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Exceptional Genetic Variability of Hepatitis B Virus Indicates That Rwanda Is East of an Emerging African Genotype E/A1 Divide

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In Western Africa, hepatitis B virus (HBV) genotype E predominates throughout a vast crescent spanning from Senegal to Namibia and at least to the Central African Republic to the East. Although from most of the eastern parts of sub-Saharan Africa only limited sets of strains have been characterized, these belong predominantly to genotype A. To study how far the genotype E crescent extends to the East, a larger number of HBV strains from Rwanda were analyzed. Phylogenetic analysis of 45 S fragment sequences revealed strains of genotypes A (n = 30), D (n = 10), C (n = 4), and B (n = 1). Twelve genotype A sequences formed a new cluster clearly separated from the reference strains of the known sub-genotypes. Thus, with four genotypes and at least six sub-genotypes and a new cluster of genotype A strains, HBV shows an exceptional genetic variability in this small country, unprecedented in sub-Saharan Africa. Despite this exceptional genetic variability, not a single genotype E virus was found indicating that this country does not belong to the genotype E crescent, but is east of an emerging African genotype E/A1 divide. ***J. Med. Virol. 81:435–440, 2009.***

KEY WORDS: molecular epidemiology; phylogeny; genotyping

INTRODUCTION

Hepatitis B virus (HBV, family Hepadnaviridae, genus *Orthohepadnavirus*) causes chronic infections in more than 350 million people worldwide, killing about one million per year [WHO, 2000]. In sub-Saharan Africa, prevalence rates of hepatitis B surface antigen (HBsAg) are often very high [Pawlotsky et al., 1995; Kurbanov et al., 2005; Makuwa et al., 2006; Kramvis and Kew, 2007a]. Apart from the eight recognized genotypes A–H of HBV [Okamoto et al., 1987; Norder et al., 1993; Stuyver et al., 2000], a new genotype, tentatively designated I, has been assigned recently to strains found in Laos and Vietnam [Jutavijittum et al., 2007; Tran et al., 2008; Olinger et al., 2008]. Most of the genotypes exhibit a distinct geographic distribution [Kimbi et al., 2004; Mulders et al., 2004; Hannoun et al., 2005; Kurbanov et al., 2005; Makuwa et al., 2006; Olinger et al., 2006]. In Northern Africa, genotype D seems to be the most prevalent genotype [Kramvis and Kew, 2007a]. However, in sub-Saharan Africa the genetic diversity and genotype distribution of HBV are somewhat puzzling. Genotype A is dominant in South Africa, Mozambique, and Kenya [Kramvis and Kew, 2007a]. From most other East African countries only few sporadic strains have been genotyped so far [Kramvis et al., 2002; Sugauchi et al., 2003; Kimbi et al., 2004; Hannoun et al., 2005; Kramvis and Kew, 2007a]. Although genotype A has been observed on all continents [Kramvis and Kew, 2007b], it is nowhere genetically as diverse as in Africa, where a variety of sub-genotypes and variants have been found. Therefore it has been suggested that genotype A evolved in Africa before spreading to other parts of the world [Hannoun et al., 2005]. Nevertheless genotype A is relatively rare

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in many parts of Africa. In fact, in West Africa, where genotype A is highly diverse genetically, genotype E is by far the most prevalent genotype. Genotype E predominates throughout a vast crescent spanning from Senegal to Namibia [Mulders et al., 2004; Kramvis and Kew, 2007a] including The Gambia [Dumpis et al., 2001], Ivory Coast [Suzuki et al., 2003], Ghana [Candotti et al., 2006], Angola [Kramvis and Kew, 2007a], Mali, Burkina Faso, Togo, Benin, Nigeria, Central African Republic, and at least the Western part of the Democratic Republic of the Congo (DRC) [Mulders et al., 2004; Olinger et al., 2006; Bekondi et al., 2007]. Except for Cameroon and Gabon, only a few sporadic but diverse strains of genotype A were found in West African countries such as Mali, Burkina Faso, and Nigeria [Mulders et al., 2004; Olinger et al., 2006]. In Cameroon, genotype A is relatively frequent, in particular in HIV carriers [Mulders et al., 2004]. In healthy Bantus and Pygmies the prevalence of genotypes A and E was similar (43.5%) [Kurbanov et al., 2005].

