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Genotyping and virological characteristics of hepatitis B virus in HIV-infected individuals in Sudan

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SUMMARY

Objectives: Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) share common routes of blood-borne transmission. In HBV mono-infected Sudanese individuals, genotypes D, E, and A circulate. The objective of this study was to molecularly characterize HBV from HBV/HIV co-infected individuals.
Methods: The polymerase overlapping the S region and the basic core promoter (BCP/PC) of HBV from 32 hepatitis B surface antigen (HBsAg)-positive and 18 HBsAg-negative serum samples were amplified and sequenced.
Results: HBV from 37 samples was successfully genotyped and the genotype distribution was 46.0% D, 21.6% E, 18.9% A, and 13.5% D/E recombinant. Compared to mono-infected individuals, the frequencies of the D/E recombinant and genotype A were higher in HBV/HIV co-infected patients, as was the intra-group divergence of genotype E. BCP/PC mutations affecting hepatitis B e antigen (HBeAg) expression at the transcriptional and translational levels were detected. Two HBsAg-positive individuals had pre-S deletion mutants. The following mutations in the S region could account for the HBsAg negativity: sM133T, sE164G, sV168G, and sS174N. No primary drug resistance mutations were found.
Conclusions: In HBV/HIV co-infected Sudanese patients, the ratio of genotype A to non-A was higher than that in mono-infected patients. The genotype E intra-group divergence in HBV/HIV co-infected individuals was significantly higher than that in HBV mono-infected patients.

1. Introduction

Hepatitis B virus (HBV), the smallest DNA virus that infects humans, is a member of the *Hepadnaviridae* family and it causes acute and chronic liver disease. Human immunodeficiency virus (HIV) is an enveloped RNA virus that replicates by reverse transcription.[1] HBV and HIV share common routes of blood-borne transmission, but they differ in efficiency of transmission and in their geographic distribution.[2] Worldwide, it has been estimated that 10% of the 34 million people infected with HIV have chronic HBV infection.[2]

In Sudan, the prevalence of HBV infection ranges from 3.9% in blood donors in Khartoum[3] to 26% in patients seen in outpatient clinics in Juba in southern Sudan.[4] The prevalence of HIV in adults in Sudan was estimated to be 1.4% in 2008,[5] and according to the Sudan National AIDS Control Programme (SNAP), this figure had decreased to 0.52% in 2011.[6] It is very difficult to surmise the true prevalence of HBV/HIV co-infection in Sudan from the data available because of the paucity of data and the wide time span between studies.

Two studies, carried out 24 years ago, determined the prevalence of HBV/HIV co-infection in Sudan, using serological techniques. The first study showed 14% HBV infection among HIV-negative individuals who were sexually active heterosexuals in the northeast of Sudan.[7] The second study, among soldiers in eastern Sudan, showed 1.7% HIV infection and 25% hepatitis B surface antigen (HBsAg) positivity, with only 0.8% being co-infected with

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HBV and HIV.[8] In a recently completed study, our group showed 225 of 358 (62.8%) HIV-infected individuals from Khartoum to have at least one HBV serological marker, with 129 (36%) being HBV DNA-negative and 96 (26.8%) being HBV DNA-positive by real-time PCR (RT-PCR).[9] HBV DNA was detected together with HBsAg in 42 (11.7%) and without HBsAg in 54 (15.1%) of the participants.[9] Of these, 35 (9.8%) had a viral load <200 IU/ml (<2.3 $\log_{10}$ IU/ml) and therefore true occult hepatitis B infection (OBI),[10] and 19 (5.3%) had viral loads >200 IU/ml (>2.3 $\log_{10}$ IU/ml) and were therefore HBsAg-covert.[11]

As a result of the lack of proofreading activity of the viral polymerase, HBV displays considerable sequence heterogeneity, leading to its classification into at least nine genotypes.[12–20] These are defined by inter-genotypic differences of more than 7.5% in the complete HBV genome.[12,21,22] It is considered that genotypes play an important role in treatment management and disease prognosis.[23–25] In Sudan, close to 60% of liver disease patients were found to be infected with genotype D (predominantly subgenotype D1), 30% with genotype E, 8.5% with genotype A, and 2.5% with D/E recombinant.[26] In contrast, in Sudanese blood donors, genotype E was found to predominate (55%), followed by genotype D (39%), D/E recombinant (4%), and genotype A (2%).[3]

No information exists regarding the distribution of HBV genotypes in HIV-infected individuals in Sudan. The objectives of this study were to genotype and molecularly characterize HBV and to determine possible virological characteristics that could differentiate isolates from HBsAg-positive and HBsAg- negative HIV-infected Sudanese patients.

