Prevalence of Transmitted Drug-Resistance Mutations and Polymorphisms in HIV-1 Reverse Transcriptase, Protease, and gp41 Sequences Among Recent Seroconverters in Southern Poland

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Background: Monitoring of drug resistance-related mutations among patients with recent HIV-1 infection offers an opportunity to describe current patterns of transmitted drug resistance (TDR) mutations.

Material/Methods: Of 298 individuals newly diagnosed from March 2008 to February 2014 in southern Poland, 47 were deemed to have recent HIV-1 infection by the limiting antigen avidity immunoassay. Proviral DNA was amplified and sequenced in the reverse transcriptase, protease, and gp41 coding regions. Mutations were interpreted according to the Stanford Database algorithm and/or the International Antiviral Society USA guidelines. TDR mutations were defined according to the WHO surveillance list.

Results: Among 47 patients with recent HIV-1 infection only 1 (2%) had evidence of TDR mutation. No major resistance mutations were found, but the frequency of strains with ≥1 accessory resistance-associated mutations was high, at 98%. Accessory mutations were present in 11% of reverse transcriptase, 96% of protease, and 27% of gp41 sequences. Mean number of accessory resistance mutations in the reverse transcriptase and protease sequences was higher in viruses with no compensatory mutations in the gp41 HR2 domain than in strains with such mutations (p=0.031).

Conclusions: Despite the low prevalence of strains with TDR mutations, the frequency of accessory mutations was considerable, which may reflect the history of drug pressure among transmitters or natural viral genetic diversity, and may be relevant for future clinical outcomes. The accumulation of the accessory resistance mutations within the *pol* gene may restrict the occurrence of compensatory mutations related to enfuvirtide resistance or *vice versa*.

MeSH Keywords: Drug Resistance • HIV Fusion Inhibitors • HIV-1 • Protease Inhibitors • Reverse Transcriptase Inhibitors

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Background

Human immunodeficiency virus type 1 (HIV-1) strains with drug resistance mutations may exhibit different levels of resistance to antiretroviral agents, and the appearance of such mutations impairs the efficacy of combination antiretroviral therapy and limits future treatment options [1,2]. These mutations can arise spontaneously as a result of high HIV-1 genetic variability, and may be further selected by antiretrovirals that incompletely suppress viral replication [3–5].

Mutations associated with resistance to the majority of currently used drug classes are well described, and the updated list of such mutations is published annually in the International Antiviral Society USA (IAS-USA) guidelines [6]. As it became apparent that HIV-1 strains harboring drug resistance mutations can be transmitted to new hosts, efforts to control and reduce the propagation of drug resistance have been initiated, including the ongoing monitoring of the transmitted drug resistance (TDR) among therapy-naïve persons with recent or newly diagnosed HIV-1 infection [7–11]. To ensure the comparability of TDR's prevalence data from different studies, a standard World Health Organization list of resistance mutations for surveillance of transmitted drug resistance has been developed according to clearly defined criteria [12]. This WHO surveillance list includes major resistance mutations and non-polymorphic mutations associated with selective pressure of reverse transcriptase and protease inhibitors.

In addition to the resistance to reverse transcriptase and protease inhibitors, there is a growing importance of resistance to the fusion inhibitor enfuvirtide (ENF) used in salvage therapy. ENF resistance is characterized by a low genetic barrier because phenotypic resistance develops with single-point mutations within the gp41 coding region [13–16]. The transmission of ENF-resistant HIV-1 variants to treatment-naïve patients has been documented [17–19], and such transmission is possible also in Poland, since there have been a number of ENF recipients in recent years [20]. Moreover, viral strains with mutations linked to ENF resistance could be imported from other countries where the number of ENF-experienced patients was higher and resistance to ENF was observed [17,19,21]. However, the prevalence of such resistance to fusion inhibitor among persons with recent HIV-1 infection in Poland is unknown.

The genome of HIV-1 strains transmitted to new individuals may contain not only major drug resistance mutations, but also treatment-associated accessory (compensatory) polymorphisms or mutations compensating for losses in viral fitness related to the emergence of major drug resistance mutations [11,22,23]. Such polymorphic accessory changes are excluded from the TDR surveillance recommendations, as they may reflect either a transmission from a person harboring HIV-1

strains with previous drug-selective pressure experience, or may appear as a consequence of wild-type virus genetic diversity. Nevertheless, it was recently indicated that the higher number of polymorphic and compensatory mutations in the protease coding region correlates with increased estimated viral fitness, higher viral load, and lower $CD4^+$ T cell count in newly-diagnosed treatment-naïve patients, irrespectively of the occurrence of TDR mutations [24]. Furthermore, the occurrence of multiple polymorphisms in the reverse transcriptase was associated with an increased risk of virological failure in patients with first-line therapy containing non-nucleoside reverse transcriptase inhibitors [25]. Hence, it is likely that HIV-1 strains with fitness improved due to the presence of such polymorphic changes will be more virulent at a population level [24].

