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Genetic Analysis of HIV Type 1 Strains from Newly Infected Untreated Patients in Cyprus: High Genetic Diversity and Low Prevalence of Drug Resistance

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Abstract

The molecular epidemiology of HIV-1 infection was first studied in Cyprus in the mid-1990s, but the extent of HIV-1 diversity and the prevalence of drug resistance have remained elusive. In an effort to address this issue, the present study examined HIV-1 strains isolated from 37 newly diagnosed untreated HIV-1 patients, representing 72% of the total number of newly diagnosed and drug-naive patients in the period 2003 to 2006. DNA sequences encoding the *gag* (p17, p24, p2, p7, p1, and p6), *pol* (protease and reverse transcriptase), and *env* (gp160) regions were amplified by RT-PCR from plasma HIV-1 RNA from all patients and sequenced using a newly designed methodology. All amplified products were studied according to established genetic methodologies to determine the genetic subtype and the prevalence of drug-resistance-associated mutations to currently available antiretroviral drugs. Analyses of the obtained viral sequences indicated that subtype A was the most common subtype present and accounted for 38% of the infections followed by subtype B (35%), subtype C (13%), CRF02_AG (8%), and subtypes D and CRF01_AE (3% each). One patient (2.7%) had an M41L/M and another patient (2.7%) an M184V amino acid substitution in the reverse transcriptase (RT) associated with high-level resistance to RT inhibitors. There were no patients with resistant mutations to protease inhibitors (PI). Additionally, one patient (2.7%) had an L44M amino acid substitution within the HR1 region of gp41 conferring resistance to the enfuvirtide (T20) fusion inhibitor. Similar to results of the 1994 molecular epidemiological study, these data demonstrate the extensive heterogeneity of HIV-1 infection in Cyprus and the low prevalence of transmitted resistance to current HIV-1 antiretroviral drugs. Taken together, these findings demonstrate that HIV-1 infection in Cyprus is being replenished by a continuous influx of new strains from many countries, establishing an ever-evolving and polyphyletic infection in the island.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is able to develop extensive genetic diversity, and by this means acquires mutations to resist inhibitory pressures by antiretroviral drugs. The global genetic diversity of HIV-1 and the spread of variants with resistance to current antiretroviral drugs are being studied through the collective efforts of multinational consortia and many other groups worldwide. Genetic analysis of HIV-1 strains isolated from many countries thus far has revealed nine distinct phylogenetic subtypes (A–D, F–H, J, and K) and at least 21 intersubtype circulating recombinants forms (CRFs) among the isolates within the major group, M.[1–6] The constant tracking of the genetic diversity of HIV-1 infection has important implications for monitoring the global evolution of HIV-1 and ensuring the efficacy of viral RNA quantitation methods[7–12] and future vaccine development.[13–16] The reported prevalence of HIV-1 infection in Cyprus is lower (about 0.05%) than other European countries and is comparable to that of neighboring countries in the eastern Mediterranean region.[17–21] The first AIDS patient in Cyprus was reported in 1987 and the first molecular epidemiological study of HIV-1 infection in Cyprus was conducted in 1994.[22] Based on phylogenetic analyses of

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obtained viral sequences encoding the C2 to V3 *env* gp120 from 24 patients infected from 1987 to 1994, subtype B was identified as the most prevalent subtype accounting for 63% of the infections, followed by subtype A (21%), subtype F (8%), and subtypes C and I (4% each).[22] Subtype I was later determined to be a complex mosaic comprising subtypes A, G, and I and has been named CRF04_cpx.[23]

Combination antiretroviral therapy (cART) with recently approved highly active antiretroviral drugs has significantly improved the quality of life for HIV-infected patients.[3] However, infection with drug-resistant HIV-1 variants with reduced susceptibility to drugs can impair the response to cART and may have important implications for the clinical management of HIV-1-infected patients. Based on reported worldwide studies, the prevalence of transmitted drug-resistant HIV-1 variants ranges from 1.4% to 28.9%.[3,24–30] The prevalence of HIV-1 variants with drug-resistant mutations in newly diagnosed individuals from western Europe and Israel is approximately 9%.[31] cART using protease and reverse transcriptase inhibitors was first introduced in the late 1990s, and until now there have been no studies investigating the extent and impact of transmission of drug-resistant HIV-1 variants in Cyprus. As part of a continuing effort to monitor the genetic diversity of HIV in Cyprus, in this study we determined the genetic diversity and the prevalence of antiretroviral drug resistance mutations among HIV-1 strains isolated from 37 newly diagnosed untreated HIV-1 patients, representing 72% of the total number of newly diagnosed patients in the period 2003 to 2006.

Materials and Methods

Study subjects

Between 2003 and 2006 blood samples were obtained from 37 HIV-1 newly diagnosed patients at the Cyprus Reference AIDS Clinic, Larnaca National Hospital, with the informed consent of all participants and the approval of the Cyprus National Bioethics Committee. All patients were living permanently in Cyprus at the time of sampling. The majority of study subjects were Greek-Cypriots, although a number reported traveling or living abroad in the past. The HIV-1 serostatus of each subject was previously established by commercial enzyme-linked immunoassay and confirmed by Western blotting. Blood was drawn within 3 months of HIV-1 diagnosis. A description of the clinical profile of each patient is presented in Table 1. All blood samples were processed at the Laboratory of Biotechnology and Molecular Virology of the University of Cyprus on the same day of sampling.

HIV-1 plasma RNA and cell-associated DNA isolation

Patients' blood (16 ml) was collected in CPT tubes (Becton Dickinson, Annapolis, MD) and peripheral blood mononuclear cells (PBMCs) and plasma were isolated using the CPT vacutainer procedure. HIV-1 RNA was extracted from 200 μl plasma and genomic DNA from about 10^7 uncultured PBMCs using QIAamp silica-gel-membrane technology with the QIAamp UltraSens Virus Kit and QIAamp DNA Blood Mini Kit, respectively (Qiagen, Valencia, CA). Genomic DNA was quantified by ultraviolet (UV) absorbance spectrophotometry using the Nanondrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR and sequencing

