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Molecular characterization of hepatitis B virus in blood donors in Botswana

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Abstract

Hepatitis B virus (HBV) poses a significant threat to blood transfusion safety in sub-Saharan Africa (SSA) where allogeneic blood donations are screened serologically, and more sensitive nucleic acid tests (NATs) are utilized infrequently. HBV strains circulating among blood donors in Botswana are not yet characterized. We designed a cross-sectional study to determine the HBV subgenotypes and prevalence of hepatitis B surface antigen (HBsAg) among blood donors between November 2014 and October 2015. A total of 12,575 blood donations were screened for HBsAg and 50 consecutive plasma samples were selected for genotyping from confirmed HBsAg^{+}

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Contributions

WTC wrote the first draft of the manuscript. WTC and MA collaborated in the lab work, primary data analysis. SM and PM were involved in statistical analysis. TM and BBP were involved in conducting some of the lab work and manuscript editing before submission. EZ, TKS and IK supervised the project and edited the manuscript prior to submission and data evaluation. MKK provided with samples and their demographics, and also edited the manuscript before submission. JTB developed the secondary analysis plan and performed the phylogenetics of the study. ME and RMM edited the manuscript prior to submission and complemented it with contextual data. SG developed the study, main directions of the analysis plan, overall interpretation of results and edited manuscript before submission. All co-authors read and authorized the final manuscript.

Participants' Consent and Ethical Approval

The participants' samples used in the study were residual from blood donors, obtained already de- identified and anonymous. These were a sub-population of HBV+ screened samples from National Blood Transfusion services in Botswana (NBTS). The study was approved by the University of Botswana Institute Review Board and the Health Research Development Division (HRDD) at the Botswana Ministry of Health and Wellness (HPDME 13/18/1)

Conflict of Interest: The authors declare that they have no conflict of interest.

donations. Overlapping *Pol* and complete *S (Pol/S)* open reading frames (ORFs) were sequenced from extracted HBV-DNA. To identify any signature amino acids, mutations were compared to sequences from a cohort of chronic HBV patients co-infected with HIV and were treatment naïve. The prevalence of $HBsAg^+$ blood donors was 1.02% [95% CI: 0.9% – 1.2%], and the circulating subgenotypes were A1 serotype *adw2* (36.1%), D2 serotype *ayw*2 (2.9%) and D3 serotypes *ayw* 1/2 (58.3%). Prevalence of escape mutations was 14% from HBV isolates of blood donors and 15% from isolates of HBV/HIV co-infected patients (p = 0.6926). The escape mutations sP120L, sG130R, sY134H, and sD144A were identified predominantly among HBV isolates from blood donors. These escape mutations have been associated with accelerated HBV sequelae (e.g. liver cirrhosis (LC) and hepatocellular carcinoma (HCC)), failure to detect HBsAg, inability to respond to immunoglobulin (Ig) therapy, and HBV vaccine escape. Characterizing the HBV burden, circulating subgenotypes, and clinically relevant mutations among blood donors in Botswana is important to elucidate the efficacy of currently available vaccines, predicting HBV-transmission patterns, understanding the cohort's risk to HBV related complications, and to developing prevention strategies and effective genotype-based antiretroviral therapies.

Keywords

HBV; blood donors; Botswana; subgenotypes; mutations

Introduction

Hepatitis B virus (HBV) infection is a significant global health problem with approximately 2 billion people with positive hepatitis B core antibody [$anti\text{-}HBc^+$] confirming exposure at some point in their lives. One eighth (257 million) of these have a chronic infection, and only a fraction is aware of their serostatus [1]. With an HBV prevalence of ≥ 8%, sub-Saharan Africa (SSA) is considered a region of high HBV endemicity [2–4]. HBV is transmitted through contact with infected blood or body fluids, including horizontal transfer (e.g. from sexual intercourse) and vertical transfer (e.g. from mother to child). Preventative strategies for HBV infection include testing of blood for active HBV infection and vaccination against HBV. Despite stringent guidelines on blood safety to exclude potential donors at high risk of transfusion-transmitted infection (TTIs), post transfusion-transmitted HBV infection is a major concern in resource-limited countries. In SSA, the median risk of being infected with HBV from a blood transfusion has been estimated at 4.3 infections per 1,000 units, and 28,595 HBV infections per year are estimated to occur [5–7]. The transmission risk of HBV remains even after HBV NAT has been performed on each donated blood unit [8].

Botswana is one of the 46 African countries that serologically tests all blood donations for the 4 mandatory TTIs [human immunodeficiency virus (HIV), HBV, hepatitis C virus (HCV) and syphilis [9]. In the 2000/2004 and 2010/2011 reports by the Centers for Disease Control and Prevention (CDC), the HBV prevalence among blood donors in Botswana was 4.21% and 2.21%, respectively [10]. Therefore, it is crucial to conduct routine epidemiological surveillance to obtain current data on HBV to help policy-makers improve HBV elimination strategies towards 2030 HBV elimination goal.

