# Human immunodeficiency virus type 1 recombinant B/G subtypes circulating in Coimbra, Portugal

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An increasing prevalence of HIV-1 non-B variants is being noticed in several European regions, particularly in countries such as Portugal, which have closer contacts with African endemic areas, where multiple HIV subtypes cocirculate. HIV-1 subtyping by phylogenetic analyses of reverse transcriptase, protease and *env* (C2–V3) genomic regions was carried out in plasma collected from 18 HIV-1-infected subjects living in Coimbra, Portugal, and suspected to be infected with non-B variants. Three (16.7%) subjects carried recombinant B/G viruses ($B^{V3}/B^{RT}/G^{pro}$; $G^{V3}/U^{RT}/B^{pro}$; $A^{V3}/G^{RT}/B^{pro}$), whereas all the remaining individuals were infected with HIV-1 subtype B. This is the first report of recombinant B/G subtypes in Portugal.

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HIV-1 can be divided into three distinct and highly divergent groups: M (major), O (outlier), and N (new). There are at least 21 major genetic forms within HIV-1 group M, including nine subtypes (A, B, C, D, F, G, H, J, K) and 12 major circulating recombinant forms [1–4]. Classification of HIV into subtypes is based primarily on the analysis of genetic sequences coding for the envelope (*env*) and other structural (*gag*, *pol*) proteins [1]. Subtype B circulates throughout the world, and is predominant in North America and Europe. However, non-B subtypes have been recently noticed in different European countries [5–8], and are widely present in Africa [1,9]. The proportion of HIV-1 subtypes and its distribution is not uniform in Sub-Saharan African regions [9–11], where a large variety of recombinant forms is circulating [2,9].

Knowledge of the circulating HIV genetic subtypes in a community may have important implications for diagnosis. For instance, the sensitivity of some serologic tests may be compromised [12], as well as the performance of nucleic acid testing [13–15], including viral load assays [16,17]. Less probably, HIV transmission and disease progression may be subtype dependent [18,19].

An extensive relationship between Portugal and distant regions in other continents has existed for many centuries, especially with the former Portuguese territories situated in west (Guinea-Bissau), central (Angola) and south (Mozambique) Sub-Saharan regions of Africa, as well as in South America (Brazil). Travel for tourism or business reasons to these areas continues to be common for the Portuguese population. The objective of this work was to seek the presence of HIV-1 non-B subtypes in Coimbra, Portugal. To our knowledge, this is the first study evaluating this issue in our country.

We selected a group of 18 HIV-infected subjects, probably infected overseas and undergoing regular follow-up in one outpatient clinic located in Coimbra. Thirteen (72%) were males. Eleven (61%) had acquired HIV-1 infection in Sub-Saharan Africa, four in western Europe, and three in South America. Ten (55%) subjects were born in Portugal, and the rest were foreigners, mostly coming from countries with Portuguese as the official language (Brazil, Mozambique, Angola, and Guinea-Bissau), where non-B subtypes are circulating [1,9]. Their main epidemiologic and clinical data are given in Tables 1 and 2, respectively.

The diagnosis of HIV infection in these patients was made between 1987 and 1999 by ELISA, and was confirmed by Western blot. Quantification of

**Table 1** Main epidemiologic features of the study population

| Patient | Age (years) | Gender | Country of birth | Country of infection | Route of infection | Year of diagnosis | Subtype | GenBank accession number |
|---|---|---|---|---|---|---|---|---|
| P2 | 43 | M | Guinea-Bissau | Guinea-Bissau | Htsex | 1998 | B | – |
| P4 | 58 | M | Portugal | Tanzania | Htsex | 1999 | B | AF204117 |
| P5 | 54 | M | Portugal | Rwanda | Htsex | 1991 | B | AF204118 |
| P6 | 26 | F | New Zealand | Germany | Htsex | 1996 | B | AF204119 |
| P8 | 40 | M | Portugal | Italy | IDU | 1987 | B | AF204121 |
| P9 | 55 | M | Guinea-Bissau | Guinea-Bissau | Htsex | 1999 | B | AF204122 |
| P10 | 29 | F | Brazil | Brazil | IDU | 1993 | B | AF204123 |
| P12 | 52 | M | Portugal | Angola | Htsex | 1996 | B | AF204125 |
| P13 | 38 | M | Portugal | Zaire | Htsex | 1997 | B | AF204126 |
| P18 | 56 | M | Portugal | Namibia | Htsex | 1999 | B | AF204131 |
| P19 | 60 | M | Mozambique | Mozambique | Htsex | 1998 | B | AF204132 |
| P21 | 30 | F | Italy | Italy | IDU | 1998 | B | AF204134 |
| P23 | 41 | M | Mozambique | Mozambique | Htsex | 1998 | B | AF204136 |
| P24 | 36 | F | Portugal | Switzerland | IDU | 1987 | B | AF204137 |
| P25 | 31 | F | Angola | Angola | Htsex | 1997 | B | AF204138 |
| P28 | 28 | M | Portugal | Cuba | Htsex | 1997 | B | AF204141 |
| P29 | 38 | M | Portugal | Zaire | Htsex | 1998 | G | AF204142 |
| P31 | 30 | M | Portugal | New Zealand | Htsex | 1996 | B | AF204144 |

