the risk of hepatocellular carcinoma and liver-related mortality is increased. Furthermore, when choosing the combination of antiretroviral drugs (ARVs) for patients with HIV/HBV co-infection, it is important to consider that some of these agents have dual activity against both viruses and that HBV is able to develop resistance to lamivudine (LAMr) at rates of 20%
per year, when this drug is used as the only HBV-active agent of the combination. For these reasons, European and American guidelines recommend that all HIV-infected individuals are screened for HBV co-infection and that those needing highly-active antiretroviral therapy (HAART) receive fully active HBV treatment as well. The same also applies for resource-constrained countries. Since 2010, WHO guidelines for the treatment of HIV infection recommend a first-line HAART regimen including tenofovir and either lamivudine or emtricitabine in HBV co-infected patients. Unfortunately, these recommendations have not yet been fully implemented in sub-Saharan Africa, where many HIV-infected patients are still not routinely screened for HBV infection and a large proportion of co-infected patients, whose HBV status is unknown, remain on regimens containing only lamivudine as the HBV-active agent.

In this study, we determined the prevalence of hepatitis B in a large cohort of HIV-infected patients in southwest Uganda, investigated the risk factors for HBV infection in this population and assessed rate and pattern of HBV LAMr in patients receiving HAART for their HIV infection.

Materials and methods

Study design

In this cross-sectional study, all HIV-infected patients aged ≥16 years that were seen at the Mbarara Regional Referral Hospital, southwest Uganda, between June 2009 and December 2011 were asked to: complete a questionnaire assessing risk factors for HBV infection; be screened for the presence of HBsAg in blood with the Determine HBsAg Rapid Test (Alere Inc., MA, USA); and have samples of blood or plasma spotted onto filter paper cards (Whatman 903 Protein Saver Card, GE Healthcare Ltd., NJ, USA) for virological molecular analysis.

Only patients who underwent all three of the above steps and for whom all data were available were included in the final analysis. In the virological analysis we focused on patients found to be HBsAg-positive. Taking into account the kinetics of HBV and HIV viral load (VL) decay following the start of treatment, we excluded from our virological analysis patients with ≤12 months of HAART. Dried plasma or blood spots (DBS/DPS) samples of the HBsAg-positive patients on HAART for their HIV infection for >12 months were tested for HBV-DNA by quantitative PCR, HBV drug resistance testing by sequencing and HIV-RNA by quantitative PCR. The presence of a detectable HIV viraemia was used as a surrogate marker of inadequate adherence to HAART.

HBsAg testing

Screening for HBsAg was performed using the Determine HBsAg Test, a rapid immune-chromatographic test that is performed manually and has a visual readout. This test has been shown to have excellent sensitivity and specificity, in the low-endemicity European setting as well as in the high-endemicity context of sub-Saharan Africa and also in HIV co-infected patients. Because of the test’s consistent excellent performances with both plasma and whole blood, either types of samples were used alternatively. The test procedure was carried out in accordance with manufacturer’s instructions.

Dried plasma or blood spot collection and storage

Dried plasma or blood spot have been shown to be a reliable alternative to plasma specimens for HIV and HBV VL quantification and for genotypic resistance testing. Previous studies showed that the two types of dried samples yield concordant VL results and for this reason we used them alternatively. For each patient, 50 µl of blood or plasma were spotted in each of the five pre-printed circles of a filter paper cards. After application, the cards were left to dry overnight at room temperature and were then stored at −80°C in the Epicentre Research Base in Mbarara, Uganda. In September 2012, the cards were dispatched to the Institute of Infection and Global Health of the University of Liverpool, UK, where they were stored at −80°C and analysed between October 2012 and January 2013.

Methods of elution, extraction and PCR quantification of HBV-DNA and HIV-RNA

An in-house protocol for elution, extraction and PCR quantification of HBV-DNA and HIV-RNA from DPS and DBS was developed, validated and used. We conducted preliminary validation experiments on mock DPS and DBS samples that were made by diluting NIBSC second HBV DNA international standard and third HIV-1 RNA international standard (WHO, Health Protection Agency, UK) in negative plasma and negative EDTA anti-coagulated blood. Our preliminary experiments showed high correlation between the known input values of the dilutions and the values obtained from DPS and DBS (Pearson correlation coefficients 0.99; p<0.001 for both HIV and HBV) and also high correlation between the values obtained from the two types of dried samples (Pearson correlation coefficients 0.99; p<0.001 for both HIV and HBV). For HBV VL quantification, the mean difference from the known input value was 0.02 log10 IU/ml (95% CI 0.05–0) for DBS and 0.1 IU/ml (95% CI 0.17–0.03) for DPS and the lower limit of detection was 250 IU/ml. For HIV VL quantification, the mean difference from the known input value was 0.4 log10 IU/ml (95% CI 0.49–0.40) for DBS and 0.29 log10 copies/ml (95% CI 0.35–0.22) for DPS and the lower limit of detection was 500 copies/ml. When using our protocol with the actual study samples, we accounted for the fact that, different from DPS, DBS have a cellular fraction in addition to plasma. For this reason, using a standard haematocrit value derived from the literature of 28.3% for people living with HIV in sub-Saharan Africa, we applied a correction factor of 1.39 to adjust the VL results obtained from DBS.

