A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon

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Blood samples ($n=544$) from two different populations (Pygmies and Bantus) in Cameroon, West Africa, were analysed. Serological tests indicated that the anti-hepatitis C virus (HCV) prevalence in Bantus (20·3 %) was higher than that in Pygmies (2·3 %, $P<0·0001$), whereas the distribution of hepatitis B virus (HBV) serological markers was equally high in both populations: in total, 9·4, 17·3 and 86·8 % for HBsAg, anti-HBs and anti-HBc, respectively. HBV genotype A (HBV/A) and HBV/E were predominant (43·5 % each) in both populations, and HBV/D was found in a minority (13 %). The preS/S region was sequenced in nine cases (five HBV/A and four HBV/E) and the complete genome in six cases (four HBV/A and two HBV/E). Subsequent phylogenetic analysis revealed that the HBV/A strains were distinct from the subtypes (subgenotypes) described previously, Ae (A2) and Aa (A1), and in the preS/S region they clustered with previously reported sequences from Cameroon. Based on the nucleotide difference from Aa (A1) and Ae (A2), more than 4 % in the complete genome, the Cameroonian strains were suggested to represent a new subtype (subgenotype), designated HBV/Ac (A3). A high (3·9 %) nucleotide divergence in HBV/Ac (A3) strains suggested that the subtype (subgenotype) has a long natural history in the population of Cameroon. One of the HBV/Ac (A3) strains was found to be a recombinant with an HBV/E-specific sequence in the polymerase reverse transcriptase domain. Further cohort studies will be required to assess detailed epidemiological, virological and clinical characteristics of HBV/Ac (A3), as well as its recombinant form.

INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is one of the major global public health problems. Of the two billion people who have been infected with HBV worldwide, more than 350 million are at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection (Kane, 1995).

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this study are AB194947–AB194955.

Based on a genomic sequence divergence in the entire genome exceeding 8 %, HBV strains have been classified into seven genotypes, denoted A (HBV/A) to G (HBV/G) (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype has been proposed with the tentative designation 'H' (Arauz-Ruiz *et al.*, 2002), which is, however, closely related to genotype F phylogenetically, with a complete genome difference of around 8 % (Kato *et al.*, 2005).

Research on HBV genotypes during the last decade has

demonstrated significant associations between the HBV genotypes and the severity of liver disease, clinical outcomes and the response to antiviral therapies (Kramvis & Kew, 2005). Moreover, it was also demonstrated that the clinical and virological characteristics may also differ among patients infected with the same genotype (Miyakawa & Mizokami, 2003). The existence of different subtypes (subgenotypes) within same genotype helps to explain this for HBV/B, where one of the subtypes (subgenotypes) (widespread in Asia; Ba) possesses a recombination with genotype HBV/C, while another (indigenous to Japan; Bj) does not (Sugauchi *et al.*, 2003). Similarly, two subtypes (subgenotypes) have been reported for HBV/A: one of them, Aa (A′/A1) prevails in sub-Saharan Africa and South Asia, while the other, Ae (A2), is widely distributed in Europe and the USA (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). The subtypes (subgenotypes) of HBV/A show no evidence of distinguishing recombination; nevertheless, they are associated with differences in replicative activity, and in the mechanisms of HBeAg seroconversion as a result of specific nucleotide substitutions in the core promoter and precore regions (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004).

The characterization of isolates from indigenous populations, especially in Africa where HBV is hyperendemic, may assist in revealing the origin of HBV and clarify the many questions about its evolutionary history (Kramvis *et al.*, 2005). The genetic diversity and distribution of HBV genotypes in Central West Africa, particularly in Cameroon, are poorly documented. No data were available for the HBV strains from Pygmies in this region. The objectives of the present study were to assess the prevalence of HBV and hepatitis C virus (HCV) markers among Bantus and Pygmies, to compare the distribution of HBV genotypes and to analyse the genomic characteristics of the HBV/A strain in Cameroon. Six full genome sequences, including four representing a new subtype (subgenotype) of HBV/A and two HBV/E strains from the Cameroonian Pygmies, were analysed.

METHODS

Blood serum samples. Blood serum samples were collected in 1994 from 544 voluntary donors, including representatives of two relatively isolated populations (Bantu and Pygmies) in Cameroon, Central West Africa. The Pygmies enrolled were from two forest encampments in the East province, and the Bantu were enrolled from five provinces across the country (Central, South, North, West and East). None of the donors had clinical symptoms of liver disease. Written informed consent was obtained from all subjects enrolled. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied in each group, their ages and sexes are summarized in Table 1.

Serological assays for hepatitis virus markers and HBV genotyping. HCV (anti-HCV) and HBV serological markers (HBsAg, HBeAg, anti-HBs and anti-HBc) were examined using a chemiluminescent immunoassay (Ortho Clinical Diagnostics).

HBsAg-positive samples were subjected to HBV genotyping using an enzyme-linked immunoassay (EIA) with monoclonal antibodies to type-specific epitopes of the preS2 region (Usuda *et al.*, 1999), using commercial kits (HBV Genotype EIA; Institute of Immunology Co.).

