

Analysis of a Local HIV-1 Epidemic in Portugal Highlights Established Transmission of Non-B and Non-G Subtypes

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The existing data support Portugal as the western European country with the highest HIV-1 subtype diversity. However, detailed phylogenetic studies of Portuguese HIV-1 epidemics are still scarce. Thus, our main goal was to analyze the phylodynamics of a local HIV-1 infection in the Portuguese region of Minho. Molecular epidemiological analysis was applied to data from 289 HIV-1-infected individuals followed at the reference hospital of the province of Minho, Portugal, at which isolated viruses had been sequenced between 2000 and 2012. Viruses of the G (29.1%) and B (27.0%) subtypes were the most frequent, followed by recombinant forms (17.6%) and the C (14.5%), F1 (7.3%), and A1 (4.2%) subtypes. Multinomial logistic regression revealed that the odds of being infected with the A1 and F1 subtypes increased over the years compared with those with B, G, or C subtypes or recombinant viruses. As expected, polyphyletic patterns suggesting multiple and old introductions of the B and G subtypes were found. However, transmission clusters of non-B and non-G viruses among native individuals were also found, with the dates of the most recent common ancestor estimated to be in the early 2000s. Our study supports that the HIV-1 subtype diversity in the Portuguese region of Minho is high and has been increasing in a manner that is apparently driven by factors other than immigration and international travel. Infections with A1 and F1 viruses in the region of Minho are becoming established and are mainly found in sexually transmitted clusters, reinforcing the need for more efficacious control measures targeting this infection route.

Globally, at the end of 2012, 35.3 million people were estimated to be infected with HIV-1, and AIDS remains one of the world's most serious health challenges (1). Phylogenetically, HIV-1 is divided into four groups: M, O, N, and P. Most HIV-1 infections are globally caused by M group viruses that can be further divided into at least nine subtypes, A to D, F to H, J, and K, as well as different circulating and unique recombinant forms (2). Although clinical evidence is still limited, and current antiretroviral regimens appear to have comparable efficacies with all subtypes, there is presently evidence showing that particular HIV-1 subtypes may have a transmission advantage (3–6), higher replicative efficiency, or altered drug susceptibilities (7–11). The geographic patterns of the M group subtypes are continuously changing in response to human population migrations and active transmission networks, thus inciting constant vigilance. Although several reports suggest that the prevalences of non-B subtypes are increasing in western Europe (12–17), the B subtype remains the most prevalent. Portugal contrasts with the rest of western Europe in its distribution of HIV-1 subtypes. In addition to the B subtype, Portugal also has a high prevalence of the G subtype (18–23). The high prevalences of the B and G subtypes are thought to have promoted the appearance among intravenous drug users (IDU) of different types of B/G recombinant strains, namely, CRF14_BG, which is considered to have emerged in Portugal in the early 1990s and then spread to Spain and other European countries (24–26). The association between HIV-1 subtype and risk-behavior patterns has been complex to define, mainly due to difficulties in obtaining large numbers of each viral subtype and transmission route within a homogeneous study population (27).

Portugal has one of the highest HIV-1 prevalence rates in western Europe and, following a decrease in the last decade of HIV-1

infection in IDU, heterosexual contact is now estimated to be the most relevant transmission route in Portugal (28). The reconstruction of viral transmission networks is a relevant tool to monitor disease transmission and the effectiveness of adopted preventive measures, as well as to suggest more adequate control strategies for specific populations. Several studies have shown that in addition to a patient interview, phylogenetic analysis of the genetic sequences from the isolated viruses can provide valuable insights to help identify events of onward transmission and evaluate the spread of the virus (29–31). Nonetheless, limited information is available to understand HIV-1 transmission clusters in Portugal.

The aim of this study was to perform a molecular epidemiologic characterization of a cohort of 289 patients followed in the

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reference hospital in Minho province, Portugal. Specifically, we aimed to identify local transmission networks and possible relationships with previously described transmission clusters. In line with previous studies in Portugal, we found a large diversity of subtypes. In addition, our analysis supports that the transmission of nonpredominant subtypes among the local population began more than a decade ago, providing valuable insights into the dynamics of infection in this geographic area.

MATERIALS AND METHODS

Study population. From 2000 to 2012, a total of 792 HIV-1 patients were followed at Hospital de Braga (HB), representing 60.3% of the HIV-1-infected individuals in the Portuguese region of Minho (28). Two hundred eighty-nine individuals from this cohort were selected for this study, according to two criteria: (i) availability of a plasma sample or plasma-derived viral sequence sampled from 2000 to 2012 and (ii) the absence of previous antiretroviral treatment at the time of sampling. The following information was collected anonymously from the clinical files of each individual: presumed transmission route, gender, age, nationality, presumed country of infection, and date of diagnosis. HB is a university-affiliated hospital serving as the reference hospital for the 1,093,021 inhabitants of the northwest Portuguese province of Minho. The prevalence of HIV-1 infection in the Minho region (0.12%) is lower than the overall prevalence in Portugal (0.31%) (28). According to transmission mode, the HB population presented significant differences from the data for the Portuguese HIV-1-infected individuals, with more IDU and fewer men who have sex with men (MSM) being infected. Also, in HB, there were more men and fewer patients >40 years old who were infected (see Table S1 in the supplemental material). The frequencies of individuals reporting heterosexual transmission are similar in a comparison of HB HIV-1 patients with the overall country data (see Table S1).

