MOLECULAR VIROLOGICAL ANALYSIS OF THE TRANSMISSION CLUSTERS AND RESISTANCE MUTATIONS OF HIV-1 SUBTYPE B IN BULGARIA (2012-2020)

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ABSTRACT

HIV-1 infection in Bulgaria is known for its high level of genetic diversity. Previous studies have indicated that subtype B is the most common strain in Bulgaria, particularly among men who have sex with men, who are at a high risk of transmission. The primary objective of this study was to identify any transmission clusters and transmission resistance in individuals newly diagnosed with HIV-1 who have not yet received antiretroviral therapy (ART).

To this end, we sequenced the HIV-1 pol gene in the samples from the study participants using either the Viroseq HIV-1 Genotyping Test (Abbott) and the Applied Biosystems 3130xl genetic analyzer or the TruGene DNA Sequencing System (Siemens Healthcare) and an OpenGene DNA sequencing system. We then subtyped the HIV-1 pol sequences, and further analyzed those that met the criteria for subtype B.

The study included a total of 595 HIV-1 subtype B sequences. Our analysis revealed that the majority of those diagnosed with HIV-1 subtype B were male and lived in Sofia region. The most common transmission mode was through sexual intercourse among men who have sex with men, followed by heterosexual transmission. We also observed the presence of multiple transmission clusters , and a low percentage of transmitted drug resistance mutations (TDRMs).

Overall, our study confirms that HIV-1 subtype B remains the most dominant strain in Bulgaria.

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INTRODUCTION

There is a wide variety of HIV-1 subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs) in Bulgaria, which can complicate the understanding of HIV-1 epidemiology [1,2]. Due to multiple introductions of various HIV-1 genotypes from abroad into Bulgaria and random founder events, numerous HIV-1 subtypes have spread unevenly into different transmission risk groups [3]. For instance, subtype B infections are mostly found in men who have sex with men (MSM), while CRF01_AE and CRF02_AG affect two geographically distinct subgroups of people who inject drugs (PWIDs). The greatest diversity of HIV-1 subtypes was identified in persons reporting heterosexual (HET) transmission [4,5,6,7,8].

It is crucial for individuals living with HIV to receive appropriate antiretroviral therapy (ART). ART helps to reduce morbidity, mortality, and viral transmission by modulating the amount of viral load in the body [9]. However, due to its high mutation rate, HIV-1 can easily develop drug resistance mutations, which can negatively impact the effectiveness of ART [10]. HIV drug resistance mutations (DRMs) can be transmitted and adversely affect HIV-1 therapy [11].

Therefore, before administration of therapy, a HIV drug resistance test is carried out to identify any transmission resistance mutations as recommended by the European and Bulgarian national guidelines. There is a list of 93 non-polymorphic HIV-1 DRMs in the surveillance DRM (SDRM), that require monitoring due to their ability to be transmitted to newly infected individuals; these are known as transmitted DRMs (TDRMs). These 93 mutations include 34 DRMs associated with nucleoside reverse transcriptase inhibitors (NRTIs) at 15 positions in the reverse transcriptase (RT) gene, 19 DRMs associated with non-nucleoside reverse transcriptase inhibitors (NNRTIs) at 10 positions in the RT gene, and 40 DRMs associated with protease inhibitors (PIs) at 18 positions in the protease gene (PR).

In our previous studies, we found a wide variety of different HIV-1 subtypes, CRFs, and URFs in Bulgaria [1,3,4,5,6]. The presence of multiple HIV-1 subtypes further complicates the understanding of HIV-1 epidemiology and the interpretation of DRM [1,2].

Our current study aims to determine the transmission clusters in HIV-1 subtype B between 2012 and 2020 in Bulgaria using newly developed bioinformatics tools. Our findings will help to focus intervention efforts more effectively to control the ongoing spread of the HIV-1 epidemic in Bulgaria.

