MOLECULAR EPIDEMIOLOGICAL ANALYSIS OF THE TRANSMISSION CLUSTERS OF THE HIV-1 CIRCULATING RECOMBINANT FORMS CRF01_AE AND CRF02_AG IN BULGARIA

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ABSTRACT

Background
The purpose of this study was to analyse the underlying HIV transmission clusters of individuals from different vulnerable groups infected with the recombinant forms of HIV-1 – CRF01_AE and CRF02_AG, between 1986 and 2011 using sequencing and phylogenetic analysis.

Material and methods
Blood samples from randomly selected 242 individuals diagnosed with HIV-1 CRF01_AE and CRF02_AG in Bulgaria were analysed. HIV-1 pol gene fragment was sequenced using the ViroSeq HIV-1 Genotyping Test (Abbott) and/or TruGene DNA Sequencing System. The phylogenetic tree reconstruction was performed with the IQ-TREE program. Identification of the phylogenetic clusters was performed using the ClusterPicker program with two parameters to identify recent and distant infection.

Results
Two main local independent epidemics confined to different geographical regions of the country were caused by HIV-1 CRF01_AE and CRF02_AG. The various viral strains circulate predominantly in two major separate regions – CRF01_AE in Sofia and CRF02_AG in Plovdiv. Most of the individuals infected with viral strains analysed in this study were people who inject drugs (PWID) or heterosexuals and only a few were men who have sex with men. The phylogenetic analysis revealed transmission clusters in both recombinant forms, few in number when confined within a short period of time and multiple clusters over an extended timeframe.

Conclusions
The introduction and rapid spread of two different strains of HIV-1 into geographically distant groups of PWID triggered local epidemic outbreaks. The phylogenetic analysis indicated the accelerated transmission of HIV, which is a characteristic of the spread through injection practices. Our study demonstrated that transmission cluster monitoring is important for better understanding of the development of epidemic and could be used as a tool for the identification of risk indicator populations.

INTRODUCTION
HIV-1 is a result of cross-species transition from SIV in chimpanzees to HIV in humans (1, 2). HIV-1 has several major groups: M, H, O and P. Group M is the most significant for the current pandemic and contains several phylogenetically distinct subtypes (A, B, C, D, F, G, H, J and K), circulating recombinant forms (CRFs) and numerous unique recombinant forms (URFs) (3). HIV-1 subtypes and CRFs are unevenly distributed in the world. This phenomenon is the result of different founder effects followed by local spread of a specific subtype within certain socioeconomic conditions.
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environment and circulation within specific vulnerable groups (3, 4). Subtype B is dominant in North America and Western Europe, and subtype A – in some countries of Eastern Europe and Central Asia, including Russia (3). Subtype C is the most abundant HIV-1 subtype in the world and is prevalent mostly in South and Eastern Africa and Southeast Asia (3). CRFs and URFs are widely distributed in Africa and in countries where different subtypes co-circulate (3, 5).

In Bulgaria, multiple HIV-1 subtypes and recombinant forms have been introduced from different countries of the world. Subsequently, the introduced strains were disseminated unequally among individuals from different transmission groups, including heterosexuals (HET), men who have sex with men (MSM) and people who inject drugs (PWID) (4, 6, 7, 8). Due to random events, certain HIV-1 strains have a chance of spreading sharply after being introduced into vulnerable groups forming expanding transmission clusters representing local outbreaks. Such events have been observed since 2005 when two different CRFs (CRF01_AE and CRF02_AG) were independently introduced and rapidly disseminated among two geographically distinct subgroups of PWID, resulting in local HIV-1 outbreaks (4, 9).

Subtype B is the most widespread in Bulgaria but found in less than half of the HIV-1+ individuals in the country. The remaining over 50% of the introduced strains are non-B subtypes, CRFs and URFs, of which CRF01_AE and CRF02_AG are the most prevalent. Although various subtypes were initially introduced in the country, subtype B, CRF01_AE and CRF02_AG are the three major HIV-1 strains in the country. HIV-1 CRF01_AE and CRF02_AG have been found to be the most prevalent among the most vulnerable groups, such as PWID. In addition, according to our epidemiological data, vulnerable individuals represent a significant proportion of the current HIV+ population in Bulgaria and since 2005 there has been a sharp increase in the incidence of HIV among PWIDs, leading to an outbreak with significant involvement of the CRF01_AE and CRF02_AG viruses (4, 10, 11).

