Resistance to Nonnucleoside Reverse-Transcriptase Inhibitors and Prevalence of HIV Type 1 Non-B Subtypes Are Increasing among Persons with Recent Infection in Spain

Carmen de Mendoza,[1] Carmen Rodriguez,[2] Javier Colomina,[3] Concepción Tuset,[4] Federico Garcia,[5] Jose M. Eiros,[6] Angelica Corral,[1] Pilar Leiva,[7] Jesús Aguero,[8] Julián Torre-Cisneros,[9] José Pedreira,[10] Isabel Viciana,[11] Jorge del Romero,[2] Ana Saez,[8] Raúl Ortiz de Lejarazu,[6] and Vincent Soriano,[1] on behalf of the Spanish HIV Seroconverter Study Group[a]

[1]Service of Infectious Diseases, Hospital Carlos III, and [2]Centro Sanitario Sandoval, Madrid, [3]Service of Microbiology, Hospital de la Ribera, and [4]Service of Immunology, Hospital General, Valencia, [5]Service of Microbiology, Hospital Universitario, Granada, [6]Service of Microbiology, Hospital Clinico, Valladolid, [7]Service of Microbiology, Hospital Central de Asturias, Oviedo, [8]Service of Microbiology, Hospital Marques de Valdecilla, Santander, [9]Department of Internal Medicine, Hospital Reina Sofia, Córdoba, [10]Department of Internal Medicine, Hospital Juan Canalejo, La Coruña, and [11]Service of Microbiology, Hospital Virgen de la Victoria, Malaga, Spain

The prevalence of drug resistance mutations was 12.1% among 198 persons who experienced human immunodeficiency virus (HIV) seroconversion identified in Spain during 1997–2004. There was a significant increase of K103N and of non-B subtypes over time. Transmission of HIV infection around the time of seroconversion was shown in 8 couples and in 2 clusters of 3 individuals.

Several studies of surveillance of drug resistance among persons who have recently experienced HIV-1 seroconversion (hereafter, "HIV-1 seroconverters") have been conducted in the United States and Europe over recent years [1, 2]. The overall prevalence of primary drug resistance is ~10%–15%, with some differences among regions and time periods. In Spain, studies conducted during the 1990s demonstrated a steadily decrease in the rate of genotypic resistance among drug-naive individuals with chronic HIV-1 infection up to the year 2000 [3, 4]; the rate has remained stable since then [5].

Studies of persons with recent HIV-1 infection are of interest, considering the implications of primary drug resistance for designing first-line therapies [6]. Moreover, it may provide a unique opportunity to monitor the spread of new HIV-1 variants within a region [7, 8] and to track the source of new infections [9]. Herein, we analyzed the prevalence of drug resistance mutations, non-B subtypes, and transmission events during episodes of acute infection among a relatively large population of HIV-1 seroconverters identified in Spain since 1997.

Patients and methods. All consecutive individuals with new HIV-1 infection seen during the period of January 1997 through December 2004 at 15 different hospitals distributed across Spain (Valencia, Elche, Granada, Málaga, Córdoba, Valladolid, La Coruña, Santiago de Compostela, Oviedo, Santander, Tarragona, Barcelona, Badalona, Santa Cruz de Tenerife, and Madrid) were examined. Recent HIV-1 seroconverters were defined as patients with laboratory evidence of acute primary HIV-1 infection (detectable plasma HIV RNA level together with negative or indeterminate HIV antibody test result); reactivity on the Combo assay, with positive results for antigen detection and negative results for antibodies; or seropositivity for HIV-1 infection (by reactive ELISA and Western blot) having had negative results of a prior test performed within the previous 12 months.

Measurement of plasma HIV RNA level was performed using the third-generation branched DNA assay (Versant v3.0; Bayer). The lower limit of detection of this assay is 50 copies/mL. The $CD4^+$ T lymphocyte count was determined by flow cytometry (Coulter) using fluorescein-labelled antibodies. Genetic sequence analyses of both HIV-1 reverse transcriptase and protease genes were performed with plasma specimens using the Viroseq HIV-1 kit (Abbott Laboratories) using an automatic sequencer (ABI Prism 3100; Celera Diagnostics). For the purpose of this study, only major or primary drug resistance mutations recorded in the latest International AIDS Society–USA panel list were considered [10].