F, female; M, male; Htsex, heterosexual; IDU, intravenous drug user.

HIV-1 RNA in plasma was performed with the Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Lisbon, Portugal). All patients were classified according to the Centers for Disease Control (CDC) criteria. The $CD4^+$ lymphocyte count was measured by flow cytometry. Samples were taken during September 1998 and April 1999.

HIV-1 RNA was extracted from 500 μL of each plasma specimen. A 297-bp nested PCR of the *protease* gene was performed, under conditions previously described [20]. The analysis of a 550-bp region within the *RT* gene and of a 318-bp fragment within the *env* gene covering the V3 region was done as previously reported [21].

**Table 2** Main clinical features of the study population

| Patient | $CD4^+$ lymphocyte count (cells/$mm^3$) | Viral load (HIV RNA copies/mL) | Antiretroviral therapy | CDC category | Reason for HIV testing |
|---|---|---|---|---|---|
| P2 | 264 | 24 612 | D4T + 3TC + IDV | B.2 | Routine screening |
| P4 | 18 | 206 000 | AZT + 3TC + NFV | C3 | *Pneumocystis carinii* pneumonia |
| P5 | 352 | 370 086 | d4T + ddI + NFV | C3 | Herpes zoster ophtalmicus |
| P6 | 242 | 80 481 | d4T + 3TC + IDV | A3 | HIV-positive partner |
| P8 | 528 | <400 | d4T + 3TC + NFV | C3 | Routine screening |
| P9 | 48 | 703 000 | Naive | C3 | Herpes zoster ophtalmicus |
| P10 | 154 | 245 000 | Naive | B3 | Pregnancy screening |
| P12 | 513 | 1997 | AZT + 3TC + IDV | A2 | Acute viral syndrome |
| P13 | 88 | <400 | AZT + 3TC + IDV | C3 | Kaposi's sarcoma |
| P18 | 1224 | 953 | d4T + 3TC + NVP | A1 | Acute viral syndrome |
| P19 | 312 | <400 | AZT + 3TC + NFV | C3 | Fever of unknown origin |
| P21 | 465 | 8070 | Naive | A2 | Routine screening |
| P23 | 330 | 14 000 | Naive | A2 | Routine screening |
| P24 | 78 | 150 689 | AZT + 3TC + NFV | C3 | Routine screening |
| P25 | 135 | <400 | d4T + 3TC + SQV | A3 | Pregnancy screening |
| P28 | 648 | 1140 | Naive | A2 | Routine screening |
| P29 | 114 | 10 051 | AZT + 3TC + NFV | A3 | Routine screening |
| P31 | 15 | 86 613 | d4T + 3TC + IDV | C3 | HSV chronic genital ulcers |

ZDV, zidovudine; ddI, didanosine; 3TC, lamivudine; d4T, stavudine; NVP, nevirapine; RTV, ritonavir; IDV, indinavir; SQV, saquinavir; NFV, nelfinavir.

Characterization of HIV-1 subtypes was carried out by direct automatic sequencing of the nested PCR fragments on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Twelve HIV-1 reference sequences belonging to HIV-1 groups M and N isolates, having full-length genomes available at GenBank, were selected for comparative analysis to produce a phylogenetic tree. The tree topology was obtained using the neighbor-joining program, and confirmed with PUZZLE, a maximum likelihood method. Pairwise distance matrices were estimated with the Kimura two-parameter model with the DNADIST program, as implemented in the PHYLIP package. Bootstrap re-sampling (1000 data sets) of the multiple alignment was performed to test the statistical robustness of the tree.

