DRUG RESISTANCE MUTATIONS AND TRANSMISSION CLUSTERS OF THE HIV-1 CRF01_AE SUB-EPIDEMIC IN BULGARIA

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ABSTRACT

Background. In Bulgaria the second most predominant HIV-1 strain after subtype B is CRF01_AE.

Material and Methods. 270 HIV-1 polymerase (pol) CRF01_AE sequences collected between 1995–2019 were analyzed with the phylogenetic approach. To identify transmission clusters, we used two different genetic distance thresholds (d), i. e. 1.5% and 0.5%.

Results. Genetic distance d=1.5% defined more distant infections in a huge cluster of 154 sequences composed mostly of people, who inject drugs (PWID), whereas when (d) was reduced to 0.5%, we determined more recent transmissions and the large cluster disintegrated into smaller ones. Different drug resistance mutations (DRM) were detected in patient's HIV-1 pol sequences and were most common in male heterosexual (HET) single sequences.

Conclusions. Our data showed repeated introduction of CRF01_AE in Bulgaria and rapid spread of the infection among PWID groups. Molecular monitoring of the epidemic among PWID communities could help reduce the spread of HIV-1 infection.

Keywords: *HIV-1, CRF01_AE, drug resistance, transmission clusters*

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by two lentiviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (1). HIV-1 is more prevalent in the world. The strains of HIV-1 can be classified into four groups: M (major), N (new), O (outlier) and P (2). Group "M" is the main group that is responsible for the global HIV-1 pandemic and contains at least 10 different subtypes (A-F, G, H, J, K and L), multiple circulating recombinant forms (CRFs) and various unique recombinant forms (URFs). HIV-1 subtype CRF01_AE was first isolated in Thailand and now predominates in South Asia and has spread throughout the world (3). The global prevalence of CRF01_AE is 5%, and it is responsible for up to 4% of the HIV-1 infections in Europe according to the SPREAD study (4).

In previous studies, CRF01_AE was found to be one of the most disseminated clades in Bulgaria with around 20%, which is likely the highest percentage across Europe (5, 6, 7). Within the country, CRF01_AE is unevenly distributed and mainly affects the vulnerable population of PWID in the region of the capital of Bulgaria, Sofia, with 35% of all HIV-1 infections (8). With this study, we aim to determine the dynamics of distribution and transmission of CRF01_AE in Bulgaria. We intend to use epidemiological data and to link it

to the data obtained by sequencing the samples. Thus, we aim to clarify the events that have led to the transmission and production of resistance mutations. In addition, we will analyze HIV-resistant mutations in this strain. We hope that our study results will help to develop better strategies for public health prevention in Bulgaria.

MATERIALS AND METHODS

- **Patient samples, research ethics and consent**

Plasma samples were collected in the National Reference Confirmatory Laboratory of HIV (NRCL of HIV) of the National Center of Infectious and Parasitic Diseases (NCIPD) in the period 1995-2019. Demographic and epidemiological information was collected during the diagnostic process in accordance with national regulations. Through anonymous codes, blood samples, demographic and clinical information was linked following the ethical standards of Bulgaria (6). The Ethics Committee of NCIPD (NCIPD IRB 00006384) approved the study.

- **Sequence generation and analysis**

Viral RNA extraction was performed following the protocol of Abbott ViroSeqTM HIV-1 Genotyping Test v2.0 from serum or plasma. A fragment of the HIV-1 pol gene was generated using a ViroSeq HIV-1 genotyping test using an Applied Biosystems 3130xl genetic analyzer (8). HIV-1 subtypes were determined using Internet-based tools REGA HIV-1 subtyping tool version 3.0 (9) and COMET v2.3 (10). We used the Genotypic Resistance Interpretation Algorithm of the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/hivdb/by-sequences/) to determine HIV-1 resistance mutations (DRMs) and their type. By using MUSCLE algorithm implemented in AliView version 1.23 (11, 12) and MAFFT version 7 (13, 14) sequences were aligned and some of the sequences were additionally manually aligned. The bioinformation program MicrobeTrace (15) was used to identify transmission clusters. Phylogenetic analysis was conducted with the Tamura-Nei algorithm (d) and clusters were determined at a genetic distance of 0.5% and 1.5%. Each pol sequence was represented graphically as a separate node. When two or more sequences were connected by certain threshold, they were labeled as a cluster, while nodes that did not connect to others were entitled as single sequences.