To determine the HIV-1 subtype and to explore possible transmission events during episodes of acute infection, *pol* sequences from this population were compared with reference strains, as well as among them. All sequences were aligned with HIV-1 group M reference sequences [11] using the Clustal X method (MegAlign, Lasergene; DNASTAR). Phylogenetic analyses were performed using the Phylip software package, version 3.5c (J. Felsenstein, University of Washington, Seattle). Evolutionary dis-

Received 19 March 2005; accepted 13 June 2005; electronically published 29 September 2005.

[a] The members of the Spanish HIV Seroconverter Study Group are listed at the end of the text.

Reprints or correspondence: Dr. Vincent Soriano, Dept. of Infectious Diseases, Hospital Carlos III, 28029 Madrid, Spain (vsoriano@dragonet.es).

Clinical Infectious Diseases 2005; 41:1350–4

1058-4838/2005/4109-0024$15.00

tances were estimated with Dnadist (Kimura 2-parameter method), and phylogenetic relationships were determined by Neighbor (neighbor-joining method). The branch reproducibility of trees was evaluated using Seqbot (1000 replicates) and Consense.

To confirm transmission events during episodes of acute HIV-1 infection, in addition to *pol* sequences, the v2v3 region from the *env* gene was additionally sequenced and analyzed, with use of conditions described elsewhere [12]. As recommended, all samples were run separately, and a new specimen obtained from each patient was used to exclude cross-contamination [13].

Baseline characteristics of the study population are recorded as percentages, means with SDs, or median values with 25%–75% interquartile ranges. The rate of drug-resistance mutations was expressed as percentage. Student's *t* test was used to compare quantitative variables, and the χ^2 test was used to compare qualitative parameters. The Cochran Mantel-Haenszel test was used to determine the significance of changes in the rate of drug-resistance mutations by year. Nonparametric tests were used to compare the proportions of patients infected with HIV-1 non-B subtypes by route of infection. Significant differences those with a *P* value of <.05. All reported *P* values were 2-sided.

Results. A total of 198 recent HIV-1 seroconverters were identified. The main characteristics of the study population are shown in table 1. Overall, 83.2% of subjects were men, and 70% were infected through male-male sex. The mean estimated duration of HIV-1 infection was 8 months. The mean plasma HIV RNA level and $CD4^+$ cell count at the time of the analysis were 4.6 log copies/mL and 570 cells/μL, respectively.

The overall prevalence of drug-resistant viruses in this population was 12.1% (24 of 198 patients). With regard to drug types, the prevalences were 9.6% (19 patients), 4% (8 patients), and 2% (4 patients) for nucleoside reverse-transcriptase inhibitors (NRTIs), nonnucleoside reverse-transcriptase inhibitors (NNRTIs), and protease inhibitors, respectively (table 1). By calendar year, the prevalences were 33.3% for 1997, 29.4% for 1998, 20% for 1999, 14.3% for 2000, 3.3% for 2001, 14.3% for 2002, 10% for 2003, and 7.7% for 2004 (figure 1). A linear-by-linear association showed a significant reduction in the overall prevalence of drug resistance among recent HIV-1 seroconverters over time ($P = .007$). This was mainly driven by a significant decrease in the rate of NRTI resistance mutations in recent years ($P = .001$). In contrast, a significant increase in the prevalence of NNRTI resistance mutations was noticed in recent years, mainly driven by K103N. Although this mutation was absent before the year 2002, the prevalence increased to 4% in 2003 and to 7.7% in 2004 ($P = .036$). Drug resistance to protease inhibitors remained relatively stable during the whole study period, with identification of mutations at codons 46, 82, and 90 as the main transmitted protease resistance mutations (table 1).

Table 1. Main characteristics of the study population of patients with recent HIV-1 seroconversion.

Variable	Value
No. of patients	198
Male sex, % of patients	83.2
Age, mean years	31
Risk group, % of patients	
Men who have sex with men	70
Heterosexual	19.5
Intravenous drug user	10
Blood transfusion recipient	0.5
Time from infection to study enrollment, median months (IQR)	8 (4–11)
CD4 cell count, median cells/mm^3 (IQR)	570 (403–734)
Viral load, median log HIV-RNA copies/mL (range)	4.63 (4.14–5.1)
No. of patients with drug-resistance mutations	
All	24 (12.1)
NRTI mutations	
All	19 (9.6)
T215Y[a]	12 (6.1)
M41L	9 (4.5)
L210W	3 (1.5)
V118I	3 (1.5)
M184V	2 (1)
D67N	2 (1)
T69N	1 (0.5)
L74V	1 (0.5)
K219Q	1 (0.5)
NNRTI mutations	
All	8 (4)
K103N	6 (3)
Y181C	2 (1)
PI mutations	
All	4 (2)
M46L/I	3 (1.5)
V82A/T/S	2 (1)
L90M	2 (1)

NOTE. Data are no. (%) of patients, unless otherwise indicated. IQR, interquartile range; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitors.

