JOURNAL OF VIROLOGY, May 2006, p. 4220–4226 Vol. 80, No. 9
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.9.4220–4226.2006

Investigating the Origin and Spread of Hepatitis C Virus Genotype 5a†

Jannick Verbeeck,[1] Piet Maes,[1] Philippe Lemey,[1,2] Oliver G. Pybus,[2] Elke Wollants,[1] Ernie Song,[3] Frederik Nevens,[4] Johan Fevery,[4] Wayne Delport,[5] Schalk Van der Merwe,[6] and Marc Van Ranst[1]*

Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Leuven, Belgium[1]; Department of Zoology, University of Oxford, Oxford, United Kingdom[2]; Department of Internal Medicine, University of the Witwatersrand, Witwatersrand, South Africa[3]; Division of Hepatology, University Hospital Gasthuisberg, Leuven, Belgium[4]; Molecular Ecology and Evolution Program, Department of Genetics, University of Pretoria, Pretoria, South Africa[5]; and Hepatology/GI-Research Laboratory, Institute of Pathology, Basic Medical Sciences, University of Pretoria, Pretoria, South Africa[6]

Received 2 September 2005/Accepted 16 January 2006

Epidemiological and phylogenetic studies of hepatitis C virus (HCV) have identified six major HCV genotypes and have attempted to characterize their origin and spread worldwide. Putative regions of endemic infection have been identified for all HCV genotypes except HCV genotype 5a. Although HCV genotype 5a was previously thought to be largely restricted to the northern part of South Africa, this study reports an unexpected cluster of the genotype in West Flanders Province in Belgium. To investigate the molecular epidemiology of this cluster and of HCV genotype 5a in general, a rigorous phylogenetic analysis of Belgian and South African HCV genotype 5a samples was performed. Remarkably, the Belgian and South African strains form two distinct clusters of similar diversity. We used a Bayesian coalescent method to estimate the rate of virus spread through time for HCV genotype 5a in both regions. Our results indicate that HCV genotype 5a strains have been spreading independently in Belgium and South Africa for more than 100 years, with a rate of spread characteristic of an epidemic genotype. These findings have major implications for tracing the origin of HCV genotype 5a. Here, we speculate about the possible origins of these clusters.

With an estimated 170 million people infected worldwide, hepatitis C virus (HCV) is responsible for most cases of parenteral non-A, non-B hepatitis and is therefore the leading cause of chronic liver disease and hepatocellular carcinoma (38). The virus is responsible for 10,000 deaths per year in the United States alone, and this number is expected to increase substantially in the forthcoming decades (1). HCV is a bloodborne pathogen; risk factors for infection include injecting drug use, receipt of a blood transfusion before 1990, use of inadequately sterilized medical equipment, and use of scarification and tattooing tools (14). Sexual and perinatal transmissions rarely occur, except in human immunodeficiency virus-HCV-coinfected individuals (4). A nonnegligible proportion (about 20%) of HCV infections have an "undefined" route of transmission.

HCV is a small, enveloped human virus containing a single-stranded RNA genome of about 9,600 nucleotides (nt) (36). HCV displays a high degree of genetic variability (22) and is classified into six major genotypes that show sequence similarities of only 66% to 69%. Each genotype contains multiple subtypes with >75% nucleotide sequence similarity (29). In comparison, the degree of similarity among different virions in a single infected patient is >95% (11).

Importantly, numerous studies have revealed a relationship between the HCV genotype and the response to interferon or pegylated interferon therapy, alone or in combination with ribavirin. Patients infected with HCV genotype 1 respond less to therapy, while patients infected with HCV type 2 or 3 show the best response (23). Since HCV genotype 5a infections are scarce, treatment responses for HCV genotype 5a are largely unknown (15). For HCV genotype 6a infections, treatment response data are also poor (12).

The six HCV genotypes have different geographical distributions. Some strains are distributed worldwide, whereas others are found only in specific geographic regions. HCV genotypes 1a, 1b, and 3a are highly prevalent "epidemic" strains that are found globally. These strains spread swiftly around the world during the 20th century, most likely through infected blood and blood products and injecting drug use, and have relatively low levels of genetic variation. In contrast, other HCV strains are highly divergent but are found in restricted geographic areas. These "endemic" strains reflect long-term transmission at low levels in particular populations (26, 30) and represent the source populations for the epidemic strains. Genotypes 1, 2, and 4 appear to be endemic to regions of West and Central Africa and the Middle East, whereas divergent endemic strains of genotypes 3 and 6 are found in Southeast Asia (22).

In contrast to other HCV strains, the origin, endemic source, and epidemic spread of HCV genotype 5a are unknown. Genotype 5a is commonly found in the northern part of South Africa (19), but it is also sporadically found elsewhere, e.g., in Australia, Brazil, Canada, Ireland, The Netherlands, and Spain (2, 3, 13, 16). In 2004, a high prevalence of HCV genotype 5a was reported in Central France (9), where it was the third most frequent genotype at 14.2%.

* Corresponding author. Mailing address: Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, BE-3000 Leuven, Belgium. Phone: 32-16-347908. Fax: 32-16-347900. E-mail: marc.vanranst@uz.kuleuven.ac.be.

† Supplemental material for this article may be found at http://jvi.asm.org.

