Molecular Diversity of HIV in Albania

Massimo Ciccozzi,1 Caterina Gori,2 Stefano Boros,1
Maria José Ruiz-Alvarez,2 Arjan Harxhi,6 Marijeta Dervishi,3
Shpetim Qyra,3 Nicola Schinina,3 Roberta D’Arrigo,2
Francesca Ceccherini-Silberstein,2 Silva Bino,4
Carlo Federico Perno,4,5 and Giovanni Rezza1

1Epidemiology Unit, Department of Infectious Diseases, Istituto Superiore
di Sanità, 2Istituto Nazionale Malattie Infettive L. Spallanzani, and 3Department
of Experimental Medicine, Tor Vergata University, Rome, Italy; 4National
Institute of Public Health (NIPH), 5Department of Infectious Disease Control,
NIPH, and 6Infectious Disease Hospital, Mother Theresa Medical Center,
Tirana, Albania

Little information is available on circulating human immunodeficiency virus (HIV) subtypes and resistance to antiretroviral drugs in Albania. To fill this gap, we studied 72 plasma samples from HIV-infected individuals from throughout the country. Subtype classification and genotypic resistance analysis were performed on the HIV pol gene region. The analysis was successfully performed on 66 (91.6%) plasma samples and showed that 43 (65.2%) strains were non-B subtypes (mostly subtype A, as determined by analysis of pol gene sequences). No major mutations in the protease gene were found, whereas analysis of the reverse transcriptase gene revealed a few major mutations associated with resistance. In conclusion, non-B subtypes are predominant in Albania, and the prevalence of resistance to antiretroviral drugs is still low.

The epidemic of HIV infection and AIDS seems to have had a limited impact on the Balkan countries, which, with the exception of Greece, are considered to be part of central Europe [1]. In recent years, most countries in the western Balkans have been affected by war, dramatic political changes, and socioeconomic disruption, which are considered to be potential determinants of the spread of HIV infection. However, in most of these countries, the incidence of AIDS has remained relatively low, ranging from 0.7 cases/1 million population in Bosnia-Herzegovina to 7 cases/1 million population in Montenegro [2].

In Albania, where migration and urbanization, together with increasing use of injection drugs, may have fueled the spread of infections such as HIV, the incidence of AIDS increased from 0.3 cases/1 million population in 1996 to 3.2 cases/1 million population in 2002. During this same period, a 3-fold increase in newly diagnosed cases of HIV infection was also reported (from 2.2 cases/1 million population to 8.2 cases/1 million population) [2]. Although these data may have resulted from changes in the pattern of HIV testing and should be interpreted with caution, the upward trend in diagnoses of HIV infection in eastern Europe is likely to be indicative of an increased incidence of infection [1].

Although some basic information on the main characteristics of individuals affected by HIV/AIDS in the Balkans is available, little is known about which HIV subtypes are circulating. A study conducted in the former Yugoslavia identified >90% of the strains as being subtype B [3], which is the prevalent subtype in western Europe. To the best of our knowledge, there are nearly no data on the subtypes circulating in Albania. Moreover, no information is available on the prevalence of resistance to antiretroviral drugs in HIV-infected individuals in Albania, nearly all of whom are antiretroviral drug naive, during 10 consecutive years (1994–2003). Because antiretroviral drugs will become more widely available in the country in the very near future, data on drug resistance will be of utmost importance.

The objective of the present study was to describe the molecular diversity of the HIV subtypes circulating in Albania. To this end, we conducted an analysis of plasma samples, including an analysis of genotypic resistance to antiretroviral drugs, from ~50% of the country’s known individuals with HIV/AIDS.

Subjects, materials, and methods. In Albania, the first case of HIV/AIDS was diagnosed in 1992, and, since that time, new diagnoses of HIV infection and AIDS have been subject to mandatory reporting to the National Institute of Public Health (NIPH) in Tirana. Since 1994, the NIPH has also acted as the national reference center for Western blot confirmation of ELISA-reactive serum samples. Cases are reported using an anonymous questionnaire that records the individual’s age, sex, place of birth, residence, HIV exposure category, date of diagnosis, and disease stage (classified as “AIDS” or “AIDS-free HIV infection”). The study was conducted in accordance with the ethical standards of the NIPH.

