African, Amerindian and European hepatitis B virus strains circulate on the Caribbean Island of Martinique

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Ten Hepatitis B virus (HBV) genotypes, as well as numerous subgenotypes, have been described in well-characterized ethnogeographical populations. Martinique has been at a crossroads between Africa, Europe, India and the Americas because of the slave trade (17th–19th centuries), followed by an important immigration of Indian and West African workers. In this work, we aimed to study the molecular epidemiology of HBV infection in Martinique according to this unique settlement pattern. To that end, blood samples from 86 consecutive HBV-infected patients from the main hospitals of the island, were retrospectively analysed. Direct sequencing of the pre-S1 or pre-C-C region or complete genome sequencing, followed by phylogenetic analyses were performed. HBV genotypes were: HBV/A1 (68.6 %), HBV/A2 (10.5 %), HBV/D, mainly HBV/D3 and HBV/D4 (8.1 %), HBV/F (3.5 %), and also HBV/E (2.3 %), two strains isolated from two West-African patients. Moreover, 74 % of the HBeAg-negative strains harboured classical pre-C-C mutations, and most HBV/A1 strains also containing specific mutations. Finally, various patterns of deletion mutants in pre-S and pre-C-C regions were found. In conclusion, our findings point to historical and migration-related issues in HBV-genotype distribution suggesting that HBV/A1, but not HBV/E, was imported from Africa during the slave trade, and further supporting the hypothesis that HBV/E has emerged recently in West Africa (<150 years). Potential origins of ‘European’ HBV/A2 and HBV/D3, ‘Amerindian’ HBV/F, and HBV/D4 strains are also discussed. Such HBV genetic diversity, beyond its epidemiological interest, may have a clinical impact on the natural history of HBV infection in Martinique.

INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is a major global health care problem: two billion people have been infected by HBV, and more than 350 million are chronically infected and thus at high risk of developing cirrhosis or hepatocellular carcinoma (Shepard et al., 2006).

Ten Hepatitis B virus (HBV) genotypes, as well as numerous subgenotypes, have been described in well-characterized ethnogeographical populations. Martinique has been at a crossroads between Africa, Europe, India and the Americas because of the slave trade (17th–19th centuries), followed by an important immigration of Indian and West African workers. In this work, we aimed to study the molecular epidemiology of HBV infection in Martinique according to this unique settlement pattern. To that end, blood samples from 86 consecutive HBV-infected patients from the main hospitals of the island, were retrospectively analysed. Direct sequencing of the pre-S1 or pre-C-C region or complete genome sequencing, followed by phylogenetic analyses were performed. HBV genotypes were: HBV/A1 (68.6 %), HBV/A2 (10.5 %), HBV/D, mainly HBV/D3 and HBV/D4 (8.1 %), HBV/F (3.5 %), and also HBV/E (2.3 %), two strains isolated from two West-African patients. Moreover, 74 % of the HBeAg-negative strains harboured classical pre-C-C mutations, and most HBV/A1 strains also containing specific mutations. Finally, various patterns of deletion mutants in pre-S and pre-C-C regions were found. In conclusion, our findings point to historical and migration-related issues in HBV-genotype distribution suggesting that HBV/A1, but not HBV/E, was imported from Africa during the slave trade, and further supporting the hypothesis that HBV/E has emerged recently in West Africa (<150 years). Potential origins of ‘European’ HBV/A2 and HBV/D3, ‘Amerindian’ HBV/F, and HBV/D4 strains are also discussed. Such HBV genetic diversity, beyond its epidemiological interest, may have a clinical impact on the natural history of HBV infection in Martinique.

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The GenBank/EMBL/DDBJ accession numbers for the complete and partial Martinican HBV sequences are HE974362 to HE974384 and HG329753 to HG329841.

Two supplementary figures are available with the online version of this paper.

Based on a genomic sequence divergence >7.5 % over the entire genome, HBV strains have been classified to date into eight genotypes (A to H), and recently, two new genotypes have been proposed, I and J, identified respectively in South-East Asia and Japan. Subgenotypes have also been identified, to date for genotypes A, B, C, D and F, based on inter-nucleotide divergences between 4 % and 7.5 %, sustained by robust topology in phylogenetic analyses (Kramvis et al., 2008). As investigations progress, the number of proposed HBV subgenotypes isolated from well-characterized ethnogeographical populations has been growing: at least 6 in genotype A (HBV/A); 9 in HBV/B; 14...
in HBV/G; 6 in HBV/D and 4 in HBV/F (Araujo et al., 2011; Devesa et al., 2004; Pourkarim et al., 2010; Yousif & Kramvis, 2013). Numerous inter-genotype recombinant strains have also been reported worldwide.

