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Genotyping of Occult Hepatitis B Virus in Sudanese Blood Donors by RFLP in the Pre-S Region: Identification of Novel Patterns D-Del 1, D-Del 2, and E-Del

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Abstract

Background: Hepatitis B virus (HBV) infection remains a significant public health concern in developing countries. HBV genotypes influence viral evolution and genetic diversity. This study aimed to genotype occult HBV strains among Sudanese blood donors using RFLP analysis of the Pre-S region. **Methodology:** This study was conducted for RFLP Genotyping of occult HBV by analysis of patterns obtained after amplifying a fragment in the Pre-S region and digestion of the amplicon by AvaII and DpnII. **Results:** Out of a total of 44 HBV DNA-positive samples from HBsAg-negative donors with occult hepatitis B, one sample failed to amplify during PCR, likely due to DNA degradation or low viral load resulting from storage or suboptimal sample quality. The remaining 43 samples were successfully genotyped by RFLP after amplification of the pre-S region and digestion of the amplicon by AvaII and DpnII and showed genotype D was the most prevalent (51.16%; 95% CI: 36.5 - 65.5%), comprising subtypes D1, D2, D3, and two novel deletion patterns, D-Del 1 and D-Del 2. Genotype E accounted for (44.19%; 95% CI: 30.2 - 59.1%), including E1, E2, E3, and a novel deletion pattern, E-Del. Genotype A1 was detected at low frequency (4.65%; 95% CI: 0.6 - 15.5%). Pre-S deletion mutants were found in genotypes D and E. D-Del 1, D-Del 2, and E-Del were never reported and showed unique truncated patterns and expanding the understanding of HBV genetic variability in Sudan. **Conclusion:** The integration of molecular techniques such as PCR and RFLP techniques effectively detected occult HBV genotypes and revealed novel Pre-S deletion variants. These find-

ings enhance knowledge of HBV diversity and may inform future molecular surveillance in blood donors.

Keywords

Molecular Detection, Occult HBV, Blood Donors, Sudan

1. Introduction

Sudan is an African country with a high Hepatitis B virus (HBV) seroprevalence of greater than 8% HBsAg-positivity, ranging from 6.8% in central Sudan to 26% in southern Sudan. HBV infection is the most common cause of chronic hepatitis disease with a high risk of developing cirrhosis and hepatocellular carcinoma (HCC) [1].

The clinical manifestations of HBV infection vary in both acute and chronic diseases. During the acute infection, patients can have subclinical or anicteric hepatitis, icteric hepatitis, or, less commonly fulminant hepatitis. In chronic infection, patients can have an asymptomatic carrier state, chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Initial symptoms are nonspecific and may include anorexia, nausea, vomiting, abdominal pain, and jaundice. In cases of severe liver damage, patients can develop jaundice, hepatic encephalopathy, ascites, gastrointestinal bleeding secondary to esophageal varices, coagulopathy, or infections [2]. Compared to other conventional DNA viruses, HBV is characterized by the complexity of its replication and a high degree of genetic variability, which gives rise to the well-recognized subtypes and genotypes of the virus. HBV genotypes have distinct geographical distributions [3]. The HBV genome DNA is a relaxed-circular DNA (rcDNA) of approximately 3.2 kb in length with a complete minus strand and an incomplete plus strand. The viral genome encodes four overlapping open reading frames (ORFs), C, P, S, and X, from which functional viral proteins are produced [4]. The presence of replication-competent HBV genome in the blood and/or liver of individuals who test negative for HBsAg by the currently available testing methods is called Occult Hepatitis B Infection (OBI) [4]. OBI is a serious public health concern. Its clinical significance stems from the possibility of its transmission and reactivation, in addition to its potential contribution to the development of progressive liver disease and HCC. Sudan was found to have a high prevalence of OBI among high-risk groups, specifically blood donors, hemodialysis patients, renal transplant recipients, HIV patients, and febrile patients [5].

Defining the epidemiology of OBI can be difficult because it relies on the sensitivity of HBsAg and HBV DNA assays. OBI is the potential risk of HBV transmission through blood transfusion, and organ transplantation, as well as from occult infected mothers to newborns [6]. In low- and middle-income countries where anti-HBc and/or NAT tests have not been implemented, HBV transmission from OBI blood donors remains a major health issue [7]. Reasons leading to OBI

include genomic regulatory regions mutations that may negatively affect viral replication and incomplete control of HBV under the host immune system [8].

