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Prevalence of Human Immunodeficiency Virus Type 1 (HIV-1) Non-B Subtypes in Foreigners Living in Madrid, Spain, and Comparison of the Performances of the AMPLICOR HIV-1 MONITOR Version 1.0 and the New Automated Version 1.5

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Plasma specimens collected in 1999 from 32 human immunodeficiency virus type 1 (HIV-1)-infected foreigners living in Madrid, Spain, were examined for the presence of non-B subtypes. Furthermore, plasma viremia was quantified using two different AMPLICOR HIV-1 MONITOR tests, version 1.0 and the new upgraded and automated version 1.5 (COBAS). Most patients came from Africa, where they most likely had acquired HIV-1 infection through sexual contact. HIV-1 genetic subtyping was based on the phylogenetic analysis of the *protease* gene. Twenty-two subtype B, six subtype G, two subtype C, one subtype A, and one D subtype infection were found. Overall, non-B subtypes represented 31.25% of the study population. Irrespective of the HIV-1 variant, viral load values above the detection limit (200 HIV RNA copies/ml) increased from 56.2 to 71.9% for results obtained using MONITOR version 1.0 and COBAS, respectively. Moreover, significant differences in viral load values (>0.5 logs) were recognized in up to 37.5% of samples. In summary, COBAS seemed to be more reliable for testing plasma viral load in HIV-infected immigrants living in Spain, one third of whom carried non-B subtypes.

Human immunodeficiency virus type 1 (HIV-1) mutates rapidly, contributing to its high degree of genetic heterogeneity in vivo (3). Methods based on nucleotide sequence analyses allow the recognition of phylogenetic relationships between different sequences. So far HIV-1 can be divided into three distinct and highly divergent groups: M (major), O (outlier), and N (new) (19, 25, 30). At least fourteen major genetic variants can be recognized within HIV-1 group M, including several subtypes (A, B, C, D, F, G, H, J, and K) and four major circulating recombinant forms (CRF01-AE, CRF02-AG, CRF03-AB, and CRF04-cpx, "complex") (19, 33). Classification of HIV-1 into subtypes is based primarily on the analysis of genetic sequences coding for the envelope (*env*) and other structural (*gag, pol*) proteins.

In Spain, as in North America and other Western European countries, HIV-1 subtype B is the most prevalent HIV-1 variant (15). Non-B subtypes have been reported mainly in Africa, where a large diversity of HIV-1 variants has been found (18). However, the prevalence of HIV-1 non-B subtypes seems to be increasing in North America (24, 34) and Europe (6, 10, 15, 16, 24), and limitations of the current commercial HIV-1 quantitation assays examining these specimens have been pointed out recently (1, 2, 17, 32), since these tests were originally designed on the basis of HIV-1 subtype B sequences. Herein we investigate the prevalence of HIV-1 subtypes in a group of 32 infected foreigners living in Madrid, Spain, and analyze the performance of two different versions of the AMPLICOR HIV-1 MONITOR test in samples belonging to these subjects.

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MATERIALS AND METHODS

Blood specimens from 32 HIV-1-infected immigrants attending one HIV unit located in Madrid were collected in 1999. Twenty-seven (84.4%) were from Africa, four (12.5%) were from South America, and one (3.1%) was from Eastern Europe. Epidemiological and clinical data are summarized in Table 1. Plasma aliquots were separated from blood cells within 4 h following phlebotomy and were frozen at −80°C until the time of analysis. The $CD4^+$ lymphocyte count was analyzed by flow cytometry (Coulter, Barcelona, Spain).

The characterization of HIV-1 subtypes was performed by phylogenetic analysis of the *protease* gene as previously described (16). We used 21 HIV-1 reference sequences belonging to HIV-1 groups M and N having full-length genomes available at GenBank. The tree topology was obtained using the neighbor-joining program (27). Alignment of DNA sequences was performed using the CLUSTAL W method (31). Pairwise distance matrices were estimated using the Kimura two-parameter model with the DNADIST program, as implemented in the PHYLIP software package (13). Bootstrap resampling (1,000 data sets) of the multiple alignment was done to test the statistical robustness of the tree.