MATERIALS AND METHODS

EDTA plasma samples (n=1053) were obtained from patients diagnosed with HIV in Bulgaria between 2012 and 2020. At the time of diagnosis, demographic and epidemiological data were collected and linked to an anonymous code [1]. Viral RNA was extracted from plasma samples according to the standard protocol of Abbott Viroseq HIV-1 Genotyping Test and/or QIAmp Viral RNA Mini Kit (Qiagen). The HIV-1 pol gene was sequenced utilizing Viroseq HIV-1 Genotyping Test (Abbott), Applied Biosystems 3130xl genetic analyzer, TruGene DNA Sequencing System (Siemens Healthcare), and OpenGene DNA sequencing system [7]. The sequences were subtyped using the automated subtype tool COMET v2.4 [12], the REGA HIV-1 subtyping tool v3.0 [13], and the jumping profile Hidden Markov Model (jpHMM) [14]. Only subtype B sequences underwent further manual analysis. The sequences were aligned using MAFFT v7 [15,16] and were manually edited using the AliView v1.23 program [17]. The phylogenetic tree was reconstructed with a set of Bulgarian subtype B sequences and reference sequences from the Los Alamos HIV Sequence Database using the maximum likelihood (ML) method. [18]. Fisher's exact test was used to compare categorical variables. Specifically, the summation method of small p-values was employed to evaluate the significance of the findings, with a statistically significant p-value being defined as less than 0.05.

RESULTS

Epidemiological analysis

A total of 595 ART-naïve patients diagnosed with HIV-1 subtype B were included in the study. Epidemiological and demographic data is presented inTable 1. The analysis revealed a significant prevalence of men infected with HIV-1 subtype B. Of all tested samples, 92.1% belonged to male patients, while only 7.9% belonged to female patients. The mean age at diagnosis was 34 years, with the youngest patient being 16 years old and the oldest 78 years old. Regarding the most probable location of acquiring the infection with HIV-1 subtype B virus, 83.4% of the sequences belonged to Bulgarian citizens who were infected within the country, 14.1% were Bulgarians most likely infected abroad, and 2.5% were foreigners who were diagnosed in Bulgaria.

Our study demonstrated a low prevalence of TDRMs in HIV-1 subtype B in Bulgaria, with only 5.2% of individuals infected with HIV-1 subtype B having any of the SDRMs of interest.

At the time of diagnosis, each individual was demanded to fill out a questionnaire assessing the risk factors and the probable route of HIV transmission. Approximately, while the MSM group was the most strongly represented, with 63.4% of infections, followed by 34.1% of heterosexual individuals, and 2.0%. of PWIDs with HIV-1 subtype B virus. Only 0.5% of the studied individuals belonged to the MSM+PWIDs subgroup.

Table 1. Characteristics of individuals infected with HIV-1 subtype B in Bulgaria.

HIV-1 subtype B	Total number	(%)
	595	100
Gender		
Women	47	7,9
Men	548	92,1
Likely country of infection		
Bulgarians infected in Bulgaria	496	83,4
Bulgarians infected abroad	84	14,1
Foreigners diagnosed in Bulgaria	15	2,5
Transmission category		
HET	203	34,1
MSM	377	63,4
PWIDs	12	2
MSM+PWIDs	3	0,5

Table 2. Statistical analysis of HIV-1 subtype B patients according to the country of infection and transmission category criteria.

