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Polymorphisms associated with resistance to protease inhibitors in *naïve* patients infected with hepatitis C virus genotype 1 in Argentina: Low prevalence of Q80K

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ABSTRACT

Incorporation of direct acting antivirals (DAA) in the treatment of Hepatitis C Virus (HCV) significantly increases sustained virologic response rates. However, despite the greater potency offered by these antivirals, drug resistance plays a key role in patients with failure to DAA. Nevertheless, there is no information about the prevalence of resistance-associated substitutions (RASs) in Argentina.

The aim of this study was to analyze HCV variants resistant to protease inhibitors (PI) in *naïve* patients infected with HCV genotype 1 from Argentina.

In this retrospective cross-sectional study, 103 patients infected with HCV-1 were included. Eighteen positions related with RASs were analyzed by Sanger at baseline and phylogenetic analysis was performed to determine the diversification of this samples.

The analyzed RASs were present in 38 out of 103 patients (36.9%) infected with HCV-1. Patients infected with subtype HCV-1b had higher prevalence of baseline RASs than patients infected with HCV-1a [51.6% vs. 12.8%, respectively ($p < 0.001$)]. The Q80K polymorphism was not found in HCV-1a samples, even when 51% of them belonged to cluster 1, which is associated with a high frequency of Q80K. Phylogenetic analysis showed that Argentinean samples were intermingled with sequences from other geographic regions.

RASs to PI were highly prevalent and subtype dependent in treatment-*naïve* Argentinean patients. Surprisingly, Q80K polymorphism was not detected in our study population. The phylogenetic analysis showed no relationship between our samples and other samples from Brazil which also present a low prevalence of Q80K. This study supports the need for surveillance of resistance in patients who will be treated with DAA in each particular country since the observed RASs have very different prevalence worldwide.

1. Introduction

The development of direct-acting antivirals (DAA) significantly increased sustained virologic response (SVR) rates in the treatment of chronic hepatitis C virus (HCV), while lowering the rates of adverse events (Bacon et al., 2011; Zeuzem et al., 2011; Forns et al., 2014; Jacobson et al., 2014; Lawitz et al., 2014; Forns et al., 2015; Krishnan et al., 2015). However, despite the significantly greater efficacy and tolerability offered by these antivirals, patients are still failing to reach virologic curation. In this context, drug resistance plays a key role in patients with failure to DAA-containing therapies (Zeuzem et al., 2011; Forns et al., 2014; Jacobson et al., 2014; Lawitz et al., 2014; Poveda et al., 2014; Forns et al., 2015; Krishnan et al., 2015; Sarrazin, 2016). Since selection of resistant variants did not represent an important issue in the era of therapy based on pegylated interferon plus ribavirin alone, the use of DAA is leading to a paradigm shift in the management of HCV treatment.

HCV NS3 protease was the first target used for therapeutic

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intervention, however it shows a high degree of genetic variability (Poveda et al., 2014; Sarrazin, 2016). Several resistance-associated substitutions (RASs) to NS3 protease inhibitors (PIs) have been described with generally low frequency in HCV genotype 1-infected patients (Poveda et al., 2014). Still, some polymorphisms, such as Q80K, which is highly prevalent and confers no substantial loss of replicative fitness in many patients, seem to be an exception for the treatment with simeprevir (SMV) and asunaprevir (ASV) (Bae et al., 2010; Sarrazin et al., 2015).

In particular, SMV was recently approved for its use in Argentina in patients with chronic HCV infection. It has been widely reported that the antiviral efficacy of SMV is adversely affected by the RASs, especially by Q80K polymorphism in patients infected with HCV genotype 1a (HCV-1a) (Bae et al., 2010; Jacobson et al., 2014; Poveda et al., 2014; Sarrazin et al., 2015; Sarrazin, 2016). Nevertheless, the prevalence of Q80K is diverse and varies by geographic region. In this sense, Q80K variant is a pre-existing RAS frequently found in HCV-1a-infected patients from North America (48.1%) and Europe (19.4%) but it was rarely detected in Brazil and Uruguay (1.8%) (Bae et al., 2010; Peres-da-Silva et al., 2012; de Carvalho et al., 2014; Lisboa-Neto et al., 2015; Sarrazin et al., 2015; Echeverría et al., 2016). However, there is no information on the prevalence of Q80K and other RASs in Argentina. For this reason, there is a big controversy about the necessity of testing the RASs in patients infected with HCV-1a in our country before the initiation of therapy including PIs.

