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High Prevalence of Hepatitis B Virus Drug Resistance Mutations to Lamivudine among People with HIV/HBV Coinfection in Rural and Peri-Urban Communities in Botswana

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Abstract: (1) Background: We aimed to determine the prevalence of hepatitis B virus (HBV) resistance-associated mutations (RAMs) in people with HBV and human immunodeficiency virus (HBV/HIV) in Botswana. (2) Methods: We sequenced HBV from participants with HBV/HIV from the Botswana Combination Prevention Project study (2013-2018) using the Oxford Nanopore GridION platform. Consensus sequences were analyzed for genotypic and mutational profiles. (3) Results: Overall, 98 HBV sequences had evaluable reverse transcriptase region coverage. The median participant age was 43 years (IQR: 37, 49) and 66/98 (67.4%) were female. Most participants, 85/97 (87.6%) had suppressed HIV viral load (VL). HBV RAMs were identified in 61/98 (62.2%) participants. Most RAMs were in positions 204 (60.3%), 180 (50.5%) and 173 (33.3%), mostly associated with lamivudine resistance. The triple mutations, rtM204V/L180M/V173L and was the most predominant (17/61 [27.9%]). Most participants (96.7%) with RAMs were on antiretroviral therapy for a median duration of 7.5 years (IQR: 4.8, 10.5). Approximately 27.9% (17/61) of participants with RAMs had undetectable HBV VL, 50.8% (31/61) had VL <2000IU/mL and 13/61 (21.3%) had VL ≥2000 IU/mL. (4) Conclusions: The high prevalence of lamivudine RAMs discourages the use of ART regimens with 3TC as the only HBV-active drug in people with HIV/HBV.

Keywords: hepatitis B virus; drug resistance; people living with HIV; Botswana; Africa

1. Introduction

Hepatitis B virus (HBV) remains a global health concern even in the era of potent vaccines and antiretroviral therapy (ART) that can greatly reduce morbidity and mortality. HBV global prevalence is 3.8% and HBV infection causes 820,000 deaths annually [1]. Of 296 million people living with chronic hepatitis B (CHB) globally, 82 million are in Africa [1]. There is a call to eliminate HBV by the year 2030 with specific targets to reduce HBV incidence by 95% and mortality by 65% [1]. The use of

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nucleos(t)ide analogues (NA) can greatly contribute to these targets, but the development of resistance-associated mutations (RAMs) remains one of the challenges that hinder HBV elimination. Prolonged antiviral use without adequate monitoring may lead to the selection of variants with RAMs that reduce ART susceptibility [2].

The World Health Organization recommends treatment of CHB patients who have a viral load of greater or equal to 20,000IU/mL[3]. NAs with a high barrier to drug resistance are highly recommended for treatment. These include entecavir for children aged 2-11 years and tenofovir (TFV) for individuals aged 12 years or older [3]. In individuals with HBV/HIV coinfection, TFV + lamivudine (3TC) or emtricitabine (FTC) is recommended. Although other drugs such as 3TC and telbivudine are active against HBV, they have a low barrier to resistance and therefore are not recommended for HBV treatment [4,5]. TFV is considered the most effective drug against HBV, however, there is emerging evidence of possible amino acid substitutions that may reduce susceptibility to TFV, such as rtS78T, rtA194T and rtN236T [6–9].

Botswana has a HIV prevalence of 20.8% in the general population [10]. Hepatitis B surface (HBsAg) prevalence ranges from 1.1% to 10.6% while occult hepatitis B infection (OBI) prevalence ranges from 6.6% to 33% with predominant subgenotypes being A1, D3 and E [11–15]. Occult hepatitis B is defined as the presence of replication-competent HBV DNA in the blood and/or liver of individuals who test negative for HBsAg [16]. The country has a robust HIV treatment program that has seen the country surpass the United Nations Programme on HIV/AIDS (UNAIDS) 95-95-95 targets at 95-98-98 [10]. In 2016, Botswana adopted the HIV Treat-all program with the first line regimen being Truvada and dolutegravir [17]. The prior regimen was TFV/FTC/EFV as the first line, while the earliest first-line regimen had 3TC as the only HBV active drug. The widespread use of ART in the country has improved patient health outcomes among people living with HIV (PLWH), however, HBV viral load and RAMs are not monitored in this setting. We therefore aimed to determine the prevalence of HBV RAMs in PLWH coinfected with HBV in Botswana.

