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Molecular typing of the local HIV-1 epidemic in Serbia

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ABSTRACT

Worldwide HIV-1 pandemic is becoming increasingly complex, with growing heterogeneity of subtypes and recombinant viruses. Previous studies have documented HIV-1 subtype B as the predominant one in Serbia, with limited presence and genetic diversity of non B subtypes. In recent years, MSM transmission has become the most frequently reported risk for HIV infection among newly diagnosed patients in Serbia, but very little is known of the network structure and dynamics of viral transmission in this and other risk groups. To gain insight about the HIV-1 subtypes distribution pattern as well as characteristics of HIV-1 transmission clusters in Serbia, we analyzed the genetic diversity of the *pol* gene segment in 221 HIV-1-infected patients sampled during 2002–2011. Subtype B was found to still be the most prevalent one in Serbia, accounting for over 90% of samples, while greater diversity of other subtypes was found than previously reported, including subtypes G, C, A, F, CRF01 and CRF02. In total, 41.3% of analyzed subtype B sequences were found associated in transmission clusters/network, that are highly related with MSM transmission route.

1. Introduction

The defining feature of HIV is its extreme genetic variability. The continuous increase of HIV genetic diversity is driven by its rapid turnover in infected individuals, by high mutation and recombination rate and short generation time (Castro-Nallar et al., 2012; Boutwell et al., 2010; Onafuwa-Nuga and Telesnitsky, 2009). Phylogenetic analysis of human immunodeficiency virus type one (HIV-1) strains revealed four distinct genetic groups in HIV-1 global pandemic so far: M (main), O (outlier), N (new) and the recently identified group P in Cameroon (Plantier et al., 2009). Group M is the most diversified one, responsible for the global pandemic of HIV-1/AIDS (Peeters and Sharp, 2000), whereas the other three groups remain mostly restricted to West Africa. Group M is further divided into ten pure subtypes and sub-subtypes (McCutchan, 2000; Paraskevis and Hatzakis, 1999; Robertson et al., 2000) while a number of circulating recombinant forms and unique recombinant forms has also been described (http://www.hiv.-lanl.gov/ content/ sequence/ HIV/CRFs/CRFs.html). The expansion of HIV epidemic has resulted in non-uniform distribution ofsubtypesand recombinants, with the greatest diversity in sub-Saharan Africa (Tatem et al., 2012).

On a global scale, according to recent studies, the most prevalent HIV-1 genetic form is subtype C accounting for almost 50% of all HIV-1 infections worldwide (Buonaguro et al., 2007; Butler et al., 2007; Hemelaar et al., 2011). Molecular epidemiology of HIV-1 in Europe is complex and geographic distribution of different subtypes is influenced by transmission risk and social factors. In Western and Northern Europe, the HIV-1 epidemic is still mainly associated with subtype B, although the prevalence and diversity of non B subtypes is increasing, partly due to individuals who have traveled to other continents or immigrated from them (Descamps et al., 2010; Giuliani et al., 2009; Hemelaar et al., 2011). On the other hand, in many Eastern European countries, a range of non B subtypes constitutes the most prevalent strains. According to epidemiological data, in former Soviet Union countries the HIV-1 epidemic is mainly sustained by subtype A (Smolskaya et al., 2006). Local situation in the Balkans, where Serbia is situated, is marked by significant diversity, with non-uniform presence of non Bclades (Stanojevic et al., 2012).

In Serbia, duration of HIV epidemic is similar to the one in Western European countries, with first cases registered in 1985. By the end of 2012 the cumulative number of reported HIV/AIDS cases was close to 3000 (http://www.batut.org.rs/download/aktuelno/Epidemija%20HIV%20infekcije%20u%20Srbiji%202012.pdf). HIV epidemic in Serbia was first recognized among intravenous drug users (IDUs) and this transmission route was the most prevalent one over the coming years. By the end of nineties, intravenous drug use was still the most prevalent risk among cumulative number of

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HIV/AIDS cases, around 48.9%, followed by sexual transmission, 33% (Stanojevic et al., 2002). According to the latest epidemiological data, among newly diagnosed HIV infections in 2012, proportion of IDUs has dramatically decreased to around 2%, while infection rate among men who have sex with men (MSM) has increased, becoming the most prevalent transmission route among newly diagnosed infections in Serbia, with around 62% (http://www.batut.org.rs/download/aktuelno/Epidemija%20HIV%20infekcije%20u%20Srbiji%202012.pdf). HIV infection cases are not evenly distributed throughout the country, with majority of cases reported in Belgrade metropolitan area. Studies on HIV subtype distribution in Serbia so far have found predominant presence of subtype B, with other subtypes much less represented (Stanojevic et al., 2002, 2012).