Table 1. The distribution of HBV and HCV serological markers and HBV genotypes among two populations in Cameroon

Population	Bantu ($n=370$)	Pygmies ($n=174$)	P
Male/Female*	177/188	87/82	NS†
Age (years); mean ± SD	34·2 ± 14·5	29·9 ± 9·2	< 0·05
Anti-HCV	75 (20·3 %)	4 (2·3 %)	< 0·0001
HBsAg	33 (8·9 %)	13 (7·5 %)	NS
HBeAg	5 (15·2 %)	0	NS
Anti-HBs	77 (20·8 %)	17 (9·8 %)	0·001
Anti-HBc	322 (87·0 %)	150 (86·2 %)	NS
HBV/A	15 (45·5 %)	5 (38·5 %)	NS
HBV/E	13 (39·4 %)	7 (53·8 %)	NS
HBV/D	5 (15·1 %)	1 (7·7 %)	NS

*Gender and age data were not available for some of the specimens.
†NS, Not significant.

Amplification, quantification of HBV DNA and nucleotide sequencing. DNA was extracted from 27 serum samples, in which HBV/A and HBV/E had been identified by genotyping EIA: 20 (15 from Bantu and five from Pygmies) and seven (only from Pygmies), respectively. Total DNA was extracted from 100 μl serum using a QIAamp DNA mini kit (Qiagen) and suspended in 100 μl storage buffer (supplied by the kit manufacturer). A real-time PCR assay, allowing detection of up to 100 viral DNA copies ml^{-1} (Abe *et al.*, 1999), with slight modifications (Tanaka *et al.*, 2004), was used for HBV DNA screening.

Two overlapping HBV DNA fragments covering the entire genome sequence were amplified using specific primers and PCR conditions that have been described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using a Prism Big Dye v3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX v11.0 (Software Development). The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers AB194947–AB194955.

Sequence analysis. Sequences were aligned using the CLUSTAL W software program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining (NJ) analysis incorporating the six-parameter distance correction method (Gojobori *et al.*, 1982) with bootstrap test confirmation performed on 1000 resamplings using the Online Hepatitis virus database (http://s2as02.genes.nig.ac.jp/). Preliminary trees were constructed for Cameroonian HBV strains obtained in this study and corresponding data of 632 HBV genome sequences available from the GenBank/DDBJ databases (the trees are available from the authors). The final trees presented herein were constructed for Cameroonian strains together with the selected GenBank/DDBJ references including the HBV/A strains of various geographical origins, and representatives of other known human HBV genotypes.

Nucleotide divergence over complete genomes was calculated using the CLUSTAL method implemented in the MEGALIGN software (Clewley & Arnold, 1997).

Detection of recombination. All Cameroonian strains' complete genome sequences were examined for the presence of recombination with other HBV genotypes, as described previously (Robertson *et al.*, 1995). Bootscan analysis implemented in the SimPlot software program (Lole *et al.*, 1999) was performed for each of the strains.

Statistical analysis. All statistical values were calculated using the Mann–Whitney U test, Fisher's exact test and the χ^2 test with Yate's correction, implemented in the STATA v8.0 software program (Stata). Differences were considered significant for *P* values less than 0·05.

RESULTS

Hepatitis virus serological markers and HBV genotypes in Cameroon

Table 1 summarizes results of the serological screening and HBV genotyping. The overall anti-HCV seroprevalence was very high (14·5 %), and was significantly higher in Bantus (20·3 %) than in Pygmies (2·3 %, $P<0{\cdot}0001$), demonstrating that transmission networks of the infection are relatively isolated between two populations (blood transfusion and medical procedures probably contributed to transmission in the Bantus but not in the Pygmies; Kowo *et al.*, 1995). Nevertheless, HBsAg prevalence was equally high in both

