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Abstract: (1) Background: Hepatitis B virus (HBV) sequencing data are important for monitoring HBV evolution. We aimed to molecularly characterize HBV sequences from participants with HBV surface antigen (HBsAg+) positive serology and occult hepatitis B infection (OBI+). (2) Methods: We utilized archived plasma samples from a cohort of people living with (PLWH) in Botswana. HBV DNA was sequenced, genotyped and analyzed for mutations. We compared mutations from study sequences to those from previously generated HBV sequences in Botswana. The impact of OBI-associated mutations on protein function was assessed using the Protein Variation Effect Analyzer. (3) Results: Sequencing success was higher in HBsAg+ than OBI+ samples [86/128 (67.2%) vs 21/71 (29.2%)]. Overall, 93.5% (100/107) sequences were genotype A1, 2.8% (3/107) were D3 and 3.7% (4/107) were E. We identified 13 escape mutations in 18/90 (20%) sequences with HBsAg coverage, with K122R having the highest frequency. The mutational profile of current sequences differed from previous Botswana HBV sequences, suggesting possible mutational changes over-time. Mutations deemed to have an impact on protein function were $_{tp}$Q6H, $_{surface}$V194A and $_{preC}$W28L. (4) Conclusions: We characterized HBV sequences from PLWH in Botswana. Escape mutations were prevalent and were not associated with OBI. Longitudinal HBV studies are needed to investigate HBV natural evolution.

Keywords: Hepatitis B virus; genotypes; escape mutations; Botswana; Africa

1. Introduction

Hepatitis B virus (HBV) infection remains a significant global health concern particularly in Africa, which has the highest HBV prevalence globally at 5.8% [1]. In Botswana, the prevalence of hepatitis B surface antigen (HBsAg) varies by region, reaching levels as high as 22% in certain areas of the country [2]. Only three studies have reported occult hepatitis B infection (OBI) prevalence in Botswana, with a range from 6.6% to 33% in adults [2–4]. OBI is defined as the presence of replicative

competent HBV deoxyribonucleic acid (DNA) in the blood and/or liver of individuals testing negative for HBsAg [5].

Understanding the genetic diversity of HBV within specific geographic locations is important for devising effective prevention and control strategies as HBV genotypes have clinical relevance. HBV genotypes are associated with vaccine efficacy [6], treatment response [7] , tendency of chronicity [8], HBsAg and hepatitis B e antigen (HBeAg) seroconversion [9]. Previous studies have identified HBV subgenotypes A1, D2, D3, and genotype E in Botswana [3,10–13]. Notably, the prevalence of subgenotype A1 varies among different demographic groups [3,10,11,14]. There is need for HBV surveillance and molecular characterization efforts especially in settings with high HIV prevalence and widespread antiretroviral treatment (ART) use, in which HBV drug resistance can be common [15]. Furthermore, HBV genomics are important in studying vaccine and treatment response, as well as transmission dynamics within the country.

OBI is not reported in national, regional, and global reports however it has clinical relevance. OBI was determined to be an independent risk factor for hepatocellular carcinoma (HCC) in one study [16]. Drug resistance associated mutations have been identified in participants with OBI [15]. Furthermore, infants born to mothers with positive HBsAg (HBsAg+) were diagnosed with OBI in one study [17]. One mechanism postulated to lead to the OBI phenotype is the presence of mutations that impair HBsAg detection. Some OBI associated mutations in different HBV open reading frames (ORFs), (pres1, pre2, surface, core, pre-core, X, and the polymerase domains) have been identified and studied in Botswana and South Africa [18,19]. The impact of these mutations on protein function was assessed using available online in silico tools as screening methods for potential candidates of functional in vitro studies. The Protein Variation Effect Analyzer (PROVEAN) was more accurate than other tools being studied [18,19].

In Botswana, the HBsAg positivity remains high, and the OBI prevalence is 3 to 4 times that of HBsAg positivity as prior studies have reported an adult OBI prevalence of 6.6% to 33% [2–4]. Therefore, the genetic diversity of HBV in both HBsAg+ and OBI participants needs to be further studied. We aimed to molecularly characterize HBV sequences from people living with HIV (PLWH) who tested positive for HBsAg and OBI, and to determine the impact of OBI-associated mutations on protein function in a cohort of PLWH in Botswana.

2. Materials and Methods

2.1. Study population

Archived plasma samples from participants in the Botswana Combination Prevention Project (BCPP), that had previously tested positive for HBsAg (HBsAg+), and OBI (OBI+) were used [20]. Details of the BCPP study are described elsewhere [21,22]. Briefly, the BCPP study was a pair-matched cluster randomized study that enrolled 12,610 all consenting adults residing in a random sample of ~20% of households in 30 geographically dispersed villages throughout Botswana between the years 2013 and 2018. The main aim of the BCPP study was to assess if a combination of HIV prevention strategies would reduce HIV incidence at a community level compared to the standard of care. At baseline 3,596 BCPP study participants were PLWH and (83% of whom knew their HIV status) [21]. Our study was approved by the Human Research Development Committee (HRDC) at the Botswana Ministry of Health (HRDC number: 01028).

2.2. Laboratory procedures

HBV screening has been described in our previous report [2]. Briefly, available plasma samples from 3304/3596 (91.9%) of PLWH in the BCPP cohort were screened for HBsAg and total core-antibodies (anti-HBc). HBsAg+ samples were further screened for HBeAg and immunoglobulin M core antibodies (anti-HBc IgM). HBV viral load was quantified for HBsAg+ samples with sufficient sample volume and performed in samples that tested negative for HBsAg to determine OBI prevalence using the Roche COBAS Ampliprep/Taqman Analyzer [2].

The QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) was used to extract DNA from 200μL of HBsAg+ and OBI+ plasma samples according to the manufacturer's protocol with a final

elution volume of 30μL. HBV DNA was amplified using tiling primers adopted from Choga's protocol [23] (Table S1). Briefly, two 10μM pools of tiling primers were prepared. A master mix for each primer pool with 5μL of DNA template was prepared, and HBV DNA was amplified using a protocol from our previous reports [15,24]. After amplification, these PCR products were combined, and library preparation followed suite [15,23,25]. The library was loaded into flow cells version R9.4.1 (Oxford Nanopore Technologies, Oxford, UK) and the GridION platform was used for sequencing.

