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Hepatitis C virus genotype 4 in Southern and Central Spain does not come from recent foreign migration waves

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Title Page

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Short Running Title: Hepatitis C Virus genotype 4 diversity in Spain

Abstract

Hepatitis C virus genotype 4 (HCV-4) is highly prevalent in Spain, but the information on the molecular characterization of HCV-4 in this region is scarce. Due to this, the molecular characteristics and the evolution of HCV-4 infection in Seville were analyzed (Southern Spain) and compared them with samples from Madrid. HCV genotype was determined by LIPA 2.0 assay and confirmed by sequence analysis of NS5B. Phylogenetic tree was estimated by MEGA 5.10. Bayesian coalescent-based methods were used to estimate the substitution rate and the age of the most recent common ancestor (MRCA). In the phylogenetic analysis of 50 NS5B HCV-4 from Seville and 11 from Madrid, two clusters were distinguished: The first cluster (HCV-4a) included 48% of the sequences from Seville and 9% of sequences from Madrid. The second cluster included the remaining sequences belonging to HCV-4d. The mean estimated substitution rate was 2.39 $x10^{-3}$ for HCV-4a and 1.81 $x10^{-3}$ for HCV-4d for Seville and 2.32 $x10^{-3}$ for HCV-4d from Madrid. The date for MRCA was estimated to be around 1981 to 1984 for HCV-4 from Seville. The dates for MRCA were dated before the recent flow of immigration in Spain. Therefore, the results presented in this study argues against the possibility of a foreign introduction of the HCV-4 from other regions with high prevalence, at least during the last two, decades in which there was a great flow of immigrants. Additionally, an unusual high prevalence of subtype 4a was observed in Seville.

Keywords: Hepatitis C virus; diversity; phylogeny; Beast; evolution.

Introduction

Hepatitis C virus genotype 4 (HCV-4) is considered a difficult-to-treat genotype in patients infected with HCV and patients infected with HCV and HIV, because the rate of sustained virologic response (SVR) to pegylated interferon plus ribavirin is poorer than that for HCV genotypes 2 or 3 (Torriani *et al*., 2004; Carrat *et al*., 2004; Pineda *et al*., 2007; Nuñez *et al.,* 2007; Mira *et al*., 2012). Recent studies carried out in Europe have reported an increase in the prevalence of HCV-4 (Medrano *et al.,* 2011). Thus, in some Mediterranean countries the rates of prevalence for this genotype are between 10 to 24% (Morice *et al.,* 2001; Van Asten *et al.,* 2004; Nicot *et al.,* 2005; Payan *et al.,* 2005; Ansaldi *et al.,* 2005; Fernandez-Arcas *et al.,* 2006; Ramos *et al.,* 2007; Esteban *et al.,* 2008). Similar figures are observed in Spain, depending on the geographical areas and the risk group the analyzed patients belong to. Accordingly, rates from 10.2 to 18% have been reported in Southern Spain (Fernandez-Arcas *et al.,* 2006; Cifuenes *et al.,* 2012), 8.5 to 20% in Central Spain (Pérez-Olmeda *et al.,* 2002; Medrano *et al.,* 2011; Poveda *et al*., 2012) and 8.2 to 19% in Northern Spain (Rubio *et al.,* 2001; Ramos-Sanchez *et al.,* 2003; Martinez *et al.,* 2005).

Most cases of HCV-4a infection in Europe have been attributed to the immigration from Africa (more precisely Egypt) and Middle East (de Bruijne *et al.,* 2009; Eriksen *et al.,* 2010). Egypt is one of the countries with highest prevalence of HCV infection (15%). HCV-4a accounts for 90% of infections in Egypt (Egyptian Ministry of Health, 2007; Khattab *et al.,* 2011). However, recent studies have also reported a high prevalence of HCV-4d, which has European origin (de Bruijne *et al.,* 2009; Eriksen *et al.,* 2010) and that it is spreading throughout European countries mainly in relation to illicit intravenous drug using (Fernandez-Arcas *et al.,* 2006; de Bruijne *et al.,* 2009; Eriksen *et al.,* 2010).