Genotypes A and D occur frequently in Africa and Europe [9], while genotypes B and C are prevalent in Asia [10]. Genotype E is almost entirely restricted to Africa, and F is found preferentially in Central and South America [11]. Genotype G was reported in France and the United States [12]. Recently, the eighth genotype H has been described in Central America [13]. HBV preS/S-gene mutation is one of the major causative factors for OBI. HBV envelope protein is encoded by the preS/S gene, which includes the preS1, preS2 and S genes. Promoter SPI [nucleotide (nt) 2219 - 2780] regulates the transcription of a 2.4-kb mRNA and encodes the large (L) protein. Promoter SPII (nt 2809 - 3152) regulates the transcription of a 2.1-kb mRNA and encodes the middle (M) and small (S) proteins. The main protein includes glycosylated GP27 and non-glycosylated P24. The region of amino acids (aa) 99 - 169 is termed the major hydrophilic region (MHR), and it contains the major conformational epitope exposed on the external surface of the viral particle [14]. MHR N-glycosylation mutations may influence viral characteristics [15]. There is a relatively conserved region (aa 124 - 147) within the MHR called the "a" determinant, which is the target of neutralizing B cell responses [16].

The present study aims to investigate the genotypes of occult Hepatitis B virus (HBV) using RFLP genotyping by analyzing amplification patterns in the Pre-S region and assessing the impact of these genotypes on the clinical presentation and treatment response in Sudan.

2. Materials and Methods

2.1. Inclusion Criteria and Serological Background

Prior to donation, all participants underwent a standard pre-donation clinical evaluation, including hemoglobin measurement, blood pressure check, body weight recording, and a general physical examination to ensure they met the national criteria for safe blood donation.

The age range of the donors included in this study was 25 to 55 years. Due to logistical and resource constraints, additional serological markers such as anti-HBc and anti-HBs were not routinely tested. As such, occult hepatitis B infection (OBI) in this study was defined based solely on the detection of HBV DNA in individuals who were negative for HBsAg by ELISA.

Inclusion criteria:

- HBsAg-negative blood donors as confirmed by ELISA.
- Adults aged 25 - 55 years.
- Eligible for blood donation per national guidelines.

Exclusion criteria:

- Individuals with known liver disease or hepatitis history.
- HBsAg-positive donors by ELISA.
- Donors who declined to participate or withdrew consent.
- Hemolyzed or insufficient samples.

2.2. Ethical Consideration

The study received ethical approval from the Ministry of Health Research Department in Port Sudan (Approval dated: 23 September 2023), the Port Sudan Central Blood Bank, and the Research Board at the Faculty of Medical Laboratory Sciences, Karary University. Participants were fully informed of the study purpose, and informed consent was obtained. Participation was voluntary, with the right to withdraw at any stage without consequences. Data confidentiality was ensured using coded questionnaires. Remaining samples were not reused for other studies.

2.3. Sample Size Calculation

The required sample size for estimating the prevalence of occult hepatitis B virus (HBV) infection among HBsAg-negative blood donors was calculated using the single proportion formula:

$$n = (z)2p(1-p)/d2$$

where:

- n = required sample size.
- Z = 1.96 (Z value for 95% confidence level).
- P = estimated prevalence of occult HBV infection (15.51% or 0.1551) based on previous study.
- d = margin of error (precision), set at 5% (0.05).

By substituting the values:

$$n =^2 (1.96)\times 0.1551\times(1-0.1551)/^2(0.05) = \approx 201$$

To account for potential sample loss or PCR failure, the sample size was increased to 250 blood donor samples.

2.4. DNA Extraction

DNA was extracted from the serum of HBV DNA-positive, HBsAg-negative patients according to the standard inorganic protocol for HBV DNA isolation described by Changotra and Sehajpal (2013) with some modifications. 50 µl of serum was mixed with 300 µl of 1X Tris-EDTA (TE) buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), 4 µl of 100 mg/ml proteinase K (Promega, USA), and 12 µl of 20% Sodium dodecyl sulfate (SDS) solution (Merck, USA). The mixture was vortexed and incubated at 56˚C for 2 hours in a ThermoCell Mixing Block (Bioer, China). The solution was cooled to room temperature, followed by the addition of 185 µl of 7.5 M ammonium acetate (Merck, USA). For protein precipitation, the mixture was vortexed and centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a fresh 2 ml tube and mixed with 1.5 µl of 20 mg/ml glycogen (Thermo Fisher Scientific, USA). Subsequently, the mixture was vortexed, and 1.4 ml of cold absolute ethanol was added. The samples were kept overnight at −20˚C. The tubes were centrifuged at 12,000 rpm for 30 minutes at 4˚C, and the superna-

tants were discarded. After that, 2 ml of cold 70% ethanol was added to each tube, and the tubes were vortexed briefly to wash the DNA pellet. Then, the tubes were centrifuged at 12,000 rpm for 15 minutes at 4˚C, and the supernatants were discarded. Finally, the pellet was air-dried and dissolved in 20 µl of TE buffer and stored at −20˚C for further use [17].

