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Research paper

Molecular epidemiology of hepatitis B virus in Paraguay

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ABSTRACT

Hepatitis B virus (HBV) infection is a leading cause of severe chronic liver disease worldwide. The HBV epidemiology in Latin American countries is complex and the data is still scanty and fragmentary. The aim of this study was to investigate the distribution of HBV genotypes in Paraguay and to estimate the viral population dynamic and spread pattern of the main phylogenetic group. To this end, partial and complete genome sequences were obtained from 60 blood donor candidates and analysed by phylogenetic and Bayesian phylodynamic approaches.

The phylogenetic analysis based on sequences of partial Polymerase/Pre-S1 overlapping region showed a predominance of the Native American subgenotype F4 (81.7%), the presence of the European subgenotypes A2 (1.7%) and D3 (8.3%), the African subgenotype A1 (3, 5%) and the Asian subgenotypes B2 (1.7%) and C2 (1.7%). The distribution of HBV genotypes was in accordance with the ethnic composition of the population.

The phylogeographic analysis of subgenotype F4 complete genomes suggests that this lineage emerged and spread in the last 300 years. Paraguay was the most probable location of the common ancestor. The lineage diverged into two main clades and spread to neighbor regions, mainly Bolivia and Northwest Argentina, and Buenos Aires. The phylogeny showed a scanty geographical structure and a complex migratory pattern.

In conclusion, the HBV genotypes circulating in Paraguay reflect the ethnic origin of the population. The distribution of genotypes and the phylogeographic reconstruction showed the impact of both global and local migrations in shaping the HBV molecular epidemiology in the region.

1. Introduction

Hepatitis B virus (HBV) infection is a leading cause of severe chronic liver disease. An estimated 257 million people worldwide carry hepatitis B virus infection (World Health Organization, 2018). The disease progression to severe forms, including the development of hepatocellular carcinoma and their complications, implies a heavy burden to low-income countries. Furthermore, political and socio-economic problems difficult, at times impossible, to deal with the prevention, management, and treatment of HBV infection and associated diseases (Yuen et al., 2018; Zampino et al., 2015). In Latin America, HBV endemicity ranges from low to high accounting for at least 7 to 12 million people infected (Ott et al., 2012; Roman et al., 2014).

HBV is classified into at least nine genotypes (A to I) and several subtypes, with a heterogeneous global distribution (Kramvis, 2014; Locarnini et al., 2013; Shi et al., 2013). The HBV genotypes in a population generally reflect their demographic history. In Latin American countries, the circulation of three main genotypes has been reported: genotype F, related to the Native American population, and genotypes A and D, as a signature of the European immigration and the slave trade from Africa during colonial times (Alvarado-Mora and Pinho, 2013; Campos et al., 2005; Roman et al., 2014). The impact of genotypes in the outcome of infection is still controversial, although an increasing number of studies suggests a significant role in the progression and severity of liver disease, seroconversion rate and antiviral treatment outcome (González López Ledesma et al., 2015; Rajoriya et al., 2017).

Paraguay is a landlocked country in central South America, bordered by Argentina to the south and southwest, Brazil to the east and

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northeast, and Bolivia to the northwest. Based on estimates by the United Nations, the total population is around 6.8 million, most of whom are concentrated in the southeast region of the country (United Nations Department of Economic and Social Affairs, Population Division, 2017). The population of Paraguay is an admixture of Guarani Native American descent and immigrant from different regions of the world. The admixture process began in the 16th century when the Spaniards started the colonization of the territory. Thereafter, two major events stand out on the demographic history of the country. First, the population collapse caused by the War of the Triple Alliance (1864–1870), when nearly 50% of the population died. Second, the European and Asian immigration actively promoted by the government to repopulate the country after the war. Small groups, mostly Germans, Ukrainians, Slavs, and Japanese, arrived the territory, with a major influx during the first half of the 20th century (Fischer et al., 1997). Europeans and Asian groups have continually arrived Paraguay until the last decades (Oddone et al., 2011). Nowadays, it is estimated that there are > 450,000 foreigners inhabiting the country. Most came from neighbor countries: immigrants from Brazil and their descent represent the largest and most prominent group (67%), and Argentineans constitute a significant proportion (19%). Notwithstanding the complex demographic process, the Paraguayan population has maintained a high proportion of Native American genetic background (Simão et al., 2017).

