Distribution of HIV-1 subtypes circulating in Slovakia (2009−2012)

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Summary. – Slovakia is one of the European countries reporting a low prevalence of human immunodeficiency virus 1 (HIV-1) infection. The HIV-1 subtype B was previously the most prevalent in Slovakia and mostly associated with men having sex with men (MSM) (Habekova *et al.*, 2010). The aim of this study was to determine the distribution of individual HIV-1 subtypes among the virus-positive individuals diagnosed in 2009–2012 in Slovakia and to compare these data with those from 2004–2008. The HIV-1 subtype B exhibited the highest prevalence (86.1%) and was mostly associated with MSM as stated before. HIV-1 non-B subtypes were found in 13.9% of patients, led by CRF01_AE (6.3%) and followed by subtype C (2.8%), F1 (1.4%), CRF03_AB (1.4%), G (0.7%), CRF02_AG (0.7%), and CRF12_BF (0.7%). The incidence of non-B subtypes varied in 2009–2012, showing a maximum in 2012. MSM represented the main risk group for total HIV-1 (68.1%) as well as B subtype (63.9%) infections in Slovakia. In contrast, the heterosexual contact represented the main route of HIV-1 acquisition of the non-B subtype virus infection. Together with the increase in the number of patients infected with the viruses of non-B subtype also other changes were observed: infections with the viruses of non-B subtype became more common among MSM and not so strongly associated with heterosexual contact. All these findings may help to design the right strategy for retaining of HIV epidemic in Slovakia at low level.

Keywords: HIV-1; B subtype; non-B subtypes; Slovakia

Introduction

HIV infection represents a global health problem. By the end of 2012 UNAIDS estimated that 35.3 million people were infected by HIV and 2.3 million people had only been infected during that year (UNAIDS, 2013). An extensive genetic diversity and rapid evolution are the main characteristics of HIV-1 which contribute to unsuccessful fight with HIV/AIDS. Factors such as high rate of recombinations caused by reverse transcriptase enzyme (Ramirez *et al.*, 2008), which lacks a proof-reading mechanism (Roberts *et al.*, 1988), together with high replication rate (Ho *et al.*, 1995),

are responsible for genetic variability of HIV-1 (Hemelaar *et al.*, 2012). Insertions, deletions, and duplications contribute to the genetic heterogeneity of HIV-1 (Hu and Temin**,** 1990) and, moreover, they result in the generation of genetically diverse viral populations differing by up to 10% within each infected individual (Korber *et al.*, 2001).

On the basis of phylogenetic analysis of numerous isolates obtained from diverse geographic origins, HIV-1 is divided into four groups: M (main), O (outlier), N (non-M, non-O) and P group. The groups represent four separated introductions into the human population by independent cross-species transmissions of SIV in West and Central Africa (Roques *et al.*, 2004; Van Heuverswyn *et al.*, 2006; Plantier *et al.*, 2009; Vallari *et al.*, 2011). Within HIV-1 group M, which accounts for the majority of infections worldwide, most sequences fall into a limited number of phylogenetically associated groups, allowing the classification of HIV-1 group M into the clades called subtypes (A-D, F-H, J-K) and sub-subtypes (A1-A4, F1, F2). 58 CRFs (circulating recombinant forms) and some

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Abbreviations: CRF = circulating recombinant form; **IVDU =** intravenous drug user; HIV-1 = human immunodeficiency virus 1; MSM = men having sex with men; PRO = protease; RT = reverse transcriptase; SIV = Simian immunodeficiency virus

URFs (unique recombinant forms) had been described by December 2013 (Brun-Vezinet and Charpentier, 2013; HIV and SIV Nomenclature).

Hemelaar *et al.* (2011) published results of the global and regional distributions of HIV-1 subtypes and CRFs in 2011 worldwide. HIV-1 subtype C was determined as the most prevalent genetic form globally and was responsible for nearly half (48%) of all global infections. Viruses of subtype A were accounted for 12% of HIV-1 infections, followed by viruses of subtype B (11%), CFR02_AG (8%), CRF01_AE (5%), subtype G (5%) and subtype D (2%). HIV-1 subtypes F, H, J and K caused less than 1% of infections worldwide (Hemelaar *et al.*, 2011).

