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Transmitted HIV Drug Resistance in Treatment-Naïve Romanian Patients

Aura Temereanca^{1,2}, Luminita Ene³, Sanjay Mehta⁴, Loredana Manolescu^{1,2}, Dan Duiculescu³, and Simona Ruta^{1,2,*}

¹“Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania

²“Stefan S. Nicolau” Institute of Virology, Bucharest, Romania

³“Dr. Victor Babes” Hospital for Infectious and Tropical Diseases, Bucharest, Romania

⁴University of California, San Diego, California

Abstract

Transmitted HIV drug resistance (TDR) remains an important concern for individuals unexposed to antiretroviral treatment. Data on the prevalence of TDR, available mainly for HIV-1 subtype B, are now also emerging for other subtypes. In Romania, a steady predominance of subtype F was reported among both long-term survivor children and newly infected adults. The pol gene of 61 drug-naïve patients infected with HIV, diagnosed between 1997 and 2011 was sequenced in order to analyze the prevalence of primary resistance mutations and to correlate these with the infecting genotype. Only 5/61 specimens were classified as infected recently using the BED-Capture Enzyme Immunoassay. Subtype F1 was prevalent (80.3%), however, other HIV-1 clades are increasingly identified, especially in the group of subjects infected recently. An HIV transmission cluster, associated to injecting drug use was identified by phylogenetic analysis. The overall prevalence of TDR was 14.75%, mainly associated with NRTI resistance (13.11%), TAMs and M184V being the most common mutations. A declining trend of TDR was recorded from 26.08% in 1997–2004 to 7.89% in 2005–2011. No primary resistance was identified among recent seroconvertors. All HIV-1 strains had minor mutations in the protease and RT genes, often detected at polymorphic positions. The declining rates of TDR might be related to the high efficacy of HAART and to the increasing number of treated patients with virological success who have a low risk of transmission. The recent increase of HIV-1 infections which involve other subtypes impose a continuous surveillance of the genetic composition of the epidemic.

Keywords

HIV-1; transmitted drug resistance; subtype F; Romania

INTRODUCTION

Transmitted drug resistance (TDR) remains an important concern for the management of HIV-1 infection among individuals unexposed to antiretroviral treatment (ART). The presence of HIV-1 drug resistance prior to ART initiation is an important predictor of

virological response to therapy [DeGruttola et al., 2000; Bennett et al., 2008], as primary resistance can lead to a delay in viral suppression [Grant et al., 2002; Little et al., 2002] and can increase the risk of earlier virologic failure [Kuritzkes et al., 2008]. Therefore, international treatment guidelines recommend that resistance testing should be performed in all drug naive patients, before beginning a first line antiretroviral regimen [Hirsch et al., 2008; Vandamme et al., 2011]. Individualized resistance testing and therapy, with the preferential use of antiretroviral drugs with a high genetic barrier, may prevent failure of the first-line therapy, even after infection with an HIV-1 drug-resistant strain.

Several large studies have reported the prevalence of transmitted drug resistance in Europe and the United States [Vercauteren et al., 2009; Wheeler et al., 2010]. In United States, where subtype B continues to be prevalent, although there are no significant trends reported, a higher prevalence of TDR rate (14.6%) has been documented in newly diagnosed HIV patients, especially associated with non-nucleoside reverse transcriptase inhibitors (NNRTIs) mutations (7.8%), and, at lower levels, with nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) associated mutations (5.6% and 4.5%, respectively) [Wheeler et al., 2010]. On the contrary, in Western Europe, after a continuous increase in the rate of TDR, more recent epidemiological studies suggest a relative stable [Chaix et al., 2009; Vercauteren et al., 2009] or declining trend [UK Collaborative Group on HIV Drug Resistance, 2007; Yebra et al., 2011] of primary resistance, from 10.4% during 1996–2002 [Wensing et al., 2005] to 8.4% during 2002–2006 [Vercauteren et al., 2009].

However, reported data are available mainly for HIV-1 subtype B viruses, most commonly found in North America, Europe, and Australia. More recently, data on the prevalence of transmitted drug resistance in other subtypes has been reported. This is particularly interesting as some non-B clades could harbor polymorphic mutations that can be associated to resistance to specific antiretroviral drugs [Dumans et al., 2009].