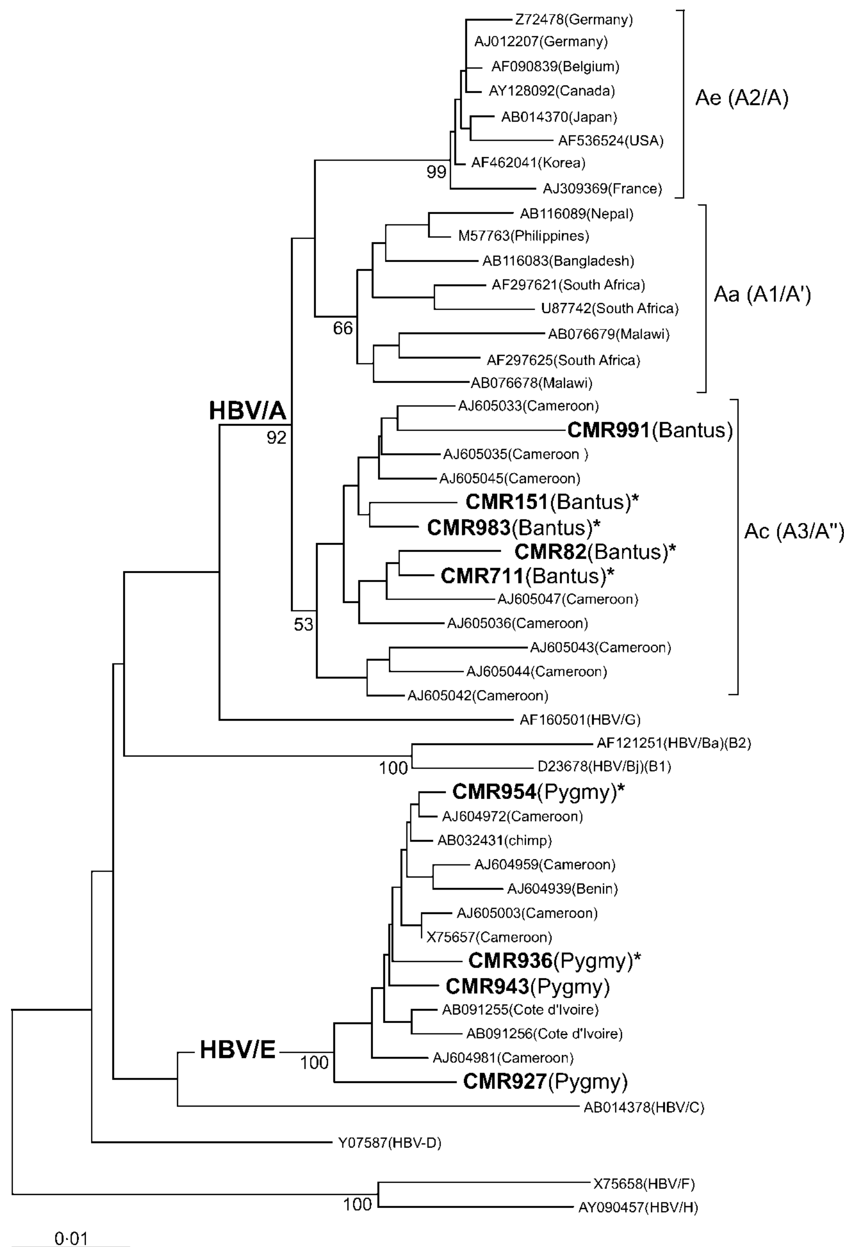


Fig. 1. A phylogenetic NJ tree constructed using the HBV preS2/S nucleotide sequences. Nine strains from Cameroon isolated in this study are indicated in bold. Reference sequences were retrieved from GenBank/EMBL/DDBJ with their accession numbers and origin (in parentheses) indicated. Bootstrap values are indicated in the tree roots. Asterisk (*) marked strains, four HBV/A and two HBV/E, from Cameroon were used for further analyses based on the complete genome sequences.

populations (8·9–7·5 %). HBeAg examined among HBsAg-positive carriers was determined in 10·9 % of cases, all of which were in the Bantus infected with HBV/E (mean age 21·2 years, range 1–30 years). The mean age of HBeAg-negative carriers in the Bantus was 31·6 years, range 17–90 years. All HBsAg-positive carriers among the Pygmies were negative for HBeAg (mean age 29·5 years, range 27–38 years). Thus, the mean age of the HBeAg-negative group was relatively young for HBsAg carriers in both populations in Cameroon, suggesting early HBeAg seroconversion. Anti-HBc seroprevalence was very high in both populations (mean 86·7 %), with no significant difference (86·2 vs 87 %), concordant with a previous report (Ndumbe *et al.*, 1993) and indicating a high incidence of HBV infection in both populations, probably attributable to effective horizontal transmission at a young age, as reported previously in African countries (Kramvis *et al.*, 2005). There was no significant difference in the distribution of the examined viral markers among the Bantu population in different provinces, or among the Pygmies population in the different encampments. A total of 46 serum samples found to be positive for HBsAg were subjected to HBV genotyping using the EIA method. Genotypes A and E identified in 43·5 % of cases were equally predominant in both of the populations, and genotype D was found in a minority (13 %) of cases. No significant difference in distribution of the genotypes was found within the same population in different provinces or between the two populations.

In order to study the molecular genetic characteristics of the prevalent HBV genotypes in Cameroon, 20 HBV/A and seven HBV/E samples, for which sufficient volume was available, were subjected to further investigation. Of the