2.5. Polymerase Chain Reaction (PCR)

The target pre-S region was amplified using primers P1 (5'-TCACCATATTCTTGGGAACAAGA-3') and P2 (5'-TTCCTGAACTGGAGCCACCA-3'), as previously described by Lindh *et al.* (1998). PCR was performed in a 50 µl reaction volume, containing 25 µl of DreamTaq PCR 2X Master Mix (Thermo Fisher Scientific, USA), 5 µl of P1 primer (5 pmol/µl), 5 µl of P2 primer (5 pmol/µl), and 5 µl of H2O. Amplification was carried out in a thermocycler, programmed as follows: an initial denaturation step at 94˚C for 3 min; 35 cycles of amplification, including denaturation at 94˚C for 45 s, annealing at 53˚C for 1 min, and extension at 72˚C for 1 min; and a final extension cycle at 72˚C for 10 min [17].

2.6. Agarose Gel Electrophoresis

2% agarose gel was prepared by adding 2 g of agarose (Merck, USA) to 100 ml of 1X Tris- borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA; pH 8.3) in a 250 ml flask. The flask was heated in a microwave oven until the agarose particles were completely melted. The flask was left to cool until the agarose solution temperature reached 55˚C, and then 2 µl of ethidium bromide (10 mg/ml) was added. The gel solution was mixed thoroughly. The appropriate gel comb was inserted into the gel tray, and the warm agarose solution was poured into the tray. The gel was allowed to solidify completely (30 minutes at room temperature). The gel comb was carefully removed. The gel was placed into the electrophoresis tank containing sufficient 1X TBE electrophoresis buffer to cover the gel. The sample mixture was loaded into the gel wells using a micropipette. The lid of the gel tank was closed, and it was attached to the electric power source. Gel electrophoresis was performed at 100 V and 60 mA for approximately 45 min. When the dyes had migrated a sufficient distance through the gel, the electric current was turned off, and the lid of the gel tank was removed. The gels were examined under UV light and photographed using the Gel Doc XR + Gel Documentation System (Bio-Rad, USA) [18].

2.7. HBV Genotyping by Restriction Fragment Length Polymorphism (RFLP)

The amplified PCR product of the pre-S region was digested separately with AvaII and DpnII restriction enzymes (New England Biolabs, USA). The digestion reaction was performed in a 50 µl reaction volume containing 20 µl of PCR product, 1 µl (10 units) of AvaII or DpnII enzyme, 5 µl of 10X buffer (10X rCutSmart Buffer for AvaII or 10X NEBuffer for DpnII) and 24 µl of water. The reactions were in-

cubated at 37˚C for 2 h. 18 ul of the digestion reaction were mixed with 2 µl of the 10X loading dye, and the sample mixture was loaded into the gel wells using a micropipette. The digestion products were separated by electrophoresis in a 3% agarose gel in 1X TBE buffer stained with ethidium bromide, as stated above. The gel was visualized with a UV transilluminator and photographed using a Gel Doc XR + Gel Documentation System [19].

3. Results

This study included 250 voluntary blood donors who tested negative for hepatitis B surface antigen (HBsAg) using enzyme-linked immunosorbent assay (ELISA), which is the standard method for routine blood screening in Sudan. The study was conducted at the Port Sudan Central Blood Bank during the period from January 2022 to March 2025.

Table 1. Demographic characteristics of the study population (n = 250).

Variable	Category	Frequency	Percentage
Age group (years)	25 - 34	67	26.8%
	35 - 44	139	55.6%
	45 - 55	44	17.6%
Sex	Male	250	100.0%
	Female	0	0.0%
Education Level	Primary education	58	23.2%
	Secondary education	65	26.0%
	University student or higher	127	50.8%
Occupation	Informal sector (e.g., market)	117	46.8%
	Unemployed	80	32.0%
	Government/Office work	53	21.2%

The viral genome was extracted from the serum of 44 HBV-positive patients using the standard inorganic protocol for HBV DNA isolation. The target pre-S regions were amplified by PCR using specific primers for this region, and the PCR products were evaluated by horizontal electrophoresis in 2% agarose gel. We successfully detected the PCR products of pre-S regions in 43 samples on the agarose gel, while we failed to amplify the target pre-S regions in one sample, which could be explained by degradation of the viral DNA during serum storage. **Figure 1** & **Figure 2** and **Table 1** showed that, the PCR products had a molecular size of 479 (2 samples, 4.65% of total samples), 446 (20 samples, 46.51% of total samples), 425 (1 sample, 2.33% of total samples), 389 (1 sample, 2.33% of total samples), 476 (18 samples, 41.86% of total samples) or 433 bp (1 sample, 2.33% of total samples).

