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PREVALENCE AND GENETIC VARIANTS OF HEPATITIS GB-C/HG AND TT VIRUSES IN GABON, EQUATORIAL AFRICA

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Abstract. The distribution of Hepatitis GB-C/HG (GBV-C/HGV) and TT viruses (TTV) infections was investigated in selected populations from Gabon using Polymerase Chain Reaction (PCR) and Enzyme Linked Immunosorbent Assay (ELISA) for anti-Envelop 2 (anti-E2) GBV-C/HGV antibodies. Among pregnant women, 29 of 229 (12.6%) were Hepatitis GB virus-C and Hepatitis G virus (GBV-C/ HGV) RNA positive (+) and 32 of 81 (39.5%) anti-E2 + versus 8 of 39 (20.5%) TTV DNA +. Among sickle cell anemia patients, 9.7% (3/31) were GBV-C/HGV RNA + versus 22.5% (7/31) TTV DNA +. For tuberculosis patients, the figures were 11.5% (4/35) and 0%. A study of hepatocellular carcinoma cases (n = 27) versus controls (n = 66) did not show significant differences for GBV-C/ HGV RNA (10.7% versus 12.1%) and TTV DNA (44.4% versus 30.3%). According to phylogenetic analysis, the 15 GBV-C/HGV strains investigated clustered in group 1, the most common in sub-Saharan Africa whereas TTV sequences (n = 4) mostly clustered in genotypes G1 and one close to genotype G3. In the Gabonese populations investigated, GBV-C/HGV and TTV infections were highly endemic. These data are consistent with the low pathogenicity of these agents.

INTRODUCTION

Several hepatitis viruses have been identified (Hepatitis A, B, C, D, and E Viruses HAV, HBV, HCV, HDV, and HEV), and several of them (HBV, HCV, HDV) can induce the chronic carrier state. Hepatitis C virus in particular has been shown to be a major cause of parenterally transmitted non-A non-B (NANB) hepatitis.¹⁻³ However, the cause of 10% to 20% of hepatitis cases remains unknown.⁴ Recent studies searching for new viral agents responsible for non-A non-E (NANE) hepatitis have resulted in the discovery of new human viruses. Thus, a flavivirus, Hepatitis GB-C/HG virus (GBV-C/HGV), has been isolated from the serum of patients with non-A non-C hepatitis.^{5,6} Hepatitis GB-C/HG virus (GBV-C/HGV) is distantly related to HCV. It has a mutation rate of 3.9×10^{-4} base substitutions per site per year, about one quarter that of HCV.^{7,8} A classification of GBV-C/HGV samples from around the world into three or more groups has been suggested.^{7,9} A correlation has been found between such "genotypes" and the geographical origin of the variants.¹⁰⁻¹² Several investigations have demonstrated that GBV-C/HGV can be transmitted by blood products,^{6,13} and a high prevalence (22% to 38%) of viral RNA has been reported for populations with parenteral risk.^{14,15} However, a high prevalence of GBV-C/ HGV infection has also been observed in groups with little or no parenteral risk, such as blood donors.¹³⁻¹⁵ Furthermore, despite several studies carried out in recent years, the role of GBV-C/HGV in pathogenesis is unclear.^{16,17}

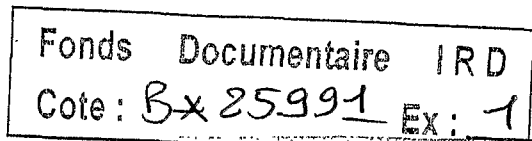
A new viral agent, the TT virus or TTV, has recently been isolated in Japan from the serum of a patient (TT) with post-transfusion hepatitis.^{18,19} TT virus (TTV) has a circular single-stranded DNA genome, 3,852 base pairs (bp) in size, the organization of which is similar to that of genomes of the *circoviridae*. TT virus belongs to a new family of viruses called

circoviridae.^{20,21} Isolates from different geographic areas are genomically heterogeneous and at least three major genotypes have been identified.^{18,19} Several studies have investigated the prevalence of TTV DNA in patients from different geographical areas.²²⁻²⁴ In Japan, 40 to 50% of patients with fulminant and cryptogenic liver diseases test positive for TTV DNA.¹⁸ However, other studies have reported lower rates of infection in such patients.^{22,24} These investigations have also demonstrated a high prevalence of TTV DNA among blood donors and suggest that TTV has a limited pathogenic impact. The route of TTV transmission is also unclear, although there is strong evidence for blood transmission and some studies have suggested a fecal-oral route.^{25,26}