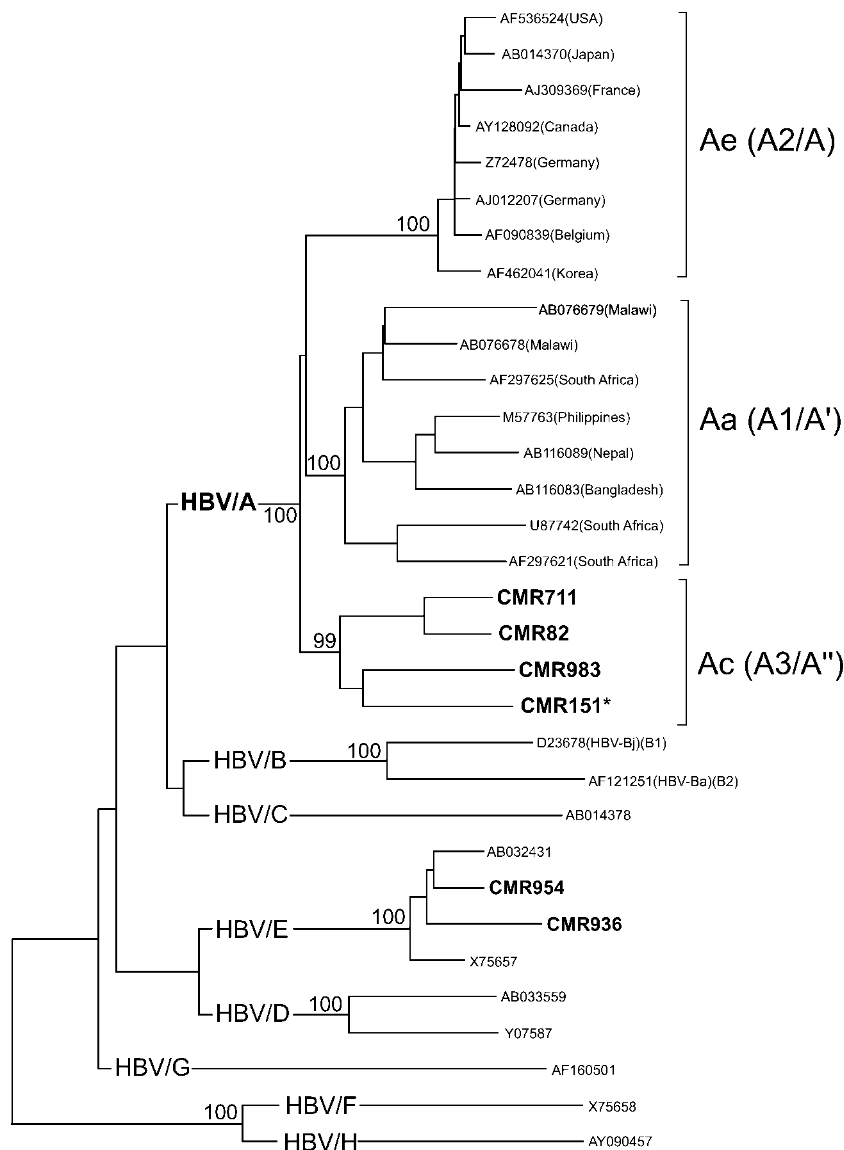
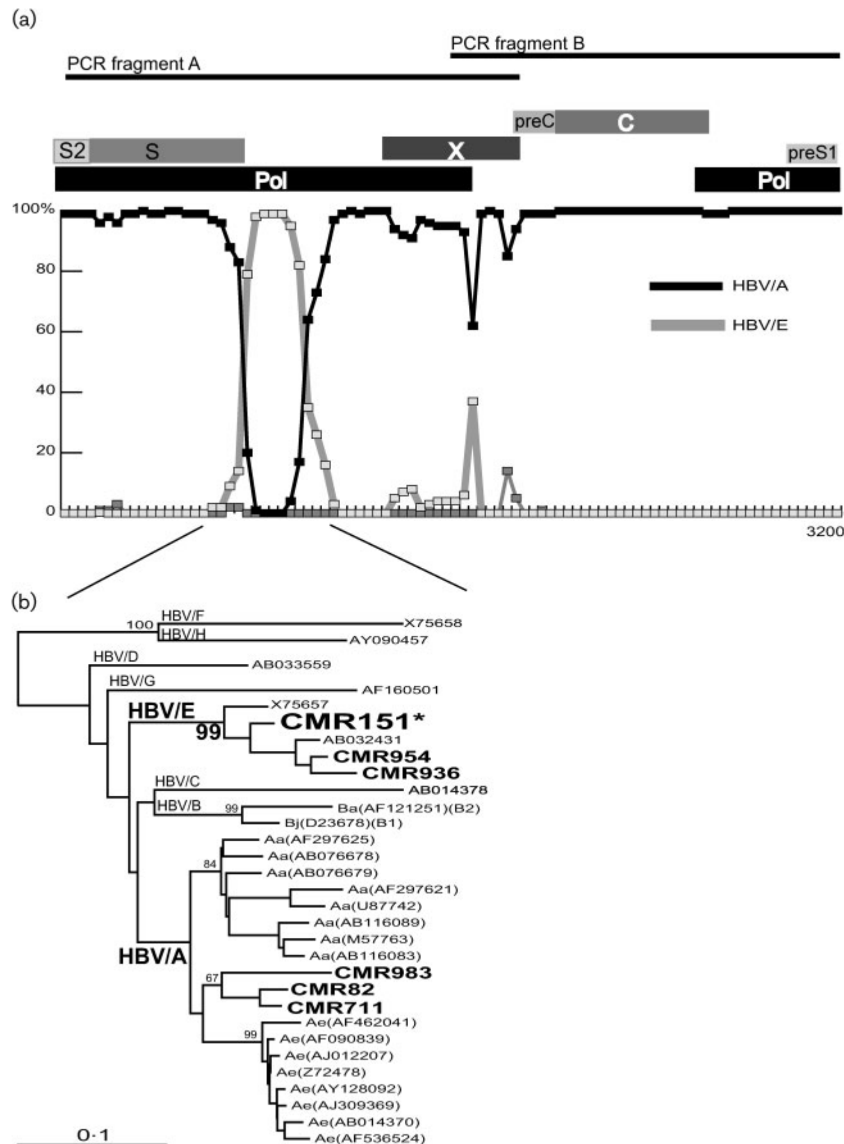


