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Epidemiological and molecular features of hepatitis B and hepatitis delta virus transmission in a remote rural community in central Africa

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ABSTRACT

Hepatitis B virus (HBV) and hepatitis delta virus (HDV) occur worldwide and are prevalent in both urban and remote rural communities. In a remote village in Gabon, central Africa, we observed a high prevalence of HBsAg carriage and HDV infection, particularly in children and adolescents. The prevalence of HBsAg differed significantly by gender and age, females being more likely than males to carry the HBsAg during the first 10 years of life, while the prevalence was higher among males than females aged 11–20 years. We also characterised HBV and HDV strains circulating in the village. The principal HBV strains belonged to genotype HBV-E and subgenotype QS-A3. Complete genome analysis revealed for the first time the presence of the HBV-D genotype in Gabon, in the form of an HBV-D/E recombinant. Molecular analysis of HDV strains and their complete genomic characterisation revealed two distinct groups within the dominant HDV clade 8. Molecular analysis of HBV and HDV strains did not reveal vertical transmission within the families studied but rather horizontal, intrafamilial transmission among children aged 0–10 years. Our findings indicate that HBV is transmitted in early childhood by body fluids rather than by sexual contact. Health education adapted to the different age groups might therefore help to reduce HBV transmission. Young children should be vaccinated to control HBV infection in areas of extremely high prevalence.

1. Background

Hepatitis B virus (HBV) occurs worldwide, with marked variations in prevalence. Highly endemic areas are found in sub-Saharan Africa, Asia, the Far East, the Mediterranean basin and some regions of South America (Kiire, 1996; Kramvis and Kew, 2007). The prevalence of antibodies to HBV core antigen (anti-HBc), which reflects exposure to HBV, increases with age in Africa, from 1.8% to 98% (Kramvis and Kew, 2007). Similarly, the prevalence of HBV surface antigen (HBsAg) in Africa varies among groups of carriers, the lowest rates being found in asymptomatic blood donors and the highest in patients with clinical manifestations of HBV infection (Kramvis and Kew, 2007).

In Africa, despite the existence of eight HBV genotypes (A–H) and of two proposed, invalidated genotypes (I and J) with distinct geographical distribution (Kramvis and Kew, 2007; Tatematsu et al., 2009), only two major HBV genotypes, A and E, predominate in central, southern and western Africa, while genotype D predominates in northern Africa (Kramvis and Kew, 2007; Miyakawa and Mizokami, 2003). Previously, genotype D was classified into eight subgenotypes (D1 to D8) (Banerjee et al., 2006; Kramvis et al., 2008; Meldal et al., 2009), but new analyses have identified only six distinct subgenotypes (D1–D6) (Yousif and Kramvis, 2013). The HBV genotypes circulating in Gabon have been described (Makuwa et al., 2008; Makuwa et al., 2006), and a subgenotype, HBV-A3, has been characterized in Cameroon and Gabon (Kurbanov et al., 2005; Makuwa et al., 2006), completing the HBV-A genotype, which was previously divided into two subgenotypes, A1 and A2 (Kramvis et al., 2002). Two further HBV-A subgenotypes, A4 and A5, were subsequently reported in Cameroon, (Olinger et al., 2006), and subgenotype HBV-A5 was detected in a Rwandan population (Hubschen et al., 2009). Recent genotype analyses have modified the HBV-A classification into subgenotypes A1, A2, A4 and quasi A3 (QS-A3) (Kramvis, 2014; Pourkarim et al., 2014; Pourkarim et al., 2010). In countries in which the two genotypes circulate, co-infection with more than one genotype has been detected, including recombinants. The first cases of recombination between genotypes A and E were reported in Cameroon and Gabon (Kurbanov et al., 2005; Makuwa et al., 2006) and recombinants of genotypes A and D have been described in South Africa (Owiredu et al., 2001). Despite the hyperendemicity of HBV-E throughout West Africa, little genetic diversity has been reported in this genotype (Bekondi et al., 2007; Kramvis et al., 2005b; Makuwa et al., 2008; Mulders et al., 2004; Suzuki et al., 2003). A study of intra-genotype nucleotide divergences in genotypes A (3.69% ± 1.75), D (2.43 ± 1.16) and E (1.73 ± 0.83) suggests that genotype A has been endemic in the African population for longer than

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Table 1
Prevalence of HBV and HDV in Dienga, by age group and sex.

Sex	HBV						HDV				
	Age group (years)	Total no. tested	HBsAg + (%)	95% CI	OR	95% CI	Total no. tested	Anti-HDV (%)	95% CI	OR	95% CI
Male	0–10	31	0				0	0			
	11–20	67	23 (34.3)	23.0–45.7	3.6	1.8–7.1	23	11 (47.8)	27.4–68.3	2.7	0.9–7.8
	21–30	35	13 (37.1)	21.1–53.2	3.2	1.5–7.0	13	7 (53.8)	26.7–81.0	3.0	0.9–10.3
	31–40	24	6 (25.0)	7.7–42.3	1.5	0.6–4.0	6	3 (50.0)	11.8–88.2	2.2	0.4–11.7
	41–50	11	2 (18.2)	2.3–51.8	1.0		2	1 (50.0)	1.3–98.7	2.1	0.1–34.4
	51–>60	21	0				0	0			
Total		189	44 (23.3)	17.3–29.3			44	22 (50.0)	35.2–64.8		
Female	0–10	22	5 (22.7)	5.2–40.2	2.8	1.0–8.2	5	3 (60.0)	14.7–94.7	5.3	0.7–37.1
	11–20	68	8 (11.8)	4.1–19.4	1.2	0.5–2.8	8	2 (25.0)	3.2–65.1	0.9	0.2–5.3
	21–30	60	7 (11.7)	3.6–19.8	1.2	0.5–2.8	7	3 (43.0)	9.9–81.6	2.4	0.5–13.3
	31–40	35	5 (14.3)	2.7–25.9	1.5	0.5–4.2	5	2 (40.0)	5.3–85.3	2.0	0.3–13.9
	41–50	27	2 (7.4)	0.9–24.3	0.7	0.2–2.9	2	1 (50.0)	1.3–98.7	2.9	0.2–50.8
	51–>60	41	3 (7.3)	1.5–19.9	0.6	0.3–2.2	3	0			
Total		253	30 (11.9)	7.9–15.8			30	11 (36.7)	19.4–53.9		
Both		442	74 (16.7)				74	33 (44.6)	33.3–55.9		

HBV, hepatitis B virus; HDV, hepatitis delta virus; HBsAg, hepatitis B surface antigen; OR, odds ratio; CI, confidence interval.

genotypes D and E and that E is the most recently introduced genotype (Kramvis and Kew, 2007). In Niger, however, where HBV-E is widespread, intra-genotype analysis showed higher variability than previously described, (Abdou Chekaraou et al., 2010) and circulation of HBV-D/E recombinants was confirmed.

Hepatitis delta virus (HDV) is an enveloped RNA virus which requires the helper function of HBV to replicate (Sureau et al., 1993). Together, the two viruses can cause severe acute and chronic liver disease. *Delta* hepatitis is distributed worldwide, with the highest endemicity in some South American countries, the Mediterranean basin, Romania, and parts of south and central Africa (Husa et al., 2005). Previous molecular phylogenetic analyses point to broad, ancient distribution of HDV in Africa, with at least seven major clades (Radjef et al., 2004). An eighth major HDV clade was described in 2005 (Le Gal et al., 2005). We subsequently confirmed that this subtype originated from and was endemic to Central Africa (Makuwa et al., 2008). Interestingly, the variation of this HDV subtype is similar to that of human HBV, with eight distinct genotypes (Kramvis et al., 2005a).