2. Patients and methods

2.1. Ethical considerations

This study was a continuation of a previous study on HBV/HIV co-infection among adult Sudanese HIV patients.[9] The study was originally approved by the Ethics Committee of the Faculty of Medicine, University of Khartoum and the National Ethics Committee at the Federal Ministry of Health. All patients provided signed informed consent, and the clinical report forms were completed by qualified practitioners. The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand.

2.2. Serum samples

Serum samples were collected from 358 HIV-positive treatment-naïve patients and 96 were positive for HBV DNA using RT-PCR.[9] Fifty of these 96 were selected randomly for downstream genotyping and molecular analysis of the HBV.

2.3. DNA extraction

DNA was extracted from 200 μl of serum using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Germany), in accordance with the manufacturer's instructions, and eluted in 100 μl of elution buffer.

2.4. PCR

Two regions of the HBV genome were amplified. The basic core promoter/precore (BCP/PC) region (1742–1900 from *Eco*R1) was amplified using a minor modification of the method described by Takahashi et al.;[27] primers 1606(+) (1606–1625 from the *Eco*R1 site) and 1974(−) (1974–1955 from the *Eco*R1 site) were used for the first round and 1653(+) (1653–1672 from the *Eco*R1 site) and 1959(−) (1959–1940 from the *Eco*R1 site) for the second round. Region 2624–1240 from *Eco*R1, covering part of the polymerase region and overlapping the complete S region (2848–835 from the *Eco*R1 site) (P/S) was amplified using primers 2410(+) (2410–2439 from the *Eco*R1 site) and 1314(−) (1314–1291 from the *Eco*R1 site) for the first round and 2451(+) (2451–2482 from *Eco*R1site) and 1280(−) (1280–1254 from *Eco*R1 site) for the second round.[28]

2.5. Sequencing

The BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) was used and sequencing was performed with an ABI 3130XL Genetic Analyzer (Applied Biosystems). The three overlapping fragments of the region 2624–1240 from *Eco*R1 obtained were assembled using the Fragment Merger tool.[29] Sequences were deposited in GenBank under accession numbers KM108588–KM108626.

2.6. Phylogenetic analysis

A neighbour-joining phylogenetic tree with a bootstrap of 1000 replicates was constructed using MEGA 5[30] or PHYLIP (Phylogeny Inference Package, version 3.69). DNADIST consecutively with NEIGHBOR (neighbour-joining) were used to generate dendrograms, which were viewed in TreeView.[31,32]

3. Results

3.1. HBV serology, amplification, and quantification of HBV DNA

Of the 50 HBV DNA-positive patients selected randomly, 28 were males. The mean patient age ± standard deviation (SD) was 35 ± 8.1 years. The median alanine aminotransferase (ALT) level was 6.15 (interquartile range (IQR) 3.3–11.8) IU/l (reference range 5–40 IU/l)[33] and the median HBV DNA viral load was 4.2 (IQR 3.56–6.67) log IU/ml. Thirty-two (64%) were HBsAg-positive and 18 (36%) were HBsAg-negative. Only six were hepatitis B e antigen (HBeAg)-positive. No difference in age, gender, HBsAg prevalence, or ALT levels was observed between the 50 samples (selected randomly for further analyses) and the original 96 HBV DNA-positive samples.[9] Of the 18 HBsAg-negative patients, 10 had viral loads <200 IU/ml (<2.3 $\log_{10}$ IU/ml), and thus according to the Taormina definition, were true OBI.[10] The remaining eight had viral loads >200 IU/ml (>2.3 $\log_{10}$ IU/ml) and therefore were HBsAg-covert[11] (Table 1). No difference in age, gender, or ALT levels was observed between the true OBI and HBsAg-covert cases.

3.2. HBV genotyping and phylogenetic analysis

Sequences were obtained for 46 of the 50 samples: 27 samples in both the BCP/PC and the P/S region, 12 in the BCP/PC alone, and seven in the P/S region only. For three samples, however, the sequences of the S region were not of good quality and therefore could not be used for genotyping.

In total, 37 HBV isolates were successfully genotyped using one of the two methods; 31 of 37 were genotyped using phylogenetic analysis of the P/S region (Figure 1).

The BCP/PC region sequences could be used to differentiate between genotype A and non-A. Six out of 37 had 1858C characteristic of genotype A and all belonged to subgenotype A1 because they had 1809–1812 TCAT and 1888A. Six samples had 1858T characteristic of non-A genotypes, but this region cannot differentiate between genotype D and E and thus these samples could not be genotyped.