Fig. 2. A phylogenetic NJ tree constructed using the complete HBV genome. Six strains from Cameroon isolated in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. The origins of the previously published HBV/A strains are indicated in parentheses. Bootstrap values are indicated in the tree roots. The strain from Cameroon with the recombination between HBV/A and HBV/E is marked with an asterisk (*).

samples, only 1/27 was HBeAg-positive (HBV/E by EIA), which was obtained from a 1-year-old child, and the rest (26/27) of the HBsAg-positive carriers had undergone HBeAg seroconversion.

HBV DNA quantification, sequencing, phylogenetic relation and genetic diversity of HBV/A subtypes (subgenotypes)

HBV DNA was detected in only 10/27 serum samples: 5/15 Bantus and 5/12 Pygmies. The highest HBV DNA level ($3{\cdot}4 \times 10^{10}$ copies ml^{-1}) was detected in the sample obtained from a 1-year-old child. The rest of the nine positive samples were obtained from (mean) 26-year-old carriers (range 21–30 years), with HBV DNA levels ranging from $1{\cdot}1 \times 10^{3}$ to $7{\cdot}8 \times 10^{5}$ copies ml^{-1}. HBV DNA-negative carriers were (mean) 30·4 years old, range 17–50 years, showing a general tendency of HBV DNA level to decline with age (not statistically significant, probably due to small numbers). HBV large S coding region sequences were successfully amplified from 9/10 samples. The sequences were subjected to a similarity search throughout GenBank/DDBJ using the BLAST search engine, and the most similar strains were used for phylogenetic analysis together with the reference sequences of all known human HBV genotypes. The phylogenetic relationship of the ~800 nt (positions 31–835) preS2/S sequences of the HBV strains is represented in Fig. 1. Within the HBV/A phylogenetic cluster, the HBV/Aa (A1) and HBV/Ae (A2) strains separated out into two clusters and the five Cameroonian strains sequenced in this study together with other Cameroonian strains retrieved from GenBank/DDBJ clustered separately. The Cameroonian strains retrieved from GenBank/DDBJ were previously designated A″ cluster according to partial (Large S) genome sequence (Mulders *et al.*, 2004). The Cameroonian and HBV/Aa (A1) sub-clusters, however, did not have significant bootstrap indexes.



The complete genome of six strains (four HBV/A and two HBV/E) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A and HBV/E were 3221 and 3212 nt, respectively. The phylogenetic analysis of the complete genome sequences (Fig. 2) revealed three distinct bootstrap test supported groups within the HBV/A cluster: HBV/Aa (A1), HBV/Ae (A2), and the third group formed by samples from Cameroon. We denoted the third phylogenetic group as 'HBV/Ac', where 'c' stands for Cameroon and Central Africa. The distinctive grouping of HBV/Ac (A3) strains was also confirmed when preS1/S2, preC/C, and complete Pol genes were analysed phylogenetically. S and X genes were phylogenetically related between the HBV/Aa (A1) and HBV/Ac (A3) groups. Estimated inter-group percentage nucleotide divergence over complete genome sequences consisted of [mean $\pm$SD (range)]: $4{\cdot}9\pm0{\cdot}4$ (4·2–6·1), Aa (A1) versus Ae (A2); $5{\cdot}1\pm0{\cdot}5$ (4·0–6·0), Aa (A1) versus Ac (A3); and $5{\cdot}2\pm0{\cdot}3$ (4·7–5·8), Ae (A2) versus Ac (A3). On the other hand, intra-group percentage nucleotide divergence was similar for HBV/Aa (A1) and HBV/Ac (A3) [mean $\pm$SD (range)]: $3{\cdot}6\pm0{\cdot}8$ (4·0–4·6) and $3{\cdot}9\pm1{\cdot}1$ (1·8–4·8), respectively, and lowest for Ae (A2): $0{\cdot}9\pm0{\cdot}3$ (0·4–1·6) ($P<0{\cdot}0001$).