HIV-1 sequences encoding approximately 1722 bp of the *gag* region, 1461 bp of the *pol* (*protease and RT*) region, and 2927 bp of the *env* (*gp160*) region were amplified from each sample by nested polymerase chain reaction (PCR) using PBMC-associated HIV-1 DNA. For any samples where the PCR product of genomic DNA was problematic, reverse transcription nested PCR (RT-PCR) using plasma HIV-1 RNA was performed instead. Primers used in the first round of PCR were 623 and 2501 for *gag*,[32] 1832 and 3583 for *pol* (*protease and RT*), and 5955 and 9181 for *env* (*gp160*). Inner primers were 684 and 2406 for *gag*, 2078–3539 for *pol* (*protease and RT*), and 6111 and JL88 for *env* (*gp160*). The primer positions corresponding to the HXB2 strain (accession number K03455) are described in Table 2. In the first round of PCR, approximately 60–100 ng of patient PBMC DNA was used, as were 20 pmol of each primer and 1.1X Platinum PCR SuperMix (Invitrogen Corp., San Diego, CA) in a 50-μl volume. DNA amplifications were carried out in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). For *gag* amplification, the thermocycling conditions were one cycle at 94°C for 2 min, 40 cycles at 94°C for 20 s, 53°C for 30 s, 72°C for 2 min, and one cycle at 72°C for 7 min. For *pol* (*protease and RT*) and *env* (*gp160*), the thermocycling conditions were the same except the hybridization temperatures were 52 and 54°C, respectively. In the second round of PCR, 3 μl samples of primary reaction products were used, along with 20 pmol of each primer and 1.1X Platinum PCR SuperMix (Invitrogen Corp., San Diego, CA) in a 50-μl volume. The amplification conditions were identical to those described for the first round of PCR, except the hybridization temperatures were 56°C for *gag* and 52°C for *pol* (*protease and RT*) and *env* (*gp160*) and the polymerization times were 2.20 min for *gag*, 2 min for *pol* (*protease and RT*), and 3.20 min for *env* (*gp160*). The detailed experimental method for the amplification of the *pol* (*protease and RT*) region by RT-PCR using plasma HIV-1 RNA is described below in this section. For *gag* and *env* RT-PCR amplification, the experimental conditions were the same as those described in the nested PCR methods with the following modifications: the Invitrogen SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity was used instead of the 1.1X Platinum PCR SuperMix according to the manufacturer's specifications (Invitrogen Corp., San Diego, CA); in the first round *env* RT-PCR amplification there was an additional reverse transcription cycle at 54°C for 1 h; for *gag* RT-PCR amplification, the reverse transcription cycle was at 52°C for 1 h; in both RT-PCR amplifications, the polymerization temperature was 68°C instead of 72°C.

Amplified products from the second-round PCR were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), their lengths were analyzed by 1% agarose gel electrophoresis, and the concentrations were quantified by UV absorbance spectrophotometry using the Nanondrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). In each sample, the DNA sequences encoding the *gag*, *pol* (*protease and RT*), and *env* (*gp160*) regions were determined by directly sequencing both complementary strands in separate reactions, using the second-round-amplified PCR product as the template and sequencing primers as follows (Table 2): primers 684, 1173, 1985, 2406 for *gag*; 2136, 3462 for *pol* (*protease and RT*); and 6111, 6438, 6858,

Table 1. Clinical and Epidemiological Information for Study Patients

Patient[a]	*Sex*[b]	*Age (years)*	*Weeks since diagnosis*[c]	*Country of origin*[d]	*Transmission risk group*[e]	*CD4 (cells/mm^3)*	*Plasma HIV-1 RNA (copies$\times 10^4$/ml)*	*Epidemiological information*[f]
CY004	M	47	1	Cyprus	MSM	363	1.3	N/A
CY005	M	32	1	Cyprus	MSM	357	2.0	N/A
CY006	M	33	1	Cyprus	MSM	593	2.9	N/A
CY007	M	36	1	Cameroon	MSM	842	1.2	Political refugee from Cameroon
CY008	F	36	1	Cyprus	HSX	556	0.2	N/A
CY009	M	24	1	Cyprus	MSM	290	2.7	HBV positive
CY010	M	45	1	Cyprus	MSM	442	12.0	N/A
CY012	M	30	8	Cyprus	MSM	461	30.0	N/A
CY013	M	54	1	Cyprus	MSM	79	100.3	N/A
CY014	M	N/A	1	Cyprus	MSM	671	1.7	N/A
CY015	F	19	4	Georgia	HSX	369	7.7	Possibly infected by heterosexual partner CY009
CY016	M	59	3	Cyprus	HSX	202	17.0	N/A
CY035	M	27	2	Cyprus	MSM	191	55.0	Diagnosed with *Pneumonocystis carinii* pneumonia
CY048	M	30	1	Burkina-Faso	MSM	181	27.0	Political refugee from Burkina-Faso
CY057	F	28	1	Georgia	HSX	N/A	2.8	Heterosexual partner of CY058; HCV positive
CY058	M	34	1	Georgia	HSX	N/A	3.2	Heterosexual partner of CY057; HCV positive
CY066	M	28	1	Cyprus	MSM	N/A	0.6	N/A
CY073	M	26	1	Pakistan	Unknown	425	6.4	Diagnosed with tuberculosis lymphadenopathy
CY089	F	20	1	Cyprus	HSX	262	5.6	Possibly infected by heterosexual partner CY008
CY109	M	36	12	Cyprus	MSM	339	2.3	Possible infected by homosexual contact in Germany
CY111	F	37	1	Sweden	HSX	32	200.0	Heterosexual partner of CY112
CY112	M	N/A	1	U.K.	HSX	292	6.0	Heterosexual partner of CY111; HCV positive
CY142	M	53	1	Cyprus	MSM	32	31.0	Diagnosed with pneumonia recurrent
CY153	M	20	2	Cyprus	MSM	223	5.9	Diagnosed with pneumonia recurrent
CY158	M	37	1	Burkina-Faso	OHPC	78	6.5	Political refugee from Burkina-Faso
CY159	M	34	4	Cyprus	MSM	1390	0.2	N/A
CY166	M	57	4	Cyprus	HSX	134	100.0	Diagnosed with non-Hodgkin's lymphoma
CY169	F	33	1	Georgia	HSX	72	12.0	Heterosexual partner of CY171; currently on treatment for lymphoma
CY170	M	36	2	Cyprus	HSX	473	13.0	Diagnosed with infectious mononucleosis and herpes genital
CY171	M	47	2	Cyprus	HSX	447	11.0	Heterosexual partner of CY169, diagnosed with gonorrhea
CY172	M	24	12	Cameroon	HSX	363	8.8	Political refugee from Cameroon
CY173	F	27	4	Ukraine	HSX	598	1.8	Infected by heterosexual partner CY170 six months before
CY175	M	30	8	Cyprus	HSX	320	1.2	Heterosexual partner of CY176
CY176	F	24	4	Ethiopia	HSX	595	2.1	Heterosexual partner of CY175
CY177	M	47	8	Cyprus	MSM	641	4.7	HBV positive
CY178	M	42	1	Cyprus	MSM	785	0.1	N/A
CY179	M	36	2	Cyprus	HSX	498	3.3	N/A

[a]Indicates the laboratory code for each study subject.
[b]F, female; M, male.
[c]Indicates the duration from the first known positive HIV antibody test.
[d]Country of birth of the study subjects.
[e]MSM, men who have sex with men; HSX, heterosexual contact; OHPC, origin from a high prevalence country.
[f]Information provided by the study subjects. N/A, not available; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2. PCR and Sequencing Primers