The genotype distribution of the 37 isolates was as follows: 17 (46.0%) genotype D, eight (21.6%) genotype E, seven (18.9%) genotype A, and five (13.5%) putative D/E recombinant. If we include the 13 (26%) samples that could not be genotyped, the

Table 1
Characteristics of HBV isolated from HIV-positive individuals

HBsAg status	Isolate	Patient characteristics			Virus characteristics			HBV infection
		Gender	Age, years	ALT IU/l	Genotype	Subgenotype	Viral load IU/ml log	
Positive	SDH001	Male	50	24	D	D1	6.82	Overt
	SDH020	Female	26	19.9	E	NA	4.25	Overt
	SDH025	Female	23	7.6	D	D2	6.86	Overt
	SDH028	Male	31	10.6	-	-	4.74	Overt
	SDH043	Female	34	4.6	-	-	3.46	Overt
	SDH049	Male	45	11.8	D	D1	5.65	Overt
	SDH050	Male	48	0.4	D	D1	9.16	Overt
	SDH052	Male	38	16.1	D	D6	7.28	Overt
	SDH065	Male	32	18.2	D	D1	8.19	Overt
	SDH071	Male	35	1.2	D/E	NA	4.56	Overt
	SDH075	Female	32	5.5	D	D1	3.73	Overt
	SDH078	Male	25	7.6	-	-	4.08	Overt
	SDH079	Male	25	11.7	-	-	3.34	Overt
	SDH084	Male	25	8.9	E	NA	6.23	Overt
	SDH097	Female	23	6.4	D/E	NA	4.53	Overt
	SDH111	Female	36	7.6	-	-	3.67	Overt
	SDH113	Female	42	27.5	D/E	NA	3.37	Overt
	SDH116	Male	23	5.5	E	NA	4.19	Overt
	SDH120	Male	27	17.8	E	NA	7.83	Overt
Positive	SDH122	Female	38	8.4	-	-	4.00	Overt
	SDH123	Female	35	15.2	D	D1	7.20	Overt
	SDH146	Female	33	8	-	-	4.17	Overt
	SDH153	Female	28	5.9	D	D1	4.16	Overt
	SDH184	Female	30	5.9	A	A1	7.00	Overt
	SDH188	Male	28	4.2	E	NA	4.95	Overt
	SDH189	Female	30	0.8	-	-	3.42	Overt
	SDH193	Male	45	5.9	E	-	8.16	Overt
	SDH194	Female	37	0.8	D	D6	3.14	Overt
	SDH195	Male	38	3.3	A	A1	3.59	Overt
	SDH196	Male	38	13.1	-	-	3.13	Overt
	SDH199	Female	35	3.8	A	A1	3.08	Overt
	SDH200	Male	45	5.6	A	A1	7.12	Overt
Negative	SDH005	Female	45	12.3	D	D1	<2.3	OBI
	SDH007	Female	30	14	D	D2	3.55	HBsAg-covert
	SDH022	Male	27	7.2	D	D1	<2.3	OBI
	SDH036	Female	30	5.9	-	-	<2.3	OBI
	SDH042	Male	41	0.8	D	D1	<2.3	OBI
	SDH058	Male	42	8.4	D	D6	2.33	HBsAg-covert
	SDH061	Male	50	1.4	D	D6	<2.3	OBI
	SDH087	Male	22	11.8	D	D2	<2.3	OBI
	SDH119	Female	35	11.8	D/E	NA	<2.3	OBI
	SDH126	Male	45	2.9	A	A1	<2.3	OBI
Negative	SDH130	Male	37	2.2	-	-	3.92	HBsAg-covert
	SDH147	Female	23	4.6	-	-	5.45	HBsAg-covert
	SDH152	Female	38	2.1	E	NA	4.62	HBsAg-covert
	SDH160	Male	37	1.2	-	-	4.01	HBsAg-covert
	SDH172	Female	35	3.8	A	A1	<2.3	OBI
	SDH174	Male	40	3.3	E	NA	4.38	HBsAg-covert
	SDH192	Male	54	7.9	D/E	NA	2.78	HBsAg-covert
	SDH197	Male	40	2.5	A	A1	<2.3	OBI

(-), no genotype assignment; NA, not applicable; Overt, HBsAg-positive; OBI, occult hepatitis B infection, HBsAg-negative with viral loads <200 IU/ml (<2.3 $\log_{10}$ IU/ml); HBsAg-covert, HBsAg-negative with viral loads >200 IU/ml (>2.3 $\log_{10}$ IU/ml).

distribution in the 50 samples was 34% (17/50) infected with genotype D, 16% (8/50) with genotype E, 14% (7/50) with genotype A, and 10% (5/50) were infected with a putative D/E recombinant. The genotype distribution did not differ between HBsAg-positive and HBsAg-negative sera, or between HBsAg-covert and OBI patients. Comparing the genotype distribution of HBV isolates in the Sudanese HBV/HIV co-infected individuals to mono-infected liver disease patients,[26] the frequencies of genotype A and the D/E recombinant were higher in HIV co-infected patients ($P < 0.05$) (Figure 2).