Currently, there are almost 6 million of foreign-born residents in Spain. This numbers correspond to 12.1% of the total population (Instituto Nacional de Estadística 2012). Of these, 3.3 million (7% of the total population) were born outside the European Union, including a large proportion of immigrants from Africa (Instituto Nacional de Estadística 2012), where genotype 4a is highly prevalent. The rest of the 2.4 million (5.1% of the total population) were born in another European Union member state (Instituto Nacional de Estadística 2012), where there are also several countries with high prevalence of genotype 4d. Consequently, due to the elevated immigration rate from endemic areas to Spain, it is important to assess the molecular epidemiology and evolution of HCV-4 infection in this area, which was the purpose of this study. To extend the evolutionary analysis, the coalescence-based population genetic method was used in order to estimate the origin and diversification of HCV-4 in Spain.

Methods

Design and study population

From November 1992 to November 2011, a total of 1523 patients with positive serology for HCV were seen at the Infectious Diseases Units of a tertiary care center in Seville, Spain. HCV genotype by LIPA was available in 970 (63.7%) of these patients. In this retrospective cross-sectional study, those 150 (15.5%) patients infected with HCV or patients infected with HCV and HIV who bore HCV genotype 4 were included. A phylogenetic analysis of NS5B partial gene sequences obtained from HCV-4 was performed in 50 patients selected randomly from de above population. On the other hand, 11 patients infected with HCV-4 and HIV, belonging to a cohort of 424 subjects, including 85 patients infected with HCV-4 and followed in the Department of Infectious Diseases of a tertiary care center in Madrid, were also included in the phylogenic analysis, in order to compare with sequences from Seville.

Laboratory determinations

HCV genotype was determined using a RT-PCR hybridization assay (Versant HCV Genotype 2.0 LIPA; Siemens, Tarrytown, NY, USA).

The HCV NS5B partial region (positions 7816-8218 according to reference HCV-1a sequence AF009606) was amplified using specific primers. For both HCV-4 subtypes, the primers used were: POLEF4 (5´-GGA TCR GAG GAY GTM GTR TG- 3´) and POLER4 (5´TGT GAT AAA TGT CYC CCC CG 3´) as outer primers and POLIF4 (5´-CTG CCM ATY ARC CCC CTG AG- 3´) and POLIR4 (5´-GGC AAT GGA GTG AGT YTG- 3´) as inner primers. PCR amplicons were directly sequenced in both senses using the ABI PRISM 3100 Genetic Analyser using the ABI PRISM Rhodamine Terminator reaction kit (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

NS5B sequences were subjected to alignment with CLUSTALX v2.0 software (Larkin *et al.,* 2007). A Maximum Likelihood (ML) phylogenetic tree were estimated by MEGA 5.10 (Kumar *et al.,* 2008). Evolutionary models were inferred according to the Akaike Information Criterion (AIC) statistics obtained with the jModeltest program (Posada and Crandall 1998).The bootstrap resampling test with 10,000 replicates was performed for the NJ tree and 1,000 for ML to assess the robustness of the tree arrangement. All HCV-4 sequences available in GenBank and that corresponded to the amplified region in this work were included in the phylogenetic analysis. D90208 (HCV-1b), AF009606 (HCV-1a), AY746460 (HCV-2a) and D17763 (HCV-3a) were added as out-groups.

Molecular evolutionary rate

Bayesian coalescent-based methods were used to estimate the substitution rate. The estimates of the rate of nucleotide substitutions per site per year (s/s/y) were obtained by means of the Bayesian Markov Chain Monte Carlo (MCMC) techniques implemented in the BEAST v1.7.2 program (Drummond *et al.,* 2007). Both strict and relaxed (uncorrelated lognormal and uncorrelated exponential) molecular clocks were enforced to estimate the rate of nucleotide substitution and the age of the most recent common ancestor (MRCA). Five demographic models were applied as coalescent priors: constant population size, exponential growth, expansional growth, logistic growth and Bayesian skyline plot. These analyses were performed using the general time reversible substitution model with gamma-distributed rates across sites and a proportion of sites assumed to be invariable (GTR+G+I). The best-fitted model analyzed in this study was selected with the Akaike Information Criterion (AIC) by using Modeltest Version 3.06 (Posada *et al.,* 1998). The length and number of MCMC were chosen, so that the effective sample sizes (ESS) were above 100, indicating that the parameter space was

explored sufficiently. The convergence of the parameters to a stationary distribution was assessed with the TRACER v1.5 program (Rambaut *et al.,* 2011) and the statistical uncertainties were summarized from the 95% highest probability density (HPD) intervals. Model comparisons were performed by a Bayes Factor (BF) analysis (Suchard *et al.,* 2001).