Genetic subtyping in the *protease* gene was performed in all 18 samples processed and amplified on different days. Surprisingly, HIV-1 subtype B was the predominant variant (94.4%). Only one sample (P29) showed a protease sequence clustering within clade G. The phylogenetic tree in Figure 1 shows only HIV-1 subtypes A, B, C and G as reference strains, taken as a root one isolate of HIV-1 group N. Additional phylogenetic trees including all HIV-1 subtypes (A–H) were constructed, and confirmed these results (data not shown). Patient 29 was born in Portugal, but was infected while living in Zaire. Additional genetic sequence analyses of other genomic regions (*env* and *RT*) showed a discrepant topology, clustering within subtype B (Table 3), suggesting that this individual carried a recombinant G/B virus.

The finding of this recombinant virus suggested the possibility that others could also exist. To confirm this hypothesis, we selected those individuals most probably infected in Africa, where most recombinant forms circulate [1,2,9]. Genetic sequence analyses of the *env and RT* regions were performed. Again, a discrepant topology was found in another two samples, P2 and P12 (Table 3). The *RT* region of P12 could not be amplified, and therefore could not be assigned to a specific subtype. Patient 2 was most probably infected in Guinea-Bissau, and patient 12 in Angola.

The purpose of this study was to look for the presence of HIV-1 non-B subtypes in Coimbra, Portugal. Our findings confirm that B/G recombinants are currently circulating and represent the greater proportion of non-B subtypes, being found in all instances among subjects infected overseas.

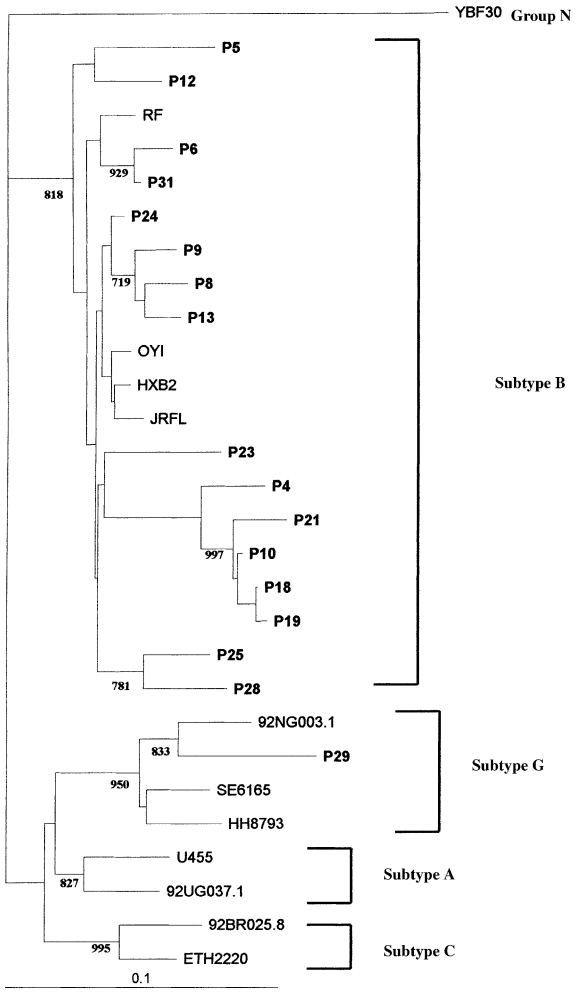


**Figure 1** Phylogenetic tree of the HIV-1 protease-coding region in 18 subjects living in Coimbra, Portugal (in bold).

HIV-1 non-B subtypes and recombinant forms have been previously reported in Spain, our neighboring country [8,21]. Interestingly, subtype G recombinants are the most frequent non-B variants in Spain, where two different clade G variants seem to split epidemiologically. Whereas A/G recombinants predominate among African immigrants [22], B/G recombinants represent the most common non-B variant among natives (mainly

**Table 3** Recombinant nature of viruses in three patients

| Patient | Protease | *RT* | Envelope |
|---|---|---|---|
| P2 | B | G | A |
| P12 | B | NA | G |
| P29 | G | B | B |

NA, not amplified genetic material.

drug users) in some regions such as Galicia [22,23], which is on the Portuguese border. A link between our Portuguese B/G viruses and those found in Galicia is unlikely, considering the large genetic distance reported between virus isolates.

Although HIV-1 non-B subtypes may currently be uncommon in Coimbra, the future public-health impact of their presence is unclear, as is their existence in other Portuguese regions. In the best case, these variants and their recombinants may be confined to their hosts, but further dissemination of these minority HIV-1 variants needs to be investigated. There is growing concern that some HIV-1 subtypes might be less susceptible to antiretroviral drugs, as has been suggested for subtype D [24] and for subtype G [25].

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