Sequencing of viral samples. Viral RNA was extracted using MagNA Pure total nucleic acid isolation kits (Roche Applied Science). Reverse transcriptase PCR (RT-PCR) and DNA sequencing were performed with the Trugene HIV-1 genotyping system (Siemens Healthcare Diagnostics). The sequenced regions include part of the coding sequences of Gag (492 to 501), p6 (44 to 53), Pol (60 to 402), p2p7p1p6 (129 to 138), protease (4 to 99), and reverse transcriptase (RT) (1 to 127) (the reported positions are amino acid positions relative to the protein start in the HXB2 reference genome, GenBank accession no. [K03455.1](#)). The subtyping of the 289 sequences was made using REGA 3.0 (32). The subtyping results were confirmed using SimPlot and RDP (33, 34). The sequences were uploaded to GenBank and assigned the accession numbers [KM205831](#) to [KM206119](#).

Phylogenetic analysis. The 289 HIV-1 sequences obtained in this study and 88 sequences from the databases, including the M group consensus and a previously defined set (32) of comprehensive subtype reference sequences with at least two reference sequences from each M group subtype (A1, A2, B, C, D, F1, F2, G, H, J, and K) and from 26 different circulating recombinant forms (CRF) (CRF01_AE, CRF02_AG, CRF03_AB, CRF04_CPX, CRF05_DF, CRF06_CPX, CRF10_CD, CRF11_CPX, CRF12_BF, CRF13_CPX, CRF14_BG, CRF18_CPX, CRF19_CPX, CRF20_BG, CRF24_BG, CRF25_CPX, CRF27_CPX, CRF29_BF, CRF31_BC, CRF35_AD, CRF37_CPX, CRF39_BF, CRF40_BF, CRF42_BF, and CRF47_BF), were aligned using MUSCLE (35). The phylogenetic analysis of the 377 sequences was conducted using RAxML 8.0.9 to produce a maximum likelihood tree using 1,000 bootstrapping replicates (36). The analysis was repeated with PhyML (37), computing the approximate likelihood ratio test (aLRT) support of all tree branches, and by Bayesian analysis using BEAST (38). The best-fitting nucleotide substitution model was estimated using jModeltest version 2.1.2 (39) to be the general time reversible (GTR) model, with a proportion of invariant site (I) and gamma distribution of rates (G), selected among 88 different models according to the Akaike information criterion (AIC), the Bayesian information criterion (BIC), and the decision theoretic framework (DT). An even-

TABLE 1 Demographics of the study population

Variable ^a	No.	%
Gender		
Male	222	76.8
Female	67	23.2
Age at diagnosis (yr)		
≤20	17	5.9
21–40	190	65.7
41–50	41	14.2
>50	41	14.2
Patient nationality		
Portuguese	260	90.0
Other	29	10.0
Ethnicity		
White	274	94.8
Black	15	5.2
Presumed country of infection		
Portugal	280	96.9
Other	9	3.1
Route of transmission		
Heterosexual contact	161	55.7
MSM	26	9.0
IDU	99	34.2
Other	3	1.1
Total	289	100

^a IDU, intravenous drug users; MSM, men who have sex with men.

tual bias introduced by convergent evolution due to the presence of drug-resistant mutations was discarded by repeating the analysis after the removal of codons associated with drug resistance in the standardized list of mutations for the surveillance of transmitted drug resistance established by the World Health Organization (40). The general topology of the trees and identification of the clustering remained unchanged. The identification of transmission clusters was based on the maximum likelihood tree, selecting the clusters from at least three individuals, with a bootstrap support of ≥95% and an average genetic distance of ≤0.03 substitutions per site (29, 41). We also used Bayesian inference, and all identified clusters showed a posterior probability equal to 1 (42).

Estimation of evolutionary dates. Estimates of the time of the most recent common ancestor (MRCA) were performed by simultaneously inferring population parameters, substitution parameters, and tree topology using Bayesian Markov chain Monte Carlo (MCMC) inference, as implemented in BEAST version 1.8.0 (38). Three independent runs of 260 million replicates were performed under a Bayesian skyline relaxed molecular clock model, using a general time reversible nucleotide substitution model, with heterogeneity among the sites modeled with a gamma distribution. An examination of the MCMC samples with Tracer version 1.6 indicated convergence and adequate mixing of the Markov chains. After inspection with Tracer, we discarded an appropriate number of steps from each run as burn-in and combined the resulting MCMC tree samples for a subsequent estimation of posteriors using TreeAnnotator version 1.8.0. We summarized the MCMC samples using the maximum clade credibility tree, with the branch length depicted in years.