HBV is a circular DNA virus belonging to the genus *Orthohepadnavirus* of the family *Hepadnaviridae*. It has four partial overlapping open reading frames (ORFs) – P, C, X and S – that encode for 7 proteins, including polymerase, precore, core, X protein, and 3 (L, M, and S) surface proteins [11]. The HBV surface antigen (HBsAg) (226 aa) includes the '*a*' determinant region (aa 124 – 147) within the major hydrophilic region (MHR) which is targeted by antibody-based assays to screen for chronic HBV and elicits antibodies which confer protection against HBV infection.

Major mutations within the S region result in viral escape from vaccination, immunotherapy and diagnosis. Since the P ORF overlaps the S ORF, variability within P can affect the **aa** sequence of the HBsAg and vice versa. Major mutations in the polymerase may lead to drug resistance [12,13]. HBV consists of multiple genotypes (A-J) with genetic variability that differ by > 8%. Some genotypes can be further divided into subgenotypes that differ by >4% [13,14]. Genotypes frequently occur in specific geographic locations and represent an important determinant of treatment response and disease progression [11]. Despite the high endemicity of HBV infection in Botswana, few studies have reported the burden of HBV among HIV co-infected patients [15–17]; however there are no data on distribution of circulating sub-genotypes of the virus in HBV mono-infected including blood donors. Moreover, there is still a gap in understanding the mutation profiles of HBV isolates of HBV mono-infected compared to HBV/HIV co-infected patients. This study sought to determine the burden of HBsAg positivity among blood donors in Botswana and to identify circulating HBV subgenotypes and clinically relevant mutations.

Methods and Materials

Study Design and Study Population

This is a cross-sectional prospective study utilizing 12,575 specimens from blood donors collected by Botswana National Blood Transfusion Service (NBTS) in Gaborone, between November 2014 and October 2015 as part of the routine blood donation screening. A subset of 50 anonymous first-time blood donors were consecutively ELISA-screened using Murex HBsAg version 3 kit [Murex Biotech, Dartford, UK] and further confirmed using Monolisa HBsAg ULTRA [Bio-Rad, France]. ELISA tests were performed on individual (rather than pooled) blood donations. The $HBsAg^{+}$ confirmed samples were evaluated for HBV subgenotypes and clinically relevant mutations. Demographic data including age and gender were obtained. Additionally, data on HIV, HCV and syphilis seropositive samples were available for investigation. The study was approved by the University of Botswana Institute Review Board and the Health Research Development Division (HRDD) at the Botswana Ministry of Health and Wellness.

Polymerase Chain Reaction (PCR) and Sequencing

Total nucleic acid was extracted from 1000 μl of subjects' sera using the Qiagen UltraSense kit (QIAGEN, Hilden, Germany). An elution volume of 50 μl was used and extracts were stored at −80ºC until use. For HBV DNA amplification, the master mix was prepared from the Superscript III Platinum One-Step kit (Invitrogen, USA) with a modification of the number of cycles to 35 and template volume of 10 μl per each reaction. We amplified a 2100

base pair fragment covering nucleotides 2400 – 1150 according to the numbering of the *Eco*RI that included the full *S* gene overlapping with part of *Pol* using HBV primers Core-F and Werle-AS as previously described [15]. The PCR conditions included preheating at 95°C for 2 minutes, denaturing at 95°C for 30 seconds, annealing at 62.5°C for 30 seconds, and extension at 72°C for 4 minutes. Amplicons were confirmed on 1% agarose gel and purified using QIAquick (Qiagen, Hilden, Germany). The BigDye Terminator v3.0 kit (Applied Biosystems; Foster City, CA, USA) was used for sequencing. Six overlapping primers were used to prepare separate master-mixes for PCR. The thermocycling conditions were denaturing at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and final extension at 60°C for 4 minutes for 25 cycles. Direct sequencing was performed using the automated Sequencer (ABI PRISM 3130xl; Applied Biosystems).