Romania has a unique HIV/AIDS epidemic, due to (1) the high frequency of HIV-1 subtype F, not present in other European countries [Cernescu et al., 1994; Chitu et al., 1999; Paraschiv et al., 2007], that seem to be phylogenetically related to strains from Angola and the Democratic Republic of Congo [Guimarães et al., 2009] and also (2) the large number of long-term survivors, patients infected parenterally during their childhood between 1988 and 1992, who are today young adults with a long history of treatment. According to the National Report of the HIV/AIDS Monitoring and Evaluation Department in Romania (<http://www.cnlas.ro>), at the end of 2011, a cumulative total of 17,435 cases of HIV/AIDS infection had been recorded, with 10,903 persons living with HIV/AIDS. From 2001, Romania provided universal access to HIV/AIDS treatment and care.

The aim of this study was to assess the prevalence of transmitted drug resistance mutations in treatment-naïve HIV-1 Romanian patients and to correlate these with the infecting genotype.

MATERIALS AND METHODS

Study Patients

Plasma samples from 61 patients, diagnosed with HIV-1 infection between 1997 and 2011, were analyzed. All subjects were naïve to antiretroviral therapy at the time of resistance testing. Written informed consent was given by all participants (or the patient's parent or legal guardian) and the study was approved by the Institutional Review Board of the Institute of Virology, Bucharest, Romania.

Virological and Immunological Tests

HIV viral load was determined with a commercial nucleic acid amplification test (COBAS TaqMan HIV-1 Test Version 2.0, Roche Molecular Systems, Branchburg, NJ), with a lower detection limit of 20 copies of HIV RNA/ml and a linear range between 34 and 10,000,000 copies HIV RNA/ml. CD4+ cell counts were performed by flow cytometry using the TRIT-EST three-color reagent CD4/CD8/CD3 with TRU-COUNT tubes (Becton Dickinson, San Jose, CA).

Assessment of Recent HIV-1 Infection

In order to differentiate recent HIV-1 infections (acquired 4–6 months prior to sampling) from chronic infections (acquired at least 12 months before sampling), all patients were tested with a capture enzyme immunoassay (BED-CEIA; Calypte Biomedical, Lake Oswego, OR), according to the serological testing algorithm for recent HIV seroconversion (STARHS) [Zetola and Pilcher, 2007]. This test measures the proportion of HIV-1-specific IgG, directed to a branched trimeric synthetic oligopeptide, with respect to the total IgG in the sample. Early seroconverters have a lower proportion of HIV-specific IgG in their plasma than those with long-term infection.

HIV-1 Sequencing

Drug resistance genotyping was performed using the ViroSeq HIV-1 Genotyping System (Abbott Laboratories, Des Plaines, IL), according to the manufacturer's instructions. Briefly, after RNA isolation from plasma and reverse transcription, the entire protease gene and two-thirds of the RT gene were amplified to generate a 1.8 kb amplicon. The purified amplicon was sequenced utilizing ABI Prism Big Dye Terminator Ready Reaction Kit version 3.0 and analyzed using the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Finally, all sequences were assembled, aligned to the HXB2 reference sequence, and manually edited using the ViroSeq v2.8 software provided by the manufacturer. Genotypic drug resistance analyses were carried out with the Stanford HIV Drug Resistance Database algorithm. Mutations associated with transmitted drug resistance were identified using the WHO 2009 list of mutations for surveillance of TDR HIV strains (<http://hivdb.stanford.edu/pages/WHOResistanceList.html>).

Phylogenetic Analyses

For HIV-1 subtypes determinations, sequences were submitted to the REGA HIV-1 subtyping tool (version 2.0, available at <http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html>). Sequences (GenBank accession numbers JX966486–JX96653) were aligned using the Geneious (Biomatters, Auckland, New Zealand) software package. The alignment was then hand-curated and PHYML implemented in the Geneious package was used to generate phylogenetic trees using a TN93 nucleotide model. Trees were then annotated in FigTree v1.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Statistical Analyses

GraphPad Prism 5.0 program was used for statistical analysis. Differences in HIV RNA levels and absolute CD4 cell count were evaluated by Fisher's exact test analysis of contingency tables, using GraphPad QuickCalcs. Two-tailed *P* values were reported. Significance was set at $P < 0.05$. Analyses of baseline characteristics were performed with either a *t*-test for categorical variables or an *F*-test for continuous variables, to determine whether there were gender differences at baseline.