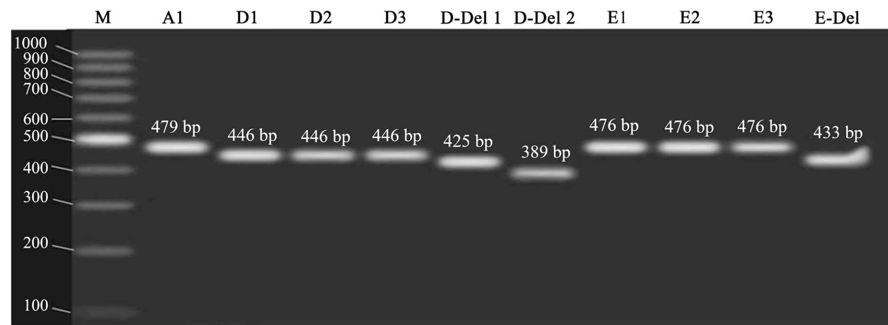

Figure 1. Image representing 2% agarose gel electrophoresis of the PCR products of the target pre-S regions within the HBV genome. M: 100 bp molecular marker (GeneRuler 100 bp, Thermo scientific, USA), A1: Genotype A with A1 pattern, D1: Genotype D with D1 pattern, D2: Genotype D with D2 pattern, D3: Genotype D with D3 pattern, D-Del 1: Genotype D with D-Del 1 pattern, D-Del 2: Genotype D with D-Del 2 pattern, E1: Genotype E with E1 pattern, E2: Genotype E with E2 pattern, E3: Genotype E with E3 pattern, E-Del: Genotype E with E-Del pattern.

Subsequently, the PCR product was digested with AvaII and DpnII enzymes restriction enzyme and the pattern of RFLP was analyzed by horizontal electrophoresis in 3% agarose gel. Figure 2 and Table 1 reveal a low prevalence of the A genotype, with only 2 samples (4.65% of total samples) displaying this genotype. In contrast, genotype D was the most abundant genotype among the studied samples, with 22 samples (51.16%) exhibiting his characteristic RFP pattern. RFLP analysis revealed that a significant proportion of the samples (19 samples, 44.19%) were classified as genotype E.

The 2 samples belonging to the A genotype had an A1 pattern (2 samples, 4.65% of total samples), as described by Lindh *et al.* (1998). This pattern consists of AvaII- and DpnII-RFLP fragments with (301, 121, 57 bp) and (318, 109, 52 bp), respectively. Genotype D includes the D1, D2, D3, D-Del 1 and D-Del 2 patterns with a prevalence of (11 samples, 25.58%), (8 samples, 18.6%), (1 sample, 2.33%), (1 sample, 2.33%) and (1 sample, 2.33%), respectively. Pattern D1 and D2 were described previously by Lindh *et al.* (1998) and had DpnII-RFLP fragments with (306, 67, 52, 21 bp) and (306, 88, 52 bp), respectively. D3, D-Del 1 and D-Del 2 are novel patterns and were classified as patterns within genotype D in addtion to D1 and D2 patterns because the 5 patterns had a unique AvaII-RFLP pattern characteristic of the D genotype, which was distinguished by undigested PCR products using AvaII. Pattern D3 had a DpnII-RFLP pattern consisting of only one DpnII restriction site. Therefore, the fragments of D2 pattern with molecular weights of 306 and 52 bp merged in one D3 fragments with molecular weight of 358 bp. This resulted in an RFLP pattern consisting of 2 fragments with 358 and 88 bp. Patterns D-Del 1 and D-Del 2 represent truncated forms of the amplified pre-S regions. Digestion of D-Del 1 and D-Del 2 patterns with DpnII resulted in fragments with sizes of (306, 88, 31 bp) and (261, 88, 40 bp), respectively. D-Del 1 pattern share the fragments with molecular weights of 306 and 88 bp with D2 pattern, while D-Del 2 pattern shares only one fragments with the molecular weight of 88 bp with

D2 pattern. Genotype E comprises the E1, E2, E3, and E-Del patterns with a frequency of 4 samples (9.3%), 13 samples (30.23%), 1 sample (2.33%) and 1 sample (2.33%), respectively. Patterns E1 and E2 were designated earlier by Lindh *et al.* (1998) and had the same AvaII-RFLP pattern (319, 108, 49 bp) but had different DpnII-RFLP patterns of (306, 118, 52 bp) and (180, 126, 118, 52 bp), respectively. E3 is novel pattern and was classified as a pattern within genotype E because it had a AvaII-RFLP pattern consisting of only one AvaII restriction site. Therefore, the fragments of E1 and E2 pattern with molecular weights of 319 and 108 bp merged in one E3 fragments with molecular weight of 427 bp. This resulted in an RFLP pattern consisting of 2 fragments with 427 and 49 bp. D3 had AvaII- and DpnII-RFLP fragments with (427, 49 bp) and (178, 141, 118, 39 bp), respectively. E-Del is a novel pattern and was classified as a pattern within genotype E because it shares with D2 the fragments of the AvaII- and DpnII-RFLP patterns with molecular weight of (108 and 49 bp) and (126, 118, 52 bp), respectively. The E- Del pattern is truncated form of the amplified pre-S regions. It is characterized by AvaII- and DpnII-RFLP fragments of (276, 108, 49 bp) and (137, 126, 118, 52 bp), respectively.