[a] Revertant forms were considered at position 215.

A total of 15 individuals (7.6%) were infected with HIV-1 non-B subtypes, distributed as follows: CRF14_BG (7 patients), F (2 patients), BF (2 patients), C (2 patients), and CRF02_AG (2 patients). Interestingly, the recognition of all of these non-B variants was restricted to the last 3 years of the study period. Moreover, a significant association between infection with non-B subtypes and acquisition of HIV-1 through injection drug use (38.9% of patients; $P < .001$) and heterosexual sex (11.4%; $P < .05$) was noticed, compared with patients who acquired HIV-1 through male-male sex (1.6%). Lastly, CRF14_BG vi-

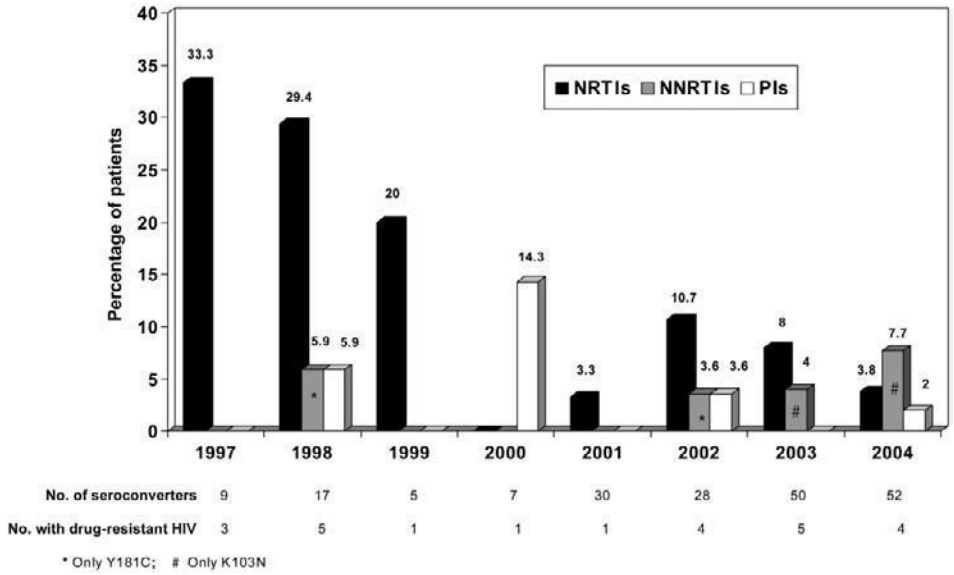

Figure 1. Yearly prevalence of viruses with resistance mutations to the different antiretroviral drug classes during the study period. NNRTIs, nonnucleoside reverse-transcriptase inhibitors; NRTIs, nucleoside reverse-transcriptase inhibitors; PIs, protease inhibitors.

ruses were identified in 5 injection drug users and 2 individuals infected via heterosexual sex. Of the latter 2 patients, one was a Spanish man who admitted to having multiple sex partners, including partners who were injection drug users, and the other was a Portuguese woman with a former sex partner from Mozambique who was known to be HIV-1 seropositive.

Phylogenetic analyses of *pol* gene sequences identified 8 clusters that involved 2 subjects and 2 clusters that involved 3 individuals, suggesting that transmission of HIV-1 occurred around the time of primary HIV-1 infection or soon thereafter (figure 2). These results were further confirmed with the analysis of the v2v3 region and in prospective samples obtained from these individuals thereafter (data not shown). Overall, subtype B represented 86% of viruses (19 patients) involved in these clusters, 68% of which (15 patients) occurred among men who have sex with men. Interestingly, 1 cluster involved 3 injection drug users, all of whom acquired virus with CRF14_BG within an interval of 4 weeks. Another cluster involved an unfortunate transfusion-transmitted infection with a clade B virus from a donor who was in the earlier phases of acute HIV-1 infection, the details of which have been reported elsewhere [14]. Geographical linkage was confirmed for all clusters, whereas an epidemiological relationship was only proven for 6 of them (figure 2). Interestingly, no drug-resistance mutations were recognized in viruses recovered from subjects involved in clusters of recent HIV-1 infection.

