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BRIEF REVIEW

# Changing epidemiology of hepatitis C virus genotypes in the central region of Argentina

**Federico Alejandro Di Lello · Adrián Alejandro Farias · Andrés Carlos Alberto Culasso · Paula Soledad Pérez · María Belén Pisano · Marta Silvia Contigiani · Rodolfo Héctor Campos · Viviana Elizabeth Ré**

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**Abstract** The aim of this study was to analyze the prevalence of hepatitis C virus (HCV) genotypes in Córdoba province, Argentina, over a 12-year period and to study the changes at the molecular level. The HCV genotype was determined in 357 HCV-infected patients, and the phylogeny and demographic reconstruction for HCV-1 was assessed. A significant reduction in HCV-2 prevalence with respect to HCV-1 in Córdoba after 2003 was observed. These findings are consistent with the epidemiological changes observed in South America. Nevertheless, the consequences of these changes remain to be elucidated.

Approximately 200 million people worldwide are thought to be infected with hepatitis C virus (HCV) [1], and a significant number of them develop cirrhosis and progress to hepatocellular carcinoma [2]. HCV is caused at least by seven different viral genotypes, each divided into different subtypes [3]. The outcome of antiviral therapy is influenced by several factors, such as viral load at baseline, host factors, and also HCV genotype. The epidemiology of HCV shows some geographical features for the different genotypes and subtypes. For instance, subtypes 1a, 1b, 2a and 3a have a worldwide distribution, whereas subtype 4a shows an endemic distribution in Africa and Europe [4–7]. Some subtypes, such as 1b and 2c, are in a demographic plateau [4, 8], while 1a is in expansion and is associated with intravenous drug use (IDU) [8]. A recent review indicates that in Latin America, including Argentina, genotype 1 is dominant [5, 9]. However, the occurrence of HCV-2c has been reported in high prevalence in Córdoba province (the second most populated region of Argentina), especially in older adults [10, 11]. On the other hand, HCV-1 has been found consistently over time and in different age groups (30-39 years old) [12, 13]. Ré et al. have suggested that HCV-2c came into Córdoba province during the migration process, mainly from Europe, which is compatible with the history of Argentina in the early 20th century [9, 11]. The the process of HCV-1 diversification is more recent, having occurred approximately 60 years ago [14, 15]. Thus, with the development of the coalescent theory and molecular evolution it is possible to understand the characteristics of virus evolution and migration. Consequently, further understanding of the prevalence of HCV and the evolutionary characteristics of various genotypes is highly important for the prediction of the future incidence of these genotypes.

Therefore, the aim of this study was to assess the prevalence of HCV genotypes in Córdoba province over a 12-year period and to analyze the changes of the molecular epidemiological status in this region.

From January 1999 to January 2010, a total of 380 patients living in Córdoba City and other small cities of Córdoba province with positive serology for HCV were

F. A. Di Lello and A. A. Farias contributed equally to this work.

F. A. Di Lello · A. C. A. Culasso · P. S. Pérez · R. H. Campos
Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

F. A. Di Lello · A. C. A. Culasso · R. H. Campos · V. E. Ré
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

A. A. Farias (✉) · M. B. Pisano · M. S. Contigiani · V. E. Ré
Laboratorio de Hepatitis y Arbovirus, Instituto de Virología "Dr. J.M. Vanella", Facultad de Ciencias Médicas, Universidad Nacional de Córdoba (UNC), Enfermera Gordillo Gómez s/n, Ciudad Universitaria, CP 5016 Córdoba, Argentina
e-mail: adrianalefarias@hotmail.com; adrianalefarias@gmail.com

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seen at the Virology Service of the ''Instituto Dr. J. M. Vanella'' in Argentina. HCV genotype was determined using a RFLP analysis of the 5′UTR region in 357 (93.9 %) of these patients, who were included in this retrospective cross-sectional study.