HIV-1 subtype B	Bulgarians infected in Bulgaria n=496 (%)	Bulgarians infected abroad n=84 (%)	Foreigners diagnosed in Bulgaria n=15 (%)	p value Bulgarians infected in Bulgaria / Bulgarians infected abroad	p value Bulgarians infected in Bulgaria / Foreigners diagnosed in Bulgaria	p value Bulgarians infected abroad / Foreigners diagnosed in Bulgaria
HET	63 (12,7)	36 (42,9)	3 (20)	0,0001	0,4257	0,1504
MSM	319 (64,3)	47 (55,9)	12 (80)	0.1445	0.2778	0.0942
PWID	11 (2)	1 (1,2)	0 (0)	1	1	1
MSM +PWID	2 (0,4)	1 (1,19)	0 (0)	0.3751	0.0910	1

Statistical analysis

In this study, we categorized patients into three groups based on the country of their infection: Bulgarians infected in Bulgaria, Bulgarians infected abroad, and foreigners diagnosed in Bulgaria. We analyzed each patient's transmission category within these groups (Table 2). The percentage of HET Bulgarians infected with HIV-1 subtype B was significantly higher among those infected abroad than those infected in Bulgaria (42.9% compared to 12.7% p=0.0001). No significant statistical differences were found between the other groups.

Analysis of transmission categories

After conducting a summary analysis of transmission categories, we proceeded with analysis based on the year of diagnosis for patients with HIV-1 subtype B (Figure 1). Throughout the study period, the percentage of PWIDs and MSM+PWIDs remained constant and approached zero, with the exception of 2018, where we observed a rise in PWIDs from zero to 5.9%.

In contrast, HETs exhibited fluctuations during the studied period. In 2012, the percentage of diagnosed HETs was 39.4%, with a 1 percent increase in 2013 and a 1 percent decrease in 2014. The percentage of HETs diagnosed with HIV-1 subtype B was at its lowest in 2015, with 22.7%. However, the rates increased to 37.5% in the following year, decreased to 24.6% in 2018 and then rose again to 43.1% in 2020. In the MSM group, there were also some fluctuations in the rate of infections. At the beginning of the period, they accounted for 60.6%, and over the following two years, this percentage remained relatively consistent. In 2015, we observed a peak with 76% diagnoses, while the percentage of diagnosed MSM in 2016 returned to its original levels of 60.7%. In 2017 and 2018, an increase in the percentage of infected individuals was noted, with 68.6% in 2018. In the last two years of the study, there was a decrease in the percentage of diagnosed cases, reaching its lowest point in 2020, with 52.9%.

Demographic analysis

The demographic analysis presented in Table 2 revealed that the majority of patients (57.5%), were residents of Sofia region. Plovdiv and Varna districts represented 5.9% and 4.0% of the patients respectively, while the remaining 32.6% were

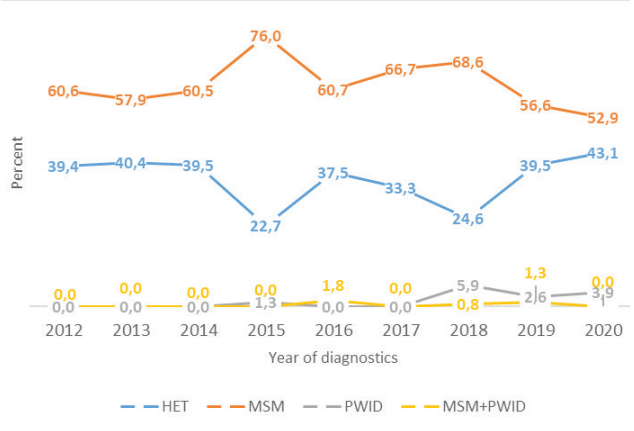

Figure 1. Percentage distribution of HIV-1 subtype B transmission categories in Bulgaria by year from 2012 to 2020.

Table 3. Regional distribution of individuals infected with HIV-1 subtype B in Bulgaria.

Region in the country	Total number of patients infected with HIV-1 subtype B	(%)
Sofia	342	57,5
Plovdiv	35	5,9
Varna	24	4,0
Other	194	32,6

dispersed across other regions in the country in an uneven manner.

Phylogenetical analysis

A phylogenetic analysis was conducted to reconstruct a tree of 595 HIV-1 subtype B sequences and 19 reference sequences [18]. Our objective was to identify clusters of sequences containing resistant mutations. Following the reconstruction of the phylogenetic tree (Figure. 2), all sequences with TDRMs were marked in red. Our analysis revealed multiple introductions of viruses with resistant mutations in clusters across the phylogenetic tree.