RESULTS

Patients Characteristics at Resistance Genotyping

A total of 61 samples from treatment-naïve, HIV-1 positive Romanian individuals, diagnosed between 1997 and 2011 were analyzed. The baseline characteristics of the study population are shown in Table I. 54.09% (33/61) of the patients were male and the median age of the entire study group was 28 years (with a range between 8 and 56 years). According to the BED-CEIA results, only 5 out of the 61 patients were classified as infected recently with HIV. More than half of the subjects (52.4%) had been infected through heterosexual intercourse. Parenteral transmission was responsible for 29.4% of the HIV cases, but only a third of these were related to injection drug use, the rest being attributable to unsafe parenteral treatments or to unscreened blood transfusions received before 1991; in 18.3% of the cases the route of transmission remained unknown due to inconsistent epidemiological information. Median baseline viral load was $5.07 \log_{10}$ RNA HIV-1 copies/ml and median CD4⁺ cell number 277 cells/mm³ (range: 147–511 cells/mm³). Male patients presented a significantly higher median viral load than female ones ($5.3 \log_{10}$ vs. $4.6 \log_{10}$ RNA HIV-1 copies/ml, $P = 0.03$) at the time of HIV resistance genotyping; no differences were recorded according to the transmission route.

HIV-1 Variants

Subtype F1 was the most prevalent HIV subtype (80.3% of the cases—49/61 patients), while non-F HIV-1 variants were recognized in the others 19.6% (12/61 patients): subtype C in 6.5% (4/61), subtype B in 4.9% (3/61), CRF 06_CPX and CRF 14_BG each in 1 patient (1.63%) and 3 cases were not assigned. Overall, the proportion of HIV infections which involved clades other than F increased from 17.4% in 1997–2004 to 21% during 2005–2011. Non-F subtype carriers were predominant in the group of individuals infected recently (three out of five subjects). There were no significant differences in HIV viremia according to HIV subtype, although patients with CRFs presented higher viral loads than the other subtypes: ($5.9 \log_{10}$ vs. $5 \log_{10}$ RNA HIV-1 copies/ml).

Prevalence of Transmitted HIV-1 Resistance

The overall prevalence of TDR was 14.75% (9/61 patients). Patients infected with HIV resistant viruses were younger than those with wild-type HIV (21 years vs. 28.5 years, $P = 0.06$) and most were males. Patients with TDR mostly belonged to the group infected in early childhood, during the early years of the nosocomial pediatric HIV Romanian epidemic, by parenteral route. Recently infected individuals, diagnosed between 2009 and 2011, mostly by injection drug use, carried no TDR mutation. All but one patients carrying resistant viruses were infected with HIV-1 subtype F1, the only exception was infected by HIV subtype C. Patients with TDR presented lower viral load values compared to those infected with wild-type HIV ($4.7 \log_{10}$ vs. $5.08 \log_{10}$ RNA HIV-1 copies/ml, $P = 0.01$); no differences were recorded in terms of immune status ($277 \text{ CD4 cell/mm}^3$ vs. $275.5 \text{ CD4 cell/mm}^3$, $P = 0.4$).

When comparing by drug class, mutations related to nucleoside reverse-transcriptase inhibitors (NRTIs) were predominant (13.11%, 8/61 patients). Global TDR to NRTIs and NNRTIs was significantly higher than to PIs for the entire study period ($P = 0.0295$). Most of the patients presented single mutations, and retained the predicted susceptibility to all antiretrovirals, irrespective of the drug class. High level resistance to all NRTIs and to the majority of the PIs was present in only one patient, while three other patients were resistant each to two drugs (AZT and D4T or 3TC and FTC). Dual class resistance was seen in only 3 out of the 61 cases (4.9%) and none of the patients presented triple-class resistance.

