

Genetic Diversity of HIV Type 1 in Montenegro

Massimo Ciccozzi,¹ Danijela Vujošević,² Alessandra Lo Presti,¹ Boban Mugoša,² Zoran Vratnica,² Alessia Lai,³ Dragan Laušević,² Nenad Drašković,⁴ Aleksandra Marjanovic,² Eleonora Cella,¹ Maria M. Santoro,⁵ Claudia Alteri,⁵ Lavinia Fabeni,⁴ Marco Ciotti,⁶ and Gianguglielmo Zehender³

Abstract

Human immunodeficiency virus type 1 (HIV-1) is characterized by high genetic variability due to its high replication rate and the lack of proofreading activity of the reverse transcriptase enzyme. On the basis of phylogenetic analysis performed on numerous isolates from all over the world, HIV-1 is subdivided into types, subtypes, subsubtypes, circulating recombinant forms, and unique recombinant forms. No data are currently available about the circulation of HIV-1 types in Montenegro. Here, we describe the genetic variability of HIV-1 strains identified in plasma samples of patients from Montenegro. Phylogenetic analysis on 32 HIV-1 sequences was carried out. The prevalent circulating HIV-1 subtype is B. The strains were interspersed within the tree. Two main clades (I and II) may suggest independent introductions of HIV-1 subtype B into Montenegro, although other epidemiological evidence will be needed to assume a small number of introductions. No obvious evidence of clustering by residence, age, or sex was found (data not shown). Nelfinavir resistance was found, though lopinavir is the only PI administered. Continuous monitoring of HIV-1-infected individuals is crucial to a better understand of the epidemiology of the B subtype in Montenegro.

Introduction

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) infection continues to spread rapidly throughout the world. According to the UNAIDS/WHO (December 2009) report, an estimated 33.4 million people are living with the virus worldwide. Recombination coupled with the lack of proofreading activity of the reverse transcriptase (RT) and the rapid turnover of HIV-1 in infected individuals are at the origin of the high genetic variability and diversification of the virus.¹⁻³ The majority of HIV-1 strains cluster within a large group called M (for Main), which includes nine subtypes (A–D, F–H, J, and K), phylogenetically distinct. Subsubtypes A and F can be further subdivided into subsubtypes A1–A4 and F1 and F2, respectively. A number of intersubtype recombinant viruses are also observed. When such recombinant viruses spread within the human population they become circulating recombinant forms (CRFs); when they remain restricted to a limited number of individuals they are called unique recombinant forms (URFs).⁴⁻⁶ The other groups are N, O, and P. Epidemiological data indicate that the impact of the HIV-1

epidemic on Balkan countries, which are part of central Europe, is still limited. By contrast, in southern Mediterranean countries, although the number of newly diagnosed cases reported among injection drug users is declining, the HIV prevalence remains high.^{7,8}

Montenegro is a young state that gained independency in May 2006. In this country the most recent official census data (2003) sets the population at 620,145 individuals. Although some basic information on the main characteristics of individuals affected by HIV/AIDS in the Balkans is available, little is known about the circulation of HIV-1 subtypes in Montenegro. The first cases of HIV-1 infection and AIDS were described in an intravenous drug user and sailor, respectively. Two cases of mother-to-child transmission were documented in 1989.⁹ Since then, new diagnoses of HIV infection and AIDS are subjected to mandatory reporting to the National Institute of Public Health (NIPH) of Montenegro. In spite of significant progress made in accessing diagnostics and treatment, no information is currently available on the circulation of HIV-1 subtypes and the prevalence of antiretroviral drug resistance in Montenegro.

¹Department of infectious Diseases, Istituto Superiore di Sanità, Rome, Italy.

²Institute of Public Health, Podgorica, Montenegro.

³Department of Clinical Sciences, University of Milan, Milan, Italy.

⁴Clinical Centre, Podgorica, Montenegro.

⁵Department of Experimental Medicine and Biochemical Sciences, University of Tor Vergata, Rome, Italy.

⁶Laboratory of Molecular Virology, Foundation Polyclinic Tor Vergata, Rome, Italy.

In Montenegro, highly active antiretroviral therapy (HAART) is provided by the Clinic for Infectious Diseases, Podgorica, and antiretroviral drugs will become more widely available in the years to come. Therefore, data on drug resistance will be of utmost importance. The objective of the present study was to investigate the molecular diversity and the epidemiology of the HIV-1 subtypes circulating in Montenegro along with the prevalence of drug resistance mutations in the protease (PR) and RT genes of both drug-naïve and drug-treated patients as reported by the International AIDS Society (IAS)–USA¹⁰ and HIV Drug Resistance Database (<http://hivdb.stanford.edu>). Updated 08/06/08).