Phylogenetic Analysis

Electropherograms were manually edited using Sequencher v5.0 software (Gene Codes Corp., Ann Arbor, MI, USA) [18]. Alignments were performed using Clustal X v.2.1 [19] and representative references of genotypes A – H retrieved from GenBank. Additional phylogenetic inference was performed using the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the Bayesian Evolutionary Analysis with the Sampling Trees software (BEAST v1.8.4) [20] with an uncorrelated log-normal relaxed molecular clock, General Time Reversible (GTR) substitution model, and gamma site heterogeneity. The MCMC was set at a chain length of 100,000,000 with parameters logged every 10,000. The tree was visualized in FigTree v1.4.3 after a 10% burn-in using Tree Annotator v1.8.4. Posterior probabilities 0.90 and above were noted as statistically significant. The Stanford Drug Resistant Database, Geno2Pheno available at http://hbv.geno2pheno.org/, and the small genome tools available at http://hvdr.bioinf.wits.ac.za/SmallGenomeTools/ were used to confirm the coverage of amplified (*Pol*, *PreS1*,*PreS2* and *S*) genes, the quality of sequences (QA), genotypes and sub-genotypes, serotypes, drug resistance and escape mutations, and translated ORFs, respectively. The sequences from this study of blood donors are available in GenBank under accession numbers MF979142 – MF979176.

Recombination and Serotype Analysis

All sequences in the study were assessed for recombination using the jumping profile Hidden Markov Model (jpHMM) tool available at http://jphmm.gobics.de/submission_hbv [21] and HBV serotypes were predicted based on the *S* gene **aa** positions: Lys/Arg→122, Arg/Lys→160, 127→Pro/Thr/Ile-Leu, Ala→159 or not Ala, and Ser →140 or not Ser using the web-based tool [22] available at http://hvdr.bioinf.wits.ac.za/serotyper/.

Mutation analysis and signature amino acids

For mutation analysis, aligned sequences were compared per genotype and ORF; *PreS1* genotype A versus D, *PreS2* (A versus D), *S* (A versus A) and *Pol* (A versus D) respectively. Comparisons were done at **aa** level in order to remove any synonymous mutations that have no effect on the HBV phenotype. Profiles and frequency of significant (non-synonymous) mutations obtained in the study were compared to those of HBV isolates from chronic HBV patients co-infected with HIV (HBV/HIV) and were treatment naïve. The cohort of

HBV/HIV co-infected treatment naïve patients has been previously reported in Botswana [15]. The comparison was done to determine genetic diversity and any signature mutations of HBV isolates that might be found in chronic HBV/HIV co-infection patients versus HBV mono-infection. Signature mutations associated with HIV/HBV co-infection were assessed using the viral epidemiology signature pattern analysis (VESPA) tool [23].

Statistical Analysis

Stata version 14 (Stata Corp, College Station, TX, USA) was used for statistical analysis. The non-parametric Wilcoxon's test was used to analyze different mutation frequencies in the *Pol/S* regions. Fischer's exact test was used for categorical data.

Results

Patient characteristics

During the period of November 2014 and October 2015, 128 of 12,575 (**1.02%**) (95% CI: 0.9% – 1.2%) blood units donated to the blood bank were $HBsAg^+$ and 50 (39.1%) specimens from the $HBsAg^+$ were selected randomly for additional analysis. The majority of blood donors were males (67.7%), and the median age was 32 ($Q_{1;}$ Q_3: 28; 35) years. Males were significantly older ($p < 0.01$) than females 28 (Q1, Q3: 26, 37) years (Fig 1).

Prevalence of HBsAg among blood donors and other TTIs

Relative to other TTIs which had the prevalence of 2.06 % (258/12,548; 95% CI: 1.81% – 2.32%) for syphilis; 1.58% (201/12,719; 95% CI: 1.37% – 1.81%) for HIV and 0.41% (52/15,744; (95% CI: 0.30% – 0.53%) for HCV, HBV was ranked the second lowest at (1.02%).

Genotypes and recombination

HBV DNA could be amplified from 36 of the 50 (72%) $HBsAg^+$ samples. Phylogenetic tree constructed showed that Botswana HBV sequences clustered with either genotype A or D regional reference sequences as shown in Fig 2. A total of 13 of 36 (36.1%) HBV sequences from blood donors belonged to subgenotype A1, 21 (58.3%) were sub-genotype D3, and 1 (2.9%) was sub-genotype D2. Sequence WC25 was excluded from the analysis as it was shorter than other sequences. The sequence WC5 was confirmed as a recombinant using online database but on the phylogenetic tree it clustered with high support to other genotype A sequences. This is because sequences used to construct the phylogenetic tree were trimmed to same size of 1650 base pairs, and that resulted in deletion of large fragment corresponding to genotype D. The recombination of WC5 was A1/D3 and had break-even point at *nt* 531.5 ±11.5 *Eco*RI shown in Fig 3.

The distribution of genotypes between HBV sequences from HIV/HBV co-infected group and blood donors was comparable. HBV subgenotype D3 was identified in majority of HBV sequences from blood donors 21(60%), whereas sub-genotype A1 was identified in 56 of 81 (69.1%) ($p < 0.05$) in the HIV/HBV co-infected group. All HBV genotype A1 sequences were serotype *adw2*. Apart from WC19, which was serotype HBV-*ayw*1, all HBV genotype D3 sequences were serotype *ayw*2.