2. Materials and Methods

2.1. Study Population

Plasma samples from PLWH recruited in the Botswana Combination Prevention Project (BCPP) (2013 – 2018) were used in this study. Details of BCPP are described elsewhere [18]. In brief, the BCPP study was a cluster-randomized trial conducted in 15 paired communities matched by size, pre-existing health services, population, age structure, and geographic location. The study enrolled 12610 participants, 3596 of whom were PLWH [18]. Participants signed written informed consent. Our study was approved by the Human Research Development Committee at the Botswana Ministry of Health (HPDME 13/18/1) with a waiver of consent.

2.2. Nanopore Sequencing

Participant plasma samples previously screened for HBsAg and OBI [13] were used. Briefly, HBsAg screening was performed using (Murex Version 2, Diasorin, Dartford, United Kingdom) while OBI screening was conducted using the COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions [13]. The QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) was used to extract DNA from 200µL of plasma with a final elution volume of 30µL. DNA concentration and quality were determined using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Library preparation was adopted from an already established protocol [19] and modified as previously described [20]. In brief, a two-step polymerase chain reaction (PCR) was performed to amplify the whole HBV genome. Master mixes were prepared for two HBV primer pools which covered the whole HBV genome. We used Q5® Hot Start High Fidelity 2× master mix (New England Biolabs, Ipswich, MA, USA) for PCR. Library preparation was carried out as per the Oxford Nanopore PCR tiling with rapid barcoding and midnight expansion protocol (version MRT_9127_v110_revH_14Jul2021) [21], replacing SARS-CoV-2 primers with HBV primers. The

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library was quantified using the Qubit fluorometer and loaded into version R9.4.1 flow cells (Oxford Nanopore Technologies, Oxford, UK). HBV sequences were generated using GridION (Oxford Nanopore Technologies, Oxford, UK).

2.3. Sequencing Analysis

Raw FASTQ files were exported and subsequently processed using Guppy, employing dual-indexed reads for base calling and demultiplexing. FASTQ were uploaded into Genome Detective (version 2.64, last accessed 19 August 2023) for reference assembly. Generated consensus HBV sequences were viewed, aligned and trimmed using AliView alignment viewer [22]. Geno2pheno (https://hbv.geno2pheno.org) (last accessed 11 December 2023) and the Stanford HBVseq (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) (last accessed 11 December 2023) online databases were used to determine HBV genotypes and RAMs. Furthermore, genotypes were confirmed using phylogenetic analysis. We constructed a maximum-likelihood tree (ML-tree) using the best-fitting model of nucleotide substitution [TVM+F+I+G4] using IQ-TREE with 1000 bootstrap replicates [23,24]. For mutational analysis, 98 HBV sequences had evaluable reverse transcriptase (RT) region coverage. We trimmed the RT position to only have amino acid positions rt1-250 and these amino acid RT sequences were analyzed for HBV RAMs using the Sandford HBVseq tool (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html).

2.4. Statistical Analysis

Participants' sociodemographic and clinical characteristics were summarized in proportions and medians with interquartile ranges (IQR). Categorical data was analyzed using Fishers' exact test or Chi-squared test where appropriate while continuous variables were compared using the Wilcoxon rank-sum test. All statistical analyses were conducted using Stata version 18.0 (StataCorp LLC, College Station, Texas, USA) and p-values less than 0.05 were deemed statistically significant.

