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High Prevalence of Hepatitis C Virus Infection and Predominance of Genotype 4 in Rural Gabon

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Hepatitis C (HCV) molecular epidemiology is documented poorly in central African countries. In response to this, a population-based study of 319 consenting adults resident in a remote village of Gabon was undertaken (mean age: 38 years; age range: 13–85+; sex ratio: 0.74). Screening for anti-HCV antibodies was performed using ELISA and recombinant immunoblot assay. Seropositive samples were assessed further with viral load and genotyping techniques. Sixty-six (20.7%) individuals were HCV seropositive. Viral loads ranged from 600 to 24.9 million IU/ml (median: 372,500). Seroprevalence and viral loads increased significantly with age ($P < 10^{-5}$ and $P < 0.003$, respectively). HCV sequences of the 5′UTR genome region were obtained from 60 (90.9%) samples and NS5B region sequences were obtained from 22 (36.6%) samples. All strains belonged to subtypes of genotype 4: 4e (72.7%), 4c (13.6%), 4p (4.5%), 4r (4.5%) and one unclassified genotype 4 strain. Evolutionary analysis of the subtype 4e sequences indicates a period of raised transmission during the early twentieth century. ***J. Med. Virol. 80:1581–1587, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HCV molecular epidemiology; NS5B; 5′UTR; genotype 4; Central Africa

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem in both developed and developing countries. More than 170 million people are infected chronically worldwide [Shepard et al., 2005]. The overall estimated HCV prevalence for the 33 countries of sub-Saharan Africa is 3.0% (median 2.2%; range: 0.1–13.8%) with variation in prevalences between west and east Africa [Madhava et al., 2002]. HCV disease burden results from the development of chronic liver diseases such as cirrhosis or carcinoma. HCV is a single-stranded, positive-sense RNA virus belonging to the Flaviviridae family. Its genome is 9.7 kb long and encodes a single long polyprotein with the following gene order: 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ [Lindenbach and Rice, 2005]. The open reading frame is flanked by short non-coding regions (UTRs) located at the 5′ and 3′ ends of the genome [Choo et al., 1991].

The HCV genome is highly variable with an average rate of evolution of 10^{-3}–10^{-4} nucleotide substitutions per site per year [Ogata et al., 1991; Pybus et al., 2001]. The lowest sequence variability among genotypes is found in the 5′UTR genome region, whereas the NS5 and E1 regions are highly variable [Smith et al., 1997]. On the basis of phylogenetic analysis, HCV has been grouped into six distinct genotypes and numerous subtypes [Simmonds et al., 1993]. Genotyping of HCV strains is an important tool for understanding differences among infections in serological reactivity, virulence, response to treatment [Halfon et al., 2003;

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Fried, 2004] and transmission route [Simmonds et al., 2005]. Furthermore, the substantial diversity of HCV represents a major challenge to future vaccine development.

Subtypes belonging to genotypes 1, 2, and 3, notably subtypes 1a, 1b, 2a, 2b, 2c, and 3a, are distributed widely throughout Western countries. Types 5 and 6 are confined largely to South Africa and southeast Asia respectively, whereas type 4 was first found predominantly in the Middle East [Ray et al., 2000] and then in central African countries [Xu et al., 1994; Fretz et al., 1995; Njouom et al., 2003a]. The recent introduction and spread of some genotype 4 strains in Europe has been highlighted [Matera et al., 2002; van Asten et al., 2004; Nicot et al., 2005; Echevarria et al., 2006; Cenci et al., 2007] and two different epidemiological profiles have been identified, with linkage either to intravenous drug users or to immigration from Africa and the Middle East [Nicot et al., 2005]. Data concerning genotype 4 prevalence and molecular epidemiology are limited in central African countries, although high levels of endemicity have been described [Njouom et al., 2003a]. Seroprevalence of up to 6.5% has been reported previously in Gabon [Delaporte et al., 1993] and the three available HCV sequences from Gabon all belong to genotype 4 [Xu et al., 1994]. Other genotypes have also been reported in nearby regions of Cameroon [Nkengasong et al., 1995; Ndjomou et al., 2002; Pasquier et al., 2005], but large-scale population-based studies are not available in Gabon. This article reports a study of HCV in a remote village of Gabon (Dienga), providing further insights into the prevalence, diversity and epidemic history of HCV in Gabon. Seroprevalence, viral loads and viral genotypes were determined and linked to population characteristics.