HBV genotypes and subgenotypes share a characteristic geographical distribution: HBV/A is ubiquitous; HBV/B and HBV/C are endemic to China, Japan and Southeast Asia; HBV/D is mainly found in the Mediterranean area, in Eastern Europe, and in a region spanning the Near East to India; HBV/E originates from West and sub-Saharan Africa; genotypes F and H are restricted to Central and South America; and HBV/G is reported in the United States and France, usually in association with genotype A. (For review see Abdou Chekaraou et al., 2011; Araujo et al., 2011; Hübschen et al., 2011; Kramvis et al., 2008; Mulyanto et al., 2011; Norder et al., 2004 and references therein).

This issue may also be of clinical interest for the management of infected patients, since different HBV genotypes and subgenotypes are increasingly associated with different features of disease, such as severity or response to antiviral therapies (see Kim et al., 2011) and references therein).

Martinique is a French island in the eastern part of the Caribbean bow. The first inhabitants, so-called ‘Amerindians’, are believed to have originated from South America (region currently comprising Venezuela and Guyana). Then, between the seventeenth and nineteenth centuries, in conjunction with French colonization, millions of people were brought to Martinique via the slave trade from Western, sub-Saharan Africa and even from Central and Eastern Africa (Thomas, 1997). After the abolition of slavery in the middle of the nineteenth century, thousands of new manual labourers migrated to Martinique. This second wave of immigration came mainly from Africa (particularly from the Republic of the Congo) and to a lesser degree from India (Flory, 2011).

Little is known about HBV infection in Martinique. Only one seroprevalence study dating from 1988 has been published. It reported an HBsAg positivity rate of 1.3 % among blood donations (Monplaisir et al., 1988). Since then, prevalence rates extrapolated from partial hospital data suggest that HBsAg seroprevalence ranges between 0.6 and 1.3 % (unpublished data, G. Lagathu, & R. Césaire, personal communication).

We thus report here unique data on the molecular characterization of HBV strains spreading in Martinique.

RESULTS

Demographics

Fifty-three men and 33 women were enrolled (sex ratio=1.61). The mean age was 41.9 ± 14.1 years for men (range 11–83) and 38.7 ± 6.1 for women (range 14–75). Most patients were from Martinique but two originated from Brazil, and one each from France, French Guyana, Haiti, Benin and Nigeria.

Virological results

Fifteen sera were HBeAg positive (17.5 %), three were positive for IgM anti-HBc. HBV viral load was <3 log IU ml⁻¹ for 38 samples (44.2 %), from 3 to 5 log IU ml⁻¹ for 31 (36.3 %), and >5 log IU ml⁻¹ for 17 (19.8 %) (Table 1). HBeAg positivity was significantly correlated with high viral load (P<10⁻³). Seventy-one patients (82.5 %) were anti-HBeAb positive. A clinical pre-C-C mutant profile was found in 45 of 61 HBeAg-negative patients (73.8 %) (See below).

Data for concomitant viral infections were documented: 9 of 65 (13.8 %) were HIV-1 positive; 1 of 67 (1.5 %) was HCV positive and 4 of 53 (7.5 %) were HTLV-1 positive. However, we found no positive sample for anti-HDV antibodies, suggesting a very low prevalence of HDV infection in Martinique.

Genotypes and subgenotypes

Eighty samples out of 86 (93 %) could be genotyped, 77 in the pre-S1 region (Fig. 1), 56 in both pre-S1 and pre-C-C regions, and three in the pre-C-C region only (Fig. 2). Complete HBV sequences were characterized for six HBV/D, the two HBV/E and the three HBV/F and for six HBV/A1 and six HBV/A2 strains randomly chosen among the HBV/A group (Fig. 3). No discordance was observed in genotyping results obtained from pre-S1 and/or pre-C-C regions or with complete genomic sequences.