2.3. Sequence analyses

2.3.1. Genotypic and mutational analysis.

Generated FASTQ files were uploaded into Genome Detective 1.132/1.133 for reference-based assembly of HBV [26] (last accessed 20 April 2023). The generated consensus sequences were downloaded and further imputed and assessed in AliView ver. 1.26 for viewing, trimming and alignment [27]. Geno2pheno (https://hbv.geno2pheno.org) (last accessed 11 December 2023), was used to assign HBV genotypes/subgenotypes. Genotypes were confirmed using phylogenetic analysis. BCPP generated sequences and reference HBV sequences from GenBank were used to construct a phylogenetic tree of the complete surface ORF using a Bayesian Markov chain Monte Carlo (MCMC) in the Bayesian Evolutionary Analysis by Sampling Trees (BEAST) v1.8.2 (BEAST Developers) program with a chain length of 100,000,000 and sampling every 10,000 generations. The analysis utilized an uncorrelated log-normal relaxed molecular clock, the Hasegawa, Kishino, and Yano (HKY) model, and the general time reversible model with gamma distributed rates of variation among sites and a proportion of invariable sites (GTR+G+I). Tracer v1.7 (BEAST Developers) was used to visualize results and confirm chain convergence. Tree Annotator v1.7.3 (BEAST Developers) was used to choose the maximum clade credibility tree after a 10% burn-in. The mutational profile of sequences generated form this study was compared to mutations in sequences generated in previous Botswana studies that were predominantly from PLWH (2009-2015) [3,10,11,28].

2.3.2. Impact of occult-associated mutations on protein function.

Sequences with a depth of >100 were used for this analysis. The Protein Variation Effect Analyzer (PROVEAN), available at http://provean.jcvi.org/index.php (accessed 24 April 2024) was used to determine the impact of occult-associated mutations on protein function Occult-associated mutations were defined as mutations identified only in OBI+ sequences and those that were overrepresented in OBI+ sequences versus HBsAg+ sequences.

3. Results

3.1. Participants clinical characteristics

Table 1 summarizes the clinical characteristics of participants whose plasma samples were used in the analysis. Most participants were female (66.4%) and had a median age of 43 (IQR: 36 – 49). Most participants had a low HBV viral load of <2000IU/ml (58.9%). Approximately 94.4% of participants were on antiretroviral therapy (ART) and were mostly on tenofovir disoproxil fumarate (TDF) containing regimen (61.6%). The TDF regimen also had emtricitabine (FTC) for all participants except for one who was on a dolutegravir/TDF regimen. The majority of participants had undetectable HIV-1 RNA (<40copies/mL) (88.8%). Median duration time on ART was 7 years (IQR: 4.7 – 9.9) Table 1.

Table 1. Participants clinical and socio-economic characteristics at enrollment.

Characteristics	Number (%) n=107
Sex	
Female	71 (66.4)
Age, years, median (IQR)	43 (36 – 49)
HBV type	
HBsAg+	86 (80.4)

OBI+	21 (19.6)
Total anti-HBc, n=104	
Positive	87 (83.7)
Anti-HBc IgM status*, n=81	
Positive	5 (6.2)
HBeAg status*, n=82	
Positive	15 (18.3)
HBV viral load	
Target not detected	21 (19.6)
<2000	63 (58.9)
≥2000	23 (21.5)
HIV viral load	
Undetectable	95 (88.8)
Detectable	12 (11.2)
ART status	
Naïve	6 (5.6)
On ART	101 (94.4)
ART regimen	
None 3TC/TDF-containing regimen	1 (1.0)
3TC-containing regimen	27 (26.7)
TDF-containing regimen#	45 (44.6)
Unknown	28 (27.7)
Duration on ART, years, n=76, median (IQR)	7.0 (4.7 – 9.9)

HBV; hepatitis B virus, IQR; interquartile range, HBsAg; hepatitis B surface antigen, OBI; occult hepatitis infection, HBeAg; hepatitis B e antigen, anti-HBc: hepatitis B core antibody, anti-HBc IgM; hepatitis B core antigen immunoglobulin M antibodies, HIV; human immunodeficiency virus, ART; antiretroviral therapy, 3TC; lamivudine, TDF; tenofovir disoproxil fumarate. *Only in HBsAg positive participants. #The TDF containing regimen also had emtricitabine (FTC) for all participants except one who was on a dolutegravir/TDF regimen.

3.2. Sequencing success

Figure 1 shows the total number of sequences generated. Sequencing was attempted on 128 samples out of the 271 HBsAg+ samples and the success rate was 67.2% (86/128). Success rate for OBI+ was 29.2% (21/72). Samples that were successfully sequenced had HBV viral loads ranging from target not detected (TND) to >1.7 x 10^8 IU/mL.
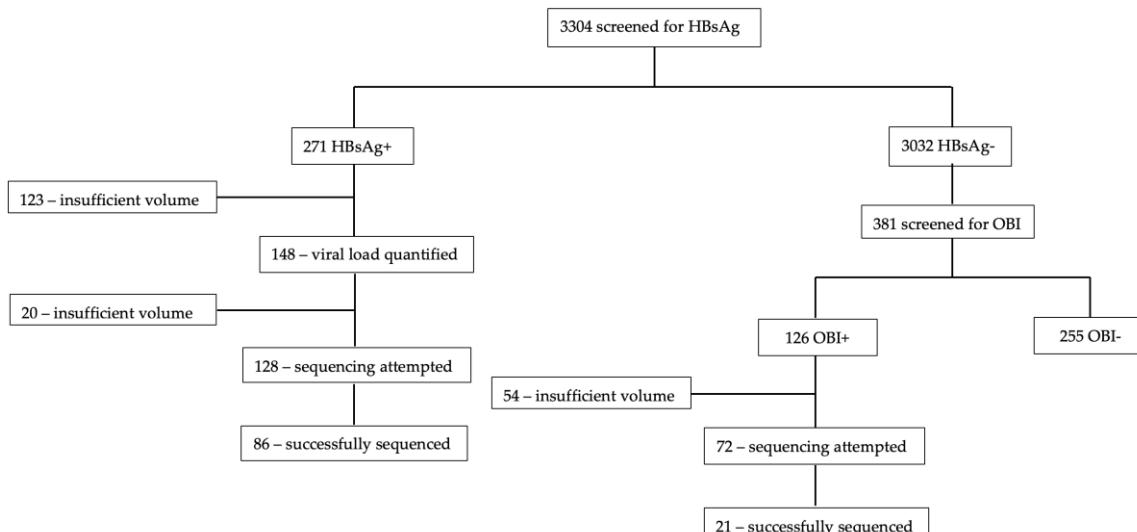