Designation[a]	*Target gene*	*Sequence*[b]	*Position*[c]	*Amplicon length (nts)*	*Reference*[d]
PCR primers					
623 (F)	*gag*	AAATCTCTAGCAGTGGCGCCCGAA	623–646	1878	32
2501 (R)	*gag*	GTTGACAGGTGTAGGTCCTAC	2481–2501		32
684 (F)[e]	*gag*	TCTCGACGCAGGACTCGGCTTG	684–705	1722	This study
2406 (R)[e]	*gag*	CTCCAATTCCYCCTATCATTTTTGGTTTCC	2377–2406		This study
1832 (F)	*pol*	CAGCATGYCAGGGAGTRGGRGGACC	1832–1856	1751	This study
3583 (R)	*pol*	GGYTCTTGRTAAATTTGATATGTCCATTG	3555–3583		This study
2078 (F)	*pol*	AGGCTAATTTTTTAGGGAARATYTGGCCTTCC	2078–2109	1461	This study
3539 (R)	*pol*	CTGTATTTCTGCTAYTAAGTCTTTTGATGG	3510–3539		This study
5955 (F)	*env*	GCTTAGGCATCTCCTATGGCA	5955–5975	3226	This study
9181 (R)	*env*	GTGTGTAGTTYTGCCAATCAGG	9160–9181		This study
6111 (F)[e]	*env*	TMATAGCAATAGTTGTGTGGACYATAG	6111–6137	2927	This study
JL88 (R)[e]	*env*	TAAGTCATTGGTCTTAAAGGTACCTG	9013–9038		32
Sequencing primers					
1173 (F)	*gag*	CAGYCAAAATTAYCCTATAGTGCA	1173–1196		This study
1985 (R)	*gag*	CCTTCYTTGCCACARTTGAAACAY	1962–1985		This study
2136 (F)	*pol*	YCAGARCAGACCAGAGCCAACAGCCCC	2136–2162		This study
2216 (F)	*pol*	AGGAGCMGAWAGACARG	2216–2232		This study
2454 (F)	*pol*	GGAMAWAARGCTATAGGTACAG	2454–2475		This study
2610 (R)	*pol*	CYTTTGGGCCATCCATTC	2593–2610		This study
2650 (R)	*pol*	AATGCTTTTATTTTYTCTTCTGTCAATGGC	2621–2650		This study
2734 (R)	*pol*	GCAAATAYTGGAGTATTRTATGGATTTTCAGG	2703–2734		This study
3003 (F)	*pol*	GGATGGAAAGGATCACC	3003–3019		This study
3019 (R)	*pol*	GGTGATCCTTTCCATCC	3003–3019		This study
3462 (R)	*pol*	CTGCCARTTCTARYTCTGCTTC	3441–3462		This study
6438 (F)	*env*	CATGCCTGTGTACCCACAGA	6438–6457		This study
6858 (F)	*env*	CCAATTCCYATACATTATTGTGCYC	6858–6882		This study
8039 (R)	*env*	GGTGCARATGWGTTTTCCAGAGC	8017–8039		This study
8530 (R)	*env*	TGGTAGCTGAAGAGGCACAG	8511–8530		This study

[a]Reverse transcriptase, primary and secondary PCR primers, and sequencing primers name as appears in the text; orientation of the PCR primer is indicated in parentheses: F, forward; R, reverse.
[b]Y, indicates equal molar mixture of C and T; R, A, and G; M, A, and C; W, A, and T.
[c]Primer positions correspond to subtype B HIV-1 HXB2 strain (GenBank accession number K03455).
[d]Salminen *et al.*[32]
[e]Used also as sequencing primers as described in the text.

8039, 8530, JL88 for *env* (*gp160*). DNA sequence reactions were performed by the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Samples were sequenced with the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Samples exhibiting partial or extensive viral diversity by direct sequencing were subsequently cloned, using the TOPO TA cloning kit for sequencing (Invitrogen Corp., San Diego, CA) and sequenced as described above.

Determination of drug-resistance-associated mutations in protease, reverse transcriptase, gag, and gp41

Genotypic resistance mutations to antiretroviral drugs to protease and reverse transcriptase were determined using a genotyping in-house assay that analyzes *protease* and *RT* genes within M-group strains (Fig. 1). HIV-1 DNA sequences encoding 1461 bp of the *pol* (*protease and RT*) region were amplified from each sample by nested RT-PCR using extracted viral RNA (Fig. 1). Primers 1832 and 3583 were used in the first round of RT-PCR; the inner primers were 2078–3539 (Table 2). In the first round of RT-PCR, approximately 15 μl of patient plasma RNA was used, as were 20 pmol of each primer and Invitrogen SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen Corp., San Diego, CA) in a 50-μl volume. Amplification was carried out in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). The thermocycling conditions were one cycle at 52°C for 1 h, one cycle at 94°C for 2 min, 40 cycles at 94°C for 15 s, 52°C for 30 s, 68°C for 2 min, and one cycle at 68°C for 5 min. In the second round of PCR, 3-μl samples of primary reaction products were used, along with 20 pmol of each primer and 1.1X Platinum PCR SuperMix (Invitrogen Corp., San Diego, CA) in a 50-μl volume. The amplification conditions were identical to those described for the second round of PCR in the nested PCR of *pol* (*protease and RT*). The DNA sequences of amplified products were determined by direct sequencing using the following sequencing primers: 2136, 2216, 2454, 2610, 2650, 2734, 3003, 3019, 3462, and 3539 (see Table 2 and Fig. 2). Genotypic resistance was defined as the presence of at least one resistance-related amino acid substitution as specified by the International AIDS Society (IAS)-USA[33] and the Stanford HIV Drug Resistance Database.[34] Assessment of the possible impact of transmitted drug resistance on the therapeutic response was performed using the Stanford drug-resistant algorithm.[35] *Gag* cleavage site-associated mutations (NC/p1/p6, corresponding to amino acids