Seventy-two plasma samples were obtained from antiretroviral drug–naive individuals with confirmed HIV infection (47...
HIV genotyping was successfully performed on 66 (91.7%) of these plasma samples. The plasma samples, stored at the NIPH, were obtained from whole blood treated with EDTA (centrifugation was at 200 g at 4°C for 10 min) and stored at −80°C before genotype analysis. All plasma samples were shipped to the Istituto Superiore di Sanità (Rome, Italy) for analysis. The samples were linked to demographic and clinical data through an anonymous numerical code, in accordance with the ethical standards of the NIPH.

HIV genotyping was performed with the Applied Biosystems Viroseq HIV Genotyping System (Abbott), in accordance with the manufacturer’s instructions, with a minor modification consisting of the extraction of viral RNA using the QIAamp Viral RNA kit (Qiagen). The polymerase chain reaction (PCR) products (1.8-kb amplicons) were sequenced using 7 different overlapping sequence-specific primers, and the sequencing samples were analyzed with a 3100 Genetic Analyzer using Viroseq analysis software (version 2.5; Applied Biosystems). Mutations were defined according to the table of mutations of the International AIDS Society [4]. For 23 samples, the volume of available plasma was too low (i.e., <100–300 µL) to obtain reliable results with the above-mentioned procedure; we thus performed a nested PCR using specific primers (gacaggctattttta-ggg [sense] and ggctcttgataaatttgatatg [antisense]) that spanned the same region that the primers of the Viroseq HIV Genotyping System did.

To rule out contamination between samples and to collect information on the most similar published gene sequences, each sequence was compared with others amplified at the same time and with published sequences by use of the Los Alamos BLAST search tool (Los Alamos HIV Sequence Database). The sequences were then aligned and compared with reference sequences of the HIV pol gene (available at: http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html) using CLUSTAL X [5]; the sequences were manually edited with the Bioedit program [6], and gaps were removed from the final alignment.

Phylogenetic trees were generated with the F84 model of substitution by use of both neighbor-joining (NJ) and maximum likelihood (ML) tree-building methods [7]. The evolutionary model was chosen as the best-fitting nucleotide substitution model in accordance with the results of the hierarchical likelihood ratio test (HLRT) implemented in MODELTEST software (version 3.0; [14]). The parameters for the nucleotide substitution model were estimated by the ML method using an NJ tree (Jukes-Cantor distance) as the base tree [15]. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed with either a bootstrap analysis using 1000 replicates, for the NJ tree, or the zero branch length test, for the ML tree. All calculations were performed with PAUP software (version 4.0; [7]). Simplot software (version 3.2; [8]) was used to generate similarity plots and bootstrap plots, for genetic diversity and intersubtype recombination analysis.

The phylogenetic tree shows that the circulating strains belonging to subtype A are monophyletic, whereas subtype B and C strains aggregate in several clusters. The mean genetic distances, measured with the Kimura-2 parameter model, was 2.2% for subtype A, 3.9% for subtype B, and 6.1% for subtype C.

No major mutations were found in the protease genes of these individuals, whereas minor mutations/polymorphisms were found in the majority of them; interestingly, polymorphisms in non-B subtypes were clustered at nucleotide positions 36, 63, and, more rarely, 10, whereas polymorphisms were found at different nucleotide positions in B subtypes. Major mutations associated with resistance (M41L, D67N, T69D/S, K101Q, V106I/M, V118I, T215L/D, and G333E) were found in the reverse transcriptase (RT) genes of 2 individuals.

**Discussion.** To our knowledge, this is the first analysis of HIV genotypes circulating in Albania. The study tested plasma samples from ~50% of the known individuals with HIV infection in Albania, where a total of 106 cases of AIDS-free HIV infection and 37 cases of AIDS had been reported as of 30 June 2003. The main finding of the study is that subtype A is predominant, which, to some extent, is surprising, given that in neighboring countries, such as Italy and Greece, which host a large number of Albanian migrants, the predominant subtype is B [9, 10].