Statistical analysis. To identify the main predictors of HIV-1 subtype group distribution, a multinomial logistic regression model was performed. Using this procedure, we assessed the association between the date of diagnosis and HIV-1 subtype group, controlling for other relevant variables. The HIV-1 subtypes with low numbers of cases were pooled,

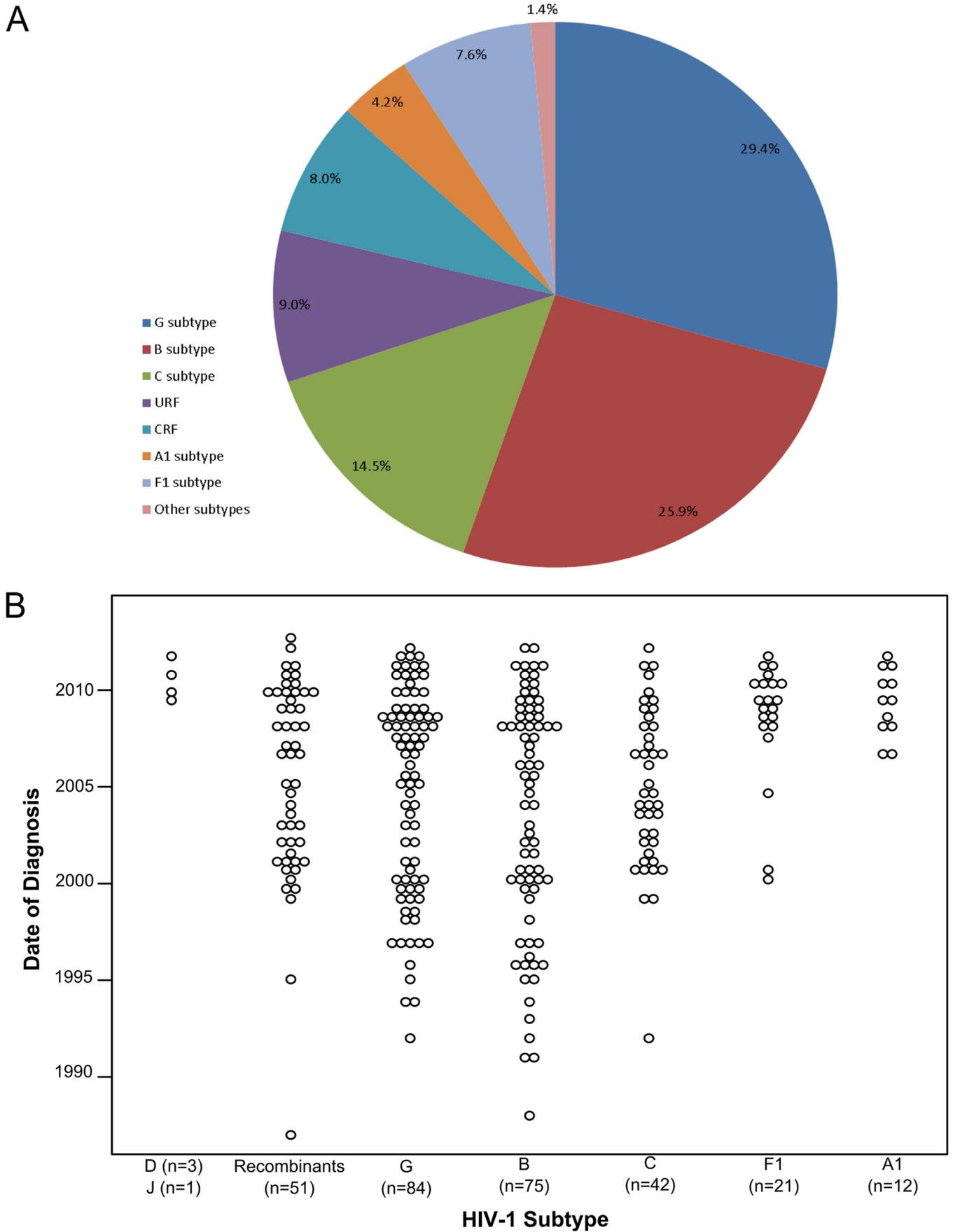


FIG 1 HIV-1 subtype diversity and temporal distribution of the date of diagnosis in the cohort of 289 HIV-1-infected individuals from the province of Minho, Portugal.