The HBV epidemiology in Latin American countries is still scanty and fragmentary. Despite being a notifiable infection, the prevalence of HBV chronic carriers in Paraguay is unknown since no population-based studies have been conducted. The data available report a prevalence ranging between 0.4%, in women in fertile age who attended the Central Public Health Laboratory, to 4.5% in a population with clinical suspicion of hepatitis (Olmedo et al., 2015; Rovira et al., 2009). On the other hand, there is a paucity of data regarding HBV molecular epidemiology in Paraguay and no information about the circulating genotypes is available to date (Velkov et al., 2018).

The genetic heterogeneity of HBV is an outstanding feature of the virus and this may have important implications in pathogenesis, treatment, and progression of liver disease (Rajoriya et al., 2017). The characterization of genotypes in a population has relevance not only in the research field but also in public health (Yuen et al., 2018). In particular, the analysis of viral sequences by phylogeographic approaches can be used to track patterns of transmission, making possible to shed light on local disease epidemiology (Kilpatrick et al., 2006; Volz et al., 2013).

The aim of this study was to investigate the distribution of HBV genotypes in Paraguay and to estimate the viral population dynamic and spread pattern of the main phylogenetic group by a Bayesian phylodynamic approach.

2. Materials and methods

2.1. Population

Serum samples from 60 HBsAg and anti-HBc positive (AxSYM, Abbott Diagnostics, USA) blood donor candidates were analysed. They were recruited from the *Red Nacional de Servicios de Sangre (RNSS),* Paraguay, between May and October 2017. The mean age of this cohort was 41.3 ± 19.2 years and 23.8% were women. The exclusion criteria were coinfection with human immunodeficiency virus or hepatitis C virus. None of the patients reported having received antiviral treatment. Informed consent was obtained from each subject. The study protocol was approved by the Bioethical Committee of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires EXP-UBA: 0069893/14.

2.2. HBV-DNA extraction, amplification and sequencing

DNA was extracted from 200 µl of serum using the High Pure Viral Nucleic acid kit (Roche Diagnostics, Germany). A region encompassing the Polymerase/Pre-S1 genes of the HBV genome (nt 2301–2972, numbered according to the reference sequence with GenBank accession no. X02763) was amplified by a nested-PCR protocol. This genomic region was used instead of the small S protein codding region, since it has shown a higher accuracy to discriminate the HBV subgenotypes (Habbal et al., 2013). For first round, primer HBV63 (sense, 5′ AGT GTG GAT TCG CAC TCC T 3′, 2269–2287) and HBV84 (antisense, 5′ TG AGC CTG AGG GCT CCA YCC 3′, 3079–3098) were used; primer HBV15 (sense, 5′ TGT TGT TAG ACG ACG AGG CA 3′, 2281–2300) and HBV86 (antisense, 5′ GTT GAA GTC CCA RTC KGG A 3′, 2973–2991) were used for the second round. In 18 out of 60 cases, full-length HBV genomes were amplified by six overlapping nested PCR protocols described elsewhere (Mojsiejczuk et al., 2016). PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing in both directions (Unidad de Genómica, INTA, Castelar, Buenos Aires, Argentina) with the same primers used in the amplification stage.

2.3. Sequences assembly

Chromatograms were individually examined to confirm the quality of sequences using MEGA6. Complete genomes were assembled with BioEdit v7.1.3.0 (Hall, 1999; Tamura et al., 2013). All sequences were screened for recombination by using the Phi-test which is available in the SplitsTree4 program (Bruen et al., 2006; Huson and Bryant, 2006). For this purpose, Polymerase/Pre-S1 region or complete genomes were aligned with a dataset of reference sequences of HBV genotypes/subgenotypes downloaded from GenBank (Supplementary Table S1). The same dataset was used for partial or complete genome sequences. Dataset was aligned with Muscle with default parameters (Edgar, 2004). Finally, recombination screening analyses were performed separately.