Discussion. We found that the current prevalence of drug resistance among recent HIV-1 seroconverters in Spain is ~10%. This frequency is in agreement with that reported in similar studies conducted in the United States and in western Europe, in which the prevalence of drug resistance is 10%–15% [1, 2]. Prior studies from Spain have highlighted a trend towards a reduction in the rate of transmission of drug-resistant HIV-1 in recent years [3–5]. Herein, we show that this prevalence seems to have been stabilized at ~10% for the past 3 years. However, a significant change was found in the genotypic profile, with the prevalence of NRTI resistance mutations steadily decreasing while the prevalence of NNRTI resistance has increased, mainly driven by an increasing rate of K103N. Similar findings have been reported among seroconverters in San Diego, California [1]. Several reasons may account for this phenomenon. First, the rate of exposure to efavirenz has increased substantially during the past few years, therefore increasing the opportunities to select K103N in the treatment-experienced population. Moreover, most NNRTI resistance mutations do not compromise significantly viral fitness and may be transmitted quite efficiently [15]. Finally, this mutation may persist for long periods after being transmitted [16], which is consistent with the establishment of widespread infection with a pure population of drug-resistant viruses.

The overall prevalence of non-B subtypes in our study of recent HIV-1 seroconverters was relatively low (7.6%). However, all infections with non-B viruses were seen within the last 3 years of the study period. Similar findings have been reported in Italy [8]. An interesting finding in our study was the association between non-B subtypes and risk behaviors other than male-male sex. A total of 7 CRF14_BG viruses were identified in our population. This new recombinant virus was originally described among injection drug users in the northern part of Spain and Portugal in 2001 [17]. To our knowledge, we report,