Frequencies were compared using the chi-square test or the Fisher's test. The Student's *t*-test and the Mann-Whitney U were used for comparing continuous variables between two groups. The statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM SPSS Inc, Chicago, IL, USA).

Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Ethic Committee of the participating Hospitals.

Results

Characteristics of the analyzed population

One hundred and fifty patients bore HCV-4 in the population from Seville. All patients were born in Spain. The median age (Q1-Q3) was 37.6 (32-44) years. Among these patients, 270 (48.2%) were infected with HCV and 279 (49.8%) were infected with HCV and HIV. With regard to the way of transmission, 434 (77.5%) were injecting drug users (IDU), in 66 (11.8%) the infections mode was unknown, in 39 (7%) blood transfusion and in 3 (0.5%) patients HCV infection was considered to have been acquired by sexual intercourse. Characteristics of the patients infected with HCV-4, separated by those with sequenced (N=50) and no sequenced (N=100) samples, are shown in Table 1.On the other hand, the median age (Q1-Q3) for patients from Madrid was 39 (37-42) years. All patients from Madrid were infected with HCV and HIV.

Phylogenetic analysis and subtype distribution of HCV-4

Fifty partial NS5B gene [position 7816 to 8227 according to HCV-1a-H77 AF009606, (412bp)] from patients infected with HCV-4 from Seville and 11 from Madrid were sequenced. The best-fit model of DNA evolution for the analyzed data was GTR + I + Γ. Phylogenetic analyses (Figure 1) distinguished clearly two groups of sequences, each forming a well-defined cluster. The first cluster (bootstrap=99) comprised 24 out of 50 (48%) sequences from Seville and 1 out of 11 (9%) from Madrid, belonging to the HCV-4a subtype and included also samples from Egypt [four of them forming a separate cluster (bootstrap=100)], Spain, France, Ireland and United States. The second cluster (bootstrap=99) comprised the remaining 26 (52%) sequences from Seville and 10 out of 11 (91%) from Madrid belonging to the HCV-4d subtype and incorporated samples from

other regions of Spain (Barcelona and Albacete), France, Canada and United States. Altogether, the subtypes determined by sequencing of NS5B were not in agreement with those obtained by Versant HCV Genotype 2.0 LIPA in 27 out of 48 (56.3%) samples infected with HCV-4.

The frequency of HIV-coinfection and the way of HCV infection was not significantly different between patients with subtype 4a and those with subtype 4d. Thus, 14 (58.3%) subjects with subtype 4a and 12 (46.2%) with subtype 4d were HIV-infected (p=0.282). Twenty-one (91.7%) patients with subtype 4a vs. 19 (73.1%) harbouring subtype 4d were IDU (p=0.089).

Molecular evolutionary rate

In samples from Seville, the BF analysis favored the relaxed uncorrelated exponential molecular clock and the exponential population size over the other models for the partial NS5B analyzed herein. However, the relaxed uncorrelated lognormal molecular clock was used since is the top-performer and the estimates are very consistent across all the models compared. Lengths of MCMC of 320 million for HCV genotypes 4a and 4d from Seville were needed to reach values of ESS above 100. On the other side, for the sequences from Madrid, the BF analysis favored the relaxed uncorrelated lognormal molecular clock and the constant population size. The MRCA date and the mean estimated substitution rate for every group of sequences are showed in table 2.

Since viral recombination could affect coalescent analyses (Schierup and Hein, 2000), the SimPlot program was used to discard this possibility. No recombination was observed and absence of recombination events was confirmed by bootscanning, using a putative recombinant sequence as a query (Worobey, 2001).

Discussion:

In this study, it has been found that the distribution of HCV-4 in Seville, Southern Spain, shows a low diversity, with only two subtypes, HCV-4a and 4d, and almost without the formation of internal clusters. In contrast with the rest of Spain, as happened in the samples from Madrid analyzed in this study, there was a high prevalence of HCV-4a in Seville. The time for the MRCA of the strains from Seville was between 1981 and 1984, that is, just before the big increase of foreign immigration in Spain.