The amplification of a portion of the HCV NS5B region was carried out as described elsewhere [16]. For the amplification of the E1/E2 region, the primers used for HCV-1a were ES_1a (5′ GGA TAT GAT GAT GAA CTG GTC-3′) and EA_1a (5′ ATG TAC AGC CGA ACC AGT TG 3′) as outer primers and IS_1a (5′ GGA CAT GAT CGC TGG TGC TCA 3′) and IS_1a (5′ TCC GCA CAC TTT GGT GAA TCC 3′) as inner primers. The primers used for HCV-1b were ES_1b (5′ GGA TAT GAT GAT GAA CTG GTC -3′) and EA_1b (5′ GTG TAA GTG GCC TCR GGR TG 3′) as outer primers and IS_1b (5′ TGG GCT AAG GTY TTG RTT GT 3′) and IS_1b (5′ GGG CAG ATC AAG GTG TTR TT 3′) as inner primers. Subsequently, the amplified DNAs were sequenced in both senses by Macrogen, Inc. (Seoul, Korea).

NS5B sequences were aligned using CLUSTALX v2.0 software [17]. A maximum-likelihood (ML) phylogenetic tree was constructed using MEGA 6 [18]. For phylogenetic analysis, 50 additional sequences were downloaded from the GenBank database for each subtype. Evolutionary models were inferred according to the Akaike information criterion statistics obtained with jModeltest v 2.1.3 [19].

The history of infections with HCV-1a and HCV-1b were reconstructed using Bayesian Skyline Plots (BSPs) [20], implemented in the BEAST 1.7.1 software package [21]. The BSPs were run under two molecular clock models: Strict and Relaxed Uncorrelated Lognormal. The runs were compared using Bayes Factors (BF). All BEAST run logs were analyzed with TRACER version 1.5 (available from http://beast.bio.ed.ac.uk/Tracer). The achievement of convergence was evaluated by checking that the effective sample size (ESS) was 200 or higher for all the parameters.

To estimate the distribution of the prior used in the actual analyses, calibration analyses were run using an external dataset. Then, the parameters of the normal, log-normal, gamma and Laplace distribution were estimated by ML. These distributions were plotted to graphically select the one that best represented the calibration run. Finally, the distribution selected and the parameters estimated were loaded in the prior section using the BEAUti tool provided by the BEAST software.

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

Nucleotide sequences for HCV have been deposited in the GenBank databse under accession numbers KJ467055–KJ467072 for E2 and KM921683–KM921739 for NS5B.

Written informed consent to participate in this study was obtained from all patients. The study was designed and performed according to the Helsinki declaration and was inscribed and approved by the ethics committee of the Health Ministry of the Province of Córdoba, Argentina (RepisN°1503).

A total of 357 out of 380 (93.9 %) subjects were successfully included in this study. The main baseline features of the patients are shown in Table 1.

During the period of this study, the prevalence of HCV-1, 2 and 3 were 55.5 %, 37.5 % and 7 %, respectively. The frequency of HCV-1 was significantly increased from 44.8 % to 61.8 %, the frequency of HCV-2 decreased from 51.2 % to 29.3 %, and the frequency of HCV-3 increased from 4 % to 8.9 % ($p < 0.001$) when the patients analyzed during 1999-2003 were compared with those from the period of 2004-2010. Additionally, when the rate of increase was compared between the subtypes with increased frequency during the analyzed period, HCV-1 showed a faster increase than HCV-3 ($p < 0.001$).

The main characteristics of the patients according to HCV genotype are shown in Table 2. Regarding differences between patients harboring the most prevalent genotypes, HCV-1 and 2, patients with HCV-2 were significantly older than patients with HCV-1 (53 [46-59] and 47 [38-57], respectively [$p = 0.001$]). Additionally, HCV-2 was less frequently found in men than HCV-1 (29 % versus 61.5 %, respectively [$p = 0.003$]). There was also significant association between the route of transmission and genotype HCV-1 or 2 ($p = 0.002$). However, in the distribution of HCV-1 or HCV-2 infection, the difference between places of residence was not statistically significant ($p = 0.122$). Furthermore, there was an association between HCV genotype (1 or 2) and the presence of HIV co-infection. We found that 32 out of 198 (16.2 %) patients infected with HCV-1 were coinfected with HIV, whereas 3 out of 134 (2.2 %) patients infected with HCV-2 were coinfected with HIV ($p < 0.001$). Concerning HCV-1 subtype, 83 (41.9 %) samples were randomly subtyped. Twenty-seven (32.5 %) HCV-1a and 56 (67.5 %) HCV-1b samples were analyzed, and no significant association with the route of transmission (p=0.525), the gender (p=0.157) or HIV coinfection (p=0.191) was found.