A few studies have reported a high prevalence of GBV-C/HGV²⁷⁻²⁹ and TTV infection³⁰ in sub-Saharan Africa. The prevalence of both types of viral infection within a single African geographical area has not been considered, and the modes of transmission of these viruses in Africa have remained elusive. We therefore assessed the prevalence of GBV-C/HGV and TTV in Gabon in equatorial Africa. Previous studies reported a high prevalence of chronic HBV and HCV infection in this population.³¹⁻³³ We therefore also carried out a case-control study to evaluate the possible effects of these viruses on hepatocellular carcinoma (HCC). Finally, a phylogenetic analysis of HGV and TTV was performed for a few Gabonese isolates.

MATERIALS AND METHODS

Study population. A total of 388 subjects from Libreville, the capital of Gabon in the west of the country, and from the vicinity of Franceville, a regional capital in the east, were enrolled between 1991 and 1995. Two hundred twenty-nine pregnant women were consecutively recruited when they



came for prenatal consultation at mother-child clinics in Libreville ($n = 150$, mean age 22.2 ± 5.1 years) and Franceville ($n = 79$, mean age 22.2 ± 5.1 years).

We evaluated the impact of parenteral transmission by studying 31 consecutively enrolled polytransfused patients with sickle cell anemia (M:F sex ratio 15/16, mean age 4.9 ± 3.2 years) from Libreville University Hospital. We also tested 35 consecutively recruited patients with tuberculosis (M:F sex ratio 20/13, mean age 33.9 ± 11.3 years) attending the Nkembo Hospital in Libreville. These patients had received multiple intramuscular injections in the past.

We also carried out a case-control study in the Department of Internal Medicine of Libreville University Hospital. This study included 27 patients with Hepatocellular Carcinoma HCC (M:F sex ratio 18/9, mean age 39.3 ± 17.1 years) and 66 hospitalized individuals without liver disease as controls (M:F sex ratio 49/17, mean age 40.0 ± 17.2 years) matched for age (± 5 years) and sex. The diagnosis of HCC was based on ultrasound scans and an alfa-fetoprotein concentration $> 1,000$ ng/ml Enzyme Immuno Assay (EIA) (Abbott, Chicago, IL). Seven of 26 tested HCC positive and 8 of 43 controls were positive for anti-HCV, 4 of 26 HCC and 2 of 33 remained indeterminate. Eleven of 26 HCC and 5 of 43 controls scored positive for Hepatitis B surface Antigen (HBsAg).

Polymerase Chain Reaction (PCR) using the 5'UTR of GBV-C/HGV RNA was carried out for all 388 samples. Three hundred and eleven randomly selected samples were also tested with NS3 specific primers. 5'UTR and NS3 PCR results agreed in 100% of cases. Sufficient serum was available to test 81 pregnant women for HGV anti-E2 antibodies and 108 randomly selected samples for TTV DNA.

METHODS

Collection of blood. Serum and plasma samples were collected and stored at -20°C . All analyses were performed blind.

RNA GBV-C/HGV extraction and cDNA synthesis. RNA was extracted from 100 μL of serum or plasma using a modification of the guanidium isothiocyanate method (RNAzol B; Bioprobe Systems).³⁴ cDNA was synthesized by incubation at 42°C with MMLV reverse transcriptase and random hexamers (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) as previously described.³⁵ The cDNA was stored at -20°C until use.

GBV-C/HGV PCR assay. cDNA (5 μL) was used for a PCR assay using degenerate primers specific for the 5'UTR (Abbott Laboratories, North Chicago, IL). Three hundred and eleven samples were then selected at random for confirmation by a second PCR assay with primers specific for non-structural region 3 (NS3) (Genelabs Technologies, Redwood City, CA). For both PCR assays, amplification conditions were as previously described.³⁵ PCR products were assessed by Southern blot hybridization.³⁵

Detection of TTV DNA by PCR. DNA was extracted from 100 μL serum using the Qiam Blood kit (Qiagen S. A., Courtaboeuf, France) and dissolved in 100 μL elution buffer. We tested the resulting DNA solution (7 μL) for TTV DNA by hemi-nested PCR as described by Okamoto¹⁹ with minor modifications. The first round of PCR was performed

with 2.5 U of Gold ampliTaQ DNA polymerase (Perkin Elmer) with RD59 sense and RD63 antisense primers¹⁹ for one annealing cycle (95°C for 10 min; 60°C for 1 min; 72°C for 1 min) then 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec followed by an additional 7 min at 72°C . This resulted in the amplification of a 286 bp fragment. The second round of PCR was carried out with the RD61 sense and RD63 antisense primers¹⁹ for 25 cycles in the same conditions, resulting in amplification of a 271 bp fragment. PCR products were subjected to electrophoresis in a 2% agarose gel and then stained with ethidium bromide.