In Europe, HIV-1 subtype B is the most common (66.1%) one. It is spread mostly among MSM, whilst strains of non-B subtypes are often associated with heterosexual transmission or immigration representing following prevalence: subtype A1 (6.9%), subtype C (6.8%), CRF02_AG (4.7%) and CRF01_AE (4.0%) (Paraschiv *et al.*, 2012; Abecasis *et al.*, 2013). HIV-1 subtype B prevails also in countries bordering Slovakia: Austria, Czech Republic, Hungary and Poland (Falkensammer *et al.*, 2007; Linka *et al.*, 2008; Mezei *et al.*, 2000, 2011; Stanczak *et al.*, 2010). Ukraine as the former USSR country is characterized by the high prevalence of subtype A and the second most common B subtype (Saad *et al.*, 2006; Bobkova *et al.*, 2013). Slovakia belongs to the European countries characterized by a low prevalence of HIV-1 infection although there is an evidence of increasing transmission, especially through sexual contact between men (ECDC, 2013). Subtype B was determined as the most prevalent genetic form in Slovakia (Mayer *et al.*, 1998; Habekova *et al.*, 2010).

The aim of this study was (*i*) to determine the current prevalence of HIV-1 B and non-B subtypes in Slovakia in 2009–2012, (*ii*) to characterize the HIV-1 patients demographically and regarding the route of their infection, (*iii*) to characterize the HIV-1 B and non-B subtype isolates phylogenetically on the basis of their RT and PRO sequences, and (*iv*) to make all possible comparisons with corresponding data for the previous period (2004–2008).

Materials and Methods

Patients. 144 HIV-1 positive patients were enrolled in this study. All patients were antiretroviral therapy naïve and newly diagnosed in the National Reference Center for HIV/AIDS Prevention in Bratislava, Slovakia. Plasma samples were collected up to 6 months after HIV-1 diagnosis between 2009 and 2012 for routine genotyping and drug resistance testing. Personal data concerning the route of transmission, country of origin and presumed country of HIV-1 acquisition were collected from patients during a pre-test or post-test counseling. Distribution of HIV-1 B and non-B subtypes was analyzed in the relationship to personal data.

Nested RT-PCR. HIV-1 RNA isolation from plasma samples was accomplished using NucleoSpin® RNA virus isolation kit (Macherey-Nagel, Germany). The nested RT-PCR and sequencing of HIV *pol* region was carried out with the use of in-house procedures according to ANRS1 protocol for RT and PRO (ANRS AC11 Resistance Study Group, 2008).

Sequencing and phylogenetic analysis. All 144 HIV-1 *pol* sequences were firstly analyzed by REGA HIV-1 Subtyping Tool (REGA HIV-1 Subtyping Tool) to obtain preliminary HIV-1 subtype classification. Nucleotide alignments were prepared by comparing a set of consensus sequences of HIV-1 group M clades (including subtypes, subsubtypes and CRFs) that are available in Los Alamos HIV Databases (Los Alamos HIV Sequence Database) using CLUSTAL W software (Labarga *et al.*, 2007), MEGA version 6.0 (Tamura *et al.*, 2013) and BioEdit software packages (Hall, 1999). Firstly, the 480 bp alignment of RT sequences was prepared and alternatively in 16 cases, the 387 bp alignment of PRO sequences was used for HIV subtyping. Molecular phylogenetic analysis was performed using ML (maximum likelihood) method implemented in MEGA version 6.0 software (Tamura *et al.*, 2013), using HKY (Hasegawa-Kishino-Yano) model as nucleotide substitution model and gamma distribution of rate variability among sites, calculated from the data with three discrete gamma categories. The bootstrap values (bootstrapping with 1000 replicates) above 60% were considered significant and shown at tree nodes. Initial trees for the heuristic search were made automatically and were obtained by applying the NJ (neighbor-joining) method to a matrix of pair wise distances estimated using MCL (maximum composite likelihood) approach. The sequences described in this article have been deposited in the GenBank Nucleotide Sequence Database under Acc. Nos. KJ483816 - KJ483943 and KJ483944 - KJ483959.