Plasma viremia was quantified using the two different versions of the AMPLICOR HIV-1 MONITOR test (Roche Diagnostics, Barcelona, Spain), a reverse transcription-PCR-based assay designed for quantifying HIV-1 RNA in plasma (22). Version 1.0 was the original commercial test. The other one was a prototype automated procedure of version 1.5 (COBAS), in which HIV-1 RNA amplification and detection take place on the COBAS AMPLICOR instrument (9). Both methods differ in the primers used for reverse transcription and PCR, the composition of the reverse transcription-PCR mixture, the thermal cycling parameters, and the internal quantification standard RNA (21, 32). Hypothetically, COBAS provides more reliable viral load data, since it is substantially less influenced by viral subtype (21).

Nucleotide sequence accession numbers. *Protease* sequences have been submitted to the GenBank database with the accession numbers AF247007 to AF247038.

TABLE 1. Epidemiological features of the study population and genetic heterogeneity in amino acid positions of the *protease* gene product associated with resistance

Sample code	Gender[a]	Age	Clinical status	Country of birth[b]	Country of diagnosis	Transmission route[c]	CD4 count (cells/μl)	Antiretroviral therapy (months)[d]	Amino acid changes of the *protease* gene product[e]	Subtype	Accession no.
M1158	M	24	A3	Morocco	Spain	Htsex	232	Naive	L63T	B	AF247007
M432	M	32	A2	Bulgaria	Spain	Htsex	531	Naive	None	B	AF247008
M2426	F	8	C2	EG	EG	Vertical	100	Naive	V77I	B	AF247009
M2131	F	24	A1	Nigeria	Spain	Htsex	988	Naive	V77I	B	AF247010
M513	M	33	A2	Peru	Spain	Htsex	478	Naive	None	B	AF247011
M2385	F	31	A2	EG	Spain	Htsex	487	Naive	None	B	AF247012
M1240	M	6	B2	Honduras	Honduras	Htsex	606	Naive	L101, L63T	B	AF247013
M927	F	28	A1	EG	EG	Htsex	785	Naive	L63S, V77I	B	AF247014
M1642	M	33	A3	Morocco	Spain	Htsex	198	Naive	L10I, L63P	B	AF247015
M963	M	37	B3	Ecuador	Spain	Homo	95	Naive	M36I	B	AF247016
M2067	F	23	A1	EG	Spain	Htsex	737	Naive	M36L, L63P	B	AF247017
M2133	F	39	A1	EG	Spain	Htsex	847	Naive	M36I	A	AF247018
M959	F	34	A2	EG	Spain	Htsex	309	Naive	K20R, M36I, L63A	C	AF247019
M2489	F	20	A1	EG	EG	Htsex	916	Naive	M36I, L63A	C	AF247020
M1296	F	31	A3	EG	EG	Htsex	94	Naive	M36I, L63S	D	AF247021
M1911	F	33	A3	Zaire	Spain	Htsex	247	Naive	L10I, K20I, M36I, V77I	G	AF247022
M2444	F	35	A2	EG	EG	Htsex	471	Naive	L10S, K20I, M36I, L63S, V77I	G	AF247023
M916	F	30	A2	EG	Spain	Htsex	406	Naive	K20I, M36I	G	AF247024
M635	F	43	C3	EG	Spain	Htsex	142	AZT + 3TC (11)	M46L, L63P	B	AF247025
M2671	F	32	A3	Nigeria	Spain	Htsex	374	AZT + 3TC (29)	M36I, V77I	B	AF247026
M2389	F	64	A2	EG	Spain	Htsex	399	AZT + ddI (24)	L10I, K20M, M36I, M46L, L63P, A71V, I84V	B	AF247027
M2558	F	30	A2	EG	Spain	Htsex	343	AZT + ddC (30)	K20I, M36I, L63P	G	AF247028
M787	M	48	A2	Nigeria	Spain	Htsex	467	3TC + d4T (13)	L10I, K20M, M36I, M46L, L63P, A71V, L90M	B	AF247029
M2814	F	39	A2	EG	Spain	Htsex	504	AZT + 3TC + NVP (9)	L10I, K20M, M36I, M46L, L63P, A71V, I84V, L90M	B	AF247030
M1303	F	9	A1	Zaire	Spain	Vertical	450	3TC + d4T + RTV (5)	M36I	B	AF247031
M430	F	27	A3	Nigeria	Spain	Htsex	140	3TC + d4T + RTV (7)	L10I, M36I, M46L, L63P, A71V, L90M	B	AF247032
M2346	F	28	B3	EG	Spain	Htsex	28	3TC + d4T + IDV (16)	L63P, V77I	B	AF247033
M2725	F	33	B3	Zaire	Spain	Htsex	10	AZT + 3TC + IDV (16)	L10I, K20M, M36I, M46L, L63P, A71V, L90M	B	AF247034
M2686	M	26	A3	Ecuador	Spain	Htsex	427	AZT + 3TC + SQV (30)	L10I, K20M, M36I, M46L, L63P, A71V	B	AF247035
M2388	F	32	A2	Cape Verde	Spain	Htsex	535	AZT + ddI + NVP (7)	L10S, K20I, M36I, L63P, V82I	G	AF247036
M2773	M	36	C3	Cameroon	Cameroon	Htsex	161	D4T + NVP + NFV (8)	K20I, M36I	G	AF247037
M1743	M	27	C3	Nigeria	Spain	Htsex	134	ddI + NVP + SQV + NFV (15)	M36I, L63P	B	AF247038