2.4. Accession numbers

Nucleotide sequences obtained in this study were submitted to GenBank under accession numbers MK139737-MK139778 and MK183630-MK183647.

2.5. HBV genotyping

Genotype or subgenotype determination was based on the phylogenetic analysis of the Polymerase/Pre-S1 region using the same dataset as for recombination screening test. Evolutionary model was inferred according to the Bayesian Information Criterion (BIC) statistics obtained with jModeltest v2.1 (Darriba et al., 2012). The TPM1uf + I + G was the best fit evolutionary model. Maximum Likelihood phylogenetic tree was constructed with RaxML software v8.2.8 in the CIPRES Science Gateway server (Miller et al., 2010; Stamatakis, 2014). The robustness of the reconstructed phylogeny was evaluated by bootstrap analysis (1000 pseudo-replicates).

2.6. Bayesian coalescent analysis

A Bayesian coalescent approach was used to estimate the phylogenetic relationship, the time to the most recent common ancestor (tMRCA), the population dynamics and the geographical spread pattern of the main subgenotype detected (subgenotype F4). The analyses were performed in the BEAST v1.8.10 software package in the CIPRES server (Drummond and Rambaut, 2007).

To this end, complete genome sequences newly generated in this work (n = 18) were combined with complete genome subgenotype F4 sequences obtained from the GenBank (n = 71). A high proportion of

sequences in the dataset (34 out of 89) displayed mutations that abolish the HBeAg expression, i.e. G1896A, other premature stop codon generated by nucleotide substitutions, insertions or deletions in the pre-Core region. It has been described that HBV evolutionary rate increases in the HBeAg negative stage, probably due to a viral adaptation to the intra-host environment, with an increased immune selective pressure (Hannoun et al., 2000; Harrison et al., 2011; Kramvis et al., 2018). Those changes are not evenly distributed throughout the HBV genome since most of the substitution accumulated in the HBeAg negative state is in the Core gene (Gauder et al., 2018). Therefore, to avoid bias in the estimation of ancestral divergence times, the Core region (nt 1814–2452) was removed from the alignment before the analyses. Dataset alignment and evolutionary model inference were performed as described above. The GTR + I + G was the best fit nucleotide substitution model.

The Bayesian skyline plot (BSP) demographic model with default parameters (10 groups with a piecewise constant function) was used as coalescent prior. The analyses were performed setting an uncorrelated lognormal (UCLN) molecular clock. Three previously published long-term evolutionary rates for HBV were tested to perform the time-scale calibration of the analyses: 1×10^{-5}, 3×10^{-5}, and 5×10^{-3} substitutions/site/year (s/s/y). These were selected from studies carried out in a similar epidemiological scale to that analysed in the present study, i.e. the analysis of a single genotype or subgenotype (Lago et al., 2014; Torres et al., 2011; Zehender et al., 2015). In addition, a phylogeographic reconstruction was performed using a discrete model with an asymmetric substitution matrix over the sampling locations. Sequences were labeled with their country of origin; in particular, Argentinean sequences were discriminated by geographic region: Buenos Aires (ARG-BsAs), Northeast (ARG-NEA) and Northwest (ARG-NOA). All the analyses were run in duplicate up to achieve the convergence of parameters. Samples were examined with Tracer v1.6 to evaluate the convergence (Effective sample size (ESS) ≥ 200, acceptable mixing without tendencies in traces), with a burn-in of 10%. Uncertainty in parameter estimates was evaluated in the 95% highest posterior density (HPD95%) interval. The Maximum clade credibility (MCC) tree was summarized using Tree Annotator v1.8.10 and visualized with FigTree v1.4.2 (available at http://tree.bio.ed.ac.uk/soft-ware/figtree/).

3. Results

3.1. Identification of HBV genotypes and SUBGENOTYPES

The genetic diversity of HBV in Paraguay was analysed by characterizing 60 HBsAg and Anti-HBc positive serum samples. Polymerase/Pre-S1 overlapping region was successfully amplified and sequenced in all samples. No statistical evidence of recombination was found.