The objective of the present study was to investigate the prevalence of reverse transcriptase, protease, and fusion inhibitors TDR mutations, as well as the frequency of compensatory mutations and natural polymorphisms, among therapy-naïve persons with likely recent HIV-1 infection diagnosed in Poland between 2008 and 2014. Selection of patients with recent infection ensures the most consistent description of the current HIV-1 mutation patterns and their possible changes over time.

Material and Methods

Patients and recent infection testing

Quantitative limiting antigen avidity enzyme immunoassay (Sedia™ HIV-1 LAg-Avidity EIA, Sedia BioSciences Corporation, Portland, Oregon, USA) was used to distinguish recent from long-term HIV-1 infection. Testing was performed according to the manufacturer's procedure and package insert [26]. Individuals with specimens with normalized optical density values (ODn) ≤1.5 in a confirmatory testing were considered to be recently infected. Such a recommended assay's ODn cut-off value (=1.5) used to classify recent and long-term infections corresponds to a mean duration of recent infection of 130 days (95% CI: 118–142) since seroconversion [27], with the false-recent ratio as low as 1.3% (0.3–3.2) [28].

The assay was performed for 298 consecutive patients. The inclusion criteria were: (1) being newly diagnosed with HIV infection, with no clinical AIDS (indicator disease) at diagnosis; (2) presenting at 1 of 4 centers for HIV Diagnostics and Therapy for AIDS in Poland located in Chorzow, Krakow, Lodz, and Wroclaw, during the center's enrolment period, between March 2008 and February 2014; (3) having their blood sample collected at first presentation; and (4) providing informed consent to participate in the study. Only patients with recent infection according to the Sedia™ HIV-1 LAg-Avidity EIA assay (n=47) were enrolled in the genetic study (Table 1).

Table 1. Characteristics of patients with new HIV-1 diagnosis, Poland, 2008–2014.

Characteristics	Patients with recent HIV-1 infection N=47 (15.8%)		Patients with long-term HIV-1 infection N=251 (84.2%)		P
	n	%	n[a]	%	
Sex					
Female	3	6.4	23	9.2	0.779[b]
Male	44	93.6	226	90.0	
Age at HIV diagnosis					
Median	29.0		29.0		0.238[c]
Interquartile range	24.0–33.0		25.0–35.0		
<30	28	59.6	127	50.6	0.340[b]
≥30	19	40.4	121	48.2	
City of HIV diagnosis					
Chorzow	17	36.2	118	47.0	0.011[d]
Krakow	3	6.4	39	15.5	
Lodz	5	10.6	34	13.6	
Wroclaw	22	46.8	60	23.9	
Year of HIV diagnosis					
2008	7	14.9	65	25.9	0.077[d]
2009	9	19.1	71	28.3	
2010	6	12.8	34	13.6	
2011	0	0	7	2.8	
2012	10	21.3	27	10.8	
2013	14	29.8	44	17.5	
2014	1	2.1	3	1.2	
Self-reported transmission route					
Sex between men (MSM)	36	76.6	165	65.7	0.673[d]
Sex between women and men (HET)	7	14.9	46	18.3	
Sex between men or women and men (BI)	2	4.3	10	4.0	
Injecting drug use	1	2.1	15	6.0	
Nosocomial	0	0	1	0.4	
Other/Unknown	1	2.1	14	5.6	

[a] – Sex was not known for 2, and age for 3 patients with long-term HIV-1 infection; [b] – two-tailed Fisher's exact test; [c] – Mann-Whitney *U* test; [d] – Pearson's chi-square test.

The research was approved by the National Institute of Public Health – National Institute of Hygiene Bioethics Committee, Poland (no. 3/2007). Written informed consent was required in order to be recruited into the study. All collected data were anonymous and coded.