[a] F, female; M, male.
[b] EG, Equatorial Guinea.
[c] Htsex, heterosexual; Homo, homosexual.
[d] AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; NVP, nevirapine; RTV, ritonavir; IDV, indinavir; SQV, saquinavir; NFV, nelfinavir.
[e] Amino acid codons at positions 10, 20, 30, 36, 46, 48, 50, 54, 63, 71, 77, 82, 84, and 90 were analyzed.

RESULTS

HIV-1 genetic subtype characterization and main epidemiological features. The presence of HIV-1 non-B subtypes in Spain has been reported previously (15, 16). All patients enrolled in this study were HIV-1-infected foreigners living in Madrid, mostly coming from African countries (84.4%) where most HIV-1 subtypes cocirculate (18). Interestingly, although all 32 subjects had probably acquired HIV-1 infection in their country of origin, in 78% of the cases their first diagnosis was in Spain.

The epidemiological features of the study population and the assignment of its genetic HIV-1 subtype are summarized in Table 1. Twenty-two subtype B, six subtype G, two subtype C, one subtype A, and one D subtype infection were found. Phylogenetic tree topology was supported by high bootstrap values (Fig. 1). Although sample no. M1296 could not be assigned initially to a specific known subtype, when the tree was performed exclusively with this sample and the reference strains, this specimen clustered within subtype D variants (data not shown).