**Figure 1.** Consort diagram of successfully sequenced samples.

In total, 27 out of the 30 BCPP study sites contributed HBV sequences to this analysis (Figure S2). Samples from participants residing in Mmadinare, Molapowabojang and Otse (BCPP study sites) were not successfully sequenced. Most sequences were generated from participants in the Central district with 50 sequences, followed by the North-West district with 26 sequences. Kgatleng district had the least number of sequences generated (n=5) (Figure 2).
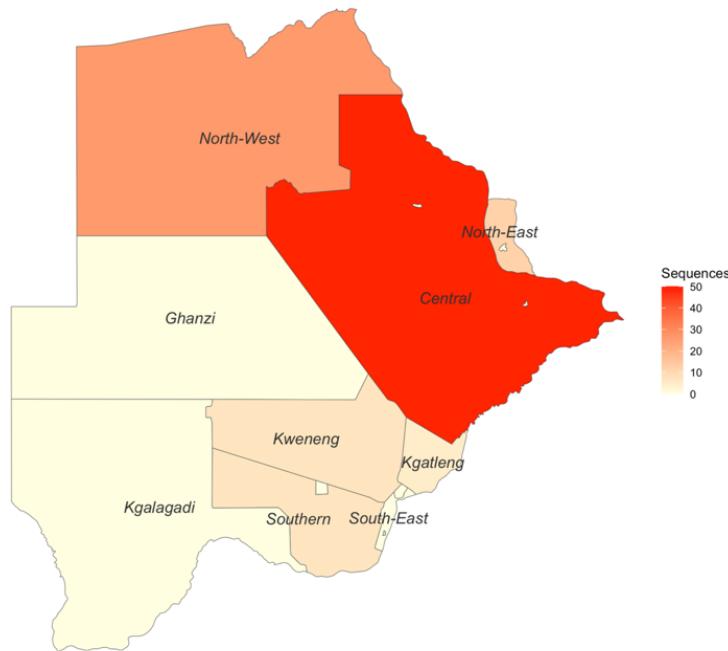
Figure 2. Generated sequences in the different districts of Botswana.

3.3. Genotypic analysis

Figure 3 shows the clustering of the study sequences with reference sequences. All previous HBV sequences from Botswana clustered together and BCPP sequences clustered randomly (Figure 3). Overall, 93.5% (100/107) sequences were genotype/subgenotype A1, 2.8% (3/107) were D3 and 3.7% (4/107) were E. Among the HBsAg+ sequences, 93.0% (80/86) sequences were genotype/subgenotype A1, 3.5% (3/80) were D3 and 3.5% (3/80) were E. For OBI sequences, 95.2% (20/21) sequences were genotype A1 and 4.8% (1/21) were E.
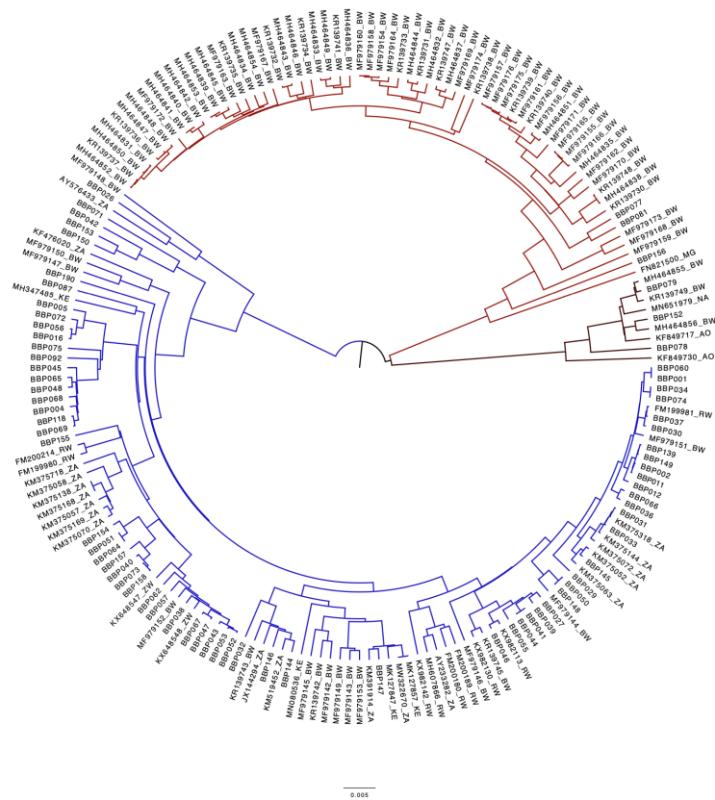
Figure 3. Bayesian phylogenetic tree of sequences generated in the BCPP cohort and reference sequences. Study sequences are denoted by BBP followed by a number while references sequences start with accession number followed by country. Sequences in blue are genotype A1, those in red are genotype D3 and those in black are E.

3.4. Mutational analysis

3.4.1. Escape mutations

A total of 13 escape mutations were detected in 20% (18/90) of sequences with surface gene coverage (Table 2). $_{surface}$K122R had the highest frequency in 9/18 (50%) of participants with escape mutations. $_{surface}$T114S, $_{surface}$S114L, $_{surface}$C139R, $_{surface}$N146S and $_{surface}$C147Y are associated with impaired virion secretion. Vaccine escape mutations ($_{surface}$G130C, $_{surface}$N131T, $_{surface}$T121N) were identified in four participants. We also identified mutations that may impact HBsAg detection ($_{surface}$T118M, $_{surface}$C121R, $_{surface}$K122R, $_{surface}$G130C) (Table 2).