Genotypic drug resistance testing

Anticoagulated venous blood samples were collected at first clinical presentation and were stored at –80°C until genomic DNA extractions with QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) were performed. In order to recognize protease and reverse transcriptase inhibitors resistance mutations, proviral HIV-1 DNA was amplified in 2 fragments of the *pol* gene, including entire protease and the 5' part of the reverse transcriptase coding region. Nested polymerase chain reactions (nested-PCR) were performed using primer pairs and cycling parameters described previously [29,30]. To examine viral resistance to fusion inhibitor (enfuvirtide, ENF), a fragment of the *env* gp41 coding region with both heptad repeat domains (HR1 and HR2) was amplified. In the first step of the nested-PCR, fragment spanning nucleotides 6201-9089 was amplified using E00-F and E01-R as outer primers [31], with the following amplification conditions: an initial denaturation at 94°C/7 minutes, followed by 40 cycles of 94°C/40 seconds, 51°C/40 seconds, and 72°C/3 minutes, with the final extension at 72°C/7 minutes, in a final volume of 50 µl. In the next step, inner primers E170-F and E25-R were used to amplify the 567 nucleotide fragment of gp41 (7799–8365) [31,32]. Amplification conditions for inner primer pairs were: 94°C/5 minutes, followed by 35 cycles of 94°C/35 seconds, 55°C/35 seconds, and 72°C/90 seconds, with the final extension at 72°C/5 minutes, in a final volume of 50 µl. All purified nested-PCR products were further subjected to the sequencing analysis with the ABI Prism Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the primers used in the inner step of nested-PCR. Sequences of both strands were determined separately using a 96-capillary 3730xl DNA Analyzer (Applied Biosystems, USA). The sequencing was successful for 45 amplified samples of reverse transcriptase and protease, and for 44 gp41 samples. The obtained sequences were manually checked and trimmed to remove primers, resulting with fragments spanning: 1) nucleotides 2231–2560 (including codons 1–99 for protease), 2) nucleotides 2565–3299 (codons 6-250 for reverse transcriptase), and 3) nucleotides 7817–8345 (codons 21–196 for gp41). All nucleotide positions are indicated according to the numbering positions of HIV-1 HXB2 (GenBank accession number: K03455).

HIV-1 subtypes were determined with the National Center for Biotechnology Information Genotyping Tool (NCBI Genotyping Tool) [33], and confirmed by the phylogenetic analysis with the set of reference sequences obtained from the Los Alamos National Laboratory HIV Sequence Database using the MEGA v5.0 [34]. All sequences were analyzed for the presence of hypermutations using HYPERMUT software v2.0 [35].

The levels of viral resistance to protease and reverse transcriptase inhibitors were predicted by the HIVdb: Genotypic Resistance Interpretation Algorithm available at the Stanford University HIV Drug Resistance Database [36]. The same software was used to identify all drug resistance mutations, as well as natural polymorphisms in the protease and reverse transcriptase coding regions. Evidence of transmitted HIV-1 drug resistance was defined as the presence of at least 1 drug resistance mutation placed on the WHO list for surveillance of transmitted drug-resistant HIV-1 strains [12]. We considered also all mutations and polymorphisms recognized in the protease and reverse transcriptase and gp41 coding regions, included in the Stanford University HIV Drug Resistance Database (2015 update, or 2008 for fusion inhibitors), and/or published in the recent International Antiviral Society USA report on drug-resistance mutations (IAS-USA, 2014 update) [6,36].

Sequence data

The HIV-1 nucleotide sequences obtained from the individuals with recent infection have been deposited in GenBank. The accession numbers for reverse transcriptase sequences were: JF837540, JF837545, JF837547, JF837554, JF837556, JF837558, JF837560, JF837565, JF837567-JF837569, JF837574, JF837580-JF837583, JF837586, KT324513-KT324540, for protease sequences: JN811569, JN811574, JN811576, JN811583, JN811585, JN811587, JN811589, JN811594-JN811597, JN811602, JN811608-JN811611, JN811614, KT324353-KT324380, and KT324381-KT324424 for gp41 sequences.

Statistical analysis

The *a priori* sample size calculation was performed in view of calculating the prevalence of TDR mutations included in the WHO TDR surveillance protocol using G*Power version 3.1.9.2 [37]. Based on prior knowledge, we expected the percent of strains harboring 1 of these mutations to vary around 9% [38,39]. We assumed the sample size needed to estimate the proportion greater than 0, with the power of 80% and the confidence level 5%. With the true proportion varying from 2% to 10%, the corresponding required sample size is 16 to 80. Further, based on our prior work, we expected approximately 40% of newly diagnosed cases to be recent infections [40]. Thus, we aimed at recruiting 200–300 patients with new diagnoses.

However, in this study the proportion of recent infections was lower than expected and the final sample size was 47. Post hoc analysis revealed that with this sample size, the power to detect that a proportion was higher than 0 was 61.3%.