The HBV genotypes were further classified into subgenotypes using the following criteria: phylogenetic analysis of the P/S region, inter-group divergence, signature amino acids, and geographical distribution.[20] Ten of 17 genotype D isolates belonged to subgenotype D1 (58.8%), two to D2 (11.7%), and five to D6 (29.5%); the one genotype A isolate belonged to subgenotype A1 (Figure 1). SDH042-OBI was an outlier of the cluster containing subgenotypes of D1–D3 and D5. However, when the analysis was repeated using only the complete S region (2848–835 from the *Eco*R1 site), SDH042-OBI clustered with subgenotype D1 (Figure 1A).

3.3. Genetic diversity of Sudanese strains

The intra-group divergence of the HBV isolated from the HBV/HIV co-infected individuals in the present study was compared to isolates from HBV mono-infected liver disease patients[26] (Figure 3). The intra-group divergence of genotype E from HBV mono-infected liver disease patients was lower than the intra-group divergence of genotype D from HBV mono-infected liver disease patients ($p < 0.05$), whereas in HBV/HIV co-infected individuals this difference was not observed (Figure 3). Moreover, only the intra-group divergence of genotype E from HBV/HIV co-infected individuals was higher than the intra-group divergence of genotype E from HBV mono-infected liver disease patients

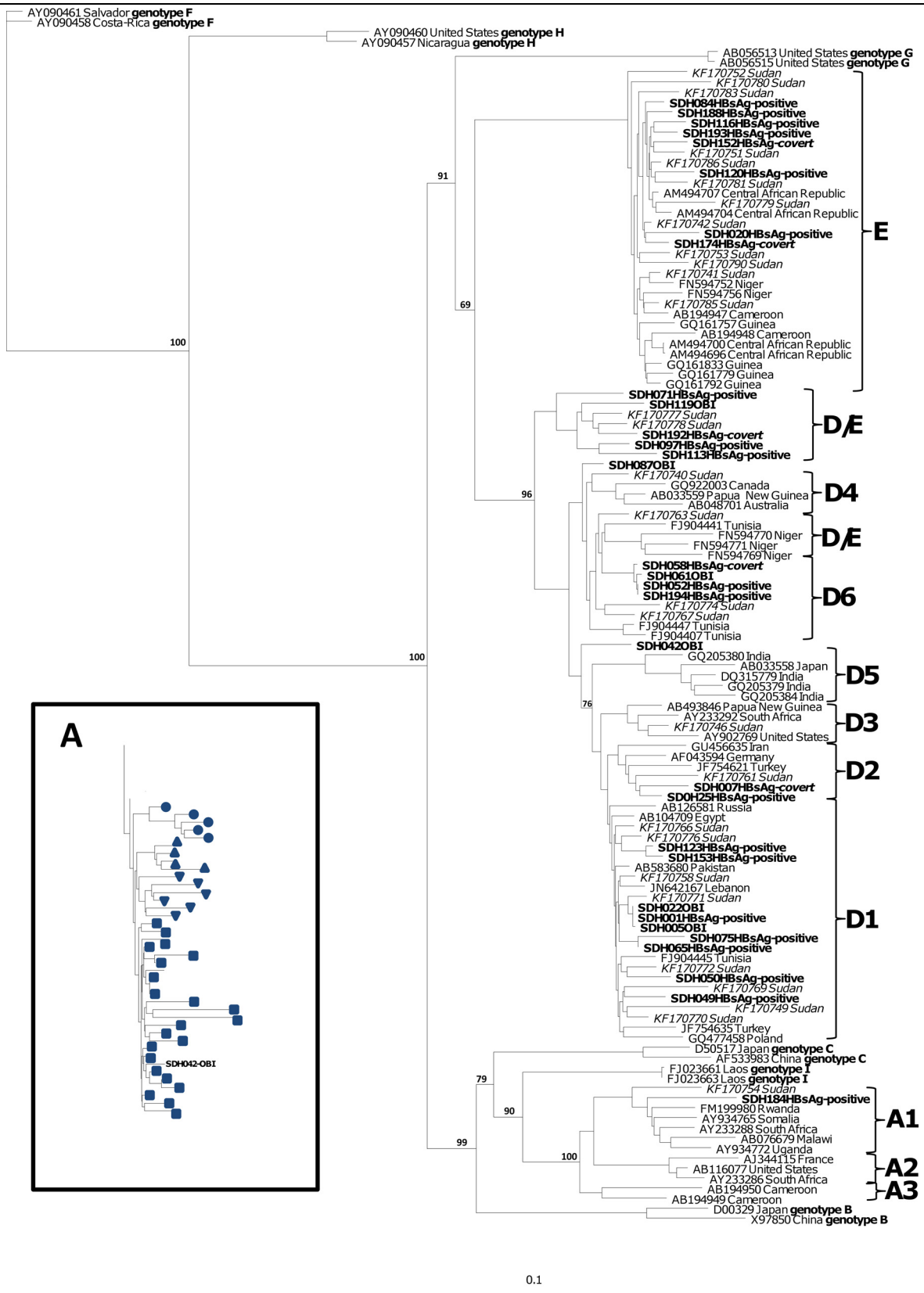

Figure 1. A rooted phylogenetic tree of 31 HBV isolates from HBV/HIV co-infected individuals; the region 2624–1240 from *Eco*R1, covering the polymerase region and overlapping complete S (2848–835 from the *Eco*R1 site). The 30 isolates sequenced in the present study have the prefix SDH and are shown in bold, followed by the clinical status (HBsAg-positive, HBsAg-covert, and occult hepatitis B infection (OBI)). These were compared using neighbour-joining with 30 HBV sequences from Sudanese liver disease patients[26] shown in italics and 55 reference sequences, labelled with their accession numbers and country of origin. Bootstrap statistical analysis was performed using 1000 datasets and the percentages are indicated on the nodes. Box A illustrates the clustering of SDH042-OBI relative to the subgenotypes of D when only the complete S (2848–835 from the *Eco*R1 site) was analyzed. The different subgenotypes are represented by different shapes: subgenotype D1 (squares), D2 (triangles), D3 (inverted triangles), and D5 (circles).