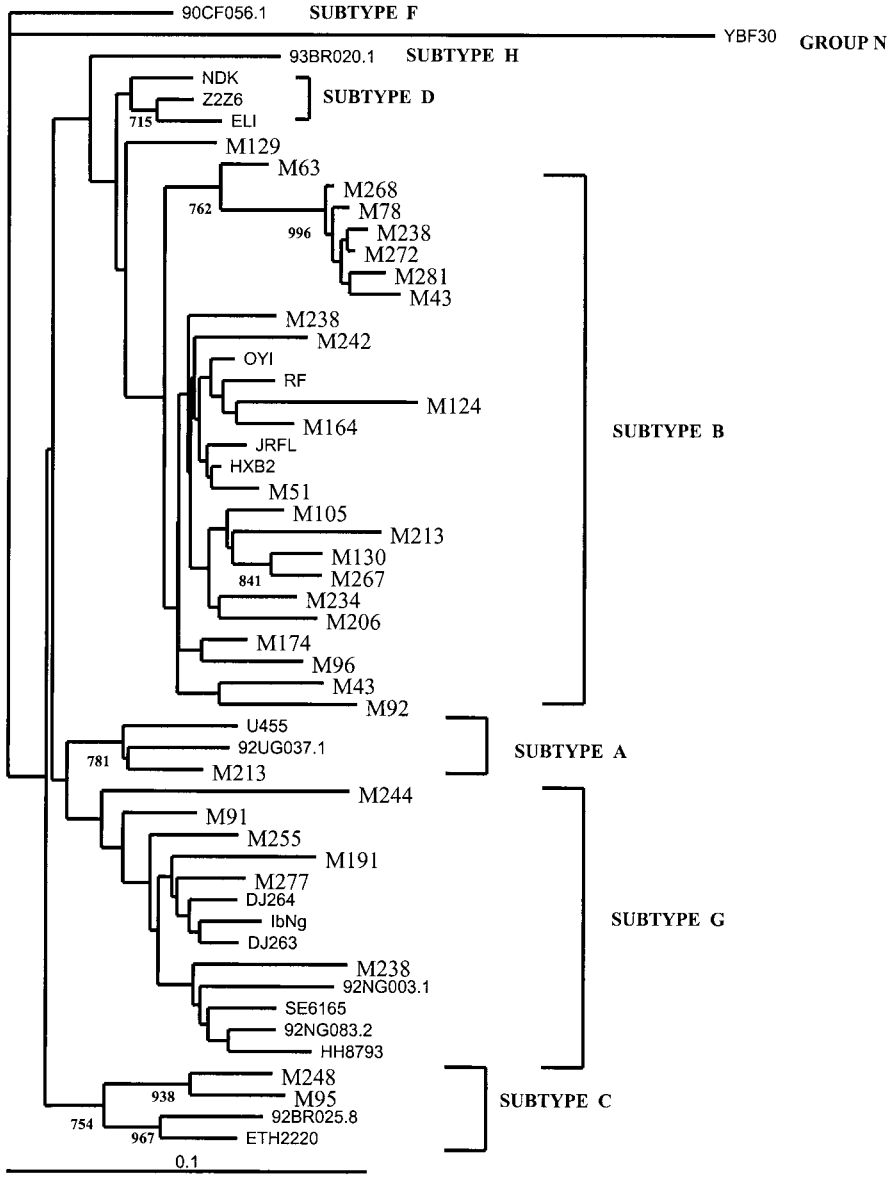

FIG. 1. Phylogenetic tree of the HIV-1 *protease* coding region from 32 foreigners living in Spain (bold). Bootstrap resampling values (1,000 sets) are indicated. The lengths of the branches are proportional to the relative evolutionary distances.

Overall, nearly one third (31.25%) of the HIV-1-infected foreigners living in Madrid carried HIV-1 non-B subtypes. All of them were African. All but one were women, and seven of them admitted to being engaged in prostitution. The origins of individuals harboring HIV-1 subtype G were Equatorial Guinea ($n = 3$), Zaire ($n = 1$), Cape Verde ($n = 1$), and Cameroon ($n = 1$). All subjects carrying subtypes A, C, and D came from Equatorial Guinea, a former Spanish colony.

Drug resistance mutations in non-B subtypes. Genetic changes associated with resistance to protease inhibitors were recognized in both naive and pretreated individuals carrying HIV-1 non-B subtypes (Table 1). For example, the secondary substitution Met36→Ile was found in all seven naive subjects infected with non-B subtypes, while the change Val77→Ile was seen in two subtype G specimens. These changes have been associated with resistance to nelfinavir and ritonavir, respectively (29). On the other hand, drug resistance mutations in subjects with non-B subtypes under antiretroviral therapy appeared at the same positions as those that were reported for subtype B (29) (Table 1).

Performance of viral load tests. Regardless of the HIV-1 variant, positive quantitative values above the detection limit (200 HIV-RNA copies/ml) increased from 56.2% for MONITOR version 1.0 to 71.9% for COBAS (Student's t test, $P < 0.05$) (Table 2). On average, the newer method outperformed version 1.0 in all aspects of HIV-1 testing. The differences between the geometric mean titers of version 1.0 (341,401) and COBAS (539,009) were found to be statistically significant (Fisher exact test, $P = 0.05$).

Differences in viral load values above 0.5 logs were consid-

TABLE 2. Differences between HIV-1 plasma viral load results provided by AMPLICOR HIV-1 MONITOR version 1.0 and COBAS

Sample code	Genetic subtype	Viral load results (titers) for:		Difference of COBAS vs. version 1.0 (log)
		Version 1.0	COBAS	
M2133	A	<200	3,650	>1.26
M2489	C	32,700	6,720	<0.68
M959	C	44,600	19,600	<0.36
M1296	D	59,300	337,000	>0.75
M916	G	<200	99,500	>2.69
M2444	G	4,300	4,230	<0.0004
M2388	G	5,800	10,300	>0.25
M1911	G	28,700	222,000	>0.88
M2558	G	146,000	82,200	<0.25
M2773	G	2,349,500	9,160,000	>0.59
M2131	B	<200	<200	0
M2067	B	<200	<200	0
M2671	B	<200	<200	0
M2389	B	<200	<200	0
M787	B	<200	<200	0
M2814	B	<200	<200	0
M2346	B	<200	<200	0
M2725	B	<200	<200	0
M2686	B	<200	<200	0
M430	B	<200	667	>0.52
M635	B	<200	8,100	>1.61
M1303	B	<200	20,500	>2.01
M927	B	8,900	4,330	<0.31
M432	B	36,700	53,700	>0.15
M2426	B	47,100	138,000	>0.46
M2385	B	60,600	176,000	>0.45
M513	B	90,300	1,530,000	>1.23
M963	B	95,350	543,000	>0.75
M1240	B	198,300	201,000	>0.03
M1158	B	213,200	182,000	<0.07
M1642	B	2,340,000	56,000	<1.61
M1743	B	5,160,700	4,260,000	<0.08