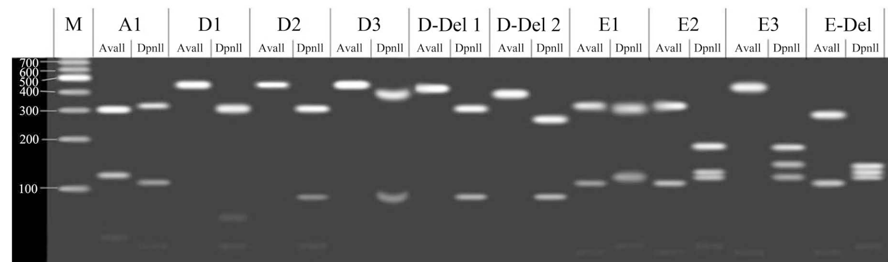

Figure 2. The RFLP pattern of the PCR products of the target pre-S regions digested with AvaII and DpnII enzymes and subjected to 3% agarose gel electrophoresis. The molecular weights of each RFLP fragment were described in Table 2. M: 100 bp molecular marker (GeneRuler 100 bp, Thermo scientific, USA), A1: Genotype A with A1 pattern, D1: Genotype D with D1 pattern, D2: Genotype D with D2 pattern, D3: Genotype D with D3 pattern, D-Del 1: Genotype D with D-Del 1 pattern, D-Del 2: Genotype D with D-Del 2 pattern, E1: Genotype E with E1 pattern, E2: Genotype E with E2 pattern, E3: Genotype E with E3 pattern, E-Del: Genotype E with E-Del pattern, AvaII: PCR prouct digested with AvaII enzyme, DpnII: PCR prouct digested with DpnII enzyme. Bands smaller than 49 bp were too diffuse to be visible in the agarose gel due to their small size [9]. Their molecular weights were calculated by subtracting the sum of the molecular weights of the visible bands from the total molecular weight of the undigested PCR products.

4. Discussion

The genotyping method used was a PCR-RFLP; compared to sequencing, this method is known to be relatively simple, fast, and not too expensive. When performed in the Pre-S region, a PCR-RFLP allowed the detection of some genotypes more easily than other techniques [20].

A total of 250 serum samples from blood donors who tested negative for HBsAg by ELISA were screened by real-time PCR for occult HBV infection. Of these, 44

samples (17.6%) were found to be HBV DNA-positive by PCR; the HBV DNA-positive samples were included in further analysis, while the negative samples were excluded. One of the positive samples failed to amplify in the pre-S region, likely due to DNA degradation or low viral load as a result of storage or poor sample quality. The remaining 43 DNA-positive samples were successfully genotyped using RFLP after amplification of the pre-S region and digestion with AvaII and DpnII enzymes. That may be caused by a mutation of HBV that affects the expression of HBsAg or by low-level replication of the virus, which does not produce enough antigens to be detected by the standard assay. The PCR product was digested with AvaII and DpnII restriction enzymes and the pattern of RFLP was analyzed by horizontal electrophoresis in 3% agarose gel revealing a low prevalence of the A genotype, with only 2 samples (4.65% of total samples) displaying this genotype. The 2 samples belonging to the A genotype had an A1 pattern (2 samples, 4.65% of total samples) which disagreed with a study reported only presence of genotype A2 in 1 strain (2%) and A1 wasn't found [21] This could be due to the small size of the cohort studied and to the northern location of Khartoum, where samples were collected [21]. Sub genotype A1 was found circulating in southern and eastern Africa and Southern Asia and A2 is found in Northern and Central Europe and North America [22].

In contrast, genotype D was the most abundant genotype among the studied samples, with 22 samples (51.16%) exhibiting his characteristic RFLP pattern. Genotype D includes the D1, D2, D3, D-Del 1 and D-Del 2 patterns with a prevalence of (11 samples, 25.58%), (8 samples, 18.6%), (1 sample, 2.33%), (1 sample, 2.33%) and (1 sample, 2.33%), respectively. Showing a disagreement with a study originating from Sudan reported the predominance of genotype E [21] and other study showed agreement with the predominance of genotype D as 60% and sub genotype D1 [23]. These results are in agreement with previous data derived from HBV infected patients originating from Tunisia, confirming a predominance of genotype D in the country [24]. Genotype D prevails in all Mediterranean regions; it was reported in more than 50% of HBV infected patients from south of Europe [25]. It seems that the genotype D is predominant in some other countries from the Maghreb like Morocco and Algeria which was observed in more than 87% and 93% of studied cases, respectively [26]. D1 was the most prevalent in 25.58%, while D2 was the most common pattern in Morocco and Turkey which was detected in 100% and 85.9% of studied population, respectively [20], However, this profile seems to be less prevalent in South Africa and Somalia where D3 and D4 sub-genotypes are predominant [20], In other countries, a co-circulation of strains with at least two different sub- genotypes, at comparable proportion, was shown. For example, in India four D sub-genotypes were described; D1 was found in 17%, D2 in 29%, D3 in 34%, and D5 in 20% of studied population. The multiplicity of patterns for the same genotype with variability in their geographical distribution should be taken into consideration especially in countries where only one genotype predominantly circulates; it plays, probably, an important role in disease progression and response to antiviral therapy [20].