Table 1. Demographic and virological data of Martinican patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
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<tbody>
<tr>
<td>Demographics</td>
<td></td>
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<tr>
<td>Age ≤29 (years)</td>
<td>22 (25.6)</td>
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<tr>
<td>Age 30–49 (years)</td>
<td>41 (47.7)</td>
</tr>
<tr>
<td>Age ≥50 (years)</td>
<td>23 (26.7)</td>
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<tr>
<td>Male (%)</td>
<td>53 (61.6)</td>
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<tr>
<td>HBV serological markers</td>
<td></td>
</tr>
<tr>
<td>IgM anti-HBcAb positive (%)</td>
<td>3 (3.5)</td>
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<tr>
<td>HBeAg positive (%)</td>
<td>15 (17.4)</td>
</tr>
<tr>
<td>HBV viral load</td>
<td></td>
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<tr>
<td>VL ≤3 log IU ml⁻¹ (%)</td>
<td>38 (44.2)</td>
</tr>
<tr>
<td>VL 3–5 log IU ml⁻¹ (%)</td>
<td>31 (36.0)</td>
</tr>
<tr>
<td>VL &gt;5 log IU ml⁻¹ (%)</td>
<td>17 (19.8)</td>
</tr>
<tr>
<td>Other serological data</td>
<td></td>
</tr>
<tr>
<td>Anti-HDVAb positive (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-HIVAb positive (%)</td>
<td>9 (13.8) *</td>
</tr>
<tr>
<td>Anti-HCVAb positive (%)</td>
<td>1 (1.5) †</td>
</tr>
<tr>
<td>Anti-HTLVAb positive (%)</td>
<td>4 (7.5) ‡</td>
</tr>
</tbody>
</table>

N, Number; VL, viral load.

* n=65.
† n=67.
‡ n=53.
 Altogether, HBV genotypes/subgenotypes were distributed as follows: HBV/A, 79.1% (HBV/A1, 68.6%; HBV/A2, 10.5%); HBV/D, 8.1% (HBV/D3, 3.4%; HBV/D4, 4.7%); HBV/E, 2.3%; HBV/F, 3.5% (HBV/F2, 2.3%; HBV/F4, 1.2%) (Figs. 1–3; Table 2).

**HBV/A.** Fifty-six pre-S1 and six complete HBV/A1 sequences were obtained. Over the complete genome sequence, HBV/A1 strains showed a mean genetic diversity of 2.06 ± 0.36%. Thirteen HBV/A1 strains (but no HBV/A2) harboured various nucleotide deletion patterns in pre-S1 and/or pre-S2 gene, ranging from 3 to 243 nt. We found: five pre-S1 deletions (three 1 aa, one 8 aa, one 81 aa); one 74 aa deletion encompassing the pre-S2 initiation codon (this sequence, B54, also possessed a stop codon mutation at aa 43 (W43*) in pre-S1 protein); and seven pre-S2 deletions (7- to 18 aa) (Fig. 4).

Kramvis and Kew reported earlier some HBV/A1-specific substitutions at position nt1809–1812, just upstream the HBeAg AUG codon (Kramvis & Kew, 2007). In accordance to their findings, all 41 Martinican HBV/A1 strains sequenced in pre-C-C region harboured double (n=32) or triple (n=9) mutations at these positions (see below).

Extensive phylogenetic analyses were performed with all the HBV/A1 sequences – both complete and pre-S1 – available in GenBank (Kramvis & Paraskevis, 2013; Pourkarim et al., 2010) (Figs. S1 and S2 available with JGV online). All complete Martinican sequences, and all but one pre-S1 sequences were randomly distributed within the so-called ‘Asian/American’ HBV/A1 cluster, and not with the ‘African’ cluster. Moreover, no Martinican clade was individualized.

Patients infected with HBV/A1 strains were more often HBeAg negative than those infected with HBV/A2 or HBV/D (P<0.01 and P<0.05 respectively) (Table 2). They were also older and had lower viral load (although not significantly). They were less often co-infected with HIV (3/59 patients) than patients with HBV/A2 or HBV/D, 4/9 and 2/7 respectively (P<0.0001 and P<0.01 respectively).

Most HBV/A1-infected patients originated from Martinique, but HBV/A1 strains were also found in two patients born in Brazil (B01, B68), one from French Guiana (B67) and one from Haiti (B33).

**HBV/A2.** Nine patients were infected with HBV/A2 strains of which six were completely sequenced. The mean genetic diversity over the entire genome sequence was 0.69 ± 0.35%.

Three strains were identical (B15, B74, B82) in the pre-S1 region, and B15 and B74 had a divergence of only 0.6% in the complete sequence, suggesting a common infecting strain. Contrary to HBV/A1, HBV/A2 strains showed no pre-S deletions.

Most HBV/A2 (7/9) infected patients were men. Five were HBeAg positive and their HBV viral load was slightly higher (but not significantly) than that of HBV/A1 patients (4.6 ± 3.8 vs 3.8 ± 1.5 IU ml⁻¹). Four of them (4/9, 44%) were co-infected with HIV, including one patient from metropolitan France.

**HBV/D.** Seven patients were infected with HBV/D strains. As described previously (Schaefer, 2005), we found the known 33 bp deletion (nt2859-nt2892) in all HBV/D sequences (Fig. 4). One strain (B58) also had one 1 aa deletion at the end of the pre-S1 protein. Three strains belonged to HBV/D3 and 4 to HBV/D4 subgenotypes. Complete genomic sequences were obtained for six of them and the mean genetic diversity was 0.44% and 1.8 ± 0.4% for the two HBV/D3 and the four HBV/D4, respectively.