In contrast to genotype A, genotype E has a surprisingly low genetic diversity, suggesting a short evolutionary history in Africa. A recent introduction of genotype E into the general human population is also compatible with its absence in the Americas despite the Afro-American slave trade until 200 years ago. However, its recent introduction would be difficult to reconcile with its vast spread throughout West and Southwest Africa and the long evolutionary history of genotype A in Africa. Thus the geographic distribution and the relative prevalence of the genotypes are of considerable interest for understanding the natural history of HBV in Africa. So far it is not clear how far the genotype E crescent extends to the east. Furthermore, for most countries in Eastern Africa little or no genotype information is available. The first molecular analysis of a larger number of HBV strains from Rwanda is described below. In this country east of the Congo genotype A, including a new variant, dominated and no genotype E was found.

MATERIALS AND METHODS

Clinical Samples

Four hundred eight serum samples were collected between 2001 and 2006 from known HIV-positive women. More than 95% were pregnant and attended one of three different health care centers in and around Kigali, Rwanda. The exact age and nationality of the patients were not known. Blood was drawn after informed consent and according to national ethical guidelines. All samples were tested for HBsAg, anti-HBs, and anti-HBc antibodies using either the Murex kits (Murex Biotech Limited, Dartford, UK) or the AxSYM® test system (Abbott Laboratories), Chicago, IL.

Amplification

DNA was extracted using the QIAamp® DNA Blood Mini Kit (Qiagen, Venlo, NL) following the manufacturer's protocol. Either part or the complete genome of HBV was amplified by two-round PCRs as described previously [Olinger et al., 2006]. In case only the S fragment PCR was positive, amplification was repeated with less stringent conditions (3 mM $MgCl_2$ and an annealing temperature lowered by 2°C).

Sequencing and Phylogenetic Analysis

The positive nested PCR products were purified and sequenced as described before [Olinger et al., 2006]. Phylogenetic analysis was performed using the MEGA v3.1 software [Kumar et al., 2004] with the neighbor-joining and Kimura 2-parameter methods. Distance calculations were also carried out with MEGA v3.1 using the pairwise and within group means options. All S fragment and complete genome sequences were submitted to EMBL/GenBank/DDBJ under accession numbers FM200180-216 and FM199974-81.

RESULTS

Serology and PCR

One hundred fifty one of the 408 samples were HBsAg positive, 252 were anti-HBc, and 203 anti-HBs positive (Table I). Eight and 19 samples could not be tested for anti-HBc and anti-HBs, respectively, due to a shortage of serum. Fifty-five of the 408 samples (13.5%) were HBV PCR positive. Thirty-seven of these were from HBsAg positive individuals, whereas about one third (18/55) of the HBV strains were obtained from patients with occult infections (Table I). Eight strains were recovered from HBsAg positive patients who were negative for both anti-HBs and anti-HBc.

Sequences and Genotypes

The S fragment was available for all but 10 PCR positive and for eight samples the complete genome

TABLE I. HBV Serology and PCR Results for the 408 HIV-Positive Patients From Rwanda

	HBsAg(+)/ anti-HBc(+)	HBsAg(+)/ anti-HBc(−)	HBsAg(+)/ anti-HBc (not tested)	HBsAg(−)/ anti-HBc(+)	HBsAg(−)/ anti-HBc(−)	HBsAg(−)/ anti-HBc (not tested)	∑
Anti-HBs positive	44	4	0	146	9	0	203
Anti-HBs negative	35	60	0	24	67	0	186
Anti-HBs not tested	1	3	4	2	5	4	19
Total	80	67	4	172	81	4	408
PCR positive	24	11	2	14	4	0	55

sequence was obtained. Phylogenetic analysis of the S fragment sequences (n = 45) revealed that 30 strains (67%) were of genotype A, 16 of which were of sub-genotype A1 (Fig. 1a) and the majority of which were most closely related to a strain from Congo (DQ020002). Strain 49063 showed the maximal similarity with a strain from Italy (EF514279). Twelve genotype A sequences clustered together, but separately from the reference strains of the different A sub-genotypes. This new cluster of genotype A was supported by a bootstrap value of 85. One strain (49057) clustered with sequences 24063 and 24074 from Nigeria, which have previously been suggested to belong to a new sub-genotype A5 [Olinger et al., 2006]. The status of this group of sequences still awaits clarification based on full genome sequences. Another strain (49008), which showed the highest similarity to strains from Mali (AM180623) and Gambia (AY934763), grouped with an A3 reference

