Brief Report

Atypical Hepatitis B Virus Serology Profile – HBsAg Posi-Tive/Anti-HBc Negative – in Hbv/HIV Coinfected Individuals in Botswana

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Atypical Hepatitis B Virus Serology Profile—HBsAg Positive/Anti-HBc Negative—In HBV/HIV Coinfected Individuals in Botswana

Bonolo B. Phinius [1,2], Motswedi Anderson [1], Margaret Mokomane [2], Irene Gobe [2], Wonderful T. Choga [1,2], Tsholofelo Ratsoma [1], Basetsana Phakedi [1], Gorata Mpebe [1], Doreen Ditshwanelo [1], Rosemary Musonda [1], Joseph Makhema [1,3], Sikhulile Moyo [1,2,3,4] and Simani Gaseitsiwe [1,3,*]

[1] Botswana Harvard AIDS Institute Partnership, Gaborone, Botswana; bphinius@gmail.com, manderson@bhp.org.bw, wchoga@bhp.org.bw, tratsoma@bhp.org.bw, goratampebe@gmail.com, basetsanaphakedi@gmail.com, dditshwanelo@bhp.org.bw, rmusonda@bhp.org.bw, jmakhema@bhp.org.bw, sikhulilemoyo@gmail.com, sgaseitsiwe@gmail.com

[2] School of Allied Health Professions, Faculty of Health Sciences, University of Botswana, Gaborone, Botswana; bphinius@gmail.com, mokomanem@ub.ac.bw, gobei@ub.ac.bw, sikhulilemoyo@gmail.com

[3] Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, Massachusetts, USA; jmakhema@bhp.org.bw, sikhulilemoyo@gmail.com, sgaseitsiwe@gmail.com

[4] School of Health Systems and Public Health, University of Pretoria, Gauteng, South Africa; sikhulilemoyo@gmail.com

* Correspondence: sgaseitsiwe@gmail.com; Tel.: (+26739002671)

Abstract: (1) Background: Hepatitis B core antibodies (anti-HBc) are a marker of hepatitis B virus (HBV) exposure; hence a normal HBV serology profile is characterized by HBV surface antigen (HBsAg) and anti-HBc positivity. However atypical HBV serologies occur and we aimed to determine the prevalence of an atypical profile (HBsAg+/anti-HBc-) in a cohort of people with HIV-1 (PWH) in Botswana. (2) Methods: Plasma samples from an HIV-1 cohort in Botswana (2013-2018) were used. Samples were screened for HBsAg and anti-HBc. Next generation sequencing was done using the GridION platform. Wilcoxon rank-sum test and Chi-squared tests were used for comparison of continuous and categorical variables respectively. (3) Results: HBsAg+/anti-HBc- prevalence was 13.7% (95% CI 10.1 – 18.4) (36/263). HBsAg+/anti-HBc- participants were significantly younger (p<0.001), female (p=0.02), ART naïve (p=0.04) and had detectable HIV viral load (p=0.02). There were no mutational differences in sequences isolated from participants with HBsAg+/anti-HBc- versus those with HBsAg+/anti-HBc+ serology. (4) Conclusions: We report a high HBsAg+/anti-HBc– atypical serology profile prevalence among PWH in Botswana. We caution against HBV testing algorithms that consider only anti-HBc+ samples for HBsAg testing as they are likely to underestimate HBV prevalence. Studies to elucidate the mechanisms and implications of this profile are warranted.

Keywords: Hepatitis B surface antigen (HBsAg); hepatitis B core antibodies (anti-HBc); HBV; HIV; Botswana; Africa

1. Introduction

Approximately 296 million people were reported to be living with chronic hepatitis B (CHB) infections in 2019 [1]. Hepatitis B virus (HBV) is one of the leading causes of liver-related mortality with up to 820 000 deaths annually [1]. Worse-off clinical outcomes may be more pronounced in people with concomitant HBV/human immunodeficiency virus (HIV) than those with either mono-infection [2,3]. Botswana has an HBV prevalence ranging from 1.1% to 10.6% in different populations [3–6] with an HBV incidence of 3.6/100 person-years in people with HIV (PWH) [7]. HIV prevalence in Botswana is 20.8% and the country has a successful national HIV management program which has resulted in Botswana surpassing the UNAIDS 95-95-95 goals [8].