On the other hand, Pickett et al. demonstrated that HCV-1a could be separated into two distinct clades, named 1 and 2 (Pickett et al., 2011). Additionally, several works associated the presence of Q80K polymorphism to clade 1 (Peres-da-Silva et al., 2012; Ehret et al., 2014; Nguyen et al., 2015). For this reason, the phylogenetic analysis of the Argentinean samples could provide important information about the clade prevalence and its relationship with sequences from other countries.

Therefore, the aim of this study was to determine the prevalence of PI resistances, in particular the Q80K polymorphism, in an Argentinean cohort of treatment-*naïve* patients with chronic HCV-1 infection.

2. Material and methods

2.1. Study population

This was a retrospective cross-sectional study that included HCV-monoinfected patients seen between January 2012 and June 2016 at the Centro de Educación Médica e Investigaciones Clínicas Norberto Quirno "CEMIC" in Argentina who fulfilled the following criteria: (1) Infected with HCV-1a or genotype 1b (HCV-1b); (2) older than 18 years; (3) *naïve* for therapy against HCV infection including any DAA.

2.2. HCV genotype/subtype determination

HCV genotype was determined by the Auto-LiPA 48 Instrument and Autoblot 3000H tests (Siemens). All procedures were carried out according to the manufacturer's instructions. Since a number of studies have detected an important proportion of discrepancies in HCV-1 subtype determination by LiPA when compared to genomic sequencing methods (Avó et al., 2013; Guelfo et al., 2014; Neukam et al., 2017), the subtype was determined by the phylogenetic analysis of NS3 region.

2.3. RT-PCR and sequencing

RNA was extracted from basal serum samples, frozen at −80 °C, using the MagNA Pure system (Roche Diagnostics, Mannheim, Germany). Reverse transcription was carried out with MMLV-RT (Promega, Madison, WI, USA) with Random Hexamer Primers using the manufacturer's protocol. The heminested amplification of HCV NS3 partial region was carried out as described as follows. For the amplification of NS3 region, the primers used were: ES3426 (5′ ATC ACG GCS TAY KCC CAR CAG AC −3′) and EA4140 (5′ CCA TGK GCC TTR GAC ATR TA 3′) as outer primers and ES3426 (5′ ATC ACG GCS TAY KCC CAR CAG AC 3′) and IA4011 (5′ CAA GTG GCC CAT CTA CAC GC 3′) as inner primers. Subsequently, the amplified DNAs (585nt) were purified from agarose gels using a commercial kit (High Pure PCR Product Purification kit, ROCHE, Mannheim, Germany) and then sequenced in both senses by INTA, (Castelar, Argentina).

2.4. RASs analysis

In order to search for NS3 substitutions at baseline, amino acids at positions 8-180 within NS3 protease were examined. It should be noted that each RAS was clearly read in the sequencing plot as the highest peak. Eighteen positions related with resistance to treatment were selected to be analyzed in this work: V36A/G/I/L/M/, Q41R, F43C/L/S, T54A/C/G/S, V55A/I, Y56F/H, Q80K/L/R, V107I, R117C/H, S122A/G/R, S138T, R155X, A156F/G/L/S/T/V, V158I, D168A/E/F/G/H/I/K/L/T/V/Y, IV170A/F/T/V, S174A/F/G/L/N/S and M175L (Howe et al., 2014; Kalaghatgi et al., 2016).

