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Genotype Distribution and Molecular Epidemiology of Hepatitis C Virus in Blood Donors from Southeast France

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The genotype distribution of hepatitis C virus (HCV) in blood donors from southeast France was tracked for a period of 13 years (1991 to 2003). Virus genomes from 321 samples were analyzed by amplification and sequencing of the NS5b and E1 regions. The most frequent genotypes were 1b (30.2%), 1a (27.7%), and 3a (22.4%). Although it was less common, genotype 2 was characterized by the presence of strains belonging to 11 different subtypes, including 5 that had never been characterized. Genotypes 1a, 1b, 3a, and 4a presented typical "epidemic" profiles, with a large number of isolates per subtype and short mean genetic distances between isolates. Type 2 isolates displayed a typical "endemic" profile, with a large number of subtypes and very few isolates in each subtype. The epidemiology of HCV infection in southeast France changed radically during the study period in relation to modifications in the etiology of infection. We observed the emergence of new epidemic subtypes (subtypes 1a and 3) linked to intravenous drug use and a decrease in the types linked to blood transfusion and nosocomial infection (epidemic subtype 1b and endemic type 2). Comparison of strains from blood donors with strains from a cohort of inpatients in the same region during 2001 and 2002 demonstrated for the first time that the monitoring of blood donors is a generally valid indicator of HCV epidemiology in terms of genotype distribution.

More than 170 million people are currently infected by the hepatitis C virus (HCV), which represents a serious cause of chronic liver disease that may progress to cirrhosis liver and hepatocarcinoma. It is an enveloped virus with a single-stranded, positive-sense, nonsegmented RNA genome of approximately 9,500 nucleotides that encodes a polyprotein of approximately 3,000 amino acids (8, 12). Analysis of the HCV genome has demonstrated extremely high heterogeneity in both structural and nonstructural coding regions and has identified at least six different genotypes that have generally been divided into several subtypes (2, 25, 32).

The prevalence and distribution of HCV genotypes depend on geographical location (19). Three broad patterns of genotype distribution have been identified to date (30). One pattern, characterized by high genetic diversity, involves geographically discrete areas of West Africa with types 1 and 2 (4, 13, 27), Central Africa with type 4 (11, 20), and Asia with types 3 and 6 (1, 29, 37, 38). This pattern is suggestive of a long period of endemic infection (19, 30). Another pattern involves areas with a few subtypes circulating in specific risk groups, e.g., subtype 3a in drug addicts (21). The third pattern involves areas where a single subtype is present, such as in Egypt with subtype 4a (6, 11, 24) and South Africa with subtype 5a (7, 31).

Previous blood donor studies in France have suggested that genotypes 1, 2, 3, 4, and 5 were the most prevalent. However, genotyping in those studies was performed by analysis of the 5′ noncoding region, which cannot discriminate subtypes (34, 35). The reference method for determination of the HCV subtype is phylogenetic analysis, based on sequencing of the NS5b and E1 genome regions (32). The aim of this study was to analyze the genotype distribution of HCV by NS5b or E1 sequencing of the viruses in 321 blood samples collected from donors between 1991 and 2003. The epidemiology of the virus in our population was analyzed as a function of sex, collection date, and year of birth. The distribution of HCV strains identified in blood donors was compared with the distributions previously reported in patients in southeast France (35).

MATERIALS AND METHODS

Blood donors. In France, blood donation practice concerns nonremunerated voluntary donors between the ages of 18 and 65 years. These donors undergo a preliminary inquiry which allows the identification and exclusion of individuals with parenteral risk factors. A total of 321 plasma samples collected from HCV-infected blood donors in southeast France between 1991 and 2003 were analyzed. There were 181 men and 140 women, with their ages ranging from 18 to 65 years (mean, 38.5 ± 11.4 years). All tested positive for anti-HCV antibody by a third-generation immunoenzymatic assay (Ortho Diagnostic Systems, Raritan, N.J.). Positive results were confirmed by the RIBA HCV 3.0 SIA (Chiron, Emeryville, Calif.). Detection of HCV RNA in plasma was performed with a commercially available Amplicor HCV kit (Roche Diagnostic Systems, Meylan, France). All tests were carried out in accordance with the manufacturers' instructions.