A portion of the partial NS5B gene (position 8,316-8,581 according to HCV-1a-H77 AF009606 [265 nt]) from 59 randomly selected patients infected with HCV-1 was sequenced. These sequences included those from 21 patients infected with HCV-1a and 38 with HCV-1b. The best-fit model of DNA evolution for the analyzed data was GTR + Γ + I for both HCV-1 subtypes. Phylogenetic

**Table 1** Epidemiological characteristics of the study population (N = 357)

| Characteristics | Population, N = 357 | % |
|---|---|---|
| *Age (Median years)*[a] | 50 (40-58) | |
| *Sex* | | |
| Male | 169 | 47.3 |
| Female | 188 | 52.7 |
| *Genotype* | | |
| 1 | 198 | 55.5 |
| 2 | 134 | 37.5 |
| 3 | 25 | 7 |
| *Sampling year*[b] | | |
| 1999 | 14 | 4 |
| 2000 | 16 | 4.6 |
| 2001 | 21 | 6 |
| 2002 | 16 | 4.6 |
| 2003 | 58 | 16.6 |
| 2004 | 63 | 18 |
| 2005 | 38 | 10.9 |
| 2006 | 22 | 6.3 |
| 2007 | 61 | 17.4 |
| 2008 | 21 | 6 |
| 2009 | 16 | 4.6 |
| 2010 | 4 | 1.1 |
| *City* | | |
| Córdoba | 185 | 51.8 |
| Villa María and Cruz del Eje | 51 | 14.3 |
| Other Locations of Córdoba | 64 | 17.9 |
| Unknown | 57 | 16 |
| *HIV* | | |
| *Yes* | 42 | 11.8 |
| *No* | 315 | 88.2 |
| *Transmission route* | | |
| Transfusion | 62 | 12.7 |
| Surgery | 43 | 18.3 |
| Sexual | 19 | 13.6 |
| IDU | 46 | 5.6 |
| Unknown | 187 | 52.4 |

[a] Available for 336 patients

[b] Available for 350 patients

analysis for HCV-1a (Fig. 1a) clearly distinguished three small groups of sequences from Córdoba province (clusters 1, 2 and 3), each forming a well-defined cluster. A similar analysis for HCV-1b showed a single cluster including 35 out of 36 sequences from Córdoba province (Fig. 1b).

A Bayesian analysis of partial E2 gene sequences (position 1,479-2,036 according to HCV-1a-H77 AF009606 [557 nt]) of HCV-1 was performed in 18 patients selected randomly from the above population. Then, nine patients infected with HCV-1a and nine patients infected with HCV-1b were included in this analysis.

The substitutions rate priors were set as normal distributions with a mean (and standard deviation) of $3.41 \times 10^{-3}$ ($4.53 \times 10^{-4}$) s/s/y for HCV-1a and $3.29 \times 10^{-3}$ ($6.36 \times 10^{-4}$) s/s/y for HCV-1b. Those rates were estimated previously [15] for the same set of samples by the analysis of external calibration datasets. In these samples, the BF analysis favored the relaxed uncorrelated log-normal molecular clock over the other models for the partial E2 sequence analyzed herein. Chain lengths of 50 million generations for HCV genotypes 1a and 1b from Córdoba province were needed to reach values of ESS above 200. The date of the most recent common ancestor (MRCA) (HDP95 %) was estimated to be around 1972 (1947-1982) for HCV-1a, and 1941 (1897-1972) for HCV1b.