The predominant route of HBV transmission in sub-Saharan Africa is reported to be horizontal, most individuals being infected by the time they reach adolescence (Candotti et al., 2007; Chiaramonte et al., 1991; Karim et al., 1991; Kramvis and Kew, 2007; McMahon, 2004; Rapicetta et al., 1991). Vertical transmission appears to play a smaller role in Africa (Candotti et al., 2007) than in other high-prevalence regions such as South-East Asia, where vertical transmission is the predominant route (Zhang et al., 2004). The increase in the seroprevalence of HBV serum markers with age indicates the importance of both childhood and adult transmission (Kramvis and Kew, 2007). Despite similarities in the mode of transmission of HBV and HDV, HDV has some particularities. Inadvertent parenteral transmission of HDV has been reported in overcrowded households, and this appears to be a common mode of spread in Africa and South America (Niro et al., 1999). The risk of vertical HDV transmission is low, and HDV infection is uncommon in populations with high rates of infantile and childhood HBV infection. Sexual contact is, however, a significant mode of HDV transmission in endemic areas (Husa et al., 2005). Although the presence of serological markers of HBV carriage in a given family can indicate intrafamilial HBV and HDV transmission, this cannot be verified by serological tests alone, and a molecular approach has thus been used in several studies of families that include members with symptomatic HBsAg carriage and/or HDV infection (Boot et al., 2008; Lin et al., 2005; Niro et al., 1999; Tajiri et al., 2007; Thakur et al., 2003). Comparison of nucleotide sequences from different HBV and HDV strains demonstrated the transmission of these viruses in several populations. High homology between sequences isolated from different individuals strongly supports the hypothesis that the viral strains originated from the same source (Boot et al., 2008; Lin et al., 2005; Tajiri et al., 2007; Thakur et al., 2003).

Table 2
Serological profiles of HBV infection by age group and sex.

Sex				HBsAg + ($n = 74$)		HBsAg – ($n = 368$)				
	Age group (years)	Total no. tested	HBsAg+ (%)	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)
Male	0–10	31	0	0	0	3 (9.7)	15 (48.4)	2 (6.5)	10 (32.3)	1 (3.2)
	11–20	67	23 (34.3)	3 (4.5)	20 (29.9)	4 (6.0)	30 (44.8)	7 (10.4)	1 (1.5)	2 (3.0)
	21–30	35	13 (37.1)	1 (2.9)	12 (34.3)	4 (11.4)	13 (37.1)	4 (11.4)	1 (2.9)	0
	31–40	24	6 (25.0)	0	6 (25.0)	5 (20.8)	11 (45.8)	1 (4.2)	0	1 (4.2)
	41–50	11	2 (18.2)	0	2 (18.2)	5 (45.5)	2 (18.2)	0	0	2 (18.2)
	51–>60	21	0	0	0	2 (9.5)	14 (66.7)	4 (19.0)	0	1 (4.8)
Total		189	44 (23.3)	4 (2.1)	40 (21.2)	23 (12.2)	85 (45.0)	18 (9.5)	12 (6.3)	7 (3.7)
Female	0–10	22	5 (22.7)	2 (9.1)	3 (13.6)	1 (4.5)	9 (40.9)	1 (4.5)	4 (18.2)	2 (9.1)
	11–20	68	8 (11.8)	3 (4.4)	5 (7.4)	9 (13.2)	41 (60.3)	9 (13.2)	1 (1.5)	0
	21–30	60	7 (11.7)	0	7 (11.7)	6 (10.0)	37 (61.7)	8 (13.3)	0	2 (3.3)
	31–40	35	5 (14.3)	0	5 (14.3)	6 (17.1)	22 (62.8)	2 (5.7)	0	0
	41–50	26	2 (7.4)	0	3 (11.5)	0	18 (69.2)	3 (11.5)	1 (3.8)	1 (3.8)
	51–>60	42	3 (7.3)	1 (2.4)	1 (2.4)	11 (26.2)	22 (52.4)	6 (14.3)	1 (2.4)	0
Total		253	30 (11.9)	6 (2.4)	24 (9.5)	33 (13.0)	149 (58.9)	29 (11.5)	7 (2.8)	5 (2.0)
Both		442	74 (16.7)	10 (2.3)	64 (14.5)	56 (12.7)	234 (52.9)	47 (10.6)	19 (4.3)	12 (2.7)

1: HBsAg replicative carrier (HBsAg+/HBeAg+/antiHBc+); 2: HBsAg non-replicative carrier (HBsAg+/anti-HBe+/anti-HBc+); 3: ongoing recent convalescence (anti-HBe+/anti-HBc+); 4: resolved infection (anti-HBc+/antiHBs+); 5: anti-HBc alone (anti-HBc+); 6: passive immunization (anti-HBs+); 7: negative (no HBV markers).

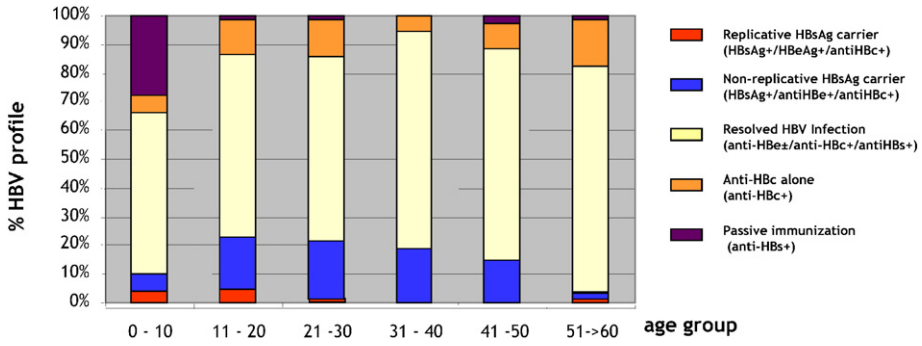

Fig. 1. HBV serological profiles by age group.

In Gabon, the prevalence of hepatitis B was first reported in 1988 and 1989 (Dupont et al., 1988; Dupont et al., 1989). In a national epidemiological study conducted in 2008 in a large population cohort of pregnant women sampled in the five main cities, the overall prevalence of HBsAg was 9.2% (Makuwa et al., 2008). A high prevalence of anti-HBc (86.8%) and HBsAg (8.6%) was also reported in rural Gabon (Makuwa et al., 2006). The first epidemiological studies of HDV in Gabon indicated a prevalence of 3.5% (Dupont et al., 1989). Our laboratory reported a prevalence of 15.6% in pregnant women, with the presence of an HDV-1 virus clade and molecular evidence that HDV clade 8 originates from and is endemic to Central Africa (Makuwa et al., 2008).

In the study reported here, we investigated the dispersal of HBV and HDV and their transmission in the general population by studying intra- and extrafamilial patterns of transmission of strains circulating in a rural area of Gabon that is highly endemic for both viruses.