Genotyping. Subtyping was accomplished by amplifying and sequencing a 339-bp amplicon of the NS5b region. A seminested PCR was performed with primers NS5-1 (sense; 5′-TAT-GAY-ACC-CGY-TGC-TTT-GAC-3′) and NS5-2 (reverse; 5′-GAG-GAG-CAA-GAT-GTT-ATC-AGC-TC-3′) for primary amplification and primers NS5-1 and NS5-3 (reverse; 5′-GAA-TAC-CTG-GTC-ATA-GCC-TCC-G-3′) for secondary amplification. Subtyping was also carried out with type-specific primers, as described previously (5).

Extraction of RNA from 200 μl of plasma, reverse transcription with random hexaprimers, and PCR of the NS5b region with generic primers and the E1-encoding region with type-specific primers were performed as reported previously (5). Amplicons in the E1 and NS5 regions were sequenced directly with the

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TABLE 1. Epidemiological features of the different subtypes

Genotype	No. of blood donors	Sex ratio[a]	Mean age (yr)[c]		
			All blood donors	Men	Women
1a	89	2.5	35.6 ± 9.5	37.1 ± 9.2	32.0 ± 9.2
1b	97	0.9	43.9 ± 12.7	38.9 ± 13.8	41.4 ± 13.4
2	35	0.9	43.3 ± 12.0	40.2 ± 15.0	46.1 ± 7.5
3a	72	1.3	35.4 ± 9.5	34.2 ± 9.4	36.9 ± 9.7
4a	8	ND[b]	39.0 ± 7.7	45.3 ± 8.3	ND
4d	16	1	34.4 ± 5.8	34.3 ± 6.6	34.6 ± 5.4
5a	4	0.33	45.5 ± 18.1	ND	ND
Total	321	1.3	38.3 ± 11.5	37.3 ± 11.3	39.7 ± 11.6

[a] Number of males to number of females.
[b] ND, not determined.
[c] Values are 95% confidence intervals.

amplification primers, a D-rhodamine DNA sequencing kit, and an ABI Prism 377 sequencer (Perkin-Elmer).

Phylogenetic analysis. The nucleotide sequences of the HCV strains from infected patients in Marseilles, France, and a panel of sequences retrieved from the GenBank database (see the phylogenetic trees in the Results section) were aligned by using the ClustalW 1.8 software package (36). Phylogenetic analysis for strain typing was focused on two genomic regions, i.e., a 357-nucleotide sequence (positions 688 to 1044) in the E1 region and a 339-nucleotide sequence (positions 8002 to 8340) in the NS5b region. The nucleotide positions refer to the subtype 2a sequence with GenBank accession number D00944. Phylogenetic analysis was performed with the MEGA software package (version 2.1, 2001; Pennsylvania State University, University Park) (16) by using the *p* distance for distance determination and the neighbor-joining method for tree drawing. The reliability of phylogenetic analysis was evaluated by a bootstrap test with 1,000 replications.

Statistical analysis. Statistical analysis was performed with the Systat v10.2 software package (Systat Software Inc.). The results are expressed as means ± standard deviations or as percentages, with 95% confidence intervals calculated according to the normal distribution or the binomial distribution, as applicable. Means between groups were compared by using the *t* test or the Student test, and group frequency was compared by the chi-square test or Fisher's exact test. The frequency distributions of the different genotypes within groups (birth year or collection year) were analyzed by the extended Mantel-Haenszel chi-square test.