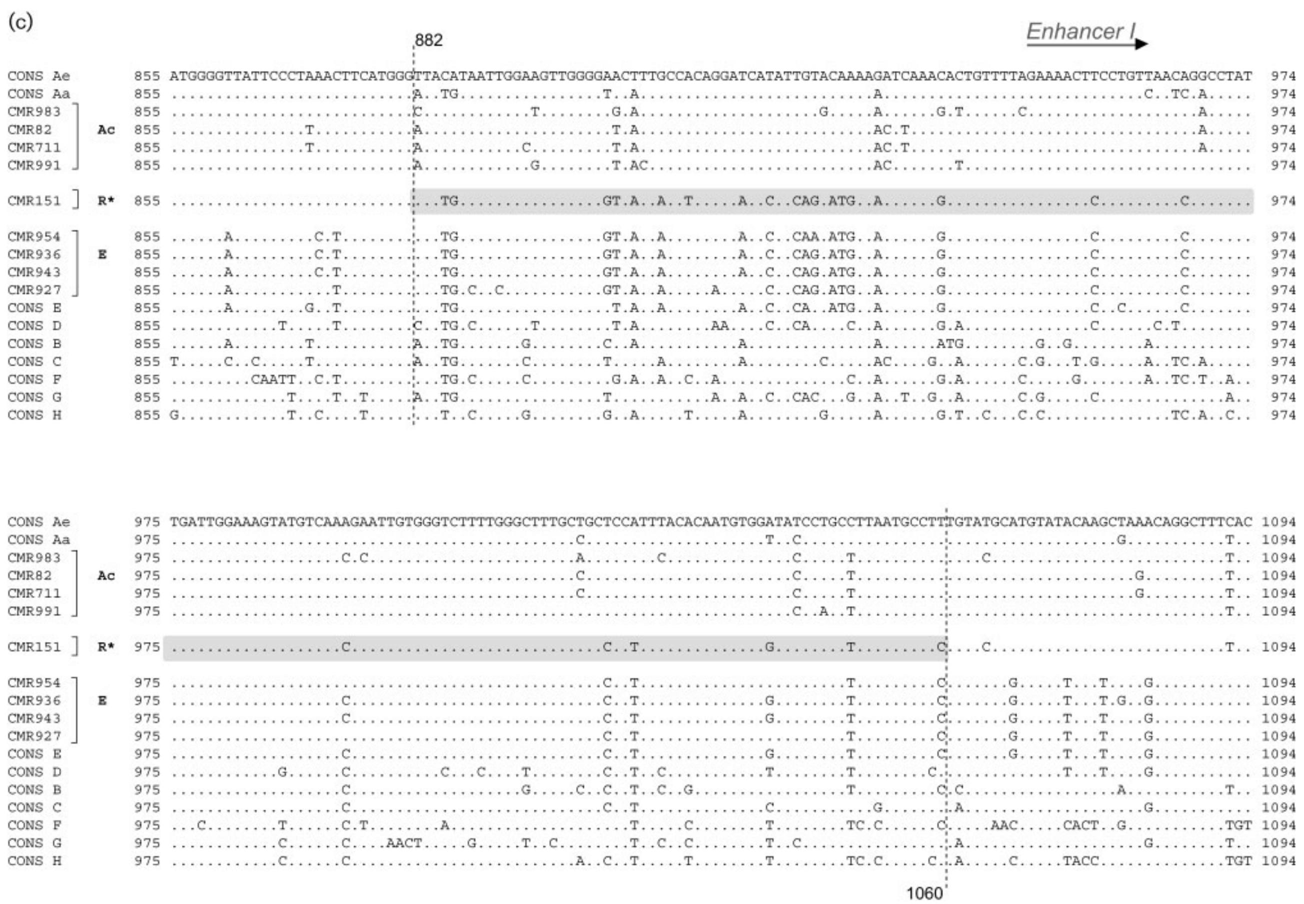


Fig. 3. (a) SimPlot analysis demonstrating the recombination in the non-overlapping part of the polymerase coding region of the CMR151 strain. The strain was subjected to bootscan analysis over the entire genome using the SimPlot program (Lole *et al.*, 1999) with a window size of 300 bp and a step size of 30 bp, under the F84 (ML) model, with bootstrap resampling performed 1000 times. Initially, consensus sequences of each human HBV genotype were used as references; after manual confirmation of the sequence alignment, the final plot was constructed using the consensus of the HBV/A, HBV/E and HBV/D genotypes only. The sequences were obtained from two overlapping PCR fragments, indicated by two lines ('A' and 'B'). HBV genome coding regions are indicated by standard abbreviations (S2, S, Pol, X, preC, C and preS1). (b) The phylogenetic NJ tree constructed using the recombinant segment. Six strains from Cameroon in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. (c) Alignment of all human HBV genotype genome nucleotide sequences in the region corresponding to the recombination in the Cameroonian strain CMR151 (shaded in grey). Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Dashed lines at 882 and 1060 represent the breakpoints.

Recombination

Evidence of recombination between HBV/A and HBV/E was observed in one of the Cameroonian strains (CMR151, marked by an asterisk in Fig. 2). The result of the bootscan analysis for the complete genome sequence of the strain is presented in Fig. 3(a). The phylogenetic tree constructed using the corresponding sequence segment confirmed the grouping of the CMR151 strain together with the HBV/E strains, with strong bootstrap support (Fig. 3b). The recombinant segment corresponded to a part of the non-overlapping HBV DNA polymerase in the reverse transcriptase (RT) domain and a part of the enhancer I–X promoter. (Fig. 3c). The breakpoints at nucleotide positions 882 and 1060 were estimated by mapping the informative sites and using χ^2 confirmation (Robertson *et al.*, 1995).