For each patient, we used one spot of the filter paper card for HBV-DNA quantification and drug resistance sequencing and a second spot for HIV-RNA quantification. Each spot was cut in six to eight pieces and was then eluted in 1 ml of elution buffer for 2 hours at room temperature with continuous gentle rotation. Based on our preliminary experiments, for the elution of HBV DNA we used a solution of phosphate-buffered saline containing 0.05% tween, whereas for the elution of HIV RNA we used the lysis buffer provided with the Abbott m2000sp kit (Abbott Molecular, Germany). After elution, samples for HBV DNA quantification and sequencing underwent off board lysis with 2 ml of the lysis buffer provided with the NucliSENS easyMAG lysis extraction system (bioMérieux, Boxtel, Netherlands) for 1 hour at 56°C, followed by automatic magnetic nucleic acid separation using the NucliSENS easyMAG platform. Amplification and quantification
of HBV DNA were performed on a ABI 7500 Fast RT-PCR system (Applied Biosystems, Warrington, UK). Amplification and quantification of HBV DNA were performed using the ABI 7500 Fast RT-PCR system (Applied Biosystems). Samples with detectable HBV DNA underwent Sanger sequencing of the polymerase gene reverse transcriptase (RT) domain (amino acids 1 to 344) and the surface (S) gene (amino acids 1 to 226) as previously described. In brief, a 1.4 kb amplicon comprising the RT and S gene was amplified by nested PCR and sequenced using a 3730 DNA analyzer (Applied Biosystems). Sequence analysis was performed using SeqScape software V 2.6 (Applied Biosystem).

HIV RNA extraction was performed on the Abbott m2000sp platform and amplification and quantification were performed using the Abbott m2000rt system.

**Statistical analysis**

Significance of the associations between demographic characteristics/risk factors for HBV infection and HBsAg status was assessed using Pearson’s $\chi^2$ test, Fisher’s exact test and Mann-Whitney U test. A multivariate logistic regression analysis including all significant ($p < 0.05$) associations found in the bivariate analysis was performed to evaluate independence of the associations. All statistical analyses were performed using STATA v12.1 (StataCorp, College Station, TX, USA).

**Results**

Of the 3022 HIV-infected patients who were asked to participate in the study, 2820 consented, completed the questionnaire, were screened for HBsAg and had DBS/DPS samples collected (93.3% uptake) (Figure 1). The dried samples used were DPS for 78.2% (2205/2820) of patients and DBS for the remaining 21.8% of patients (615/2820).

Among the 2820 enrolled patients, 109 were found to be HBsAg-positive (3.9%). The prevalence of HBsAg-positivity was significantly higher in men than women (6.2% vs 2.9%; 54/886 vs 55/1934; $p$ < 0.001). Men had a significant greater number of lifetime sexual partners compared to women ($p$ < 0.001); women were more likely to have received blood transfusions ($p$ = 0.002), to have had cosmetic cuts ($p$ = 0.009) and to have had needlestick injuries ($p$ = 0.007). The relative proportion of females was significantly higher in the HIV-monoinfected group compared to the HIV/HBV co-infected group ($p$ < 0.001 on both the bivariate and multivariate analysis) (Table 1). HBsAg-positive patients were significantly more likely to have had a higher number of lifetime sexual partners in the bivariate analysis ($p$ = 0.009) and this association was confirmed also in the multivariate analysis ($p$ = 0.022) (Table 1).