Few complete HBV/D4 sequences have been described in the literature: three from native Australians, one from Papua-New Guinea, one from Spain, and more recently three in Haiti (Andernach et al., 2009) and three in a Canadian Inuit population (Osiowy et al., 2011). More partial HBV/D4 sequences have also been described (Andernach et al., 2009, Norder et al., 2004, Osiowy et al., 2011, Santos et al., 2010) mainly in the S gene. Phylogenetic analyses with all published D4 partial sequences confirmed that Martinican strains clearly belong to the HBV/D4 subgenotype but do not form a separate cluster (data not shown).

**HBV/E.** Only 2 HBV/E strains were identified and fully characterized in our cohort from two adult patients from Benin and Nigeria.

**HBV/F.** Three strains belonged to the HBV/F genotype. HBV/F is found mainly in South American natives, i.e. the suspected original population of Martinique. Therefore, we fully explored the three HBV/F isolates, B18, B24 and B26. B18 and B26 were HBV/F2 and B24 was HBV/F4. The HBV/F2 strains harboured a high nucleotide sequence similarity (more than 97%) although B26 showed a 33 nt deletion in its pre-S2 region, and a point mutation (Methionine to Valine) in a pre-S2 initiation codon (Fig.
had low viral loads, of the actual role of these mutations on HBV replication.

India and areas of South America (Benoist, 1963). As the slaves. The remaining 10 % have roots in Western Europe, Martinican population is composed of descendants of African due to the history of the slave trade. More than 90 % of today's HBV strains spreading in Martinique. Martinique is at a

**DISCUSSION**

We report here the first data on molecular epidemiology of HBV strains spreading in Martinique. Martinique is at a crossroads between Africa, Europe, and the Americas, due to the history of the slave trade. More than 90 % of today's Martinican population is composed of descendants of African slaves. The remaining 10 % have roots in Western Europe, India and areas of South America (Benoist, 1963). As the Caribbean is thought to be an area of low- to mid-endemicity for HBV infection (Fest et al., 1993; Monplaisir et al., 1988; M. Guel-Simeon, V. Pillas, J. Deloumeaux, H. Delacroix-Maillard, G. Saint-Georges, L. Do Amaral, M. Borel, M. Laurent, E. Gordien, E. Saillard, personal communication), we thus focused on studying the molecular epidemiology of HBV strains on the island.

Samples from 86 HBV-infected patients were studied. As described in many studies worldwide, more than 80 % of them were anti-HBeAb positive. Viral co-infection (HCV, HIV, HTLV-1) was documented in few cases. However, no HDV co-infection was found, contrary to results published by Monplaisir et al. in 1988 (1.9 % in blood donors and 8.8 % in chronic hepatitis B patients) (Monplaisir et al., 1988), but in agreement with those obtained from the neighbouring island of Guadeloupe (E. Gordien, personal communication). This difference may be explained by the small size of our cohort or the evolution of HDV prevalence over the last 20 years. Also, we cannot exclude a problem with false positivity linked to the performances of first-generation anti-HDVAb assays. Therefore, there is currently no clear evidence that HDV was transmitted with HBV during the slave trade. As a result, large-scale phylogeographical studies on HDV in West, Central, and Sub-Saharan Africa would be of great interest to shed light on HDV spread in Africa.

Seven HBV genotypes/subgenotypes were characterized in Martinique. HBV/A was by far the most common (79.1 %), with most cases belonging to the ‘African-Asian’ HBV/A1 subgenotype (68.6 %) and the remaining 10.5 % to the ‘European’ HBV/A2. Such results have also been described in other countries with a history of African slave trade (Andernach et al., 2009; Santos et al., 2010), but the proportion of HBV/A1 was significantly higher in our study: 68.6 % in Martinique vs 43 % and 37 % in Haiti and Brazil, respectively (P<0.001).

HBV/A1 subgenotype, although first described in South Africa, is now known to be found predominantly in Eastern African countries (Hübschen et al., 2009; Norder et al., 2004) and in areas with a history of migration from Africa, including Indian subcontinent and Latin America (Kramvis & Paraskevis, 2013). However, new HBV/A subgenotypes (HBV/A3 to HBV/A7) have been recently described in several West African countries (for review, see (Pourkarim et al., 2010) and references therein). Interestingly, HBV/A5, which was first described in Nigeria (Olinger et al., 2006) was found with high prevalence in Haiti (19.6 %) (Andernach et al., 2009). However, no HBV/A3-HBV/A7 strains were found in Martinican patients nor in South American communities/populations of African origin (Araujo et al.; 2004; Motta-Castro et al., 2005; Quintero et al., 2002; Santos et al.,

4). Compared to the HBV/F4 strain, the similarity over the whole sequence was 94 %.