Results and Discussion

Study population

Demographic characteristics of the study population are illustrated in Table 1. The majority of all studied patients were men (93.1%). The mean age of the patients was 32.0 years (10–62 years), for men and women 32.9 (19–62 years) and 27.0 years (10–51 years), respectively. The youngest individual was 10 and the oldest was 62 at the time of HIV diagnosis. Most of the patients were infected by sexual contact between men (68.1%), followed by patients infected by unprotected heterosexual contact and intravenous drug use. Similar results were obtained in 2004–2008 when more than half of HIV-1 infected patients were MSM (Habekova *et al.*, 2010).

HIV-1 B subtype

The two phylogenetic trees (Fig. 1 and 2) were constructed from HIV-1 *pol* sequences that cluster with the consensus

Table 1. Demographic characteristics of HIV-1 patients in Slovakia in 2009-2012 and routes of the virus acquisition

		Number (%)		
		Total	B subtype	Non-B subtype
HIV-1 patients	Total	144 (100.0%)	124 (86.1%)	20 (13.9%)
	Females	10 (6.9%)	4 (2.8%)	6 (4.2%)
	Males	134 (93.1%)	120 (83.3%)	14 (9.7%)
Route of the virus acquisition	Heterosexual contact	28 (19.4%)	20 (13.9%)	8 (5.6%)
	IVDU	3 (2.1%)	2 (1.4%)	1 (0.7%)
	MSM	98 (68.1%)	92 (63.9%)	6 (4.2%)
	Unknown	15 (10.4%)	10 (6.9%)	5 (3.5%)
Patients' country of origin	Slovakia	130 (90.3%)	119 (82.6%)	11 (7.6%)
	Outland	14 (9.7%)	5 (3.5%)	9 (6.3%)
Presumed country of the virus acquisition	Slovakia	61 (42.4%)	55 (38.2%)	6 (4.2%)
	Outland	33 (22.9%)	27 (18.8%)	6 (4.2%)
	Non-european outland	13 (9.0%)	7 (4.9%)	6 (4.2%)
	Unknown	37 (25.7%)	35 (24.3%)	2 (1.4%)

of HIV-1 B subtype. The analysis showed that 124 of all 144 (86.1%) sequences were derived from patients infected with HIV-1 subtype B viruses. HIV-1 B subtype remained the most prevalent genetic form in Slovakia in 2009–2012 like in the previous period (2004–2008) (Habekova *et al.*, 2010). These findings are consistent with data coming from Europe, reporting the highest prevalence of HIV-1 B subtype (Paraskevis *et al.*, 2009). Moreover, regarding the data of route of transmission in Europe, being infected with a subtype B virus was more common among MSM than among patients in other transmission groups (Vercauteren *et al.*, 2009; Chalmet *et al.*, 2010; Abecasis *et al.*, 2013; Frentz *et a*l., 2013). According to our results, MSM still remained associated with 68.1% of all HIV-1 infections (98 of 144) in Slovakia and with 74.2% of HIV-1 subtype B infections (92 of 124). As compared to 2004–2008, prevalence of HIV-1 subtype B (66.4%) became increasing among MSM in Slovakia (Habekova *et al.*, 2010). The highest prevalence of subtype B among MSM is related to the high number of men infected with subtype B strains which was showed in Slovakia by our study and by other studies from Europe (Abecasis *et al.*, 2013).

Regarding the origin of subtype B strains, the majority (89.8%) of them circulates in European countries (Frentz *et al.*, 2013). Data of our study revealed that at least 57.0% of all patients were infected with subtype B viruses that were already found in other persons in Europe before (38.2% in Slovakia, 18.8% in other European countries). In relation to the high proportion of patients with unknown origin of subtype B strains the resulting number could be even higher.