TABLE 2 Multinomial logistic regression model relating HIV-1 subtypes with date of diagnosis, age, gender, and presumed transmission mode

Subtype comparison	Variable	B ^c	SE	Wald ^a	OR ^b	95% CI ^c	
						LB	UB
Recombinants vs A1/F1	Days (or yr) since date of diagnosis	-0.0004	0.000	6.278*	1.000 (0.852)	0.999	1.000
	Age	-0.006	0.019	0.091	0.994	0.959	1.031
	Male	0.298	0.638	0.218	1.347	0.386	4.699
	Heterosexual	-0.808	0.735	1.207	0.446	0.106	1.884
	IDU ^d	-0.346	0.820	0.178	0.708	0.142	3.533
G vs A1/F1	Days (or yr) since date of diagnosis	-0.001	0.000	10.774**	0.999 (0.824)	0.999	1.000
	Age	0.016	0.016	1.100	1.016	0.986	1.048
	Male	-0.847	0.476	3.167	0.429	0.169	1.090
	Heterosexual	1.450	1.161	1.559	4.264	0.438	41.537
	IDU	2.012	1.214	2.747	7.475	0.693	80.672
B vs A1/F1	Days (or yr) since date of diagnosis	-0.001	0.000	17.319***	0.999 (0.781)	0.999	1.000
	Age	-0.016	0.017	0.932	0.984	0.952	1.017
	Male	-0.473	0.512	0.856	0.623	0.229	1.698
	Heterosexual	-1.234	0.663	3.459	0.291	0.079	1.069
	IDU	-1.593	0.771	4.269*	0.203	0.045	0.921
C vs A1/F1	Days (or yr) since date of diagnosis	-0.000	0.000	5.154*	1.000 (0.862)	0.999	1.000
	Age	0.017	0.019	0.815	1.017	0.980	1.056
	Male	-0.646	0.579	1.242	0.524	0.168	1.632
	Heterosexual	-0.267	0.949	0.079	0.766	0.119	4.919
	IDU	1.216	0.996	1.491	3.374	0.479	23.754

^a *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

^b OR, odds ratio.

^c 95% CI, 95% confidence interval; LB, lower bound; UB, upper bound.

^d IDU, intravenous drug users.

^e B, regression coefficient.

resulting in 5 groups that were used for statistical analysis: G ($n = 84$), B ($n = 75$), C ($n = 42$), other subtypes (A1, $n = 12$; F1, $n = 21$; total, $n = 33$), and recombinants ($n = 42$). The least represented subtypes, J ($n = 1$) and D ($n = 3$), had no influence on the results and were excluded from the analysis. The independent variables analyzed were date of diagnosis, age, gender, and transmission route. The IBM SPSS package (version 19) was used to conduct all statistical analysis, and the results were considered to be significant at a P value of < 0.05 .

Ethics. The project was approved by the ethics committee of the HB. Written consent was obtained from all patients enrolled in the study. The clinical data were codified to ensure the confidentiality of the patients.

RESULTS

High HIV-1 subtype diversity. Among the 289 individuals who met the inclusion criteria, 76.8% were male, and the average age at diagnosis in the study population was 44.5 years (range, 18 to 87 years) (Table 1). The most frequently reported route of infection was heterosexual contact ($n = 161$ [55.7%]), followed by IDU ($n = 99$ [34.2%]) and MSM ($n = 26$ [9.0%]) (Table 1). In accordance with the population of Minho, the study population was highly homogeneous, with $> 90\%$ of the individuals being Portuguese, of white ethnicity, and presumed to have been infected in Portugal. The most frequent subtypes found were G ($n = 85$ [29.4%]), B ($n = 75$ [26%]), and C ($n = 42$ [14.5%]), followed by the F1 ($n = 22$ [7.6%]) and A1 ($n = 12$ [4.2%]) subtypes. Only 1.4% of the studied individuals were infected with other “pure”

subtypes (D, $n = 3$; J, $n = 1$). The most frequently found CRF was CRF14_BG ($n = 15$ [5.2%]), followed by CRF02_AG ($n = 4$ [1.4%]). Individuals infected with unique recombinant forms (URF) constituted 9.0% of the population (Fig. 1A).

Increasing incidence of infection with A1 and F1 HIV-1 subtypes. The dates of HIV-1 diagnosis in the study population spanned the period 1987 through 2012. We investigated the proportion of infections diagnosed each year with different subtypes. The less frequent subtypes were pooled to allow for statistical analysis (Fig. 1B), and a significant multinomial logistic regression model was obtained ($\chi^2_{(df = 20)} = 77.3$; $P < 0.001$; pseudo- $R^2_{Nagelkerke} = 0.245$). The model (Table 2) shows that the chance of being infected with the A1 or F1 subtype increased during the period of time studied compared to that with the other subtypes (odds ratio [OR], 0.852 and $P < 0.05$ for recombinant viruses; OR, 0.824 and $P < 0.01$ for G subtypes; OR, 0.781 and $P < 0.001$ for B subtypes; and OR, 0.862 and $P < 0.05$ for C subtypes). Overall, these data support the hypothesis that infection with A1 and F1 subtypes began to appear recently in Minho province but are becoming established.