RESULTS

Distribution of HCV genotypes. All 321 HCV RNA-positive samples collected between 1991 and 2003 were amplified and sequenced. The type-specific strategy in the E1 region was used for four samples for which sequencing of the NS5b region failed (because of the absence of PCR amplification). The results demonstrated the presence of subtype 4a in all four cases. Table 1 details the distributions of the different types together with demographic data. The distributions of the HCV subtypes can be summarized as follows: type 1b, 30.2%; type 1a, 27.7%; type 3a, 22.4%; type 4d, 5%; type 4a, 2.5%; and type 5a, 1.3%.

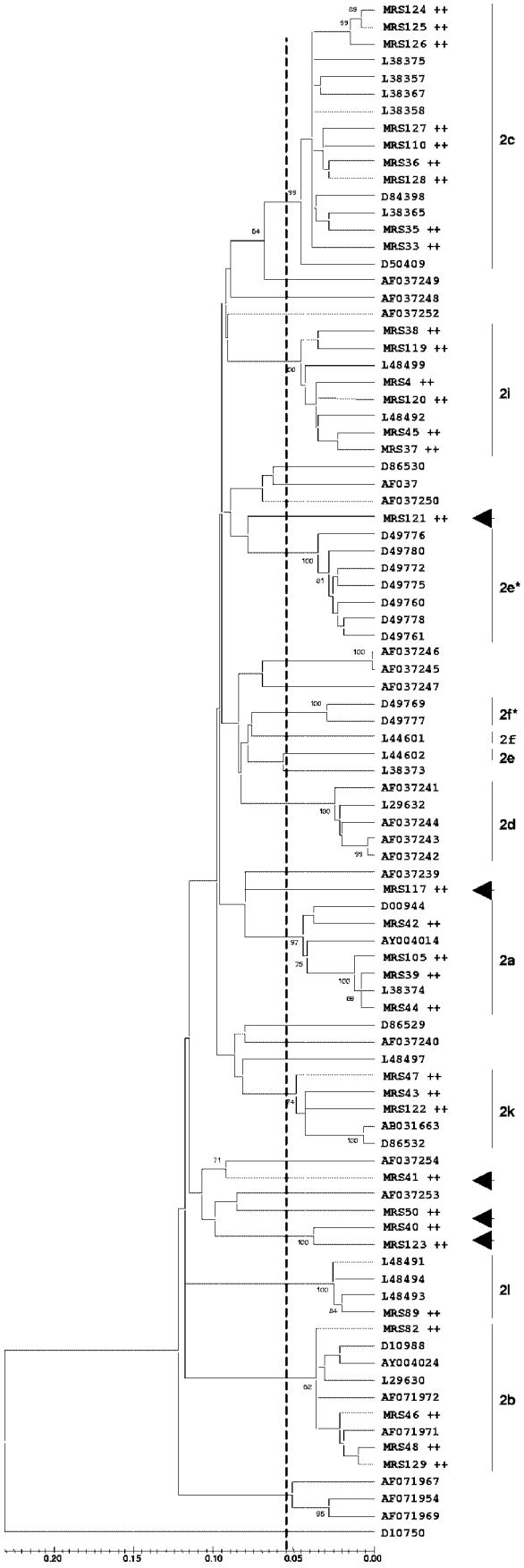
Genotype 2, with an overall prevalence of 10.9%, presented a special subtype distribution. As shown in Fig. 1, phylogenetic analysis of the NS5b region identified a total of 35 type 2 strains. Twenty-nine strains clustered within six previously described subtypes, i.e., 2a, 4 strains (11.4%); 2b, 4 strains (11.4%); 2c, 10 strains (28.6%); 2i, 7 strains (20%); 2k, 3 strains (8.6%); and 2l, 1 strain (2.9%). The remaining six strains, i.e., MRS40, MRS123, MRS41, MRS50, MRS117, and MRS121, were unassignable to any previously described subtype and represented five putative new subtypes.