Of particular interest was a cluster containing 15 sequences, each of which harbored the TDRM L90M. This cluster comprised 11 sequences from patients living in Sofia, 2 from Kyustendil region, and 1 each from Plovdiv and Blagoevgrad regions. Thirteen patients self-identified as MSM, while two identified as HET. While most of the patients were infected in Bulgaria, the introduction of viruses from other European countries was also evident. Two of the patients believed to have contracted the virus in Spain, while other two - in Serbia and Germany. Additionally, we analyzed the presence of other sexually transmitted infections (STIs) from the information obtained during the diagnostic process and found that 46.7% of the sequences forming the cluster belonged to individuals with other STIs. The

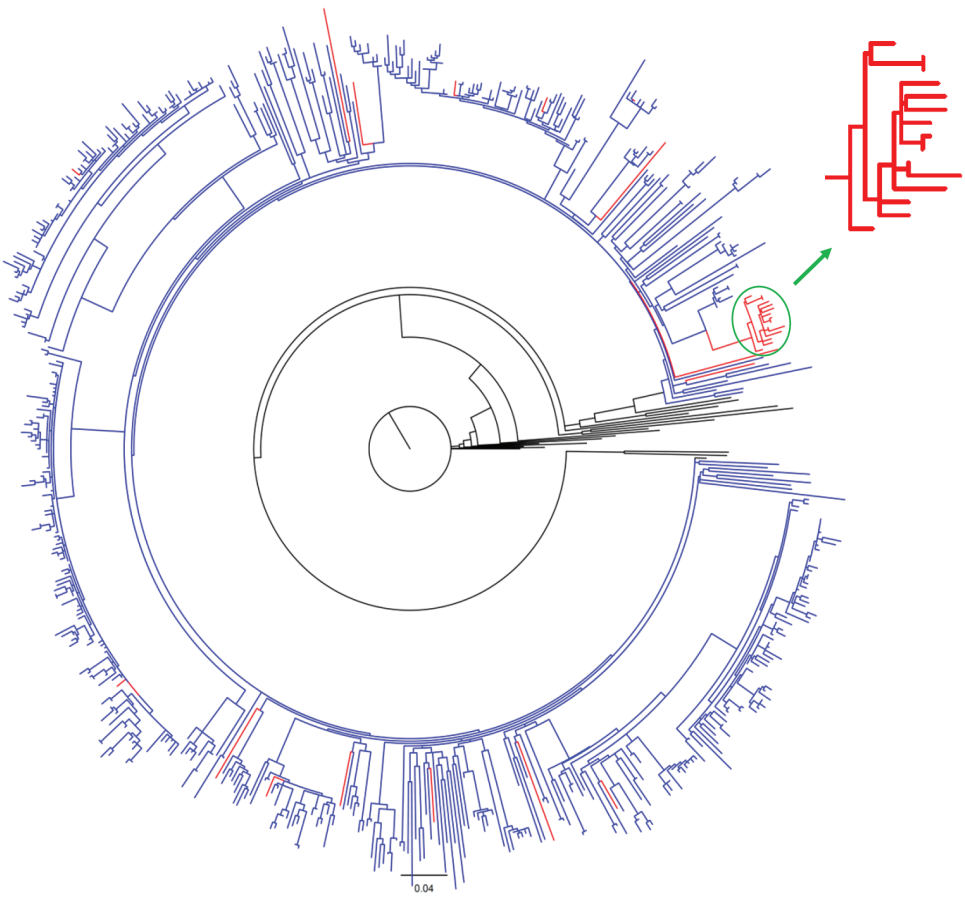

Figure 2. Phylogenetic tree of sequences from HIV-1 subtype B in Bulgaria. The phylogenetic tree was composed of 614 sequences, 595 were Bulgarian isolates and 19 - reference sequences. Reference sequences are colored in black, Bulgarian sequences are colored in blue and sequences containing resistance mutations are colored in red. The red cluster is composed of 15 resistance sequences.

most frequently reported STI was syphilis, followed by genital herpes, chlamydia, and hepatitis B.