Genotype and subgenotype was assessed by phylogenetic analysis (Fig. 1). The following subgenotypes were identified: A1 (n = 3, 5.0%), A2 (n = 1, 1.7%), B2 (n = 1, 1.7%), C2 (n = 1, 1.7%), D3 (n = 5, 8.3%) and F4 (n = 49, 81.7%).

3.2. Bayesian coalescent analyses

In order to estimate the tMRCA, the viral population dynamic and the spread pattern of the main strain, full-length HBV genomes were obtained in 18 out of 49 subgenotype F4 cases and analysed by phylodynamic methods. The Core region was removed from the alignment before the analyses, as it was stated in the Material and Method section.

Three alternative evolutionary rates of 1×10^{-5}, 3×10^{-5} and 5×10^{-5} s/s/y were tested, which dated the MRCA of subgenotype F4 to 958, 304 and 167 years ago, respectively. The intermediate rate of 3×10^{-5} s/s/y reproduced a suitable time-frame for subgenotype F4 evolution and provided the best fit with historical and epidemiological data. This means that the common ancestor of the currently circulating F4 strains dated back to the year 1713 (HPD95% = 1462–1853) and the viral population started to grow and spread around 250 years ago (Fig. 2 and Supplementary Fig. S1).

Paraguay was the estimated location for the most recent common ancestor (posterior probability = .65) and basal lineages of subgenotype F4 (Fig. 2). A first divergent event in the phylogeny gave rise to two separated groups. The first group had an MRCA dated in 1762 (HPD95% = 1568–1878) and located in Paraguay. From there, the lineage spread to Bolivia and Northwest Argentina (NWA), mainly. This group exhibits certain geographic structure with several monophyletic clusters. Firstly, a clade (Paraguayan clade, Fig. 2) clustered most of the sequences from Paraguay and one isolate from Buenos Aires, Argentina, and had a common ancestor in 1868 (HPD95% = 1790–1980). Secondly, it is also remarkable a monophyletic clade from Bolivia, with an MRCA in 1814 (HPD95% = 1655–1917). This clade contained 8 out of 11 Bolivian isolates included in the dataset, although it did not have a statistical support (pp = 0.53). Finally, a monophyletic clade containing four sequences from NWA is also observed in the MCC tree.