The purpose of this national representative study was to analyse the underlying HIV-1 transmission clusters of the two most widespread recombinant forms of HIV-1 CRF01_AE and CRF02_AG in Bulgaria using the most up-to-date methods for phylogenetic analysis. Using the ClusterPicker program, we analysed the transmission clusters of the CRF01_AE and CRF02_AG and the participation of different transmission groups in the accelerated spread of these viruses in Bulgaria.

MATERIAL AND METHODS

Study design and specimen preparation

Blood samples from all individuals diagnosed with HIV-1 CRF01_AE and CRF02_AG between 1986 and 2011 were analysed during a clinical follow-up at the National Reference Confirmatory Laboratory of HIV in Sofia, Bulgaria. Plasma samples were linked to epidemiological data through an anonymous numerical code according to the established ethical standards of Bulgaria as previously described (7).

Phylogenetic analysis and cluster identification

The HIV-1 pol gene fragment was sequenced using the ViroSeq HIV-1 Genotyping Test (Abbott) and/ or TruGene DNA Sequencing System (Siemens Healthcare) and either the Applied Biosystems 3130xl genetic analyser or an OpenGene DNA sequencing system following the manufacturer’s protocol (4).

HIV-1 CRF01_AE and CRF02_AG of the analysed sequences was determined using the automated subtype identification tool COMET v2.2 (12) and REGA HIV-1 subtyping tool version 3.0 (13). Sequence alignments were performed using the MUSCLE algorithm implemented in AliView version 1.23 (14, 15). Additional quality control of the subtype purity and possible presence of gaps in the sequence was performed. After the clean-up and preliminary quality analysis, the complete dataset contained 141 CRF01_AE sequences comprised of 901 nucleotides in length and 101 CRF02_AG sequences comprised of 918 nucleotides in length. The phylogenetic tree reconstruction was performed with IQ-TREE v1.6.11 program and was built through the construction of an initial parsimony tree by the phylogenetic likelihood library and search for the best model among 88 DNA models included in the program. The best-fit model was found to be: TP3M + F + I + G4 according to the Bayesian information criterion (16, 17, 18). Verification of the topology of the phylogenetic tree was performed with generating 1000 samples for ultrafast bootstrap (18). The phylogenetic tree was rooted with midpoint root and was used for further analysis of the phylogenetic clusters. The tree was visualised using FigTree v1.4.4.
Identification of phylogenetic clusters was performed using the ClusterPicker program at a genetic distance of 0.5% and 1.5% corresponding to 0.005 and 0.015 nucleotide substitutions/site, respectively.

RESULTS

Study population demographics

In this study, we generated and analysed 242 HIV-1 pol gene sequences from the second and third most prevalent HIV strains in Bulgaria – CRF01_AE and CRF02_AG (Table 1). Men were 74.4% and women 25.6%, most of them in the age group 20-29. According to the permanent address, patients were focused in three major regions: Sofia, Plovdiv and the town of Peshtera. Sofia and Plovdiv were the dominant regions with almost identical proportion of infected individuals (38.4% and 37.6%, respectively). It is curious that completely different viral strains circulated in these two major regions: CRF01_AE in Sofia and CRF02_AG in Plovdiv. In addition, although Peshtera is close to Plovdiv, CRF01_AE that is typical for the more remote region of Sofia has been distributed in Peshtera.