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The HBV genome is partially double-stranded with four overlapping open reading frames (ORFs), namely the Polymerase which codes for the reverse transcriptase enzyme, *precore/core (preC/C)* coding for the hepatitis B e antigen (HBeAg), and hepatitis B core antigen (HBcAg) respectively, *preS1/preS2/S* coding for the small, middle and large surface antigens, and *X* gene for the transcriptional trans-activator protein [9]. HBV biomarkers such as hepatitis B surface antigen (HBsAg), HBeAg, and HBV deoxyribonucleic acid (DNA) are used for diagnosis and disease monitoring. These biomarkers are produced at different levels throughout the course of an HBV infection [10] and some are detected using serological assays. Typically, HBsAg, which is a diagnostic marker can be detected 4-10 weeks after infection, and the detection of this antigen in blood within 6 months of infection is deemed an acute infection, while its persistence post 6 months is deemed a chronic infection. Anti-HBc immunoglobulin M (IgM) antibodies are detected early on because the HBcAg is a highly immunogenic [11,12]. As anti-HBc IgM levels decline, anti-HBc immunoglobulin G (IgG) predominates and normally persists for life indicating exposure to HBV infection [11]. Anti-HBc levels differ at different stages of HBV infection, normally being high during active infection [13].

Anti-HBc positivity is commonly used to select participants to screen for HBsAg in HBV surveillance studies [14,15]. Anti-HBc loss has been reported in CHB patients [16,17] thereby resulting in an atypical HBV serology profile characterized by HBsAg positive serology in the absence of anti-HBc (HBsAg+/anti-HBc-). This serology profile has also been observed during an acute HBV infection in a non-Hodgkin lymphoma patient [18]. Several studies attribute this serology profile to the immune suppression [16–18] while one case report attributes it to mutations in the core region of the HBV genome [19]. Other atypical HBV serology profiles and mechanisms behind them have been reviewed elsewhere [20]. A few studies have been undertaken to describe the HBsAg+/anti-HBc- atypical profile in sub-Saharan Africa [21], none in Botswana. We aimed to determine the prevalence of this atypical HBV serology profile (HBsAg+/anti-HBc-) and its associated factors in a large cohort of PWH in Botswana.

2. Materials and Methods

2.1. Study population

In this cross-sectional study, stored plasma samples of PWH who participated in the Botswana Combination Prevention Project (BCPP) (2013-2018) were used. The BCPP study enrolled 12,610 consenting participants with the main aim of evaluating the impact of a combination prevention package on HIV incidence [22]. A random sample of 20% of adults aged 16-64 enrolled in 15-paired communities were used. These communities were matched by size, pre-existing health services, population age structure and geographic location. Of the 12610 participants, 3596 were PWH [22]. This study was approved by the University of Botswana Institutional Review Board (UBR/RES/URB/BIO/267) and Health Research Development Division (HRDD) at the Botswana Ministry of Health (HPDME 13/18/1).

2.2. Serology screening and viral load quantification

Participant plasma samples that were previously screened for various HBV serology markers were used [6]. Briefly, plasma samples were initially screened for HBsAg (Murex Version 2, Diasorin, Dartford, UK) and total core antibodies (anti-HBc) using the Monolisa anti-HBc PLUS ELISA kit (Bio-Rad, Marnes-la-Coquette, France). Samples with positive HBsAg serology (HBsAg+) were then screened for HBeAg and anti-HBc IgM using the Monolisa HBe Ag/Ab and Monolisa anti-HBc Plus 1 Plaque (Bio-Rad, Marnes-la-Coquette, France) respectively. HBV DNA was quantified using the COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions which has a broad linear range of 20IU/mL – 1.7 x 10^8IU/mL.

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2.3. Sequencing methods

The QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) was used to extract DNA from plasma according to manufacturer's instructions with a final elution volume of 30mL (Qiagen, Hilden, Germany). A two-step PCR was performed to amplify the whole HBV genome. Briefly, master mixes were prepared for two primer pools. The first-round master mixes composed of 1.5mL of nuclease free water, 0.05mL of primer pool, 6.25mL of Q5â Hot Start High Fidelity 2x master mix (Oxford Nanopore Technologies, Oxford, UK), and 5mL of template volume. PCR conditions were an initial denaturation at 98°C for 30 seconds, 35 cycles of a denaturation step at 98°C for 15 seconds, annealing and extension steps at 65°C for 5 minutes, and finally, a hold step at 4°C for ¥. Second round PCR master mixes for each primer pool were composed of 0.5mL of the primer pool, 3.75mL of nuclease free water, 6.25mL of the Q5â Hot Start High Fidelity 2x master mix and 2.5mL of the template. First round PCR conditions were used for second round PCR. Library preparing was done as per the Oxford Nanopore PCR tiling with rapid barcoding and midnight expansion protocol (version MRT_9127_v110_revH_14Jul2021) [23], replacing SARS-CoV-2 primers for HBV primers.