Here, we report a molecular epidemiological analysis of Belgian HCV genotype 5a infections from West Flanders, an area with a previously unrecognized and unusually high prevalence and diversity of HCV genotype 5a. By comparing these strains with new sequences from South Africa, we show that the Belgian and South African populations have sustained independent populations of HCV genotype 5a for at least 100 years. Furthermore, we use coalescence-based population genetic methods (5) in order to estimate the epidemic history of HCV genotype 5a strains. Such methods have proven useful in reconstructing the history of HCV infections prior to the identification of the virus in 1989 (20, 24, 25, 26, 31, 32, 33). The results shed new light on the global origin and transmission history of HCV.

MATERIALS AND METHODS

Study population. A total of 980 Belgian patients who had been admitted to the University Hospital Gasthuisberg of Leuven were found to be chronically infected with HCV in a prospective study from January 2001 to December 2004. Of these, 52 patients were infected with HCV genotype 5a. Serum samples from 41 HCV genotype 5a-infected patients were collected for further research. Age, gender, and sampling date were known for all these patients. All Belgian patients had Caucasoid ethnicity. Additionally, we collected serum samples from 46 South African HCV genotype 5a patients between January 1997 and December 2004. Ethnicity was known for all of them (32 Caucasoid, 10 Negroid, 2 Mongoloid, 1 Indian, and 1 mixed). All these patients came from the north-central part of South Africa, in the area of Johannesburg and Pretoria. Serum samples from 31 South African HCV genotype 5a-infected patients were used for further research. Table S2 in the supplemental material summarizes the demographic characteristics of all of the patients.

Genotyping of HCV strains. Genotyping of HCV RNA-positive patients was carried out with the Versant HCV Genotype Line Probe Assay (Bayer Corporation, Tarrytown, New York; manufactured by Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. The Versant HCV Genotype Line Probe Assay was validated for the genotyping of HCV genotype 5a strains in a previous study (37). Our results showed that this assay is a reliable method for genotyping of the infrequently found HCV genotype 5a strains.

Sequencing of 5′ UTR, E1-E2, NS4B, and NS5B. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Leusden, The Netherlands) according to the manufacturer's instructions. Using a one-step reverse transcription-PCR (QIAGEN OneStep RT-PCR kit), we amplified 262 nt in the 5′ untranslated region (UTR), 584 nt in the E1-E2 hypervariable region 1 (HVR-1), and 573 nt in the NS4B region. PCR cycling was performed with the following primers in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA): forward primer 1FB (5′-CTAGCCATGGCGTTAGTATGAGTGT-3′) and reverse primer 2RB (5′-GGTGCACGGTCTACGAGACCT-3′) for the 5′ UTR; forward primer HVR1-F (5′-TGCTGGGTCCARRTYACCCC-3′) and reverse primer HVR1-R (5′-GCTGTCATTACAGTTAAGGGCA-3′) for the E1-E2 (HVR-1) region; and forward primer NS4B-F (5′-ATCAACATCGACGCYCACATG-3′) and reverse primer NS4B-R (5′-CCCACTGACAAAGTTCCACAT-3′) for the NS4B region. For amplification in the 5′ UTR, the following PCR cycling program was used: an initial 50°C hold for 30 min; a 95°C hold for 15 min; 45 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a 72°C hold for 10 min. For amplification of the E1-E2 region (HVR-1), we used the same basic program with 40 cycles, with an annealing temperature of 57°C. For the amplification of the NS4B region, we performed 45 cycles with an annealing temperature of 55°C. PCR products were purified using the QIAquick PCR Purification kit (QIAGEN). Purified DNA fragments were directly sequenced on both strands using the ABI Prism BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems). Additionally, we amplified and sequenced a 401-bp fragment of the NS5B region of the HCV genome as described by Sandres-Saune and colleagues (28) for the samples from patients for whom sera were still available.

Phylogenetic reconstruction. Multiple alignments of the nucleotide sequences were created for the E1 region, the NS4B region, and the concatenated E1/NS4B region using the Clustal X program version 1.83 (10). For all further analyses of the E1 region, we did not include HVR-1, which is the target of strong positive selection and difficult to align unambiguously. Maximum likelihood phylogenetic trees were inferred under the Hasegawa-Kishino-Yano model of nucleotide substitution with gamma distributed rate variation among sites, using the program PhyML (8). Bootstrap analysis was performed using 500 replicates. Mean pairwise nucleotide diversities were calculated using maximum likelihood trees that included only a single viral strain per patient.

Inference of genotype 5a epidemic history. (i) Estimating the HCV evolutionary rate. For the E1 gene region, we obtained an evolutionary-rate estimate from the core-E1-E2 data serially sampled by Tanaka et al. (32). Evolutionary rates were estimated using the BEAST v. 1.2 software, and a specific substitution rate for the E1 region of interest was inferred (http://evolve.zoo.ox.ac.uk/Beast). Since there are no appropriate serially sampled data available for the NS4B gene region, the NS4B rate was obtained by scaling the E1 rate using a relative rate analysis, performed in PAML (39).