Evidence for local transmission clusters. The phylogenetic analysis of the viral sequences allowed the identification of 17 transmission clusters (Fig. 2). This analysis was performed using a maximum likelihood tree and confirmed by Bayesian inference (see Table S2 in the supplemental material). Of all the sequences,

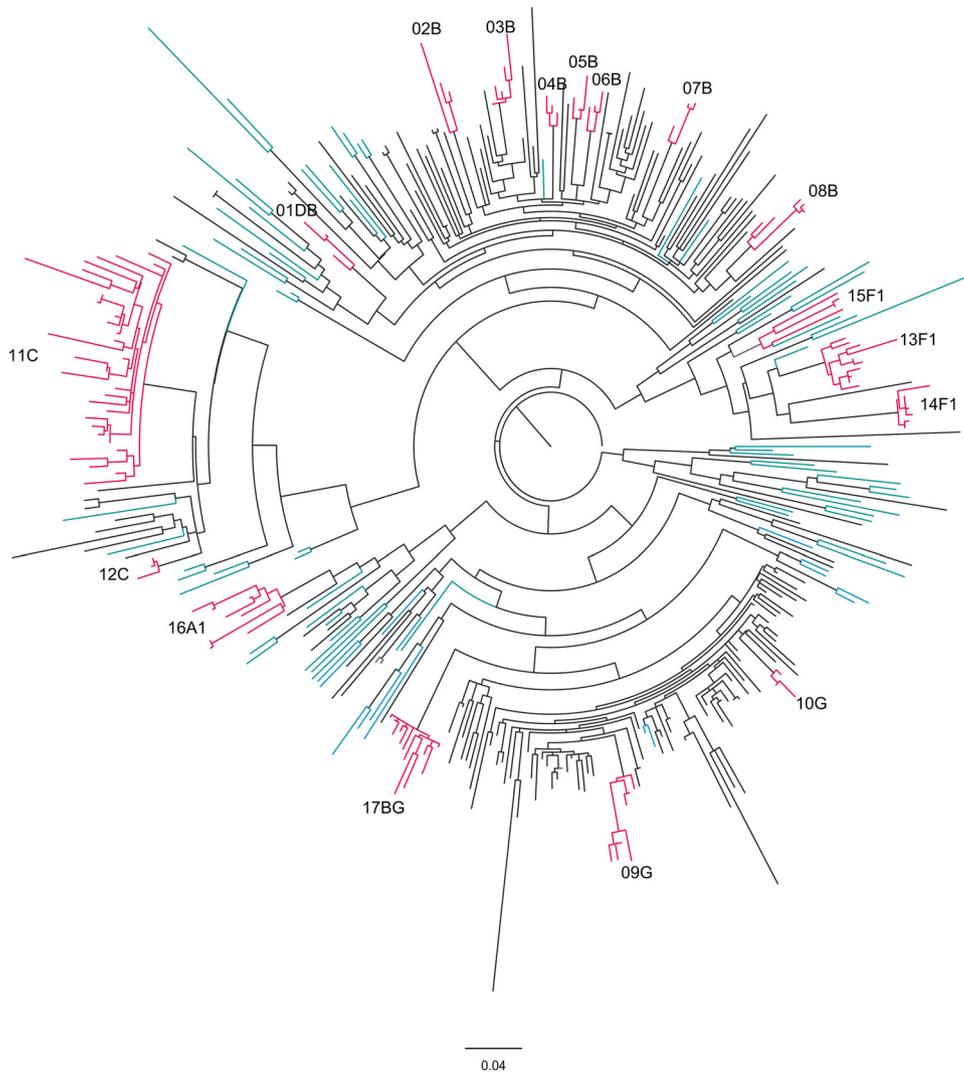


FIG 2 Phylogenetic relationships among the HIV-1 sequences isolated from 289 infected individuals from Minho province, Portugal. Maximum likelihood (ML) phylogenetic analysis was performed using 289 partial HIV-1 sequences obtained in this study and 89 subtype reference sequences (colored in blue) and rooted using the M-group consensus sequence. The branch lengths are expressed as the number of nucleotide substitutions per site. The transmission clusters (colored in pink) were supported by an ML bootstrap support of $\geq 95\%$ based on 1,000 replicates, an average genetic distance of ≤ 0.03 substitutions per site, and a Bayesian posterior probability equal to 1.