We checked the sensitivity of the procedure by adding a dilution of a positive sample to each experiment. To avoid false positive results, we systematically introduced negative controls at each stage of PCR and dTTP was replaced by dUTP in the PCR mixture.³⁵ All positive and randomly selected negative samples gave the same result in two independent assays.

5'UTR GBV-C/HGV and TTV sequencing and phylogenetic analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen S. A.). The nucleotide sequences of these products were directly determined on both strands using PCR primers.

A 214 bp amplified product from the 5'UTR GBV-C/HGV, excluding primer sequences, (from nucleotide -172 to -386 relative to the AUG codon near the start of the long open reading frame),⁵ obtained from 15 pregnant women, was used for phylogenetic analysis. The sequences obtained were aligned with the corresponding region of 35 5'UTR GBV-C/HGV sequences from GenBank.

A 224 bp amplified fragment (excluding primer sequences, from nucleotide 1938 to 2162, in open reading frame 1)¹⁹ of TTV DNA was directly sequenced. This fragment was amplified from the DNA of 4 individuals (2 HCC patients and 2 matched case-control samples) and was used for phylogenetic analysis. The sequences obtained were aligned with the corresponding region of 39 TTV sequences from GenBank. For both GBV-C/HGV and TTV, the sequences were aligned with CLUSTAL W 1.7 software³⁶ and a phylogenetic tree was created with DNADIST and NEIGHBOR, PHYLIP package version 3.53.³⁷ The data set were bootstrapped, resampling 1,000 times with SEQBOOT software.

Anti E2 GBV-C/HGV antibody ELISA assay. Antibodies directed against the E2 envelope protein of GBV-C/HGV (anti-E2 antibodies) were detected using an ELISA research kit (μPlate Anti-Hgenv, Boehringer-Mannheim, Germany). The procedure was carried out and the results interpreted in accordance with the manufacturer's recommendations.

STATISTICAL METHODS

Data were analyzed using EPIINFO 6 (Center for Disease Control and Prevention, Atlanta, GA). Results are expressed as means with standard deviations (SD) and as prevalence rates with 95% confidence intervals (95% CI). The Chi Square test and, if necessary, the Fisher exact test were used to compare HCC patients and their controls. $P < 0.05$ was regarded as statistically significant.

TABLE 1
Hepatitis GB-C/HG (GB-C/HG) virus RNA and TT virus DNA distribution and coinfection in Gabon

	Pregnant women						Parental risk factors					
	Libreville			Franceville			Sickle cell anemia			Tuberculosis		
	<i>n</i>	<i>n</i> +	%+	<i>n</i>	<i>n</i> +	%+	<i>n</i>	<i>n</i> +	%+	<i>n</i>	<i>n</i> +	%+
HGV/GBV-C RNA	150	20	13.3	79	9	11.4	31	3	9.7	35	4	11.5
Anti-E2	81	32	39.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
TTV DNA	ND	ND	ND	39	8	20.5	31	7	22.5	35	0	0
HGV/GBV-C RNA + TTV DNA	ND	ND	ND	39	0	0	31	0	0	35	0	0

ND = not done.
n = number of samples.
n + = number of positive samples.
n - = number of negative samples.

RESULTS

The results of PCR tests for the GBV-C/HGV and TTV genomes and serological tests for the anti-E2 antibody are reported in Tables 1 and 2.

Pregnant women. Twenty-nine of 229 (12.6%) pregnant women (20 of 150 from Libreville and 9 of 79 from Franceville) tested positive for GBV-C/HGV RNA. Anti-E2 GBV-C/HGV antibodies were found in 32 of 81 (39.5%) of the pregnant women tested. All anti-E2 antibody-positive women tested negative for GBV-C/HGV RNA and only one woman testing positive for GBV-C/HGV RNA tested negative for anti-E2 antibodies.