3. Results

3.1. Participant Description

Participant plasma samples with positive HBsAg and OBI were used. From 271 samples with positive HBsAg, we attempted to sequence 128 and 79/128 (61.7%) had a valuable RT region. Out of the 72 samples with OBI, only 19/72 (26.4%) had a valuable RT region, hence a much lower sequencing success rate for samples with OBI. Therefore, we had a total of 98 RT sequences for downstream analysis (Figure 1).

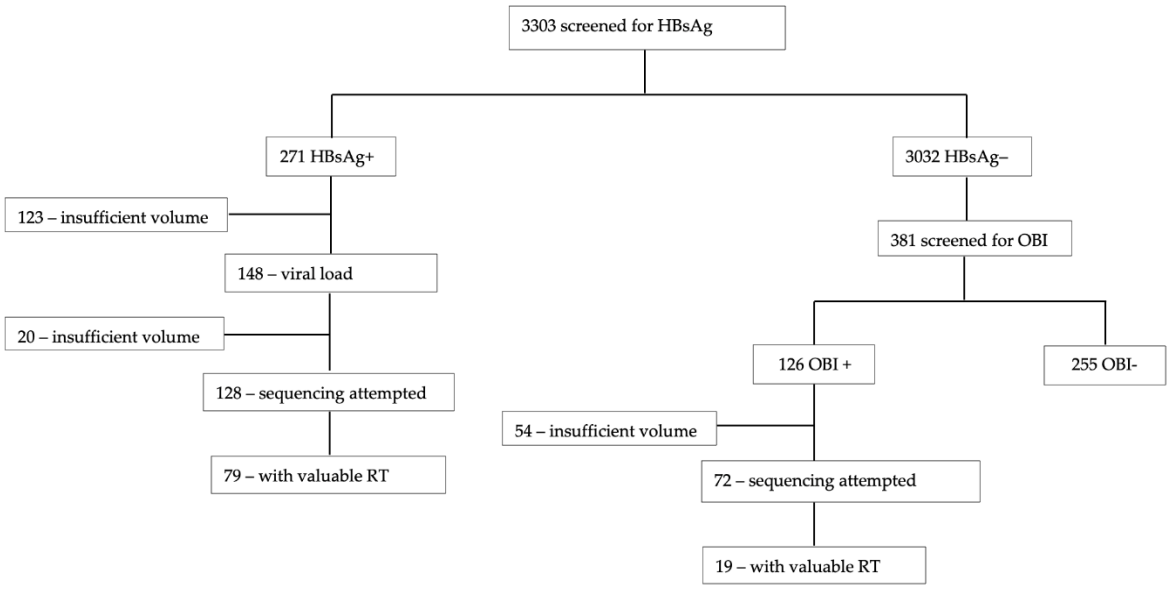

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Figure 1. Laboratory flow diagram.

Most participants had suppressed HIV viral load (VL) (85/97, 87.6%), were on ART (93/98, 94.9%) and mostly on tenofovir disoproxil fumarate (TDF)-containing regimen (40/67, 59.7%). There was no statistically significant difference between participants with and without RAMs in all variables except for HBV viral load categories, Table 1. HBV sequences with RAMs were also generated from participants with low HBV viral loads and some whose viral load results indicated "target not detected", Table 1.

Table 1. Participant demographic and clinical characteristics.