2. Materials and methods

2.1. Area and population

The study was conducted in a remote Gabonese village called Dienga (Ogooué-Lolo Province), located near the border with the Congo, 180 km from Franceville. Since 1994, the Franceville International Centre for Medical Research (CIRMF) has maintained a community clinic in Dienga, with laboratory facilities for studies of malaria. Of the 2500 registered residents, 778 were permanent (44.1% men, 55.9% women; mean age, 31 years). A map was drawn, the village was divided into six neighbourhoods, and all houses were identified with a number. Available data for each household included the number of members, their age and sex, as well as family relationships and occupations. Family trees were constructed with CraneFoot v3.2 software (2006).

The study protocol was reviewed and approved by the Gabonese Ministry of Health (research authorization No. 00,093/MSP/SG/SGAQM). The Health Director and the Governor of the province received written information, as did the traditional chief of the village. The planned studies were described orally to all participants, and individual written consent was obtained for blood sampling; parents' written consent was obtained for the participation of children and minors. A free medical examination and basic medicines were provided to all participants and non-participants.

2.2. Blood samples

Blood samples were collected in the village health care centre into 7-ml Vacutainer tubes containing EDTA (VWR International, France) and then centrifuged for 10 min (2000 *g*). Plasma was stored in aliquots at −20 °C, then transferred on dry ice to CIRMF and kept at −80 °C until analysis. A total of 442 blood samples were collected for serological analysis of HBV and HDV markers. A second blood sample was taken from families of HBsAg carriers, which comprised 132 household contacts.

2.3. HBV and HDV serological tests and molecular detection of HBV

Anti-HBc was detected with Monolisa anti-HBc Plus, HBsAg with Monolisa HBsAg Ultra and anti-Hbs with Monolisa anti-HBs (Biorad, Marnes la Coquette, France). All samples that were positive for anti-HBc and/or HBsAg were tested for HBeAg and anti-HBe (Monolisa HBe, Biorad).

As detection of total antibodies to HDV is sufficient to diagnose HDV infection, total antibodies were determined in all HBsAg-positive samples by the Murex anti-delta (total) assay (Abbott/Murex Diagnostic Division, Wiesbaden, Germany).

The PrimerDesign™ Quantification Kit for Hepatits B Virus (HBV) genomes (HBV-Core Protein Region standard kit, PrimerDesign Ltd., Southampton, United Kingdom) was used for molecular detection of HBV. Under optimal PCR conditions the PrimerDesign pathogen detection kit has a very high priming efficiency (>95%) and can detect between 1×10^8 and 1×10^2 copies of target template.

2.4. DNA and RNA extraction, amplification and sequencing

DNA and RNA were extracted from clarified plasma (after centrifugation at 700 *g* for 10 min) with the Qiamp DNA viral Mini Kit and

Table 3
Genotypes of HBV and HDV in the infected individuals.

Samples numbers	HBV genotype (565-bp partial S gene)	HBV genotype (522-bp core gene)	HDV genotype (327 bp sHD gene)	Age	Sex
DN31	QS-A3	E	–	9	F
DN32	QS-A3	na	–	14	M
DN103	QS-A3	na	na	15	M
DN409	QS-A3	na	na	5	M
DN410	QS-A3	na	–	28	M
DN415	QS-A3	na	HDV-8	13	M
DN453	QS-A3	na	–	23	M
DN560	QS-A3	na	HDV-8	16	M
DN603	QS-A3	QS-A3	HDV-8	11	M
DN604	QS-A3	na	na	4	M
DNG878	QS-A3	na	HDV-8	15	M
DN17	E	na	na	10	F
DN77	E	E	na	14	F
DN111	E	na	na	16	M
DN113	E	E	na	15	M
DN177	E	na	HDV-8	15	M
DNG187	E	E	–	15	M
DN284	E	QS-A3	–	29	M
DN342	E	E	HDV-8	20	M
DN495	E	na	na	27	F
DN502	E	E	HDV-8	6	M
DN514	E	na	–	17	M
DN171	D	E	–	12	M
DN437	D	E	–	5	F
DN600	na	QS-A3	HDV-8	8	M

na: Not available; HDV ELISA negative.

Qiamp RNA viral Mini Kit, respectively (Qiagen, Courtaboeuf, France), as recommended by the manufacturer.

2.5. HBV amplification

Extracted DNA was used for PCR amplification of a 565-bp fragment of the *S* gene in 24 samples, of a 568-bp fragment of the core gene in 10 samples, and of one complete HBV genome, as described previously (Hu et al., 2000; Makuwa et al., 2006; Olinger et al., 2006).

The core region was amplified under previously described PCR conditions (Olinger et al., 2006). One complete HBV genome was amplified by semi-nested PCR as previously described (Hu et al., 2000).

2.6. HDV amplification

Extracted RNA was used to amplify a 327-bp fragment of the *sHD* gene of HDV and also one complete genome, as described previously (Radjef et al., 2004).

Briefly, using the SuperScript VILO cDNA synthesis kit (Invitrogen, USA), we generated first-strand cDNA as recommended by the manufacturer. Then, we used the primers HDV-889S (889–911) CATGCCGA CCCGAAGAGGAAAG and HDV-1289AS (1289–1265) GAAGGAAGGC CC-TCGAGAACAAGA, which encompass the *R0* region (400 nucleotides) covering the 3 end of the *HD* gene, and successfully amplified 16 samples. The complete HDV genome was obtained with previously described specific primers (Radjef et al., 2004).

2.7. HBV and HDV sequencing

The PCR products were sequenced directly (GATC Biotech, Konstanz, Germany).

2.8. Phylogenetic analysis

Pairwise alignments were performed for the partial HBV-S gene region, the partial HBV-pre-core/core gene, HDV-*R0* fragment and both HBV and HDV complete genomes with Clustal W software (1.7). Phylogenetic analyses were performed by the maximum likelihood method (MEGA v6 software, Boostrap resampling by 1000 replicates) (Kumar et al., 2004) and then by the Bayesian method. As the two methods gave the same result, we present only the results of the Bayesian method. Phylogenetic trees were reconstructed in MrBayes version 3.1.2 software (2005) (Ronquist and Huelsenbeck, 2003) using the generalised time reversible (GTR) model of evolution with a gamma distribution of variable site, run for 8,000,000 generations with a burn-in of 25%. Parameters were examined with the Tracer programme (http://evolve.zoo.ox.ac.uk/software.html/id=tracer), and all the estimated sample sizes were >1200. The tree datasets were computed with strict consensus and majority rules algorithms to evaluate the posterior