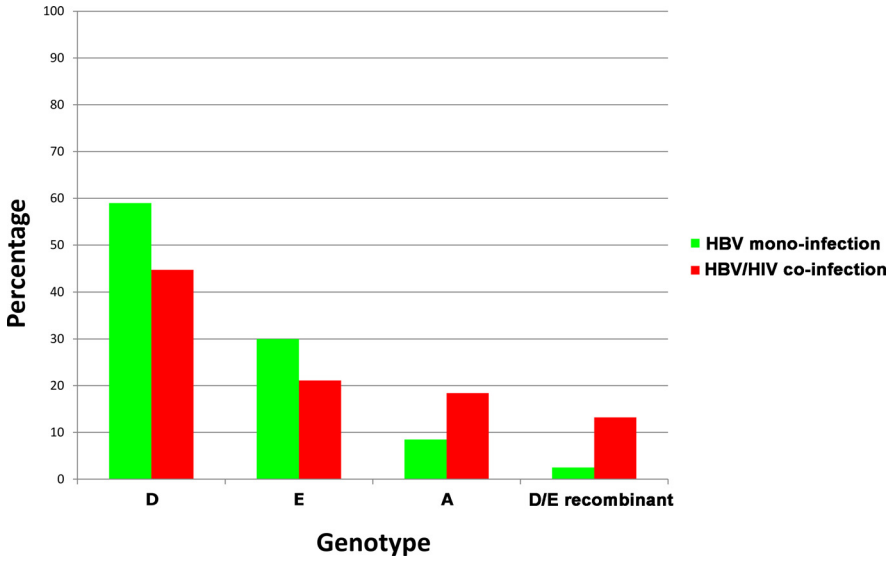

Figure 2. Ratio of the distribution of HBV genotypes in mono-infected liver disease patients[26] compared to HBV/HIV co-infected individuals.

($p < 0.05$). The intra-group divergence of genotype E from HBV/HIV co-infected individuals was 83% higher than from HBV mono-infected liver disease patients, whereas it was only 3.7% higher for genotype D.

3.4. Analysis of the BCP/PC region

BCP/PC mutations can down-regulate or abolish the expression of HBeAg at the transcriptional, translational, or post-translational levels.[34] Of the six isolates from HBeAg-positive sera, two had mutations at the BCP: SDH052 (HBsAg-positive, genotype D) had A1762G, and SDH084 (HBsAg-positive, genotype E) had A1762T/G1764A. The remaining four isolates, from HBeAg-positive patients, had wild-type BCP/PC. Thirty of the 44 isolates from HBeAg-negative individuals (17 HBsAg-positive and 13 HBsAg-negative) were successfully amplified and sequenced in the BCP/PC region. Twenty-five isolates had genotype assignments: 11 belonged to genotype D, five to genotype E, six to genotype A, and three were D/E recombinants. The mutations found in the isolates from HBeAg-negative patients are shown in Table 2. Three isolates had A1762T alone and three isolates had start codon mutations at 1814–1816. Ten of the 26 isolates had G1896A. Even though 1858T is found in both genotypes D and E and allows for G1896A to occur, G1896A was found more frequently in genotype E and the D/E recombinant compared to genotype D ($p < 0.05$). Moreover, G1896A occurred more frequently in isolates from HBsAg-positive compared to HBsAg-negative individuals ($p < 0.05$). G1896A occurred together with A1762T/G1764A in two genotype E isolates. The mutation C1766T was found in five genotype A isolates only ($p < 0.05$).