Epidemiology. Determination of the frequency distribution of HCV genotype according to the year of collection showed a significant increase in the prevalence of subtype 3a from 0% in 1990 to 28% in 1999 ($\chi^2 = 16.05$; $P < 0.01$). The prevalence of subtype 1b dropped significantly from 50% in 1990, when it was predominant, to 23% in 1998 ($\chi^2 = 6.34$; $P < 0.05$). A slight evolution of the distribution of subtype 1a has been observed since 2000 (Fig. 2); however, this was not found to be statistically significant.

The sex ratios for the different subtypes were also determined and were compared with the sex ratio of the entire population. Significant differences were found for subtype 1a strains, which showed an overrepresentation among males ($\chi^2 = 7.5$; $P < 0.005$), and for subtype 1b strains, which showed an overrepresentation among females ($\chi^2 = 10.8$; $P < 0.002$). The other subtypes showed no significant differences with regard to sex ratio, but it is noteworthy that subtype 4a was identified only in males ($n = 8$).

Analysis of the strain distribution according to age showed that donors infected by types 2 (mean age, 43.3 ± 12.0 years) and 1b (41.4 ± 13.4 years) were significantly older than donors infected by types 1a (35.6 ± 9.5 years), 3a (35.4 ± 9.5 years), and 4d (34.4 ± 5.8 years) (1a versus 1b, $P < 0.001$; 1a versus 2, $P < 0.001$; 3a versus 1b, $P < 0.001$; 3a versus 2, $P < 0.001$; 4d versus 1b, $P < 0.01$; and 4d versus 2, $P < 0.01$). The differences between types 1a, 3a, and 4d and between types 1b and 2 were not significant. The paucity of genetic data regarding subtype 5a strains (four strains of which were characterized) does not permit judgment of the statistical significance of the differences in the mean age of the donors infected with subtype 5a (45.5 ± 18.1 years) compared to the mean age of the donors infected with the other subtypes.

The mean age of men infected with subtype 1a was statistically higher than that of women (37.1 ± 9.2 years versus 32.0 ± 9.3 years; $P = 0.025$). Indeed, subtype 1a was identified in approximately 35% of men born between 1941 and 1970 and increased from 8 to 38% among women born during the same period. The opposite results were obtained for subtypes 1b and 2, for which the mean female age was higher than mean male age (43.9 ± 12.7 years and 38.9 ± 13.8 years, respectively, and 46.1 ± 7.5 years and 40.2 ± 15.0 years, respectively), However, statistical significance was low for subtype 1b ($P = 0.071$) and null for type 2.



Comparison of strains in blood donors and patients from the same region. The distribution of the 96 strains identified in our plasma samples collected from blood donors between 2001 and 2002 was compared with the distribution of strains identified in samples collected from 184 patients living in the same region during the same period (35). The distributions were similar for subtypes 1 (56.2% versus 61.4%), 2 (6% versus 8.3%), and 3 (24.5% versus 28.1%). Conversely, subtype 4 was significantly more prevalent in patients than in donors (13.6% versus 7.3%; $\chi^2 = 8.32$; $P < 0.01$). It should be noted that the sex ratio was significantly different in the two populations (1.3 in blood donors versus 2.2 in patients; $P < 0.001$).

Analysis of HCV strain clustering in a phylogenetic tree built with sequences identified in donors and patients showed no striking pattern. No typical subtype pattern could be associated with the two populations.

The distribution of the strains and the mean genetic distances between subtypes were compared between donors and patients. The mean genetic distances were significantly lower for subtypes 1a (0.041 ± 0.008 versus 0.047 ± 0.009) and 1b (0.053 ± 0.007 versus 0.063 ± 0.007) in patients than in donors ($P < 0.01$). This was not the case for subtype 3a (0.050 ± 0.009 versus 0.049 ± 0.006), which showed comparable mean genetic distances in donors and patients.