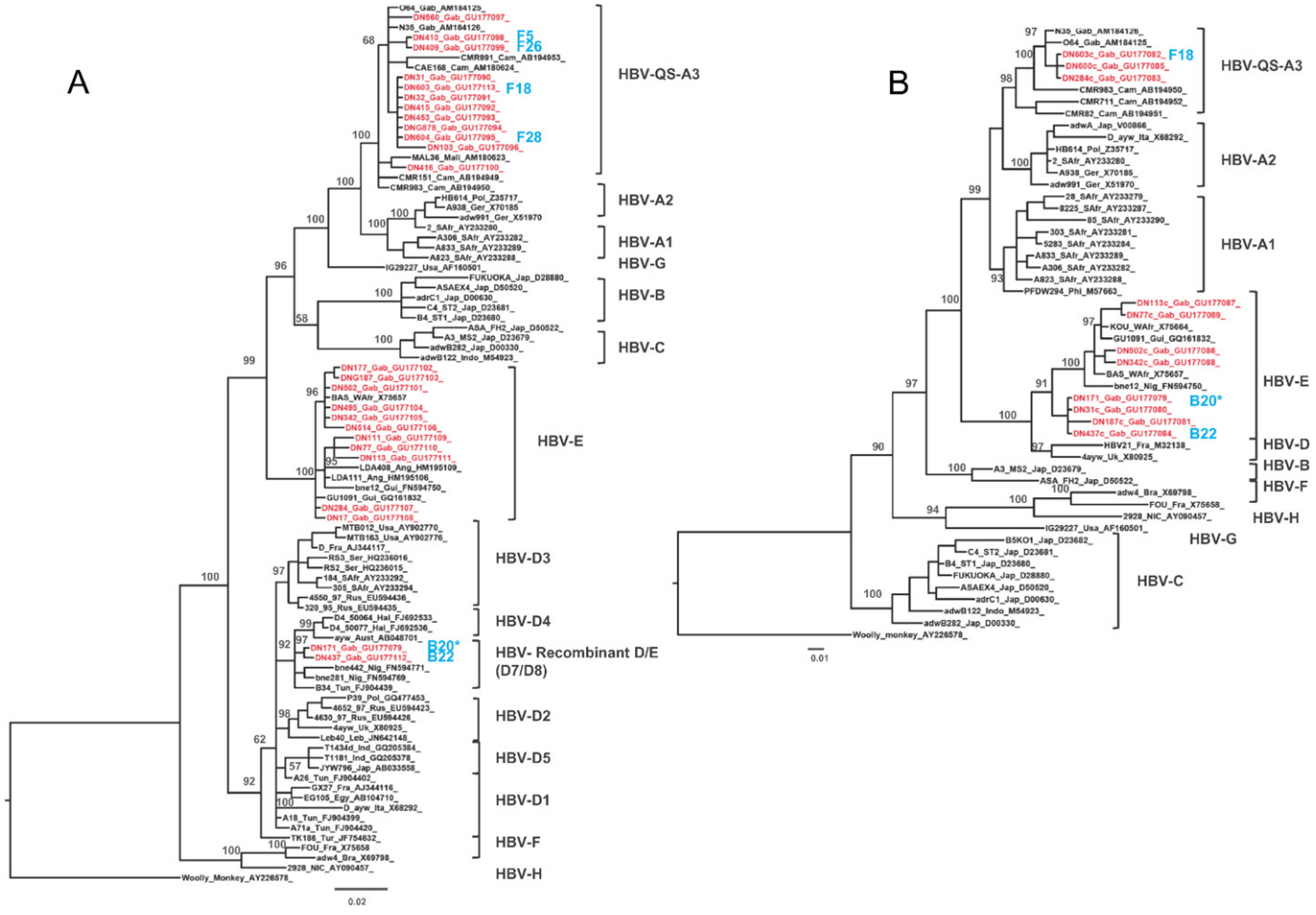

Fig. 2. (A) Phylogenetic analysis of a 565-bp HBV-*S* gene and (B) a 522-bp HBV-core gene from different HBV isolates, with woolly monkey strain (AY226578) as the root. The phylogenetic trees were inferred by the Bayesian method in the GTR model, with gamma-distributed rates at sites, 8 million generations and effective sample sizes greater than 1200 and 2600, respectively. BPP (Branching Posterior Probability) is represented by bullets >0.9. Samples sequenced in this study are represented in red. Correspondence with the numbers of the family members is represented in blue. Asterisks show the sample entirely sequenced.

probabilities of branching pattern (BPP). The trees were visualized in the FigTree programme.

Recombination points were sought with Simplot software (v 3.5). The complete genome of DN171-Gab was compared with each representative sequence of the HBV-D genotype (M32138, X80925) and the HBV-E genotype (X75657, X75664) with the settings window 200 bp, step 20 bp, GapStrip on, Kimura 2 parameters. In addition, we performed neighbour-joining analyses separately on each area. Sequence identity between the HBV strains obtained was evaluated with MEGA 5 software by computing the pairwise distances between the strains.

2.9. Statistical analysis

Statistica software v7.1 (StatSoft France, www.statsoft.fr) was used for statistical analysis. HBV and HDV serological profiles were analysed according to age group in a two-sided chi-square test (Fisher exact test when appropriate) and odds ratios.

3. Results

3.1. HBV and HDV are highly endemic in a rural Gabonese population, particularly in males

We enrolled 442 people (mean age, 29.4 years; range, 1–90 years; male:female ratio, 0.74) who were present in the village at the time of blood sampling. All were found to be to clinically healthy, with no major clinical symptoms of HBV or HDV infection.

The overall prevalence of HBV markers was 97.3%. Only 12/442 people (2.7%) had no markers. As shown in Table 1, there was a high overall prevalence of HBsAg (16.7%, 74/442) and of antibodies to HDV (44.6%, 33/74).

The prevalence of HBsAg differed significantly by sex, being higher in males (23.3%, 44/189) than in females (11.9%, 30/253) ($p = 0.002$). Interestingly, among children aged 0–10 years, HBsAg was present only in girls (≥6 years old) and not in boys, in whom the highest level of passive immunization was recorded (32.3% versus 18.2%); however, the prevalence was significantly higher in boys aged 11–20 years (34.3%, 23/67) than in girls of the same age group (11.8%, 8/68) ($p = 0.003$). No significant age or gender difference in the prevalence of antibodies to HDV was observed. As shown in Table 2 and Fig. 1, only 10 of the 74 HBsAg carriers (13.5%) were in the replicative phase of HBV infection, with a characteristic serological profile of HBsAg, HBeAg and anti-HBc positivity. The remaining 64 (86.5%) HBsAg carriers had undergone spontaneous conversion from the HBeAg-positive chronic phase of infection to the inactive HBV carrier phase, with a typical serological profile (HBsAg, anti-HBe and anti-HBc). The commonest serological profile (±anti-HBe, anti-HBc and anti-HBs) was found in 290/430 individuals (67.4%), who had thus resolved their HBV infection. Anti-HBc was the sole marker in 10.6% of the population and anti-HBs was the sole marker in 4.5% (n = 20), the latter being a marker of HBV vaccination. We noted that 14 (73.7%) of the 19 individuals with anti-HBs were aged 0–10 years, probably because of a recent vaccination campaign.