The library was loaded into flow cells version R9.4.1 (Oxford Nanopore technologies, Oxford, UK). Sequencing was performed using the GridION platform (Oxford Nanopore Technologies, Oxford, UK), with base calling and demultiplexing carried out using the latest MinKNOW Release as of April 2023 in high accuracy mode. Raw FASTQ files were exported and subsequently processed using Guppy, employing dual indexed reads for base calling and demultiplexing. For reference-based assembly of HBV, we utilized the Genome Detective 1.132/1.133 [24] (https://www.genomedetective.com/, last accessed 15 April 2023).

2.4. Sequence diversity and mutational analysis

To assess for any known mutations or signatures amino acids that may be associated with immune pressure among HBV patients in Botswana, 110 (14 HBsAg/anti-HBc- and 96 HBsAg/anti-HBc+) aligned core sequences were included in the analysis. Generated HBV sequences were viewed and aligned using AliView ver. 1.26 [25]. Geno2pheno (https://hbv.geno2pheno.org) was used to determine HBV subgenotypes. Babylon Translator was utilized to extract the core ORF and the sequences were translated into amino acids [26]. Genetic diversity and any signature mutations were investigated by comparing the sequences isolated from patients with HBsAg+/anti-HBc- versus those with HBsAg+/anti-HBc+ serology.

2.5. Statistical analyses

We calculated the Wilson 95% confidence interval (95% CI) around prevalence estimates. Continuous variables were presented in medians and interquartile ranges and compared between atypical profile carriers (HBsAg+/anti-HBc-) and normal profile carriers (HBsAg+/anti-HBc+) using the Wilcoxon rank-sum test. Categorical variables were presented in proportions and the Chi-squared test was used to compare variables between atypical profile carriers and normal profile carriers. A proportion test was done to determine the association between HBV serology profile and sex, HIV suppression and ART status. All statistical analyses were done in Stata version 18.0 (StataCorp LLC, College Station, Texas, USA) and p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Atypical profile prevalence and participant demographics

A total of 263 participants were screened for both HBsAg and anti-HBc. Most participants were female 63.1% (166/263) and their median age was 42 (IQR: 35 – 50). Most of the participants had undetectable HIV viral load [VL] (< 40copies/mL) (211/262, 80.5%). Most participants were on antiretroviral therapy (ART), 229/260 (88.1%), with 92/163 (56.4%) being on tenofovir-containing regimen. Thirsty-six, 13.7% (95% CI 10.1 – 18.4) of the 263 participants were HBsAg+/anti-HBc-. There

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was an association between sex and serology profile (p= 0.02), Table 1. With a proportion test, the prevalence of this profile was significantly higher in females than males (17.5% vs 7.2%, p = 0.02). Participants with the atypical profile were significantly younger than those with the normal serology profile [35 (30 – 44) vs 43 (36 – 50), p<0.001]. The prevalence of the atypical profile was higher among ART naïve participants than ART experienced participants (25.8% vs 12.2%, p = 0.04). Furthermore, there was an association between serology profile and HIV VL (p = 0.02) Table 1, with a high prevalence observed among participants with detectable VL as opposed to those with undetectable VL (23.5% vs 11.4%, p = 0.02). There was no statistically significant difference between participants with HBsAg+/anti-HBc- and those who were HBsAg+/anti-HBc+ serology profiles in nadir CD4+ T-cell count, ART-regimen and duration on ART, Table 1.

Table 1. Participant demographics.

Characteristics	(HBsAg+/anti-HBc–) N = 36	(HBsAg+/anti-HBc+) N = 227	*p-Value*
Sex, N (%)			
Female	29 (81)	137 (60)	
Male	7 (19)	90 (40)	0.02
Age, years, median (IQR)	35 (30 – 44)	43 (36 – 50)	<0.001
Nadir CD4, cells/mm^3, n = 113, N (%)			
<200	8 (47)	34 (35)	
≥200	9 (53)	62 (65)	0.36
HIV VL			
Undetectable	24 (67)	187 (83)	
Detectable	12 (33)	39 (7)	0.02
ART status, n = 260, N (%)			
ART-naïve	8 (22)	23 (10)	
On ART	28 (78)	201 (90)	0.04
ART duration, years, n=196, median (IQR)	7.0 (3.8 – 11.3)	6.6 (4.6 – 9.5)	0.79
ART regimen, n = 163, N (%)			
TFV–containing	11 (65)	81 (56)	
3TC–containing	5 (29)	59 (50)	
Non TDF, non 3TC-containing	1 (6)	6 (4)	0.67
HBeAg, n = 231, N (%)			
Positive	2 (7)	24 (12)	
Negative	27 (93)	178 (88)	0.43
IgM, n = 244, N (%)			
Positive	3 (10)	13 (6)	
Negative	28 (90)	200 (94)	0.45
HBV VL IU/mL, n = 144, N (%)			
TND	8 (44)	29 (23)	
≤2000	6 (33)	72 (57)	
>2000	4 (22)	25 (20)	0.11