RESULTS

- **Characteristics of the CRF01_AE sub-epidemic in Bulgaria**

The first case of HIV-1 CRF01_AE infection in Bulgaria was detected in 1995 in a heterosexual man. The first mother-to-child transmission (MTC) was found 4 years later. The next important event in this sub-epidemic was the introduction of the CRF01_AE in the group of PWID in 2002. In 2009 in Sofia, 26 individuals were diagnosed with CRF01_AE, 18 (69.2%) of whom were PWID, indicating ongoing outbreak among PWIDs. In 2011, 31 cases were identified, 17 of whom PWID, 12 HET and 2 MSM.

The total number of people diagnosed with HIV-1 subtype CRF01_AE by the end of 2019 was 270 or 16% of all patients with HIV. Analysis of gender distribution showed higher number of men than women. In CRF01_AE, men were 187 (69.3%) and women 83 (30.7%) with a ratio of 2.25:1, while the ratio found in other subtypes was with a higher weight of men 4:1. The age at diagnosis varied, youngest diagnosed was a newborn (0 years), the oldest was 63 years old. The largest share of those diagnosed was between 20-29 (40%), followed by 30-39 (33.3%) years of age, while the infected young (≤19) and older individuals (≥50) represented a smaller share. Patients indicating that the infection was acquired in Bulgaria were 251 (93%) and those infected abroad (mainly Western European countries) were 19 (17%). Analysis of the geographic distribution demonstrated that most of the infections were found in individuals from Sofia, 164 (60,7%), and the rest were dispersed across the country. When comparing transmission groups with CRF01_AE, we found that PWID were 141 (52.2%), followed by HET – 101 (37.4) and MSM – 13 (4.8) (Table 1).

Table 1. Characteristics of individuals infected with HIV-1 CRF01_AE compared to infections with other HIV-1 subtypes and CRFs in Bulgaria.

Characteristic	Subtype CRF01_AE	Other subtypes
	number (%)	number (%)
Total	270	1413
Gender		
Men	187 (69.3)	1195 (84.6)
Women	83 (30.7)	218 (15.4)
Age (years)		
≤19	28 (10.4)	68 (4.8)
20-29	108 (40.0)	538 (38.1)
30-39	90 (33.3)	493 (34.9)
40-49	35 (13.0)	204 (14.4)
≥50	9 (3.3)	110 (7.8)
Country of Origin		
Bulgaria	269 (99.6)	1361 (96.3)
Other country	1 (0.4)	52 (3.7)
Likely Country of Infection		
Bulgaria	251(93.0)	1176 (83.2)
Other country	19 (7.0)	237 (16.8)
Region in Bulgaria		
Sofia	164 (60.7)	625 (44.2)
Other regions	106 (39.3)	788 (55.8)
Transmission category		
HET	101 (37.4)	592 (41.9)
MSM	13 (4.8)	630 (44.6)
PWID	141 (52.2)	158 (11.2)
Other	15 (5.6)	33 (2.3)

- **Analysis of CRF01_AE transmission clusters**

In order to characterize transmission clusters among the sub-epidemic of HIV-1 CRF01_AE in Bulgaria we used MicrobeTrace program applying two thresholds of genetic distance, i.e. 0.5% delimited more recent infections and 1.5% for more distant infections (Fig. 1). Analysis of genetic distance of 1.5% revealed the presence of 6 clusters and 98 single sequences. One of the clusters contained 154 sequences, 75.3% were men and 24.7% women. Most of them were PWID – 70.1%, followed by HET – 19.5%. The dissemination between genders was more evenly distributed in the group of single sequences, where HET were 65.3%. DRMs were 12.3% and 4.55% of them were NRTI and 7.8% NNRTI. DRMs were 23.3%. 2% of all single sequences had PR DRM, 20.4 % NRTI and 19.4% NNRTI.