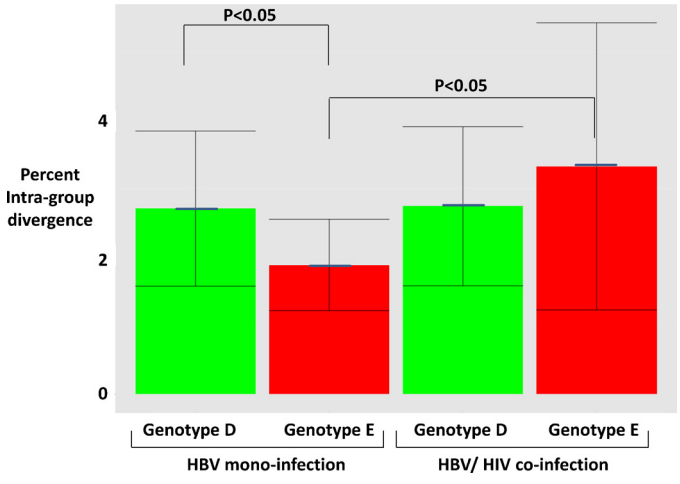

Figure 3. Comparison of the genetic diversity of genotype D and E isolated from HBV mono-infected liver disease patients and from HBV/HIV co-infected individuals.

3.5. Analysis of the pre-S1, pre-S2, surface, and polymerase regions

The sequence of the P/S region (2624–1240 from the *Eco*R1 site) was obtained for 31 HBV isolates from HBV/HIV co-infected individuals. Of the 31 isolates, 20 were from HBsAg-positive and 11 from HBsAg-negative patients. Pre-S deletions were found in two genotype E isolates. SDH084 HBsAg-positive had a 93-nucleotide deletion in the pre-S1 and pre-S2 regions, from 3178 to 58 from the *Eco*R1 site, which led to an 8 amino acid deletion from position 111 to 118 in the pre-S1 and 23-amino acid deletion from position 1 to 23 in the pre-S2. SDH120 HBsAg-positive had a 36-nucleotide deletion in the pre-S2 starting from nucleotide 5 to 40 from the *Eco*R1 site, which resulted in a 12-amino acid deletion from position 6 to 17. Table 3 shows the various mutations in the polymerase and overlapping S regions.

4. Discussion

In our recently completed study on 358 HIV-positive participants, 225 (62.8%) had serological evidence of past or current HBV infection. HBV DNA was detected in 96 (26.8%): 42 (11.7%) with an overt HBsAg-positive infection and 54 (15.1%) who were HBsAg-negative with hepatitis B core antibodies (anti-HBc) as a marker of past HBV infection.[9] Fifty of the 96 HBV DNA-positive samples were randomly selected for molecular characterization of the HBV.

The distribution ratio of HBV genotypes D and E found in HBV/HIV co-infected individuals (Figure 2), did not differ from that found in HBV mono-infected liver disease patients and there was no difference in their clustering in the phylogenetic tree (Figure 1).[26] However, genotype A and the putative D/E recombinant were found at a higher frequency in HBV/HIV co-infected individuals compared to HBV mono-infected liver disease patients (genotype A, 18.4% vs. 8.6%; D/E, 13.2% vs. 2.5%; $p < 0.05$).

Table 2
Molecular characteristics of the BCP/PC region of HBV isolated from HBeAg-negative HIV co-infected individuals