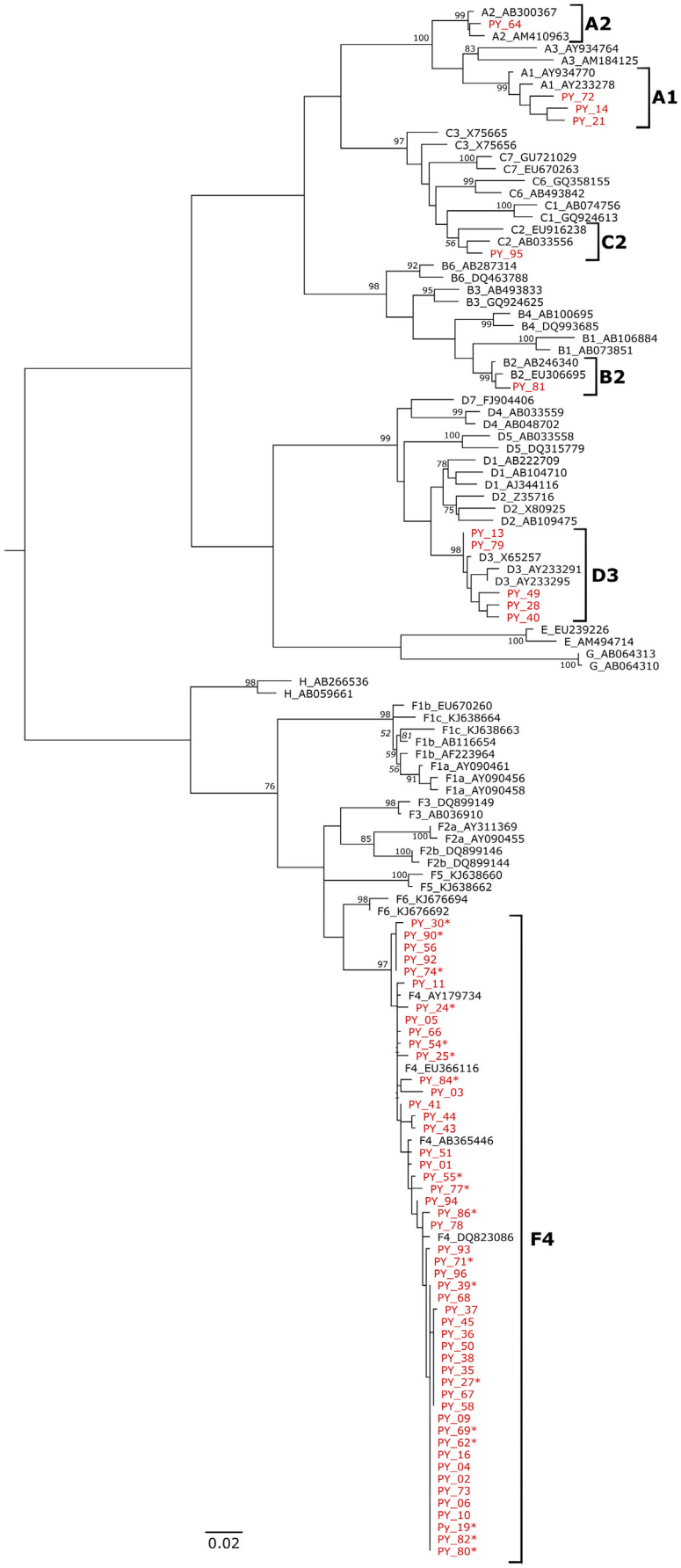
On the other hand, the second group had a common ancestor located in Paraguay in 1753 (HPD95% = 1548–1874) and showed a broader geographical distribution without a defined geographic structure. It is mainly constituted by isolates from Buenos Aires but also included sequences from Paraguay and Northern Argentina, one isolate from Brazil and one from Peru. Unlike the first group, in this "Cosmopolitan" F4 lineage, sequences from the different geographical locations are intermingling. The only significant monophyletic group observed is a clade constituted by isolates from Buenos Aires with an MRCA dated in 1848 (HPD95% = 1696–1943).

4. Discussion

The HBV epidemiology in Latin America is complex and continually changing. Historical, cultural and socio-economic factors have determined differences in the prevalence of infection, patterns of transmission and circulating genotypes. In this study, we analysed the HBV genotypes in a blood donor population from Paraguay. The circulating genotypes reflected the ethnic composition of the population. As in other Latin America countries, Native American and European genotypes were mainly detected, along with a minority of African and Asian genotypes. The subgenotype F4 represented the largest proportion of HBV infections and showed a high genetic diversity. This suggests that it would be the autochthonous strain, which is also supported by the phylogeographic analysis.

The distribution of HBV genotypes in Paraguay reflects the ethnic origin of the population. The subgenotype F4 was largely prevalent, as expected from the major Native American genetic background in the country and neighbor regions (Piñeiro y Leone et al., 2008; Simão et al., 2017; Torres et al., 2011). Besides, the presence of foreign strains of European, Asian and African origin reflected the influence of the modern-day human migration, from colonial times to the present, into the HBV epidemiology.