Table 2. Genotypes and their associated patterns of the HBV virus which were detected in the serum of HBV-positive patients based on RFLP analysis. The molecular weights of the PCR products and RFLP fragments from the studied samples were determined.

Genotype	Number of samples	% of total samples	Pattern	Size of the PCR product (bp)	RFLP pattern (bp)*		code of the Samples	Number of samples	% of total samples
					AvaII	DpnII			
A	2	4.65	A1	479	(301, 121, 57)	(318, 109, 52)	799, 962	2	4.65
D	22	51.16	D1	446	446	(306, 67, 52, 21)	461, 500, 575, 595, 732, 781, 826, 833, 987, 990, 996	11	25.58
			D2	446	446	(306, 88, 52)	427, 480, 590, 721, 778, 789, 825, 832	8	18.6
			D3	446	446	(358, 88)	973	1	2.33
			D-Del 1	425	425	(306, 88, 31)	709	1	2.33
			D-Del 2	389	389	(261, 88, 40)	786	1	2.33
E	19	44.19	E1	476	(319, 108, 49)	(306, 118, 52)	494, 717, 807, 994	4	9.3
			E2	476	(319, 108, 49)	(180, 126, 118, 52)	505, 613, 698, 726, 760, 804, 841, 850, 861, 966, 971, 977, 995	13	30.23
			E3	476	(427, 49)	(178, 141, 118, 39)	852	1	2.33
			E-Del	433	(276, 108, 49)	(137, 126, 118, 52)	862	1	2.33

*Bands smaller than 49 bp were too diffuse to be visible in the agarose gel due to their small size (24). Their molecular weights were calculated by subtracting the sum of the molecular weights of the visible bands from the total molecular weight of the undigested PCR products.

RFLP analysis revealed that a significant proportion of the samples (19 samples, 44.19%) were classified as genotype E. Genotype E comprises the E1, E2, E3, and E-Del patterns with a frequency of 4 samples (9.3%), 13 samples (30.23%), 1 sample (2.33%) and 1 sample (2.33%), respectively. Which is different to other study showed a higher percentage of genotype E identified in 57.5% [21] and agreed with other study showed Genotype E observed in 24% [23] Pre-S deletion mutants were found in genotype E isolates from ASCs from Guinea [25] Pre-S deletion/mutations affect the progression to serious liver disease in patients infected with either genotype B or C [27].

This study represents the first molecular characterization of occult HBV infection and the Pre-S deletion mutants were found in genotypes D and E in blood donors in Sudan. The influx of different HBV genotypes is due to the fact of Sudan being a central position in Africa with waves of migration from surrounding territories and continuous [28]. The identification of the **D-Del 1**, **D-Del 2**, and **E-**

Del patterns provides novel insights into HBV genetic diversity and emphasizes the need for PCR-based screening methods to detect occult HBV infections in blood donors. These findings have significant implications for transfusion medicine, as occult HBV remains a major risk factor for transfusion-transmitted infections.

5. Conclusions

This study investigated occult HBV infection among ELISA HBsAg-negative Sudanese blood donors using PCR-RFLP analysis of the pre-S region. Genotype D was the most prevalent (51.16%), followed by genotype E (44.19%), while genotype A was rare (4.65%). Importantly, three previously unreported RFLP patterns D-Del 1, D-Del 2, and E-Del were identified, suggesting the presence of novel pre-S region deletions. These findings contribute to understanding the genetic diversity of HBV in Sudan and emphasize the need for molecular surveillance in blood banks, especially in regions where standard serological testing may miss occult infections.

Future research should include full sequencing of these novel variants to confirm deletions and determine their clinical and epidemiological relevance. In addition, larger population studies incorporating clinical data and serological markers (anti-HBc, anti-HBs) are necessary to evaluate the potential impact of these variants on disease progression, transmission risk, and response to therapy.