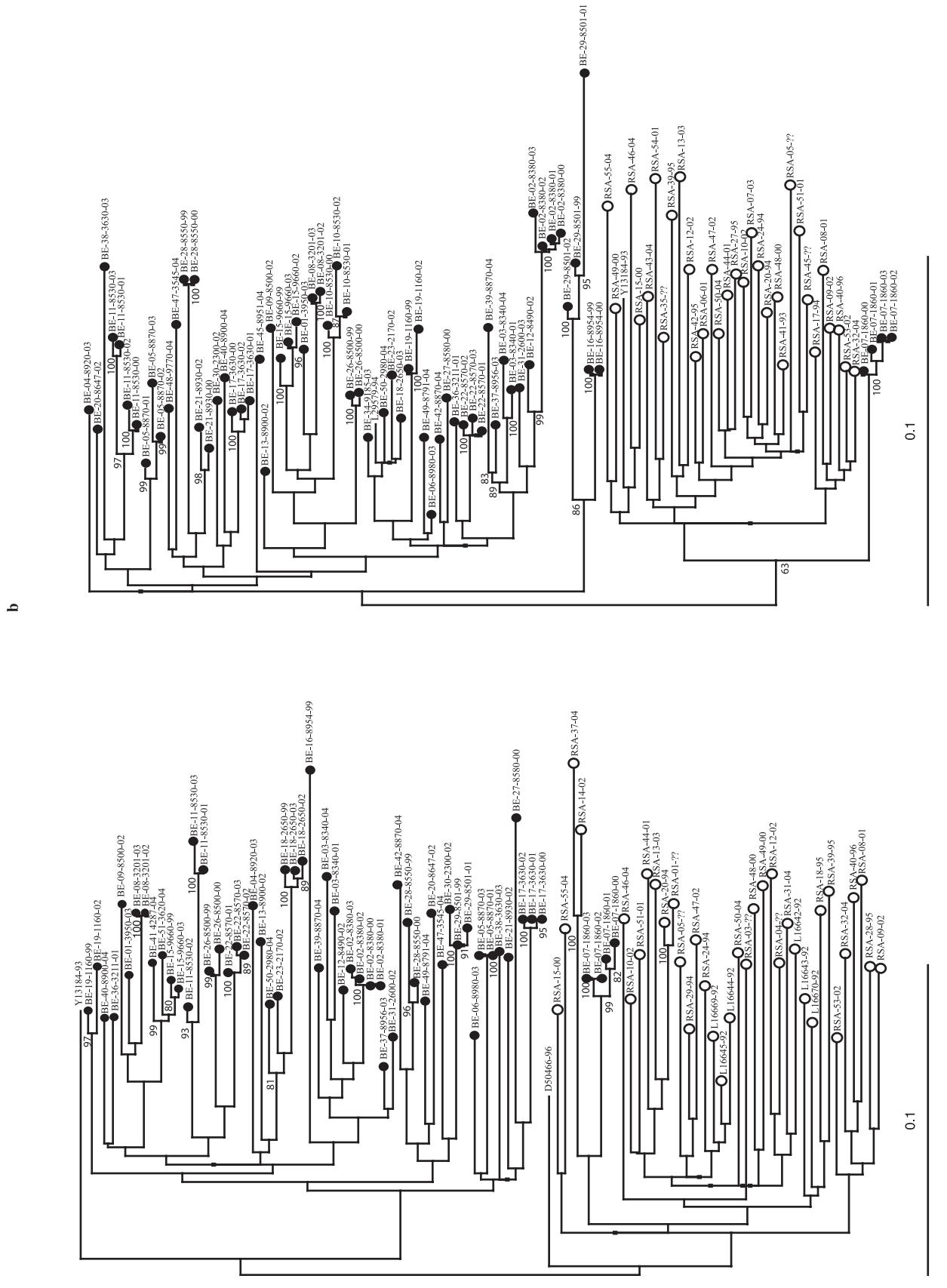
(ii) Estimating HCV population history. HCV demographic history was inferred using a Bayesian coalescent method implemented in BEAST v. 1.2 (6). Since we had no a priori reason to assume a particular model of change in the viral population size over time, we used the recently developed Bayesian skyline plot to estimate epidemic history (5). Because the sequences were sampled over a limited time frame and therefore did not contain sufficient information about the evolutionary rate, we used the nucleotide substitution rates estimated as described above as informative prior distributions ($7.2 \times 10^{-4} \pm 1.5 \times 10^{-4}$ substitutions/site/year for E1 and $5.4 \times 10^{-4} \pm 1.5 \times 10^{-4}$ substitutions/site/year for NS4B). Samples for which the isolation date was not established were included by using the following strategies. A uniform prior distribution for the sampling year was used for the Belgian and South African samples, constrained by the earliest and most recent sampling years, and an exponential distribution was used for database sequences with a mean of 1.5 years and an offset based on the year of publication. Markov Chain Monte Carlo analyses were run for 15×10^6 or 25×10^6 states, depending on the length of the data set, and posterior distributions were calculated after a burn-in of 10%. The posterior samples were analyzed using the program Tracer (http://evolve.zoo.ox.ac.uk/Tracer).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper were deposited in GenBank using the National Center for Biotechnology Information (Bethesda, MD) Sequin v. 5.26 submission tool under accession numbers DQ164538 to DQ164777 and DQ333614 to DQ333690.

RESULTS

The HCV genotype 5a cluster in Belgium. Investigation of the HCV genotype distribution in Flanders, Belgium, indicated that HCV genotype 5a was the second most prevalent genotype in West Flanders Province at 27.7%, after HCV genotype 1 (50.9%). In Belgium, 57.4% of the patients infected with HCV genotype 5a lived within a 20-km radius in West Flanders Province. HCV genotype 5a was significantly more prevalent in West Flanders than in the other provinces of Belgium ($P < 0.0001$; Pearson chi-square test), where HCV genotype 5a occurs in less than 2% of the cases and HCV genotype 1b is the dominant strain. No difference could be observed in the gender distributions of HCV genotype 5a-infected patients (59.9% male and 40.1% female; $P = 0.339$; Pearson chi-square test). As was noted for HCV genotype 5a in France (9), we found that HCV genotype 5a-infected patients in Flanders were significantly older than patients infected with other HCV genotypes (mean ages, 58.1 years and 49.8 years, respectively; $P < 0.0001$; Mann-Whitney test). These results suggest that HCV genotype 5a strains have circulated in Belgium for some time.