Enhancer/promoter elements and amino acid characteristics of the HBV/Ac (A3) strains

A comparison of the nucleotide substitutions within the *cis*-acting elements among the four HBV/Ac (A3) strains and the consensus sequences of the HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes) as well as the other HBV genotypes (including HBV/Ba, Bj, B2, B1) are summarized in Table 2. Nine specific nucleotide substitutions were found in HBV/Ac (A3) strains: $G^{1173}A$ (enhancer I–X promoter), $C^{1473}G$, $G^{1512}A$ and $C^{1703}T$ (enhancer II–core promoter), $A^{2742}G$ (S1–promoter), $C^{3021}T$, $C^{3042}T$,

Table 2. Subtype (subgenotype) specific sites (bold) within enhancers and promoter regions of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Consensus sequences were composed according to 60 % or higher incidence at the corresponding nucleotide position.

Region	Position (nt)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
Enhancer I–X promoter (950–1350)	963	**C**	T	T	T	T	T
	1041	T	A	A	A	G	**A**
	1173	G	–	**A**	**A**	**A**	–
	1320	A	A	A	A	A	**G**
	1350	**T**	–	–	–	–	C
Enhancer II–core promoter (1400–1850)	1404	**T**	–	–	–	–	C
	1464	**G**	T	T	T	T	T
	1473	C	**G**	**G**	–	**T**	–
	1484	A	–	–	–	–	**C**
	1511	G	–	–	–	–	**A**
	1512	**T**	**A**	**A**	–	**A**	**G**
	1703	C	**T**	**T**	**T**	A	–
	1727	A	–	–	–	–	**G**
	1740	T	–	–	–	–	**C**
	1809–1812	**TCAT**	-TC-	--C-	----	G--C	G--C
Encapsidation signal (1846–1908)	1888	**A**	G	G	G	G	G
S1–Promoter (2716–2806)	2720	**A**	T	T	T	T	**G**
	2742	A	**G**	**G**	**G**	**G**	–
	2744	C	**A**	**A**	–	–	–
	2777	G	C	T	T	T	**T**
S2–Promoter (2999–3219)	3013–3014	**CA**	--	--	--	--	**GC**
	3021	C	**T**	**T**	**T**	**T**	–
	3042	C	**T**	**T**	**T**	–	–
	3052	T	–	–	–	–	**C**
	3057/60	T/C	C/T	C/T	C/T	C/T	**C/T**
	3069	A	–	–	–	–	**C**
	3072–3073	TG	A-	C-	--	--	**-A**
	3076	T	**C**	**C**	**C**	**C**	–
	3111	**T**	**T**	**T**	**T**	**T**	**C**
	3118	C	–	–	–	–	**T**
	3121	G	–	–	–	–	**A**
	3124	G	–	–	–	–	**A**

T[3076]C and C[3111]T (S2–promoter). Interestingly, three of four strains had substitutions in the Kozak sequence (1809–1812) (Ahn *et al.*, 2003; Tanaka *et al.*, 2004), and two had a basal core promoter double mutation (1762/1764).

Although HBV/Ac (A3) amino acid motifs in general were more similar to HBV/Aa (A1) than to HBV/Ae (A2) motifs, HBV/Ac (A3) strains had some specific sites: Thr[84] in preS1, Ala[146] in Pol/terminal protein, Ser[239], Trp[246], Ser[257] in Pol/spacer, Asp[356], Arg[501], Ser[607] in Pol/RT, and Thr[47] in X proteins when compared with consensus sequences composed according to 60 % or higher incidence at the corresponding amino acid position (Table 3). Pre-core/core amino acid patterns had no specific substitutions among HBV/A subtypes (subgenotypes).

Table 3. Subtype (subgenotype) specific sites (bold) in amino acid sequences of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Consensus sequences were composed according to 60 % or higher incidence at the corresponding amino acid position.

ORF	Position (aa)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
PreS1	54	**Q**	–	–	–	–	**A**
	67	F	–	–	–	–	**L**
	74	**V**	–	–	–	–	I
	84	I	**T**	**T**	**T**	–	–
	86	**A**	T	T	T	T	T
	89	P	–	–	–	–	**S**
	90	A	–	–	–	–	**T**
	91	**V**	–	–	–	–	**I**
PreS2	32	**L**	V	V	V	V	V
	47	S	–	–	–	–	**A**
S	209	L	–	–	–	–	**V**
Pol/terminal protein	17	**E**	G	G	G	G	**G**
	33	E	–	–	A	A	**A**
	74	P	**Q**	**Q**	–	–	–
	102	T	**N**	**N**	–	–	–
	120	N	–	–	–	–	**T**
	146	T	**A**	**A**	**A**	**A**	–
Pol/spacer	236	**T**	–	–	–	–	S
	239	P	**S**	**S**	**S**	**S**	–
	246	R	**W**	**W**	**W**	–	–
	257	F	**S**	**S**	**S**	**S**	–
	269	**Y**	–	–	–	–	H
	271	A	–	–	–	–	**V**
	273	S	–	–	–	–	**N**
	308	S	K	K	–	–	**C**
	334	**Q**	–	–	K	K	K
	338	**K**	E	E	E	E	E
	348	L	–	–	–	–	**R**
Pol/RT	356	E	**D**	–	**D**	**D**	–
	501	W	**R**	**R**	**R**	**R**	–
	607	T	**S**	**S**	**A**	**S**	–
	617	I	**L**	L	–	–	–
	619	H	–	–	**D**	**D**	–
	666	K	**R**	**R**	–	–	–
X region	11	**S**	–	–	–	–	P
	31	**A**	S	S	S	S	S
	34	L	**V**	**V**	–	**F**	–
	47	**S**	**T**	**T**	–	**T**	A
	146	**S**	F	–	–	A	A
	147	**S**	–	–	–	P	P