Table 2. Escape mutations in the surface region.

Mutation	Frequency	Genotype	HBV type	Reported impact	Reference
T114S	2	A1	HBsAg	Other substitutions at position 114 (R) reported to impair virion secretion	[29]
S114L	1	E	HBsAg	Other substitutions at position 114 (R) reported to impair virion secretion	[29]
T118M	1	A1	OBI	Impair antigenicity, detection escape	[30]
C121R	1	A1	OBI	Other substitutions at position 121 (S) reported to reduce antigenicity and impair HBsAg detection	[31,32]

K122R	9	A1	HBsAg	Decreased HBsAg expression, detection failure	[33–35]
Q129C	1	A1	OBI	Other Q129 (N) mutations lead to impaired antigenicity and immunogenicity, Q129R leads to impaired virion/S protein secretion, Q129H leads to decreased virion secretion	[29,36,37]
G130C	1	A1	OBI	Other G130 mutations to lead to diagnostic escape, vaccine/ immunoglobulin therapy escape, altered antigenicity	[38,39]
N131T	2	A1	HBsAg	Vaccine escape	[40]
T131N	1	D3	HBsAg	Vaccine escape, diagnostic escape, hepatitis B immunoglobulin resistance	[41–43]
C137I	1	A1	HBsAg	Other C137 mutations are reported to decrease antigenicity	[31]
C139R	1	A1	HBsAg	Impair virion/S protein secretion	[37]
N146S	1	E	OBI	Impair virion secretion	[29,44]
C147Y	1	A1	HBsAg	Impair virion secretion	[29]

HBsAg; hepatitis B surface antigen, OBI; occult hepatitis infection.

3.4.2. Comparison of Botswana reference HBV sequences and BCPP HBV sequences.

For all downstream analyses, only subgenotype A1 sequences were used as they constituted >93% of the sequences. Tables 3 and 4 show previously generated Botswana sequences (REFERENCE-unique) and BCPP unique mutations with a prevalence of >20%. The full list of mutations is shown in Tables S3 and S4. BCPP sequences tended to have more unique mutations in all ORFs with a high prevalence (>20%) that were not observed in previous Botswana sequences. Some amino acid substitutions unique to BCPP sequences had a much higher prevalence, such as $_{preC}$V17F (53.6%), $_x$P33S (42.2%), and $_{surface}$I195M (55.7%). Among mutations that were identified in both sets of sequences, the prevalence of $_{pres2}$A7T and $_{pres2}$A11T was higher in the BCPP sequences (19.9% and 10.3% vs 5.3% for both mutations in the reference sequences). However, $_{pres2}$T38I was lower in the BCPP sequences (30.3% vs 41%). For the transcriptional transactivator protein (HBx), the prevalence of $_x$G22S, $_x$A21T and $_x$S46P was higher in the BCPP sequences than in the reference sequences (43.8%, 28.1%, and 67.2% vs 8.3%, 8.3% and 41.7%). In the surface protein, $_{surface}$N131T and $_{surface}$V194A had a lower prevalence in the BCPP sequences compared to the reference sequences (2.2% and 7.7% vs 37.5% and 14.8%) while the opposite was observed for $_{surface}$K122R (10.1% vs 2.3%).

Table 3. Mutations in HBV ORFs.

	REFERENCE SEQUENCES			BCPP SEQUENCES			COMMON MUTATIONS		
	Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence	Mutation	REFERENCE	BCPP
PreS1	A90P	9/37	24.3%	I48V	21/99	21.2%	T94P	44.4%	50.5%
				A90V	23/99	23.2%			
PreS2	None			I45T	18/89	20.2%	A7T	5.3%	16.9%
				L54S	21/89	23.6%	A11T	5.3%	10.3%

	Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence		Reference	BCPP
							T38I	41.0%	30.3%
							A53V	12.8%	4.5%
preC	None			V17F	15/28	53.6%	None		
Core	None			None			None		
X	V131I	4/19	21.1%	R26C	13/64	20.3%	S11P	12.5%	18.3%
				P29S	17/64	26.6%	G22S	8.3%	43.8%
				P33S	27/64	42.2%	A31T	8.3%	28.1%
				T36A	14/64	21.9%	S46P	41.7%	67.2%
Surface				E164D	25/87	28.7%	K122R	2.3%	10.1%
				I195M	39/70	55.7%	N131T	14.8%	2.2%
							V194A	37.5%	5.7%

BCPP; Botswana Combination Prevention Project.

Table 4. Mutations in the polymerase domains.

	BW REFRENCE SEQUENCES			BCPP REFERENCES			COMMON MUTATIONS		
	Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence		REFERENCE	BCPP
TP				Q138H	15/72	20.8%	V71I	28.2%	34.7%
							Q87H	53.8%	47.2%
							H182Q	5.1%	21.7%
Spacer	Q6K	15/38	39.5%	S18P	12/59	20.3%	P64A	44.7%	36.3%
	A7T	23/38	60.5%	H47R	19/91	20.9%	I84T	13.2%	12.1%
	S89T	9/38	23.7%	P127S	12/52	23.1%	Y86H	13.2%	26.4%
	L158I	10/38	26.3%				H93S	44.7%	45.1%
							S125N	5.6%	22.9%
							S129N	5.3%	12.3%
RT	R110G	12/40	30.0%	N124H	19/84	22.6%	V7A	40.0%	16.3%
	Q139H	12/40	30.0%	Y126H	20/84	23.8%	L53I	35.0%	11.4%
	H271C	13/40	32.5%	V173L	23/84	27.4%	I103V	37.5%	23.5%
				L180M	41/87	47.1%	P109S	20.0%	14.1%
				M204V	37/69	53.6%	H122N	35.0%	2.4%
							W153R	7.5%	19.0%
							K266V	27.5%	14.5%
							K266I	65.0%	65.2%
							N332S	39.3%	15.9%
							Q333K	44.4%	14.5%
Rnase H				V128D	18/70	25.7%	S2P	41.7%	61.9%
				V148A	24/70	34.3%	Y116F	8.3%	30.0%
							R151K	8.3%	7.8%

BCPP; Botswana Combination Prevention Project, TP; terminal protein, RT; reverse transcriptase.