Phylogenetic analysis of the HCV genotype 5a cluster. Figure 1 shows the phylogenetic trees of the E1 region (Fig. 1a), the NS4B region (Fig. 1b), the concatenated gene regions (Fig. 1c), and the NS5B region (Fig. 1d). The phylogenetic analyses showed that samples obtained from individual patients over different consecutive years were more closely related to each other than to those obtained from other patients, as reported previously (18). The phylogenies indicate that two subclades can be distinguished, one formed by the Belgian samples and one formed by the South African samples. One exception was the Belgian patient BE-07-1860. In the E1 phylogeny, the sam-


a
b
0.1
0.1

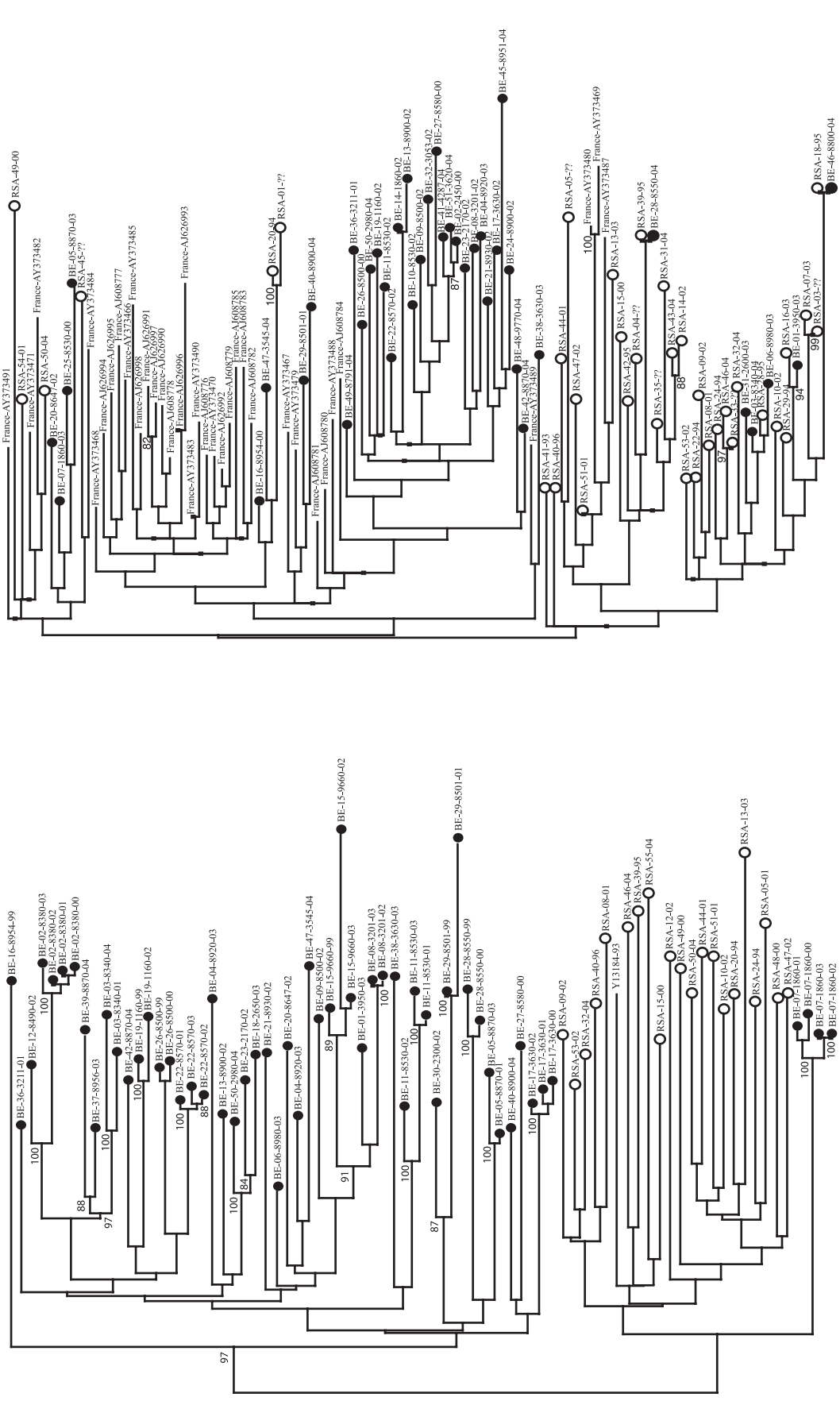


FIG. 1. Maximum likelihood phylogenetic trees reconstructed for the South African (○) and Belgian (●) samples. (a) Phylogeny for a 408-bp fragment in the E1 region. (b) Phylogeny for a 543-bp fragment in the NS4B region. (c) Phylogeny for the concatenated gene regions. (d) Phylogeny for a 401-bp fragment in the NS5B region. Each sample is indicated by a code representing the origin of the sample (RSA, Republic of South Africa; BE, Belgium), a patient number, and the year of sampling (the symbol "??" is used when the year of sampling is unknown). We also included HCV genotype 5a reference sequences available in the GenBank database. Their GenBank accession numbers, years of sampling, and countries of origin (if known) are shown. The numbers at the nodes represent the bootstrap support for 500 replicates; only values over 80% are shown, with the exception of the two major clades (>50%).