According to literature, the HIV-1 epidemic is mainly driven within country transmission (Kouyos *et al.*, 2010; Frentz *et al.*, 2013). Our data indicate that it is at least 38.2% of HIV-1 B subtype infections having Slovak origin in Slovakia and this number could be higher. On the other hand,

significant number (23.7%) of HIV-1 infections originating from outland (18.8% from other European countries, 4.9% from non-European outland) suggests that many HIV-1 strains were introduced into the Slovak population.

HIV-1 non-B subtypes

Based on the phylogenetic analysis (Fig. 3), 20 of all 144 HIV-1 *pol* sequences were classified into the HIV-1 non-B clades, which represent the prevalence of 13.9%. 7 strains were pure subtypes (C, F1, G) and 13 strains clustered with the consensuses of different CRFs (CRF01_AE, CRF02_AG, CRF03_AB, CRF12_BF). The CRF01_AE was the most prevalent (6.3%) among non-B subtypes, followed by subtype C (2.8%), F1 (1.4%), CRF03_AB (1.4%), G (0.7%), CRF02_AG (0.7%) and CRF12_BF (0.7%). As compared to the period of 2004–2008, prevalence of non-B subtypes increased almost twice (Habekova *et al.*, 2010). These findings correspond with results published by Paraskevis *et al.* that showed an increasing prevalence of HIV-1 non-B subtypes in Europe (Paraskevis *et al.*, 2009).

Moreover, distribution of non-B subtypes varied in 2009–2012 as follows: 7.7% in 2009, 14.8% in 2010, 10.0% in 2011 and 23.7% in 2012 (Fig. 4). A significant increase was recorded in 2012 when 9 of all 20 patients infected with HIV-1 non-B viruses were diagnosed (Table 2). These data suggest that HIV-1 epidemic in Slovakia became more variable. This reality could be the consequence of travel and migration that play a key role in the early spread of HIV due to easier international traveling within Europe (Frentz *et al.*, 2013).

Regarding the route of transmission, heterosexual contact was the most common among patients infected with non-B strains, as it was found in 2004–2008 in Slovakia (Habekova
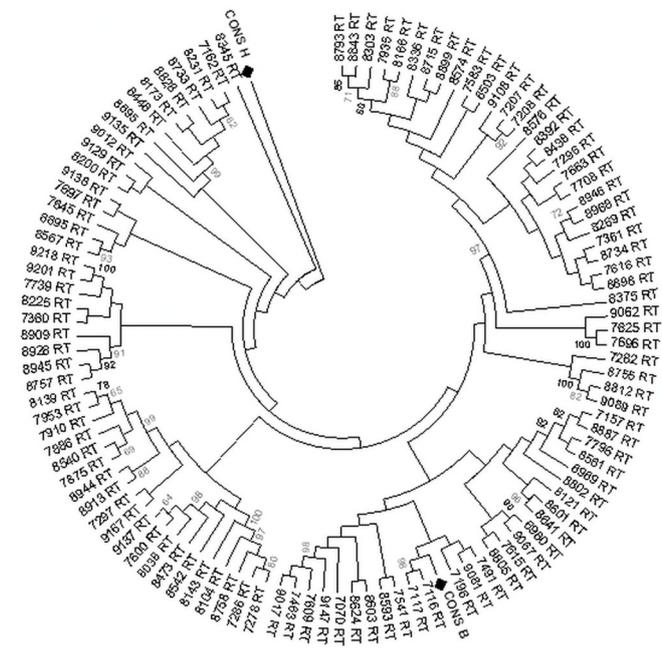
Fig. 1
RT region-based phylogenetic tree of HIV-1 subtype B isolates
The tree was constructed using the ML method based on the HKY model. The subtype H served as outgroup. Subtype B and H consensus sequences (full squares) are included.
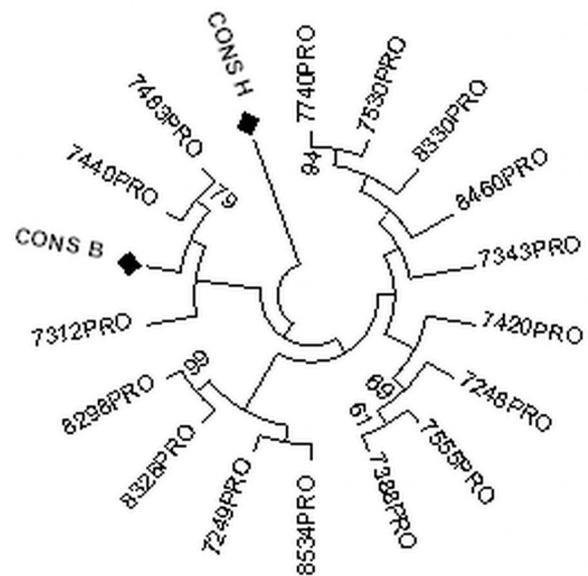