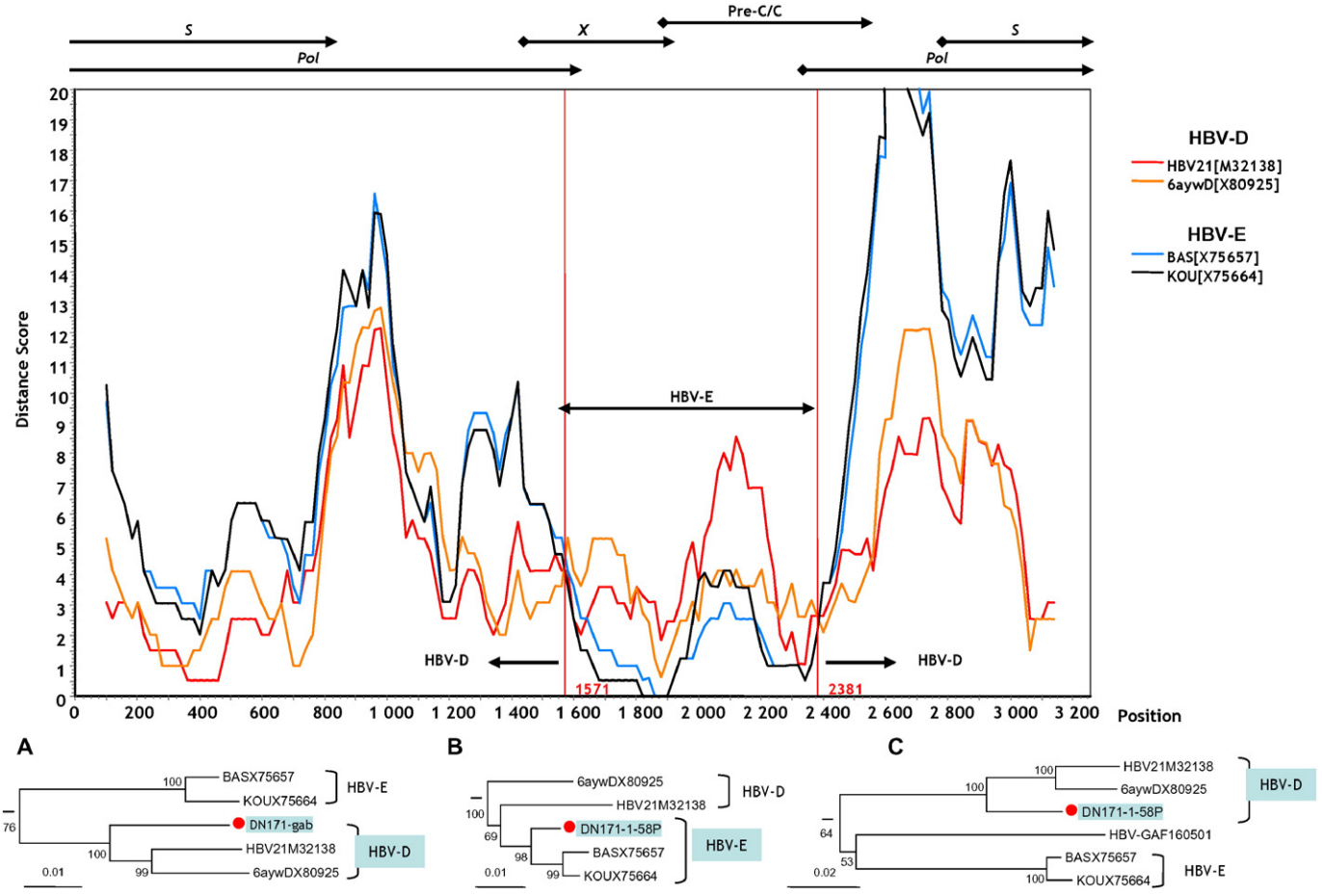

Fig. 3. SimPlot analysis of the complete genome of DN171-Gab against representative sequences of the HBV-D genotype (M32138, X80925) and HBV-E genotype (X75657, X75664), with the settings window 200 bp, step 20 bp, GapStrip on, Kimura 2 parameters and the neighbour-joining method. Inserted trees were obtained from the 5–3 segment, as follows: A (1–1570), B (1571–2380) and C (2381–3221) of the DN171-Gab sequence with Mega3 software, Kimura 2 parameters and the neighbour-joining method. Numbers on branches are boostrap values (●for values >75%).

3.2. Predominance of HBVQS-A3 and -E and detection of a newly introduced HBV-D strain among circulating HBV strains

HBV DNA was detected in 46 of the 74 HBsAg-positive plasma samples, and a 565-bp portion of the HBV-S gene could be amplified in 25 samples (Table 3). Some of our samples could not be amplified in regions S and C. This may be due a certain variability of HBV strains in central Africa, which has still not been studied, and also by low viral loads in some individuals. As shown in Fig. 2A, three HBV genotypes were found to be circulating in the village: genotype HBV-A ($n = 12$; BPP = 100%) and genotype HBV-E ($n = 11$; BPP = 100%). The last two amplified sequences were closely related to genotype D (BPP = 92%).

All the HBV-A strains identified here belonged to sub-genotype QS-A3 (HBV QUASI-A3). All but one of the new HBV strains in this cluster were closely related to two HBV strains (AM184125 and AM184126) previously described by our group. The remaining HBV strain, DN416, was most closely related to an HBV QS-A3 strain from Mali. The new HBV-E sequences obtained in this study formed a homogeneous cluster with no evidence of sub-genotype.

The two strains related to the HBV-D genotype (DN171 and DN437) were closely related to each other and were related to the same sub-genotype (BPP = 92%), formerly designated D7/D8. This group is now considered to be a recombinant group and is named D/E. This recombinant group had never previously been detected in Gabon.