	No RAMs, n=36	RAMs present, n=61	P-value
Sex, n (%)			
Female	24 (64.9)	42 (68.9)	
Male	13 (35.1)	19 (31.2)	0.82
Age, years, median (IQR)	41 (34 – 47)	45 (40 – 50)	0.68
Region			
South	7 (18.9)	10 (16.4)	
Central	17 (46.0)	23 (37.7)	
North	13 (35.1)	28 (45.9)	0.56
Nadir CD4 T cell count, cells/μL, n (%), n=19			
<350	7 (70.0)	3 (33.3)	
≥350	3 (30.0)	6 (66.7)	0.11
HIV viral load, copies/mL, n (%) n=97	29 (80.6)	56 (91.8)	
Suppressed	7 (19.4)	5 (8.2)	0.12
Unsuppressed			
HBV type, n (%)			
HBsAg	28 (75.7)	51 (83.6)	
OBI	9 (24.3)	10 (16.4)	0.43
HBV viral load, n (%)			
TND	3 (8.1)	17 (27.9)	
<2000	27 (73.0)	31 (50.8)	
>2000	7 (18.9)	13 (21.3)	0.04
HBeAg status, n (%), n=76			
Negative	24 (88.9)	39 (79.6)	
Positive	3 (11.1)	10 (20.4)	0.30
Anti-HBc IgM, n (%), n=75			
Negative	25 (92.6)	46 (95.8)	
Positive	2 (7.4)	2 (4.2)	0.55
Total anti-HBc, n (%) n=95			
Negative	7 (19.4)	9 (15.3)	
Positive	29 (80.6)	61 (85.9)	0.44
ART status, n (%)			
ART-naive	3 (8.1)	2 (3.3)	
On ART	34 (91.9)	59 (96.7)	0.29
Current ART regimen, n (%) n=67			
3TC containing, without TDF	8 (38.1)	19 (41.3)	
TDF containing*	13 (61.9)	27 (58.7)	1.00
Duration on ART, years, median (IQR), n=74	5.9 (4.2 – 10.3)	7.5 (4.8 – 10.5)	0.55

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Notes: *all but 1 participant were on TDF/FTC containing regimen, the participant did not have RAMs. **Abbreviations:** RAMs, resistance-associated mutations; HBV, hepatitis B virus; TND, target not detectable; HIV, human immunodeficiency virus; ART, antiretroviral therapy; 3TC, lamivudine; TDF, tenofovir disoproxil fumarate; IQR, interquartile range; HBsAg, hepatitis B surface antigen; OBI, occult hepatitis B infection; HBeAg, hepatitis B e antigen; Anti-HBc IgM, hepatitis B core immunoglobulin M antibodies; anti-HBc, hepatitis B core antibodies.

3.2. Resistance Associated Mutations

Overall, subgenotypes A1 (93.9%), D3 (3.1%) and E (3.1%) were identified among the 98 participants. A similar distribution was observed among participants with RAMs (n=61); A1 (90.2%), D3 (4.9%), E (4.9%). Phylogenetic analysis based on maximum-likelihood with 1000 bootstrap replicates was used to construct a tree. Some of the sequences with RAMs were clustering closely together (supported by posterior probability >0.90), suggesting a possible transmission, Figure 2.