DISCUSSION

The first donor samples in this study were collected in 1991 during the first year after implementation of serological screening for HCV in French blood banks. At that time the epidemiological distribution featured a large predominance of subtype 1b strains (~50%), followed by types 1a and 2 (~20% each), and a low prevalence of other types (<5% each). In 2003 a radically different distribution was observed, with a predominance of subtype 1a (~40%), followed by subtypes 3a and 1b (~30% each). Over the 12-year study period, genotypes 1a and 3a emerged while genotypes 1b and 2 regressed.

To our knowledge, this is the first study which has analyzed the evolution of the HCV subtype distribution as a function of the year of blood collection. To date, previous studies have analyzed the subtype distribution as a function of the age of the patients. For example, a shift in the distribution was observed in northeast Italy, where subtype 1b and 2 were practically replaced by subtypes 1a and 3a (9). Another study with German patients showed that subtype 1b was predominant among elderly patients, while subtype 1a was predominant among the younger population (26). A third study with Russian patients showed that subtype 1b was progressively replaced by subtype 3a (14).

FIG. 1. Phylogenetic tree of partial NS5b nucleotide sequences of 33 HCV strains isolated from blood donors (++) and a panel of reference strains identified by their GenBank accession numbers. New subtypes are indicated by arrowheads. The numbers at the right correspond to prototype strain subtypes as previously published by Stuyver et al. (33) for subtypes 2a, 2b, 2c, 2d, 2e, 2f, 2k, and 2l and Tokita et al. (39) for subtypes 2e* and 2f*. The numbers at the nodes are the percentages of 1,000 bootstrap replicates higher than 50%. The scale bar indicates the *p* distances. The vertical dotted line represents separation between subtypes.

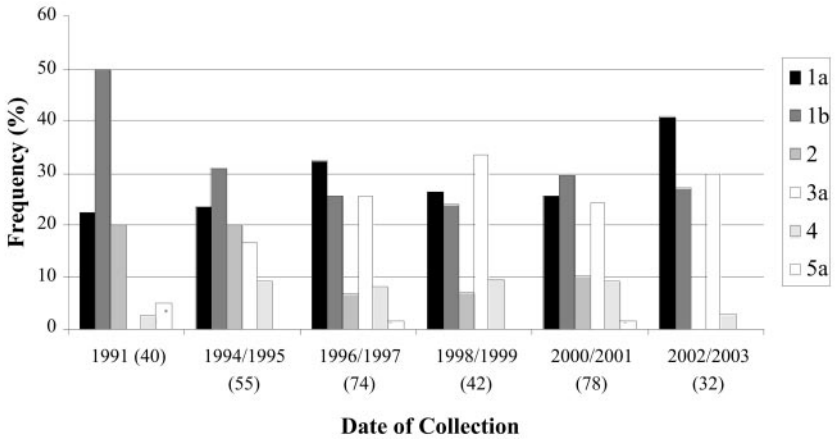


FIG. 2. Distribution of hepatitis C virus types according to date of collection. The number of cases is indicated in parentheses.

Past studies have shown that the main risk factor for infection by genotypes 1a and 3a is intravenous drug use (10, 18, 35) and that the main risk factor for infection by genotypes 1b and 2 is blood transfusion (10, 18). This epidemiological scenario was corroborated by analysis of the age data for the different groups. Patients infected by genotypes 1b and 2 were older than those infected by genotypes 3a and 1a. This finding suggests that at least two successive changes occurred. The first, implicating genotypes 1b and 2, was linked to blood transfusion; and the second, implicating genotypes 3a and 1a, was linked to intravenous drug use.