Functional mutations and genetic variability

The comparison for percentage proportions for genetic diversity, and total **aa** differences stratified by subgenotypes and protein of HBV sequences from HBV/HIV co-infected patients and HBV mono-infected blood donors are shown in Table I. We compared the mutations in S gene between sequences obtained from HBV/HIV co-infected patients [15] and blood donors. Twelve mutations including diagnostic escape mutations (sY100C, sR122K, sT123A, sC124R, sM133T), vaccine escape mutations (sT126N, sQ129R, sM133L, sF134V) and immunoglobulin therapy failure (sG119R, sG130N, sT140S) were exclusive to isolates obtained from HBV/HIV patients versus n = 4 including sP120L, sG130R, sY134H, and sD144A) (Fig 4) obtained from some isolates from blood donors. Three of 5 (60%) escape mutations isolated from sequences from blood donors resided within the 'a' determinant's first loop (**aa** 124–137), 1 (20%) within the second loop (**aa** 138–147), and P120L was present in the mini loop outside the range (**aa** 124 −147).

Similarly, 58.3% of mutations identified in the sequences from HBV/HIV co-infected group were within the first loop. Except for the substitution at position 130 (G → R) in HBV sequences from blood donors and from (G → N) in HBV/HIV co-infected individuals, there were no mutations at **aa** level that distinguished the two groups. There were no drug resistance mutations identified in either groups.

Discussion

This is the first study to characterize subgenotypes and mutations in *Pol/S* genes among HBV mono-infected blood donors in Botswana. Our study showed that the prevalence of $HBsAg^{+}$ among blood donors in Botswana decreased to 1.02% in 2014 – 2015 from 2.21% in 2010 – 2011 [24]. This decrease is expected and results from many factors including the implementation by the NBTS of stringent interventions recommended by WHO to exclude high risk potential blood donors and HBV/HIV treatment programs. Similar trends have been observed in neighboring countries [25,26]. However, these data may not be applicable to the general population as they excluded persons vaccinated at birth who were less than 16 years during the time of study and high-risk populations who could not donate blood [27]. HBV subgenotypes have been associated with varied clinical outcomes and treatment efficacy [28,29,11]. Most HBV genotyping studies have been done among HIV/HBV co-infected cohorts and have shown that A, D, and E circulate in southern Africa, with genotype A being the most common and genotype E the least common [30,31,17,16,15].

This study observed that sub-genotypes A1, D2, and D3 circulate among blood donors in Botswana in agreement with other studies conducted in the country among non-blood donor cohorts [17,16,15]. To our knowledge, this is the first description of D2 and recombinant A1/D3 in our setting. Distribution of genotype D2 has been reviewed and shown to circulate in Northern Africa (Morocco, Tunisia), Europe, Asia, and the Middle East [32,33,11,34]. It is not surprising to observe this degree of variability in genetic epidemiology in Botswana since the blood donor cohort includes diverse ethnic groups. Furthermore, this pilot study shows the current HBV burden in Botswana might be underrepresented as no study has reported HBV molecular epidemiology among HBV mono-infected including blood donors. This information may enlighten decisions on choice of regimens to be used in HBV risk

groups in Botswana since HBV genotypes influence treatment responses, and this information may inspire new interventions to screen for HBV especially among blood donors. A proportion of samples could not be amplified, likely due to low viral loads [<20 IU/ml] as previously seen in previous studies [35]. In this study, we had limited sample volumes available for HBV DNA quantification and analysis of other genomic regions. The presence of immune escape variants is of high clinical significance, as these mutants may facilitate HBV reactivation even in anti-HBs^{+} patients, transmission despite proper active/passive vaccination strategies, and missed diagnosis when commercial HBsAg diagnostic kits fail to detect the escape variants[36]. sD144A was the predominant mutation among genotype D sequences isolated from blood donors. Previous studies found that this mutation was associated with impaired HBsAg antigenicity, immune escape, (thereby decreasing the sensitivity of commercial HBsAg immunoassays), and escape from vaccine and immunoglobulin therapy. [37]. Although it was found exclusively among HBV isolates from blood donors, the change from Asp to Glu at position s144 (D144E) has been described also in HBV isolates from HBV/HIV infected patients [38]. Additionally, WC33 had the dual secondary escape mutations sY134H and sD144A associated with HBV reactivation. Together with sD144A they reside in the immunogenic segment (**aa** 139 −149) and have been extensively discussed by Verheyen *et al* in correlation with reactivation [39,38]. sY134H causes a secondary co-variation with rtV142A in RT domain and is associated with failure to respond to immunoglobulin therapy in addition to HBV re-activation [40,39]. sP120L mutation in genotype D sequences has been functionally characterized and linked with occult HBV (OBI) [41]. However, in the current study, sP120L was observed in HBV mono-infected as well. Furthermore, mutations at position 120 of the S (P120) have also been reported in several studies of HBV isolates from blood donors and other chronic HBV patients of different risk groups [42,43]. The impact of different escape mutations found in the isolates from the HBV/HIV co-infected group have been discussed [15]. Although several studies have confirmed that there is no association between HBV infection and HIV status [44,17], our findings suggest that the genetic diversity may differ between HBV isolates from these two groups. Except for the **aa** substitutions at position 130 in the *S* gene that overlapped in 2 groups, the sample size and clinical information required to establish statistical inferences of any signature mutations that may be associated with multi-virulence (HIV/HBV) compared to mono-infection were limited. The escape mutations between variants of subgenotype A1 and D3 were not statistically significant across the *S* region for both cohorts (Table I).

