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. 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 and future vaccine development. 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. 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. Based on phylogenetic analyses of

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obtained viral sequences encoding the C2 to V3 \textit{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).\textsuperscript{32} Subtype I was later determined to be a complex mosaic comprising subtypes A, G, and I and has been named CRF04_cpx.\textsuperscript{33}

Combination antiretroviral therapy (cART) with recently approved highly active antiretroviral drugs has significantly improved the quality of life for HIV-infected patients.\textsuperscript{34} 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%.\textsuperscript{35–38} The prevalence of HIV-1 variants with drug-resistant mutations in newly diagnosed individuals from western Europe and Israel is approximately 9%.\textsuperscript{39} 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 10⁶ 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 Nanodrop 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 PFMB-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 \textit{gag},\textsuperscript{32} 1832 and 3583 for \textit{pol} (protease and RT), and 5955 and 9181 for \textit{env} (gp160). Inner primers were 684 and 2406 for \textit{gag}, 2078–3539 for \textit{pol} (protease and RT), and 6111 and JL88 for \textit{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 \textit{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 \textit{pol} (protease and RT) and \textit{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 \textit{gag} and 52°C for \textit{pol} (protease and RT) and \textit{env} (gp160) and the polymerization times were 2.20 min for \textit{gag}, 2 min for \textit{pol} (protease and RT), and 3.20 min for \textit{env} (gp160). The detailed experimental method for the amplification of the \textit{pol} (protease and RT) region by RT-PCR using plasma HIV-1 RNA is described below in this section. For \textit{gag} and \textit{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 \textit{env} RT-PCR amplification there was an additional reverse transcription cycle at 54°C for 1 h; for \textit{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 Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). In each sample, the DNA sequences encoding the \textit{gag}, \textit{pol} (protease and RT), and \textit{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 \textit{gag}; 2136, 3462 for \textit{pol} (protease and RT); and 6111, 6438, 6858,
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<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Weeks since diagnosis</th>
<th>Country of origin</th>
<th>Transmission risk group</th>
<th>CD4 (cells/mm³)</th>
<th>Plasma HIV-1 RNA (copies × 10⁴ /ml)</th>
<th>Epidemiological information</th>
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<td>HSX</td>
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<td>U.K.</td>
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<td>Heterosexual partner of CY171; currently on treatment for lymphoma</td>
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<td>2</td>
<td>Cyprus</td>
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<td>447</td>
<td>11.0</td>
<td>Heterosexual partner of CY169, diagnosed with gonorrhoea</td>
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<td>F</td>
<td>27</td>
<td>4</td>
<td>Ukraine</td>
<td>HSX</td>
<td>598</td>
<td>1.8</td>
<td>Infected by heterosexual partner CY170 six months before</td>
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<td>30</td>
<td>8</td>
<td>Cyprus</td>
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<td>4</td>
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*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

<table>
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<tr>
<th>Designation a</th>
<th>Target gene</th>
<th>Sequence b</th>
<th>Position c</th>
<th>Amplicon length (nts)</th>
<th>Reference d</th>
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<td>623 (F)</td>
<td>gag</td>
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<td>gag</td>
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<td>2481–2501</td>
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<tr>
<td>684 (F) e</td>
<td>gag</td>
<td>TCTGACGAGGAATCCGTTGTC</td>
<td>684–705</td>
<td>1722</td>
<td>This study</td>
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<tr>
<td>2406 (R) e</td>
<td>gag</td>
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<td>2377–2406</td>
<td>This study</td>
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<td></td>
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<tr>
<td>2650 (R)</td>
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<td>This study</td>
<td></td>
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<td>2703–2734</td>
<td>This study</td>
<td></td>
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<tr>
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<td>This study</td>
<td></td>
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<td></td>
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<td>8017–8039</td>
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<tr>
<td>8530 (R)</td>
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<td>TGTAGCTGAGGAGGCCAGAC</td>
<td>8511–8530</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

aReverse 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.
bY, indicates equal molar mixture of C and T; R, A, and G; M, A, and C; W, A, and T.
cPrimer positions correspond to subtype B HIV-1 HXB2 strain (GenBank accession number K03455).
dSalminen et al.32

eUsed 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. Sequences were compared with the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences 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)-USA33 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
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-USA33 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 database1 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; A1-98UG37136, AF484509; A2-97CDKTBB48, AF286238; A2-94CY017, AF286237; B-83HXB2, K03455; B-981058, AY331295; B-90BK132, AY173951; B-00671, AY423387; C-9292BR025.8, U52953; C-86ETH220, U46016; C-04SK164B1, AY772699; C-95IN21068, A067155; D-01A280, AY253311; D-944UG114.1, U88824; D-83ELI, K03454; D-014412HAL, AY371157; F1-93VI997, AF190128; H-93VI991, AF190127; J-94SE9280.9, AF082394; K-97EQTB11C, 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 Geno2pheno41 and PSSM42 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.
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/mm³ 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. Four subjects were presumably infected in Germany by homosexual contact. One man was infected 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

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

Table 3. Characteristics of the Study Subjects: (a) IQR, interquartile range. (b) Information available for 35 patients. (c) Information available for 34 patients.

A 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 was infected in Thailand by heterosexual contact. One of the men 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 based on 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; CRF01_AE, one sequence; 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, L63F/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
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 em-
tricitabine and PLR for abacavir. Furthermore, 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 efa-
virenz and it has an impact on the sensitivity to etravirine
when it coexists with other mutations.33,41

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 sub-
type B sequences (CY013 and CY014) and one subtype A se-
quence (CY178) had the P453L (p1p/p 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 in-
hibitor-associated mutations were analyzed from 33 Cypriot
env sequences as previously established.33,38 The env se-
quences from four patients (CY006, CY066, CY109, and
CY177) were excluded due to incomplete sequencing as pre-
viously 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 re-

tance 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 posi-
tions of the two cysteine residues in this region (correspond-
ing 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 resi-
dues, 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 gly-
cosylation 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 APCR. 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 sub-
type D sequence and the CRF01_AE and CRF02_AG se-
quences.

The PSSM algorithm detected all the viruses as R5 (using
CCR5 coreceptor). The Geno2pheno algorithm predicted
34 viruses as R5, and two (CY003 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, re-
respectively (Table 1). It is important, however, to note that both
interpretation coreceptor algorithms may not be fully com-
patible 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 contri-
bution to the molecular epidemiology of HIV-1 infection in
Europe and the Mediterranean countries and to the evalua-
tion 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 in-
trasubtype 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 diver-
sity (5.1%) among these sequences in comparison with the
overall intrasubtype diversity (11.0%) suggests that the iso-
lates 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 in-
formation 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 in-
fected 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 Uk-
ainewho were infected in Cyprus.

This is the first time the prevalence and patterns of anti-
retroviral drug resistance-associated mutations were estimated
dealing with B- and non-B subtypes has been successfully es-
tablished and used to determine transmitted drug resistance.
Cyprus has one of the lowest levels of transmitted drug resis-
tance (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.31,18,27 No primary mu-
tations 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.60,61 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 occurrence 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.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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