Eight of the 39 enrolled pregnant women from Franceville (20.5%) tested positive for TTV DNA. We observed no coinfection with GBV-C/HGV and TTV viruses in this group.

Patients with parenteral risk factors. Three of the 31 (9.7%) children with sickle cell anemia tested positive for GBV-C/HGV RNA and 7 (22.5%) tested positive for TTV DNA. We observed no coinfection with the two viruses in this group of patients. Four of 35 (11.5%) patients with tuberculosis tested positive for GBV-C/HGV RNA; none tested positive for TTV DNA.

Patients with HCC and matched controls. Three of 27 (10.7%) patients with HCC and 8 of 66 (12.1%) controls tested positive for GBV-C/HGV RNA (not significant). Twelve of the 27 (44.4%) patients with HCC and 20 of the 66 (30.3%) matched controls tested positive for TTV DNA (not significant). Three of the 66 (4.5%) controls and 2 of the 27 (7.4%) HCC cases had GBV-C/HGV/TTV coinfection.

Correlation between clinical parameters and GBV-C/HGV/TTV status. There was no significant difference in the numbers of positive or negative tests for GBV-C/HGV and

TTV according to type of patients, age or sex, in any of the groups.

Genetic analyses of GBV-C/HGV isolates. We analyzed GBV-C/HGV 5'NCR sequences for 15 samples obtained from pregnant women from Franceville. These sequences were compared with GBV-C/HGV sequences (available from databases) of isolates from around the world. All 15 strains appeared to belong to group 1 (Figure 1).

Genetic analyses of TTV isolates. TTV DNA sequence analysis was performed using 4 samples obtained from Gabonese subjects (2 patients with HCC and 2 matched controls). The 224 bp sequences were aligned and compared with known TTV sequences (genotypes 1 to 3). Phylogenetic analysis showed that 3 of the 4 isolates (2 HCC and 1 control) clustered in genotype G1. The TTV sequence obtained from one of the controls was closely related to genotype 3 (Figure 2).

DISCUSSION

This study provides information about GBV-C/HGV and TTV infection in central Africa. We found that the prevalence of infection was high for these two viruses in this area. Although our results showed the parenteral transmission was operative, we could not demonstrate that it was the exclusive factor in acquisition of these viruses. In this area where the incidence of HCC is high, we observed that GBV-C/HGV and TTV infection were not associated with HCC.

The prevalence of GBV-C/HGV was as great as 12.6% and that of TTV 20.5%, based on detection of viral DNA by PCR. This observation is consistent with previous data describing the prevalence of HCV (6.5% of adults have anti-HCV antibodies)³¹ and HBV (9.6 to 19% are HBsAg carriers).

TABLE 2

Hepatitis GB-C/HG virus RNA and TT virus DNA prevalence and coinfection in hepatocellular carcinoma (HCC) cases and matched controls from Gabon

	HCC			Controls			<i>P</i>
	<i>n</i>	<i>n</i> +	%+	<i>n</i>	<i>n</i> +	%+	
HGV/GBV-C RNA	27	3	10.7	66	8	12.1	NS
TTV DNA	27	12	44.4	66	20	30.3	NS
HGV/GBV-C RNA + TTV DNA	27	2	7.4	66	3	4.5	

P = χ^2 test.
NS = not significantly different.
n = number of samples.
n + = number of positive samples.