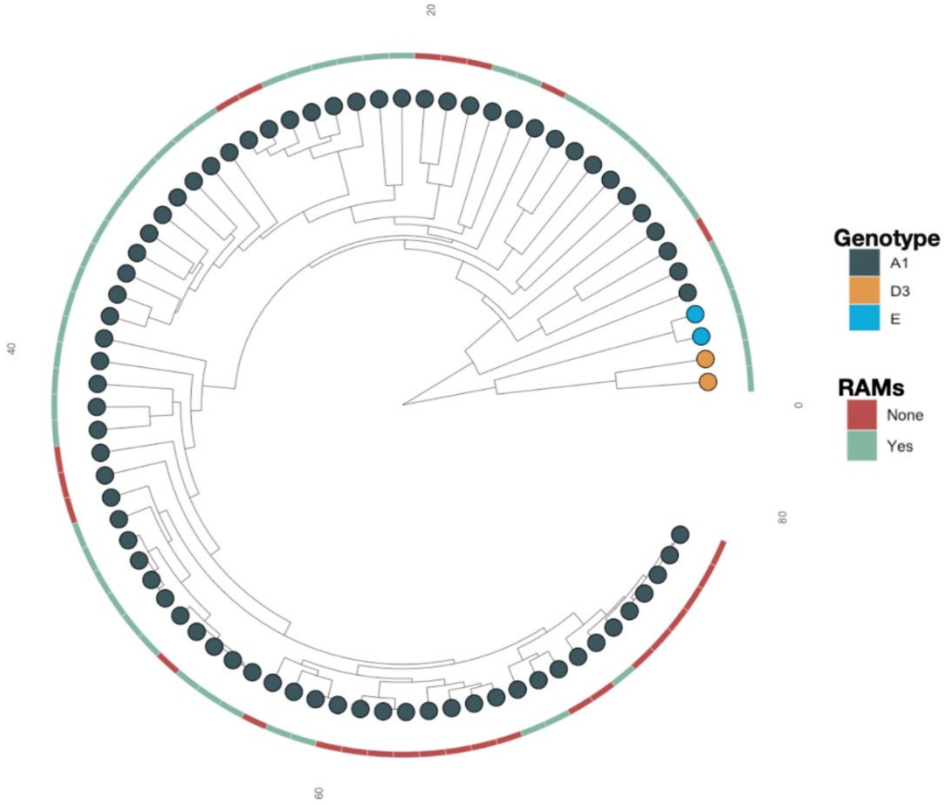

Figure 2. ML phylogenetic tree of representative sequences with and without RAMs by HBV subgenotype.

In this study, we identified RAMs which confer resistance to 3TC in 61/98 (62.2%) participants. There were no RAMs identified which confer resistance to tenofovir in our study, therefore, among participants on TDF-containing regimen, none had HBV TDF RAMs. However, there was one participant who had an amino acid substitution ((N236K) at a position that has been reported to confer resistance against tenofovir, rt236. Amino acid substitutions were identified at 7 RT positions, most being at position rt204 (60.3%), rt180 (50.5%), rt173 (33.3%) and rt250 (6.9%), Figure 3A and B. We observed already characterized amino acid substitutions, rtM204V, rtL180M and rtV173L being the most prevalent. Other amino acid substitutions were observed in positions known for resistance associated mutations, however, they had not been well characterized, Figure 3B.

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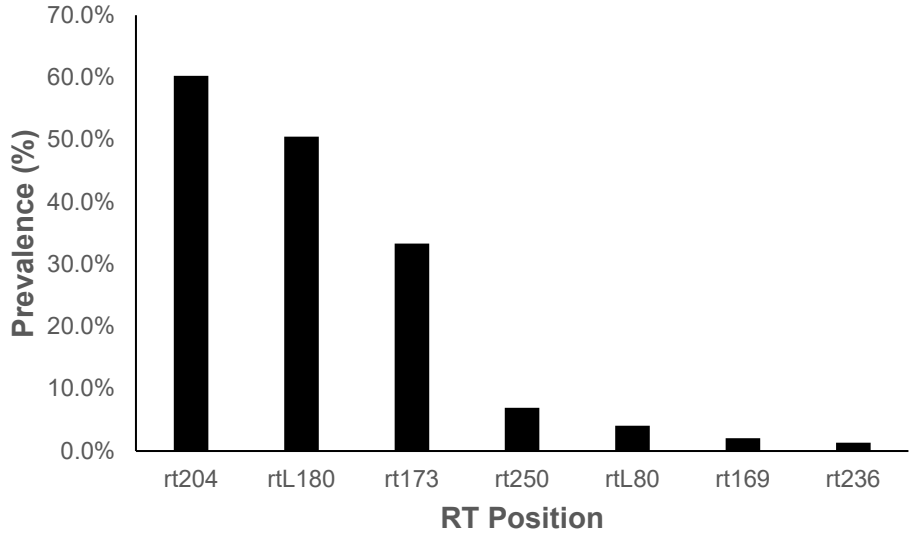
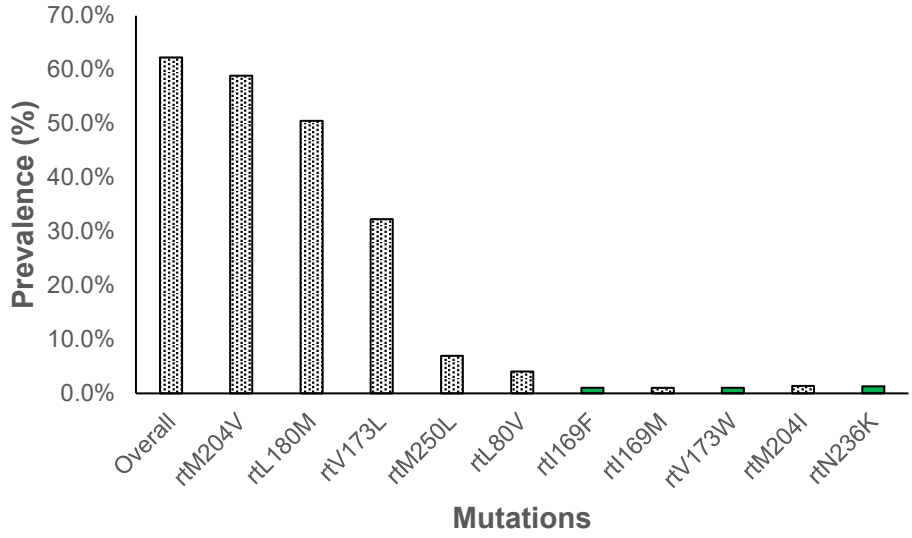