With genetic distance of 0.5% 10 transmission clusters and 218 single sequences were found. The largest cluster consisted of 18 sequences, all of which were isolated from PWID, 55.6% men and 44.4% women. With exception of one NRTI DRM falling into a dyad, none of the sequences of the newly formed clusters had DRM. At 0.5% cutoff male single sequences were twice as many as women and the number of HET individuals and PWID were relatively equal. All DRMs were found among single sequences, 1% PR DRM, 12.8% NRTI and 14.7% NNRTI.

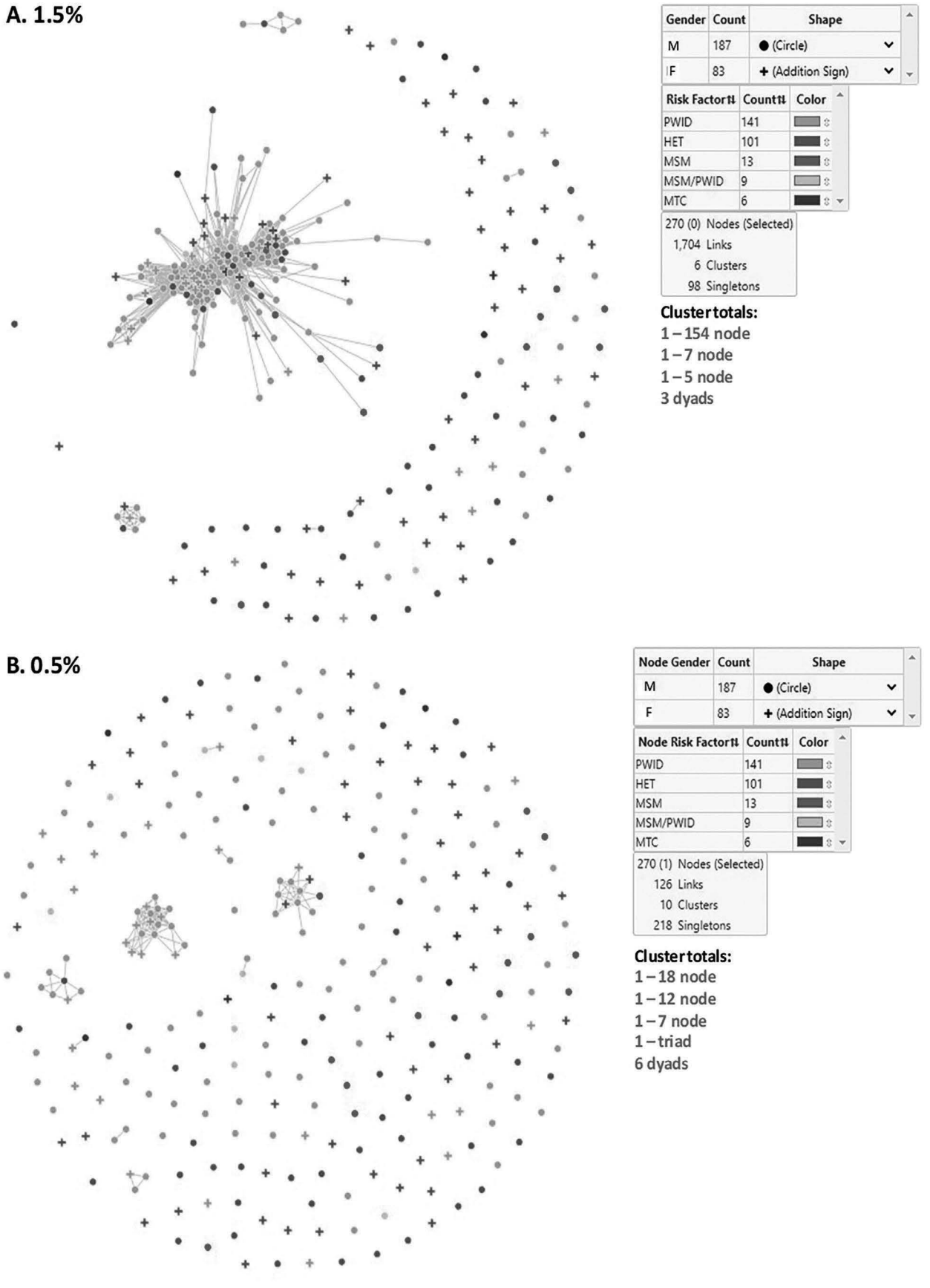

Figure 1. Graphical representation of CRF01_AE clusters in Bulgaria using MicrobeTrace.
A. genetic distance 1.5% **B.** genetic distance 0.5%. Gender is marked with circles (male) and plus sign (female). Color indicates transmission category: green PWID, blue HET, red MSM, yellow MSM/PWID and purple MTC. Cluster totals is provided.

- **Identification of drug resistance mutations in CRF01_AE infections in Bulgaria**

We identified 51 (18.9%) patients with DRMs, including 2 persons (1 HET and 1 MTC) with multiple protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI), and non-nucleoside reverse transcriptase inhibitor (NNRTI) DRMs (Table 3). Eight additional persons had both NRTI and NNRTI DRMs, including 4 HET, 3 PWID and 1 MTC transmission. The most prevalent NRTI and NNRTIs DRMs were M184V (17/57, 31.6%), and E138A (8/42, 19.0%), respectively (Table 2). Twenty-nine patients had NRTI DRMs, including 2 patients with 5 mutations, 3 with 4 mutations, 2 with 3 mutations, 7 with 2 mutations, and 15 patients with a single NRTI DRM. Thirty-two persons had mutations to NNRTIs, including 2 with 3 mutations, 6 with 2 mutations, and 24 had single mutations.