Access to the database previously described by Tamalet et al. (35) gave us a unique opportunity to compare HCV infection in blood donors and hospitalized patients from the same region during the same time period (2001 and 2002). Despite the differences in ages, sex ratios, and clinical presentations, the distribution of genotypes in the two populations was globally similar for strains belonging to types 1, 2, and 3 (accounting for 86.4 and 92.7% of HCV strains in patients and donors, respectively). This finding suggests that the monitoring of blood donors provides a relatively unbiased picture of the epidemiological situation. The logical exception to this rule involves donor population underrepresentation of genotype 4, which was associated with human immunodeficiency virus infection in the study of Tamalet et al. (35).

Comparison of the donor and the patient data provided two other important insights. The first is that the mean genetic distances between subtype 1a and subtype 1b isolates were shorter in the patient population. A probable explanation for this difference in subtype 1a strains is that drug addicts are often treated in public hospitals but rarely donate blood. Regarding subtype 1b, the most likely explanation is association with nosocomial transmission, in particular, in hemodialysis patients (35). Another important insight obtained by analysis of the phylogenetic reconstructions was the absence of clustering between the patient and the donor strains. This finding refutes the hypothesis of a phylogenetic basis for pathogenicity in each subtype.

Analysis of the HCV genotypes within a defined population is a useful epidemiological tool for the study of the evolution of HCV infection in different geographical regions and risk groups. However, the utility of genotype analysis depends on the technique used. Until now, most data have been obtained by analysis of the viral 5′ noncoding region. This method allows good discrimination between the six HCV types but not differentiation between subtypes due to the high degree of conservation of the 5′ noncoding region. Prior comparison of the typing results based on the 5′ noncoding region (34) or the NS5b region (data not shown) in our population showed that ~30% of the strains assigned to subtype 1b by using the 5′ noncoding region were in fact subtype 1a strains. Based on that preliminary finding, subtyping in the current study was based on analysis of the NS5b region or the envelope sequence.

The epidemiological patterns of the different viral subtypes were of special interest. Genotypes 1a, 3a, 4a, and 1b presented typical "epidemic" profiles, with a large number of isolates for a given subtype and short mean genetic distances between isolates. All four of these genotypes are related to parenteral transmission, associated with the use of blood products and intravenous drug abuse over the last 50 years in Europe, North America, Japan, and Australia (19, 21, 30). The most likely explanations for this pattern insofar as subtypes 1a, 3a, and 4a are concerned are the explosive epidemiological spread of HCV through intravenous drug use and the recent nature of their emergence. The slightly greater mean distances between subtype 1b isolates were probably due to slower spread through blood transfusion and to the fact that transmission peaked prior to 1990, meaning that this subtype has been evolving longer than subtypes 1a, 3a, and 4a.

Genotype 2 isolates from donors and patients displayed a typical "endemic" profile, with a large number of subtypes (11 genotype 2 subtypes) and few isolates in each subtype. Infection by type 2 was also associated with the older age bracket in our study. Remarkably, type 2 accounted for more than 20% of strains collected before 1995 but was never found between 2001 and 2002. This finding suggests that this endemic genotype is progressively being replaced by epidemic genotypes. The most prevalent subtypes were 2a, 2b, 2c, 2i, and 2k, all of which presented more than three strains each. Among the various subtype 2 isolates found in our study, two, i.e., subtypes 2i and 2l, were exclusive to

France (23). Subtype 2k has previously been observed in Moldavia and Russia (28) and is one of the two subtypes responsible for the recombinant strain identified in St. Petersburg, Russia (15). Subtype 2a, 2b, and 2c are common in East Asia, North America, and Europe (mainly Italy) (3, 17, 22). Six type 2 strains could not be assigned to any previously described subtypes and belonged to five newly identified subtypes (Fig. 1).

Overall, this study provides strong evidence that the molecular epidemiology of HCV infection in southeast France has changed radically in relation to the changing etiology of infection over the last 15 years. The main change involved the emergence of new epidemic genotypes and regression of endemic type 2 genotypes. This study also demonstrated for the first time that monitoring of blood donors is a globally valid epidemiological indicator of HCV genotype distribution and demography markers.

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