39.4% grouped in the 17 transmission clusters. In terms of HIV-1 subtype distribution, 7 clusters included subtype B viruses, three included subtype F1, two included subtype G, two included subtype C, one included subtype A1, one included CRF14_BG, and one included DB URF (Table 3). The distribution of the clustered sequences per subtype showed that 95.5% of all F1 sequences (21 out of 22) were incorporated in clusters. Respectively, 66.7%, 80.9%, and 80% of all A1, C, and CRF14_BG sequences were found in clusters. In contrast, the B and G subtype clusters include only 35.9% and 11.8% of the B and G subtype sequences, respectively. The mean number of individuals per cluster was 6.9. Among the clusters with a number of individuals that was above the mean, the largest cluster was 11C. It was composed almost entirely of IDU (27 out of 31 individuals), and 4 of these individuals reported sharing injection material. The second largest cluster was 17BG, and it was also composed mainly of IDU (11 out of 12 individuals), with the non-IDU individual in the cluster reporting

sexual contact with one of the other members of the cluster. For all F1 clusters (13F1, 14F1, and 15F1), the transmission mode was predominantly sexual (Table 3). In the 13F1 cluster, 1 patient out of 10 was of Brazilian nationality. Furthermore, the phylogenetic analysis shows that the viral sequences from the 13F1 and 14F1 clusters have common ancestors with reference sequences collected in Brazil (GenBank accession no. [AY173957.1](#), [EU735538.1](#), and [EU735540.1](#)). Additionally, BLAST analysis identified 5 sequences highly related to the 14F1 cluster (GenBank accession no. [GQ251099](#), [GQ251280](#), [GQ251075](#), [GQ251072](#), and [GQ251122](#); Fig. 3) viruses that were isolated in Italy and phylogenetically linked to those in Brazil (43). Cluster 16A1 was a sexually transmitted cluster (5 heterosexual and 3 MSM).

Established local transmission of non-B/G HIV-1 subtypes. In order to estimate the evolutionary dates of the reported transmission clusters, we performed a Bayesian MCMC analysis. All independent runs converged to almost identical values for all pa-

TABLE 3 Characterization of the 17 HIV-1 transmission clusters identified in the study population of the Portuguese region of Minho

Cluster name	No. of individuals	HIV-1 subtype	Yr of diagnosis (mean, range)	Route of transmission (n) ^a	Time of MRCA	
					Yr	95% HPD
01DB	3	DB URF	2009, 2006–2012	IDU (4), heterosexual (1)	2002	1998.2–2006.1
02B	3	B	2006, 2003–2008	IDU (2), heterosexual (1)	1999	1994.2–2003.3
03B	5	B	2003, 2000–2009	Heterosexual (2), MSM (1)	2004	2001.9–2006.5
04B	3	B	2007, 2006–2009	Heterosexual (4)	2004	2000.7–2006.9
05B	3	B	2006, 2003–2008	MSM (3), heterosexual (2)	2003	2000.0–2005.6
06B	3	B	2008, 2005–2010	Heterosexual (3)	2002	1998.4–2004.8
07B	3	B	2009, 2008–2010	Heterosexual (3)	2004	2000.0–2006.9
08B	5	B	2007, 2000–2010	Heterosexual (2), MSM (1)	2002	1998.1–2004.4
09G	7	G	2006, 1999–2009	Heterosexual (5), IDU (2)	2000	1997.1–2003.2
10G	3	G	2010, 2008–2011	Heterosexual (2), IDU (1)	2008	2004.8–2010.4
11C	31	C	2004, 1999–2011	IDU (27), heterosexual (4)	1994	1990.4–1998.1
12C	3	C	2006, 2001–2009	Heterosexual (3)	2005	2001.6–2007.6
13F1	10	F1	2006, 2000–2011	Heterosexual (7), IDU (3)	2000	1993.2–2003.5
14F1	7	F1	2009, 2007–2011	Heterosexual (6), IDU (1)	2005	2002.7–2007.2
15F1	4	F1	2010, 2009–2010	Heterosexual (4)	1994	1987.5–2001.6
16A1	8	A1	2009, 2006–2011	Heterosexual (5), MSM (3)	1999	1994.0–2003.3
17BG	12	CRF14_BG	2002, 2000–2006	IDU (10), heterosexual (2)	1999	1994.5–2002.8

^a IDU, intravenous drug users; MSM, men who have sex with men.

^b MRCA, most recent common ancestor; HPD, high posterior density.

rameters (data not shown). The mean substitution rate was 2.16×10^{-3} (95% highest posterior density [HPD] interval, 1.85×10^{-3} to 2.49×10^{-3}) substitutions per site per year. The date of the most recent common ancestor (MRCA) was determined for all the clusters and ranged from 1994 to 2008 (Table 3). There were no marked differences in the MRCA dates among the different subtypes. Among the clusters of nonprevalent HIV-1, the 11C, 15F1, 16A1, and 17BG subtypes were the ones with older MRCA dates, ranging from 1994 to 1999. These clusters are composed of viruses from Portuguese individuals presumed to be infected in Portugal (Table 3). These results support the existence of clusters of nonprevalent subtypes that started transmitting among the local population more than a decade ago. To gain insights into the activities of the clusters over the years, the date of HIV-1 diagnosis for all cluster-included individuals was analyzed. For the majority of the clusters, the results are suggestive of continuous onward transmission (Table 3). A possible exception is cluster 17BG, since it showed an earlier mean date of diagnosis (2002) and since in the 6 years from 2006 to 2012, no viruses belonging to this cluster were found in the study population, thus suggesting a decrease in transmission.