In Table 4, we report mutations specific to the polymerase domains. It is noticeable that resistance associated mutations (rtM204V, rtL180M, rtV173L) were unique to the BCPP cohort. In the same RT region, some mutations had a noticeably higher prevalence in the reference sequences as compared to the BCPP sequences. These are rtV7A (40.0% vs 16.3%), rtL53I (35.0% vs 11.4%), rtH122N (35.0% vs 2.4%), rtN332S (39.3% vs 15.9%) and rtQ333K (44.4% vs 14.5%). In the terminal protein (TP) region, tpH182Q had a noticeably higher prevalence in the BCPP cohort than in the reference sequences (21.7% vs 5.1%). spY86H, spS125N and spS129N in the spacer domain occurred more frequently in the BCPP sequences than the reference sequences. In the RNase H domain, RNaseHY116F was observed at a much higher prevalence in the BCPP cohort compared to the reference sequences (30.0% vs 8.3%).

3.4.3. Impact of occult-associated mutations on protein function.

For this analysis, we focused on sequences that had a depth of >100 and we identified mutations that were in sequences isolated from OBI participants only ($_{core}$T142S, $_{tp}$E88R, $_{tp}$Q6H, $_{rt}$M250L, $_{preC}$W28L). Other mutations were overly represented in OBI+ sequences compared to HBsAg+ sequences. $_{surface}$V194A and $_{surface}$S55P appeared in 3/13 (23.1%) OBI participants each versus 1/53 (1.9%), HBsAg participant each, p-value 0.004. Using PROVEAN, three mutations were deemed deleterious, that is were deemed to affect protein function negatively: $_{tp}$Q6H, $_{surface}$V194A and $_{preC}$W28L.

Table 5. Impact of mutations identified only/overrepresented in participants with OBI.

ORF	Mutation	PROVEAN Prediction
Core	$_{core}$T142S	Neutral
Terminal protein	$_{tp}$E88R	Neutral
	$_{tp}$Q6H	Deleterious
Surface	$_{surface}$V194A	Neutral
	$_{surface}$S55P	Deleterious
Reverse transcriptase	$_{rt}$M250L	Neutral
Precore	$_{preC}$W28L	Deleterious

ORF; open reading frame, PROVEAN; Protein Variation Effect Analyzer.

4. Discussion

In this study, we identified HBV subgenotypes A1, D3, and E across a wide geographic area in Botswana, with subgenotype A1 representing more than 93% of all sequences. We also report the mutational profile of HBV in the Botswana population including mutations with deleterious impact on protein function in participants with OBI.

Our findings are consistent with prior HBV studies in Botswana which identified the same subgenotypes however with varying genotype prevalence [3,10,11]. We also report immune, vaccine, and diagnostic escape mutations in this population, some of which have been identified in other populations in Botswana [10,11,28]. $_{surface}$K122R was the most prevalent escape mutation and was identified only among participants with HBsAg positive serology. This mutation is associated with decreased HBsAg expression and HBsAg detection failure [33–35], however it was not detected among the OBI samples. We did not perform quantitative HBsAg ELISA which could have revealed HBsAg levels in samples with these mutations compared to those without. We also identified known mutations ($_{surface}$T118M, $_{surface}$N146S), and uncharacterized mutations at positions associated with immune escape ($_{surface}$C121R, $_{surface}$Q129C, $_{surface}$G130C,) in OBI participants although these mutations are not unique to individuals with OBI in other studies [30,41,45,46]. All these mutations were identified in the major hydrophilic region (MHR) (position 99 to 169) of the HBsAg with the majority of these being found in the "α" determinant of the MHR (position 124 to 147) which is a major cluster of antigenic epitopes [47]. We also identified vaccine escape mutations (VEMs) in 16.7% of participants with escape mutations, which is a cause for concern as these may counter-act vaccination efforts in the country. This was at position 131 of the surface region also in the "α" determinant of the MHR known for mutations that allow the virus to evade vaccine induced immune response [48]. We have identified a potential vaccine escape mutation, G130C which was reported as a novel mutation in 2017 [49]. Mutations at position 130 are reported to be vaccine escape mutations and have been isolated in vaccinated individuals [38,39].

There was a change in mutation patterns between sequences previously generated in Botswana (2009-2015) [3,10,11,28] and sequences we generated in the BCPP study (2013–2018). For example, the RT region of BCPP sequences harbors more drug resistance associated mutations than the reference sequences. Over 90% of PLWH among the BCPP participants were ART-experienced while in the previous studies, participants were mostly ART-naïve [3,10,11,28]. Due to the overlapping pattern of the HBV genome, some of the mutations observed in the RT region of the polymerase affected the surface region. Position $_{surface}$I195 corresponds to the $_{rt}$M204 [50], therefore its prevalence is higher in BCPP sequences compared to previous Botswana sequences. Furthermore, $_{surface}$E164D is known to

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alter HBsAg antigenicity and tends to occur with surfaceI195M, as observed in our study and a previous study [51].

Most of the mutations identified in our study are uncharacterized. Other mutational variations of interest between BCPP and previously generated Botswana sequence are the xP33S in the X region. This mutation was only observed in the BCPP sequences and had not been previously identified in Botswana. It is a B-cell epitope mutation that has been shown to result in increased endoplasmic reticulum (ER) stress [52] and reduced protein stability in combination with other mutations [53]. There were some mutations that were common to both sequence datasets; however, they were more prevalent in the BCPP sequences. For example, the xG22S, which is reported to be an HCC-related HBx mutation [54]. BCPP sequences also had the xT36A, a functionally characterized HCC-associated mutation at >20% prevalence. This mutation is reported to enhance viral genome integration into the host cell resulting in insertion mutations and a 3'-terminal truncation of HBx [55,56]. While previously generated Botswana sequences and the BCPP sequences were generated from different parts of the country, we cannot rule out the possibility that more people are progressing to chronicity and HCC in the population.