Overall, the highest prevalence of DRMs were found in MTC (3/6, 50%), followed by HET (29/81, 35.8%), MSM (3/13, 23.1%), MSM/PWID (1/9, 11.1%), and much less in PWID (15/141, 1.1%). For HIV-1-infected HET, 10.9% and 12.9% had NRTI and NNRTI DRMs, respectively. For HIV-1-infected MSM, 7.7% and 15.4% had NRTI and NNRTI DRMs, respectively, while 2.0% of PWID had both NRTI and NNRTI DRMs. All patients with PI or NRTI DRMs reported acquiring HIV-1 infection in Bulgaria, while 3/32 (9.4%) cases (2 HET and 1 MSM) with NNRTI DRMs specified that they acquired their infections abroad in either Germany, Turkey, or Spain. Although 60.7% of our study population resided in the capital Sofia, 52.6% and 54.5% of those with NRTI and NNRTI DRMs, respectively, were from other regions in Bulgaria.

Table 2. Drug Resistance Mutations (PR/NRTI/NNRTI) of HIV-1 subtype CRF01_AE persons in Bulgaria.

PR Major Mutation	Number of Patients with Mutation	NRTI Mutations	Number of Patients with Mutation	NNRTI Mutations	Number of Patients with Mutation
M46I	1	K65R	1	K101E	1
I54V	1	D67G	1	K103KN	2
V82A	1	D67N	3	K103N	5
I84V	1	K70N	1	V106I	3
		K70KT	1	V106VI	4
		K70R	4	V108VI	1
		L74LI	1	E138A	8
		L74V	5	E138G	1
		V75M	4	E138EG	1
		F77L	1	E138K	1
		Y115F	1	V179VD	3
		Q151M	1	Y181C	1
		M184MV	17	Y188L	3
		M184V	1	G190Q	1
		L210W	1	G190GA	1
		T215Y	1	G190GE	1
		T215I	1	G190A	1
		T215A	1	H221Y	1
		T215TA	2	P225PH	1
		T215TS	1	M230L	1
		T215F	2	K238RT	1
		K219E	3		
		K219KQ	1		
		K219Q	2		

Table 3. Multiple drug resistance mutations of HIV-1 subtype CRF01_AE persons in Bulgaria.