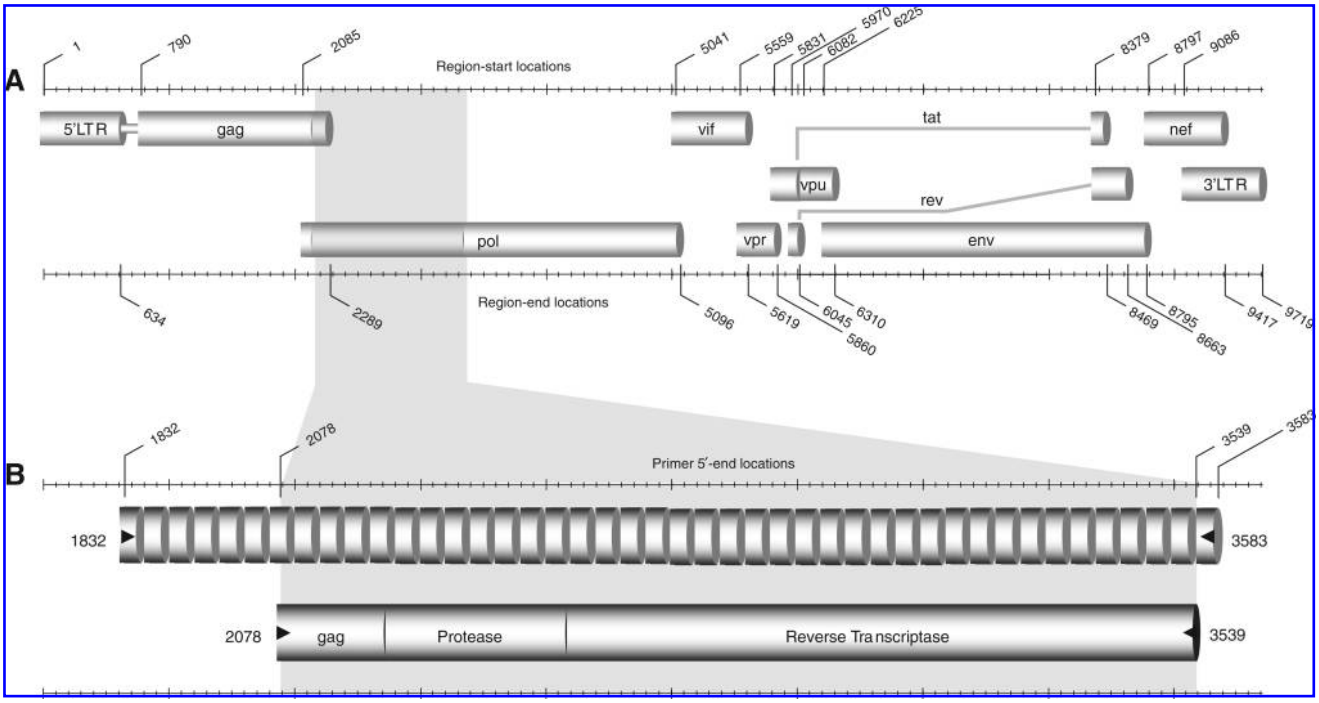


FIG. 1. Schematic representation summarizes the amplification of the 5′-end-half *pol* region within HIV-1 M Group subtypes used in a novel genotypic drug-resistant assay of *protease* and *reverse transcriptase* described in this study. (**A**) The upper diagram summarizes the overall HIV-1 genetic organization exemplified by the HXB2 genome (GenBank accession number K03455). The beginning and end of each major genetic region are indicated by a corresponding number above and below the diagram, respectively, using the HXB2 numbering. The shaded area in the HIV-1 gene map indicates the *gag* (*p6*) and *pol* (*prot and p51 RT*) region amplified in the genotypic drug-resistant assay. (**B**) The lower scheme summarizes the nested RT-PCR designed for the amplification of *protease* and *RT* genes. Black arrows indicate the orientations and 5′-end-primer-binding positions of the primary (1832 and 3583) and secondary (2078 and 3539) PCR primers with respect to the HXB2 genome. The intermittent and solid cylinders indicate the product of the primary RT-PCR and secondary PCR, respectively.

428–454 of the HXB2 gag precursor protein) were analyzed from the Cypriot gag sequences as specified by Maguire *et al.*[36] and Nijhuis *et al.*[37] Fusion-inhibitor-associated mutations of gp41 (heptad repeat 1, corresponding to amino acids 30–51 of the HXB2 gp41) were analyzed from Cypriot gp41 sequences as specified by the IAS-USA[33] and Van Laethem *et al.*[38]

Phylogenetic tree analysis

We used DNA alignment, distance calculation, and phylogenetic tree construction programs from the Molecular Evolution Genetic Analysis (MEGA) software.[39] Patients' DNA sequences encoding the *pol*, *gag*, and *env* viral regions were aligned against corresponding sequences of genetically characterized HIV-1 strains obtained from the Los Alamos database[1] using the alignment method described in the MEGA program run with default conditions. Pairwise distance matrices were calculated using the Kimura two-parameter distance estimation approach with a transition/transversion ratio of 2.0 and phylogenetic trees were constructed using the neighbor-joining method. The consistency of the phylogenetic clustering was tested using bootstrap analysis with 100 replicates. Bootstrap values above 70 were considered adequate for subtype assignment. The subtype assignment was confirmed using the REGA algorithm.[40] GenBank accession numbers for control reference sequences in phylogenetic analyses are as follows: A1-94SE7253, AF069670; A1-9292UG037.1, U51190; A1-94Q23, AF004885; A1-98UG57136, AF484509; A2-97CDKTB48, AF286238; A2-94CY017, AF286237; B-83HXB2, K03455; B-981058, AY331295; B-90BK132, AY173951; B-00671, AY423387; C-9292BR025.8, U52953; C-86ETH2220, U46016; C-04SK164B1, AY772699; C-95IN21068, AF067155; D-01A280, AY253311; D-9494UG114.1, U88824; D-83ELI, K03454; D-014412HAL, AY371157; F1-93VI850, AF077336; F1-93FIN9363, AF075703; F1-9393BR020-1, AF005494; F1-96MP411, AJ249238; F2-95MP255, AJ249236; F2-95MP257, AJ249237; F2-020016BBY, AY371158; F2-97CM53657, AF377956; G-93HH8793.1.1, AF061640; G-96DRCBL, AF084936; G-92NG083, U88826; G-93SE6165, AF061642; H-93VI997, AF190128; H-90056.1, AF005496; H-94VI991, AF190127; J-94SE9280.9, AF082394; J-93SE9173.3, AF082395; K-97EQTB11C, AJ249235; K-96MP535, AJ249239; 01AE-90CM240, U54771; and 02AG-IBNG, L39106.

Coreceptor usage

The coreceptor usage of HIV-1 was predicted from DNA sequences encoding the *env* (V3 loop) region using the Geno2pheno[41] and PSSM[42] coreceptor prediction algorithms running with default conditions.

Results

Clinical and epidemiological features of the study patients

The study group consisted of 37 HIV-1-seropositive patients from the Cyprus Reference AIDS Clinic of Larnaca