The current study has shown a significant reduction in HCV-2 and the concomitant increase of HCV-1 prevalence in Córdoba province, Argentina, after 2003. Thus, HCV-2 was predominant in patients attending the hospital between 1999 and 2003, whereas HCV-1 was predominant in the patients attending from 2004 to 2010. The finding of a more recent MRCA (1941-1972) for HCV-1 compared to the previous published estimation for HCV-2 in Córdoba province (around 1875) [11] would support these results.

The results of this study indicate a significant increase in the prevalence of HCV-1 since 2004 in Córdoba province. This kind of shift has been observed worldwide and represents the changing epidemiology of HCV throughout the world [4, 22, 23]. The prevalence of HCV genotypes around the world has changed during the last 20 years due to a combination of factors. The most important are probably the eradication of transfusion-associated infections, improvement in health-care-related standards, continuous expansion of IDU, and the population movement that increases some genotypes through the immigration from endemic areas. Particularly, it has been proposed that the initial spread of HCV in Argentina started during the last century through the use of unsafe parenteral injections, surgical procedures and transfusion of blood products [9]. For instance, several studies carried out in small towns in Argentina and Europe showed a high rate of HCV-1b among the elderly as a result of distant iatrogenic transmission in a rural area where intravenous drug use and sexual promiscuity were absent [24–26].

It is also known that age of patients is an important component in the preferential spread of each genotype of HCV. In the studied population, patients infected with HCV-2 were older than patients infected with HCV-1. Consequently, the age of the patients analyzed was strongly related to the genotype distribution. These findings are consistent with those of Ansaldi et al., who observed and

**Table 2** Characteristics of the patients with HCV infection according to genotype (N=357)

| Characteristics | Genotype 1 N=198 | Genotype 2 N=134 | Genotype 3 N=25 | *p*-value |
|---|---|---|---|---|
| *Age, no. (%)*[a] | | | | |
| 18-29 | 13 (65) | 7 (35) | 2 (8) | |
| 30-39 | 42 (84) | 8 (16) | 6 (24) | |
| 40-49 | 44 (65.7) | 23 (34.3) | 11 (44) | |
| 50-59 | 50 (45.9) | 59 (54.1) | 4 (16) | |
| 60-69 | 32 (60.4) | 21 (39.6) | 2 (8) | |
| 70-80 | 4 (33.3) | 8 (66.7) | 0 (0) | 0.001 |
| *Sex, no. (%)* | | | | |
| Male | 104 (52.5) | 49 (36.6) | 16 (64) | |
| Female | 94 (47.5) | 85 (63.4) | 9 (36) | 0.004 |
| HIV | | | | |
| Yes *no.* (%) | 32 (16.2) | 3 (2.2) | 7 (28) | |
| No *no.* (%) | 166 (83.8) | 131 (97.8) | 18 (72) | <0.001 |
| *Sampling years, no. (%)*[b] | | | | |
| 1999-2003 | 56 (28.7) | 64 (51.1) | 5 (20) | |
| 2004-2010 | 139 (71.3) | 66 (49.2) | 20 (80) | <0.001 |
| *City, no. (%)* | | | | |
| Córdoba | 107 (54) | 63 (47) | 15 (60) | |
| Villa María and Cruz del Eje | 23 (11.6) | 28 (20.9) | 0 (0) | |
| Other city | 36 (18.2) | 20 (14.9) | 8 (32) | |
| Unknown | 32 (16.2) | 23 (17.2) | 2 (8) | 0.026 |
| *Transmission route, no. (%)* | | | | |
| Transfusion | 32 (16.2) | 21 (15.7) | 9 (36) | |
| Surgery | 22 (11.1) | 15 (11.2) | 6 (24) | |
| Sexual | 7 (3.5) | 12 (8.9) | 0 (0) | |
| IDU | 34 (17.2) | 5 (3.7) | 7 (28) | |
| Unknown | 103 (52) | 81 (60.4) | 3 (12) | <0.001 |