The HCV-4 subtype distribution observed in this study in Southern Spain is different to that reported previously in other regions of Spain, where subtype 4d is very predominant (Ramos-Sanchez *et al.,* 2003; Martinez *et al.,* 2005; Fernandez-Arcas *et al.,* 2006). However, in Seville, a prevalence of subtypes similar to that reported in other countries of Western Europe, such as France, Greece or Cyprus, was found (Morice *et al.,* 2001; Nicot *et al.,* 2005; Katsoulidou *et al.,* 2006; de Bruijne *et al.,* 2009; Demetriou *et al.,* 2009). In previous studies, the presence of HCV-4a in Europe was associated strongly to immigration from Egypt or Middle East (Morice *et al.,* 2001; Rubio *et al.,* 2001; Nicot *et al.,* 2005; Demetriou *et al.,* 2009). This is not the case observed in Seville, since all the patients included in this study were native from Spain and, as the phylogenetic analysis suggested, seems to be not related with the Egyptian samples.

In the sequence analysis of NS5B, which is the reference method for HCV genotyping, a perfect concordance with LIPA at genotype level was observed, but discrepant results were observed in a very high proportion of samples at subtype level. Direct sequencing of the NS5B region is preferable for the precise identification of HCV subtypes (Simmonds *et al.,* 2005). The inability of LIPA to identify the correct subtype has been

reported by other studies, especially, regarding subtypes 1a and 1b, but also for genotype 4 subtypes (Chen *et al.,* 2002; Nolte *et al.,* 2003; Zekri *et al.,* 2005). In this work, more than one half of discordant subtypes between both methods for HCV-4 samples from Seville and Madrid were found. This is a critical point, as differences regarding the response to therapy reported for subtypes HCV-1a and HCV-1b (Rallon *et al.,* 2012) could also happen for different subtypes of HCV-4.

During the last two decades, Spain has been a transit place or the final destination for a very important number of immigrants from other areas of the world with elevated prevalence's of HCV. In spite of this fact, the infection with both subtype 4a and 4d were dated between 1981 and 1984 in samples from Seville, just before the big increase of immigration in Spain, which began in the middle of the nineties (Instituto Nacional de Estadística 2012). As the MRCA represents the time of the diversification process of all sequences included in the analysis, in the case of HCV-4 sequences from Seville (a non-monophyletic group), this would imply the time at which currently circulating virus in Seville started diversifying. This epidemiological information is important because the evolutionary reconstructions indicate that several HCV-4a and HCV-4d strains with a common ancestor originating in the early 1980 entered in Seville. This would not imply that the diversification has occurred in Seville, but the polyphyletic nature of the group would suggest multiple viral introductions instead. All these results suggest that the high prevalence of HCV-4 found in Seville is not due to the large immigration received in the last 10 to 15 years. Furthermore, the countries that contributed most to the immigrant community in the recent years, such as Morocco, Romania, Germany, UK and some Latin American countries, have a low prevalence of HCV-4 infection (Fay *et al.,* 2006; Quarleri *et al.,* 2007; Brant *et al.,* 2010; Mora *et al.,* 2010; Sultana *et al.,* 2011; Brahim *et al.,* 2012). Consequently, there was not a potential risk for a growth in the prevalence of

HCV-4 due to this issue. Nonetheless, periodical surveillance studies of the prevalence of HCV-4 infection should be considered in the context of patients infected with HCV and HIV and, principally, among IDUs.

This study has a limitation. The sequences of all HCV-4 infected patients could not be analyzed. However, the analyzed samples were obtained at random and significant differences between patients with and without sequenced virus were not observed (Table 1). Moreover, the sample size was large enough to make a good molecular characterization of distribution of HCV-4 subtypes and the estimation of MRCA in this area.

In summary, the substitution rate calculated for HCV-4 is between the published previously range (Di Lello et al, 2012) and the date for MRCA for samples from Seville was dated before the big increasing of immigration in Spain. Accordingly, the results presented in this study argue against the possibility of a foreign introduction of the HCV-4 from other regions with high prevalence, at least during the two last decades in which there was a great influx of people. Additionally, an unusual high prevalence of subtype 4a was observed in Seville (Southern Spain), but not in Madrid (Central Spain).