Characterization of HIV-1 Resistance Mutations

Genotyping reverse transcriptase (RT): TDR mutations associated with NRTI resistance were observed in eight out of the nine HIV resistant strains, all belonging to F1 subtype (Table II). Thymidine analogues mutations (TAMs), selected mostly by ZDV and d4T, were prevalent (6/9; 66.66%), with type II TAMs being more frequently detected (4/9; 44.44%): K70R and K219Q (each in three cases), D67N and T215F (each in two cases). The type I TAMs (M41L and L210W) were observed each in only 1 case. M184V, the 3TC-selected mutation was identified in three HIV resistant strains, alone in two cases and together with all the above mentioned type II TAMs, as well as other NRTI conferring resistance mutations (T69D and L74I) in one case. The Q151M multinucleoside resistance complex, which causes high or intermediate-level resistance to all NRTIs [Shirasaka et al., 1995; Iversen et al., 1996; Rhee et al., 2006] was not present.

TDR associated with NNRTIs (Table II) was identified only in 2 HIV-1-subtype F1 strains (2/9): one isolate carried V179F and Y181C mutations, and the other one harbored mutations Y188C and Y106M, all conferring resistance across the entire NNRTIs class.

Several atypical substitutions at key positions known to be linked with drug resistance were detected also within the RT gene: for NRTI-T69S (1/61 patients), T69N (2/61 patients) and for NNRTI-E138A/ G (5/61 patients), H221Y (1/61 patients).

Genotyping the protease (PR) gene revealed TDR mutations in only two HIV strains, one belonging to subtype C and the other to subtype F1 (Table II). Both isolates presented the M46L mutation-conferring intermediate-level resistance to nelfinavir and potential low-level resistance to lopinavir and atazanavir-alone in the subtype C strain and along with multiple other resistance mutations, such as L24I, I47V, F53L, I54V, and V82A in the F1 strain.

All the studied HIV-1 isolates carried a high frequency of secondary mutations in the PR gene, detected often at polymorphic positions. The most common accessory PI resistance mutations found were M36I (56/61 patients) and R41K (56/61 patients), followed by mutations L89M (51/61), I15V (50/61), and L63T (41/61). The analysis of the RT gene also revealed many accessory mutations presented in all studied sequences, encountered most frequently at positions V35T, T39A, V60I, I135L, A272P, I293V.

Temporal Trends of TDR

To assess the TDR prevalence over the calendar years, all the study participants were grouped in two distinct periods according to the year of HIV diagnosis (Table I). The prevalence of TDR to any antiretroviral drug recorded a calendar-year declining trend from 26.08% in 1997–2004 to 7.89% in 2005–2011 ($P = 0.09$).

Regarding drug class, transmitted NRTI-associated resistance showed a decline over time from a rate of 21.73% in 1997–2004 to 7.89% in 2005–2011 ($P = 0.1$). NNRTI and PI associated resistance was present only in patients diagnosed during 1997–2004 (each in 8.69% of the cases), while no NNRTIs and PIs related mutations were detected during the last observational period (2005–2011) ($P = 0.07$).

Phylogenetic Analyses

A phylogenetic tree of all samples demonstrated grouping of all the sequences subtyped as F1, and a group of CRF sequences clustering between the subtype B and subtype C sequences (Fig. 1). Within the subtype F sequences, a small cluster of four sequences was identified that had a genetic distance of less than 1% from each other. Given the genetic

similarity, this likely represents a HIV transmission cluster, associated to injecting drug use. All these four IDU related infections were diagnosed during 2010–2011 time period.

DISCUSSION

A relatively moderate and constantly decreasing rate of transmitted drug resistance was recorded in a group of treatment-naïve HIV-1 positive Romanian patients, diagnosed between 1997 and 2011. The global TDR prevalence was 14.75%, mainly associated with NRTI resistance; most of the patients infected with HIV variants harboring TDR were diagnosed before 2004 (26.08% during 1997–2004 vs. 7.89% during 2005–2011). The predominance of mutations associated with reverse transcriptase inhibitors is due to a broader and longer use of these drugs in Romania and to the low genetic barrier of NNRTI-containing regimens [Johnson et al., 2007]. Most of the subjects harbored HIV strains resistant to only one drug class; triple class resistance was not observed. All individuals carrying TDR mutations, except one case, were infected with HIV-1 sub-subtype F1. Although infection with non-B HIV variants was associated with a lower prevalence of TDR [Chilton et al., 2008], a recent study reported an increased rate of resistance for the F1 subtype, especially in South American subjects, infected by homosexual route, but also, at a lower frequency, in ART naïve Romanian subjects infected heterosexually [Franzetti et al., 2012]. It is worth mentioning that, although subtype F is still predominant, other HIV-1 clades are increasingly identified, especially among individuals infected recently. A previous study has already demonstrated the spread of subtype C among MSMs in Romania [Paraschiv et al., 2012].