**Pre-C-C mutants**

Pre-C-C mutants were assessed in 61/71 (86 %) anti-HBeAb positive patients, either by direct sequencing (n=45) or by the Sangtec technique (n=16). The remaining 10 samples had low viral loads, <3 log IU ml\(^{-1}\). The overall prevalence of classical BCP/PC mutants was 74 % (45/61). BCP were more frequent (41 of 61 samples, 67 %) than PC stop-codon mutations (nine of 61 samples, 15 %). Additionally, the G1899A mutation was found in 12/45 tested samples (27 %): four HBV/A1, three HBV/D, the two HBV/E, and the three HBV/F. The mean viral load value was higher in the G1899A subgroup (5.28 log IU ml\(^{-1}\)) than in BCP or PC mutants (4.58 and 4.61 log, respectively) although insignificantly. Moreover, in four HBV/A1 cases, one 8 nt deletion encompassing nt1764 to nt1771 was identified. According to the different genotypes, we found that most HBV/A1 sequences (36 of 49; 73.4 %) had BCP mutations. PC mutation was common in genotypes HBV/D (4/4), HBV/E (2/2) and HBV/F (2/3), but rare in genotype HBV/A1 (1/49).

No PC/BCP mutants were found in HBV/A2-infected patients. It is noteworthy that HBV/F strains carried all the well-described pre-C-C mutants: BCP, PC, G1899A.

Using site-directed mutagenesis, Kramvis and Kew found that for HBV/A1 strains mutations at positions nt1809–1812, 1862 or 1888 could affect HBsAg expression, either at the translational or at post-translational level. (Kramvis & Kew, 2007). These positions could be analysed, in our cohort, for 34 HBsAg-negative HBV/A1 strains sequenced in pre-C-C region: all were mutated, with 25 double mutations (1809T/1812T), 9 triple mutations (1809T/1811C/1812T), 33 1862T and 25 1888A point mutations.

However, pre-C-C mutants were also found in 5 of the 15 HBsAg positive strains: all five had BCP mutations and one among them carried the PC stop-codon mutation. Moreover all seven HBsAg-positive HBV/A1 strains also contained subgenotype-specific mutations (1809T/1812T, n=7; 1862T, n=7; 1888A, n=6). Therefore, this finding raises the question of the actual role of these mutations on HBV replication.
This may be related to the fact that the areas of Africa concerned by the slave trade differed over time and colonies. In this view, HBV/A5 strains that form a close cluster may have been imported to Haiti from a particular community in Nigeria or Rwanda (Andernach et al., 2009; Santos et al., 2010).

According to a recent study by Kramvis & Paraskevis (2013), HBV/A1 subgenotype originated from South Africa, and can be separated into an 'African' clade, and an 'Asian/American' one. When focusing specifically on the Martinican HBV/A1 sequences, we found that the majority of them belong to the 'Asian/American' HBV/A1 clade, along with most of the Haitian strains recently published by Andernach et al. (2009). However, this clade appears to be very heterogeneous, including Somalian, South African, Haitian, South American, and Asian isolates randomly distributed within this cluster (Pourkarim et al., 2010). Moreover, bootstrap values were somewhat low, suggesting frequent population movements and multiple virus introductions. The slave trade lasted throughout the 17th and mid-19th century and, due to high mortality, there were regular arrivals of slaves from the African continent, with concomitant importation of their viruses (Van Brussel et al., 1999).

Another main observation is the absence of the HBV/E genotype in Martinican patients. The two HBV/E strains in this study were found in two adult African patients. Currently, HBV/E is limited to sub-Saharan Africa and is the predominant genotype in a vast crescent of Western African countries ranging from Senegal to Angola (Mulders et al., 2004), i.e. the major historical slave trading posts (Thomas, 1997). Therefore, as others have postulated, our results furthermore support the hypothesis of a recent introduction of HBV/E strains in the human population, very likely in West Africa. Considering the important post-slavery migrations from Africa to the French West Indies and Guiana between 1854 and 1889 (Flory, 2011), it is probable that HBV/E appeared in Africa within the last 150 years. To date, these observations provide the best explanation for the absence or very low prevalence of HBV/E in populations descending from African slaves (Andernach et al., 2009; Araujo et al., 2004; Motta-Castro et al., 2005; Quintero et al., 2002; Santos et al., 2010).