MATERIALS AND METHODS

Study Population

The study took place in February 2003. Dienga is located near the Congo border, 180 km from Franceville. Among 2,500 registered residents, 778 were permanent residents (men 44.1%; women 55.9%; mean age 31 years). Since 1994 the International Centre of Medical Research, Franceville (CIRMF) has maintained a community clinic with laboratory facilities for studies on malaria. Ethical clearance was obtained from the Gabonese Ministry of Health and from the CIRMF ethics committee. Procedures were performed in accordance with the Helsinki declaration. All subjects (age $\geq$13 years) were informed of the study and only those who signed an informed consent were included.

Collection and Transport of Samples

Blood samples were collected in EDTA tubes. Three aliquots of plasma were stored frozen at -20°C overnight then transferred at -160°C until they were used at CIRMF (serological tests and PCR) and at Bichat Claude Bernard Hospital, Paris France (viral load measurements).

Serological Tests

All samples were screened for anti-HCV antibodies using a commercial third-generation ELISA (Monolisa, anti-HCV PLUS version 2, Bio-Rad, Marne-La-Coquette, France). Positive and doubtful plasma samples with 10% $\pm$ deviation from the threshold value were then tested with the third-generation Recombinant Immunoblot Assay-SIA (RIBA; Chiron Corporation, Emeryville, CA). Subjects were considered to be HCV-seropositive if both ELISA and RIBA were positive.

RNA Extraction and HCV Viral Load Determination

All positive samples were analyzed further. RNA was extracted from 140 µl of plasma using the Qiamp® viral mini kit (QIAGEN, Courtaboeuf, France). Samples were eluted with 60 µl elution buffer and stored at -80°C. HCV viral load was determined using a quantitative RT-PCR-based Cobas AMPLICOR™ MONITOR test, version 2.0 (linear range, 600 to 500,000 IU/ml; 1 IU/ml = 2.7 copies/ml; Roche, Meylan, France).

Amplification and DNA Sequencing

Subsequently, RNA was amplified by RT-PCR and nested PCR with a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Waltham, MA) using primers specific to the 5′UTR and NS5B regions (primers provided in supplemental material). Briefly, reverse transcription and first amplifications were performed using Titan One Tube RT-PCR kit (Roche Diagnostics, Mannheim, Germany). The thermal cycle was 43°C for 60 min for the RT step, then the amplification cycles were performed with a hybridization step of 52–60° according to the primers used. Five microliters of the first amplification product were used as template for the nested second round of PCR in a final volume of 100 µl.

PCR products were purified in columns (QIAquick PCR purification kit) and sent to Macrogen Inc. (Seoul, Korea) for sequencing. Sequence accession numbers are as follows: AM177500 to AM177508 and AM888292 to AM888304 for the NS5B sequences, and AM177509 to AM177562 for the 5′UTR sequences.

Sequence and Phylogenetic Analysis

Unaligned 5′UTR and NS5B sequences were edited by hand and aligned using Clustal W version 1.8 (http://www-igbmc.u-strasbg.fr/bioinfo) and Se-al version 2.0 (http://evolve.zoo.ox.ac.uk). Sequences were compared to reference sequences from the European HCV database (http://euhcvdb.ibcp.fr/euhcvdb/) and from the Los-Alamos HCV database (http://hcv.lanl.gov/). Phylogenetic trees were estimated and assessed using bootstrapping, maximum parsimony and minimum evolution methods under the Kimura 2 parameter model, as implemented in PAUP* version 4 [Swofford, 2002]. Bootstrap values above 66% were considered significant. Reference 5′UTR or NS5B sequences belonging to genotype 4 and sampled from central African

countries were also selected from the database, for the purposes of subtype identification and phylogenetic analysis [Kuiken et al., 2005].