2.5. Phylogenetic analysis

Because of the large number of sequences available for the NS3 region for both HCV-1a and HCV-1b, a reduced and manageable dataset for phylogenetic analysis was created. Along with the sequences obtained for this study, reference sequences have been included under the following criteria. First, a database search was performed using our sequences as query in the BLASTN program (https://blast.ncbi.nlm.nih.gov) and the three most similar sequences to each one in our dataset were retrieved (lower E number). Second, sequences from relevant studies carried out in two neighbor countries (Peres-da-Silva et al., 2012; de Carvalho et al., 2014; Echeverría et al., 2016) were included. Due to the large number of sequences from Brazil, fifteen sequences for HCV-1a and twenty for HCV-1b were randomly picked. Finally, in the case of HCV-1a, 10 additional reference sequence were included, five for each of the two clades defined by Pickett and colleagues (Pickett et al., 2011).

The resulting two datasets were aligned with MUSCLE V3.8.31 (Edgar, 2004) and trimmed out to our sequenced region using BioEdit V 7.2.5 (Hall, 1999). Then, they were subjected to a model selection procedure using jModelTest 2.1.9 (Darriba et al., 2012). Finally, the phylogenetic trees were obtained by maximum likelihood procedure with PhyML V 3.0.1 (Guindon and Gascuel, 2003) by setting the model parameters according to those suggested by Bayesian Information Criterion in the jModelTest analysis. For branch support, a non-parametric bootstrap analysis was carried out with 1000 pseudoreplics. Additionally, the SH-like support was also calculated.

The produced trees were visualized with FigTree V 1.4.0 (available at http://tree.bio.ed.ac.uk/software/figtree/).

2.6. Statistical analysis

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. The statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM SPSS Inc, Chicago, IL, USA).

2.7. Nucleotide sequences accession numbers

Nucleotide sequences for the HCV have been deposited in GenBank under accession numbers KY614603 – KY614705.

2.8. Ethical aspects

Written informed consents to participate in this study were obtained from the patients. The study protocol was approved by the ethics committee of the "Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires" (record number 732575/2010) in accordance with the 1975 Helsinki Declaration.

3. Results

3.1. Study population

A total of 103 HCV-monoinfected patients were included in this study. In subtype classifications determined by NS3 direct sequence, 39 out of 103 (37.9%) patients were infected with HCV-1a and 64 (62.1%) were infected with HCV-1b. Sixty two patients (60.2%) were male, the median [interquartile range (IQR)] age was 54 (46–62) years and the median (IQR) viral load was 5.94 (5.44-6.47) $\log_{10}$ IU/mL.

3.2. RASs analysis

The 18 analyzed RASs were present in 38 out of 103 patients (36.9%) infected with HCV-1. When other polymorphisms not reported as resistance associated, but in the same analyzed positions, namely S122T, IV170G and S174G/L, were considered, the number was 42 out of 103 (40.8%). The most common NS3 polymorphisms identified in patients at baseline were V36L/M (6.8%), Y56F (12.6%), R117C/H (5.8%), S122G/N (6.8%) and SN174A/F/T (6.8%). Patients infected with HCV-1b had a higher prevalence of baseline NS3 RASs than patients infected with HCV-1a [33/64 (51.6%) vs. 5/39 (12.8%), respectively ($p < 0.001$)]. Eleven patients (10.7%) infected with HCV-1b harbored two or three RASs simultaneously, while none in the HCV-1a subtype presented more than one RAS ($p = 0.004$).

Q80K polymorphism was not detected in patients infected with HCV-1a. However, variants at position 80 were detected in 2 out of 64 (3.1%) HCV-1b infected patients (Q80L/R). Frequencies of individual polymorphisms by subtype are shown in Table 1. Additionally, no significant differences were found between presence or absence of RASs and HCV viral load [5.93 (5.26–6.65) $\log_{10}$ IU/mL vs. 5.95 (5.46–6.39) $\log_{10}$ IU/mL $p = 0.942$].