Similar to other Latin American countries, the presence of A2 and D3 subgenotypes is related to the European ancestry of the population (Alvarado-Mora and Pinho, 2013; Campos and Mbayed, 2005; Mojsiejczuk et al., 2016; Roman et al., 2014), since the subgenotype A2 is mainly present in Northwest Europe, while subgenotype D3 is prevalent in the Mediterranean region and Western Europe (Kostaki et al., 2018; Norder et al., 2004; Zehender et al., 2012). These strains would have been directly introduced by the Europeans immigrants that have arrived in Paraguay since the 16th century. In addition to this direct immigration, the movement of relatively large groups from and to neighbor countries, due to historical and socio-economic factors, have characterized the demographic dynamics of the country (Oddone et al., 2011). This regional migratory process seems also have played a significant role in the distribution of HBV genotypes in Paraguay. For instance, a high prevalence of subgenotype D3 was observed in South and Southeast Regions of Brazil, and in the Province of Misiones, Argentina,



(caption on next page)

Fig. 1. Maximum-likelihood phylogenetic tree constructed on the Pol/PreS1 sequences.
The analysis includes reference sequences retrieved from GenBank, indicated by their accession numbers and the respective genotype/subgenotype, and 60 sequences from Paraguay generated in this study (in red). Isolates from which full-length genome was sequenced are labeled with an asterisk (*). The numbers at nodes correspond to bootstrap values (≥ 70%) obtained with 1000 pseudo-replicates. The scale bar indicates genetic distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