[a] Available for 336 patients

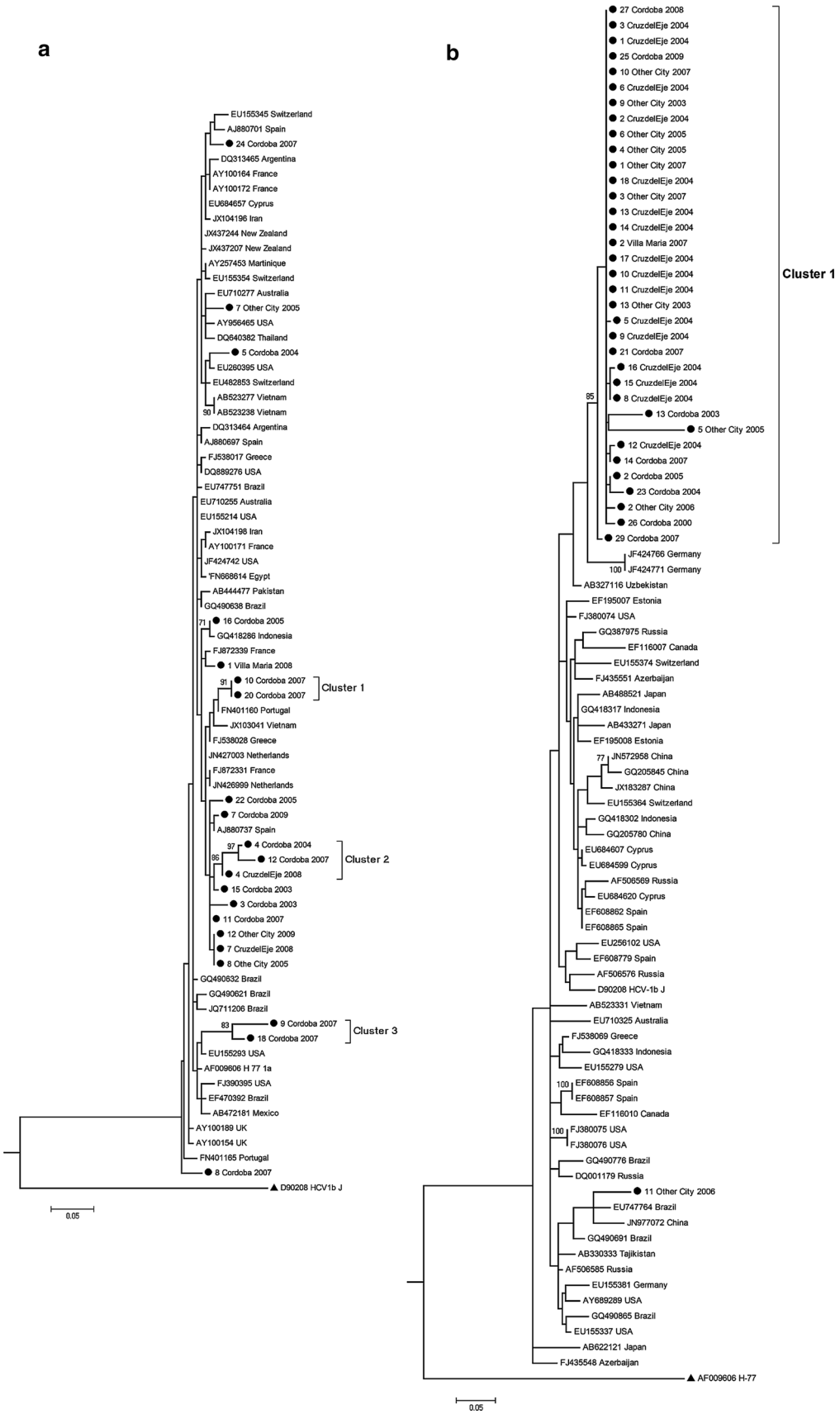
[b] Available for 350 patients

association between HCV-2 infection and advanced age [27]. Furthermore, the preferential spread of each genotype of HCV is also related to multimodal transmission routes [8, 28]. Here, the transmission route was significantly associated with HCV genotype distribution (p=0.002). Generally, the frequency of IDU was higher in patients infected with HCV-1. However, these data must be interpreted with caution due to the large proportion of patients (55.4 %) for whom the route of transmission was unknown. On the other hand, the genotype distribution was not statistically different with respect to location (p=0.122), but it was statistically associated with gender (HCV-2, 36.6 % in males versus 63.4 % in females [$p = 0.003$]). This result may be explained by the high prevalence of HCV-2 genotype in women in some towns of Córdoba province, such as Cruz del Eje, a finding that was reported previously by our group [10, 11].

In this study, the phylogenies of HCV-1a and HCV-1b, which represent, at the present, the most prevalent infecting genotypes in the region, were also characterized. Sequences from Córdoba and the most closely related ones in GenBank showed a higher diversity of HCV-1a with an intermingling of most of the samples and the formation of three small clusters, revealing the polyphyletic origin of this subtype. In contrast, one single cluster including all samples but one was observed for HCV-1b, which indicates a minor diversification of this subtype with respect to HCV-1a in Córdoba.

The change in HCV genotypes over time was corroborated by Bayesian analysis, and it showed differences between the two genotypes. The HCV-1a and HCV-1b population history showed a two-stage process. The first stage started with the MRCA around 1970 for HCV-1a and

**Fig. 1** Phylogenetic tree of HCV-1 sequences from Córdoba province (NS5B-265nt). Fifty sequences from the rest of the world were included in each tree. The numbers at each node correspond to bootstrap values from a ML tree obtained with 100 replicates (bootstrap values lower than 70 are not shown). Filled circles indicate samples from Córdoba province. a) Twenty-one HCV-1a sequences, where AF009606 H-77 was added as prototype and D90208 HCV-J 1b (HCV-1b) as an out-group. b) Thirty-six HCV-1b sequences from Córdoba, where D90208 HCV-J 1b was added as the prototype and AF009606 H-77 (HCV-1a) as an out-group