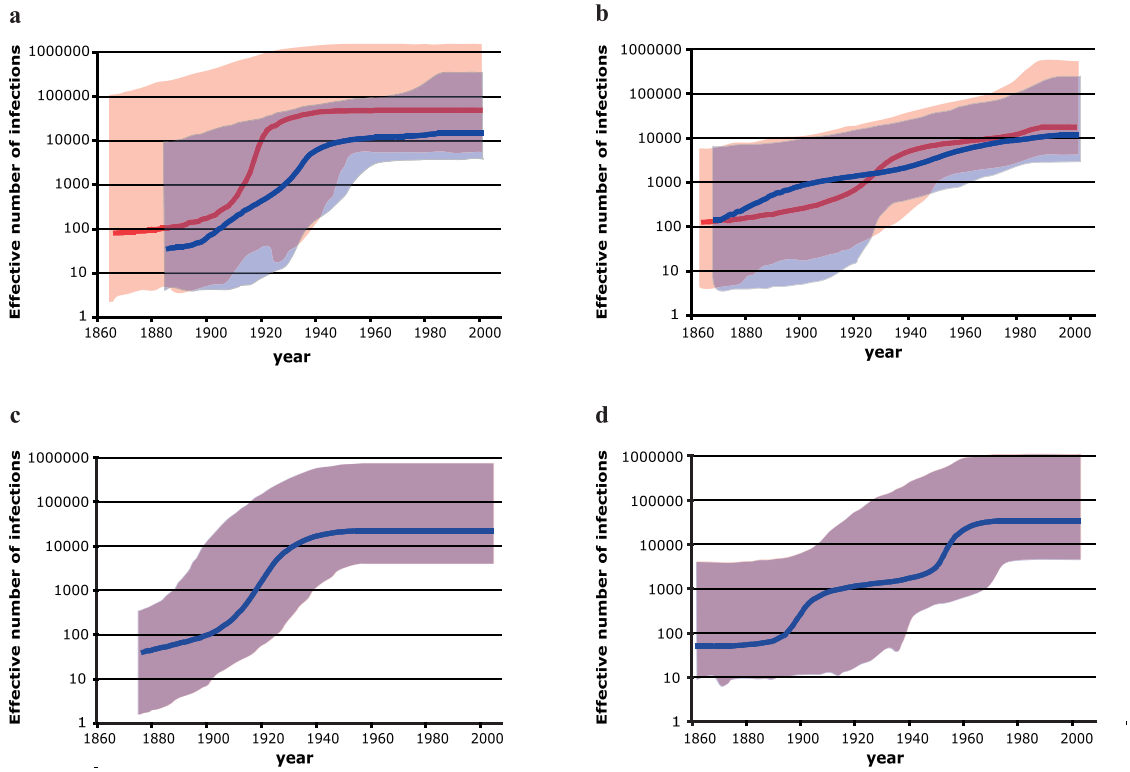


FIG. 2. Superimposed Bayesian skyline plots estimated from the South African (a) and Belgian (b) samples in the E1 (blue) and NS4B (red) regions of the HCV genome. Bayesian skyline plots for the concatenated data sets for South Africa (c) and Belgium (d) are also shown. The dark lines are the median estimates, and the shadings indicate the 95% highest posterior density intervals.

ple from this patient was more closely related to the African samples than to the other Belgian samples (Fig. 1a). In the NS4B region and the concatenated regions, this patient clustered separately from both major clades (Fig. 1b and c). It is therefore likely that this patient was epidemiologically unrelated to the other Belgian patients. The separation of the South African and Belgian strains was not highly supported by bootstrap analysis in the separate genes (49.6% in the E1 tree and 63% in the NS4B tree). However, the concatenated data set, which contains more phylogenetic signals, significantly supported the monophyletic clades (97%).

The existence of the two clades suggests that the virus has spread independently in two different locations. Furthermore, both clades also have similar nucleotide diversities (mean pairwise nucleotide diversities, 0.14 ± 0.03 and 0.16 ± 0.04 in E1 for the Belgian and South African sequences, respectively; 0.10 ± 0.03 and 0.12 ± 0.03 in NS4B).

Since the NS5B genes of a number of genotype 5a strains from a French population have been sequenced (9), we also sequenced part of the NS5B gene for the remaining Belgian and South African samples using the protocol described by Sandres-Saune and colleagues (28). A phylogenetic reconstruction of the NS5B region indicated that the Belgian, South African, and French samples were not monophyletic (Fig. 1d), although samples from the same country did tend to cluster together. The loss of monophyletic relationships for both Belgian and South African sequences in this gene region is very likely due to the lower phylogenetic signal of the NS5B region, which has approximately half the E1 and NS4B substitution rates (27). This was investigated further using the samples for which sequences were available in all three gene regions. The NS5B data were unable to reject a tree in which the sequences were forced to cluster according to geographic origin (see the supplemental material for further details).

Estimation of epidemic history. In order to investigate the origin and spread of HCV genotype 5a in both populations, we estimated divergence dates and demographic parameters in a Bayesian coalescent framework, as implemented in BEAST v. 1.2.