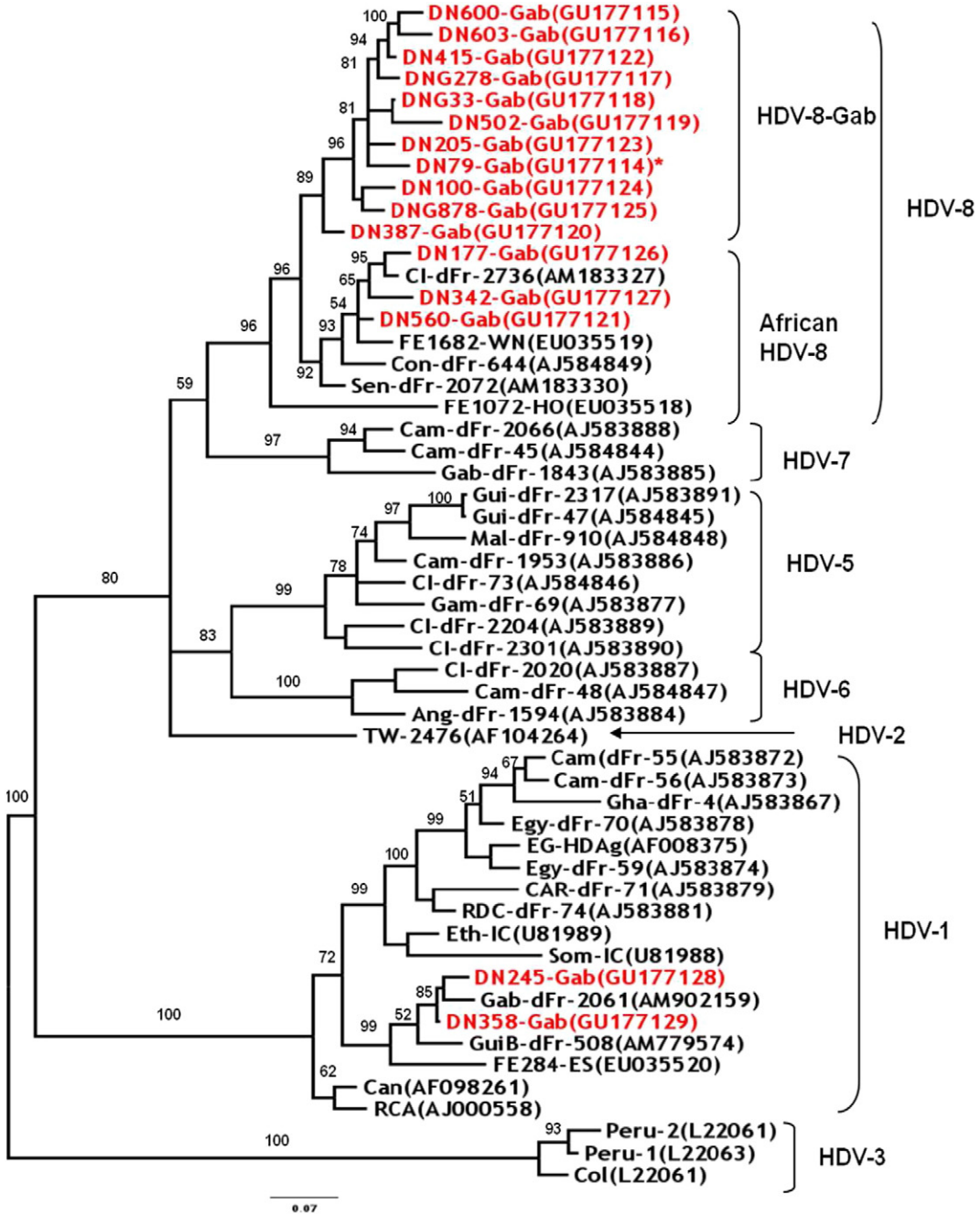

Fig. 4. Phylogenetic analysis of a 327-bp fragment of the *sHD* gene of HDV from various HDV isolates, with HDV clade 3 as the root. The phylogenetic trees were inferred by the Bayesian method in the GTR model, with gamma-distributed rates at sites with 1 million generations and an effective sample size >355. BPP (Branching Posterior Probability) is shown along the main nodes (by bullets for values >0.9). The samples sequenced in this study are represented in red. Asterisks show the samples entirely sequenced.

A 522-bp portion of the HBV core gene was also amplified in 11 samples (Fig. 2B). Three of the 11 HBV strains belonged to the HBV-QS-A3 sub-genotype and the remaining eight to the HBV-E genotype.

A comparison of the HBV-S and core regions in the 11 HBV strains showed that one strain (DN31) was a QS-A3/E recombinant. One of the other eight HBV E strains, DN284, was also an E/QS-A3 recombinant. The last two HBV-D strains were D/E recombinants, confirming the recombinant status of this sub-genotype.

3.3. Characterization of the new HBV recombinant by complete genomic phylogenetic analysis

As stated above, we detected the recombinant HBV D/E sub-genotype for the first time in Gabon, by analysis of two portions of the HBV-S and HBV-core genes. Sequencing and phylogenetic analysis of the complete HBV genome (3221 bp) of strain DN171 showed that it was closely related to the HBV-D reference strains (bootstrap value, 100%). Furthermore, SimPlot analysis of complete genome sequences and phylogenetic trees constructed with the corresponding sequence fragments revealed a recombinant pattern (HBV-D/E) of this new strain, with two breakpoints (Fig. 3). In the phylogenetic tree generated by SimPlot analysis from sequences of fragment A (1–1570 bp), strain DN171 was found to belong to the HBV-D genotype (bootstrap value, 100%) (Fig. 3A). In the phylogenetic tree generated with sequences of fragment B (1571–2381 bp), strain DN171 was closely related to the HBV-E genotype (bootstrap value, 98%) (Fig. 3B). In the tree generated with sequences of fragment C (2381–3221 bp), the strain was again identified as belonging to the HBV-D genotype (bootstrap value, 100%) (Fig. 3C).

Separate phylogenetic analyses of the partly overlapping open reading frames coding for selected genes confirmed these findings, as follows: *Pol* (HBV-D; bootstrap value, 100%), *Core* (pre-core/core; HBV-E; bootstrap value, 95%), X (HBV-E; bootstrap value, 58%) and S (HBV-D; bootstrap value, 100%). The HBV-A strain (pFDW294, M57663) was used as the reference strain (data not shown). The two breakpoints in the X gene and pre-core/core region have been traced to strains described in Niger and Ghana.

In the pre-core/core and X amino acid patterns, the DN171 strain had no specific substitutions in the HBV-E or HBV-B–H genotype. In the *Pol* gene of the DN171 strain, only two specific nucleotide or amino acid substitutions were found: A/361/T-Ser/121/Phe and A/661/C-Lys/221/His. In the *S* gene, encompassing the pre-S1, pre-S2 and S domains, five HBV-D genotype-specific nucleotide or amino acid substitutions were found: C/194/T–Pro/65/Leu, T/253/A–Leu/85/Ile, G/256/C–Ala/86/Gln, G/340/A–Asp/114/Asn and C/658/A–Pro/220/Thr.

3.4. Identification of two distinct groups within the dominant HDV clade 8, related to virus dispersal in the village

For sequence and phylogenetic characterization, 16 HDV strains were successfully amplified (327 bp of the *sHD* gene), and one complete genome was obtained. Most (87.5%) of the HDV strains circulating in the village belonged to the HDV-8 clade.

Only two strains belonging to the HDV-1 clade were found, in persons aged 6–20 years. In the HDV-8 clade, we identified two groups (bootstrap 96%): the first included HDV reference strains, together with three of the HDV strains from this study, while the second contained only HDV strains originating in the village. The estimated inter-group nucleotide divergence in the 327-bp fragment was 8.0% (Fig. 4).

We also sequenced the first complete HDV strain (DN79) from Gabon, which was 1679 bp long and related to clade 8. As expected, this new HDV-8 strain contained the two overlapping open reading frames that are translated into small and large HDV proteins, with corresponding, mostly conserved regions, such as a nuclear localization signal, arginine-rich motifs and a highly variable proline-rich domain at the carboxy terminal of the large hydrophilic domain (data not shown). Phylogenetic analysis confirmed the genetic affiliation of this strain to the HDV-8 clade (bootstrap value, 100%) (data not shown).

3.5. Molecular epidemiological analysis suggests horizontal transmission of HBV and HDV within families and in the village

For the study of HBV and HDV transmission in the village, we selected two families (B and F, see Figs. 5 and 6). Family B was composed of 14 individuals (Fig. 5). The genotypes identified in this family were HBV-QS-A3, —E and -D. By sequence analysis of a portion of the HBV-S and core genes, we found no cases of vertical transmission in family B but detected