Table 1. Study population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CRF01_AE</th>
<th>CRF02_AG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n) (%)</td>
<td>(n) (%)</td>
<td>(n) (%)</td>
</tr>
<tr>
<td>Total</td>
<td>141 100.0</td>
<td>101 100.0</td>
<td>242 100.0</td>
</tr>
<tr>
<td>Men</td>
<td>96 68.1</td>
<td>84 83.2</td>
<td>180 74.4</td>
</tr>
<tr>
<td>Women</td>
<td>45 31.9</td>
<td>17 16.8</td>
<td>62 25.6</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤19</td>
<td>16 11.3</td>
<td>14 13.9</td>
<td>30 12.4</td>
</tr>
<tr>
<td>20-29</td>
<td>69 48.9</td>
<td>58 57.4</td>
<td>127 52.5</td>
</tr>
<tr>
<td>30-39</td>
<td>41 29.1</td>
<td>18 17.8</td>
<td>59 24.4</td>
</tr>
<tr>
<td>40-49</td>
<td>12 8.5</td>
<td>6 5.9</td>
<td>18 7.4</td>
</tr>
<tr>
<td>≥50</td>
<td>3 2.1</td>
<td>5 5.0</td>
<td>8 3.3</td>
</tr>
<tr>
<td>Region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permanent address – Sofia</td>
<td>83 58.9</td>
<td>10 9.9</td>
<td>93 38.4</td>
</tr>
<tr>
<td>Permanent address – Plovdiv</td>
<td>8 5.7</td>
<td>83 82.2</td>
<td>91 37.6</td>
</tr>
<tr>
<td>Permanent address – Peshtera</td>
<td>18 12.8</td>
<td>0 0.0</td>
<td>18 7.4</td>
</tr>
<tr>
<td>Permanent address – other</td>
<td>32 22.7</td>
<td>8 7.9</td>
<td>40 16.5</td>
</tr>
<tr>
<td>Transmission category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HET</td>
<td>63 44.7</td>
<td>23 22.8</td>
<td>86 35.5</td>
</tr>
<tr>
<td>MSM</td>
<td>2 1.4</td>
<td>0 0.0</td>
<td>2 0.8</td>
</tr>
<tr>
<td>MSM+PWID</td>
<td>3 2.1</td>
<td>1 1.0</td>
<td>4 1.7</td>
</tr>
<tr>
<td>PWID</td>
<td>69 48.9</td>
<td>77 76.2</td>
<td>146 60.3</td>
</tr>
<tr>
<td>MTCT</td>
<td>4 2.8</td>
<td>0 0.0</td>
<td>4 1.7</td>
</tr>
</tbody>
</table>

HET – heterosexuals; MSM – men who have sex with men; PWID – people who inject drugs; MTCT – mother-to-child transmission.

Epidemiological data in combination with results from the phylogenetic analysis, reviewed that the HIV-1 strain CRF02_AG was highly concentrated in the region of Plovdiv and only 17.8% of these viruses were dispersed outside this region. In contrast, CRF01_AE was more evenly distributed in the country including Sofia (58.9%), Peshtera (12.8%) and other regions (28.4%) – Fig. 1 and Table 1.
Figure 1. Distribution of CRF01_AE and CRF02_AG in different regions of the country.

There was also a significant difference in the spread of CRF01_AE and CRF02_AG among different transmission groups of the population. CRF01_AE was more evenly distributed between PWID (48.9%) and HET (47.7%), whereas CRF02_AG was found predominantly in PWID (76.2%), and much less in HET (22.8%) – Fig. 2 and Table 1.

Figure 2. Distribution of CRF01_AE and CRF02_AG in different transmission groups.
Phylogenetic analysis and cluster definition
ClusterPicker software was implemented to analyse the phylogenetic clusters of the two viral strains, CRF01_AE and CRF02_AG. The phylogenetic clusters were defined with two parameters for genetic distance: 0.5% and 1.5% corresponding to 0.005 and 0.015 nucleotide substitutions/site, indicating recent and more remote transmission events. The initial cluster support threshold was defined to have bootstrap values >0.9. Two different options were defined for sequence positions on the topology of the phylogenetic tree: single sequences and phylogenetic clusters. Clusters with threshold size of 10 or more sequences were defined as large clusters.

When analysing the 141 CRF01_AE sequences with genetic distance of 0.5%, two clusters (representing very recent transmission) were identified with two sequences each (Fig. 3 A). At genetic distance of 1.5%, 13 clusters (representing remote in time transmission) with 2 or more sequences were identified, of which 9 clusters were composed of 2 sequences, 2 clusters of 3 sequences, and 2 clusters of 4 sequences (Fig. 3 B).

![Figure 3 A and B. Phylogenetic tree of CRF01_AE strains reconstructed with ML algorithm using IQ-tree program. A. ClusterPicker analysis with genetic distance of 0.005 nucleotide substitutions/site. B. ClusterPicker analysis with genetic distance of 0.015 nucleotide substitutions/site. The red arrows indicate the location of the phylogenetic clusters with the corresponding genetic distance. One sequence isolated from HET indicated with blue arrow is at the basis of the sequences isolated from the group of the PWIDs. The size and digital expression of nucleotide substitutions/site are shown at the bottom of the phylogenetic tree.](image)

When analysing 101 CRF02_AG sequences with genetic distance of 0.5%, four clusters (representing very recent transmission) with two sequences each, were identified (Fig. 4 A). At genetic distance of 1.5%, 12 clusters (representing remote in time transmission) with 2 to 5 sequences were identified. Of these, 9 clusters were composed of 2 sequences, 1 cluster of 3 sequences and 2 clusters of 5 sequences (Fig. 4 B).
Figure 4 A and B. Phylogenetic tree of CRF02_AG strains reconstructed with ML algorithm using IQ-tree program. A. ClusterPicker analysis with genetic distance of 0.005 nucleotide substitutions/site. B. ClusterPicker analysis with genetic distance of 0.015 nucleotide substitutions/site. The red arrows indicate the location of the phylogenetic clusters with the corresponding genetic distance. The size and digital expression of nucleotide substitutions/site are shown at the bottom of the phylogenetic tree.