To calculate Bayesian skyline plots, we analyzed the Belgian and South African clusters separately and included only a single sequence per patient. The sample from patient BE-07-1860, which clustered with South African strains in E1, was excluded from this analysis. The Bayesian skyline plots depict the estimated change in the effective number of infected individuals through time and are shown in Fig. 2. In the figure, we plotted the skyline plots for the two gene regions and those for the concatenated data sets separately. The estimated dates for the most recent common ancestors for the separate analyses were as follows: 1869 (confidence interval [CI], 1792 to 1929) for E1 and 1866 (CI, 1818 to 1913) for NS4B of the Belgian subclade; 1883 (CI, 1821 to 1934) for E1 and 1863 (CI, 1794 to 1921) for NS4B of the South African subclade.

Although the dates for the most recent common ancestors and the current effective numbers of infections are similar in the Belgian and South African populations, there is some difference between the dynamics in the Belgian and the South

African plots. The skyline plots show that in Belgium, HCV genotype 5a spread with a roughly constant exponential growth through time. In the South African population, however, there is more evidence of a decrease in the rate of spread toward the present. Epidemic growth rates estimated using parametric models applied to the concatenated data sets indicate that the number of infected individuals doubled approximately every 11 years in the Belgian population (exponential-growth model), while the doubling time was about 5 years during the exponential phase in South Africa (logistic-growth model), which correspond to estimated growth rates of 0.061 $year^{-1}$ (CI, 0.044 to 0.975) and 0.128 $year^{-1}$ (CI, 0.059 to 0.208), respectively.

DISCUSSION

Statistical analysis of the HCV genotype distribution in Belgium revealed a cluster of HCV genotype 5a in the western part of Flanders, with HCV genotype 5a being the second most prevalent HCV genotype in the area. This is a remarkable finding, because this genotype was thought to be a major genotype only in South Africa, although HCV genotype 5a also has a worldwide distribution as a minor genotype. In Central France, HCV genotype 5a is the third most prevalent genotype. Phylogenetic analysis of the E1 and the NS4B regions of the HCV genome revealed two distinct clusters of HCV genotype 5a strains, one originating from South Africa and one originating from Belgium. The time to the most recent common ancestor of the Belgian HCV genotype 5a strains was shown to be approximately 120 years, suggesting that these strains have circulated in Belgium for quite a long time. Therefore, it is reasonable to assume that a recent source of infection, such as the contamination of a plasma pool with HCV genotype 5a (34), is not the cause of the introduction of this HCV genotype 5a strain, and we can only speculate about the possible introduction and transmission events that occurred many years ago. A similar evolutionary time scale was calculated for HCV genotype 5a samples originating from South Africa. The parameters estimated using the Bayesian coalescent approach have rather large confidence intervals. However, this analysis takes several sources of uncertainty into account in a statistically rigorous way. Phylogenetic uncertainty was accommodated by sampling genealogies using Markov Chain Monte Carlo analysis. Furthermore, we did not fix a particular evolutionary rate but instead imposed an informative prior distribution for this parameter.

It is very tempting to assume that the Belgian HCV genotype 5a cluster originated from the South African cluster, since HCV genotype 5a had previously been thought to be mainly restricted to South Africa. Our results, however, show that the Belgian HCV genotype 5a clade has an age similar to that of the South African clade. Moreover, the two clusters appear to have reached comparable population sizes in their respective countries. This finding sheds new light on the hypotheses concerning the emergence of HCV genotype 5a. This HCV genotype seems not to be restricted to South Africa alone, and the HCV genotype 5a genetic diversity might be equally pronounced in other populations. We also attempted to investigate the relationship between the South African and Belgian samples and a French population (9). However, the more conserved NSB5 gene region, which might contain appropriate phylogenetic signal between genotypes (27), was not informative enough alone to resolve any relationships within a subtype of a single genotype (Fig. 1).

Taking the results of this study into account, it is reasonable to assume that there has been a common ancestral HCV genotype 5a population and that the virus has spread from this common pool in two directions at the same time. It is possible that the Congo, or a neighboring area, harbors the common ancestor of the Belgian and South African HCV genotype 5a strains, since there was much trading and trafficking between Belgium and its colony Congo in the mid-1800s during the reign of King Leopold II. In order for this hypothesis to hold, there also must have been contacts between the Congo and South Africa. Since Central and South Africa were both mostly exploited in the late 1800s, we can assume that people traveled from Central Africa to South Africa and vice versa, and this may have led to the dissemination of HCV genotype 5a within Africa. It is possible that other clusters of HCV genotype 5a will be found in the future, and further sampling of HCV genotype 5a strains from other regions of the world will help us to understand the transmission history of the genotype. If further sampling of 5a strains led to either cluster losing its monophyletic nature, then it would be necessary to revaluate the conclusion that HCV genotype 5a was transmitted independently in the two countries during the 20th century.