a
EU155345 Switzerland
AJ880701 Spain
24 Cordoba 2007
DQ313465 Argentina
AY100164 France
AY100172 France
EU684657 Cyprus
JX104196 Iran
JX437244 New Zealand
JX437207 New Zealand
AY257453 Martinique
EU155354 Switzerland
EU710277 Australia
7 Other City 2005
AY956465 USA
DQ640382 Thailand
5 Cordoba 2004
EU260395 USA
EU482853 Switzerland
AB523277 Vietnam
AB523238 Vietnam
DQ313464 Argentina
AJ880697 Spain
FJ538017 Greece
DQ889276 USA
EU747751 Brazil
EU710255 Australia
EU155214 USA
JX104198 Iran
AY100171 France
JF424742 USA
FN668614 Egypt
AB444477 Pakistan
GQ490638 Brazil
16 Cordoba 2005
GQ418286 Indonesia
FJ872339 France
1 Villa Maria 2008
10 Cordoba 2007
20 Cordoba 2007
Cluster 1
FN401160 Portugal
JX103041 Vietnam
FJ538028 Greece
JN427003 Netherlands
FJ872331 France
JN426999 Netherlands
22 Cordoba 2005
7 Cordoba 2009
AJ880737 Spain
4 Cordoba 2004
12 Cordoba 2007
4 CruzdelEje 2008
Cluster 2
15 Cordoba 2003
3 Cordoba 2003
11 Cordoba 2007
12 Other City 2009
7 CruzdelEje 2008
8 Othe City 2005
GQ490632 Brazil
GQ490621 Brazil
JQ711206 Brazil
9 Cordoba 2007
18 Cordoba 2007
Cluster 3
EU155293 USA
AF009606 H 77 1a
FJ390395 USA
EF470392 Brazil
AB472181 Mexico
AY100189 UK
AY100154 UK
FN401165 Portugal
8 Cordoba 2007
D90208 HCV1b J
0.05
b
27 Cordoba 2008
3 CruzdelEje 2004
1 CruzdelEje 2004
25 Cordoba 2009
10 Other City 2007
6 CruzdelEje 2004
9 Other City 2003
2 CruzdelEje 2004
6 Other City 2005
4 Other City 2005
1 Other City 2007
18 CruzdelEje 2004
3 Other City 2007
13 CruzdelEje 2004
14 CruzdelEje 2004
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13 Other City 2003
5 CruzdelEje 2004
9 CruzdelEje 2004
21 Cordoba 2007
16 CruzdelEje 2004
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13 Cordoba 2003
5 Other City 2005
12 CruzdelEje 2004
14 Cordoba 2007
2 Cordoba 2005
23 Cordoba 2004
2 Other City 2006
26 Cordoba 2000
29 Cordoba 2007
Cluster 1
JF424766 Germany
JF424771 Germany
AB327116 Uzbekistan
EF195007 Estonia
FJ380074 USA
GQ387975 Russia
EF116007 Canada
EU155374 Switzerland
FJ435551 Azerbaijan
AB488521 Japan
GQ418317 Indonesia
AB433271 Japan
EF195008 Estonia
JN572958 China
GQ205845 China
JX183287 China
EU155364 Switzerland
GQ418302 Indonesia
GQ205780 China
EU684607 Cyprus
EU684599 Cyprus
AF506569 Russia
EU684620 Cyprus
EF608862 Spain
EF608865 Spain
EU256102 USA
EF608779 Spain
AF506576 Russia
D90208 HCV-1b J
AB523331 Vietnam
EU710325 Australia
FJ538069 Greece
GQ418333 Indonesia
EU155279 USA
EF608856 Spain
EF608857 Spain
EF116010 Canada
FJ380075 USA
FJ380076 USA
GQ490776 Brazil
DQ001179 Russia
11 Other City 2006
EU747764 Brazil
JN977072 China
GQ490691 Brazil
AB330333 Tajikistan
AF506585 Russia
EU155381 Germany
AY689289 USA
GQ490865 Brazil
EU155337 USA
AB622121 Japan
FJ435548 Azerbaijan
AF009606 H-77
0.05

1940 for HCV-1b and showed a gradual increase in the population size up to 2000. The second stage showed a plateau in the population size from 2000 up to 2010. The present estimate of the time of the MRCA seems to be consistent with the age of the patients infected with HCV-1a and HCV-1b in our population, i.e., patients harboring HCV-1a were younger than patients infected with HCV-1b (38 [31-41] versus 55 [43-63], respectively $p < 0.001$). Thus, it might be speculated that HCV-1b has been gradually replaced by HCV-1a during the past decade in Córdoba. All of these values corroborate the findings reported in much of the previous work in this field for other regions of Argentina, which also showed a MRCA around 1930-1980 [14, 15, 29]. Additionally, a comparison of the time of the MRCA for HCV-1 calculated here and that for HCV-2 in Córdoba previously reported by Re et al, revealed a more recent MRCA for HCV-1 [11].

The small number of sequences analyzed in the present work could be considered a limitation. However, the phylogenetic analysis showed a strong difference between the two subtypes, and it would be unlikely that the inclusion of more samples would change the observed pattern. Moreover, the sample size was large enough for Bayesian analysis to make a good molecular characterization of distribution of HCV-1 subtypes and the estimation of MRCA in this area.

In conclusion, these findings enhance our understanding about the changing epidemiology of HCV in Córdoba province, Argentina. Additionally, the differences in the diversity of HCV-1a (polyphyletic) and HCV-1b (monophyletic) suggest differences in the characteristics of patients infected with these subtypes that should be addressed. Finally, the epidemiological changes observed in this study are consistent with those reported worldwide. Although the consequences of these changes remain to be elucidated, the HCV genotype is an important determinant of the therapy response and pathogenesis, and therefore, active epidemiological surveillance is recommended.

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**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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