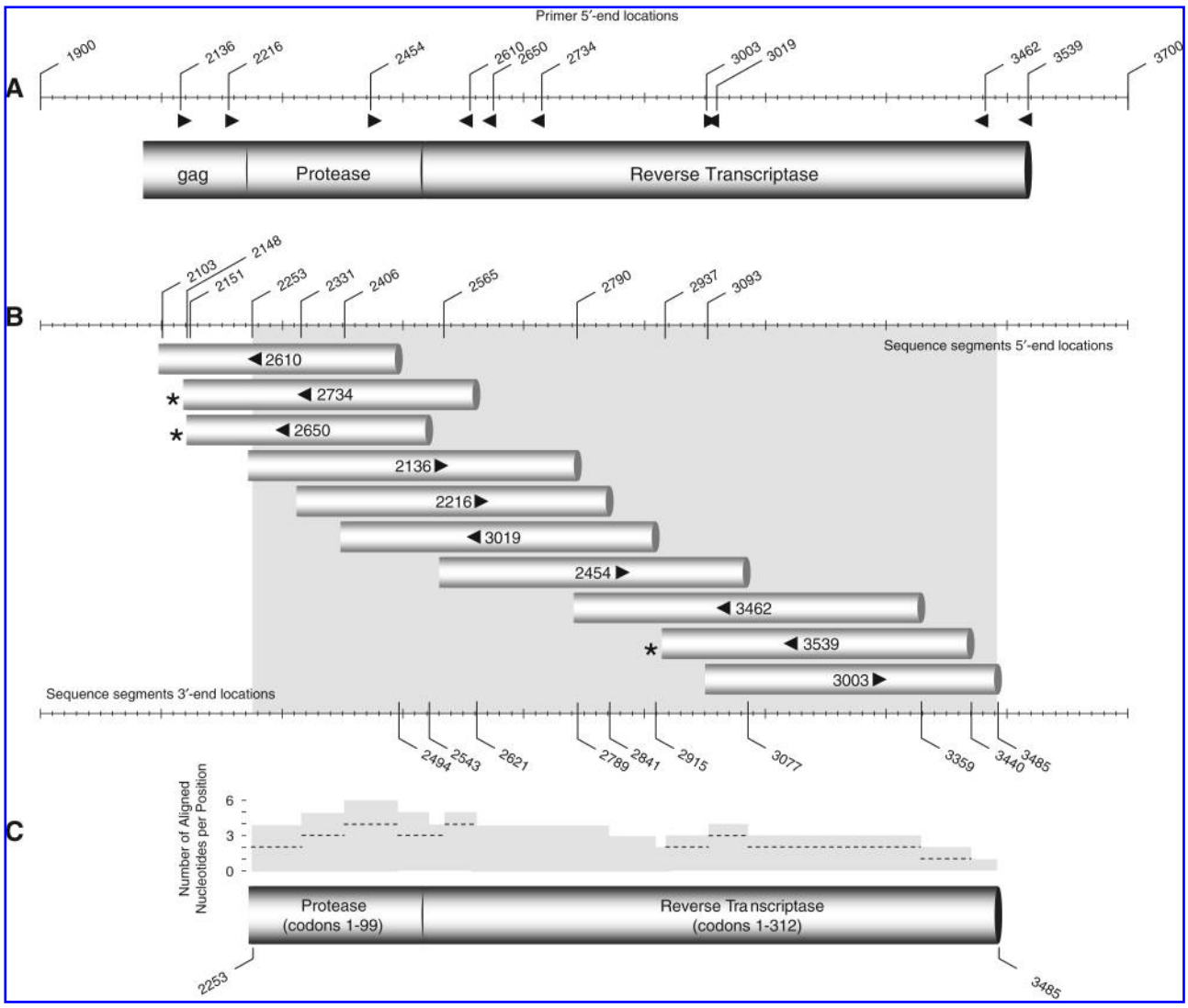


FIG. 2. Schematic diagram summarizes the sequencing procedure of the *protease-RT pol* region. (**A**) A solid cylinder represents the final amplicon containing *gag* (*p6*) and *pol* (*prot and p51 RT*) regions derived from the RT-PCR procedure (Fig. 1). Black arrows indicate the orientations and 5′-end binding positions of the sequencing primers. (**B**) Gray cylinders indicate the sequence segments obtained from each sequencing primer. The beginning and end of each segment are indicated by a corresponding number above and below the diagram, respectively. DNA segments marked by asterisks are alternates: 2734 and 2650 can be used instead of 2610 and 3539 instead of 3462. (**C**) A solid cylinder represents the final aligned DNA sequence derived from all partial segments (corresponding to nucleotides 2253 to 3485 on the HXB2 genome) containing the full *protease* (codons 1 to 99) and partial *RT* (codons 1 to 312) genes. At the bottom, the diagram indicates the number of aligned nucleotides per nucleotide position from information derived from all partial sequence segments; dashed lines indicate the limit of aligned nucleotides per aligned nucleotide position used in the genotypic analysis.

National Hospital. Each patient is identified by a laboratory registration number ascending in chronological order of blood drawn. The study subjects represent 72% of the total number of newly HIV-1-diagnosed patients monitored at the clinic from 2003 until 2006 in Cyprus. The general demographic, epidemiological, and clinical features of all study subjects are summarized in Table 3. Among the 37 patients, 29 were male and 8 were female. Their median age was 34 years. Twenty-eight patients have asymptomatic infection, whereas nine have symptomatic infections. Two patients were coinfected with hepatitis B virus (HBV) and three with hepatitis C virus (HCV). The median $CD4^+$ lymphocyte count was 363 cells/mm^3 and the median plasma HIV-load was 4.75 log HIV-1 RNA copies/ml. Twenty-four patients are Cypriots, five are from sub-Saharan Africa, five from Eastern Europe, two from western Europe, and one from Asia. Of the patients, 18 were infected by homosexual/bisexual contact, 17 were infected by heterosexual contact, one was infected in an HIV-1 high prevalence country (Burkina-Faso), and for one the route of infection is unknown.

Epidemiological features varied among the 37 patients. Sixteen subjects were infected in Cyprus: eight men by homosexual contact and three men and five women by heterosexual contact. Four subjects were presumably infected in Greece by homosexual contact. One man was infected in Germany, one in the United Kingdom, and one in the United States by homosexual contact. Two men were infected in Cameroon, one heterosexually and one homosexually. One

Table 3. Characteristics of the Study Subjects

Characteristics[a]	*Patients (N = 37)*
Gender (%)	
Male	29 (78)
Female	8 (22)
Age (years)[b]	
Median (IQR)	34 (27–42)
Region of origin (%)	
Cyprus	24 (65)
Western Europe	2 (5)
Eastern Europe	5 (14)
Sub-Saharan Africa	5 (14)
Asia	1 (3)
Route of transmission (%)	
MSM	18 (49)
HSX	17 (46)
OHPC	1 (3)
N/A	1 (3)
CDC stage number (%)	
A	28 (76)
B	3 (8)
C	6 (16)
Plasma HIV-RNA (log copies/ml)	
Median (IQR)	4.75 (4.3–5.1)
CD4 count (cells/ml)[c]	
Median (IQR)	363 (199–565)
Subtype (%)	
A	14 (38)
B	13 (35)
C	5 (13)
D	1 (3)
CRF01_AE	1 (3)
CRF02_AG	3 (8)
Coinfection (%)	
HBV	2 (5.4)
HCV	3 (8.1)

[a]IQR, interquartile range.
[b]Information available for 35 patients.
[c]Information available for 34 patients.

man was infected in South Africa by homosexual contact. Two men were infected in Burkina-Faso, one by homosexual contact and the other by an unknown route of infection. One man was infected in Thailand by heterosexual contact. One heterosexual couple was infected in Georgia. One heterosexual couple was infected in Ethiopia. One heterosexual couple (the man is from the United Kingdom and the woman from Sweden) living in Cyprus reported that the place of infection is unknown. One patient was infected by homosexual contact whose place of infection is unknown and for one patient the route and place of infection are unknown.

PCR and DNA sequencing

Uncultured PBMCs and plasma from all subjects were HIV-1 positive by nested PCR in the *gag*, *pol* (*protease and RT*), and *env* (*gp160*) regions. The positive PCR combined with the extensive genetic diversity of the HIV-1 strains, as described in the phylogenetic analysis (Fig. 3), demonstrates that the newly designed PCR primers are suitable for diverse M-group strains. All HIV-1 PCR products were further analyzed by nucleotide sequencing analysis. The complete DNA sequences from *gag* and *pol* were derived by direct (population) sequencing using PCR-amplified products from PBMC-extracted genomic DNA. The *env* regions in 10 patients were derived by direct sequencing, in 15 patients from *env* inserts of cloned PCR fragments, in five patients by RT-PCR from plasma-extracted HIV-1 RNA, and in seven patients no *env* sequences were obtained because of primer failure to bind to the DNA template.