(**a**)



(**b**)

Figure 3. Prevalence of amino acid substitutions ((a). By RT position, (b). Specific mutations, black bars-mutations have been characterized, green bars-uncharacterized amino acid substitutions).

Uncharacterized mutations mostly appeared in one participant, Table 2. Some participants [17/61 (27.9%) and 10/61 (16.4%)] had the triple mutations (rtV173L, rtL180M and rtM204V) and the double mutations (rtL180M and M204V). Of participants with the triple mutations, 5/12 (41.7%) were on the 3TC regimen and among those with the double mutations, 5/8 (62.5%) were on a regimen with 3TC-only backbone, Table 2.

Table 2. Combination of mutations in participants with RAMs.

Mutations	Frequency	ART status	ART regimen*#
rtV173L/rtL180M/rtM204V	17	All on ART	7 on TDF 5 on 3TC
rtL180M/rtM204V	10	All on ART	3 on TDF 5 on 3TC
rtM204V	8	All on ART	7 on TDF 1 on 3TC

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rtL180M	5	All on ART	2 on TDF 2 on 3TC
rtV173L/rtL180M	5	All on ART	2 on TDF 2 on 3TC
rtL180M/rtM204V/rtM250L	3	1 on ART 2 ART naïve	No data
rtV173L/rtM204V	2	1 on ART 1 ART naïve	On 3TC
rtV173L	2	1 on ART 1 ART naïve	No data
rtI169F/rtV173L/rtL180M	1	On ART	On TDF
rtV173L/rtL180M/rtM204V/rtM250L	1	On ART	On TDF
rtL180M/rtM204V/rtN236K	1	On ART	On 3TC
rtL80V/rtV173L/rtL180M/rtM204I	1	On ART	On 3TC
rtL80V/rtV173L/rtL180M	1	On ART	On 3TC
rtL80V/rtV173L/rtL180M/rtM204V	1	On ART	On TDF
rtV173W/rtL180M	1	On ART	On TDF
rtI169M/rtL180M	1	On ART	On TDF
rtM204V/rtM250L	1	On ART	On TDF

Notes: *All participants on TDF-containing regimen also had FTC on the same regimen, #Participants with ART regimen data. **Abbreviations:** ART, antiretroviral therapy; 3TC, lamivudine; TDF, tenofovir disoproxil fumarate; RT, reverse transcriptase.