Coalescent Analysis

The epidemic history of HCV subtype 4e was investigated using coalescent-based analysis, which uses a population genetic model to infer to effective number of infections through time from the pattern of genetic variation in the sequences [Pybus et al., 2001; Drummond and Rambaut, 2007]. This approach has been shown to reliably reconstruct the known epidemic history of subtype 4a in Egypt [Pybus et al., 2003] and has recently been used to infer the history of HCV in Cameroon [Njouom et al., 2007]. It also estimates phylogeny on a real time scale of years. All available NS5B subtype 4e sequences (16 from this study plus 4 database strains) were analyzed using the Bayesian skyline plot method with 4 linear steps, as implemented in the program BEAST v1.4.6 [Drummond and Rambaut, 2007]. As in previous analyses [Pybus et al., 2001, 2003] an HKY + gamma nucleotide evolution model was used with a rate of NS5B gene evolution of 0.0005 substitutions per site per year. The Bayesian analysis was run for 50,000,000 states and sampled every 10,000 states. Correct analysis behavior was investigated and confirmed using the program Tracer v1.4 (http://beast.bio.ed.ac.uk/).

Statistical Analysis

Statistical analysis was carried out using Epi-Info version 6 and Statview 5.0. Prevalences across genders and across age groups, and the relationship between viral load and age were compared using chi-square tests and the Spearman correlation test. P values below 0.05 were considered significant.

RESULTS

Population Characteristics

A total of 319 adults were included in the study. The sex ratio was 0.74 with 136 (43%) males and 183 (57%) females. Mean age was 38 years and ranged from 13 to 85 years. Table I shows the number of subjects included by age group and gender. Approximately a third of the studied population were 13–24 years old, another third were 25–44 years old, and the remaining third were more than 45 years old.

Serological Tests and Viral Loads

All samples were screened with both ELISA and RIBA; 66 subjects (20.7%; 95% CI = 16.3–25.1) were HCV-seropositive. Seroprevalence increased with age, from 1% in age group 13–24 to 62.2% in those over 65 years old (Table I; $P < 10^{-5}$). There was no significant difference in seroprevalence by gender (OR = 1.10; 95% CI = 0.94–1.30; $P > 0.5$). However, in the subset of patients under 45 years old, there was a significantly higher seroprevalence in females compared to males (OR = 0.21; 95% CI = 0.07–0.72; $P > 0.02$). HCV RNA was amplified for 60 (90.9%) samples and 6 (9.1%) were negative using the 5′ UTR region. Among those samples, 22 (36.6%) were amplified using the NS5B region. Of the 60 PCR positive samples, viral load could be measured for 59 samples. Four (6.7%) subjects had a viral load below the threshold value (600 IU/ml). Fifty five subjects had values ranging from 600 IU/ml to 24.9 million IU/ml, with a median value of 372,500 IU/ml. Statistical comparison of viral loads, log (IU/ml), with age groups revealed a significant positive correlation with age ($P = 0.0023$; Spearman correlation test shown in Fig. 1). Values above 5 million IU/ml were detected among elderly subjects (mean age 67 years; range 47–81+). No difference in viral load was noted between genders ($P = 0.089$), but among the subset of individuals under 45 years old, there is a trend towards higher viral loads in females than in males ($P = 0.047$; Mann–Whitney U-test).

Phylogenetic and Coalescent Analysis

Using the 5′UTR region, phylogenetic analysis showed that most HCV strains in this area belonged to genotype 4 and were classified into the following subtypes: 4e (43; 71.7%), 4c/4d (7; 11.7%), 4c (1; 1.7%), 4a (1; 1.7%), unclassified genotype 4 (6; 10%) and 2 (3.3%) sequences that may belong to subtype 5a (Table II). Classification was statistically supported for all samples, with the exception of the two 5a sequences (bootstrap value <50%), data not shown.