HBsAg	Isolate	Genotype	Subgenotype	Viral load IU/ml log	1753	1762–1764	1766	1768	1809–1812	1814–1816	1817	1850	1858	1862	1888	1896	1899
Positive	SDH025	D	D2	6.86	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH049	D	D1	5.65	T	AG	C	T	GCAC	ATT	C	T	T	G	G	G	A
	SDH065	D	D1	8.19	T	AG	G	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH071	D/E	NA	4.56	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH075	D	D1	3.73	T	AG	C	T	GCAC	ATG	C	T	T	G	G	G	C
	SDH097	D/E	NA	4.53	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	A
	SDH116	E	NA	4.19	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	A
	SDH120	E	NA	7.83	T	AG	G	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH123	D	D1	7.2	G	TG	T	A	GCAC	ATG	C	T	T	G	G	G	G
	SDH153	D	D1	4.16	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH188	E	NA	4.95	T	TA	C	T	GCAC	ATG	C	T	T	G	G	A	G
	SDH194	D	D6	3.14	G	AG	C	T	GCAC	ATG	C	T	T	G	G	G	G
	SDH195	A	A1	3.59	T	AG	T	T	TCAT	ATG	C	A	C	G	A	G	G
	SDH199	A	A1	3.08	T	AG	C	T	TCAT	ATG	C	A	C	G	A	G	G
	SDH200	A	A1	7.12	T	GT	T	T	TCAT	ATG	C	A	C	G	A	G	G
Negative	SDH007	D	D2	3.55	T	AG	C	T	GCAC	AAG	C	T	T	G	G	G	G
	SDH042	D	D1	<2.3	T	AG	C	T	GCAC	ATG	C	T	T	G	G	G	G
	SDH058	D	D6	2.33	G	TG	C	T	GCAC	ATG	C	T	T	G	G	G	G
	SDH061	D	D6	<2.3	G	TG	C	T	GCAC	ATG	C	T	T	G	G	G	G
	SDH126	A	A1	<2.3	T	AG	T	T	TCAT	ATG	C	A	C	G	A	G	G
Negative	SDH152	E	NA	4.62	T	AG	C	T	GCAC	ATT	C	T	T	G	G	G	G
	SDH172	A	A1	<2.3	T	AG	T	T	TCAT	ATG	C	A	C	T	A	G	G
	SDH174	E	NA	4.38	T	AG	C	T	GCAC	ATG	C	T	T	G	G	G	G
	SDH192	D/E	NA	2.78	T	AG	C	T	GCAC	ATG	C	T	T	G	G	A	A
	SDH197	A	A1	<2.3	T	AG	T	T	TCAT	ATG	C	A	C	G	A	G	G

BCP/PC, basic core promoter/precore; HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; NA, not applicable.

Table 3
S and polymerase mutations found in HBV isolated from HBV/HIV co-infected individuals

Type of mutation	HBV region	Mutation	Genotype	HBsAg status
Vaccine and immune escape	Pre-S1	I48E	A	Positive
	Pre-S2	Q10R	D/E	Positive
		R48T	E	Positive
			D	Positive
			D	Positive
			D	Negative
PreS2F22 mutation	Pre-S2	F22S	E	Positive
			D	Positive
		F22L	D/E	Positive
			E	Positive
			E	Positive
			D/E	Positive
a′ determinant mutation	S	P120T	E	Positive
		M133T	D	Negative
		G145R	E	Positive
		E164G	D/E	Negative
			E	Negative
HBV ‘reactivation’ marker	S	V168G	E	Positive
			A	Positive
			D/E	Negative
HBV ‘reactivation’ marker	S	S174N[a]	D	Negative
			D/E	Negative
			D/E	Negative
Overlapping polymerase drug resistance mutations	RT/POL	A181	D/E	Negative
		Q215S	D	Positive
			D	Positive
		I233	E	Positive

HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; S, S region; RT/POL, reverse transcriptase/polymerase; NA, not applicable.
[a] Significant association.

Genotypes D, E, A, and D/E recombinant were found in Sudanese blood donors, with genotype E predominating.[3]

With regard to the subgenotype distribution, in agreement with previous studies of HBV in Sudan,[3,26] subgenotype D1 was found to predominate in the HBV/HIV co-infected individuals (Figure 1). Thus the predominant subgenotype of D found in Sudan is D1. This is the subgenotype of D that prevails in the Mediterranean region, Arabian subcontinent, and in China.[30] Subgenotype A1 was found circulating in HBV/HIV co-infected individuals and liver disease patients,[26] whereas subgenotype A2 was found in blood donors.[3] Subgenotype A1 predominates in eastern and southern Africa and subgenotype A2 is the subgenotype of A predominating outside Africa.[35]

Following the calculation of the intra-group divergence of genotype D and E isolates from HBV mono-infected liver disease patients and HBV/HIV co-infected individuals, it was found that genotype E from HBV mono-infected liver disease patients had a low diversity of 1.8% compared to 2.7% for genotype D from HBV

mono-infected liver disease patients ($p < 0.05$) (Figure 3). Genotype E has been characterized to have a low diversity compared to other genotypes and subgenotypes of HBV. Using more than 400 sequences of HBV isolated from West African countries, the genetic diversity of genotype E was found to be 0.73% for the S gene and 1.7% for the complete genome.[36] Complete genomes of genotype E isolates from Namibia, Angola, and Madagascar were found to differ from each other by only 1.2%.[37] Genotype D has been shown to be the most divergent genotype after genotype C.[38] The significant difference in intra-group divergence of genotypes D and E seen in mono-infected individuals, however, was not seen in HBV/HIV co-infected individuals, and the intra-group divergence of genotype E in HBV/HIV co-infected individuals was significantly higher than that in HBV mono-infected liver disease patients (Figure 3). It appears that in HBV mono-infected liver disease patients, who are immune competent, the genetic diversity of genotype E is constrained, whereas in HBV/HIV co-infected individuals there is an expansion of the quasi-species. HIV infection and its associated immune suppression might alter the heterogeneity of the virus and increase the genetic variability of HBV,[39] which may be as a result of the reactivation of occult HBV that can survive in the presence of immune selection.