DISCUSSION

Since its origin in Africa approximately 100 years ago, HIV-1 has continuously been undergoing genetic diversification that is enhanced by the massive globalization of the human population (21). Despite the perception that current antiretroviral regimens have comparable efficacies across existing diverse HIV-1 subtypes, there is evidence showing that some HIV-1 subtypes may have a transmission advantage, higher replicative efficiency, or even altered drug susceptibility (7–11), raising awareness on the relevance of investigating HIV-1 diversity. Furthermore, for the effective targeting of preventive measures, it is very relevant to perform persistent monitoring of the HIV-1 pandemic using phylogenetic and epidemiological data analyses as tools in the reconstruction of viral transmission networks (27–29).

Existing data show that Portugal contrasts with the rest of west-

ern Europe in its distribution of HIV-1 subtypes (18, 21). It is important to gain a further understanding on the causes underlying this difference, also in light of the evidence supporting a recent increase in infections with non-B HIV-1 subtypes in several western European countries (15–17, 44). In this study, we analyzed 289 HIV-1-infected individuals from Minho province, Portugal. Collectively, the results obtained are consistent with previous studies in Portugal showing a high prevalence of non-B subtypes (73.0%), mainly viruses of the G subtype (29.4%), followed by those of the C (14.5%) subtype. In our study population, the heterogeneity of HIV-1 subtypes is attributable to Portuguese-born individuals presumed to be infected in the region, with only 3.8% of the cases occurring in immigrants or individuals from Portugal presumed to be infected elsewhere. Contrarily, the rising prevalence of non-B HIV-1 subtypes in western Europe has been attributed to the growing number of immigrants from sub-Saharan Africa and South America, where these variants are prevalent. As an example, 27% of the HIV-1 cases diagnosed in 2007 in Spain were of non-B subtypes, and 90% of these cases were African and South American immigrants (45). A phylogenetic analysis of our study population indicates high interindividual genetic distances for the most prevalent subtypes, B and G, suggesting old and multiple introductions of viruses of these subtypes in the region. This observation is in accordance with the predominance of the B subtype in western Europe, having probably been introduced on several occasions in the late 1970s and early 1980s (46). As for the G subtypes, it is possible that the intense human migrations between Portugal and its former African colonies in the 1970s and 1980s, due mostly to the independence wars (47), contributed to its early introduction in Portugal. These intense human migrations may have also contributed to the early introduction of other HIV-1 subtypes, namely in the case of the migration connection with Angola, where there is a large HIV-1 genetic diversity (48). The distribution of clustered sequences per subtype showed that the vast majority of the F1, C, A1, and CRF14_BG sequences were incorporated in clusters. Contrarily, the B and G subtype clusters