This pilot study focused on the volunteer blood donors and gives a more representative spectrum of the immune-pathological pattern of HBV infection in the general population than patients' samples that would bias the results toward a specific variant of the disease e.g. HIV/HBV co-infected [45]. In conclusion, this study shows evolutionary diversity between different HBV cohorts and highlights the need to establish mutations that correlate with HBV/HIV co-infection as opposed to HBV mono-infection. Additionally, HBV is still a significant burden in Botswana among other TTIs and the HBV molecular epidemiology might be more diverse in the general population than currently reported. Currently, Botswana is still using only serological assays to screen allogeneic blood donations. Use of NATs in addition to serological screening should be introduced at NBTS and utilized as the

standard screening technique to prevent TTIs transmission through blood transfusion services. As well, routine epidemiological studies of HBV among different cohorts should be conducted to provide more robust estimates of HBV prevalence.

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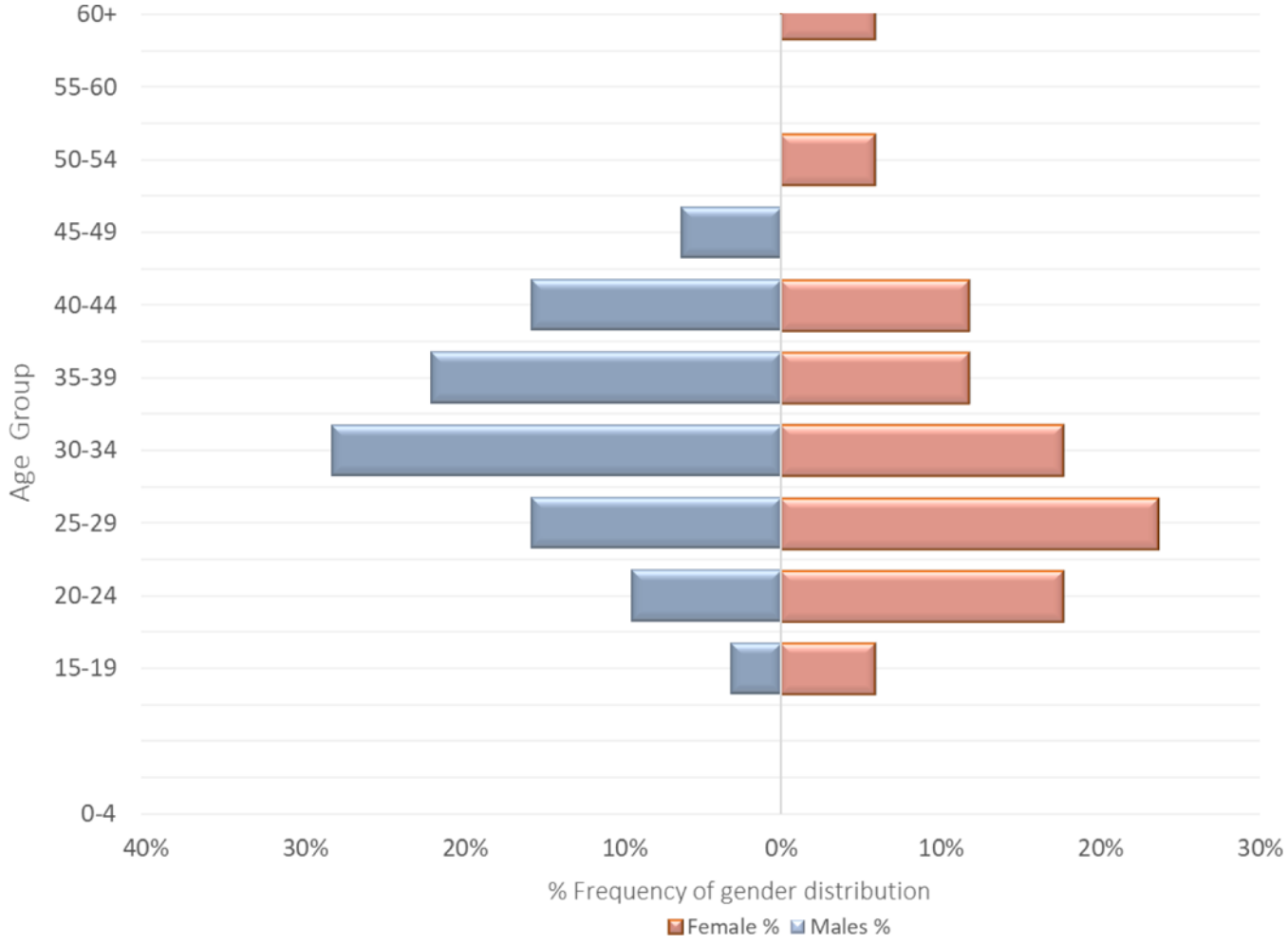