DISCUSSION

In this study, we combined demographic, molecular and virological data from 595 individuals with HIV-1 subtype B diagnosed in Bulgaria between 2012 and 2020. We analyzed the phylogenetic clusters formed, transmitted drug resistance and routes of HIV-1 subtype B transmission.

Our analysis of the ART-naive transmission network of subtype B found that most phylogenetic clusters were composed primarily of sequences isolated from MSM with the potential to facilitate the accelerated spread of resistance mutations among these individuals.

Indeed, we identified a 15-member cluster of subtype B sequences from 13 MSM and 2 male HET. The presence of two male HET sequences in a cluster of 13 MSMs indicates possible bridges of transmission of HIV infection and/or inaccurately filled self-reporting forms. The analysis of the 15-member cluster showed 14 PI SDRMs, 4 NNRTI SDRMs and one NRTI SDRM, indicating transmission among these individuals with the potential for further spread among HET. The study found that 4 s out of 15 sequences in a cluster were from patients who acquired the infection abroad, meaning that those SDRMs could be introduced into Bulgaria from other countries and spread locally.

HIV infection was introduced in the MSM community in Bulgaria around 2010, much later, than in the HET group [6]. However, infection spread much more rapidly in the MSM group, making the study of the major prevalent subtype in this group important. Half of the cases with SDRM were detected in the capital city of Sofia, where the highest number of HIV-1 cases were registered during our survey period. However, SDRMs were also detected in persons from 15 other districts of the country, including remote locations in Bulgaria, suggesting that SDRMs are widespread in the country despite the low overall prevalence. SDRMs were also identified in 25.0% of drug-naive individuals who were co-infected with other STIs and in 3.3% of individuals engaged in sex work, suggesting additional potential mechanisms for onward spread of these SDRMs.

Genetic analysis confirmed the dominance of HIV-1 subtype B found in our previous study [6]. The study revealed that Subtype B was predominant among men and was unevenly distributed among persons with transmission risk behavior in Bulgaria. The majority of these sequences belonged to MSM. These results were expected, as the HIV-1 subtype B epidemic in Western Europe has similar characteristics [5,6]. The detection of clusters containing individuals from different transmission groups indicated the presence of transmission bridges between those groups.

O study has some limitations. Some individuals with subtype B were excluded from analysis due to receiving therapy in another country before their diagnosis in Bulgaria, and others - due to the lack of HIV-1 pol sequence. It is important to note that the self-reporting process also may have some limitations. At the beginning of the epidemic, the majority of men with HIV subtype B reported HET transmission, while subsequently, an increasing number of individuals infected with subtype B reported MSM behavior. This fact may be related to a heavier stigma in the early years of the epidemic.

CONCLUSION

HIV-1 subtype B is still the most common cause of HIV infections diagnosed in Bulgaria, although previous studies have shown that a variety of HIV subtypes are distributed throughout the country, including rare subtypes. The incidence of infected women differs significantly from that of infected men, which is a likely consequence of the introduction of HIV-1 subtype B in the vulnerable group of MSM, where it spreads rapidly. Transmission clusters involving vulnerable groups can serve as a springboard for the accelerated spread of resistant mutations to the general population.

The implementation of molecular virological surveillance specifically designed for vulnerable groups can be an effective measure for limiting the transmission of HIV within a community. By closely monitoring the molecular characteristics of the virus, it becomes possible to identify and target specific groups at a higher risk of contracting and spreading the disease. This approach can help the development of better targeted and effective prevention strategies, ultimately reducing the prevalence of HIV in the community.

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