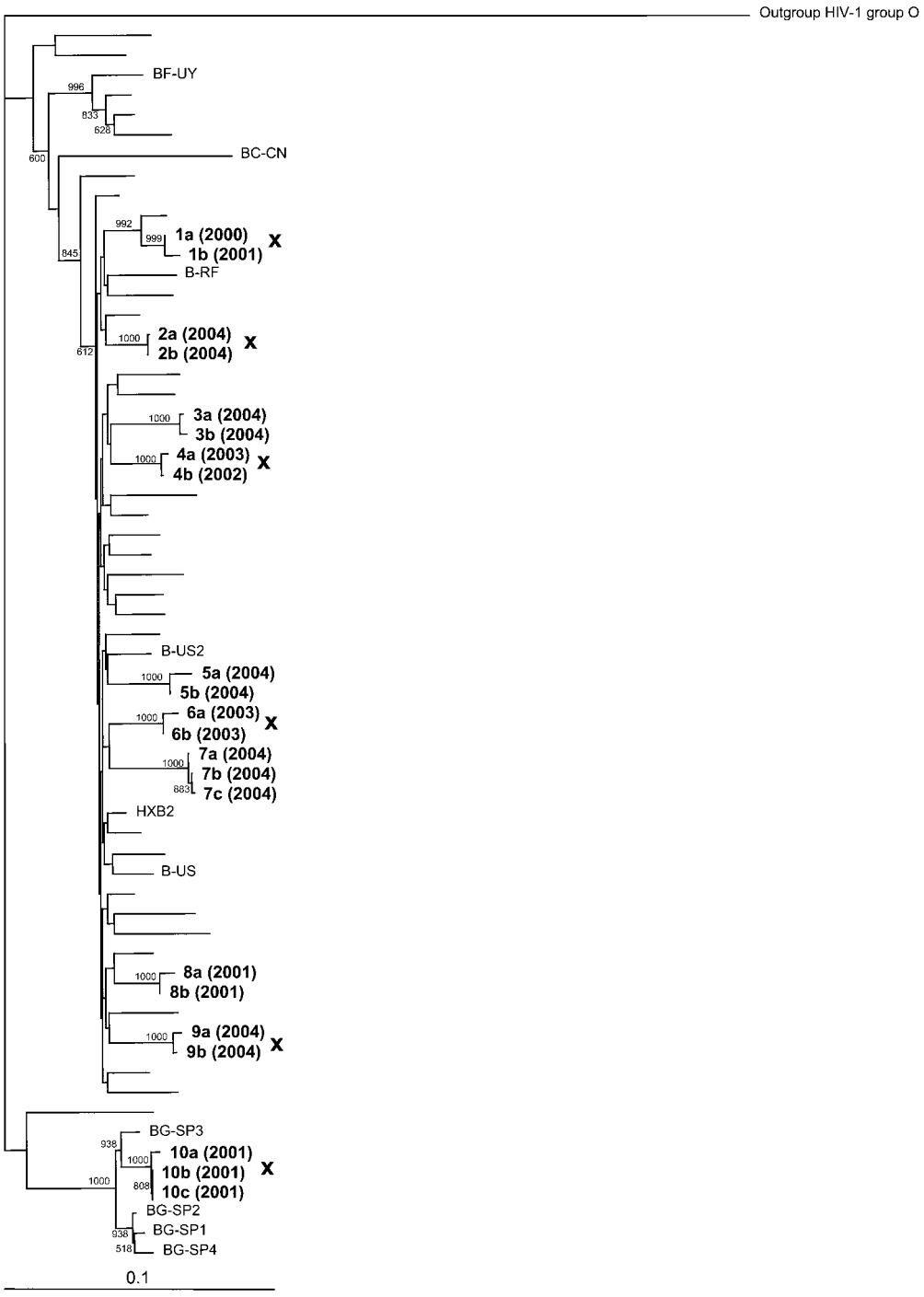

Figure 2. Phylogenetic tree based on *pol* sequences belonging to individuals with recent HIV-1 infection and possible transmission clusters. Clusters are shown in boldface. The year of HIV-1 seroconversion is marked in parenthesis. Linkages supported with epidemiological information are indicated with a cross. Reference sequences are marked first with the initials of the subtype followed by the initials of the country. Bootstrap values of >50% are indicated on the branches. GenBank accession numbers of sequences from seroconverters are AY209177, AY209178 and DQ103891 to DQ103910.

for the first time, episodes of primary HIV-1 infection caused by the CRF14_BG strain. Interestingly, 2 of these infections occurred in persons infected via heterosexual sex, whereas a cluster of 3 CRF14_BG infections were documented in a group of injection drug users who shared needles.

For 22 (11.1%) of 198 individuals, we found strong evidence in favor of transmission of HIV-1 around the time of seroconversion in the index case. Despite its relative genetic conservation, the HIV-1 *pol* gene has been shown to hold sufficient genetic variability to allow adequate subtyping by phylogenetic

analyses and tracking transmission events [18]. With use of these methods, a high rate of transmission of HIV-1 during primary infection has been described recently in the United Kingdom [9]. This is not surprising, taking into account the high viral load generally seen during acute HIV-1 infection and the risky unprotected behaviors in which may be engaged individuals who are not aware of their HIV-positive status. We identified a total of 10 transmission clusters, 2 of which involved 3 individuals each. One of the latest was represented by a group of 3 injection drug users infected with a CRF14_BG strain, as mentioned above. A blood donor in the window period transmitted HIV-1 to a transfusion recipient [14]. The remaining clusters of HIV-1 transmission were identified among men who have sex with men. In all but 4 of these paired infections, an epidemiological link could be found, supporting the theory that HIV-1 was transmitted from one to the other. In the rest, casual encounters with unknown sex partners most likely occurred.

In summary, the current prevalence of primary drug resistance among recent HIV-1 seroconverters in Spain seems to have been stabilized at ~10%, although 3 new details merit particular attention. First, resistance to NNRTIs is now the most frequently transmitted resistance mutation. Second, non-B subtypes are responsible for a growing proportion of new HIV-1 infections, and the new CRF14_BG is spreading in Spain, mainly among injection drug users and their sex partners. Third, the recognition of clusters of infections associated with transmissions that occur during acute HIV-1 infection provides emphasis that prevention strategies should be reinforced in risky populations.