Monitoring of subtypes distribution can give valuable information to a health care services with regards measures to sustain the epidemics and reduce onward transmission. With ongoing generation of viral genetic diversity and potential for the emergence of novel genetic variants, it has become increasingly important to establish potential clinical implications of subtype variation. Some studies gave evidence that different subtypes may respond differently to drug therapy and found different rates of disease progression within HIV subtypes, while others suggested limited significance of differences among subtypes with regards development and clinical interpretation of antiviral resistance (Camacho and Vandamme, 2007; Easterbrook et al., 2010; Kantor, 2006; Vijver et al., 2006). The application of phylogenetic analysis to the study of HIV have revealed the origin of HIV-1 and HIV-2 epidemics, the classification of HIV into different types, groups and subtypes (Castro-Nallar et al., 2012; Lemey et al., 2003), drug resistance mutation pathways and mechanism of drug resistance. Phylogenetic methods can provide unique insights in transmission networks and spread of the virus. So far, phylogenetic studies performed throughout the world helped to map local HIV epidemics in correlation with transmission pathway, drug resistance, risk behavior and cluster size. Many studies were based on partial pol gene phylogeny, which has shown to be adequate to infer transmission events and to characterize epidemiological patterns of public health relevance (Hué et al., 2004).

The aim of this study was to analyze molecular diversity of HIV-1, prevalence and distribution of subtypes in Serbia, as well as to better characterize local HIV epidemic spread by means of phylogenetic analysis.

2. Methods

2.1. Study population

The study included 221 consenting, HIV infected patients aged 18 or more, referred to the Center for HIV/AIDS, Institute for Infectious and Tropical Diseases in Belgrade, which is the main center treating most of HIV infected patients in Serbia. Majority of included patients were newly diagnosed (179/221), partly within the European project for monitoring of primary HIV resistance SPREAD/EuropeHIVResistence, while 42/221 patients were on treatment. Blood samples were collected from September 2002 to the end of 2011. Epidemiological, clinical and behavioral data were collected using standardized questionnaire. Transmission risk was categorized as men who have sex with men (MSM), heterosexual, intravenous drug use (IDU), transfusion, vertical transmission or unknown. For patients reporting IDU in addition to another risk, the former was considered as main risk for acquiring HIV infection.

2.2. RNA isolation, RT PCR and DNA sequencing

Genotypic resistance testing was performed by using commercial Viroseq™ HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA) (53 samples), Trugene HIV Genotyping kit (Visible Genetics, Toronto, Canada) (32 samples), according to the manufacturer's recommendations and an in-house genotypic resistance assay (136 samples). For in house processed samples, plasma samples obtained from EDTA-whole blood were stored at −80 °C prior to genotypic testing. A total of 1 mL of each plasma sample was centrifuged for 1 h at 4 °C at 20,000 rpm. The supernatant was carefully removed to the volume of 280 μL, the pellet was resuspended and used for RNA-extraction. RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocoland was subjected to genotyping by an in-house nested PCR protocol amplifying HIV-1 *pol* gene. (Snoeck et al., 2005).

Reverse transcription was performed using the One Step RNA PCR Kit (Qiagen, Hilden, Germany), followed by nested PCR protocol using Taq PCR Core Kit (Qiagen, Hilden, Germany). The amplified products, 1.6 kb in length (full-length protease and near complete reverse transcriptase), were purified using the Qiagen PCR purification kit (Qiagen Hilden, Germany) according to the manufacturer's protocol and sequenced bidirectionally on ABI Prism 310- Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) using Big Dye Terminator chemistry and six sequencing primers (Snoeck et al., 2005). Obtained sequences were visually inspected and manually edited and then assembled with SeqScape HIV-1 Genotyping System Software v 2.5 (Applied Biosystem, Foster City, CA, USA).

2.3. Datasets and reference strains

Sequence analysis included three datasets. The first dataset, including all 221 sequences analysed in the study, was used for subtype assignment, performed using the REGA HIV-1 subtyping tool (http://jose.med.kuleuven.be/genotypetool/html/subtyping-hiv.html). Further, 219/221 Serbian sequences of sufficient length (full-length protease and minimally 250 codons of reverse transcriptase) were used in ML phylogenetic analysis together with the six reference sequences downloaded from the HIV Los Alamos database (www.hiv.lanl.gov/content/index) for general phylogenetic insight into the dataset. Accession numbers of 221 sequences obtained in the study together with reference sequences used for phylogenetic analysis and the HIV-1 subtype F sequences that were used as outgroup are listed in the Supplementary data file.

In order to investigate local transmission networks, in particular within subtype B as the most prevalent one, we built the second dataset containing 219 Serbian sequences of sufficient length (full-length protease and minimally 250 codons of reverse transcriptase) plus 175 HIV-1 sequences classified as subtype B, sampled across Europe and Northern America downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Criteria for inclusion of sequences were clearly identified subtype for each sequence, and clearly established origin of each corresponding patient. The country distribution and the accession numbers of these sequences are listed in the Supplementary data file.

The third dataset included sequences from the transmission network and the most expanded transmission clusters within subtype B, and all Serbian subtype G sequences, as the non B subtype with the highest prevalence detected in the previous analysis. The third dataset was used to investigate the temporal origin of the clusters by Bayesian molecular clock analysis as implemented in BEAST version 1.5.1 (Drummond and Rambaut, 2007). The reference dataset for this analysis included sequences of the matching subtype sampled from different geographical locations (Europe and Africa) with known collection date. The country distribution and the accession numbers of these sequences are listed in the Supplementary data file.