Discussion
In this study, we analysed two of the most prevalent HIV-1 strains in Bulgaria – CRF01_AE and CRF02_AG. The spread of these two recombinant forms is significant both at the country and at the global levels as demonstrated by previous national and global worldwide distribution surveys (4). Of additional interest are the findings that these two strains have been introduced among the most vulnerable individuals to blood-borne infections such as PWID, where viruses have a chance of accelerated spread in limited geographical areas causing an epidemic outbreak among the affected populations. For example, in the late 1990s-early 2000s in Finland, HIV affected a marginalised population of PWID with high rates of imprisonment and homelessness and CRF01_AE, a prevalent variant in Southeast Asia that was circulating in Finland in the early 1990s, was the cause of the outbreak. CRF01_AE was imported from Helsinki, Finland to Stockholm, Sweden leading to an outbreak there among PWID that started probably in around 2003 and was detected in 2006. Similarly, in a PWID-related outbreak in the early 2000s in Northern Italy, HIV-1 diagnoses among PWID formed a monophyletic cluster of subtype G with origin in West Africa (19). Our phylogenetic analysis uses bioinformatics programs and a range of phylogenetic proximity and genetic distance parameters to analyse isolated viral sequences and to identify phylogenetic clusters that represent transmission events occurring at different time frame. The smaller genetic distance of 0.005 nucleotide substitutions/site allows identification of transmission events that occurred within one year. Using this time constraint, only two clusters of two
sequences each were detected in the CRF01_AE phylogenetic tree, representing transmission events within the time frame of one year (Fig. 3 A). These transmission events took place within PWIDs in Sofia. In contrast, four clusters with two sequences each were identified for the other HIV-1 strain – CRF02_AG, indicating more turbulent transmission events occurring within a short time frame among the group of PWIDs (Fig. 4 A).

At a greater genetic distance of 0.015 nucleotide substitutions/site, many phylogenetic clusters have been identified in both phylogenetic trees, representing the unfolding of broad-scale transmission events that have taken place over a longer period of time during the expansion of independent local epidemics in remote geographic regions. Individual groupings of sequences stand out on the topology of phylogenetic trees reconstructed by both recombinant forms of HIV-1. On the phylogenetic tree of CRF01_AE sequences, PWIDs are separated into a distinct group of sequences with relatively short branches generally representing short evolutionary history. The sequences from the HET individuals are positioned closer to the root of the tree. The sequences isolated from HET individuals are at the basis of all sequences and precede all PWIDs sequences showing that these viruses were first introduced and spread within HET transmission group, and later were transferred to PWIDs. This finding is in line with our epidemiological data and previous reports indicating that HIV was introduced into the vulnerable group of PWID later as compared to HET individuals (4). In addition, our data sample includes only the individuals from whom the HIV-1 pol gene was successfully generated, and not those from whom no viral sequence has been obtained due to successful therapy and low viral load.

Conclusions

A large variety of HIV-1 subtypes and recombinant forms has been introduced in Bulgaria. The two recombinant forms CRF01_AE and CRF02_AG have been introduced into geographically distant groups of PWID and triggered local epidemic outbreaks. The phylogenetic analysis indicated the presence of accelerated transmission of HIV, which is characteristic of the spread through injection practices. The presence of a reservoir of a large number of HIV-infected individuals from vulnerable groups is of concern because there is a possibility of rapid dissemination and transmission into the general population. The monitoring of transmission clusters is important for better understanding of the epidemic and for identification of risk indicator population groups.

Acknowledgments

This work was funded by a grant from the Ministry of Education and Science, Bulgaria, contract DN03/16.12.2016 and by the European
Regional Development Fund through Operational Programme Science and Education for Smart Growth 2014-2020, Grant BG05M2OP001-1.002-0001-C04 “Fundamental Translational and Clinical Investigations on Infections and Immunity”.

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