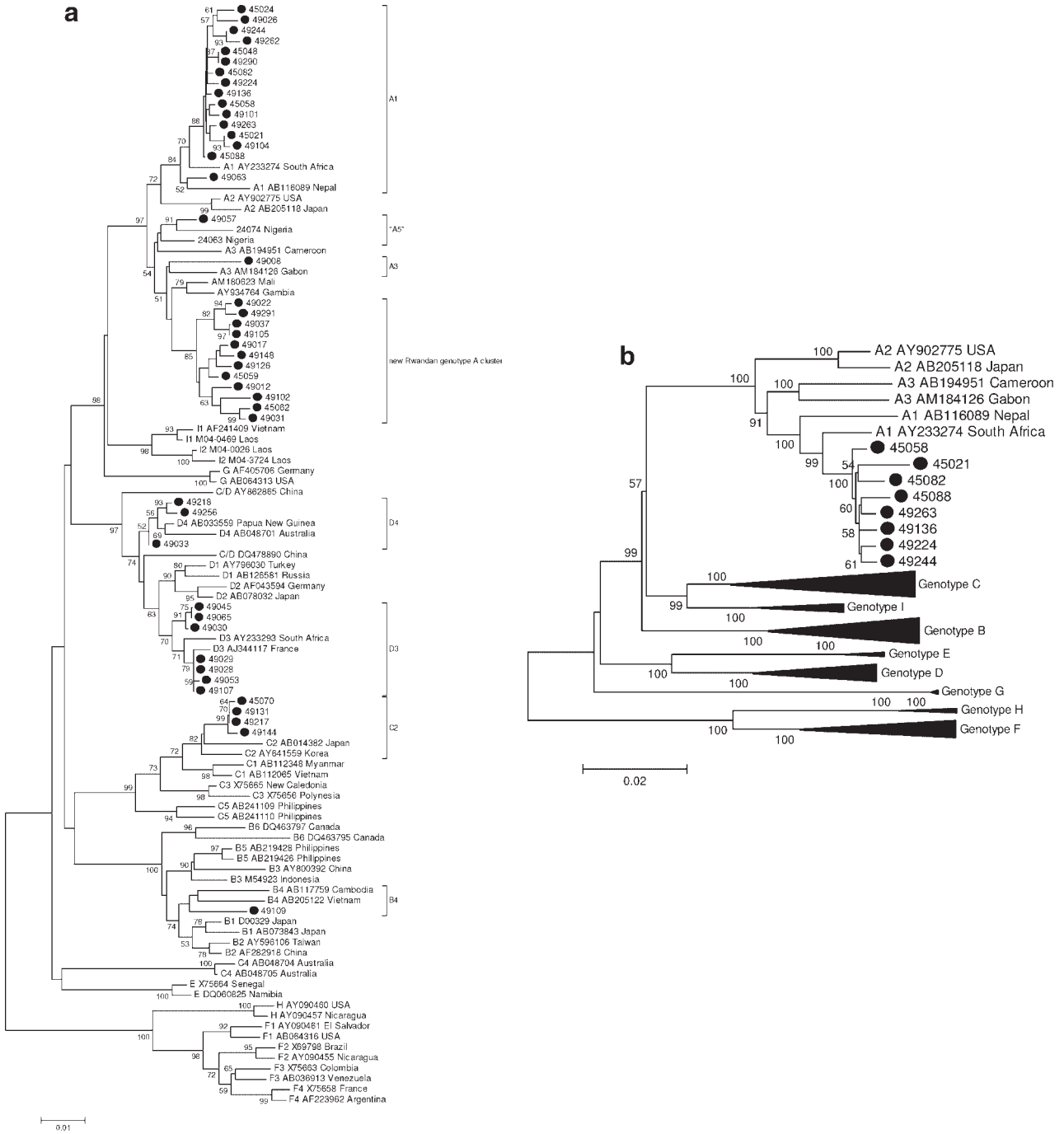

Fig. 1. **a**: Phylogenetic analysis of 45 HBV strains from Rwanda (●) based on 825 nt of the S fragment. "A5" designation according to Olinger et al. [2006]. **b**: Phylogenetic analysis of eight full genome HBV strains from Rwanda (●).

strain. A single strain (49109) belonged to sub-genotype B4 and was related most closely to sequence AY033072 and to AB031266 from Vietnam; four strains belonged to sub-genotype C2, which showed the maximal similarity to EU075342, EU075336, and EU075334 from China and 10 viruses (22%) grouped with genotype D. The latter were either sub-genotyped as D3 (n = 7), of which two different lineages were observed and which were most closely related to European strains (e.g., EU414141 and EU686583), or D4 (n = 3) strains (Fig. 1a) which were most similar to AB048703 from Australia and AB033559 from Papua New Guinea. All strains for which the full genome was available belonged to sub-genotype A1 (Fig. 1b) which was fully consistent with the phylogeny based on their S fragment only.