HBsAg; Hepatitis B virus surface antigen, anti-HBc; hepatitis B virus core antibodies, HIV; human immunodeficiency virus, VL; viral load, ART; antiretroviral therapy, HBeAg; hepatitis B virus e antigen, IgM; immunoglobulin M, 3TC; lamivudine, TFV; tenofovir, TND; target not detectable.

3.2. Sequencing results

Sub-genotypes identified among participants with HBsAg+/anti-HBc- serology profile were A1 [11/14 (78.6%)], E [2/14 (14.3%)] and D2 [1/14 (7.1%)]. We did not identify any mutational differences in sequences isolated from participants with HBsAg+/anti-HBc- serology versus those with HBsAg+/anti-HBc+ serology.

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4. Discussion

The prevalence of the atypical serology profile (HBsAg+/anti-HBc-) in our study was 13.7%. In a Zimbabwean cohort of PWH initiating ART, it was 4.3% [21], and much lower (1.8%) in France [16]. These results show that HBV prevalence screening algorithms that only test HBsAg among individuals with positive anti-HBc serology [14,15] may underestimate HBV prevalence. Participants with the atypical profile in our study were significantly younger and female. A previous study showed a higher anti-HBc prevalence in males versus females [27], while other studies showed higher anti-HBc prevalence in older participants than younger participants [28,29]. Anti-HBc production may therefore be inherently less in females and younger participants as observed in these studies and our study. Highly sensitive quantitative anti-HBc assays may be useful in determining anti-HBc titter levels by age and gender.

HBV subgenotypes A1, D2 and E were observed among atypical participants and these genotypes have been shown to be circulating in Botswana [4,30]. Several mechanisms have been put forth in describing the cause of this atypical serology profile. Diagnostic escape mutations (G2011A, A2092T, G2138A, C2139T, C2242T, A2320G) in the core region and immunotolerance of the core antigen have been put forth as some of the causes for this profile [19]. None of these mutations were identified in our study and we also did not identify any mutations in the core region of sequences generated from participants with and without the atypical serology profile similar to the study by Avettand-Fenoel et al (2006) [16]. Pre-exposure to HBV antigens *in utero* to infants born to HBeAg mothers has been said to cause an immunotolerance to HBeAg and HBcAg resulting in anti-HBc negativity [31]. In a case study of a 62-year-old non-Hodgkin lymphoma patient who had recently received immunosuppressive therapy, this presentation was also observed and associated with delayed antibody response due to her being immune compromised [18]. Lack of antibody production due to immunosuppression because of HIV has also been discussed [16,17,21], in our study on the other hand, participants with the atypical serology profile were no more immunocompromised than those with the normal serology profile. All our study participants were PWH and participants with atypical serology profile were mostly ART-naïve with detectable HIV VL which may be an indication of declining CD4+ T-cell count even though the difference in CD4+ T-cell count between participants with atypical serology profile and those with the normal profile was not observed in our study.

We used the Monolisa anti-HBc PLUS ELISA kit (Bio-Rad, Marnes-la-Coquette, France) with a sensitivity of 99.5% and specificity of 99.5% and did not confirm with a different kit. With a high specificity of the assay, it is unlikely that the assay falsely identified anti-HBc negative participants, however, there is room to query that. Anti-HBc levels are associated with HBV infection stage with participants in the immune clearance phase and having HBeAg negative hepatitis having higher median anti-HBc levels than those in the immune tolerance and low replicative phase [13]. We could not ascertain infection stages in our study as most participants were on ART for several years, we did not test for several other HBV markers and we also did not have liver enzyme tests results for our study participants.

5. Conclusions

We report for the first time in Botswana, participants with an atypical serology profile characterized by positive HBsAg and negative anti-HBc serology at a high prevalence of 13.7%. Participants with this profile were younger, female and ART naïve with detectable HIV VL. We caution against testing algorithms that only screen HBsAg among participants with positive anti-HBc serology as they may underestimate HBV burden therefore missing out on participants in need of care. Future studies to elucidate the mechanisms and implications of this profile are warranted.

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

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

Data Availability Statement: The data presented in this study are available on 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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Conflicts of Interest: The authors declare no conflict of interest.

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