Fig 1.

Age-sex index graph showing frequency distributions for the study participants. The male sex-age index is normally distributed and age range 30–34 years has highest HBV burden

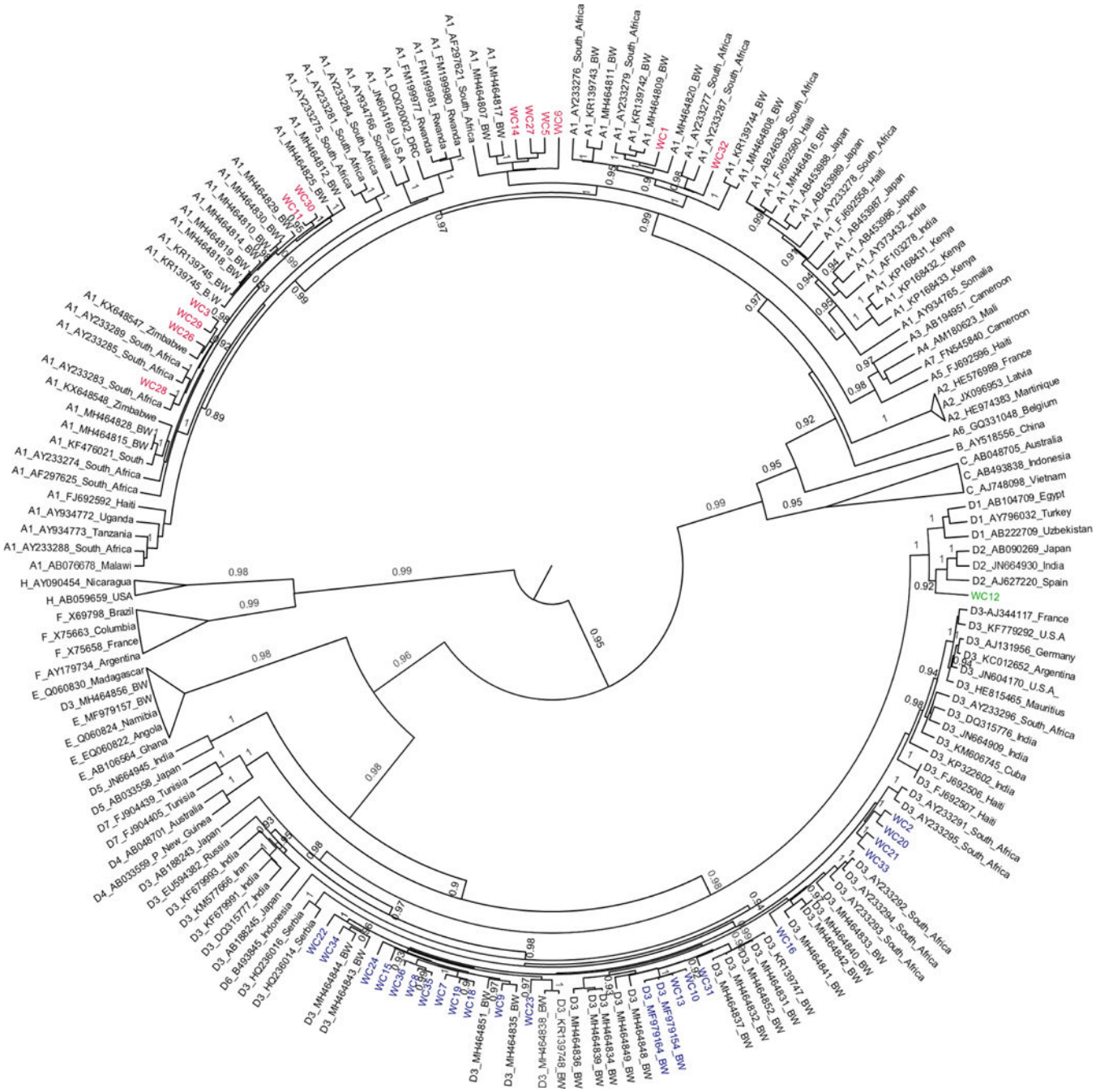

Figure 2.