Out of 109 HBsAg-positive patients, 55 (50.5%) had been on HAART for their HIV infection for >12 months (median duration 36 months, IQR 24.5-49); 96% (53/55) of those on HAART for >12 months were on HAART regimens including only lamivudine as the HBV-active agent and the remaining (4%; 2/55) were on HAART regimens including tenofovir plus lamivudine with activity against HBV. HBV-DNA was detected in 55% (30/55) patients, all of whom on HAART regimens that included only lamivudine as the HBV-active agent. None of the two patients on HAART regimens comprising tenofovir and lamivudine were found to have a detectable HBV VL. The median HBV-DNA VL value was 6.2 $\log_{10}$ IU/ml (IQR 3.8-7). HIV-RNA was undetectable in all 30 patients that were found to be viraemic for HBV.

HBV-DNA sequencing was successful in the 23 out of 30 patients (77%). In all seven cases where HBV-DNA sequencing was not successful, the HBV-DNA VL was <1000 IU/ml ($<3\log_{10}$ IU/ml). Median duration of ART in the 23 patients where HBV-DNA sequencing was successful was 36.5 months (IQR 29-46). The most common HBV sub-genotype was A1, found in 83% (19/23) of patients, followed by genotype D4 in the remaining 17% (4/23) of patients. Known LAMr mutations were detected in 17 out of 23 (74%) patients. No significant difference in terms of HBV VL or genotype was observed between patients with and patients without known LAMr mutations. Of the 17 patients with known LAMr mutations, 15 (88%) were sub-genotype A1, found in 83% (19/23) of patients, followed by genotype D4 in the remaining 17% (4/23) of patients. Known LAMr mutations were detected in 17 out of 23 (74%) patients. No significant difference in terms of HBV VL or genotype was observed between patients with and patients without known LAMr mutations.

Out of 17 patients with known LAMr mutations, 15 (88%) were sub-genotype A1 infected and two (12%) were sub-genotype D4 infected. All 17 patients presented primary LAMr mutations located in domain C of the YMDD motif, which were represented by the mutation rtM204V (YVDD sequence) in five cases and rtM204I (YSDD sequence) in five cases and at least one secondary/compensatory mutation. All 22 patients with the primary mutation rtM204V had also the secondary/compensatory mutation rtL180M; five patients had the triple mutation pattern rtM204V, rtL180M and rtV173L. Of the five patients with the primary LAMr mutation rtM204I, four presented the mutation rtL80I and one the mutation rtV173L as secondary/compensatory mutations (Table 2).
The 3.9% prevalence rate of hepatitis B observed in our HIV cohort in the southwest region of Uganda is in line with the 3.8% prevalence rate reported in the general population of the region in the national hepatitis B serosurvey.2 In our HIV cohort, men had double the prevalence of HBsAg carriage than women. This is in line with the findings of the national serosurvey of the general population and with the findings from other studies on HIV co-infected patients in sub-Saharan Africa.2,20 The reasons for this difference are not entirely clear. The fact the men are more likely to progress to chronicity following an acute infection offers only a partial explanation for this imbalance.21 We did not measure hepatitis B core antibodies in this study, however, in the Ugandan hepatitis B serosurvey, male gender was found to be an independent predictor also of lifetime HBV infection. This indicates that, compared to women, men are more likely to come into contact with HBV. The only risk factor for HBV infection that was found to be significantly over-represented in the male population of our study was a greater number of lifetime sexual partners. However, in contrast with the hypothesis that men are more likely to be HBV-infected because of greater sexual exposure, the 2004–2005 HIV/AIDS sero-behavioural survey found that Ugandan women tend to marry and become sexually active at a younger age than their male counterparts and often have older and more sexually experienced partners.22 Also in contrast with this hypothesis is the fact that, in Uganda as well as in general in the rest of sub-Saharan Africa, women have a greater likelihood of being HIV-infected than men. Further epidemiological studies are needed to clarify the reasons behind this imbalance.

In this study the presence of HBsAg among HIV-infected patients was significantly associated with a greater number of lifetime sexual partners. Sub-Saharan Africa is often collectively referred to as a macro-region of high-endemicity for hepatitis B infection and, in accordance to the classical model of transmission of hepatitis B in high-endemicity areas, it is generally believed that most individuals in this region come into contact with HBV early in childhood and that only a minority of people reach adult life remaining naïve and susceptible to the infection.23 However, different countries in sub-Saharan Africa have varying prevalence rates of hepatitis B infection and marked differences are also present within the same country, as reported in Uganda by the above-mentioned national serosurvey.2,24 Universal immunisation of infants against hepatitis B was introduced in Uganda in 2002. According to WHO/UNICEF estimates,
immunisation coverage in Uganda increased steadily until 2011 and remained stable at 78% between 2012 and 2014. While the beneficial impact of this measure is likely to become evident within a short period of time in areas, like the northeastern region, where most infections are acquired perinatally and in early childhood, it may take longer to see an improvement where sexual transmission in adult life seems to be an important modality of infection, like in the southwestern region. In the latter case, the addition of a catch-up immunisation programme of sexually-active young adults could achieve a more rapid result.