To further elucidate on OBI-associated mutations, we used a freely available online tool, PROVEAN, previously shown to be more accurate in predicting the impact of functionally characterized HBV mutations on protein function than other prediction tools [18,19]. This analysis also allows for the selection of mutations that could be candidates for further in vitro studies. Three OBI associated mutations were deemed deleterious: tpQ6H, surfaceV194A and precW28L. The tpQ6H mutation has not been characterized however it falls within the N-terminal helices of the TP. Mutations and deletions in this subdomain were shown to impact RNA packaging, DNA synthesis and protein priming [57]. precW28L has not been characterized however a stop codon at this position has been identified in a participant with OBI [58] and in patients with HCC in a much older study [59]. Other surface mutations (surfaceS55P and surfaceV194A) were overrepresented in OBI+ sequences compared to HBsAg+ sequences, and other studies also show that these mutations are not unique to OBI+ sequences [30,60]. It is worth exploring the HBsAg levels in HBsAg+ and OBI+ samples with these mutations. surfaceS55P has not been functionally characterized, while surfaceV194A is a well know mutation associated with decreased extracellular HBsAg levels [61].

Our analysis focused on the differences between sequences from HBsAg+ and OBI+ infection, but it could be that the host factors have a key role to play on the OBI phenotype. Host factors such as immune response, human leukocyte antigen classA2 and interleukin-10 have been associated with OBI persistence [31,62,63]. Some studies attribute this phenotype to viral factors such as epigenetic control mechanisms [64,65] and mutations [18,19,66] that lead to reduced HBsAg expression and DNA replication..

A strength of the study is that we used samples from the BCPP study which recruited participants from different communities in Botswana. However, we analyzed sequences from only 27 out of the original 30 BCPP study sites due to insufficient volumes and the low sequencing success rate. Sequencing success was generally low, especially for OBI+ samples, however, this study still provides the largest number of HBV sequences in Botswana. We generated HBV sequences from participants with a target not detectable viral load result, a group usually excluded during sequencing. Escape mutations, surfaceT114S, surfaceN131T and surfaceK122R were identified in these participants which shows that there may be an underrepresentation of mutations in different reports that may not sequence participants with these viral load results. There is need however, to adopt a nucleic acid testing assay with a lower limit of detection than the one used in our current study. A limitation of the study is that participants were all PLWH predominantly on ART which limits generalizability to the general Botswana population. We make comparisons between previously generated Botswana sequences and the BCPP sequences, which comes with a limitation as these datasets are not from the same communities. Therefore, we cannot rule out the impact of host-genetic factors which could limit the conclusions in the differences we note. However, the mutational profiles of these sequences are fully documented in this study.

5. Conclusions

We have molecularly characterized HBV from PLWH in Botswana and identified that subgenotype A1 is predominant countrywide. We have also reported vaccine escape mutations which shows the importance of periodic monitoring of circulating HBV strains in the population. It is essential to generate HBV sequencing data to monitor the evolution of HBV and the emergence of mutations that could evade immunity and vaccines, potentially affecting HBV prevention and management strategies.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: HBV primers used for sequencing PCR; Figure S2: Number of sequences generated per BCPP communities in the different Botswana districts; Table S3: List of mutations in HBV ORFs; Table S4: List of mutations in HBV Pol domains.

Appendix A

Table S1. HBV primers used for sequencing PCR.

Primer Name	Sequence	Positions
SC_1_LEFT	TTCCACCAAGCTCTGCAAGATC	11 - 32
SC_1_RIGHT	AGAGGAATATGATAAAACGCCGCA	384-407
SC_2_LEFT	CTCCAATCACTCACCAACCTCC	325-346
SC_2_RIGHT	AAAGCCCTACGAACCACTGAAC	692-713
SC_3_LEFT	AAATACCTATGGGAGTGGGCCT	632-653
SC_3_RIGHT	TTGTGTAAATGGAGCGGCAAAG	1655-1676
SC_4_LEFT	AGAAAACTTCCTGTTAACAGACCTATTG	949-976
SC_4_RIGHT	GGACGACAGAATTATCAGTCCCG	1326-1348
SC_5_LEFT	TCCATACTGCGGAACTCCTAGC	1265-1286
SC_5_RIGHT	TGTAAGACCTTGGGCAGGATTTG	1632-1654
SC_6_LEFT	CTTCTCATCTGCCGGTCCGTGT	1559-1580
SC_6_RIGHT	AGAAGTCAGAAGGCAAAAACGAGA	1947-1970
SC_7_LEFT	GGCTTTGGGGCATGGACATT	1890-1909
SC_7_RIGHT	ATCCACACTCCGAAAGAGACCA	2256-2277
SC_8_LEFT	GACAACTATTGTGGTTTCATATTTCT	2193-2218
SC_8_RIGHT	TTGTTGACACCTATTAATAATGTCCTCA	2576-2594
SC_9_LEFT	TGGGCTTTATTCCTCTACTGTCCC	2492-2515
SC_9_RIGHT	GGGAACAGAAAGATTCGTCCCC	2889-2910
SC_10_LEFT	TTGCGGGTCACCATATTCTTGG	2816-2837
SC_10_RIGHT	GGCCTGAGGATGACTGTCTCTT	3189-3210