ered significant and were recognized in up to 37.5% of cases. They occurred more frequently in non-B rather than subtype B specimens (60 versus 27.3%) (Table 2). One specimen belonging to subtype G (M916) yielded repeated discrepancies in plasma viremia above 2.5 logs. However, viral load values were significantly higher using version 1.0 in 4 of 10 HIV-1 non-B subtype specimens (Table 2). With respect to subtype B specimens, 41% showed significantly higher values using COBAS, and 18.2% showed higher values using version 1.0.

DISCUSSION

Performance of two different versions of the AMPLICOR HIV-1 MONITOR test. Quantitative values of plasma viremia using the currently available viral load assays can be unreliable for testing non-B subtypes or recombinant forms of HIV-1 (1, 5, 7, 11, 17). The AMPLICOR HIV-1 MONITOR test, version 1.0, was developed when little sequence information on HIV-1 subtypes was available (22). The primers used (SK431 and SK462) were designed on the basis of a subtype B consensus sequence (22). This fact explains the low performance of this assay for testing non-B subtypes. It is estimated that HIV-1 subtypes A, E, F, and G were underestimated by 10-fold or more. The use of COBAS, which was developed to minimize subtype-related variation (21), seemed to allow an equivalent quantitation of HIV-1 RNA regardless of the subtype. It uses a set of primers (SK145 and SKCC1B) which are based on a non-B subtype consensus sequence (21, 32). The lower viremia values provided by COBAS in 40% of samples from subjects carrying non-B subtypes and in 18.2% of those with subtype B found in our study was an unexpected finding. It could be due to the difference in how the amplicons are captured (microwells versus microbeads) by the detection reagents used in the prototype automated test or the presence of mismatches in sample primer binding sites. The recognition of higher levels of HIV-1 plasma viremia by either assay in two subjects carrying subtypes G (M2773) and B (M1743) and being under triple or quadruple antiretroviral combinations was also unexpected, but it might be explained by noncompliance with the prescribed treatment noticed in these subjects.

The high number of specimens showing significant differences (>0.5 log) in viral load values between the AMPLICOR quantification versions strongly reinforces the importance of always monitoring HIV-1-infected patients with the same version of any viral load quantitation technique (17).

Epidemiological implications of the spreading of non-B subtypes. The presence of HIV-1 non-B subtypes in Spain has been reported previously (15, 16). However, in our study nearly one third of the population (31.25%) carried non-B subtypes. All 10 subjects infected with non-B subtypes came from Africa, where a large variety of HIV-1 subtypes and recombinant forms are circulating (18). Seven of those 10 patients with HIV-1 non-B subtypes were identified as prostitutes. The reported promiscuity of the three other individuals or their sexual partners should remain in question. Spreading of these minor HIV variants among native individuals in Spain is of particular concern, as it seems to happen in other European countries (10) and the United States (34). The primary diagnosis of more than three-fourths of these patients occurred in Spain. This reinforces the fact that HIV testing should be offered to all persons belonging to high-risk groups and/or emigrating from regions of high endemicity where testing is not available.

The spread of different HIV-1 subtypes in a single geographic region coupled with intersubtype recombination (16, 26) has serious implications for the efforts to control the AIDS pandemic. The impacts of the different genetic subtypes on pathogenesis, the course of HIV infection, transmissibility, vaccine efficacy, and diagnosis based on serologic (20) or PCR assays (2) are not yet well known and must be further studied (12). It has been previously reported that susceptibility to antiretroviral drugs might differ between distinct subtypes (8, 23). In this study we have shown that mutations associated with resistance to protease inhibitors appear in HIV-1 non-B subtypes at the same positions that they do in subtype B strains. This observation suggests that the distinct HIV-1 subtypes evolve convergently at the genetic level when antiretroviral drugs act as selective forces (28).

Studies designed to monitor the spread of HIV-1 subtypes should be encouraged. In areas where non-B subtypes represent a significant proportion of infections, viral load quantitation tests able to appropriately recognize the different viral variants should be implemented.

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