Genetic Variability of Genotypes and Sub-Genotypes

The maximal genetic distance between the S fragment sequences of the 45 Rwanda strains was 8.36%. Genotype A sequences showed a maximal genetic distance of 4.64% (mean 2.48%), which was about twice as high as the distance between genotype D sequences (maximum 2.18%, mean 1.10%). Among the sub-genotypes and variants the maximal genetic diversity was highest within the new group of genotype A sequences, with 2.74% (mean 1.35%), followed by A1 with 2.00% (mean 0.78%), D3 with 1.15% (mean 0.59%), D4 with 0.37% (mean 0.20%), and C2 with 0.36% (mean 0.18%). By BLAST search, the closest relatives to a sequence belonging to the new cluster of genotype A (45059) were strains AY934764 with a genetic distance of 2.09% and AM180623 with 2.22%. The maximal diversity among the eight A1 strains over the complete genome was 1.73% (mean 0.98%).

Nucleotide and Amino Acid Sequence Analysis

Fourteen strains showed ambiguous nucleotides in up to seven positions (49033) in a region of 825 nucleotides of the S fragment. With one exception (49224) ambiguous nucleotides were detected in all full genome sequences (genotype A1) with a maximum of nine in sample 49263. No insertions or deletions were found. Three strains (49045, 49065, 49148) showed internal stop codons in the S gene. In the region of the a determinant (amino acids 124–147) [Zuckerman and Zuckerman, 2003], two of the most frequent vaccine- and drug-selected amino acid mutations [Table XIV.4, Carman et al., 2005] were observed in some strains: D144A (45024, 49026, 49244, 49262) and G145R (49008). Mutations in these positions are often associated with anti-HBs immune pressure [Carman et al., 2005]. While all D144A mutants were of sub-genotype A1, the G145R mutant belonged to sub-genotype A3. All but one (45024) of the samples with these mutations, were from patients who had received standard anti-retroviral therapy with stavidine, lamivudine, and nevirapine for at least 6 months. Two patients were HBsAg positive (49244 and 49262), while the other three were HBsAg negative, but anti-HBc positive. Two complete genome sequences were obtained from patients with occult infections (45082 and 45088), but no mutations typical for both strains were found.

DISCUSSION

This study shows a surprising and for sub-Saharan Africa exceptionally high diversity of HBV strains. Four different genotypes (A, B, C, and D), three of which had a prevalence above 8%, and at least six different sub-genotypes (A1, A3, B4, C2, D3, and D4) were found within and around Kigali, the capital of the small East African country Rwanda. Only the vast Republic of South Africa seems to have a similar HBV geno- and sub-genotype diversity. Most other sub-Saharan countries have essentially two genotypes: A and E. Except for Cameroon, where both genotypes have a similar prevalence [Kurbanov et al., 2005], either one of the two genotypes dominates.

In Rwanda, two-thirds of the strains belonged to genotype A. This genotype seems to dominate in all East African countries for which larger sets of HBV strains have been characterized such as South Africa, Mozambique, and Kenya [Kramvis and Kew, 2007a]. The majority of genotype A strains (16/30) in Rwanda belonged to A1, the main "African" A sub-genotype. Interestingly, this sub-genotype was also found in most countries along the eastern coast of Africa from the Republic of South Africa to the Horn of Africa, although only few sequences are available from most of these countries [Kramvis and Kew, 2007a]. Thus our results provide further evidence of a dominance of A1 sub-genotype when moving towards Eastern Africa.