DISCUSSION

A previous study carried out in Cameroon among the Bantus and the Pygmies (Kowo *et al.*, 1995) demonstrated a high (18·6 %) overall seroprevalence of HCV, which was significantly higher in Bantus (31·7 %) than in Pygmies (11·1 %). The results of the present study also indicate the very high HCV seroprevalence (14·5 %), and support the difference between the two populations. However, in our study, HCV seroprevalence among the Pygmies was lower (2·3 %), which might be attributed to the younger age of examined subjects compared with the cohort previously studied (Kowo *et al.*, 1995). The difference in HCV seroprevalence between the two populations might be explained by exposure of the Bantus to transmission routes such as medical procedures and blood transfusion, to which the Pygmies are not exposed. However, HBV seroprevalence (HBsAg and anti-HBc) was equally high among the two populations and different regions of the country, which is concordant with previous data (Ndumbe *et al.*, 1993). Further epidemiological investigation is required to evaluate factors contributing to the difference in HBV and HCV transmission in the Pygmies, in contrast with neighbouring Bantus.

The only data available on HBV genotypes in Cameroon demonstrated the predominant prevalence of HBV/A in human immunodeficiency virus-positive cohort (Mulders *et al.*, 2004). The present study revealed that both HBV/A and HBV/E are distributed equally in both native populations in Cameroon. The phylogenetic analysis revealed a close relationship in the large S coding region among the Cameroonian strains sequenced in this study and those from the same country available from previous reports (Mulders *et al.*, 2004; Norder *et al.*, 1992). Based on phylogenetic analysis of the complete genome, including four sequences in this study, the presence of a third phylogenetic cluster was confirmed within HBV/A in this study. The cluster was distinct from known HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes), and designated HBV/Ac (A3) (where 'c' stands for Cameroon and Central Africa). The inter-subtype (subgenotype) nucleotide divergence over the complete genome sequences falls within the 4–8 % range that justifies the classification of HBV/Ac (A3) into a distinct subtype (subgenotype) according to the recent proposals on HBV nomenclature (Kato *et al.*, 2005; Kramvis *et al.*, 2005). The high intra-subtype (subgenotype) nucleotide divergence of four HBV/Ac (A3) complete genomes suggests a long natural history of this subtype (subgenotype) within the native population of Cameroon, as has been reported for subtype (subgenotype) HBV/Aa (A1) in southern African Blacks (Kimbi *et al.*, 2004). On the other hand, HBV/E strains obtained from the Pygmies did not group together separately from the strains isolated in different geographical regions, even though the Pygmies represent an isolated population in Africa. The presence of low divergent HBV/E genotype among the Pygmies might not support the hypotheses proposed previously that HBV/E has a very short history in humans (Mulders *et al.*, 2004).

The newly described subtype (subgenotype) HBV/Ac (A3) possesses a combination of the sites specific for either HBV/Aa (A1) or HBV/Ae (A2) within the corresponding enhancer/promoter elements and amino acid motifs (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, the subtype (subgenotype) also has HBV/Ac (A3) unique substitutions. The recombination affecting a short, non-overlapping segment of the polymerase RT domain found in one of the Cameroonian strains is the first event documented to have occurred between HBV/A and HBV/E. The sequencing data generated in the present study could be used to design assays that can discriminate between HBV/Ac (A3) and the other subtypes (subgenotypes) of HBV/A in order to characterize its clinical–virological features. Cohort studies are required to investigate a possible association of HBV/Ac (A3) infection with early HBeAg/anti-HBe seroconversion and low HBV DNA levels in carriers indicated by the tendencies observed on the small number investigated in present study.

At the present time, investigation of HBV molecular heterogeneity, global distribution of HBV genetic forms, including recombination and mutations as well as efficient implications of the data, is one of the major directions in the field of virus research (Kramvis *et al.*, 2005). In this respect, further standardization of the HBV nomenclature and, an efficient and logical classification should be based on a consensus of the accumulated data including recent studies.

In conclusion, the complete genome of the third subtype (subgenotype) of HBV/A, identified in Cameroon, has been analysed and unique nucleotide/amino acid substitutions have been identified within this subtype (subgenotype). The high intra-group divergence suggests that this subtype (subgenotype) represents an indigenous HBV strain with a long natural history. Recombination between this subtype (subgenotype) and genotype E is described.

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