Materials and Methods

Plasma samples were obtained from whole blood treated with EDTA after centrifugation at 200×g for 10 min in a refrigerated centrifuge, and stored at –80°C until 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 according to the ethical standards of the NIPH.

An epidemiological analysis of 35 patients with HIV/AIDS attending the NIPH was performed, including genotypic resistance analysis of the virological response to PR and RT antiretroviral drugs. HIV-1 *pol* sequencing was performed on 32 plasma samples collected from 1997 to 2010 as described previously.¹¹ Three samples were not amplifiable. HIV genotyping was performed with the Applied Biosystems Viroseq HIV Genotyping System (Abbott Molecular, Abbott Park, IL), according to the manufacturer's instructions, with a minor modification consisting in the extraction of viral RNA by the QIAamp Viral RNA kit (Qiagen, Milan, Italy). The amplified products (1.8-kb amplicons) were sequenced using seven different overlapping specific primers, and the sequences were analyzed with a 3100 Genetic Analyzer using the Viroseq analysis software v 2.5 (Applied Biosystems, Foster City, CA). All 32 HIV-1 *pol* sequences were first analyzed using the REGA HIV-1 Subtyping Tool.¹² Then they were aligned using Clustal X 1.1 and manually edited by Bioedit.¹¹ The final data sets included HIV-1 *pol* from Montenegro strains, as well as specific subtypes and CRF reference sequences downloaded from the HIV Los Alamos database (<http://hiv-web.lanl.gov/>). Phylogenetic trees were generated with the F84 model of substitution by use of both neighbor-joining (NJ) and maximum likelihood (ML) tree-building methods.¹¹

The best fitting nucleotide substitution model was tested with a hierarchical likelihood ratio test.¹¹ An ML tree was then inferred with the selected model as previously described.^{11,12} Calculations were performed with PAUP* software version 4.0.¹¹

Statistical support for specific clades was obtained by bootstrapping values (1000 replicates) for the NJ trees. The tree was rooted by outgroup rooting. SimPlot and split decomposition analysis were performed for genetic diversity and intersubtype recombination analysis.¹¹ Demographic and clinical data for all 35 patients were collected anonymously according to the ethical standards of the Institute Ethics Committee of Montenegro. The median age of the patients was 30 years (interquartile range: 5–48), and 30 (85.7%) out of 35 were males. At the time of samples collection, 65.7% of the

patients were on HAART (the most used combination was AZT/3TC + LPV/RTV).

Results

Of the 32 HIV-1 patients with an available *pol* sequence, 27 were infected by B subtypes, four by C subtype, and one by A1 subtype (data not shown). The distribution of the different subtypes by age, sex, mode of transmission, residence, and year of HIV/AIDS diagnosis is reported in Table 1.

The proportion of subtype B strains increased over time (chi square for trend $p < 0.05$): this subtype represented 84% of all subtypes identified after 1999; instead, no difference was observed for non-B subtypes over the time period considered (Table 1). A phylogenetic tree was generated for B subtypes only and it showed that the strains were interspersed within the tree. Two main clades (I and II), highlighted by the box in Fig. 1, were statistically supported (bootstrap values >75%). In both clades subtype B sequences from individuals infected through sexual transmission clustered. Clades I and II are evidence of probably independent introductions of HIV-1 subtype B into Montenegro; moreover they could imply the presence of separate transmission networks for subtype B within the country. No obvious evidence of clustering by residence, age, or sex was found (data not shown). No putative recombinant forms were found. Three out of 22 (13.6%) treated patients harbored resistant viruses. One patient carried resistance only to nucleoside RT inhibitors (NRTI) (caused by the presence of the thymidine analogue mutation M41L), one resistance to PR inhibitors (PI) (L33F), and one was resistant to both NRTI (thymidine analogue mutations D67N and V118I) and PI (D30N and N88D). No non-NRTI resistance mutations were detected. No resistance was identified in drug-naïve patients.