Primers adopted from Choga et al 2023 [23]
(https://dx.doi.org/10.17504/protocols.io.5qpvo3xxzv4o/v1).
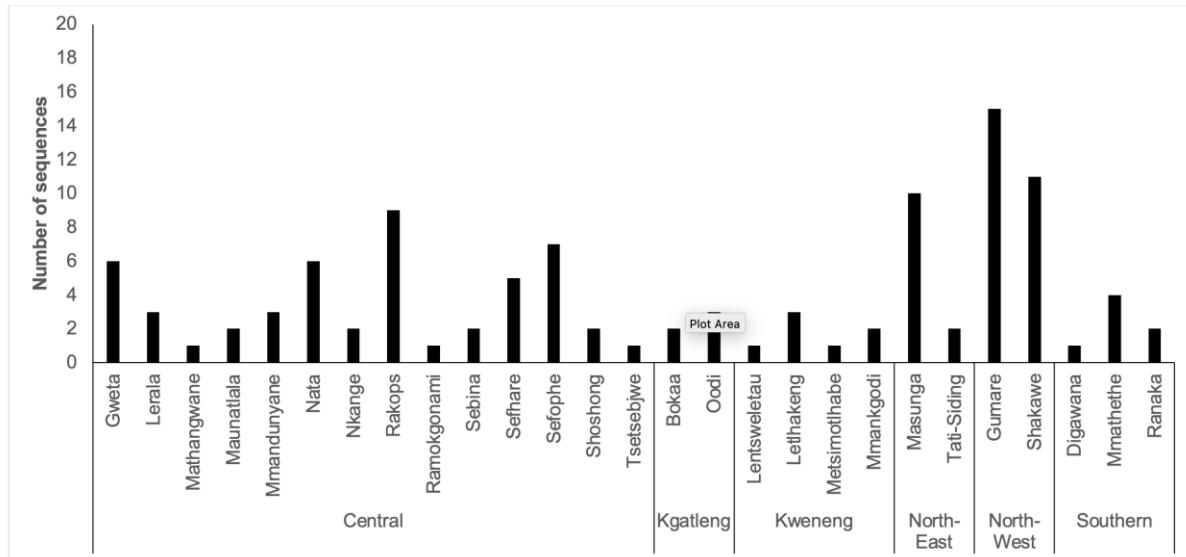
Figure S2. Number of sequences generated per BCPP communities in the different Botswana districts.

Table S3. List of mutations in HBV ORFs

	REFERENCE SEQUENCES			BCPP SEQUENCES			COMMON MUTATIONS		
	Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence		REF	BCPP
PreS1	S5P	3	8.1%	G35R	12	12.1%	T94P	44.4%	50.5%
	A6S	6	16.2%	N37Y	15	15.2%			
	K10N	3	8.1%	I48V	21	21.2%			
	F25L	5	13.5%	A90V	23	23.2%			
	V74F	2	5.4%						
	I84V	5	13.5%						
	A86T	2	5.4%						
	V88L	3	8.1%						
	A90T	6	16.2%						
	A90P	9	24.3%						
	V91S	5	13.5%						
	P92L	4	11.1%						
	P92S	5	13.9%						
	P93S	4	11.1%						
PreS2	V17I	2	5.3%	Y21C	2	2.2%	A7T	5.3%	16.9%
	Y21S	2	5.1%	L32V	8	9.0%	A11T	5.3%	10.3%
	F22L	3	7.7%	N37T	7	7.9%	T38I	41.0%	30.3%
	R48T	5	12.8%	A39V	9	10.1%	A53V	12.8%	4.5%
				H41P	2	2.2%	L54P	15.4%	14.6%
				H41L	3	3.4%			
				I45T	18	20.2%			
				S46F	8	9.0%			
				S47L	2	2.2%			
				T49I	6	6.7%			

				D51V	6	6.7%			
				L54S	21	23.6%			
preC				C14Y	2	7.14%	None		
				W28L	3	10.71%			
				V17F	15	53.57%			
Core	S35A	2	8.3%	P45S	8	9.8%	None		
	A41S	2	8.3%	C61R	2	2.4%			
	C48G	2	8.3%	G63R	2	2.4%			
	E64D	3	12.5%	E64K	3	3.7%			
	L65V	3	12.5%	M66T	2	2.4%			
	T67N	4	16.7%	N74I	3	3.7%			
	N74S	2	8.3%	S106F	2	2.4%			
	E77D	2	8.3%	T142S	2	2.7%			
	A131P	3	12.5%	V148A	2	2.7%			
	R151C	4	16.7%	R181P	3	3.7%			
	D153A	3	12.5%						
	S183P	2	8.3%						
X	E80A	2	8.3%	L5V	16	26.7%	S11P	12.5%	18.3%
	I88S	2	8.3%	Y6C	9	15.0%	G22S	8.3%	43.8%
	K130M	3	15.8%	S12A	11	18.3%	A31T	8.3%	28.1%
	V131I	4	21.1%	R26C	13	20.3%	S46P	41.7%	67.2%
				P29S	17	26.6%			
				A31S	8	12.5%			
				G32R	8	12.5%			
				P33S	27	42.2%			
				T36G	3	4.7%			
				T36A	14	21.9%			
				S47A	5	7.8%			
				D48V	6	9.4%			
				C78R	8	12.7%			
				A85S	2	3.4%			
				L93S	2	4.3%			
				V116L	2	4.3%			
				S147P	2	4.3%			
Surface	M1R	2	5.4%	N3S	3	3.5%	T114S	3.4%	2.2%
	S45P	3	8.1%	G7R	3	3.5%	K122R	2.3%	10.1%
	L49R	2	5.4%	F8L	2	2.3%	N131T	14.8%	2.2%
	L98V	3	3.4%	R24K	4	4.5%	V194A	37.5%	5.7%
	M103I	3	3.4%	G44S	2	2.3%			
	P111A	3	3.4%	S45T	2	2.3%			
	S117R	2	2.3%	S45A	4	4.5%			
	Q129R	4	4.5%	P46T	3	3.4%			

Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence
G130N	2	2.3%	S55P	9	10.2%
T140I	2	2.3%	T57I	5	5.6%
A159V	5	5.7%	V96A	9	10.1%
E164G	4	4.5%	Q101K	2	2.2%
V168A	2	2.3%	W156Q	2	2.3%
L173P	3	3.4%	A159I	2	2.3%
V184A	4	4.5%	E164D	25	28.7%
S204N	8	9.1%	F179L	2	2.3%
			V180A	6	6.9%
			W182R	2	2.3%
			F183S	3	3.5%
			V184L	2	2.7%
			G185W	2	2.7%
			I195M	39	55.7%
			W199R	2	2.9%
			Y200C	2	2.9%
			P203Q	2	2.9%
			Y206H	2	2.9%
			Y206R	3	4.3%
			I213M	2	2.9%
			P214L	4	5.7%

Table S4. List of mutations in HBV polymerase domains.