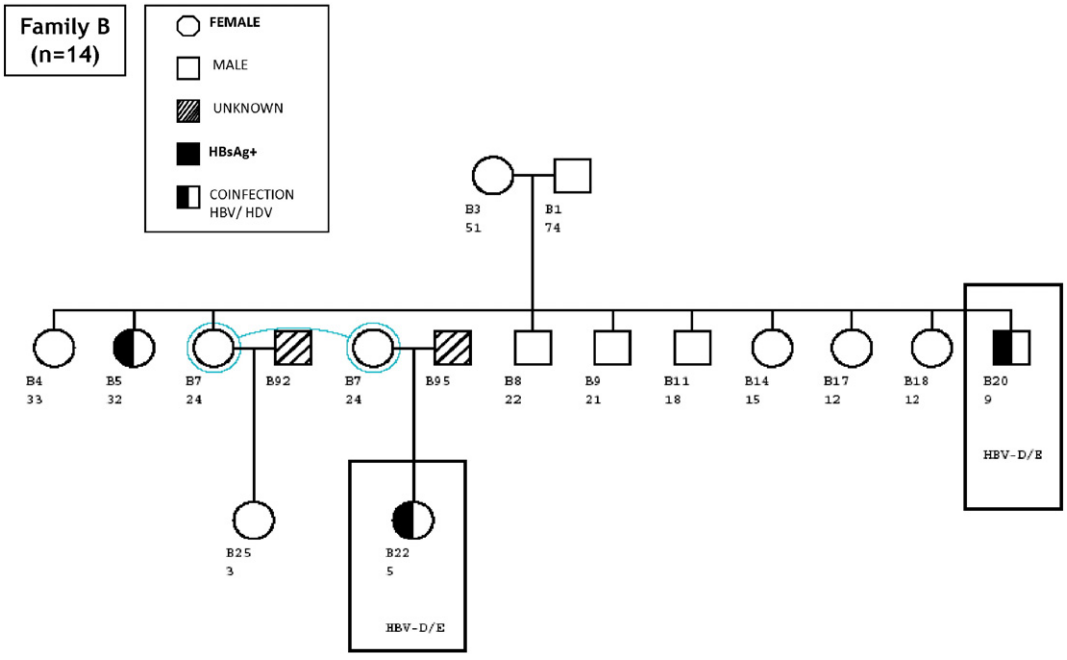

Fig. 5. HBV horizontal transmission in family B, constructed with CraneFoot v3.2 software. Family B is composed of 14 members. HBV-D/E strains (indicated in square) had 100% identity with son B20 and granddaughter B22.

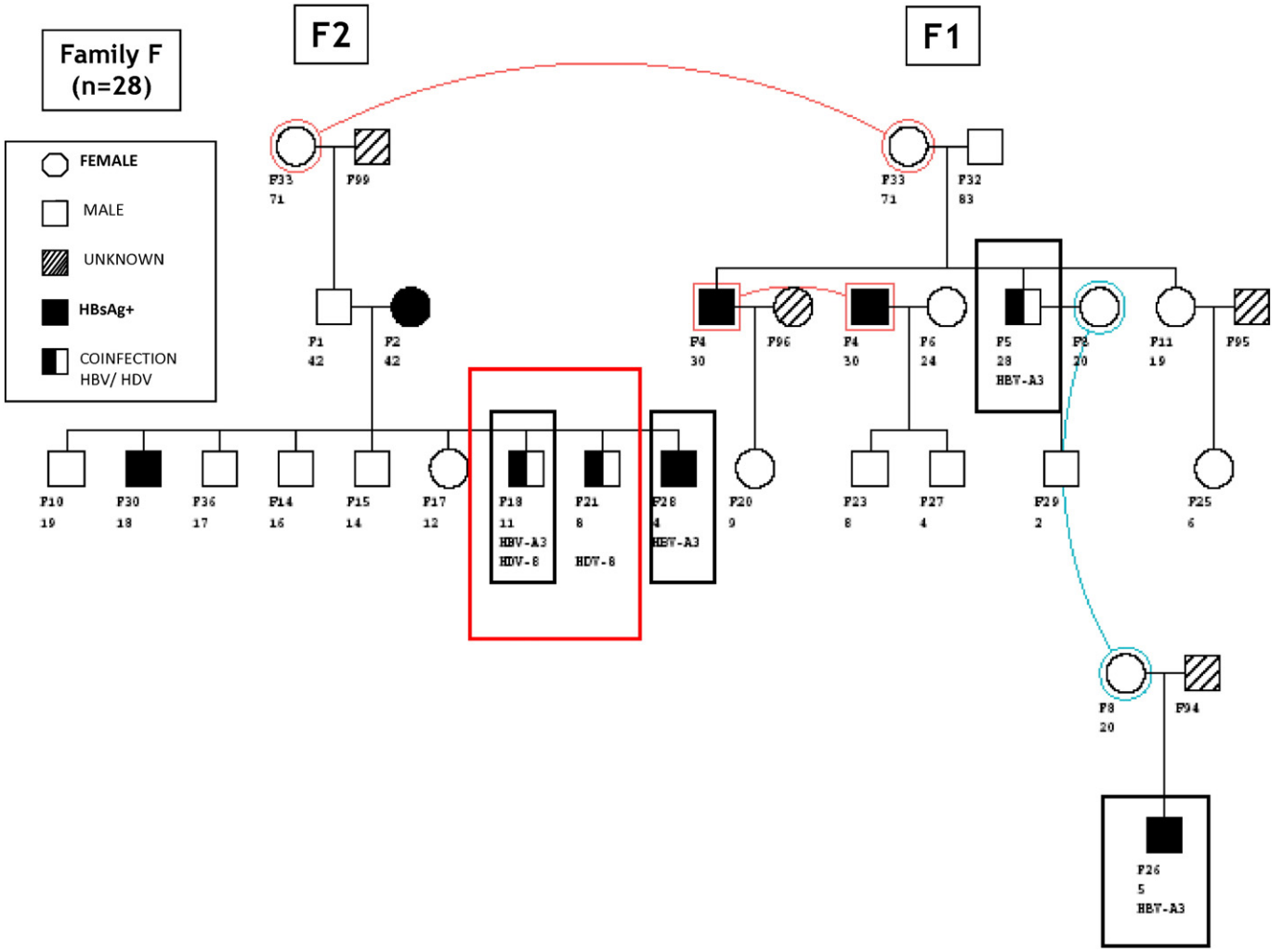

Fig. 6. HBV/HDV horizontal transmission in households F1 and F2, constructed with CraneFoot v3.2 software. Family F is composed of 28 members, divided into two households (F1 et F2). In household F1, the HBV-QS-A3 strain (in the black square) had 100% identity in son F5 and in grandson F26. In the second household (F2), the HBV-QS-A3 strains (in the black square) had 100% identity between grandsons F18 and the F28. In household F2, the HDV-8 strain (in the red square) had 100% identity in grandsons F18 and F21.

horizontal transmission of HBV strains. A son (B20, 9 years) and a granddaughter (B22, 5 years) were carriers of HBV-D/E strains with 100% sequence identity (see Fig. 5 and also Fig. 2 for the sequence identity).

Family F was composed of two households, the first founded by the mother (F33, 71 years), with 14 members, and the second founded by her son (F1, 42 years), with 11 members (Fig. 6). In the first household, the HBV-QS-A3 strains identified in a son (F5, 28 years old) and a grandson (F26, 5 years old) were 100% identical (see Fig. 2). Sequence identity (100%) was also found between HBV-QS-A3 strains detected in two grandsons of the second household (F18, 11 years and F28, 4 years). The overall sequence similarity of HBV-QS-A3 strains circulating in this family was 99.3% (Figs. 2 and 6).

The HDV-8 clade thus predominated in the two families and in the village. The divergence of strains belonging to this clade obtained from various individuals in the village was 5.9%. Molecular epidemiological analysis showed that the same HDV strain (100% sequence identity) was present in family F (see Fig. 6) in two grandsons F18 and F21 (see also Fig. 4 F18 is the strain DN603 and F21 is the strain DN600).