Sequence Name	PR Major	NRTI	NNRTI
2380208TG08	M46I, I84V	F77L, M184V	E138K
2580305TG05	I54V, V82A	D67N, K70R, T215I, K219Q	V106I
3820505TG05		D67G, L74V, V75M, Q151M, M184V	G190Q, H221Y
15930517VS17		L74V, M184V, K219E	E138G, M230L
5790415VS15		L74LI, M184MV	K103N, P225PH
8410209TG09		L74V, M184V	K103KN, Y181C, G190GA
12010914TG14		M184V, K219E	K103N, Y188L
6520112TG12		M184V	K103N, E138A, Y188L
8100313TG14		M184V	K101E, G190A
9800619VS19		K219KQ	E138A

DISCUSSION

In 1995 CRF01_AE was identified, approximately 10 years after the first detected HIV-1 infection in Bulgaria. CRF01_AE was first introduced in HET and in 2002 the strain was transferred into the PWID community. CRF01_AE was rapidly disseminated among this population leading to a local outbreak in 2009 (6, 8, 7). Thereafter CRF01_AE became the second predominant subtype in Bulgaria and nowadays it is the most prevalent among PWID (52%) and least in MSM (4.8%). In addition, we found that a significantly higher number of women were infected with CRF01_AE (30.7%) than those infected with other HIV-1 subtypes (18, 19).

In our study, we used two different thresholds of genetic distance to determine recent and more distant infections of CRF01_AE in Bulgaria. Six clusters were identified at 1.5% genetic distance, featuring a large outbreak cluster containing 154 sequences, the majority of participants, almost 41%, were young male PWIDs. The data collected in our study confirmed the understanding that CRF01_AE was distributed mainly among PWID and PWID/MSM. The dominance of a large cluster indicates the rapid spread of CRF01_AE infection in a group that does not follow safety measures, such as PWIDs. The main participants in the clusters were young male PWIDs. Very small fraction of PWIDs did not cluster, unlike HETs, which were mostly single sequences. This indicates that CRF01_AE does not spread massively among other transmission groups or leads to likely dead-end transmission. However, CRF01_AE is also spreading among other groups indicating the presence of transmission networks between risk groups and the general population. To find more recent HIV-1 CRF01_AE transmissions we lowered the genetic distance threshold to 0.5% and the large cluster collapsed. All clusters at 0.5% contained sequences mainly from PWIDs diagnosed in 2009-2019 matching the period of expansion in HIV-1 CRF01_AE infections in Sofia (Table 1). The local sub-epidemic of HIV-1 CRF01_AE in Bulgaria was maintained through the spread in PWIDs although it was probably introduced into the country through HET transmission. We identified a variety of antiretroviral DRMs in HIVs from persons with CRF01_AE infection. Most prevalent NRTIs and NNRTIs DRMs were M184V (17/57, 31.6%), and E138A (7/41, 17.1%), respectively. The NNRTI E138A DRM confers resistance to rilpivirine (RPV) and is present in 1.2% and 0.1% of treated and untreated people with CF01_AE infection, respectively, and is a polymorphic mutation that can survive for several years (16, 20).

Fortunately, as in our previous HIV-1 studies in Bulgaria, most DRMs were found in cases that did not cluster or were in transmission pairs (7, 17) and were much less prevalent in PWIDs (1.1%) compared to HET (28.7%) and MSM (23.7%).

The increased numbers of HIV-1 CRF01_AE infections in PWIDs in Bulgaria has led to a strong public response and measures by the Bulgarian Ministry of Health and NGOs (21). Campaigns have been put in motion to distribute free needles, syringes and condoms, free testing for HIV and hepatitis, and various educational initiatives. The study included only patients whose HIV-1 pol sequences were successfully obtained, thus excluding patients who had underwent a successful therapy and their viral load was low, as well as people who had not yet been diagnosed. These limitations may affect our analyzes and conclusions. Our study also included individuals who are likely infected abroad, this suggesting a more extensive study of transmission networks.

To analyze the spread and transmission clusters of HIV-1 subtype CRF01_AE we conducted a phylogenetic analysis of the available HIV-1 pol gene sequences. Our study found transmission clusters indicating local outbreak among PWID. Resistance mutations were found mostly in HET and non-clustering singleton sequences. To better understand and control HIV-1 epidemic, continuous monitoring of this and other subtypes of HIV-1 in Bulgaria is further needed.

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