Additionally, seven HBV/D-Martinican strains (8.1%) were described. HBV/D has a large geographical distribution. It is present in a span ranging from the Mediterranean basin to the Middle East and India, in Southern and Eastern Africa, and also in Aboriginal populations in Indonesia, Papua and Australia, and in people native to the Canadian Arctic (Norder et al., 2004; Osiowy et al., 2011). Recently, several HBV/D strains were described in Brazil, Haiti and Rwanda (Andernach et al., 2009; Hübschen et al., 2009; Santos et al., 2010). The three Martinican HBV/D3 strains were closely related to old European strains and this origin is coherent with the population settlement in Martinique. However, the origin of the Martinican HBV/D4 strains is less clear. Indeed, considering complete genome phylogenetic analyses (Fig. 3), one strain seems close to those recently described in Haiti, while the other three were closer to strains found in different settlements of arctic-native Dene populations living in Canada (Andernach et al., 2009; Osiowy et al., 2011). However, due to the very small number of complete HBV/D4 sequences available, no clear conclusion can be drawn. In all phylogenetic analyses, all HBV/D4 sequences formed a distinct clade with a high bootstrap value, supporting a unique origin, but the location of this oldest common ancestor remains to be found.

Finally, we found that three patients born in Martinique were infected with HBV/F strains. Two of these strains were related, belonging to HBV/F2 (subgenotype mainly described in Brazil), and the third belonged to HBV/F4 (described in Argentina and Bolivia). Although contamination mode was not available for these patients, and while we cannot formally exclude that they could have been

**Table 2. Characteristics of Martinican patients according to HBV genotype**

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A2</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>NT</th>
</tr>
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<tbody>
<tr>
<td>Number of patients [N (%)]</td>
<td>59 (68.6)</td>
<td>9 (10.5)</td>
<td>7 (8.1)</td>
<td>2 (2.3)</td>
<td>3 (3.5)</td>
<td>6 (7.0)</td>
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<tr>
<td>Male [N (%)]</td>
<td>38 (64)</td>
<td>7 (78)</td>
<td>5 (71)</td>
<td>2 (100)</td>
<td>1 (33)</td>
<td>0 (0)</td>
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<td>Median age [years (range)]</td>
<td>42 (11–83)</td>
<td>38 (28–46)</td>
<td>35 (20–56)</td>
<td>45 (39–52)</td>
<td>59 (52–60)</td>
<td>28 (17–75)</td>
</tr>
<tr>
<td>HBeAg positive [N (%)]</td>
<td>7 (12)*</td>
<td>5 (56)</td>
<td>3 (43)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>Mean HBV VL (logIU ml⁻¹ ± SD)</td>
<td>3.8 ± 1.5</td>
<td>4.6 ± 2.3</td>
<td>4.7 ± 1.8</td>
<td>3.2 ± 0.7</td>
<td>5.0 ± 2.2</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>Anti-HIVAb positive [N (%)]</td>
<td>3 (5)†</td>
<td>4 (44)</td>
<td>2 (29)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

N, Number; NT, non-typable; VL, viral load.

*HBV/A1: P<0.01 vs HBV/A2, P<0.05 vs HBV/D.
†HBV/A1: P<0.0001 vs HBV/A2, P<0.01 vs HBV/D.
infected outside the island, our findings show that HBV/F genotype circulates in Martinique. Whether or not a separate Martinican HBV/F clade exists needs to be evaluated in larger cohorts.

In summary, three main genotypes, HBV/A, /D and /F, were identified in Martinique. Of note, these genotypes have been implicated in more serious hepatic disease (Ganne-Carriè et al., 2006; Kim et al., 2011; Schaefer, 2005).

In addition, we further characterized Martinican HBV strains molecularly. Classical pre-C-C mutants were found in 74 % of HBeAg-negative samples. As described previously (Kidd-Ljunggren et al., 2002; Lok et al., 1994), based on the epsilon structure of the pregenomic RNA, the prevalence of PC mutants was clearly related to the HBV genotype, respectively 0 and 2 % for HBV/A2 and HBV/A1, 66 % for HBV/F and 100 % for HBV/D and HBV/E strains (P<0.05). Likewise the G1899A mutation was mainly found in non-HBV/A strains (67 %), whereas the classical BCP mutations were the most prevalent and found mainly in HBV/A1 strains (73 %). It is noteworthy that patients infected with classical pre-C-C mutant strains had a mean viral load of 4.5 logIU ml$^{-1}$, i.e. more than 1 logIU ml$^{-1}$ above the 3.3 log threshold for chronic active HBV infection, according to the European Association for the Study of the Liver consensus conference (EASL, 2012). Moreover, in accordance with Kramvis and Kew, we found that the vast majority of HBV/A1 strains harboured a specific pre-C-C profile, which is thought to strongly impact HBeAg expression, at translational or at post-translational level, either by leaky scanning of AUG codon (1809–1812), or by peptide signal cleavage impairment (1862T) (Kramvis & Kew, 2007). This could explain the lower VL associated with this subgenotype.