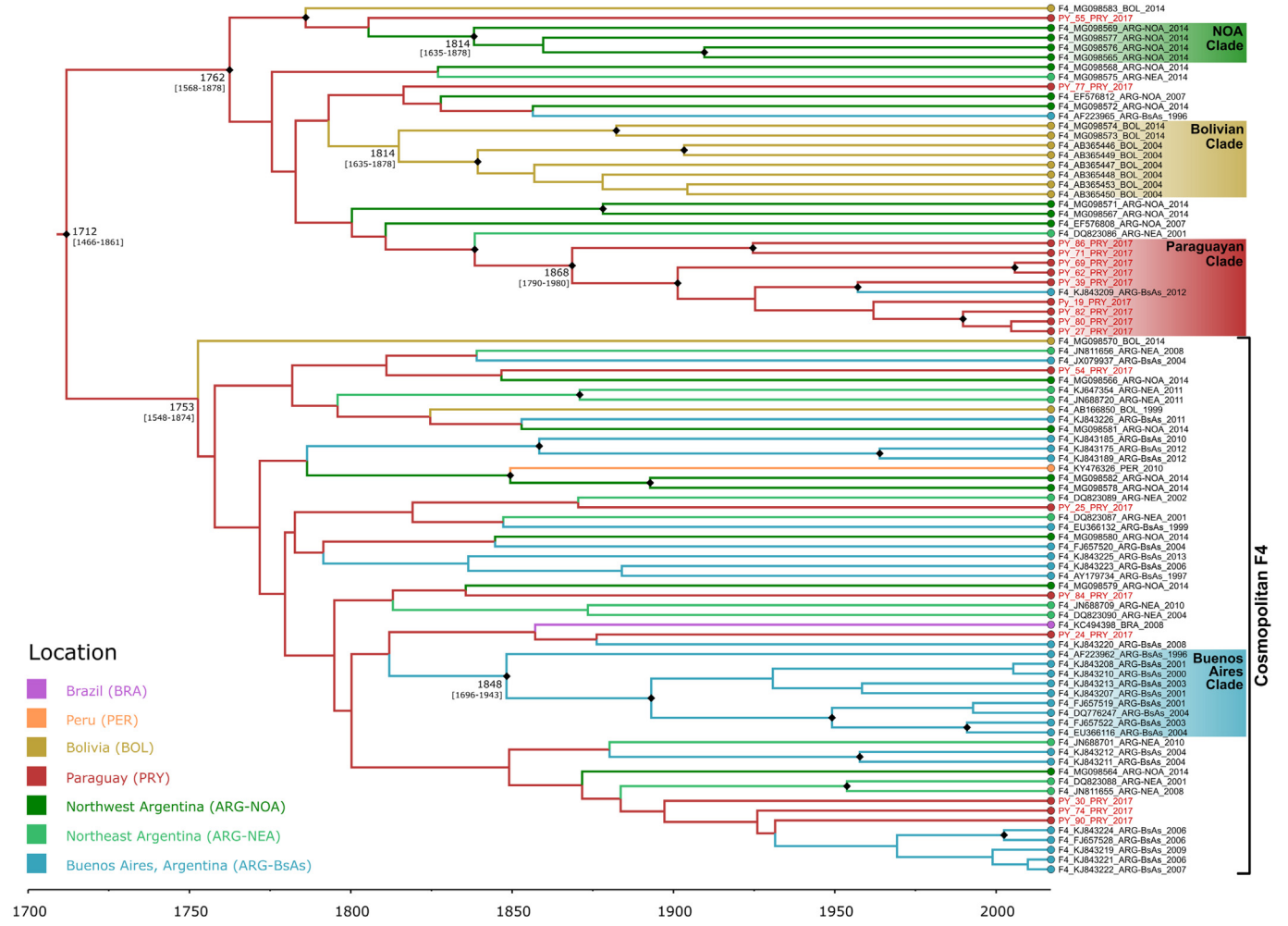


Fig. 2. Time-scaled Maximum clade credibility (MCC) tree for HBV subgenotype F4 genomes excluding core region
The time scaled phylogeny was estimated with an evolutionary rate of 3×10^{-5} s/s/y. The sequences from Paraguay reported in this study are colored in red. Branches are colored according to the most probable location of the parental node of each lineage (colour-coded in lower-left inset). The years for MRCA (median value [HPD95%]) of the lineages discussed in the text are shown in their corresponding node. All nodes marked with a diamond showed posterior probability ≥.90. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which shares borders with Paraguay. It has been related with the high frequency of Euro-descent, particularly Italians, who arrived the region at the end of the 19th and early 20th century (Bertolini et al., 2012; Mojsiejczuk et al., 2016; de Paoli et al., 2018). The subgenotype D3 strains detected in Paraguay showed a close phylogenetic relationship with those reported in Brazil and Misiones, Argentina (data not shown). It could be hypothesized that this subgenotype was introduced to the south of Brazil and then was brought to Paraguay by the Brazilian groups that settled in the country since the 1960s (Oddone et al., 2011).

Similarly to the European HBV strains, the presence of the African subgenotype A1 in Latin America is associated with colonial-times migrations, specifically with the Transatlantic slave trade (Campos and Mbayed, 2005; Lago et al., 2014; Roman et al., 2014). The introduction of subgenotype A1 in Paraguay could have happened during the colonial period or more recently from neighboring regions such as Brazil where this subgenotype circulates (Lampe et al., 2017; Motta-Castro et al., 2008). Finally, subgenotype B2 and C2, identified in one case each, would be related to immigration from East and Southeast Asia in the 20th century, where these genotypes prevail (Pourkarim et al., 2014).

Finally, it is worth mention that the subgenotype F1b was not detected in Paraguay, despite being one of the most prevalent in the Southern Cone of South America (Argentina, Chile and Uruguay) and responsible for a notable proportion of primary and chronic infection in the region (González López Ledesma et al., 2015; Rodrigo et al., 2016). This might be because we analysed a blood donor population, where the HBV infected individuals generally present long-term asymptomatic chronic infections, where subgenotype F1b is less represented (González López Ledesma et al., 2015). Although the study of blood donors provides a valuable information, it has limitations to describe the epidemiological scenario in the general population. It would be of interest to analyze additional populations of infected individuals to describe in more detail the HBV epidemiology in Paraguay.

As characterize the evolution of viruses, HBV is under a time-

dependent rate phenomenon. This implies that short-term evolution studies provide rates of evolutionary change higher than those estimated from longer-term studies (Duchêne et al., 2014; Patterson Ross et al., 2018; Zehender et al., 2015). In order to estimate the temporal frame for the evolution of subgenotype F4, we tested previously published long-term substitution rates for HBV. A rate of 3×10^{-5} s/s/y provided the best fit of subgenotype F4 evolution with the historical and epidemiological data. This rate is equivalent to that obtained by Zehender et al. (2015) when analyze the genotype A evolution, using historical data to perform the temporal calibrations. Unlike our estimation, in a previous study of the genotype F, Torres et al. (2011) proposed that the rate of 1×10^{-5} s/s/y provides an epidemiologically realistic scenario for the evolution of the lineage. This difference may be due that a higher rate fits better when the analysis focuses on a sublineage, which arose and evolved on a shorter time scale than the entire genotype F.