The decreasing trend of TDR rates reflects the higher efficacy of HAART used in the later years compared to dual- and monotherapy used earlier in the Romanian epidemic. Specific ARV treatment was introduced in Romania in 1996 as dual therapy (based on two NRTI—AZT, d4T, or 3TC) and in 1998 as triple therapy (mainly two NRTI+ 1 PI/r). Romania was the first country in Central and Eastern Europe to provide free access to treatment and care for HIV/AIDS. The introduction of ART dual- and triple therapy has resulted in a substantial increase of the mean survival period from an average of 16.6 months between 1990 and 1995 to 34 and 42 months in 1997 and 1999, respectively and to an average of 81 months between 2008 and 2011. At the end of 2011, 7,536 patients (79.9% of the total number of those in active records) were receiving state of the art ART (<http://www.cnlas.ro>). All these improved treatment strategies, together with the prompt management of treatment failure with new active ARV are accountable for suppression of viral replication in the treated population, and subsequently for the absence of TDR in newly infected individuals. This is supported by the observation that no TDR associated mutations were found in the subset of recent infections with HIV, although this fact need further confirmation by larger epidemiological studies.

Evidence of a stable or declining rate of transmitted drug resistance was already reported in several other European studies. The SPREAD Programme, the most representative surveillance program of TDR in Europe, recorded a stabilizing trend over time (2002–2006) in the overall transmitted resistance, whereas NNRTI and PI-associated mutations seem to decrease over the calendar years [Vercauteren et al., 2009]. A decline in transmitted drug-resistant HIV-1 rates was also described in Spain (from 11.3% in 2004–2006 to 8.4% in 2007–2010) [Yebra et al., 2011] and the United Kingdom (in patients with recent infection, from 10.5% in 1996–1997 to 4% in 2004) [UK Collaborative Group on HIV Drug Resistance, 2007]. A WHO survey [WHO HIV Drug Resistance Report, 2012] conducted in 20 low and middle income countries showed an overall transmitted drug resistance rate of 3.7%. Low rates of TDR are also reported in sub-Saharan Africa (from 1.1% in South Africa, to 12.3% in Uganda) despite the wider use of ART [Hamers et al., 2011].

Patients with transmitted drug resistance presented a significant lower viral load compared to those infected with wild-type HIV, suggesting a possible loss of viral fitness induced by resistance mutations [Martinez-Picado et al., 1999]. An in vivo fitness cost associated with the M184V/I mutation has been previously shown in patients naive to antiretroviral therapy [Harrison et al., 2010].

Out of the nine patients with TDR, five were exposed to HIV-1 through parenteral route, most likely in early childhood, during the nosocomial pediatric HIV Romanian epidemic. Three of these patients with TDR (aged 14, 15, and 8 years at the time of HIV testing), were diagnosed in 1999, 3 years after the introduction of ARV dual therapy in Romania, reflecting the low efficacy of dual therapy in the suppression of resistance. The other two cases were diagnosed in 2008 and 2010 at the age of 27 years and 21 years, respectively; in these cases the possibility of a superinfection with a resistant HIV-1 strain is suggested, probably by a sexual route. Among adults, heterosexual transmission is the predominant route of HIV infection (65% of the new cases).