In contrast to the long-term endemicity of HCV genotype 2 in Martinique (17), HCV genotype 4 strains in Cameroon (21), and HCV genotype 6 in Southeast Asia (35), the endemic pool of HCV genotype 5a has not yet been found. For this reason, we should be cautious in searching for an association between an ecological or historical event and the spread of HCV genotype 5a. In Belgium, the growth rates of the HCV genotype 5a strains suggest an exponential spread, nearly as fast as an established epidemic subtype like 1b (26). In contrast to the HCV genotype 5a epidemic, which is localized in a small area, the established epidemic subtypes 1a, 1b, and 3a are widespread. It is noteworthy that the HCV genotype 5a epidemic in Belgium is confined to such a small area of the country. Since the collection of blood in blood banks is organized on a regional level in Belgium, this could be a possible explanation for the localized spread of the genotype. The fact that this Belgian "hot spot" area was a rural area characterized by a close-knit society might be another explanation. In a study describing the epidemiological profile of newly diagnosed HCV patients in Belgium, a significant association was found between infection with HCV genotype 5a and a transfusion history (7). The results of our study also indicate that the transmission of HCV genotype 5a in Belgium is mainly associated with blood transfusions and hemodialysis.

In the South African population, the phase of exponential spread was also characterized by high growth rates indicative of rapid epidemic spread. The slowdown around 1950, however, does not appear to be consistent with transmission through blood products, which continued until 1990. The South African dynamics might perhaps reflect an iatrogenic intervention in the early 20th century, such as the use of unsterilized medical equipment. However, we wish to remain cautious in hypothesizing such scenarios based on coalescent analyses alone.

Investigation of the different circulating HCV genotypes and their evolution is not only crucial for epidemiological and clinical

analyses (40), but might also be helpful for the improvement of diagnostic tests and treatment regimens. Since data about the epidemiology and natural history of HCV genotype 5a and the treatment responses of HCV genotype 5a-infected patients are scarce, investigation of the clustering of these HCV strains can be a helpful tool for progress in these research areas.

ACKNOWLEDGMENTS

We thank our coworkers in the Laboratory of Clinical and Epidemiological Virology for their helpful comments and discussion.