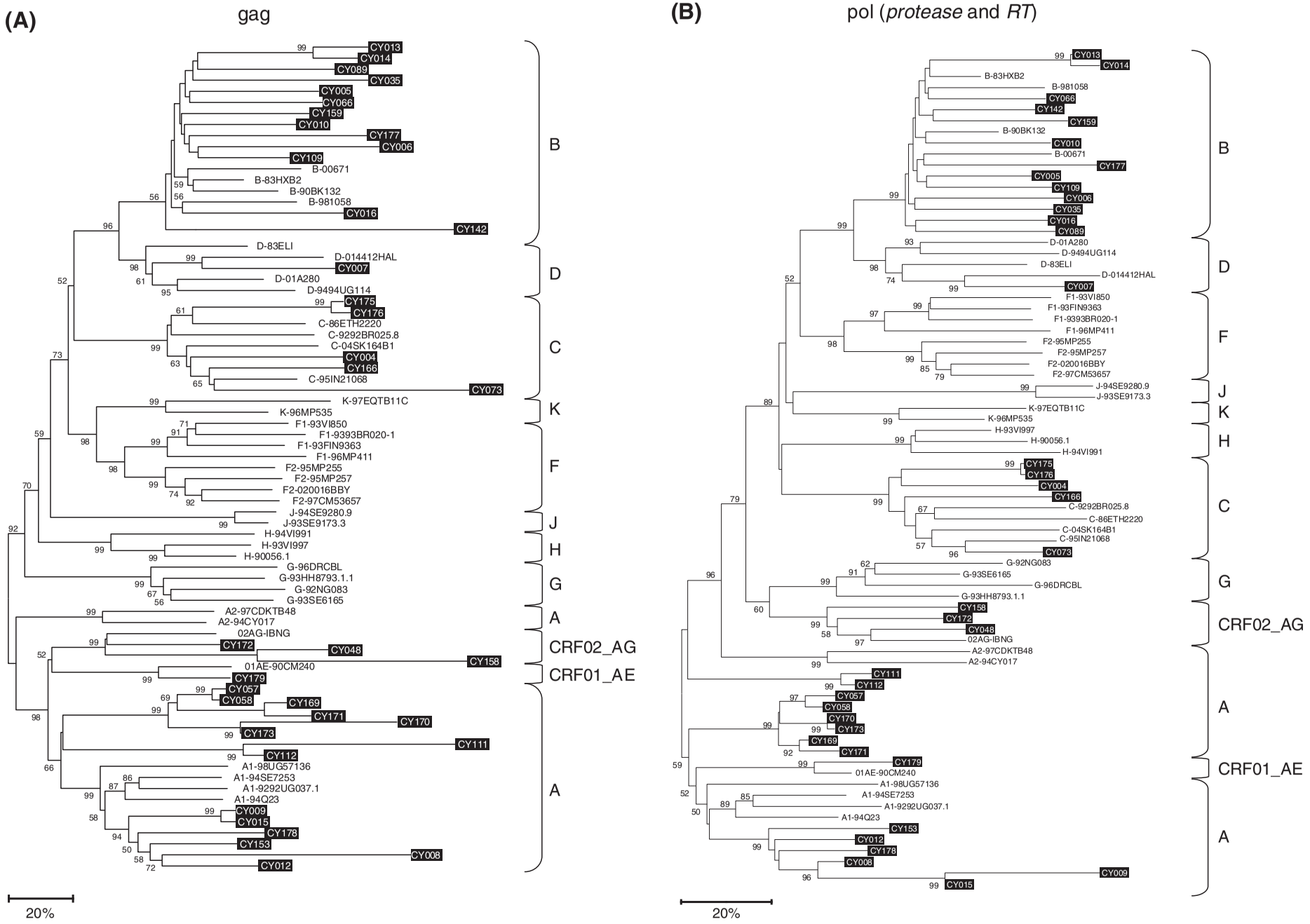
Phylogenetic analysis

The molecular epidemiological relationship between DNA sequences encoding the *gag*, *pol*, and *env* regions was analyzed by nucleotide phylogenetic analysis. Three phylogenetic trees, one corresponding to each viral region, were constructed for the 37 study subjects on the basis of 37 derived *gag* and *pol* and 30 *env* DNA sequences (see Fig. 3A, B, and C, respectively). In addition to the sequences from Cyprus, 37 previously sequenced HIV-1 isolates from diverse global locations, encompassing all nine known subtypes (A through K) and two circulating recombinant forms (CRF), CRF01_AE and CRF02_AG, were also included in the analysis.

According to the constructed phylogenetic trees shown in Fig. 3, four distinct subtypes (A, B, C, and D) and two CRFs (CRF01_AE and CRF02_AG) were identified for the Cypriot sequences within the M group: subtype A, 14 sequences; subtype B, 13 sequences; subtype C, five sequences; subtype D, one sequence; CRF01_AE, one sequence; and CRF02_AG, three sequences. In the phylogenetic tree constructed based on *env* sequences (Fig. 3C), seven sequences classified previously as *gag* and *pol* are missing: subtype A, one sequence; subtype B, five sequences; and CRF02_AG, one sequence. It is important to note that the Cypriot sequences in subtypes A (38% of patients), B (35%), C (13%), and CRF02_AG (8%) have a relatively high average intrasubtype genetic diversity. The average (range) intrasubtype nucleotide divergence among the Cypriot *gag* sequences with subtype A is 11.0% (0.6 to 27.9%); within subtype B, 10.0% (3.2 to 16.2%); within subtype C, 10.3% (1.0 to 14.0%); and within CRF02_AG, 12.6% (8.1 to 14.9%). This finding suggests that subtypes A, B, C, and CRF02_AG were transmitted to Cyprus during the study period (2003 to 2006) by multiple sources, which is consistent with the epidemiological data of the study subjects presented in Table 1.

Genotypic drug resistance

There was no protease inhibitor (PI)-associated mutation observed in the untreated study population, but a number of minor PI-associated mutations (L10I, K20M/R, M36I, L63P/F/S/A/V, A71V/T, and V77I) were observed in 36 patients (97%). The amino acid substitution M36I was found in all non-B samples, while the amino acid substitutions K20R and L10I were observed in 14% and 9% of subtype A sequences, respectively. Furthermore, amino acid substitutions L63P and A71V were seen in 17 sequences (46%) of subtype B sequences and V77I was seen in 12 sequences (31%). Nucleoside reverse transcriptase inhibitor (NRTI)-associated mutations were seen in one subtype B sequence (M41L/M mutation in CY010 patient) and in one subtype A sequence (M184V mutation in patient CY169). The M41L mutation is associated with potentially low resistance (PLR) to didanosine, abacavir, and tenofovir and low resistance (LR) to zidovudine and stavudine, whereas the M184V mutation is