Fig. 2
PRO region-based phylogenetic tree of HIV-1 subtype B isolates
The tree was constructed using the ML method based on the HKY model. The subtype H served as outgroup. Subtype B and H consensus sequences (full squares) are included.

et al., 2010) and also in Europe (Abecasis *et al.*, 2013; Frentz *et al.*, 2013). On the other hand, the number of patients with non-B strains infected by sexual contact between men had increased from 0.7% in 2004–2008 (Habekova *et al.*, 2010) to 4.2% in 2009–2012. Generally, the proportion of men infected with non-B subtype viruses increased from 3.5% (5 men) (Habekova *et al.*, 2010) to 9.7% (14 men) in this study.

The phylogenetic analysis of non-B sequences revealed some clusters that could indicate transmission events between particular patients. Based on post-test evaluation it was confirmed that patients coded 8491 and 8568 harboring subtype C strain were sexual partners. This was proved also by the phylogenetic tree and the bootstrap value showing 100%.

9 of all 20 non-B strains clustered with the consensus CRF01_AE. According to patients′ data, two strains of the cluster were obtained from a homosexual pair (coded 9092, 9122) which was consistent with the bootstrap value 100%. Other three patients with the CRF01_AE strains were infected through heterosexual contact and for the rest four patients the route of HIV-1 transmission remained unknown. In comparison with the previous period 2004–2008 (Habekova *et al.*, 2010), significant increase of CRF01_AE distribution was observed in 2009–2012. Moreover in 2012, 6 persons infected

with CRF01_AE strains were diagnosed and only three of them have an origin of HIV-1 infection in Slovakia. Despite the fact, no epidemiological association was proved among CRF01_AE infected patients, with an exception of the pair of MSM coded 9092 and 9122. Other two strains of CRF03_AB clustered together and have a common origin in Ukraine. The strains were derived from a heterosexual pair (coded 7509, 7907) which could be suggested by the similarity in the bootstrap analysis. According to literature, this newly identified variant CRF03_AB circulates in 1.3% in Ukraine (Los Alamos HIV Sequence Database/Geography).

Regarding the origin of HIV-1 non-B viruses in Europe, patients infected with these strains often came from Sub-Saharan countries (31.7%) (Chalmet *et al.*, 2013). In Slovakia, 4 of all 20 non-B infected patients (coded 7062, 7846, 8710, 8911) have probable origin of HIV-1 infection in Africa which represents 20% of all non-B infections. The strain from patient coded 7062 clustered with subtype G and originates from Nigeria where the HIV-1 subtype G circulates frequently (39.8%) (Los Alamos HIV Sequence Database/Geography). The patients coded 7846 and 8911 were infected with two African strains probably by contact with their sexual partners originating from Africa. The patient coded 7846 was infected by CRF02_AG strain originating from Cameroon where prevalence of the CRF is 39.7% (Los

Alamos HIV Sequence Database/Geography). The second strain coded 8911 clustered with HIV-1 subtype C occurring in Africa very frequently (Los Alamos HIV Sequence Database/Geography). The strain coded 8710 also clustered with the subtype C consensus and comes from South Africa.