TABLE I. Distribution of the 319 Included Adults From Dienga by Age, Gender and Anti-HCV Antibody Prevalence

Age groups (years)	Total adults, n[a]=	Total anti-HCV+[b]	%	Total female, n=	Female anti-HCV+[b]	%	Total male, n=	Male anti-HCV+[b]	%
13–24	100	1	1.0	49	1	2	51	0	0
25–34	53	1	1.9	29	0	0	24	1	4
35–44	60	11	18.3	42	10	23.8	18	1	5.6
45–54	32	13	40.6	22	9	40.9	10	4	40
55–64	37	17	45.9	22	10	45.5	15	7	46.7
>65	37	23	62.2	19	10	52.6	18	13	72.2
Total	319	66	20.7	183	40	21.9	110	26	23.6

[a]Total number.
[b]Positive cases confirmed by ELISA and recombinant immunoblot assay.

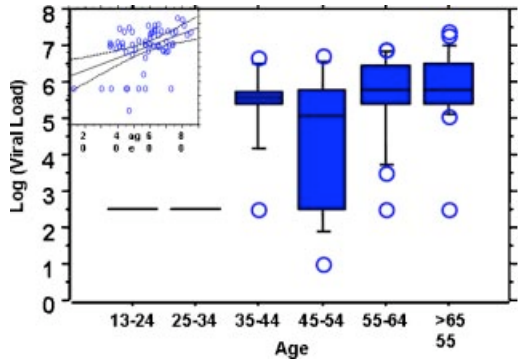

Fig. 1. Box plot of HCV viral loads (log 10 scale) for each age group. For each box the median is indicated by the central line and the first and third quartiles (inter-quartile range or IQR) are represented by the length of the box. The extreme values (within 1.5 times the IQR from the upper or lower quartile) are represented by the bars extending from the IQR box. Values more than 1.5 times the IQR from the median are plotted individually as circles. Inset: Correlation of viral load with age. Fitted model: log (viral load) = 2.92 + 0.043 × age; Spearman's non-parametric test $P < 0.003$; Rho = 0.384.

The 22 NS5B sequences were analyzed together with previously classified genotype 4 sequences that had been obtained from Gabonese or other African individuals during the past 10 years (Fig. 2). The neighbor-joining phylogeny showed that 16 (72.7%) of our isolates belonged to subtype 4e. Within this 4e cluster, 3 more closely related sequence pairs (D898–D887; D309–D257; D536–D481–D273) could be identified, while 2 patients (D778–D578) seem to harbor identical sequences. A further three (13.6%) isolates belonged to subtype 4c, one (4.5%) to subtype 4r, one (4.5%) to subtype 4p, while D828 remained unclassified within genotype 4 (Fig. 2; Table II).

Figure 3 displays the results of the coalescent-based analysis of subtype 4e. The estimated age of the subtype 4e phylogeny is 96 years, corresponding to a date of 1908 (95% credible interval = 1,878–1,930). This age is similar to that estimated for many other HCV subtypes [e.g., Pybus et al., 2001, 2003]. The phylogeny is strongly star-shaped, suggesting a history of viral population growth (Fig. 3). In agreement with this hypothesis, the graph of estimated effective number of infections through time (skyline plot) indicates rapid growth during the first half of the twentieth century, with a particularly sharp rise during the period 1920–1940 (Fig. 3). However, the large confidence intervals for this estimate (arising from the relatively small sample size) means that these data cannot exclude the hypothesis of a smoother growth of infections over the wider period 1900–1950.

TABLE II. Comparison of Genotyping Results Using 5′UTR or NS5B Regions

5′UTR subtype	Number (%)	NS5B subtype	Number (%)
4e	43 (71.7%)	4e	16 (72.7%)
4c/4d	7 (11.7%)		
4c	1 (1.7%)	4c	3 (13.6%)
4a	1 (1.7%)		
4U[a]	6 (10%)	4U[a]	1 (4.5%)
		4p	1 (4.5%)
		4r	1 (4.5%)
5a	2 (3.3%)		
Total number	60		22

[a]Unclassified subtype.