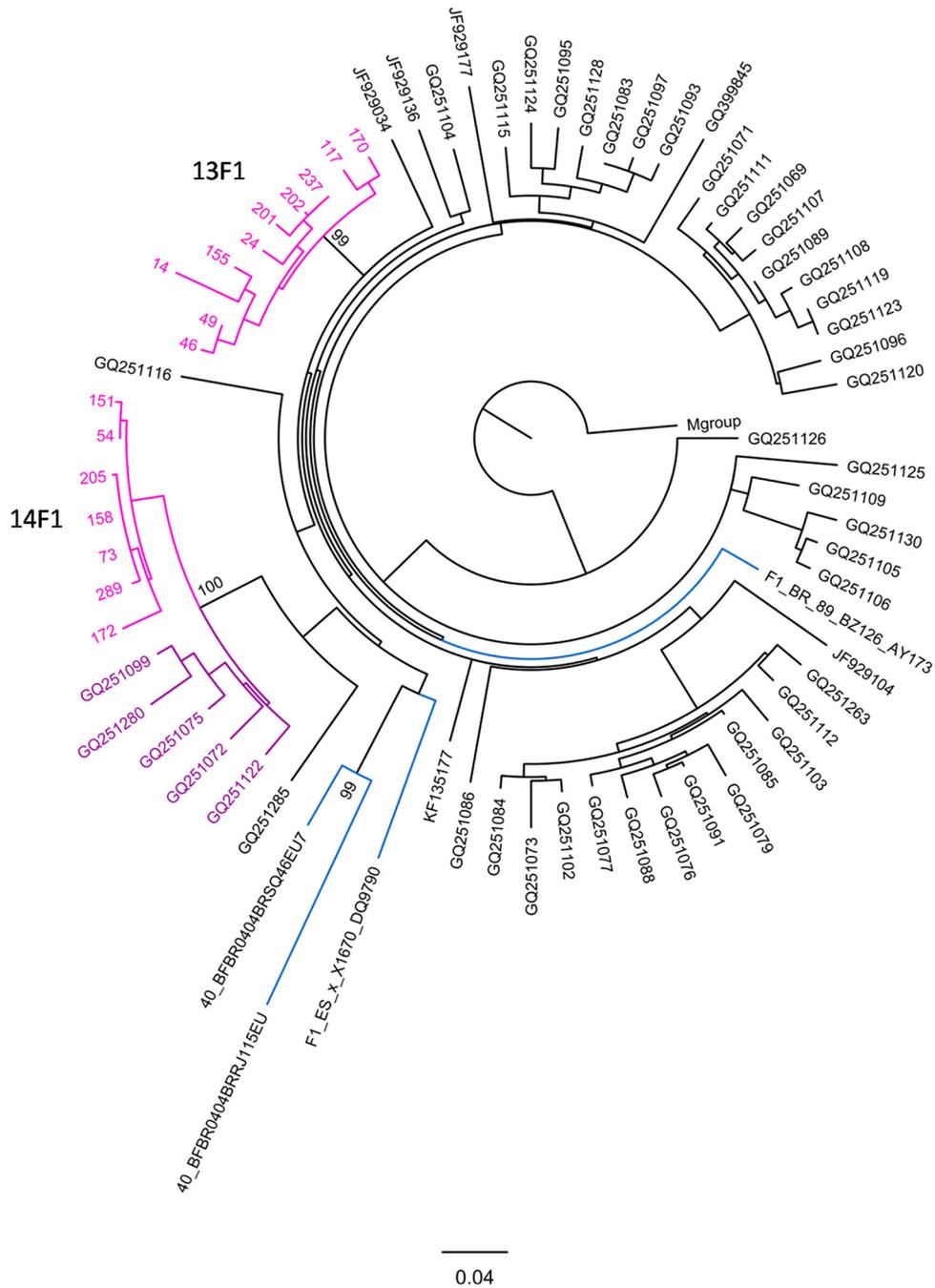


FIG 3 Phylogenetic relationships between the 13F1 and 14F1 transmission cluster sequences and public sequences available in the databases. Maximum likelihood (ML) phylogenetic analysis was performed using 13F1 and 14F1 sequences (pink), 4 subtype references (blue), and the 50 most similar sequences obtained in the BLAST query of all publically available sequences. Highlighted in purple are the public sequences that are included in cluster 14F1. The tree was rooted using the M-group consensus.

include only 35.9% and 11.8% of the respective subtype sequences, likely due to the long-time circulation of B and G subtypes among the studied population. Interestingly, clusters of non-B and non-G subtypes were also found, namely those of the C, F1, A1, and CRF14_BG viruses, with MRCA dates in the late 1990s, even when considering only Portuguese-born individuals presumed to be infected in the region. This supports the hypothesis that these HIV-1 subtypes were introduced in Minho more

than one decade ago. The two largest transmission clusters, 11C and 17BG, are in a large majority (>87%) composed of IDU, reflecting the compartmentalization and closed character of the transmission among individuals from this risk group. The analysis of the date of diagnosis of the individuals in these clusters suggests that its transmission might be decreasing, namely in the case of 17BG, since in the 6 years ranging from 2006 to 2012, no HIV-1 infection with viruses from this cluster were found in the study

population. In the last decade, other local epidemics with CRF14_BG have been described among IDU in Spain and Portugal (20, 22). Our results are in line with the data showing a decrease in the prevalence of HIV-1 among IDU in Portugal (28), consequently reducing the transmission of BG recombinant viruses. Importantly, our data support an increased incidence of infection with the F1 and A1 subtypes in the study population. The occurrence of onward transmission events of F1 and A1 HIV-1 subtypes in our study population was strongly linked to sexual transmission. We identified three F1 clusters, mainly formed by individuals who report heterosexual contact as the presumed viral transmission route. The phylogenetic analysis shows that the viral sequences from clusters 13F1 and 14F1 have common ancestors with reference sequences collected in Brazil. The analysis of the public databases allowed the identification of 5 sequences isolated in Italy that belong to the 14F1 cluster, thus suggesting a large geographic range of this transmission network, contrary to what was found in all the other clusters. In common with the 13F1 and 14F1 clusters, the previously described Italian cluster was also phylogenetically linked to Brazil (43), suggesting this country as the origin of subtype F1 viruses in southern Europe. We also identified one sexually transmitted A1 subtype cluster (16A1) that was epidemiologically linked to Mozambique, since a viral sequence isolated from one Mozambican presumed to be infected prior to immigrating to Portugal had a common ancestor with the cluster.

Overall, our study of the local HIV-1 epidemic in the Portuguese region of Minho supports that it contrasts with other regions of western Europe in its high HIV-1 diversity. This diversity is increasing due to transmission among native individuals and not in a manner driven by immigration or international travel. Our molecular and epidemiologic analyses highlight the onward transmission of the F1 and A1 subtype viruses via sexual transmission routes, supporting the need for continuous monitoring and strengthening of preventive strategies targeted at this transmission mode.

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