Consequently, a substitution rate of 3 × 10–5 s/s/y translates the MRCA of subgenotype F4 to the early 18th century, suggesting that this lineage emerged and spread in the last 300 years. It is widely accepted that the genotype F was present in the Americas in pre-Columbian times. Notwithstanding, the arrival of European conquers had a strong impact on local demography and infectious diseases epidemiology. It is estimated that the Native population fell by 80%, which might have caused the extinction of several genotypes F lineages that were previously endemic in the continent; political and armed conflicts such as the War of the Triple Alliance caused large-scale migration; the establishment of Jesuit missions gathering native populations into high-density communities. These demographic changes in the post-colonization period, among others, would have provided the conditions for the emergence of the surviving lineages, including the subgenotype F4 (Mello et al., 2013; Torres et al., 2011).

The phylogeographic analysis indicated that Paraguay is the most probable location of the MRCA. Notwithstanding, this result could be biased by the large number of samples from Paraguay. The subgenotype F4 might be autochthonous of a broader area including Paraguay, Bolivia and the north of Argentina. Specifically, the subgenotype F4 would be associated with the Native American communities of the Gran Chaco. In a previous study by Godoy et al. (2016), the subgenotype F4 was detected in two Native American communities of this area, although the number of samples was limited to one case of each. The Grand Chaco is a lowland geographical region divided among eastern Bolivia, western Paraguay, northern Argentina and a portion of the Brazilian states of Mato Grosso and Mato Grosso do Sul. Their native inhabitants generally lived in small groups of few families and followed a simple nomadic life of hunter-fisher-gatherers. In general, the biological evidence showed that the Gran Chaco communities present high genetic diversity and low differentiation among populations, suggesting intense gene flow between them (Demarchi and Ministro, 2008). This structure in the host population might explain the scanty geographical structure observed in the phylogeny of subgenotype F4 (Kostaki et al., 2018; Volz et al., 2013). Finally, it is likely that the original distribution of subgenotype F4 did not include Buenos Aires, and its dispersion to this southern latitude would be a consequence of more recent migrations. Similarly, the current intense migratory movements in South American countries explain its sporadic detection of subgenotype F4 in Brazil and Peru (Mello et al., 2013).

Finally, some limitations of this study need to be considered. Phylodynamic analyses are strongly conditioned by the sampling. In particular, the subgenotype F4 sequences available in public databases have been sampled in the last decades and from the general population of large urban centers mostly. Due to the limited temporal sampling, the use of external substitution rates has been preferred for the temporal calibration of HBV long-term studies (Zehender et al., 2015). Even when these analyses are formulated and interpreted according to the epidemiological context, the rate selection implies an arbitrary process. Moreover, the sampling also affects the accuracy of the population dynamic plot. None of the drastic demographic reductions underwent by the host population was reflected in the subgenotype F4 BSP. The BSP analysis might not be sensitive enough to detect these events from the sequences included in the dataset, because of the absence of ancient samples to display the viral diversity before these events, as well as the scanty or lacking signal preserved by contemporary sequences. At last, a geographic sampling bias might be also present, not only due to the uneven availability of samples from different regions but also by the assignation of geographic origin (based on the place of sample collection). This can result in a biased ancestral origin assignation (Frost et al., 2014). Hence, the hypothesis raised in our study represents the evolutionary history of the lineages included in the analysis. A new hypothesis about subgenotype F4 origin and dynamic may arise as new genomic data become available.

In summary, this study is the first approach to characterize the molecular epidemiology of HBV in Paraguay. The distribution of genotypes and the phylogeographic reconstruction showed the impact of both global and regional migrations in shaping the HBV molecular epidemiology in the country and Latin América in general. The new molecular data provide valuable information for the characterization of the evolution of Native American autochthonous strains, particularly to establish the origin and spread of the subgenotype F4.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.020.

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Declarations of interest

none.

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