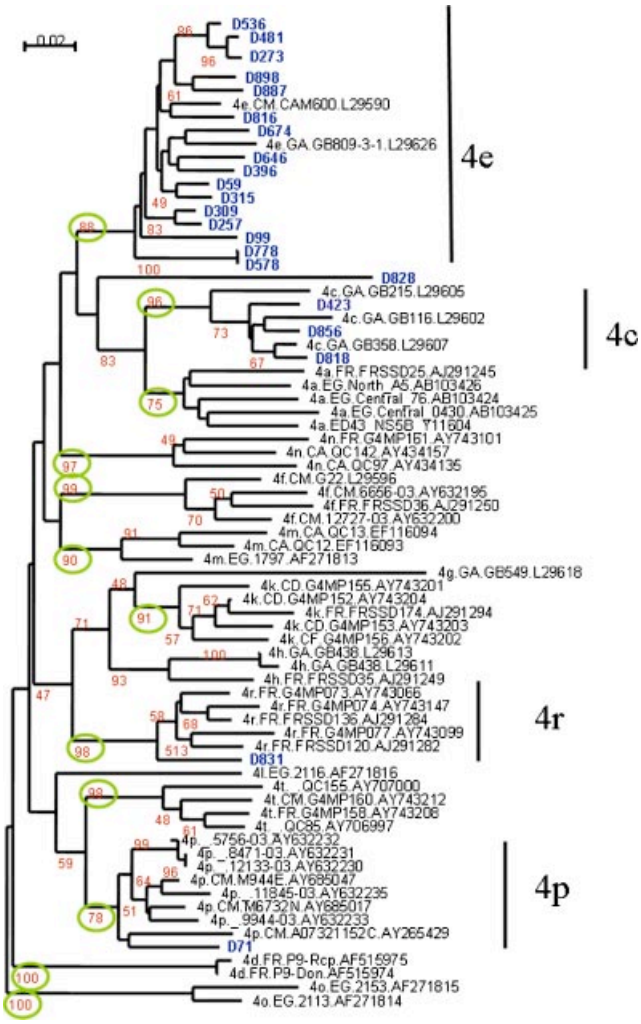

Fig. 2. Phylogenetic analysis of the 22 Dienga sequences with previously described NS5B sequences from Gabonese isolates and to genotype 4 sequences. Sequence identification uses the following nomenclature: Subtype.country.isolate.accession, according to the Los Alamos database [Kuiken et al., 2005]. For clarity, we show only 2 or 4 reference sequences for each subtype of genotype 4.

DISCUSSION

This study characterized the prevalence and genotype distribution of HCV in Dienga, Gabon. Viral prevalence (20.7%) is 10 times higher than in most non-African countries [Alter et al., 1999] but is similar to the prevalence reported (17.1%) for a remote area in Cameroon (800 km north of Dienga) [Louis et al., 1994; Njouom et al., 2003a]. Other surrounding countries