(A)
gag
CY013
CY014
CY089
CY035
CY005
CY066
CY159
CY010
CY177
CY006
CY109
B-00671
B-83HXB2
B-90BK132
B-981058
CY016
CY142
D-83ELI
D-014412HAL
CY007
D-01A280
D-9494UG114
CY175
CY176
C-86ETH2220
C-9292BR025.8
C-04SK164B1
CY004
CY166
C-95IN21068
CY073
K-97EQTB11C
K-96MP535
F1-93VI850
F1-9393BR020-1
F1-93FIN9363
F1-96MP411
F2-95MP255
F2-95MP257
F2-020016BBY
F2-97CM53657
J-94SE9280.9
J-93SE9173.3
H-94VI991
H-93VI997
H-90056.1
G-96DRCBL
G-93HH8793.1.1
G-92NG083
G-93SE6165
A2-97CDKTB48
A2-94CY017
02AG-IBNG
CY172
CY048
CY158
01AE-90CM240
CY179
CY057
CY058
CY169
CY171
CY170
CY173
CY111
CY112
A1-98UG57136
A1-94SE7253
A1-9292UG037.1
A1-94Q23
CY009
CY015
CY178
CY153
CY008
CY012
B
D
C
K
F
J
H
G
A
CRF02_AG
CRF01_AE
A
20%
(B)
pol (protease and RT)
CY013
CY014
B-83HXB2
B-981058
CY066
CY142
CY159
B-90BK132
CY010
B-00671
CY177
CY005
CY109
CY006
CY035
CY016
CY089
D-01A280
D-9494UG114
D-83ELI
D-014412HAL
CY007
F1-93VI850
F1-93FIN9363
F1-9393BR020-1
F1-96MP411
F2-95MP255
F2-95MP257
F2-020016BBY
F2-97CM53657
J-94SE9280.9
J-93SE9173.3
K-97EQTB11C
K-96MP535
H-93VI997
H-90056.1
H-94VI991
CY175
CY176
CY004
CY166
C-9292BR025.8
C-86ETH2220
C-04SK164B1
C-95IN21068
CY073
G-92NG083
G-93SE6165
G-96DRCBL
G-93HH8793.1.1
CY158
CY172
CY048
02AG-IBNG
A2-97CDKTB48
A2-94CY017
CY111
CY112
CY057
CY058
CY170
CY173
CY169
CY171
CY179
01AE-90CM240
A1-98UG57136
A1-94SE7253
A1-9292UG037.1
A1-94Q23
CY153
CY012
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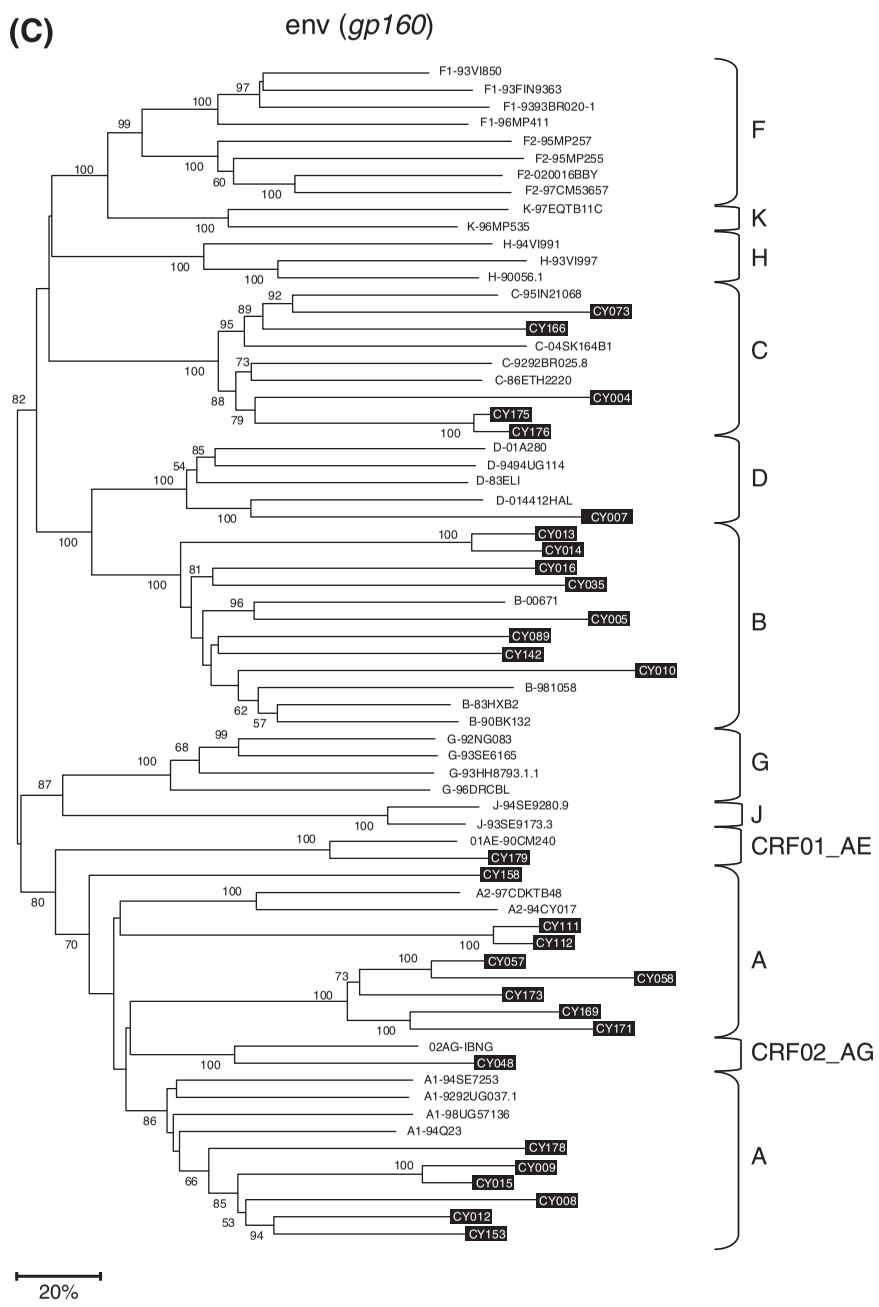

FIG. 3. Neighbor-joining phylogenetic trees for the nucleotide sequence of the *gag* region (**A**), the *pol* region (*prot and p51 RT*) (**B**), and the *env* region (*gp160*) (**C**) of HIV-1 strains obtained from newly diagnosed drug-naive patients in Cyprus, based on the Kimura two-parameter distance estimation method. In all trees representative reference sequences of HIV-1 subtypes (A–K) and CRF01_AE, CRF02_AG (shown in boldface) are included. The numbers indicated at several nodes are consensus bootstrap values out of 100 replications (only bootstrap values greater than 50% are denoted). The sequences determined in the study are shown in white on black boxes with a prefix CY for Cyprus and the number following denoting the laboratory code. The divergence between any two sequences is obtained by summing the horizontal branch length, using the scale at the lower left. The brackets on the right side of the trees indicate the determined subtypes as described in Results.

associated with high resistance (HR) to lamivudine and emtricitabine and PLR for abacavir. Furthemore, nonnucleoside reverse transcriptase inhibitor (NNRTI)-associated mutations were observed in one subtype C sequence (V179D mutation in CY004). The V179D mutation is associated with low-level (about 2-fold) resistance to nevirapine, delavirdine, and efavirenz and it has an impact on the sensitivity to etravirine when it coexists with other mutations.[33,43]

Gag cleavage site-associated mutations were analyzed from the 37 Cypriot *gag* sequences as previously established.[36,37] Genetic analysis of the *gag* sequences revealed that two subtype B sequences (CY013 and CY014) and one subtype A sequence (CY178) had the P453L (p1/p6 cleavage site) amino acid substitution previously shown to be linked with PI-associated drug resistance in combination with the presence of the protease I50V amino acid substitution.[36,37] Fusion inhibitor-associated mutations were analyzed from 33 Cypriot *env* sequences as previously established.[33,38] The *env* sequences from four patients (CY006, CY066, CY109, and CY177) were excluded due to incomplete sequencing as previously explained. Genetic analysis of the gp41 heptad repeat 1 (HR1) revealed that one subtype B sequence (CY005) had the L44M amino acid substitution, which is associated with resistance to the fusion inhibitor enfuvirtide (T20).[33,38]