The 81% prevalence of HBeAg negativity in HBV/HIV co-infected patients[9] was lower than in HIV-negative patients, where 88% were HBeAg-negative.[26] When comparing the mutation distribution in BCP/PC at 10 loci that can affect HBeAg expression (1762, 1764, 1809–1812, 1814–1816, and 1896), we found that mutations at 1762 and 1764 occurred less frequently in isolates from HBV/HIV co-infected individuals compared to those from mono-infected liver disease patients ($p < 0.05$). Mutations at 1762 and 1764 can reduce the transcription of precore mRNA, resulting in lower expression of HBeAg.[40]

Two genotype E HBV isolates from HBsAg-positive HBV/HIV co-infected individuals had deletions in the pre-S1 and pre-S2 regions. No deletions were detected in genotype D isolates. The 12 amino acid deletion mutant was found in the pre-S2, whereas the 31 amino acid deletion covered the 3′ pre-S1 and 5′ pre-S2. Similar deletion mutants were found in genotype D and E isolates from Sudanese HBV mono-infected liver disease patients,[26] but only in genotype E isolates from blood donors in Sudan[3] and Guinea.[41] The deletion mutants in HBV genotype E isolates from HBV mono-infected liver disease patients were mainly in hepatocellular carcinoma (HCC) patients, whereas this was not the case for genotype D isolates.[26] Deletion mutants in the pre-S have been implicated in the progression of liver diseases in genotypes B and C[42] and the development of HCC in genotype A.[43] The presence of deletion mutants in HBV from HBV/HIV co-infected individuals has been described in genotype A, both subgenotype A1[44] and subgenotype A2.[45] PreS2F22L was found in four isolates from HBsAg-positive HBV/HIV co-infected individuals and was found to be significantly associated with G1896A. PreS2F22L has been significantly associated with cirrhosis[46] and has been shown to be a risk factor for the development of HCC in Chinese patients infected with genotype C[47] and in Indian patients infected with genotype D.[46,48] HIV infection has been intimated to increase the hepatocarcinogenic potential of HBV.[49]

In agreement with other studies in HIV-infected individuals, HBsAg negativity was frequent.[44,50] The HBsAg negativity resulted in both OBI (viral loads <200 IU/ml (<2.3 $\log_{10}$ IU/ml))[10] and HBsAg-covert infection (viral loads >200 IU/ml (>2.3 $\log_{10}$ IU/ml)).[11] In the case of OBI, the inability to detect HBsAg could be as a result of its presence at very low concentrations, below the detection limits of the assay. In the case of the HBsAg-covert infection, the HBsAg negativity may be the result of the presence of detection escape mutations in the 'a' determinant. Although we found no mutational pattern that could differentiate the HBV isolated from HBsAg-negative individuals from those isolated from HBsAg-positive ones, there were a number of mutations that could account for the HBsAg negativity (Table 3). These mutations include ps2R48T, sM133T, sE164G, sV168G, and sS174N and have also been found in strains circulating in HBsAg-negative HIV-infected individuals infected with subgenotype A1 of HBV.[44] Of particular interest is S174N, which has only been detected in HBV from HBsAg-negative HBV/HIV co-infected individuals[44,51] and has been described as a reactivation marker in a single patient infected with subgenotype A2.[51] No primary drug resistance mutations were detected in the present study. This is not unexpected because all patients recruited in this study were treatment-naïve.

The distributions of genotypes D and E of HBV found in HBV/HIV co-infected individuals did not differ from those found in HBV mono-infected liver disease patients. However, the ratio of genotype A to non-A in HIV co-infected individuals was found to be higher than that in mono-infected patients. A predominance of genotype A of HBV in HIV co-infected individuals has also been reported by others.[44,45,52] Subgenotype A1 is the predominant strain circulating in countries neighbouring Sudan, including Kenya[53] and Uganda.[54] In a previous study, it was shown that the only factor that differentiated HBV-positive HIV-infected individuals from those who were HBV-negative was the number of lifetime sexual contacts,[11] thus implicating sexual transmission of this subgenotype.[44] Furthermore, the intra-group divergence of genotype E in HBV/HIV co-infected individuals was significantly higher than that in HBV mono-infected patients. We detected pre-S deletions, mutations associated with severe liver disease, detection-escape mutations, as well as reactivation markers, in the HBV isolated from HIV co-infected individuals. These alterations can have important implications in exacerbating the clinical manifestations of HBV infection, its detection, and clinical management.

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