In Europe, many of patients with HIV-1 non-B strains became infected likely before they moved to Europe (Frentz *et al.*, 2013). When comparing our data with those from Europe, an equivalent situation could be applicable for Slovakia. 6 of 20 patients with HIV-1 non-B viruses (coded 7062, 8710, 7869, 7907, 8467, 8982) declared that they acquired their infection in the country from which they originate. As shown, viruses of the patients coded 7062 and 8710 came to Slovakia from Africa, patient coded 7869 was from Georgia, and the patient coded 7907 from Ukraine. The CRF01_AE strains coded 8467 (from Czech Republic) and 8982 (from Vietnam) came from the countries in which they also occur (Los Alamos HIV Sequence Database/Geography). Except these already mentioned cases, there are other 6 non-B strains coming from outland (codes 7509, 7695, 7846, 8911, 9013, 9251). The CRF03_AB strain coded 7509 probably originates in Ukraine because of the possible HIV-1 transmission from the partner coded 7907. The strain CRF01_AE coded 9013 should originate from Hungary according to known epidemiological data but no previous occurrence of the CRF was recorded in that country (Los Alamos HIV Sequence Database/Geography). A homosexual man (coded 9251) was infected with CRF12_BF strain in Austria where an occurrence of the CRF is very rare (Los Alamos HIV Sequence Database/Geography). When taking to account the origin of these mentioned strains, 12 of all 20 patients infected with HIV-1 non-B subtype strains came from outland and their origin is not associated with any other Slovak HIV-1 non-B strains. On the other hand, there were strains of the three HIV-1 non-B subtypes presumed to have an origin in Slovakia: F1 (patient coded 7150), C (patients coded 8491, 8568) and CRF01_AE (patients coded 8930, 9092, 9122). All the three subtypes have already circulated in Slovakia (Habekova *et al.*, 2010). Based on these findings it was shown that the epidemic of HIV-1 non-B subtypes became a combined result of both migration and domestic transmission. Similar situation was observed in Switzerland where only <25% of all non-B infections diagnosed could be prevented by domestic interventions (Von Wyl *et al.*, 2011). Therefore as in Switzerland as well as in Slovakia, awareness should be raised among immigrants and individuals with partners from high prevalence countries to restrain the spread of non-B subtypes (Kouyous *et al.*, 2010; Von Wyl *et al.*, 2011).

In conclusion, the study described the prevalence of HIV-1 B and non-B subtypes in Slovakia in 2009–2012. Our findings confirmed the fact that distribution of HIV-1 varied in this period, showing a maximum in the year 2012. Sexual contact between men remained the main risk factor
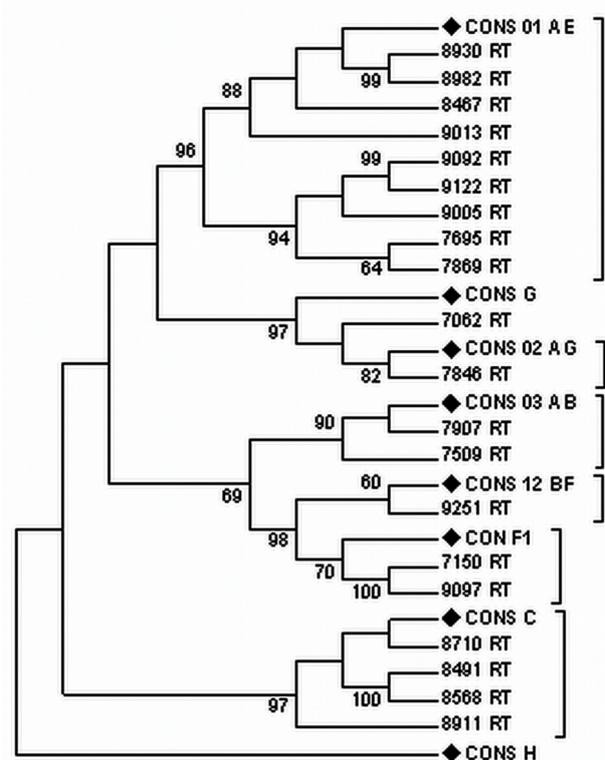