4. Discussion

The high prevalence of anti-HBc among inhabitants of Dienga confirms the heavy exposure of this population to HBV, partly owing to the geographical position of this remote Gabonese village. Exposure was as high as that reported among the Baka pygmies of eastern Cameroon and in relatively isolated populations of pygmies and Bantu throughout Cameroon (Kurbanov et al., 2005; Ndoumbe et al., 1993). The overall HBsAg prevalence in the village was also high and varied by sex and age group, as previously reported. Studies conducted in tropical and subtropical Africa have shown that HBV infection usually occurs during infancy (Kiire, 1996). Furthermore, HBsAg has been found to be more prevalent in males than females in Africa (Kramvis and Kew, 2007). In Ghana, a high seroprevalence of HBV markers was found in children aged 1–16 years (Martinson et al., 1998). In another study, conducted in Cameroonian schoolchildren, there was a significant difference between boys and girls ($p < 0.05$) (Chiaramonte et al., 1991; Rapicetta et al., 1991). In our study, the highest prevalence of HBsAg was found in young children and we found significantly higher carriage rates in men than in women ($p = 0.002$) and in boys than in girls ($p = 0.003$). As previously described, the greater chronicity of HBV infection in males may be due either to a prolonged replicative phase or to a differences in the sexual behaviour of men and women (Bekondi et al., 2007; Kramvis and Kew, 2007). It was recently reported, however, that body fluids such as saliva, urine, sweat and tears from HBV carriers are potential sources of transmission (Heiberg et al., 2009; Komatsu et al., 2012).

The proportion of HBeAg-positive carriers decreased with age. A low HBeAg prevalence (6.4%) was confirmed in 0–19-year-olds in rural

Gabon, and similar results were reported in Cameroon. Furthermore, we found only HBsAg in 4.2% of 0–10-year-old children, indicating recent HBV vaccination in the village (a campaign was conducted in Dienga village in 2005 as reported by Ouwe-Missi-Oukem-Boyer et al., 2011). The highest immunization coverage was in the age group 0–10 years, but twice as many boys as girls had HBsAg. We have no explanation for this observation, but the campaign appears to have been ineffective.

HDV is also highly endemic in some parts of southern and central Africa (Andernach et al., 2014; Husa et al., 2005). In our study, the prevalence of anti-HDV was high, with no significant difference in seropositivity by gender or age group, in contrast to HBV. This suggests either that the modes of HDV and HBV transmission are not exactly the same or that HDV infections are transmitted by super-infection rather than by co-infection with HBV.

We previously characterized HBV strains in Gabon as belonging to sub-genotype QS-A3 and genotype E, including the recombinant strain HBV-QS-A3/E and also HDV strains closely related to clades 1 and 8 (Makuwa et al., 2008; Makuwa et al., 2006). Here, we confirm the presence of these HBV genotypes and HDV clades in the rural population of Dienga. Moreover, for the first time, we identified genotype D HBV strains in the form of an HBV-D/E recombinant and showed that the new HDV strains clustered within clade 8. As previously reported, genotype E isolates clustered with and separately from genotype D, which is characterized by a 33-nucleotide deletion at the terminus of the pre-S1 domain (Bowyer and Sim, 2000; Makuwa et al., 2006). In previous studies, HBV-D and E genotypes showed very little divergence at the end of the X and most of the C region (Bowyer and Sim, 2000). In our analysis, the complete sequence of the DN171 strain was 3222 nucleotides long, and no deletion was observed in the pre-S1 domain, as described for strains classified in the HBV-D/E genotype (Abdou Chekaraou et al., 2010). Phylogenetic analysis of various HBV genes and SimPlot analysis both confirmed the existence of a D/E recombinant form of this HBV strain. A D/E recombinant strain previously described in the Central African Republic was classified as genotype E because of its uniqueness (Bekondi et al., 2007). More recently, however, additional HBV-D/E strains have been described in Niger, with different recombination breakpoints (Abdou Chekaraou et al., 2010). In Gabon, we also confirmed that the D8 genotype is a genotype regrouping of recombinant strains HBV-D/E (Yousif and Kramvis, 2013). In a very remote village, it is not surprising that there is a link between the D and E strains found in two children. The factors favouring such recombination are related to their high prevalence in Gabon, and we have presented here a new possibility of recombination with genotype D, which entered the territory recently. Furthermore, we show that they are transmitted within families, although their precise origin was not found. More studies are needed to reconstruct the natural history and origin of HBV-D and E in central Africa.

With the coexistence of genotypes A and E in West Africa and the recent spread of genotype E throughout central Africa, simultaneous analysis of HBV-S and pre-core/core sequences has revealed the presence of recombinant A/E in Cameroon and in Gabon (Makuwa et al., 2008; Makuwa et al., 2006; Olinger et al., 2006). In our previous studies in Gabon, in rural and urban populations, we found mostly genotype QS-A3 (Makuwa et al., 2008; 2009). Here, we confirm the presence of this genotype and also a considerable increase in genotype E. It is, however, difficult to reach a conclusion because of the small number of samples. The only possible explanation is horizontal transmission among HBV-E-infected children and adolescents in this close village community.

Additionally, we sequenced both the core and S regions of 10 strains and found discrepancies in the analysis of genotypes for five of them (HBV-QS-A3/E, HBV-E/QS-A3 and HBV-D/E), indicating a significant number of recombinant strains. This indicates that complete genome sequencing would be necessary. Because in the vast majority of cases of recombination the S and C regions are classified as discordant (Abdou Chekaraou et al., 2010; Bekondi et al., 2007; Garmiri et al., 2009; Yousif et al., 2014), new strategies are being developed to amplify several HBV strains in Gabon in order to map the genotypes circulating in this country accurately.

The risk of a family for HBV infection is determined mainly by the number of infectious members and by behavioural and cultural factors. In the village and families studied here, we collected information on risk factors that might be associated with virus transmission, particularly among young people, such as ear-piercing for girls, injections outside a health centre, use of sharp objects and the beginning of sexual activity. In areas where HDV is endemic in the general population, interpersonal contact in overcrowded households could favour HDV transmission. Despite the HBsAg carrier status of the mothers in the two families, we found no vertical transmission of HBV or HDV, as the same strains were not identified in the mothers and the children. The high sequence homology among the HBV strains found in the families and in other villagers indicates horizontal intra-familial and also inter-familial transmission. Similarly, HDV-8 strains predominated in the village and within families. Clearly, inter-familial contacts play a major role in dissemination of HBV and HDV in this village. Phylogenetic analyses of HDV strains showed that all except one of the amplified HDV-8 strains originated from male HBsAg carriers and that the HDV-8 strains fell into two distinct subgroups, with 7.5% nucleotide divergence. These findings indicate that there are two subgroups within the HDV-8 clade, one of which contains only strains from the village. A large survey of samples from various areas of central Africa is needed to confirm the subgroup partition of this clade.

5. Conclusion

In conclusion, this study of a remote Gabonese village shows a high prevalence of HBsAg carriage and HDV infection, particularly among children and adolescents. The prevalence of HBsAg differed significantly by gender and age, females being more likely than males to carry HBsAg during the first 10 years of life, while the prevalence was higher among males than females in the 11- to 20-year age group. Moreover, we describe for the first time the presence of the HBV-D genotype in Gabon, in the form of HBV-D/E recombinants, with previously reported A3/E recombinants. We found no vertical transmission of HBV or HDV in the families studied but observed horizontal intra-familial and inter-familial transmission of HBV and HDV among children. Our findings indicate that HBV is transmitted in early childhood via body fluids rather than through sexual contact. Health education adapted to the different age groups might help to reduce HBV transmission. All young children should be vaccinated to control HBV infection in areas of extremely high prevalence.

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