Given the similar prevalence of HBsAg-positivity in our HIV-infected cohort compared to the general population in the same region, it is possible that even among people with HIV/AIDS a relevant proportion of individuals are still naïve and susceptible to HBV infection and would benefit from vaccination.

After a median 3 years (range 12–61 months) of HAART, 55% (30/55) of co-infected patients in our cohort were found to be viraemic for HBV with a high median VL and 31% (17/55) presented mutations resulting in substitutions in the YMDD motif of the HBV reverse transcriptase. All viraemic patients had been on HAART regimens including only lamivudine as HBV-active drug. Because HIV RNA was undetectable in all patients found to have a detectable HBV VL, we assumed compliance to HAART in these patients. The rate of YMDD variants in our cohort appears lower than the 40–50% reported after 2 years of therapy with lamivudine as the only HBV-active drug in western HIV/HBV co-infected cohorts. The reason for such difference is not clear. One possible explanation is that, by using DPS/DBS rather than plasma samples in our study, we might have failed to detect some patients with low-level HBV viraemia and we might have had more failures in HBV DNA sequencing for LAMr mutations. HBV sequencing was successful in the majority of patients, all of whom had HBV VL > 4.2 log_{10} IU/ml, whereas sequencing failed in all patients with a HBV VL < 3 log_{10} IU/ml.

<table>
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<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>HAART regimen</th>
<th>Time on HAART (months)</th>
<th>HBV VL (log_{10} IU/ml)</th>
<th>HBV sub-genotype</th>
<th>HBV mutations associated with resistance to lamivudine</th>
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AZT: azidothymidine; F: female; HAART: highly-active antiretroviral therapy; HBV: hepatitis B virus; M: male; NVP: nevirapine; VL: viral load; 3TC: lamivudine.
(rtM204V, rtL180M, rtV173L) that has been associated with a vaccine escape phenotype in chimpanzees. The actual clinical relevance in humans of these HBV variants, often referred as antiviral drug-associated potential vaccine-escape mutants (ADAPVEMs), seems to be low at present. Nevertheless, from a theoretical point of view, they represent a major public health concern.

The major limitation of our study is the limited sensitivity of molecular testing performed on DPS/DBS samples. By using dried spots we might have underestimated the rate of HBV virulent patients in our cohort. On the other hand, DPS/DBS are easy to collect, store and dispatch and can represent a convenient alternative to plasma in settings with limited laboratory capacity. For the study of hepatitis B infection, dried fluid spots have been mainly used in seroprevalence studies and the available experience of their employment for genotyping and VL quantification is still limited. We have shown that this type of sample can be effectively used for molecular analysis of HBV in large epidemiological studies and this represents a major strength of this study.

In conclusion, this study demonstrated an intermediate level of hepatitis B prevalence (3.9%) among people living with HIV/AIDS in southwest Uganda, which is comparable to the prevalence observed in the general population of the same region. Similarly to other epidemiological studies conducted in sub-Saharan Africa, this study found a significantly higher prevalence of hepatitis B in men compared to women. The reasons for this difference remain largely unclear. Sexual transmission seems to be a major mode of HBV transmission among HIV-infected Ugandan adults.

Because a substantial proportion of people living with HIV/AIDS in Uganda seem to reach adulthood remaining susceptible to HBV, screening for hepatitis B infection and immunity is key to identifying individuals susceptible to HBV infection who can benefit from vaccination. Screening for hepatitis B is also important to identify patients already infected with hepatitis B that need to be promptly switched to HAART regimen containing tenofovir, avoiding the development of lamivudine-resistant HBV strains and ADAPVEMs.

### Authors’ contributions

SB, AMG, RM, YB and LAW contributed towards the conception and design of this study; RM and LAW provided and cared for the patients and participated in collection of the epidemiological data; RM, YB and LAW participated in collection of the biological samples; GC GMF and AMG and contributed to the molecular virological analysis; GC wrote the manuscript. All authors read and approved the final manuscript. GC is the guarantor of the paper.

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### Competing interests

None declared.

### Ethical approval

Approval was obtained from the ethics committee of the Mbarara University of Science and Technology before recruitment. The Ugandan Ministry of Health approved the export of DPS/DBS samples to the University of Liverpool for molecular testing.

### References