Fig. 3

RT region-based phylogenetic tree of HIV-1 non-B subtype isolates
The tree was constructed using the ML method based on the HKY model. The subtype H served as outgroup. Consensus sequences of subtype C, F1, G, H, CRF01_AE, CRF02_AG, CRF03_AB and CRF12_BF (full squares) are included.
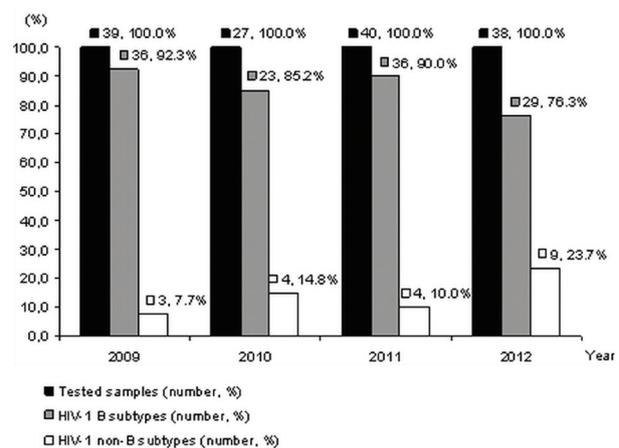

Fig. 4

Prevalence of HIV-1 isolates of subtypes B and non-B in Slovakia in 2009–2012

Table 2. Epidemiological data of patients infected with HIV-1 non-B viruses

Patients		HIV-1 infection				
Code	Sex	Country of origin	Virus subtype	Route of infection	Presumed country of virus acquisition	Year of diagnosis
8491	M	Slovakia	C	MSM	Slovakia	2011
8568	M	Slovakia	C	MSM	Slovakia	2011
8710	M	South Africa	C	Not known	South Africa	2011
8911	F	Slovakia	C	Heterosexual	Africa (?)	2012
7150	M	Slovakia	F1	IVDU	Slovakia	2009
9097	M	Slovakia	F1	MSM	Not known	2012
7062	M	Nigeria	G	Heterosexual	Nigeria	2009
7695	F	Ukraine	CRF01_AE	Heterosexual	Ukraine	2010
7869	M	Georgia	CRF01_AE	Not known	Georgia	2010
8467	M	Czech Republic	CRF01_AE	Heterosexual	Czech Republic	2011
8930	F	Slovakia	CRF01_AE	Heterosexual	Slovakia	2012
8982	F	Vietnam	CRF01_AE	Not known	Vietnam	2012
9005	M	Russia	CRF01_AE	Not known	Not known	2012
9013	M	Slovakia	CRF01_AE	Not known	Hungary	2012
9092	M	Slovakia	CRF01_AE	MSM	Slovakia	2012
9122	M	Latvia	CRF01_AE	MSM	Slovakia	2012
7846	F	Slovakia	CRF02_AG	Heterosexual	Cameroon	2010
7509	F	Slovakia	CRF03_AB	Heterosexual	Ukraine	2009
7907	M	Ukraine	CRF03_AB	Heterosexual	Ukraine	2010
9251	M	Slovakia	CRF12_BF	MSM	Austria	2012

of HIV-1 infection associated mostly with HIV-1 B subtype infection. Our findings are consistent with results of the last 2nd generation surveillance provided in MSM in Slovakia, in which 6.1% prevalence of HIV-1 infections was described among MSM (Mirandola *et al.*, 2009). Moreover, results of our study revealed multiple introductions of HIV-1 viruses into the Slovak population. All obtained data and findings could help to design the targeted prevention in high risk MSM group in Slovakia and focus on retaining HIV prevalence at low level in Slovakia.

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