Members of the Spanish Seroconverter Study Group. Javier Colomina (Hospital de la Ribera, Valencia); Concepción Tuset (Hospital General, Valencia); Felix Gutiérrez (Hospital General, Elche); Enrique Bernal (Hospital General, Elche); Federico Garcia (Hospital Universitario, Granada); Isabel Viciana (Hospital Virgen de la Victoria, Málaga); Julian Torre-Cisneros (Hospital Reina Sofia, Córdoba); Jose M Eiros (Hospital Clínico, Valladolid); Raúl Ortíz de Lejarazu (Hospital Clínico, Valladolid); José Pedreira (Hospital Juan Canalejo, A Coruña); Antonio Aguilera (Hospital Xeral, Santiago de Compostela); Pilar Leiva (Hospital Central de Asturias, Oviedo); Jesús Agüero (Hospital Marqués de Valdecilla, Santander); Ana Sáez (Hospital Marqués de Valdecilla, Santander) María Saumoy (Hospital Joan XXIII, Tarragona); Esteban Ribera (Hospital Vall d'Hebrón, Barcelona); Estrella Caballero (Hospital Vall d'Hebrón, Barcelona); Lidia Ruíz (Hospital Germans Trias i Pujol, Badalona); José Luis Gómez (Hospital Ntra Sra de la Candelaria, Santa Cruz de Tenerife); Carmen Rodríguez (Centro Sanitario Sandoval, Madrid) Jorge del Romero (Centro Sanitario Sandoval, Madrid); Carmen de Mendoza (Hospital Carlos III, Madrid); Angélica Corral (Hospital Carlos III, Madrid); Vincent Soriano (Hospital Carlos III, Madrid).

Acknowledgments

Financial support. Fundación Investigación y Educación en SIDA (IES); Red de Investigación en SIDA (RIS- project 173); and Comunidad Autónoma de Madrid (CAM).

Potential conflicts of interest. All authors: no conflicts.

References

1. Little S, Holte S, Routy J, et al. Antiretroviral drug resistance among patients recently infected with HIV. N Engl J Med **2002**; 347:385–94.
2. Wensing A, Boucher C. Worldwide transmission of drug-resistant HIV. AIDS Rev **2003**; 5:140–55.
3. Briones C, Pérez-Olmeda M, Rodriguez C, del Romero J, Hertogs K, Soriano V. Primary genotypic and phenotypic HIV-1 drug resistance in recent seroconverters in Madrid. J Acquir Immune Defic Syndr **2001**; 26:145–50.
4. De Mendoza C, del Romero J, Rodriguez C, Corral A, Soriano V. Decline in the rate of genotypic resistance to antiretroviral drugs in recent HIV seroconverters in Madrid. AIDS **2002**; 16:1830–2.
5. De Mendoza C, Rodriguez C, Corral A, del Romero J, Soriano V. Evidence for a different sexual transmission of HIV strains with distinct drug resistance genotypes. Clin Infect Dis **2004**; 39:1231–8.
6. Little S, Daar E, D'Aquila R, et al. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. JAMA **1999**; 282:1142–9.
7. Yerly S, Vora S, Rizzardi P, et al. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. AIDS **2001**; 15: 2287–92.
8. Balotta C, Facchi G, Violin M, et al. Increasing prevalence of non-clade B HIV-1 strains in heterosexual men and women, as monitored by analysis of reverse transcriptase and protease sequences. J Acquir Immune Defic Syndr **2001**; 27:499–505.
9. Pao D, Fisher M, Hue S, et al. Transmission of HIV-1 during primary infection: relationship to sexual and sexually transmitted infections. AIDS **2005**; 19:85–90.
10. International AIDS Society—USA. HIV drug mutations. Available at: http://www.iasusa.org/resistance_mutations/index.html. Accessed February 2005.
11. HIV databases. Available at: http://www.hiv.lanl.gov/content/index. Accessed February 2005.
12. Delwart E, Shpaer E, Louwagie J, et al. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science **1993**; 262:1257–61.
13. Leitner T, Albert J. Reconstruction of HIV-1 transmission chains for forensic purposes. AIDS Rev **2000**; 2:241–51.
14. Toro C, Rodes B, Colino F, et al. Transmission of HIV type 1 through blood transfusion from an antibody-negative/p24 antigen-negative donor. AIDS Res Hum Retroviruses **2003**; 19:447–8.
15. Nicastri E, Sarmati L, d'Ettorre G, et al. Replication capacity, biological phenotype, and drug resistance of HIV strains isolated from patients failing antiretroviral therapy. J Med Virol **2003**; 69:1–6.
16. Pao D, Andrady U, Clarke J, et al. Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. J Acquir Immune Defic Syndr **2004**; 37:1570–3.
17. Thomson M, Delgado E, Manjon N, et al. HIV-1 genetic diversity in Galicia, Spain: BG intersubtype recombinant viruses circulating among injecting drug users. AIDS **2001**; 15:509–16.
18. Hue S, Clewley J, Cane P, Pillay D. HIV-1 pol gene variation is sufficient for reconstruction of transmissions in the era of antiretroviral therapy. AIDS **2004**; 18:719–28.