Phylogenetic Tree of HBV isolates sequences from blood donors supported with posterior probabilities > 0.90 shown. The HBV sequences from this study are indicted by “WC” (red for genotype A and blue for genotype D), while references are indicated by sub-genotype-accession number-country.

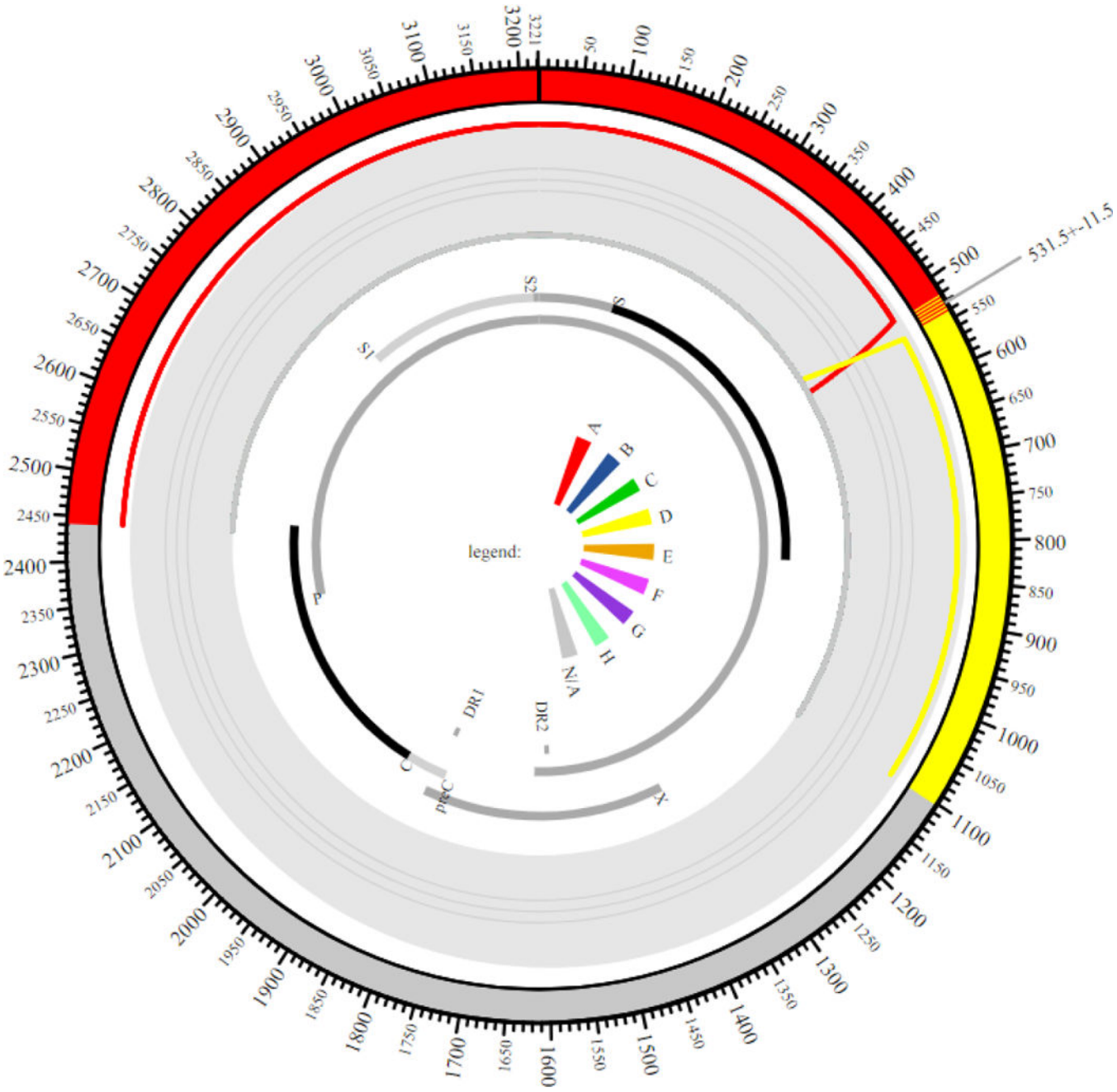

Fig 3.

Circular representation of WC5 showing a recombinant sequence, A1/D3, and a break-even point at position *nt* 531.5 ±11.5 *Eco*RI in the *S* protein. The graph was constructed using online data base jumping profile Hidden Markov Model (jpHMM).