In addition, direct sequencing allowed us to identify an 8 nt deletion in the BCP region between nt1764 and nt1771 in four anti-HBeAb positive patients, all infected with

**Fig. 4.** Pre-S1 and pre-S2 deletions in Martinican HBV isolates. Alignment of amino acid sequences of pre-S1 and the beginning of pre-S2 of Martinican HBV isolates is shown. The sequence of the M57663 strain (subgenotype HBV/A1) is considered as the wild-type reference. The genotype or subgenotype of each Martinican strain is indicated. Dots stand for amino acids that are identical to those of the wild-type, dashes for deletions, and stars for stop codons.
HBV/A1. This deletion is associated with a frameshift in HBx ORF, leading to the production of a truncated HBx protein. It should be noted that when compared to other pre-C-C mutants, the viral load seems to be lower in patients infected with these strains. Interestingly, Li et al. (2001) previously described an 8 nt deletion between nt1768 and nt1775 in HBV/B and HBV/C strains. In vitro transfection experiments demonstrated that these mutants led to diminished transcription and virion secretion (Kohno et al., 2000). Other deletion variants in the pre-C-C promoter region were also described, notably in highly immunosuppressed patients and in HIV/HBV co-infected patients (Preikschat et al., 1999; Revill et al., 2007).

Moreover, we also found several pre-S deletions in the Martinican strains. Some authors have shown that pre-S1 and pre-S2 deletions increase significantly with patient age (Huy et al., 2003) and were responsible for a severe course of hepatitis B and a higher incidence of HCC (Gerken et al., 1991; Huy et al., 2003; Sugauchi et al., 2003). In an Italian study, Pollicino et al. (1995) found no clinical significance for pre-S deletions. Other studies reported the association of pre-S deletions and pre-C-C mutations was significantly associated with the development of liver cirrhosis (Chen et al., 2007; Preikschat et al., 2002). In our cohort, pre-S deletions were found only in HBV/A1 sequences (and in one HBV/D and one HBV/F). Patients with pre-S deletions were older (47.1 vs 39.3 years of age), but the difference was not significant. The same trend persists when analysing HBV/A1-infected patients only, related very likely to an accumulation of deletions with infection duration, and/or immune/therapeutic pressure. Whether or not pre-S deletions are associated with worsened clinical course could not be assessed in our population and needs further study.

In conclusion, we report here unique data on the molecular characterization of HBV strains in Martinique. We show that African HBV/A1 is the main circulating subgenotype (near 70 %). HBV/A2, /D3 and /D4 subgenotypes, as well as the HBV/F2 and /F4, are also present. However, HBV/E is absent in the island. This HBV genotype distribution reflects the unique settlement history of the island. We also show that these potentially highly replicative strains (>4 log IU ml⁻¹) harbour several mutations and/or deletions in their genome. Together, these findings may be of great clinical concern in the natural history of hepatic disease and in the management of HBV infection in Martinique.

### METHODS

#### Samples

Serum samples from 86 consecutive HBs antigen (Ag)-positive patients who consulted at the Martinique University Hospital in Fort de France and in eight other well-distributed island hospitals collected between November 2001 and April 2004, were considered for this study. Demographic data such as, gender, place and date of birth, and virological data such as other virus co-infections were also available for these clinical samples.

These samples were part of the collection of the CeRBiM (Centre de Ressources Biologiques de la Martinique) a biological resource centre approved by the French Ministry of Research. Ethical considerations