This work was supported by the Flemish Fund for Scientific Research (Fonds voor Wetenschappelijk Onderzoek; FWO) grant G.0513.06. P.L. was supported by the European Molecular Biology Organization (EMBO). O.G.P. was funded by the Royal Society.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1998. Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. Morb. Mortal. Wkly. Rep. **47:**1–39.
2. **Chamberlain, R. W., N. J. Adams, L. A. Taylor, P. Simmonds, and R. M. Elliot.** 1997. The complete coding sequence of hepatitis C virus genotype 5a, the predominant genotype in South Africa. Biochem. Biophys. Res. Commun. **236:**44–49.
3. **Davidson, F., P. Simmonds, J. C. Ferguson, L. M. Jarvis, B. C. Dow, E. A. C. Follett, C. R. G. Seed, T. Krusius, C. Lin, and G. A. Medgyesi.** 1995. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. J. Gen. Virol. **76:**1197–1204.
4. **Dienstag, J. L.** 1997. Sexual and perinatal transmission of hepatitis C. Hepatology **26:**S66–S70.
5. **Drummond, A. J., A. Rambaut, B. Shapiro, and O. G. Pybus.** 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol. Biol. Evol. **22:**1185–1192.
6. **Drummond, A. J., G. K. Nicholls, A. G. Rodrigo, and W. Solomon.** 2002. Estimating parameters, population history and genealogy simultaneously from temporally spaced sequence data. Genetics **161:**1307–1320.
7. **Gérard, C., J. Delwaide, D. Vaira, B. Bastens, B. Servais, E. Wain, C. Bataille, G. Daenen, J. Belaïche, and the GLEVHE.** 2005. Evolution over a 10 year period of the epidemiological profile of 1,726 newly diagnosed HCV patients in Belgium. J. Med. Virol. **76:**503–510.
8. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
9. **Henquell, C., C. Cartau, A. Abergel, H. Laurichesse, C. Regagnon, C. De Champs, J. L. Bailly, and H. Peigue-Lafeuille.** 2004. High prevalence of HCV genotype 5a in central France evidenced by a prospective study from 1996 to 2002. J. Clin. Microbiol. **42:**3030–3035.
10. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73:**237–244.
11. **Hoofnagle, J. H.** 2002. Course and outcome of hepatitis C. Hepatology **36:**S21–S29.
12. **Hui, C. K., M. F. Yuen, E. Sablon, A. O. Chan, B. C. Wong, and C. L. Lai.** 2003. Interferon and ribavirine therapy for chronic hepatitis C virus genotype 6: a comparison with genotype 1. J. Infect. Dis. **187:**1071–1074.
13. **Jover, R., J. Perez-Serra, F. de Vera, J. M. Alamo, C. Munoz, C. Yago, R. Martinez-Ramirez, and J. V. Vidal.** 2001. Infection by genotype 5a of HCV in a district of Southeast Spain. Am. J. Gastroenterol. **96:**3042–3043.
14. **Kim, W. R.** 2002. Global epidemiology and burden of hepatitis C. Microbes Infect. **4:**1219–1225.
15. **Legrand-Abravanel, F., K. Saundres-Sauné, K. Barange, L. Alric, J. Moreau, P. Desmorat, J. P. Vinel, and J. Izopet.** 2004. Hepatitis C virus genotype 5: epidemiological characteristics and sensitivity to combination therapy with interferon-alpha plus ribavirin. J. Infect. Dis. **189:**1397–1400.
16. **Levi, J. E., D. T. Takaoka, R. H. Garrini, R. M. Fachini, R. Focaccia, E. de Bortholi Santos, H. P. Mitre, J. S. de Mendonça, N. de Paulo Cavalheiro, A. A. Barone, and S. Wendel.** 2002. Three cases of infection with hepatitis C virus genotype 5 among Brazilian hepatitis patients. J. Clin. Microbiol. **40:**2645–2647.
17. **Martial, J., Y. Morice, S. Abel, A. Cabié, C. Rat, F. Lombard, A. Edouard, S. Pierre-Louis, P. Garsaud, O. Béra, R. Chout, E. Gordien, P. Deny, and R. Cesaire.** 2004. Hepatitis C virus (HCV) genotypes in the Caribbean island of Martinique: evidence for a large radiation of HCV-2 and for a recent introduction from Europe of HCV-4. J. Clin. Microbiol. **42:**784–791.
18. **Munro, J., J. D. Briggs, and E. A. B. McCruden.** 1996. Detection of a cluster of hepatitis C infections in a renal transplant unit by analysis of sequence variation of the NS5a gene. J. Infect. Dis. **174:**177–180.
19. **Murphy, D. G., B. Willems, J. Vincelette, L. Bernier, J. Cote, and G. Delage.** 1996. Biological and clinicopathological features associated with hepatitis C virus type 5 infections. J. Hepatol. **24:**109–113.
20. **Nakano, T., L. Lu, P. Liu, and O. G. Pybus.** 2004. Viral gene sequences reveal the variable history of hepatitis C virus infection among countries. J. Infect. Dis. **190:**1098–1108.
21. **Ndjomou, J., O. G. Pybus, and B. Matz.** 2003. Phylogenetic analysis of hepatitis C virus isolates indicates a unique pattern of endemic infection in Cameroon. J. Gen. Virol. **84:**2333–2341.
22. **Pawlotsky, J. M.** 2003. Hepatitis C virus genetic variability: pathogenic and clinical implication. Clin. Liver Dis. **7:**45–66.
23. **Pawlotsky, J. M.** 2003. Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C. Antiviral Res. **59:**1–11.
24. **Pybus, O. G., A. Cochrane, E. C. Holmes, and P. Simmonds.** 2005. The hepatitis C virus epidemic among injecting drug users. Infect. Genet. Evol. **5:**131–139.
25. **Pybus, O. G., A. J. Drummond, T. Nakano, B. H. Robertson, and A. Rambaut.** 2003. The epidemiology and iatrogenic transmission of hepatitis C virus in Egypt: a Bayesian coalescent approach. Mol. Biol. Evol. **20:**381–387.
26. **Pybus, O. G., M. A. Charleston, S. Gupta, A. Rambaut, A. C. Holmes, and P. H. Harvey.** 2001. The epidemic behaviour of the hepatitis C virus. Science **292:**2323–2325.
27. **Salemi, M., and A. M. Vandamme.** 2002. Hepatitis C virus evolutionary patterns studied through analysis of full-genome sequences. J. Mol. Evol. **54:**62–70.
28. **Sandres-Saune, K., P. Deny, C. Pasquier, V. Thibaut, G. Duverlie, and J. Izopet.** 2003. Determining hepatitis C genotype by analyzing the sequence of the NS5b region. J. Virol. Methods **109:**187–193.
29. **Simmonds, P.** 1995. Variability of hepatitis C virus. Hepatology **21:**570–583.
30. **Simmonds, P., and D. B. Smith.** 1997. Investigation of the pattern of diversity of hepatitis C virus in relation to times of transmission. J. Viral. Hepat. **1:**69–74.
31. **Tanaka, Y., K. Hanada, E. Orito, Y. Akahane, K. Chayama, H. Yoshizawa, M. Sata, N. Ohta, Y. Miyakawa, T. Gojobori, and M. Mizokami.** 2005. Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan. J. Hepatol. **42:**47–53.
32. **Tanaka, Y., K. Hanada, M. Mizokami, A. E. Yeo, J. W. Shih, T. Gojobori, and H. J. Alter.** 2002. A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc. Natl. Acad. Sci. USA **99:**15584–15589.
33. **Tanaka, Y., S. Agha, N. Saudy, F. Kurbanov, E. Orito, T. Kato, M. Abo-Zeid, M. Khalaf, Y. Miyakawa, and M. Mizokami.** 2004. Exponential spread of hepatitis C virus genotype 4a in Egypt. J. Mol. Evol. **58:**191–195.
34. **Thomas, I., T. Branckaert, E. Mathys, R. Vranckx, and R. Laub.** 2000. PCR detects HCV RNA in a plasma pool contaminated by a single preseroconversion donation of genotype 5a. Vox Sang. **79:**69–71.
35. **Tokita, H., H. Okamoto, H. Iizuka, J. Kishimoto, F. Tsuda, L. A. Lesmana, Y. Miyakawa, and M. Mayumi.** 1996. Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh (11a) genetic groups. J. Gen. Virol. **77:**293–301.
36. **Tsukiyama-Kohara, K., N. Iisuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:**1476–1483.
37. **Verbeeck, J., P. Maes, E. Wollants, G. Maertens, S. Van der Merwe, F. Nevens, and M. Van Ranst.** 2005. Genotyping of hepatitis C virus 5a strains using the VERSANT HCV genotype assay. J. Clin. Microbiol. **43:**6117–6119.
38. **World Health Organization.** 1997. Hepatitis C: global prevalence. Wkly. Epidemiol. Rec. **72:**65–72.
39. **Yang, Z.** 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. **15:**555–556.
40. **Zein, N. N.** 2000. Clinical significance of hepatitis C virus genotypes. Clin. Microbiol. Rev. **13:**223–235.