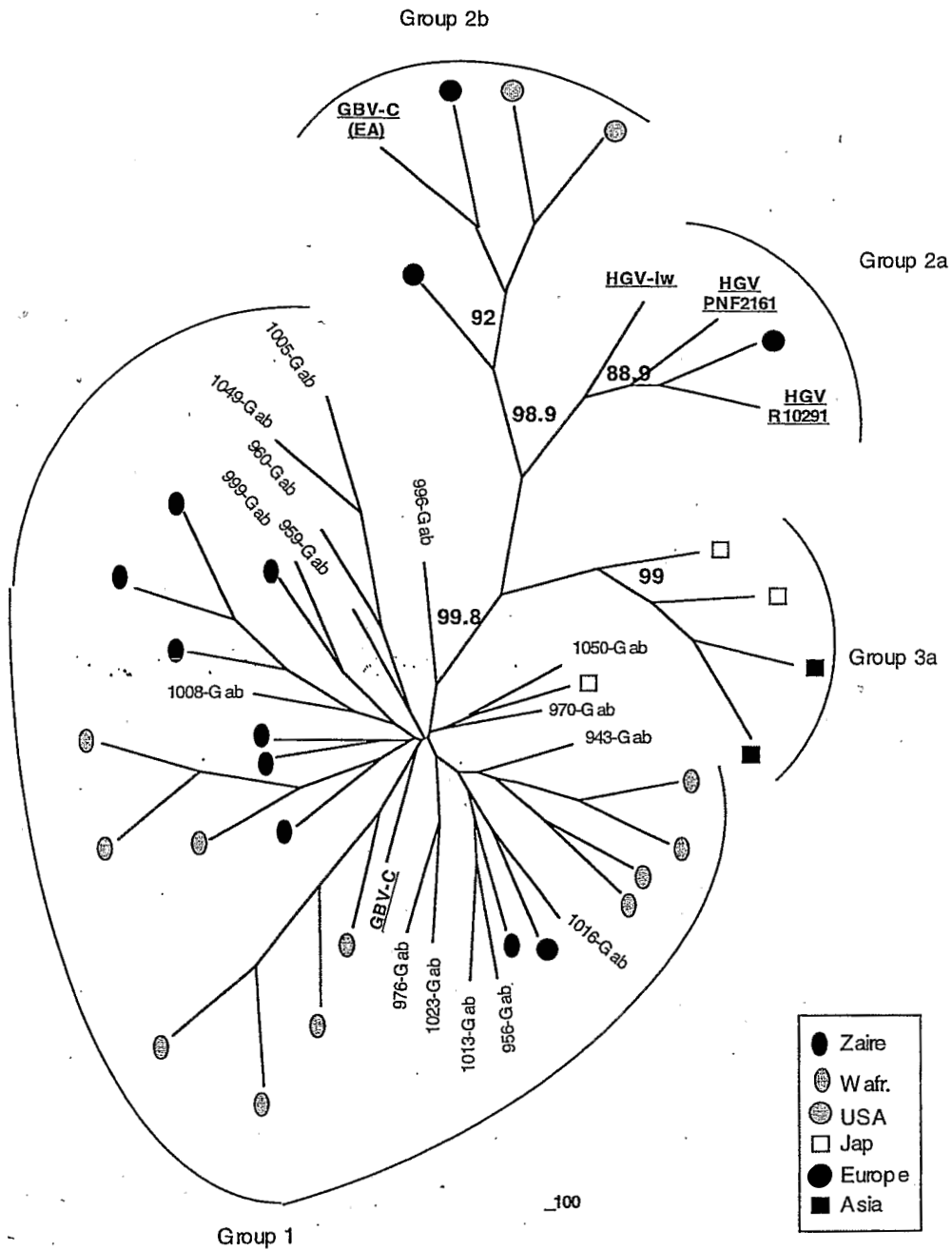


FIGURE 1. Phylogenetic tree of Hepatitis GB-C/HG viruses (GBV-C/HGV) sequences from 15 Gabonese isolates. Consensus phylogenetic tree for 14 isolates constructed on a 214 nucleotide fragments [position -172 to -386 of GBV-C (EA) and HGV PNF2161 prototypes] indicated with a number followed by "Gab". The tree was generated from an alignment of 35 sequences of various geographical origins. The geographic origin is indicated by a symbol in the figure. Sequences from Zaire, Ghana (W Afr.), Europe, USA, and Japan were available from GenBank and are described elsewhere.^{9,11,48} The sequences of a few isolates have not yet been published (accession numbers: D87249 and D87250 from Japan, AF058746 from China, Asia). Bootstrap values obtained from 1,000 replicates are given as percentages of the branching forks if significant.

ers)^{32,33} in the Gabonese population. The prevalence of GBV-C/HGV in Gabon is similar to that reported in Ghana,²⁷ South Africa^{28,29} and central Africa.¹² We found no significant correlation between GBV-C/HGV infection and age or sex. This is consistent with some³⁸ but not all²⁷ previous reports. We evaluated GBV-C/HGV infection in pregnant women from urban and semi-rural areas of Gabon and found no significant difference between these two areas. All preg-

nant women that tested positive for anti-E2 antibody were negative for GBV-C/HGV RNA. As in other series, the absence of detectable GBV-C/HGV RNA in anti-E2 antibody-positive subjects indicates previous exposure to and clearance of the virus.^{16,39} Also consistent with this view, the prevalence of previous exposure (anti-E2 antibody = 39.5%) was three times higher than that of viremia (12.6%). This figure is three times higher than that for pregnant women in