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Legend Figure:

Figure 1: Phylogenetic tree of 50 HCV-4 sequences from Seville (412 nt) and 11 from Madrid. The numbers at each node correspond to bootstrap values from a ML tree obtained with 1,000 replicates (bootstrap values lower than 70 are not shown). HCV-4 sequences from France (27), Ireland (1), USA (8), Canada (2), Spain (3), Egypt (11) and Portugal (1) were included in the phylogenetic analysis. D90208 (HCV-1b), AF009606 (HCV-1a), AY746460 (HCV-2a) and D17763 (HCV-3a) were added as out-groups. Full circles indicated samples from Seville and Empty circle indicated samples from Madrid. The end of each sample name corresponds to the HCV subtype.

Table 1: Characteristics of the patients with chronic hepatitis C genotype 4 (n=150) separated by those with sequenced and no sequenced virus.

Parameter	No Sequenced (N=100)	Sequenced (N=50)	P
Age (years)*	37 (31.7-42.8)	38 (34.8-44.1)	0.256
Male gender no. (%)	87 (87)	39 (78)	0.156
HIV Positive no. (%)	61 (61)	26 (52)	0.412
Subtype by LIPA no. (%)			
A	2 (2)	3 (6)	
A/B	1 (1)	0 (0)	
A/C/D	28 (28)	8 (16)	
B	2 (2)	2 (4)	
C/D	35 (35)	23 (46)	
E	1 (1)	1 (2)	
Undetermined	31 (31)	13 (26)	0.404
Transmission no. (%)			
IDU	87 (87)	39 (78)	
Unknown	10 (10)	6 (12)	
Sexual	0 (0)	2 (4)	
Transfusion	1 (1)	1 (2)	0.242

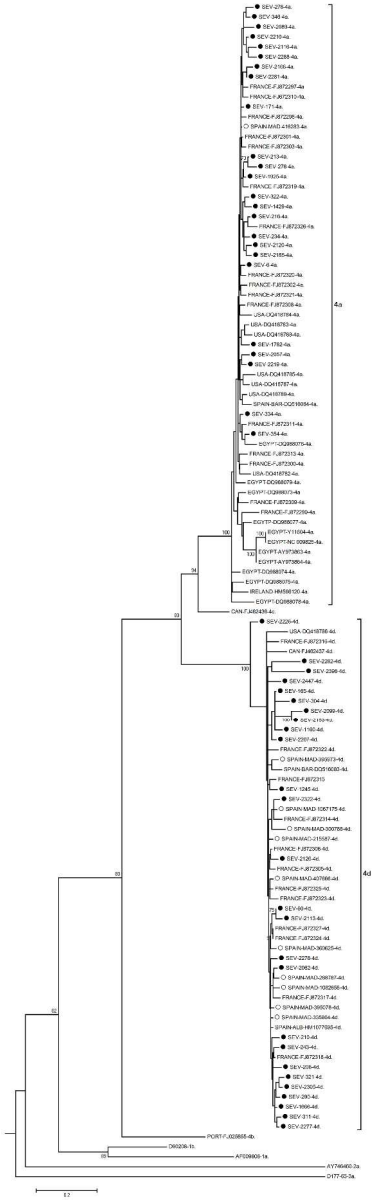
*Median (quartile 1-quartile 3).

Table 2: Estimates of the MRCA for the NS5B gene by Bayesian coalescent.

Nº of NS5B Sequences	Place	HCV-4 Subtype	MRCA (HPD 95%)	Mean Substitution Rate (HPD 95%)
24	Seville	4a	1981 (1947 / 1999)	1.32×10^{-3} (2.81×10^{-4}-2.63×10^{-3})
26	Seville	4d	1984 (1940 / 1999)	1.77×10^{-3} (2.16×10^{-4}-3.24×10^{-3})
10	Madrid	4d	1988 (1966 / 2002)	2.72×10^{-3} (5.91×10^{-4}-4.82×10^{-3})

MRCA: Most Recent Common Ancestor

HPD 95%: Highest probability density 95%.



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