Injection drug users (IDUs) have no transmitted drug resistance mutations, but seem to form an epidemiologically related cluster, based on the viral strain analysis. This most probably reflects the new introduction of this route of HIV transmission in Romania, in the last years. Indeed, in 2011, out of the 619 newly diagnosed HIV/AIDS cases, 113 (18.2%) were intravenous drug users (<http://www.cnlas.ro>), a marked increase compared to 2010—12 cases (2.72%) out of 440 new infections or to 2009—5 cases (1.16%) out of the total of 428 new infections. This is attributable to a recent rise in the combined use of opioids with new psychotropic substances, allowing increased injecting frequency (6–10 injections/ day vs. 3–5 injections/day for heroin only). Several neighboring countries (Bulgaria, Greece) together with other European countries such as Lithuania, Italy, and Luxembourg reported increases in HIV case reports or prevalence among IDUs, during 2010–2011, signaling the potential emergence of a new wave of HIV infections, similar to the outbreaks registered during 2000–2001 in the Baltic States (Estonia, Latvia, and Lithuania) [Joint United Nations Programme on HIV/AIDS, 2004].

Thymidine analogue resistance mutations (TAMs) were the most common type of TDR mutation detected in treatment-naïve, Romanian patients infected with HIV-1, a finding consistent with the results of the European TDR surveillance program [Vercauteren et al., 2009]. This fact can reflect the extensive use of zidovudine or stavudine in the pre-and early HAART era. The TAM-2 pathway was the better represented, in contrast with the available data obtained from subtype B strains [Yahi et al., 1999]. Type II TAMs cause lower levels of phenotypic and clinical resistance to the thymidine analogs and cross-resistance to abacavir (ABC), didanosine (ddI), and tenofovir (TDF), than do the type I TAMs [Shafer and Schapiro, 2008].

The next most frequent mutation was M184V, which confers high-level resistance to lamivudine and emtricitabine. The phenotypic and clinical significance of M184V is influenced by the presence or absence of other NRTI resistance mutations. Four TAMs in combination with M184V cause high-level resistance to both ABC and ddI [Whitcomb et al., 2003; Rhee et al., 2004; Lanier et al., 2004; Marcelin et al., 2005; Vermeiren et al., 2007].

All the studied HIV-1 strains carried minor mutations with high frequencies in the protease and RT genes. These mutations, often found at polymorphic positions, appear to have minimal effect on treatment outcomes. An optimum response to first-line regimens has been reported for patients with pretreatment drug resistance that received a combination of at least three fully active antiretroviral drugs, however a reduced increase in the CD4 cell number

was noted [Hamers et al., 2012]. Regardless, these minor mutations should be considered when assessing the possible impact on the therapy response as they have been reported to facilitate the emergence of major mutations [Montes et al., 2004; Vergne et al., 2006; Bennett et al., 2009]. In addition, differences in the sensitivity to antiretrovirals have been recorded in patients infected with non-B subtypes. The most common accessory PI resistance mutation observed in this study was M36I, and this has been reported to increase viral fitness and confer resistance to ritonavir and nelfinavir [Clemente et al., 2003; Johnson et al., 2008]. A high frequency of minor PI resistance mutations (M36I, L63P, and K20R/I) has been reported in newly diagnosed immigrants from Spain, infected with non-B subtypes [de Felipe et al., 2011]. More relevant to Romania, a high level of genetic diversity, with the frequency of PR polymorphisms reaching 61.5% and that of RT polymorphisms 34.8% [Bártolo et al., 2009], has been found among the HIV F1 isolates from Angola which are closely related to the Romanian epidemic. The E138A-RT mutation, a polymorphism that has been added recently to the list of mutations associated with decreased etravirine response [Tambuyzer et al., 2011] was recorded in the study group.

Phylogenetic analysis found that 5/6 sequences from intravenous drug users were clustered together. Using a conservative genetic distance threshold of 1%, four of these sequences formed a putative transmission cluster [Smith et al., 2009]. All of the members of this subtype F IDU cluster were diagnosed in the same period (2010–2011), and the monophyletic nature of the cluster reflects the possibility of a single source of transmission. However this cluster must be further investigated to support this conclusion. Similar data have been reported for clade-A HIV infection among IDUs in Saint Petersburg, Russia [Masharsky et al., 2010] and in Greece [Para-skevis et al., 2011].

It was also found that the subtype F sequences from subjects with an unknown or parenteral route of infection clustered separately from those with a sexual route of infection ($P = 0.031$ F-ST [Hudson et al., 1992], $P = < 0.05$ Slatkin–Maddison Test [Slatkin and Maddison, 1989]), suggesting that these might be separate epidemics.