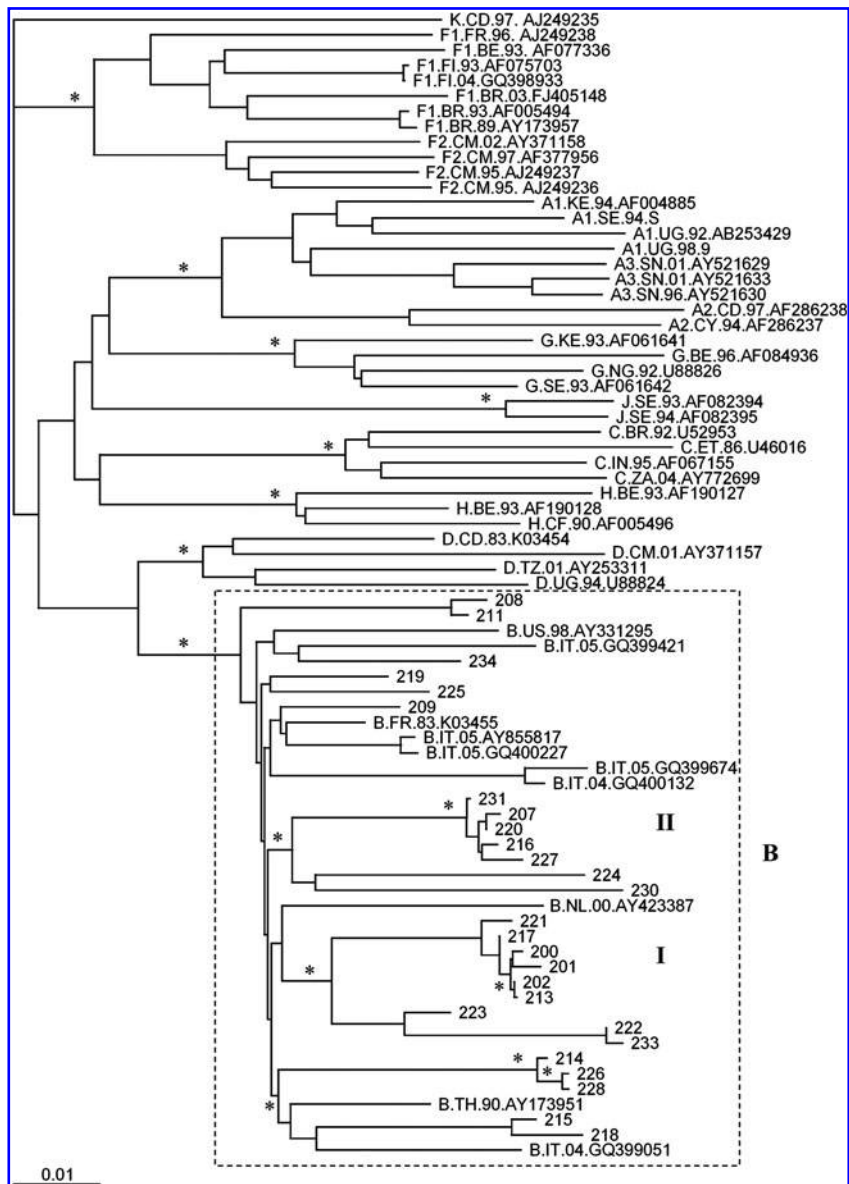
Discussion

To our knowledge, this is the first study on HIV-1 genotypes circulating in Montenegro. The study tested plasma samples from about 44% of the known individuals with HIV-1 infection in Montenegro, where a total of 71 cases of HIV/AIDS cases were registered by the end of 2006; 40 out of 71

TABLE 1. DISTRIBUTION OF HIV-1 SUBTYPES BY AGE, SEX, MODE OF TRANSMISSION, AND YEARS OF DIAGNOSIS IN MONTENEGRO

	B	Non-B
Age		
<30	15 (88,2)	2 (11,8)
>30	12 (80,0)	3 (20,0)
Sex		
Male	24 (88,9)	3 (11,1)
Female	3 (60,0)	2 (40,0)
Mode of transmission		
Sexual	26 (83,9)	5 (16,1)
Vertical	1 (100,0)	—
Years of diagnosis		
1996–2000	2 (7,4)	—
2001–2006	6 (22,2)	3 (60,0)
2007–2010	19 (70,4)	2 (40,0)

FIG. 1. Phylogenetic relationships based on a 1330-nt region of the *pol* gene in 27 HIV-1 B subtype strains from Montenegro and 48 subtype-specific reference sequences (subtypes A to K labeled as gene bank). The Montenegrin sequences are numbered. The main supported clades are marked as I and II. One (*) along a branch represents significant statistical support for the clade subtending that branch ($p < 0.001$ in the zero-branch-length test and bootstrap support $> 80\%$). The scale bar indicates 1% of nucleotide divergence. The tree was rooted using one HIV-1 K strain as the outgroup.



developed AIDS. The B subtype was the predominant one. This finding is not surprising, given that in neighboring countries, such as Italy, Greece, and Serbia, the predominant subtype is B.^{13,14} In contrast to findings in Albania and Bulgaria,^{11,12} in Montenegro the introduction of the HIV-1 non-B subtype did not appear to be more recent than the B subtypes. Probably this is a bias, because previous data on HIV-1 were collated with data from Serbia, where the HIV-1 B subtype constituted more than 90% of the infected individuals¹⁴; in addition, because Montenegro has been independent only since 2006, the number of HIV-1 cases could be underestimated. All clusters mainly included viral strains from individuals infected exclusively through sexual contact (only one was for vertical transmission). Moreover, the presence of two distinct and well-supported monophyletic clades within the HIV-1 B phylogeny might suggest that separate transmission clades have been evolving along independent phylogenetic lineages. It is also possible to have dozens of introductions of Clade I and II from different countries. Other

epidemiological evidence would be needed to determine whether it is reasonable to assume a very small number of introductions to Montenegro.