	REFERENCE SEQUENCES			BCPP SEQUENCES			COMMON MUTATIONS		
	Mutation	Frequency	Prevalence	Mutation	Frequency	Prevalence		REF	BCPP
TP	D16A	3	7.7%	Q6H	2	2.8%	V71I	28.2%	34.7%
	T18L	3	7.7%	A20V	2	2.8%	Q87H	53.8%	47.2%
	V71N	4	10.3%	A20G	2	2.8%	H182Q	5.1%	21.7%
	E75D	4	10.3%	L23P	2	2.8%			
	N120K	2	5.1%	D32G	2	2.8%			
	S121G	2	5.1%	A40I	2	2.8%			
	K161Q	2	5.1%	G46Q	2	2.8%			
	L181I	3	7.7%	W54L	2	2.8%			
				F61L	2	2.8%			
				S80P	2	2.8%			
				P82H	2	2.8%			
				E88R	2	2.8%			
				I91S	13	18.1%			
				R93K	2	2.8%			

				Q95E	2	2.8%				
				M113I	2	2.8%				
				D128N	2	2.8%				
				Q138H	15	20.8%				
				L153T	2	3.8%				
				G157R	2	3.8%				
				R162K	2	3.8%				
				S167G	2	4.3%				
Spacer	V4A	3	7.9%	S15P	7	11.9%	P64A	44.7%	36.3%
	S5I	6	15.8%	S18P	12	20.3%	I84T	13.2%	12.1%
	Q6K	15	39.5%	V29I	6	6.7%	Y86H	13.2%	26.4%
	A7T	23	60.5%	R34Q	11	12.2%	H93S	44.7%	45.1%
	R10W	3	7.9%	Q36L	15	16.7%	S125N	5.6%	22.9%
	Q19K	5	13.2%	H47R	19	20.9%	S129N	5.3%	12.3%
	P20S	2	5.3%	R63W	2	2.2%			
	L24P	5	13.2%	F74L	2	2.2%			
	V29T	3	7.9%	S91A	11	12.1%			
	P46S	3	7.9%	H93P	2	2.2%			
	P56S	4	10.5%	K102E	3	3.3%			
	H83R	5	13.2%	F126I	4	8.2%			
	G85D	2	5.3%	P127S	12	23.1%			
	S87I	2	5.3%	P128S	5	9.6%			
	S89N	6	15.8%	S130F	2	3.2%			
	S89T	9	23.7%	A131S	9	12.7%			
	S90K	5	13.2%	Q136E	2	2.5%			
	S91F	5	13.2%	Q136K	5	6.2%			
	S92L	3	7.9%	G137R	12	14.5%			
	L108F	3	8.1%	P138L	2	2.4%			
	V121M	2	5.4%	S141P	3	3.6%			
	F123L	2	5.6%	T150I	2	2.2%			
	S129G	2	5.4%	T150S	7	7.9%			
	S135N	2	5.4%	Q151E	5	5.6%			
	F140S	4	10.5%	K155D	6	6.7%			
	L158I	10	26.3%	S159T	4	4.5%			
	L161I	2	5.3%						
RT	E1D	5	12.5%	V7T	2	2.3%	V7A	40.0%	16.3%
	H9Q	3	7.5%	E11K	3	3.5%	L53I	35.0%	11.4%
	S105T	5	12.5%	R15K	3	3.5%	I103V	37.5%	23.5%
	S106C	3	7.5%	I16T	2	2.3%	P109S	20.0%	14.1%
	R110G	12	30.0%	A21T	2	2.3%	H122N	35.0%	2.4%
	S119C	3	7.5%	R51G	2	2.3%	W153R	7.5%	19.0%

Mutation	n	%	Mutation	n	%	Mutation	%	%
Q139H	12	30.0%	G52E	2	2.3%	K266V	27.5%	14.5%
R242K	3	7.5%	L53N	2	2.3%	K266I	65.0%	65.2%
H271P	2	5.0%	L53S	2	2.3%	N332S	39.3%	15.9%
H271S	3	7.5%	T54Y	2	2.3%	Q333K	44.4%	14.5%
H271C	13	32.5%	T54S	4	4.5%			
T322I	3	9.1%	V63A	7	8.0%			
			H122L	2	2.4%			
			N124H	19	22.4%			
			Y126H	20	23.5%			
			L129M	2	2.4%			
			N131D	16	18.8%			
			Y151F	2	2.4%			
			R167H	2	2.4%			
			V173L	23	27.4%			
			L180M	41	47.1%			
			I187A	2	2.3%			
			V190A	2	2.3%			
			R192S	2	2.8%			
			R192L	3	4.2%			
			R193V	2	2.8%			
			M204V	37	53.6%			
			V207A	2	2.9%			
			V214A	2	2.9%			
			T222A	2	2.9%			
			A223V	2	2.9%			
			T225A	2	2.9%			
			L229F	2	2.9%			
			N238T	9	13.0%			
			S246F	3	4.3%			
			M250L	4	5.8%			
			V253I	10	14.5%			
			S256G	6	8.7%			
			T259S	8	11.6%			
			Q262R	2	2.9%			
			I282V	8	11.6%			
			C287Y	2	2.9%			
			R289E	2	2.9%			
			R289K	2	2.9%			
			I290F	2	2.9%			

				Y312H	2	2.9%			
				M336 V	2	2.9%			
				Y339C	2	2.9%			
				Y339L	2	2.9%			
RNase H	V138D	3	12.50%	R1Q	2	9.5%	S2P	41.7%	61.9%
				C5M	2	9.5%	Y116F	8.3%	30.0%
				T37A	2	9.1%	R151K	8.3%	7.8%
				A38T	2	9.1%			
				K53N	2	4.1%			
				L54I	2	3.7%			
				G75S	3	4.7%			
				T77A	10	15.6%			
				G84R	2	3.1%			
				A93V	2	2.9%			
				P100L	2	2.9%			
				L107V	4	5.7%			
				Y108S	3	4.3%			
				R113S	2	2.9%			
				L114P	2	2.9%			
				P115L	5	7.1%			
				R117H	2	2.9%			
				R117C	2	2.9%			
				R117Q	6	8.6%			
				T119S	8	11.4%			
				V128D	18	25.7%			
				F142S	3	4.3%			
				V148A	24	36.9%			

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