3.3. Phylogenetic analysis

The phylogenetic tree reconstruction revealed that all Argentinean HCV-1a sequences obtained in this study were distributed in the two known clades in similar proportion [20 sequences were included in clade 1 (51.3%) and 19 in clade 2 (48.7)] (Fig. 1). None of the Argentinean samples obtained here from clade 1 had the Q80K polymorphism and were intermingled with samples from countries of Asia, Europe, North America and Latin America independently of the presence of Q80K. Most HCV-1a Argentinean samples of the clade 1 (16 out of 20) belonged to a sub-cluster with a bootstrap value of 85% and constituted by samples from Brazil (1), Uruguay (6) and those retrieved from BLAST analysis (8).

Additionally, S174N polymorphism was identified as significant phylogenetically-informative, contributing to clade differentiation since it was present in all samples belonging to clade 1 but none from clade 2. Alternatively for the clade 2 this position was wild type (S174) in 16 (84.2%) and S174G in 3 (15.8%).

The HCV-1b sequences did not constitute groups of related sequences and were distributed randomly in the phylogenetic tree among sequences from other geographic regions (Fig. 2).

4. Discussion

This manuscript represents, to our knowledge, the first study that estimates the prevalence of PI RASs in Argentina in a considerably high number of individuals. In this context, a high prevalence (36.9%) of known RASs was detected by Sanger sequencing in HCV-1 infected patients. However, the highest prevalence was observed for HCV-1b, with more than 50% of patients infected with HCV containing RASs, in contrast to less than 15% in those patients infected with HCV-1a. In addition, a high prevalence of double mutants (17.2%) was detected in HCV-1b but not in HCV-1a. Surprisingly, the Q80K polymorphism was not detected in patients carrying HCV-1a, even in those patients belonging to clade 1, and only two changes (Q80L and Q80R) were observed in patients infected with HCV-1b. Furthermore, the phylogenetic analysis showed that Argentinean samples of both subtypes were distributed randomly among sequences around the world without formation of clusters.

The findings of the current study showed a prevalence of over 30% of natural occurring NS3 resistance-associated variants, detected by Sanger. When compared to most previous studies from the region and the rest of the world, the prevalence observed in this work was higher (Paolucci et al., 2012; Peres-da-Silva et al., 2012; Vicenti et al., 2012; Hoffmann et al., 2013; de Carvalho et al., 2014; Applegate et al., 2015; Lisboa-Neto et al., 2015; Altunok et al., 2016; Echeverría et al., 2016). On the other hand, these results are in agreement with a receent study conducted by Chen et al. which showed a high prevalence of PI RASs in HCV-1. However, most of the HCV-1 PI RASs observed by Chen et al. were due to a high prevalence of Q80K (37.6% in HCV-1a), which was not present in our samples (Chen et al., 2016).

Contrary to expectations, this study found a higher prevalence of RASs in HCV-1b than in HCV-1a. Most previous studies have shown higher prevalences in HCV-1a (Paolucci et al., 2012; Vicenti et al., 2012; Margeridon-Thermet et al., 2014; Larrat et al., 2015; Chen et al., 2016). However, in most of these reports, the values were biased by the

Table 1
Prevalence of the most important NS3 RASs in the study population (n = 103).

NS3 RAS	Frequency of naturally ocurring RASs (%)		p
	HCV-1a (n:39)	HCV-1b (n:64)	
V36L	1 (2.6)	5 (7.8)	0.269
V36M	1 (2.6)	0	0.246
Q41R	0	0	–
F43C/L/S	0	0	–
T54S	1 (2.6)	0	0.198
V55A/I	0	0	–
Y56F	0	13 (20.3)	0.003
Q80K	0	0	–
Q80L	0	1 (1.6)	0.433
Q80R	0	1 (1.6)	0.537
V107I	0	0	–
R117C	0	3 (4.7)	0.170
R117H	0	3 (4.7)	0.144
S122G	0	6 (9.4)	0.048
S122N	1 (2.6)	0	0.198
S122T*	0	2 (3.1)	0.079
S138T	0	1 (1.6)	0.433
R155S	S:1 (2.6)	0	0.198
A156F/N/S/T/V	0	0	–
V158I	0	0	–
D168T	0	1 (1.6)	0.433
▲I/V170A	0	1 (1.6)	0.433
▲I/V170G	1 (2.6)	0	0.324
◆S/N174A	0	3 (4.7)	0.170
◆S/N174F*	0	4 (6.2)	0.111
◆S/N174G*	3 (7.7)	0	0.024
◆S/N174L*	0	1 (1.6)	0.620
◆S/N174T	0	4 (6.2)	< 0.001
†M175L	39 (100)	0	< 0.001