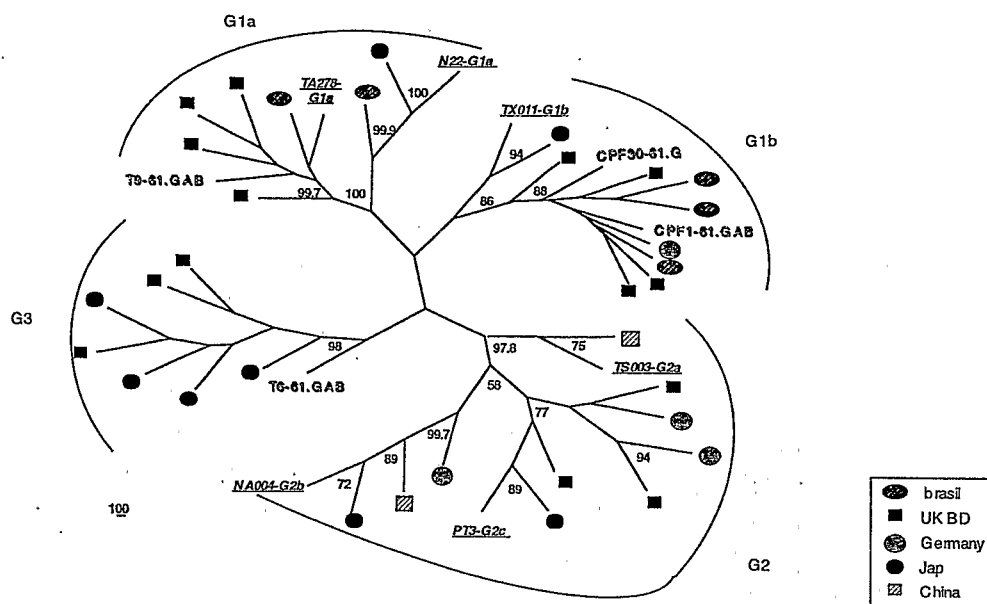


FIGURE 2. Phylogenetic tree for TT virus (TTV) sequences from Gabon. Consensus phylogenetic tree for 4 Gabonese isolates based on comparison of partial nucleotide sequences of the 224 nucleotide fragments (position 1938–2162 in ORF1)²⁵ indicated with "Gab". The phylogenetic tree was constructed by the neighbor-joining method with the reference sequences (N22, TA 278:G1a), (Tx011:G1b), (Ts003:G2a), and (NA004:G2b) previously reported²⁵ and sequences originating from various geographical areas: United Kingdom,²⁴ Japan,^{25,49} China (AB018447, AB018475), and Germany.⁵⁰ Classification into genotypes (indicated by Arabic numbers) was based on an evolutionary distance > 0.30 and that into subtypes (lower case letters) on a distance > 0.15 . Bootstrap values obtained from 1,000 replicates are given as percentages of the branching forks.

France (16%), and four times higher than for pregnant women in England (11.1%).^{15,40} The prevalence of GBV-C/HGV viremia in patients with sickle cell anemia (9.7%) was lower than that observed in other African polytransfused patients (e.g., South African hemophiliacs = 23.8%).²⁹ These results suggest parenteral transmission although other routes could not be excluded.^{15,16,35}

We observed a high prevalence of TTV infection, consistent with previous reports.^{22,24} However, the prevalence of TTV in our population was lower than that reported for pregnant women in Congo.³⁰ We found no correlation between the prevalence of TTV infection and age or sex, also consistent with reports from Congo. Similar prevalences of TTV have been reported in pregnant women and unselected blood donors in Thailand.⁴¹ Thus, the high prevalence of TTV viremia in pregnant Gabonese women probably reflects extensive exposure of the general population in this area. As there is currently no serological assay for TTV infection, we were only able to investigate active TTV infection. The overall prevalence of exposure to this virus is therefore likely greater. Previous reports have suggested a fecal-oral route of transmission for TTV.^{25,26} It is unclear whether TTV is efficiently transmitted by sexual intercourse but recent data obtained in France suggest that this is the case.⁴²