With regard to antiretroviral drug resistance, the level of resistance observed in Montenegro was low in HIV-1 drug-treated patients and completely absent in drug-naive patients compared to the other European countries.¹⁵ This finding may be explained by the recent and partial introduction of antiviral therapy in Montenegro. Of interest, one patient harbored the typical mutations related to resistance to nelfinavir (D30N and N88D), while the only PI administered in Montenegro is lopinavir. This finding suggests that this patient was infected by a resistant virus from an HIV-1-infected individual coming from another country. Better characterization and continuous monitoring of HIV-1 B-infected individuals in Montenegro are crucial in order to understand the epidemiology of the B subtype in this country and to assess the efficacy of prevention and therapy in controlling the epidemic and in keeping the local epidemic at a low level.

Sequence Data

The nucleotide sequences obtained in this study have been submitted to GenBank under accession numbers HQ655112 to HQ655143.

Author Disclosure Statement

No competing financial interests exist.

References

- Hahn BH, Shaw GM, Arya SK, Popovic M, Gallo RC, *et al.*: Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature* 1984;312: 166–169.
- Seillier-Moisewitsch F, Margolin BH, and Swanstrom R: Genetic variability of the human immunodeficiency virus: Statistical and biological issues. *Annu Rev Genet* 1994;28: 559–596.
- Peeters M and Sharp PM: Genetic diversity of HIV-1: The moving target. *AIDS* 2000;14(Suppl 13):S129–S140.
- Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, *et al.*: HIV-1 nomenclature proposal. *Science* 2000;288: 55–56.
- McCutchan FE: Understanding the genetic diversity of HIV-1. *AIDS* 2000;14:S31–S44.
- Salminen MO, Carr JK, Burke DS, and McCutchan FE: Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Res Hum Retroviruses* 1995;11:1423–1425
- HIV/AIDS Surveillance in Europe: Mid-year report, 2005.
- EuroHIV: HIV/AIDS Surveillance in Europe. Mid-year report 2007. Saint-Maurice: Institut de Veille Sanitaire, 2007; 76.
- Annual Report on HIV/AIDS in Montenegro for 2009 (Institute of Public Health, Center for Disease Control and Prevention, Podgorica, Montenegro, May 2010).
- Johnson VA, Brun-Vezinet F, Clotet B, Gunthard HF, Kuritzkes DR, Pillay D, *et al.*: Update of the drug resistance mutations in HIV-1: December 2009. *Topics HIV Med* 2009;17(5):138–145.
- Ciccozzi M, Gori C, Boros S, Ruiz-Alvarez MJ, Harxhi A, Dervishi M, *et al.*: Molecular diversity of HIV in Albania. *J Infect Dis* 2005;192:475–479.
- Salemi M, Goodenow MM, Montieri S, de Oliveira T, Santoro MM, Beshkov D, *et al.*: The HIV type 1 epidemic in Bulgaria involves multiple subtypes and is sustained by continuous viral inflow from West and East European countries. *AIDS Res Hum Retroviruses* 2008;24(6):771–779.
- Paraskevis D, Pybus O, Magiorkinis G, Hatzakis A, Wensing A M, Van de Vijver D, *et al.*: Tracing the HIV-1 subtype B mobility in Europe: A phylogeographic approach. *Retrovirology* 2009;20(6):49.
- Stanojevic M, Papa A, Papadimitriou E, Zerjav S, Jevtovic D, Salemovic D, *et al.*: HIV-1 subtypes in Yugoslavia. *AIDS Res Hum Retroviruses* 2002;18(7):519–522.
- Wheeler WH, Ziebell RA, Zabina H, Pieniazek D, Prejean J, Bodnar UR, *et al.*: Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010;24(8):1203–1212.

Address correspondence to:

Massimo Ciccozzi

Department of Infectious

Parasite and Immune-Mediated Diseases

Istituto Superiore di Sanità

Viale Regina Elena, 299

Rome 00161

Italy

E-mail: massimo.ciccozzi@iss.it