*Not reported effect on treatment. ▲I170 is the major amino acid in subtype 1a and V170 is the major amino acid in subtype 1b. ◆ S174 is the major amino acid in subtype 1a Clade 2 and Subtype 1b and N174 is the major amino acid in subtype 1a, clade 1. †The major amino acid in subtype 1a (L175) is the resistant variant (M175L) in subtype 1b.

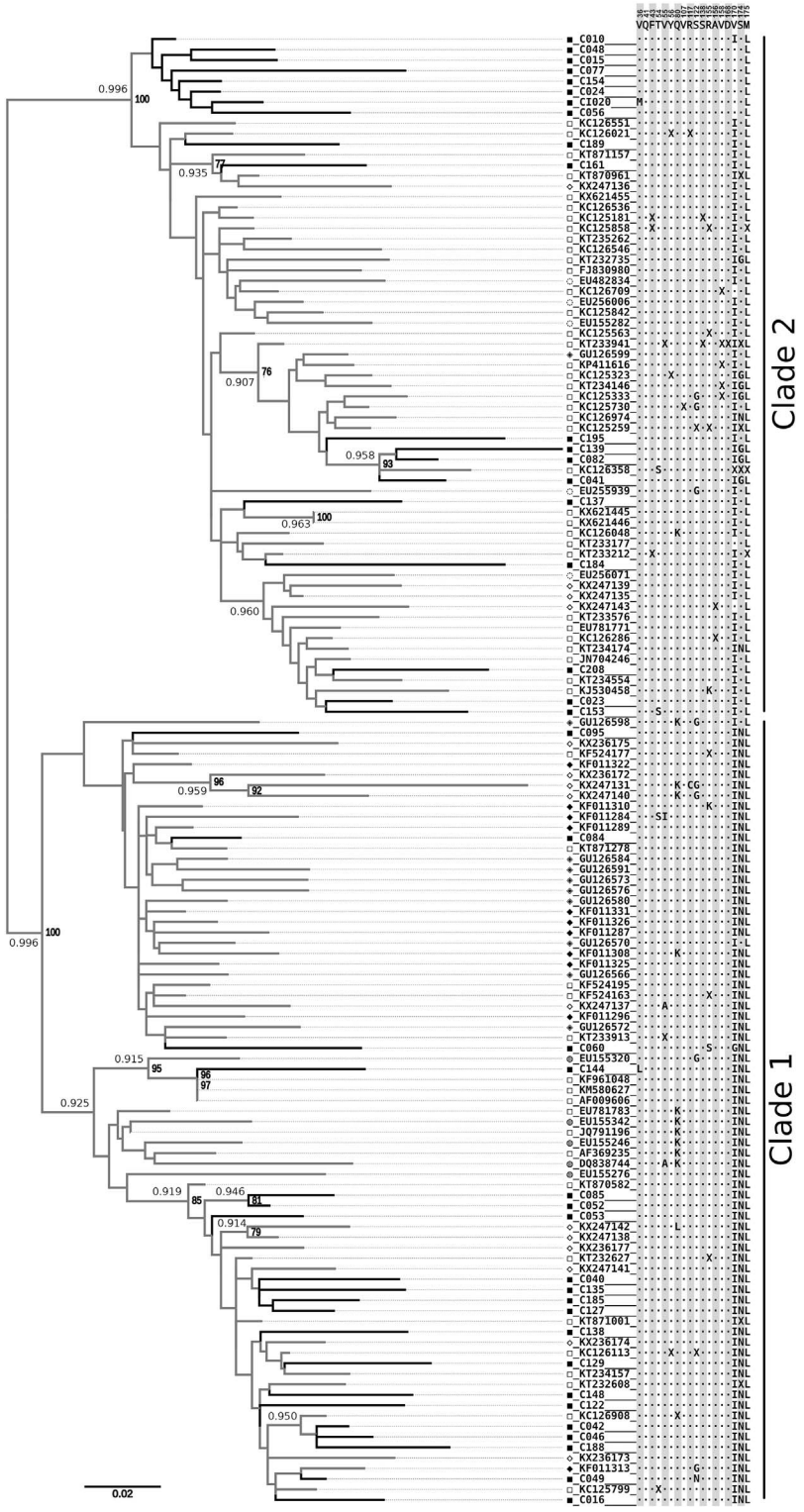

Fig. 1. HCV-1a maximum likelihood phylogenetic tree (GTR + Γ + I model for nucleotide substitutions). The branch lengths are proportional to the evolutionary distance between the samples. The numbers after each node represent the bootstrap support value as percentage. The symbols before the sequence name represent the source of the sample. Squares: solid for sequence from Argentina and hollow for those retrieved from BLAST analysis. Circle: Clade reference, stripped for clade 1 and hollow for clade 2. Diamonds: Sequence from relevant bibliography from bordering countries, hollow for Echeverria et al. (Uruguay); solid for Carvalho et al. (Brazil) and double for Peres da Silva et al. (Brazil). Bootstrap values lower than 75 and aLTR values lower than 0.9 are not shown. The amino acids at relevant positions are indicated after the sequence name in one letter amino acid code (X for undetermined codon). The top sequence represents the wild type amino acid at each position. A dot in the alignment represent that this amino acid match the most common one, otherwise the one letter code for the mismatched amino acid is represented.

high presence of Q80K in HCV-1a. A possible explanation for the observed difference in the RAŚs prevalence is the absence of Q80K in the Argentinean samples analyzed in this study. Moreover, the present findings seem to be consistent with other researches which also found a low prevalence of RASs in HCV-1a in Brazil (Peres-da-Silva et al., 2012; de Carvalho et al., 2014). Additionally, in this work, double mutants were only observed in HCV-1b and at a high frequency. It is known that the combination of specific RASs in the same viral genome resulted in an increase in the fold change of EC_{50} values and/or replication capacity respect to the single mutants (Verbinnen et al., 2010; McPhee et al., 2012; Sullivan et al., 2013). These findings were unexpected and suggest that, for the Argentinean samples, HCV-1b infection may have a worse treatment prognosis than the expected for other geographical regions. Moreover, the use of SMV would be recommended for patients with HCV-1a infection since Q80K is absent. In this context, since the presence of RAS is subtype dependent, and taking into account the influence of HCV-1 subtype in DAA treatment response, HCV subtyping must be determined by genomic sequencing methods.