Most studies on TTV in North America and Europe have shown no significant impact of TTV on liver disease. However, a few studies have suggested an association with acute and chronic hepatitis and TTV and HCC.^{43,44} Our case-control study enabled us to assess the impact of GBV-C/HGV and TTV in Gabonese patients with HCC. Although the prevalence of GBV-C/HGV and TTV viremia tended to be slightly higher in patients with HCC, it was not significantly different from that of age and sex matched controls. Thus,

our results are not consistent with an association between viral infection and HCC.^{16,17,45}

We were able to analyze for a large proportion of the study population the presence of both the TTV and GBV-C/HGV genomes. We found that simultaneous infection by GBV-C/HGV and TTV was rare in the Gabonese population. GBV-C/HGV and TTV coinfection was detected only in HCC cases, and there was no significant difference from the matched controls. The relatively small number of samples available for testing of both GBV-C/HGV and TTV did not permit a more in depth investigation of this issue.

A phylogenetic analysis of GBV-C/HGV and TTV isolates was performed. Most of the Gabonese TTV isolates clustered in group 1. Previous phylogenetic analyses have suggested that variants of GBV-C/HGV can be assigned to three or more groups or genotypes based on geographical origin.^{46,47} A comparison based on other sequences, such as those for the NS3 helicase or NS5b, could not distinguish between isolates of different geographical origins.^{7,10} For GBV-C/HGV sequences, we analyzed a restricted region of 5'NC and the dendrogram obtained was similar to those obtained for full-length sequences.^{11,48} We therefore used a fragment of the 5'NCR to analyze the phylogenetic relationship between the Gabonese isolates and other sequences from around the world.^{11,46} Our isolates clustered in group 1, together with the few previously reported African sequences and the original GBV-C isolate. This indicates that the most frequent group in circulation in Gabon is the same as that in west Africa.¹²

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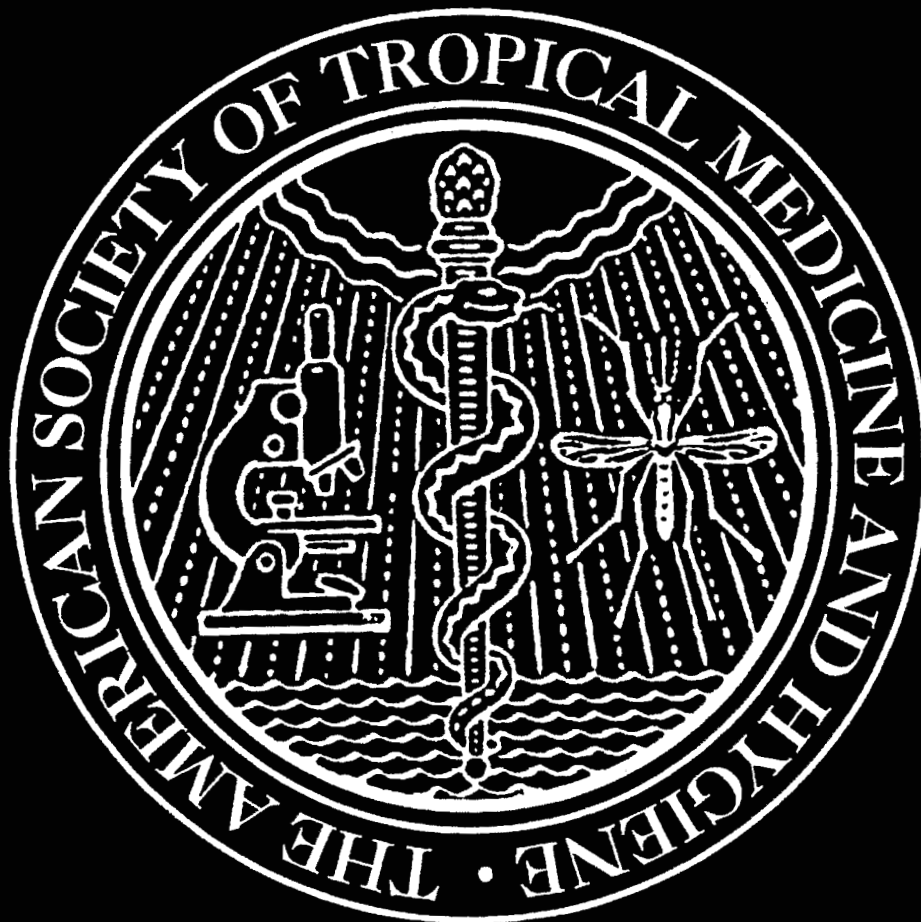
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