One A strain clustered with strains of sub-genotype A3, which was so far found, for example, in Cameroon, DRC, Gabon, and the Gambia [Mulders et al., 2004; Hannoun et al., 2005; Makuwa et al., 2006]. Another single strain was most closely related to strains from Nigeria, which, have previously been suggested to form a provisional new sub-genotype ("A5") [Olinger et al., 2006]. Forty percent of genotype A strains in Rwanda, however, belonged to a separate cluster supported by a high bootstrap values and distinct from all known genotype A sub-genotypes. At least on the S gene the between-group mean genetic distance from the closest sub-genotype (A3, six published sequences from Cameroon and Gabon) was 2.11%. The maximal genetic distance of 3.01% was observed between strains AM184126 from Gabon and 49291. The phylogenetic characteristics and the geographic confinement of the large number of strains may well be suggestive of a new sub-genotype which can, however, only be confirmed by full-length sequences. In any case, these strains from Rwanda extend further the genetic variability of genotype A, possibly beyond sub-genotype A3 [Mulders et al., 2004] and the other new variants found in West Africa ("A4" and "A5") [Olinger et al., 2006]. Interestingly, except for A1, A2, and to a lesser extent A3 strains, the other genotype A variants seem very confined

geographically. This pattern is confirmed further by the distinct variant of genotype A found in Rwanda. The high genetic diversity of genotype A, which is higher in Africa than anywhere else suggests that this genotype has a long evolutionary history in Africa and probably has spread from Africa to other parts of the world [Hannoun et al., 2005]. On the other hand the low prevalence and the geographical limitation of the new genotype A variants [Olinger et al., 2006] in Africa suggest their compartmentalized evolution in different regions in Africa. This study provides important evidence to support this view. Thus, genotype A is in contrast to the high prevalence and the low genetic diversity of genotype E in Western Africa.

Another notable result is the conspicuous absence of genotype E from Rwanda. In the West-African bulge and along the West-African coastal countries genotype E is the most prevalent genotype. While at least in the Western part of Congo the majority of the few strains reported belonged to genotype E [Mulders et al., 2004], not a single strain of this genotype was found in neighboring Rwanda. Thus, Rwanda is not part of the vast African genotype E crescent. The geographical location of the country provides further important evidence of a clear genotype E/A1 divide between West and East Africa with Rwanda on the east of this divide, and running through Congo. Thus our results also suggest that east of the genotype E/A1 divide pockets of genotype A variants can be found similar to those described in Western Africa [Olinger et al., 2006]. While the diversity of genotype A viruses found in Africa continues to increase, this is not the case for genotype E, which has no sub-genotypes.

Interestingly, more than 20% of the sequences from Rwanda belong to sub-genotypes D3 and D4 which otherwise in Africa were only reported sporadically from, for example, South Africa [Kimbi et al., 2004; Kew et al., 2005], Ghana (D4) [Candotti et al., 2006], and Somalia (D4) [Norder et al., 2004]. Genotype B and C strains were only reported rarely from African countries such as South Africa [Bowyer et al., 1997] and Egypt [Zekri et al., 2007] and now also from Rwanda where C2 strains represent nearly 9% of all strains. This sub-genotype is normally observed in Far Eastern countries [Huy et al., 2004; Chan et al., 2005; Tanaka et al., 2005] and has to our knowledge not yet been reported from any other African country.

Despite the observed co-circulation of several HBV genotypes, no obvious mixed infection of genotypes was detected based on the electropherograms of the S fragment and the complete genome sequences. Although several samples showed ambiguous nucleotides, their number and positions indicated the presence of quasi-species rather than mixed infections.

One hundred fifty one samples were positive for HBsAg, but surprisingly only about one quarter of these (37/151) were PCR positive. About two-thirds of the latter (25/37) were from patients that were positive for at least one antibody (anti-HBs, anti-HBc) and only eight were PCR positive among the HBsAg positive and antibody double-negative (8/60). This suggests that in particular in the latter group a number of samples were false positive in the HBsAg ELISA. Frequent false-positivity in HBsAg ELISA tests has been reported before in Rwanda [Pirillo et al., 2007], but also during pregnancy [Weber et al., 1999]. Nevertheless, eight HBsAg positive patients were PCR positive but had no antibodies, a constellation which may be explained by either early infection or HIV-associated immune-suppression (or a combination of both). Among the 172 HBsAg negative patients with anti-HBc 14 had occult infections. Among the four weak PCR positives in HBsAg and anti-HBc double negative samples, three were also anti-HBs negative, a pattern compatible with very early acute infection. Antiretroviral treatments of the patients may have further complicated the serological status of this cohort. No association between genotype and serological pattern could be observed.

In summary, Rwanda does not belong to the genotype E crescent, but is east of an African genotype E/A1 divide. Strains from Rwanda show an exceptional genetic variability, unprecedented in sub-Saharan Africa, with four genotypes, at least six sub-genotypes and a new cluster of genotype A strains.

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