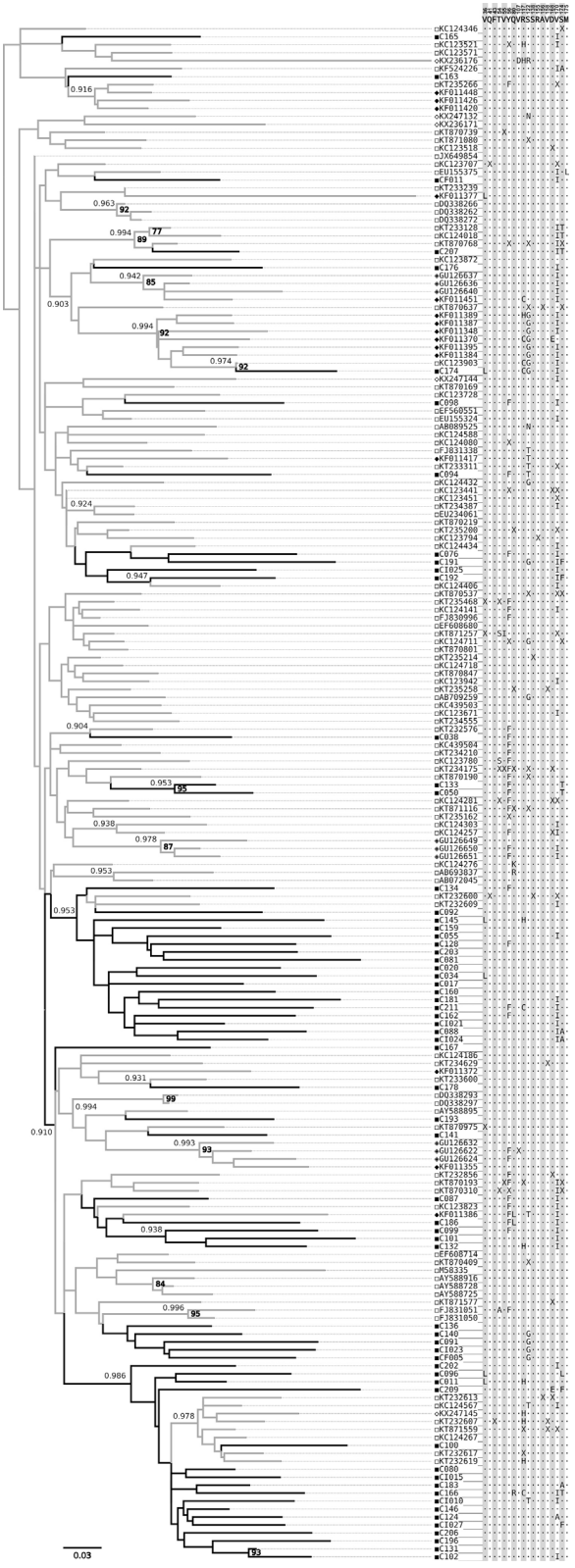

Fig. 2. HCV-1b maximum likelihood phylogenetic tree (TPM2 + Γ + I model for nucleotide substitutions). The branch lengths are proportional to the evolutionary distance between the samples. The numbers after each node represent the bootstrap support value as percentage. The symbols before the sequence name represent the source of the sample. Squares: solid for sequence from Argentina and hollow for those retrieved from BLAST analysis. Diamonds: Sequence from relevant bibliography from bordering countries, hollow for Echeverria et al. (Uruguay); solid for Carvalho et al. (Brazil) and double for Peres da Silva et al. (Brazil). Bootstrap values lower than 75 and aLTR values lower than 0.9 are not shown. The amino acid at relevant positions are indicated after the sequence name in one letter amino acid code (X for undetermined codon). The top sequence represents the wild type amino acid at each position. A dot in the alignment represent that this amino acid match the most common one, otherwise the one letter code for the mismatched amino acid is represented.

Due to high prevalence of Q80K around the world, routine Q80K screening in HCV-1a was recommended as part of the FDA's adoption of SMV (Alves et al., 2013; Lin and Chung, 2014; Morel et al., 2014; Beloukas et al., 2015; Nguyen et al., 2015; Sarrazin et al., 2015; Shepherd et al., 2015; Jimenez-Sousa et al., 2016; Kliemann et al., 2016; Gozlan et al., 2017; Newsum et al., 2017).Taking this into consideration, one of the most significant findings emerging from this study is the low prevalence of Q80K polymorfism in HCV-1a infected patients from Argentina.