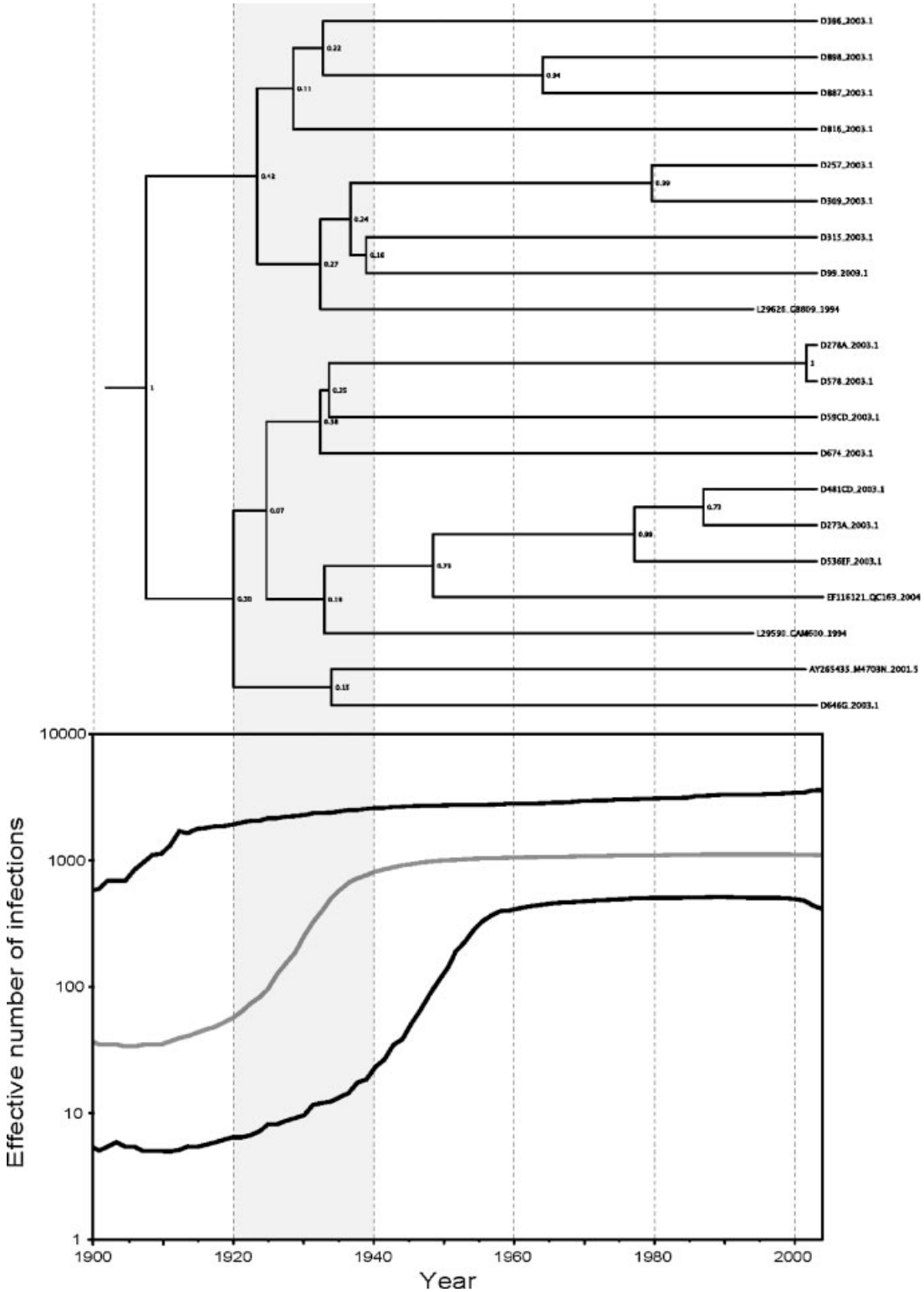

Fig. 3. Results of the coalescent-based evolutionary analysis. Top: the "maximum clade credibility" phylogeny of HCV subtype 4e, estimated on a real time scale of years using BEAST (see methods). The years 1920–1940 are highlighted by a grey box. Bayesian posterior probabilities (measure of support for each cluster) are given and are generally low for more ancestral nodes, in agreement with the bootstrap results (see Fig. 2). Bottom: estimated Bayesian Skyline Plot, showing the growth in the effective number of infections through time, placed on the same timescale as the phylogeny above. The grey curve is the median estimate of this estimate; the black lines represent the upper and lower 95% credible intervals. This plot was calculated using BEAST and TRACER (see Materials and Methods Section).