6. Limitation of the Study

This study was limited by the lack of advanced diagnostic infrastructure in Sudan, which made it challenging to conduct molecular analyses locally. Consequently, some laboratory procedures particularly PCR amplification and genotyping were outsourced to facilities abroad. Additionally, the study did not include sequencing of the novel deletion variants, which limits the ability to confirm genotype assignment and map exact breakpoints. These constraints reflect broader resource limitations in low-income settings and highlight the need for improved molecular diagnostics capacity in the region.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] Perz, J.F., Armstrong, G.L., Farrington, L.A., Hutin, Y.J.F. and Bell, B.P. (2006) The Contributions of Hepatitis B Virus and Hepatitis C Virus Infections to Cirrhosis and Primary Liver Cancer Worldwide. *Journal of Hepatology*, **45**, 529-538. https://doi.org/10.1016/j.jhep.2006.05.013

[2] Sivakrishnan, S. and Pharm, M. (2019) Liver Disease Overview. *World Journal of Pharmacy and Pharmaceutical Sciences*, **8**, 1385-1395.

[3] Lau, K.C.K., Burak, K.W. and Coffin, C.S. (2020) Impact of Hepatitis B Virus Genetic Variation, Integration, and Lymphotropism in Antiviral Treatment and Oncogenesis. *Microorganisms*, **8**, Article 1470. https://doi.org/10.3390/microorganisms8101470

[4] Tsukuda, S. and Watashi, K. (2020) Hepatitis B Virus Biology and Life Cycle. *Antiviral Research*, **182**, Article ID: 104925. https://doi.org/10.1016/j.antiviral.2020.104925

[5] Eltom, K., Albeely, A., El Hussein, A.R.M., Elkhidir, I.M. and Enan, K. (2020) Occult Hepatitis B Virus Infection in Sudan: A Systematic Review and Meta-Analysis. *JGH Open*, **4**, 800-807. https://doi.org/10.1002/jgh3.12411

[6] Candotti, D., Assennato, S.M., Laperche, S., Allain, J. and Levicnik-Stezinar, S. (2018) Multiple HBV Transfusion Transmissions from Undetected Occult Infections: Revising the Minimal Infectious Dose. *Gut*, **68**, 313-321. https://doi.org/10.1136/gutjnl-2018-316490

[7] Raimondo, G., *et al.* (2019) Update of the Statements on Biology and Clinical Impact of Occult Hepatitis B Virus Infection. *Journal of Hepatology*, **71**, 397-408.

[8] Candotti, D., Grabarczyk, P., Ghiazza, P., Roig, R., Casamitjana, N., Iudicone, P., *et al.* (2008) Characterization of Occult Hepatitis B Virus from Blood Donors Carrying Genotype A2 or Genotype D Strains. *Journal of Hepatology*, **49**, 537-547. https://doi.org/10.1016/j.jhep.2008.04.017

[9] Baptista, M., *et al.* (2003) Follow up of Infection of Chacma Baboons with Inoculum Containing a and Non-A Genotypes of Hepatitis B Virus. *World Journal of Gastroenterology*, **9**, 731-735. https://doi.org/10.3748/wjg.v9.i4.731

[10] Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R.I., Imai, M., Miyakawa, Y., *et al.* (1988) Typing Hepatitis B Virus by Homology in Nucleotide Sequence: Comparison of Surface Antigen Subtypes. *Journal of General Virology*, **69**, 2575-2583. https://doi.org/10.1099/0022-1317-69-10-2575

[11] Norder, H., Hammas, B., Lee, S., Bile, K., Courouce, A., Mushahwar, I.K., *et al.* (1993) Genetic Relatedness of Hepatitis B Viral Strains of Diverse Geographical Origin and Natural Variations in the Primary Structure of the Surface Antigen. *Journal of General Virology*, **74**, 1341-1348. https://doi.org/10.1099/0022-1317-74-7-1341

[12] Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F., *et al.* (2000) A New Genotype of Hepatitis B Virus: Complete Genome and Phylogenetic Relatedness. *Microbiology*, **81**, 67-74. https://doi.org/10.1099/0022-1317-81-1-67

[13] Arauz-Ruiz, P., Norder, H., Robertson, B.H. and Magnius, L.O. (2002) Genotype H: A New Amerindian Genotype of Hepatitis B Virus Revealed in Central America. *Journal of General Virology*, **83**, 2059-2073. https://doi.org/10.1099/0022-1317-83-8-2059

[14] Norton, P.A., Menne, S., Sinnathamby, G., Betesh, L., Cote, P.J., Philip, R., *et al.* (2010) Glucosidase Inhibition Enhances Presentation of De-N-Glycosylated Hepatitis B Virus Epitopes by Major Histocompatibility Complex Class I *in Vitro* and in Woodchucks. *Hepatology*, **52**, 1242-1250. https://doi.org/10.1002/hep.23806