Previously, only a few studies have reported the prevalence of Q80K in the region but none in Argentina. (Peres-da-Silva et al., 2012; de Carvalho et al., 2014; Lisboa-Neto et al., 2015; Echeverría et al., 2016). In this context, the studies from Brazil had also observed low prevalences of this polymorphism in HCV-1a infected patients (Peres-da-Silva et al., 2012; de Carvalho et al., 2014; Lisboa-Neto et al., 2015). These findings along with our results suggest that the prevalence of Q80K (around 9%) previously reported for this region (Sarrazin, 2016) should be reconsidered and further studies should be carried out. Moreover, RAS Q80L and Q80R were detected in two patients infected with HCV-1b. Minimal or low levels of resistance to ASV and SMV were observed with Q80L or Q80R substitutions (McPhee et al., 2012; Lenz et al., 2015) and the detected low prevalence is in agreement with other studies in western patients (Krishnan et al., 2016).

Regarding the prevalence of Q80K in HCV-1a clades, an Irish study reported a high prevalence of Q80K (40.2%) in clade 1 vs. 0% in clade 2 (Nguyen et al., 2015). Similarly, other studies in Germany and Italy also showed high prevalence of Q80K in clade 1 (Ehret et al., 2014; De Luca et al., 2015). Conversely, in concordance with our results, one Brazilian study has reported low prevalence of Q80K in HCV-1a, clade 1 (Peres-da-Silva et al., 2012).

However, the Brazilian, Uruguayan and Argentinean samples do not seem to be phylogenetically related, despite the geographic proximity. In addition, the Argentinean samples did not constitute any group, as observed by Peres-da Silva et al. for samples belonging to HCV-1a clade 1 without Q80K in Brazil (Peres-da-Silva et al., 2012).

Recently, McCloskey et al. have suggested that S174N substitution may interact with Q80K but it is likely that, as the author noted, this result is influenced by sampling bias, as the vast majority of the sequences were from United States where Q80K has a high prevalence (McCloskey et al., 2015). In contrast to this finding, the present work has identified S174N as a significant phylogenetically-informative polymorphism contributing to HCV-1a clade 1 differentiation but not related to Q80K. It is important to note that, to our knowledge, S174N substitution was not associated with PI resistance per se.

Another interesting result from this work is that there were no significant differences between HCV viral load and the presence of RASs in NS3. The present findings seem to be consistent with other research which found no obvious effect of baseline RASs on viral load (Larrat et al., 2015; Beloukas et al., 2015). These results suggest that the presence of RASs in NS3 was not enough to affect their ability to replicate and, probably, they transmit *in vivo*.

Finally, a number of limitations need to be considered. First, the sequence information to detect NS3 RASs was not determined by next-generation sequencing. The sequencing technology used was the Sanger method, so the presence of minor variants at frequencies $< 15–20\%$ cannot be excluded. However, some works have reported that not only the presence of RASs but also the RAS dominancy ($> 15–25\%$) in the quasispecies infecting a patient has an impact on treatment outcomes (Kinugasa et al., 2016; Sarrazin et al., 2016; Ikeda et al., 2017). Second,

this study has the limitation that the number of analyzed sequences was relatively low and the samples belong only to patients from Buenos Aires (Capital city of Argentina) and the metropolitan area. Nevertheless, there is a large number of studies conducted on a similar sample size (Paolucci et al., 2012; Vicenti et al., 2012; Hoffmann et al., 2013; Altunok et al., 2016; Echeverría et al., 2016). Due to the small analyzed sample size, caution must be applied, as the findings might not be transferable to the whole country.

5. Conclusion

In conclusion, results described in this paper provide the first information on a genetic profile of PIs RASs from Argentina. The analysis of natural HCV polymorphisms associated with resistance to DAAs indicates that natural RASs are relatively common in HCV isolates from treatment- *naïve* patients from Argentina and that Q80K appears to be rare-to-absent in our country. This research serves as a basis for future studies that include other viral regions such as NS5A and NS5B in order to generate a global map of RASs, since each country would seem to present a particular situation. Finally, this data show that it is crucial to define the resistance testing in order to optimized the management of HCV-infected patients in Argentina

Competing interest

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

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