It has been estimated that, during the past 10 years, thousands of Albanian women and girls have been working as sex workers in other Balkan countries and in western Europe, especially in Italy and Greece. However, Albania is also a main transit country for the trafficking of women from central and eastern Europe [11]. Although it is highly likely that most HIV
Figure 1.  Phylogenetic relationships based on a 1330-nt region of the pol gene in 66 HIV strains from Albania and representative strains of HIV-1 group M (subtypes A–D, F1, F2, G, H, J, K) from the Los Alamos HIV Sequence Database. Bootstrap values <70% are not shown. The sequences of the Albanian strains are designated as nos. 03, 05–12, 15–34, and 37–73. The subtype clusters are differentiated by typeface (bold, underlined, bold italic, and underlined italic, for subtype A, B, C, and D, respectively). Sequence 62 is in bold and is double underlined because, in the detailed analysis, it was determined that it was not a pure subtype, and it was classified as a putative BA recombinant form. The scale bar indicates 10% nucleotide sequence divergence. *P<.001, zero length branch test.
Type A strain, as determined by the full genome should be performed to confirm that our sub-
the eastern European subtype A strains. However, analysis of that the Albanian strains may be closer to the African than to
in most countries [12]. A comparison of subtype A sequences primarily from eastern Europe, where subtype A is prevalent
Italy or Greece, it is possible that subtype A infections come
subtype B infections diagnosed in Albania were acquired in Italy or Greece, it is possible that subtype A infections come
primarily from eastern Europe, where subtype A is prevalent
in most countries [12]. A comparison of subtype A sequences from Albania with those from other geographical areas showed that the Albanian strains may be closer to the African than to the eastern European subtype A strains. However, analysis of the full genome should be performed to confirm that our subtype A strain, as determined by pol gene sequences, is a pure subtype A (i.e., subtype A as determined by analysis of env gene sequences also). Our data suggest that HIV entered Albania through different routes. HIV subtypes showed different dynamics; in particular, subtype A, which had lesser genetic divergence than the other subtypes, tended to spread more rapidly and to be more commonly detected in women.

With regard to antiretroviral drug resistance, the mutations found in the HIV protease gene are consistent with the low frequency of minor mutations expected for B and non-B subtypes at nucleotide positions associated with subtype-specific polymorphisms. However, analysis of the RT gene revealed some mutations that are not compatible with the total absence of antiretroviral drug treatment. In fact, mutations at nucleotide positions 41, 67, 69, and 215 are poorly represented in antiretroviral drug–naive individuals; as was confirmed by a study of the prevalence of major mutations in antiretroviral drug–naive individuals in Italy [13], these mutations are likely to be transmitted by an antiretroviral drug–treated individual. The presence of the mutations in subtypes D and A probably indicates the occurrence of antiretroviral drug pressure on subtypes other than B, and this suggests that these mutations originate from countries with access to nucleoside and nonnucleoside RT inhibitors. Mutations at nucleotide position 215 (D or L, which usually appear during treatment with thymidine analogues) can be considered revertants from a typical Y or F originally present in the viral donor. Their relatively good fitness, however, is often associated with their persistence, and, therefore, we cannot exclude the possibility that these mutations were transmitted directly by the viral donor.

These results highlight the importance of resistance testing in antiretroviral drug–naive individuals. As a whole, the information on mutations suggests that resistant HIV strains are beginning to circulate in Albania, perhaps because the infection is being spread from individuals who acquired it abroad and return frequently to Albania. Follow-up analyses will be necessary to confirm and expand on this hypothesis.

Some limitations on the results of this study and some possible biases should be mentioned. First, we could not obtain full genome sequences, and, therefore, subtyping at this stage is only putative. Second, although we obtained plasma samples from ~50% of the known HIV-infected individuals in Albania, a recruitment bias that possibly affected the representativeness of our study population may have occurred nonetheless. Third, information on sexual behavior and travel history was limited and, thus, was of no use in tracing the origin of infection with the different HIV subtypes or the way in which these subtypes were introduced in Albania. Fourth, we had no information on the duration of HIV infection in the study participants. This may have created a bias in the calendar-year analysis, the results of which indicated that non-B subtypes started to spread in Albania. However, given that there is no definitive evidence that non-B subtypes and B subtypes differ in terms of virulence, it is unlikely that there was a selective effect on the time of reporting HIV infection with different subtypes. Overall, given the high rate of success in amplifying and sequencing the genetic material in processed plasma samples, a selection bias can probably be ruled out.

Our findings show that multiple HIV subtypes are circulating in Albania and that non-B subtypes are predominant. Antiretroviral drug resistance is still limited because of the lack of access to treatment, although major mutations in the RT gene have been observed. Our analysis is important because antiretroviral drug treatment will become more widely available in Albania in the near future.

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