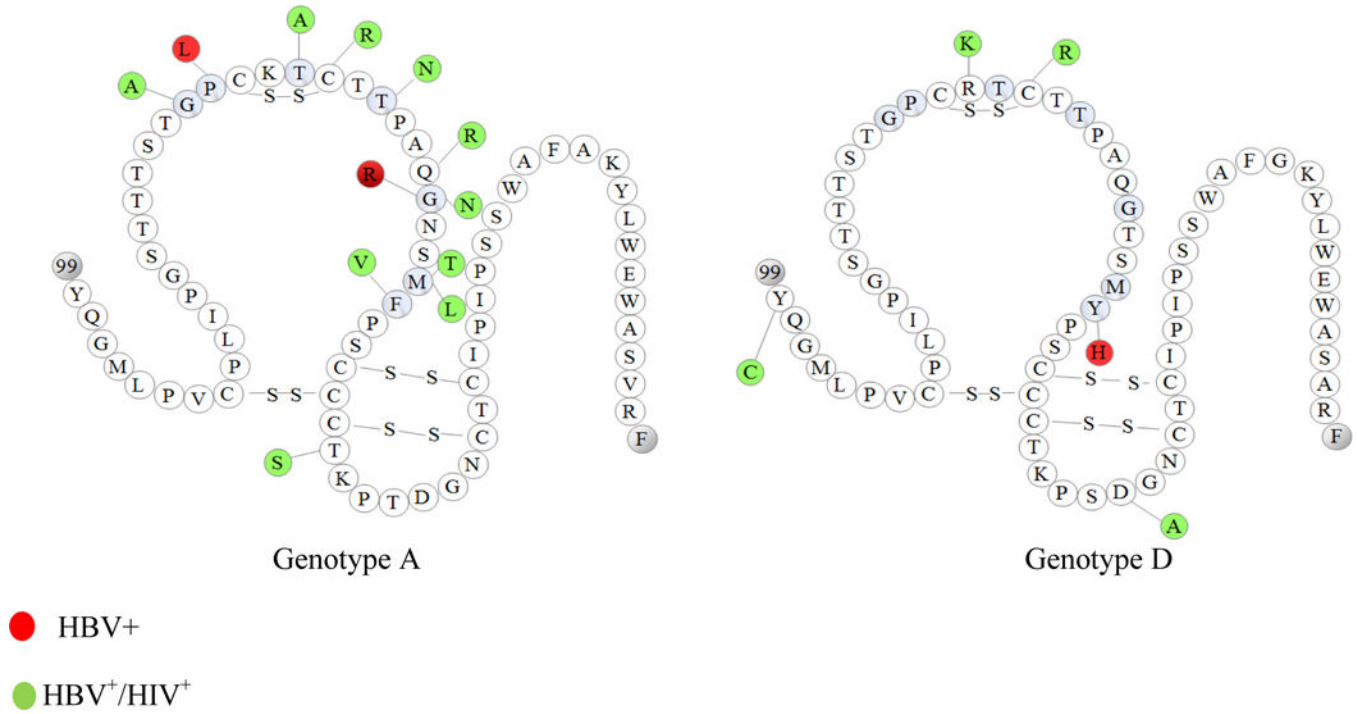

Fig 4.

Showing consensus sequences for HBsAg per genotype; (a) Left: genotype A and (b) Right: genotype D. Escape mutations indicated for the two groups; (HIV/HBV) co- infected significant variants are shown in green and for (HBV) mono-infected variants are shown in red

Table I

Evolutionary differences within the surface proteins of sequences from HBV mono-infected versus HBV/HIV co-infected

ORF	Genotype A (n = 12) D (n = 23)	Genetic Variability[¥] (mean ± SE)	*p-Value*	Distribution of Escape Mutations
PreS1	**A**	0.12 ± 0.04	0.29	None DRM
	D	0.03 ± 0.02		
PreS2	**A**	0.09 ± 0.04	0.54	
	D	0.04 ± 0.02		
S	**A**	0.02 ± 0.02	0.67	
	D	0.02 ± 0.02		
Pol	**A**	0.05 ± 0.03	0.77	
	D	0.03 ± 0.02		
HBV/HIV coinfected[≠] A (n = 54) D (n = 14)	**A**	0.013 ± 0.01	0.87	G119R, T123A, C124R, T126N, Q129R, G130N, M133L/T, F134V, T140S
	D	0.018 ± 0.01		Y100C, R122K
HBV monoinfected[≠] A (n = 12) D (n = 23)	**A**	0.019± 0.01	0.97	P120, G130R
	D	0.02 ± 0.02		Y134H, 2*D144A

[≠] the sequences used for this comparison were 415bps covering the surface region. No mutations that confer resistance to known HBV antiviral drugs were observed: None drug resistance mutations (DRM) where observed in all sequences analysed.

[¥] Represents mean **aa** genetic variations observed in HBV sequences.