[15] Yu, D., Li, X., Mom, V., Lu, Z., Liao, X., Han, Y., *et al.* (2014) N-Glycosylation Mutations within Hepatitis B Virus Surface Major Hydrophilic Region Contribute Mostly to Immune Escape. *Journal of Hepatology*, **60**, 515-522. https://doi.org/10.1016/j.jhep.2013.11.004

[16] Mathet, V.L., Feld, M., Espínola, L., Sánchez, D.O., Ruiz, V., Mandó, O., *et al.* (2002) Hepatitis B Virus S Gene Mutants in a Patient with Chronic Active Hepatitis with Circulating Anti-HBs Antibodies. *Journal of Medical Virology*, **69**, 18-26. https://doi.org/10.1002/jmv.10267

[17] Babakhanzadeh, E., Danaei, H., Abedinzadeh, M., Reza Ashrafzadeh, H. and Ghasemi, N. (2021) Association of miR-146a and miR196a2 Genotype with Susceptibility to Idiopathic Recurrent Pregnancy Loss in Iranian Women: A Case-Control Study. *International Journal of Reproductive BioMedicine*, **19**, 725-732. https://doi.org/10.18502/ijrm.v19i8.9620

[18] Changotra, H. and Sehajpal, P.K. (2013) An Improved Method for the Isolation of Hepatitis B Virus DNA from Human Serum. *Indian Journal of Virology*, **24**, 174-179. https://doi.org/10.1007/s13337-013-0155-y

[19] Lindh, M., Gonzalez, J.E., Norkrans, G. and Horal, P. (1998) Genotyping of Hepatitis B Virus by Restriction Pattern Analysis of a Pre-S Amplicon. *Journal of Virological Methods*, **72**, 163-174. https://doi.org/10.1016/s0166-0934(98)00026-3

[20] Ouneissa, R., Bahri, O., Ben Yahia, A., Touzi, H., Azouz, M.M., Ben Mami, N., *et al.* (2013) Evaluation of PCR-RFLP in the Pre-S Region as Molecular Method for Hepatitis B Virus Genotyping. *Hepatitis Monthly*, **13**, e11781. https://doi.org/10.5812/hepatmon.11781

[21] Mahgoub, S., Candotti, D., El Ekiaby, M. and Allain, J. (2011) Hepatitis B Virus (HBV) Infection and Recombination between HBV Genotypes D and E in Asymptomatic Blood Donors from Khartoum, Sudan. *Journal of Clinical Microbiology*, **49**, 298-306. https://doi.org/10.1128/jcm.00867-10

[22] Kramvis, A. and Paraskevis, D. (2005) Subgenotype A1 of HBV—Tracing Human Migrations in and out of Africa. *Antiviral Therapy*, **18**, 513-521. https://doi.org/10.3851/imp2657

[23] Yousif, M., Mudawi, H., Bakhiet, S., Glebe, D. and Kramvis, A. (2013) Molecular Characterization of Hepatitis B Virus in Liver Disease Patients and Asymptomatic Carriers of the Virus in Sudan. *BMC Infectious Diseases*, **13**, Article No. 328. https://doi.org/10.1186/1471-2334-13-328

[24] Ayed, K., Gorgi, Y., Ayed-Jendoubi, S., Aouadi, H., Sfar, I., Najjar, T., *et al.* (2007) Hepatitis B Virus Genotypes and Precore/Core-Promoter Mutations in Tunisian Patients with Chronic Hepatitis B Virus Infection. *Journal of Infection*, **54**, 291-297. https://doi.org/10.1016/j.jinf.2006.05.013

[25] Zehender, G., Ebranati, E., Gabanelli, E., Shkjezi, R., Lai, A., Sorrentino, C., *et al.* (2012) Spatial and Temporal Dynamics of Hepatitis B Virus D Genotype in Europe and the Mediterranean Basin. *PLOS ONE*, **7**, e37198. https://doi.org/10.1371/journal.pone.0037198

[26] Khelifa, F. and Thibault, V. (2009) Characteristics of Hepatitis B Viral Strains in Chronic Carrier Patients from North-East Algeria. *Pathologie Biologie*, **57**, 107-113. https://doi.org/10.1016/j.patbio.2008.07.031

[27] Ali, M.M., Hasan, F., Ahmad, S. and Al-Nakib, W. (2010) Comparative Evaluation of INNO-LiPA HBV Assay, Direct DNA Sequencing and Subtractive PCR-RFLP for Genotyping of Clinical HBV Isolates. *Virology Journal*, **7**, Article No. 111. https://doi.org/10.1186/1743-422x-7-111

[28] Salih, E.Y., Saeed, W.S., Satti, M.M., Abbas, G., Suleiman, I.A. and Khalil, E.A. (2018) Hepatitis B Virus Genotypic Patterns in Sudan Reflects Population Movements. *Global Journal of Virology and Immunology*, **3**, 176-180.