<table>
<thead>
<tr>
<th>Region, primer name</th>
<th>Primer sequence (5'→3')</th>
<th>Position (nt)</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-S1</td>
<td>TCACCATATTTCTGGGAACAAGA</td>
<td>2823–2845</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>TTCTGTGAACCTGGAGCCACCA</td>
<td>80–60</td>
<td>Antisense</td>
</tr>
<tr>
<td>Pre-S-Pol</td>
<td>CCGCTGTTGGCTGCAGTTTC</td>
<td>56–75</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>GAAACCTCAACAAATGGGCGC</td>
<td>704–685</td>
<td>Antisense</td>
</tr>
<tr>
<td>PolBCD</td>
<td>TCAAGGTATGTTGGCCCG</td>
<td>457–473</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>TAACCCACTTTTTGTTTT</td>
<td>866–847</td>
<td>Antisense</td>
</tr>
<tr>
<td>Pol-X</td>
<td>TTTCTGTATTCTGTGGGTATCCAT</td>
<td>808–831</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>TCGAGGCGCCGACGGGCAC</td>
<td>1445–1429</td>
<td>Antisense</td>
</tr>
<tr>
<td>X-Pre-C</td>
<td>TGCCACACTGGATCTGGGAGGGACGTCCTT</td>
<td>1392–1421</td>
<td>Sense</td>
</tr>
<tr>
<td>Pre-C-C2</td>
<td>AGGAGTCGCTGGAGCTTGAAC</td>
<td>1884–1862</td>
<td>Antisense</td>
</tr>
<tr>
<td>HBV104UB</td>
<td>GTTCAGGCTCCCAAGCTGTG</td>
<td>1862–1881</td>
<td>Sense</td>
</tr>
<tr>
<td>Pre-C-F</td>
<td>GAGGAGGGAGGTCTCTCT</td>
<td>2390–2371</td>
<td>Antisense</td>
</tr>
<tr>
<td>C-Pre-S</td>
<td>CACACTTCCGGAACCTACTGTT</td>
<td>2323–2344</td>
<td>Sense</td>
</tr>
<tr>
<td>HBVC/F</td>
<td>AGAGGTGCTCCATGCTGTA</td>
<td>2861–2842</td>
<td>Antisense</td>
</tr>
</tbody>
</table>

**Table 3.** Primer sequences used for amplification and sequencing of HBV/F strains

HBV/F sequences (n=40) from subgenotypes HBV/F1 to /F4 were retrieved from GenBank to design specific primers for amplification and sequencing of 7 overlapping fragments covering the entire genome sequence.
for this study are covered by the French law on the constitution of biological collections and the powers of the national reference centres.

**Study design.** These 86 clinical samples were sent to the Virology Laboratory of Avicenne University Hospital (Bobigny, France), associated with the French National Reference Centre for Hepatitis B, C and Delta (LA-FNRC), in order to study the molecular epidemiology of HBV infection on this Caribbean Island characterized by a unique settlement pattern.

Further serological and molecular virological tests were performed according to routine practice at French centres for diagnosis of hepatitis B and hepatitis Delta infection. Further serological and molecular virological tests were performed according to routine practice at French centres for diagnosis of hepatitis B and hepatitis Delta infection.

**Serological analyses.** Anti-HCV, anti-HIV-I/II and anti-HTLV I/II Ab status was recorded for 67, 65 and 53 patients respectively.

HBV serological markers: HBsAg, anti-HBs antibodies (Ab), total and IgM anti-HBc Ab (Abbd); HBeAg and anti-HBe Ab (bioMérieux) and anti-hepatitis delta virus (HDV) Ab (ETI-AB-DELTAK-2 test; Diasorin) were performed on all samples.

**Molecular analyses.** HBV viral load was measured by bDNA Versant HBV 3.0 Assay (Siemens), or Cobas Monitor, Cobas Amplicor (Roche) according to manufacturers’ recommendations.

Pre-C-C mutants, i.e. the basal core promoter (BCP) mutations (A1762T/G1764A), the pre-Core (PC) stop codon mutation (G1896A) and the G1899A mutation were assessed via the sequencing of a 460 bp-long fragment in the pre C-C region according to a previously described technique (Sugauchi et al., 2001). For PCR-negative samples, a sensitive commercial assay was used according to the supplier’s recommendations (Affigene HBV Mutant VL 19 Test, Sangtec Molecular Diagnostics). Briefly, this test permits the detection and semi-quantification of G1764A and G1896A mutants after amplification of a pre-C-C gene region, and specific ‘mini-sequencing’ reactions with fluororescence-labelled nucleotides. For HBV/A1 samples, specific points of mutations were also analysed, according to the paper by Kramvis & Kew (2007).

HBV genotypes and subgenotypes were determined by PCR amplification of pre-S1 (479 bp for genotype HBV/A, position 2823–3002) or complete HBV genome, followed by bidirectional sequencing and by extensive phylogenetic analyses as described elsewhere (Abdou Chekarou et al., 2010). For HBV/F, new genotype-specific primers were designed (Table 3). Complete and partial Martinican HBV sequences have been submitted to EMBL/GenBank/DDBJ under accession numbers HE974362 to HE974384 and HG329753 to HG329841.

**Statistical analyses.** Results are presented as mean values ± SEM. Statistical values were calculated using ANOVA and ±2 test with Yate’s correction when necessary, using STATview software. Differences were considered significant for P-values <0.05.

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**REFERENCES**