4. Discussion

Botswana has a robust and successful HIV treatment program resulting in the country achieving UNAIDS 95-95-95 goals [10]. However, HBV screening before ART initiation is not a robust and there is also no monitoring of HBV response to ART once people with HIV/HBV are initiated on ART for HIV. This widespread and prolonged use of ART for HIV without HBV screening and monitoring has potentially led to the selection of drug resistant HBV variants. In this study we report the prevalence of HBV RAMs in participants with HBV/HIV in Botswana. We generated HBV sequences from samples with varying HBV viral loads, even those deemed to have undetectable HBV viral load by the assay used in the study. This is not common as HBV sequencing is generally done for high viral load samples limiting the identification of the full spectrum of mutations in a population [6].

HBV sequences generated in this study were mostly subgenotype A1 (93.9%) which is considerably different from other previous studies in the country. In blood donors, subgenotype A1 prevalence was 36.1% [11], 45.5% in pregnant women [25] and 48% in ART-naïve PLWH [26]. In our current study, our sample size is larger, and sequences are generated from diverse communities across Botswana as opposed to previous studies that focused mainly on the capital city Gaborone and surrounding areas.

We detected RAMs in participants with varying HBV viral load, some with a TND result from the assay that we used which has also been reported elsewhere [27,28]. Therefore, HBV drug resistance is not to only be associated with treatment failure, as it also occurs at low viraemia. Most participants in this study were on TDF-containing or 3TC-containing regimens, which reflects the first line ART regimens at the time the BCPP study was conducted as well as historic first line ART regimen. Most participants had RAMs associated with 3TC therapy. Botswana adopted the Treat-All strategy in 2016 with DTG-based ART as the first line regimen. However, earlier first-line regimens included 3TC, and many participants in our study had been on 3TC-containing regimens, especially zidovudine/3TC plus EFV or NVP. With the ART duration of our study participants, it is not surprising that they would harbor HBV variants with mutations associated with 3TC resistance.

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3TC is a low genetic barrier drug for HBV [29] , hence as expected, we identified a high prevalence of 3TC associated resistance in this study. M204V (58.9%), L180M (50.5%) and V173L (32.3%) were the predominant mutations and they are known to confer resistance to 3TC and enhance viral replication [30]. A similar pattern has been observed in Gabon [31] and also similarly to our study, the predominant RAM was M204V/I in a systematic review of HBV RAMS in Africa [9] and globally [32]. The M204V mutation as shown in our results, occurred either alone or in combination with other mutations, most commonly with L180M and V173L which are described as compensatory mutations to M204V as they enhance viral replication [31,33].

Some of the study participants were on TDF-containing regimen and we report no RAM conferring resistance to TDF. HBV resistance against TFV is still controversial. A recent review suggests that resistance to TFV may require more than one resistance mutation that confers resistance to other NAs being L180M, A181V/T, M204I/V and N236T [6]. A study in the United States showed that participants who had been exposed to 3TC took a longer time to achieve HBV viral suppression while on TFV compared to 3TC-naïve participants [34]. Therefore, it is likely that 3TC-associated mutations may eventually lead to decreased susceptibility to TFV. Some of the study participants with 3TC-associated mutations were on a TDF-containing regimen, this could be because they started on 3TC-based ART before switching to a TDF-based regimen. It could also be due to the transmission of variants with 3TC resistance mutations [35]. We report an uncharacterized polymorphism N236K in a position known to confer resistance to TFV.

5. Conclusions

In a population of ART-experienced individuals with concomitant HBV/HIV, the prevalence of HBV RAMs was high, particularly those known to confer resistance to 3TC. The high prevalence of 3TC RAMs in this population discourages the use of ART regimens with 3TC as the only HBV-active drug in people living with HIV/HBV. The presence of HBV RAMs hinders HBV elimination efforts hence the need to monitor HBV drug resistance mutations in Botswana and globally. TDF-associated resistance mutations were not observed while most participants were on TDF hence supporting the effectiveness of TDF in treating HBV.

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Institutional Review Board Statement: The study was approved by the Ministry of Health Research and Development Committee (HDPME 13/18/1, 25 June 2021).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the BCPP study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available as the sequences are currently being analyzed for other objectives of the bigger project.

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