Sequence analysis of env (V3 loop) region

Alignment of the predicted protein sequences of the V3 loop of *env* gp120 sequences from 36 Cypriot sequences was performed. For comparison, the consensus sequence from each subtype was also deduced (data not shown). The positions of the two cysteine residues in this region (corresponding to amino acid positions 296 and 331 of the HXB2 gp41) were conserved, suggesting a similar folding of this region of the protein. Thirty-one sequences have 35 amino acid residues, while five sequences (CY111, CY112, CY016, CY089, and CY166) have 34 amino acid residues, missing glycine (G) at position 24. All 36 sequences had a putative N-linked glycosylation site at the N-terminus of the V3 loop. Nine of 13 subtype B sequences had the GPGR tetrapeptide motif at the crest of the V3 loop, which is the most common motif found throughout subtype B sequences. However, in two subtype B sequences (CY005 and CY089), the first G in the GPGR motif was replaced by alanine (A), another nonpolar amino acid leading to the motif APGR. In other three B sequences (CY010, CY016, and CY035) the arginine (R) in the GPGR motif was replaced by serine (S), a polar amino acid, alanine (A), a nonpolar amino acid, and lysine (K), a basic amino acid, respectively. Additionally, the subtype B sequence CY159 had the motif RPRK. The tetrapeptide motifs at the tip of the loops of the 14 subtype A sequences were highly variable, containing 10 GPGQ, one RPGQ, and two GPRK. The five subtype C sequences had the GPGQ motif, as did the subtype D sequence and the CRF01_AE and CRF02_AG sequences.

The PSSM algorithm detected all the viruses as R5 (using CCR5 coreceptor). The Geno2pheno algorithm predicted 34 viruses as R5, and two (CY035 and CY111) as dual-tropic/mixed-tropics or X4 (using CXCR4 coreceptor). The two algorithms were in accordance in their capacity to detect, or not detect, R5 viruses in 34 of 36 cases (94.5%). The two samples detected as carrying dual-tropic/mixed-tropic or X4 viruses had a low CD4 cell count and high viral load, respectively (Table 1). It is important, however, to note that both interpretation coreceptor algorithms may not be fully compatible to non-B strains.

Discussion

In the period between January 2003 and December 2006, 52 persons were reported to be infected with HIV-1 in Cyprus, of which 65% were Cypriots and the rest were foreigners. Compared with the data already published by Kostrikis *et al.*, where the samples were studied with a heteroduplex mobility assay of the *env* (C2–V3) region,[22] the present study yields more detailed information about the epidemiological status of HIV infection in Cyprus among 72% of the newly diagnosed patients in the time period 2003–2006. Clearly, subtypes A and B are dominant, followed by subtypes C, CRF02_AG, D, and CRF01_AE, strains that dominate the global epidemic. The frequency of non-B subtypes entering Cyprus (65%) is higher than that reported a decade ago (40%).[22] This finding reinforces the observed trend of the increasing prevalence of HIV-1 non-B subtypes among newly diagnosed patients in Europe.[24] This phenomenon is most likely due to a large number of immigrants from African and eastern European countries, where non-B subtypes are predominant; this has also been observed in other European countries,[18,27,44–51] countries of the Mediterranean region,[17,52] and in the United States.[4,53] The present study represents a significant contribution to the molecular epidemiology of HIV-1 infection in Europe and the Mediterranean countries and to the evaluation of the movement of various strains across geographic regions.

The phylogenetic trees show that there are three distinct and divergent clusters within the A subtype, one in subtype B, one in subtype C, and one in CRF02_AG. The average intrasubtype diversity (range) among six Cypriot *gag* sequences within subtype A (CY057, CY058, CY169, CY171, CY170, and CY173) is 5.1% (0.6–11.9%). The relatively low genetic diversity (5.1%) among these sequences in comparison with the overall intrasubtype diversity (11.0%) suggests that the isolates were derived from epidemiologically linked individuals. This finding is further reconfirmed by the branch topologies in the phylogenetic trees from the *gag*, *pol,* and *env* sequences (Fig. 3A, B, and C), the bootstrap values associated with the cluster (99 out of 100 replicates), and the epidemiological information provided by the study subjects. Indeed, this cluster of patients consists of three heterosexual couples: a couple from Georgia (CY057 and CY058) who reported being infected in Georgia, a man from Cyprus (CY171) and a woman from Georgia (CY169) who were infected in Cyprus, and a man from Cyprus (CY170) and a woman (CY173) from Ukraine who were infected in Cyprus.

This is the first time the prevalence and patterns of antiretroviral drug resistance-associated mutations were estimated for a population in Cyprus in 2003–2006. A novel method for dealing with B- and non-B subtypes has been successfully established and used to determine transmitted drug resistance. Cyprus has one of the lowest levels of transmitted drug resistance (5.4%) worldwide,[30] similar to Brazil, Chile, Japan, Denmark, Slovenia, and Georgia,[28,46,54–57] and in contrast to Greece, The Netherlands, and Portugal.[3,18,27] No primary mutations conferring resistance to PIs or NNRTIs were observed in

the Cyprus isolates, but a high rate of minor mutations was seen. Two patients (5.4%) had RT mutations associated with reduced susceptibility to the NRTIs. The results of this study support the need for routine resistance testing before the initiation of antiretroviral therapy for HIV patients in Cyprus following the European and IAS-USA guidelines, which recommend resistance testing in chronically infected drug-naive patients when the regional prevalence of resistance is ≥10% and ≥5%, respectively.[58,59] This is also supported by the increase in newly diagnosed HIV-1-infected patients in 2007 in relation to the total number of newly diagnosed HIV-1-infected patients in 2006. Therefore, there should be more active surveillance of resistance-associated mutations in untreated individuals in order to recognize, as soon as possible, any significant change that may affect their future clinical management, as well as to plan and optimize the first line regimen and estimate of prevalence of resistance over time.

The gp41 HR1 domain is well conserved in all study subjects. However, one newly diagnosed patient out of 33 (3%) had one of the currently known mutations in the HR1 region associated with resistance to T20. The incidence of genotypic changes at amino acids 36–45 among T20-naive populations is low, demonstrating a natural conservation of the motif.[60,61] Our results reinforce the suggestion of testing the HR1 region with population sequencing before commencing therapy with T20.

Tropism predictions of the newly diagnosed patients were in accordance with the literature data, where it is well known that R5 variants are generally responsible for the establishment of primary infection.[62] To effectively monitor patient response to the new coreceptor inhibitors, such as maraviroc, a sequence-based method for predicting *coreceptor* usage should be performed prior to and after administration of the inhibitors in correlation with the CD4 cell count and viral load.[62,63]

In conclusion, the results presented in this report provide important information about HIV-1 genetic variation in Cyprus. A high genetic diversity of subtypes and CRFs in *gag*, *pol* (*protease and RT*), and *env* (*gp160*) sequences indicates multiple introductions of distinct viral variants. New data are provided for HIV-1 variation associated with resistance to protease, RT, and entry inhibitors: 5.4% had resistant mutations associated with NRTIs and 3% presented a T20-resistant mutation. Cyprus has a low prevalence of transmitted resistance compared with most other European countries in the *pol* (*protease and RT p51*) region, but the presence of resistant mutations in the genetic area of gp41 reveals that the newly diagnosed patients have a very heterogeneous genetic profile. Furthermore, these results provide important baselines prior to the introduction of combined antiretroviral therapy to newly diagnosed individuals in Cyprus.

Sequence Data

GenBank accession numbers for the sequences obtained in this study are as follows: *gag* sequences, EU673411–EU673447; *pol*, EU673374–EU673410 and *env*, EU668962–EU668991.

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Disclosure Statement

No competing financial interests exist.

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