The major limitation of this work is related to the restricted number of patients in the study group. Further and continuous TDR surveillance is necessary to gain more knowledge on the incidence and spread of TDR patterns in Romania, and to confirm the observed trend. Another limitation is the use of population sequencing, which underestimates the presence of drug resistance mutations in viral sub-populations below the detection limit [Gunthard et al., 1998; Schuurman et al., 2002; Halvas et al., 2006; Rozera et al., 2012]. Also, among antiretroviral-naïve chronically HIV positive patients, that were possibly diagnosed late during the evolution of HIV infection, reversion of the resistant strain to the wild-type phenotype may have occurred. Limited epidemiological data and the lack of identification and testing of all sexual contacts of those late presenters—considered as being infected as children during the Romanian pediatric HIV epidemics did not allow the investigation of a possible superinfection with HIV resistant strains.

In conclusion, in Romania transmitted HIV drug resistance decreased over time, following the general trend reported in Western Europe. A decrease frequency of TDR was also observed for all drug classes. The decline of primary HIV resistance might be related to the high efficacy of the HAART and to the increasing number of treated patients in virological success who have a low risk of transmission. The high frequencies of minor mutations in the protease and RT genes prompt to a careful evaluation of the long-term efficacy of future therapies. The recent increase in the number of HIV-1 infections involving other subtypes impose a continuous surveillance of the genetic composition of the epidemic.

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Fig. 1. Phylogenetic tree. A phylogenetic tree of all samples demonstrated grouping of all the sequences subtyped as F1 and a group of CRF sequences (SR14; SR31, SR44) clustering between the subtype B and subtype C sequences (black font lines). Within the subtype F sequences, a HIV transmission cluster (SR 53, 61, 63, 64), associated to injecting drug use was identified.

TABLE I**Baseline Characteristics of the Study Population**

	Total	With TDR	Wild type	<i>P</i>
Number of patients	61	9	52	
Males (n; %)	33; 54%	6; 66.6%	27; 51.9%	0.2
Median age in years (range)	28 (8–56)	21 (8–44)	28.5 (16–56)	0.06
Route of transmission (n; %)				
Sexual contact	32; 52.4%	3; 33.3%	29; 55.7%	0.5
Parenteral	18; 29.4%	5; 55.5%	13; 25%	0.2
Unknown	11; 18.3%	1; 11.1%	10; 19.2%	1
HIV variants (n; %)				
F1	49; 80.3%	8; 88.8%	41; 78.8%	1
Non-F1	12; 19.6%	1; 11.1%	11; 21.1%	1
Median CD4 count, cells/mm ³ (range)	277 (147–511)	277 (137–458)	275.5 (146–571)	0.4
CD4 count < 200 cells/mm ³	21	3	18	1
CD4 count > 500 cells/mm ³	15	1	14	0.6
Median HIV RNA, log ₁₀ copies/ml (range)	5.07 (4.3–5.3)	4.71 (4.2–5.5)	5.08 (4.3–5.6)	0.01
Year of HIV diagnosis (n; %)				
1997–2004	23; 37.7%	6; 26%	17; 73.9%	0.3
2005–2011	38; 62.29%	3; 7.89%	35; 92.1%	0.3
Status of HIV infection (n; %)				
Recent infection	5; 8.1%	0	5; 9.6%	1
Established infection	56; 91.8%	9; 100%	47; 90.3%	1

TABLE II

Transmitted Drug Resistance Mutations (TDRs) by Antiretroviral Drug Class

Patients	NRTIs	NNRTIs	PIs	Subtype
1	L210W	None	None	F1
2	M41L	None	None	F1
3	K219Q	None	None	F1
4	None	None	M46L	C
5	M184V	V179F, Y181C	None	F1
6	D67N, T69D, K70R, L74I, M184V, T215F, K219Q	None	L24I, M46I, I47V, F53L, I54V, V82A	F1
7	K70R	None	None	F1
8	D67N, K70R, T215F, K219Q	V106M, Y188C	None	F1
9	M184V	None	None	F1

NRTIs, nucleoside reverse-transcriptase inhibitors; NNRTIs, non-nucleoside reverse-transcriptase inhibitors; PIs, protease inhibitors.