show lower prevalence, with 13.9% in the Republic of Congo (general hospital, Brazzaville [Dokekias et al., 2003]). Prevalences of 1.7% in subjects <40 years up to 5.6% above 40 years were reported for a rural population of the Republic of Equatorial Guinea [Basaras et al., 1999], and of 2.8% for villages of Central African Republic [Fretz et al., 1995]. Dienga HCV seroprevalence increased significantly with age, reaching 62.2% in individuals older than 65 years, similar to previous findings in rural areas of Cameroon [Mencarini et al., 1991; Delaporte et al., 1994; Njouom et al., 2003a]. Viral loads were similarly correlated with age, in contrast with findings for genotype 1, 2, or 3 [Schijman et al., 2004]. There is a tendency towards higher seroprevalence and viral loads in females under 45 years. Gender-specific patterns of susceptibility and risk factors could be the cause and should be investigated further [Schott et al., 2007].

Among the 66 anti-HCV positive samples, 6 were PCR negative, suggesting spontaneous viral clearance, as observed in other studies [Kondili et al., 2002; Njouom et al., 2003b; Uto et al., 2006]. PCR amplification was less successful for the NS5B coding region than for the more conserved 5′UTR non-coding region (Table II). However, as viral loads were high for these samples (Fig. 1), the lack of NS5B amplification was not due to insufficient material but linked to genetic variability, as previously described [Nicot et al., 2005; Pasquier et al., 2005]. The analysis of the highly conserved 5′UTR region using neighbor-joining trees was not fully discriminative, especially between genotypes 1 and 6 [Laperche et al., 2005]. Phylogenetic analysis of the NS5B sequences highlighted a cluster of Gabonese subtype 4e infections, analogous to findings in Cameroon [Njouom et al., 2003a]. HCV subtype 4c was first described by Stuyver et al. [1994] in Gabonese patients and was found in this study in three individuals only (bootstrap value = 96%). The 2 remaining subtypes (4r and 4p) were described previously in the Democratic Republic of Congo and in France [Bukh et al., 1992; Nicot et al., 2005]. Subtype 4p has also been found in Cameroon [Morice et al., 2001; Njouom et al., 2003a]. One sequence (D828) belongs to genotype 4 but is not associated with any previous classified subtype; recombination cannot yet be excluded for this strain [Stuyver et al., 1996]. A corresponding variety of genotype 4 subtypes has recently been reported in African immigrants to Europe [Morice et al., 2001; van Asten et al., 2004; Nicot et al., 2005].

A high diversity of genotype 4 strains was found in Dienga, with a dominant cluster of strains classified as subtype 4e. This cluster, together with the high observed genetic diversity in Gabon, plus the high anti-HCV prevalence among elderly people, suggests a scenario of long-term endemic infection followed by a recent rise in transmission, giving rise to a "cohort" of infected individuals in Gabon. This scenario is strongly supported by the results of the coalescent-based analysis, which dates the period of more rapid transmission to the first half of the 20th century, most likely between 1920 and 1940. This period also correlates with the appearance of many new lineages in the subtype 4e phylogeny (shaded area in Fig. 3). This epidemic history is in agreement with that previously proposed for HCV in bordering countries, notably Cameroon [Gisselquist, 2003; Njouom et al., 2003a, 2007; Nerrienet et al., 2005].

In Egypt the HCV epidemic is characterized by a dominant subtype 4a [Ray et al., 2000]. There, the transmission factors have been identified and HCV infection is clearly linked to the use of non-sterile needles during antischistosomiasis injection campaigns from the 1920s to the 1970s [Ray et al., 2000; Pybus et al., 2003]. In central African countries, the contamination occurred perhaps earlier and has been linked to unsafe mass chemoprophylaxis for sleeping sickness in Cameroon around the 1940s [Njouom et al., 2007]. A precise transmission route could not be established in this study, as the examined population was too small. However, past practices of non-sterile medical procedures in Gabon have been reported and persistence of blood transfusion, and unsafe traditional practices (scarification, tattoo, circumcision) could also have contributed to the epidemiological picture [Prevention, 2003; Nicot et al., 2005].

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