The trend in the number of resistance-related mutations in protease, reverse transcriptase, and gp41 sequences over time was investigated with normal regression. The nonparametric Mann-Whitney *U* test was used to compare mean numbers of mutations in protease and reverse transcriptase between patients infected with HIV-1 strains containing and lacking accessory mutations in gp41 HR2 region. Comparisons between patients with recent and long-standing HIV-1 infection were performed with the Pearson's chi-square or 2-tailed Fisher's exact test for categorical variables, or the Mann-Whitney *U* test for analysis of continuous variables, as appropriate. Analyses were performed using STATISTICA v10.0 (StatSoft, Warsaw, Poland).

Results

TDR mutations and resistance-associated polymorphisms among patients with recent HIV-1 infection

Of the 298 patients recruited in the years 2008–2014 and tested with the Sedia™ HIV-1 LAg-Avidity EIA, 47 (15.8%) were classified as individuals with recent infection and were subjected to the drug-resistance analysis. The characteristics of patients are presented in Table 1. All study participants with recent infection were of Polish origin. The majority of them reported sexual contacts as a transmission route (45; 95.7%), with men who acquired infection through sex with men (MSM) accounting for 76.6% of the studied patients. The predominating HIV-1 genetic variant was subtype B, which was identified in 44 patients (93.6%); the remaining 3 persons were infected with subtypes A, F, and a putative unique recombinant form with sequences of subtypes A, B, and D in 3 different genomic regions.

Out of the list of TDR mutations recommended for surveillance by the WHO, the only mutation found within the group of recently infected individuals was a minor protease inhibitors (PIs) resistance mutation – G73S. This mutation was detected in a single subtype B sequence (2%, 95% CI 0.1–11.3%) obtained from a patient infected in 2010. However, no resistance-related mutation was found in the reverse transcriptase and gp41 sequences derived from the patient.

Mutations not included in the WHO TDR surveillance list but present in the current IAS-USA guidelines and/or at Stanford University HIV Drug Resistance Database were found in a total of 46 patients, and the prevalence of strains with at least 1 of such drug resistance-associated mutations and polymorphisms, mainly accessory, in any region of the viral genome (reverse transcriptase, protease or gp41) was 98%. These drug resistance-related mutations occurred in 11% of the reverse transcriptase samples, 96% of protease samples, and 27% gp41 sequences. According to the HIVdb: Genotypic Resistance

Interpretation Algorithm, altogether, resistance of different levels to reverse transcriptase inhibitors and protease inhibitors was detected for 4 (9%) HIV-1 strains. There were no HIV-1 strains with cross-resistance to different drug classes among patients with recent HIV-1 infection.

Mutations in the reverse transcriptase coding region according to IAS-USA guidelines and/or Stanford University HIV Drug Resistance Database

Among 111 mutations in 64 positions of the amplified region of reverse transcriptase sequences, only one accessory nucleoside reverse transcriptase inhibitors (NRTIs)-resistance-related mutation (V118I) was detected in a single sample (Figure 1). In addition, 3 mutations described as non-nucleoside reverse transcriptase inhibitors (NNRTI)-selected were identified in this gene. Polymorphic accessory mutation V106I was found in 2 sequences, while E138A and non-polymorphic E138K were present in a single specimen, each. All of these reverse transcriptase resistance-related mutations occurred separately in 11% of samples, and there was no significant trend in their occurrence over time.

Analysis of the reverse transcriptase sequences with the HIVdb: Genotypic Resistance Interpretation Algorithm, performed to assess the potential impact of identified mutations on the level of drug resistance, revealed susceptibility to all NRTIs, for all sequences examined. Two subtype B strains (4%) exhibited different levels of resistance to NNRTIs attributed to the presence of mutation in the 138 codon of reverse transcriptase. In 1 HIV-1 sequence obtained from a patient diagnosed in 2010, mutation E138K was related to intermediate level of resistance to rilpivirine (RPV) as well as potential low-level to efavirenz (EFV), etravirine (ETR), and nevirapine (NVP). The E138A mutation in the second sequence derived from a patient diagnosed in 2012 was associated with low-level resistance to RPV and potential low-level resistance to ETR.

Mutations in the protease coding region according to IAS-USA guidelines and/or Stanford University HIV Drug Resistance Database

Among 68 mutations identified in 35 codons of the entire protease coding region sequences, 21 mutations in 16 positions were related to PIs resistance (Figure 1). These positions were: 10, 16, 20, 33, 36, 60, 62-64, 69, 71, 73, 74, 77, 89, and 93. The most frequent were accessory mutations I62V, L63P, and I93L, each occurring in more than 50% of the sequences analyzed. Subsequently, M36I and V77I mutations were present in over 20% of the protease samples each. More than 10% of protease sequences harbored 1 of the following mutations: L10I, G16E, M36L, I64L, I64V, and A71V; whereas over 5% contained D60E, A71T or L89M. The least frequent (<5%) mutations were
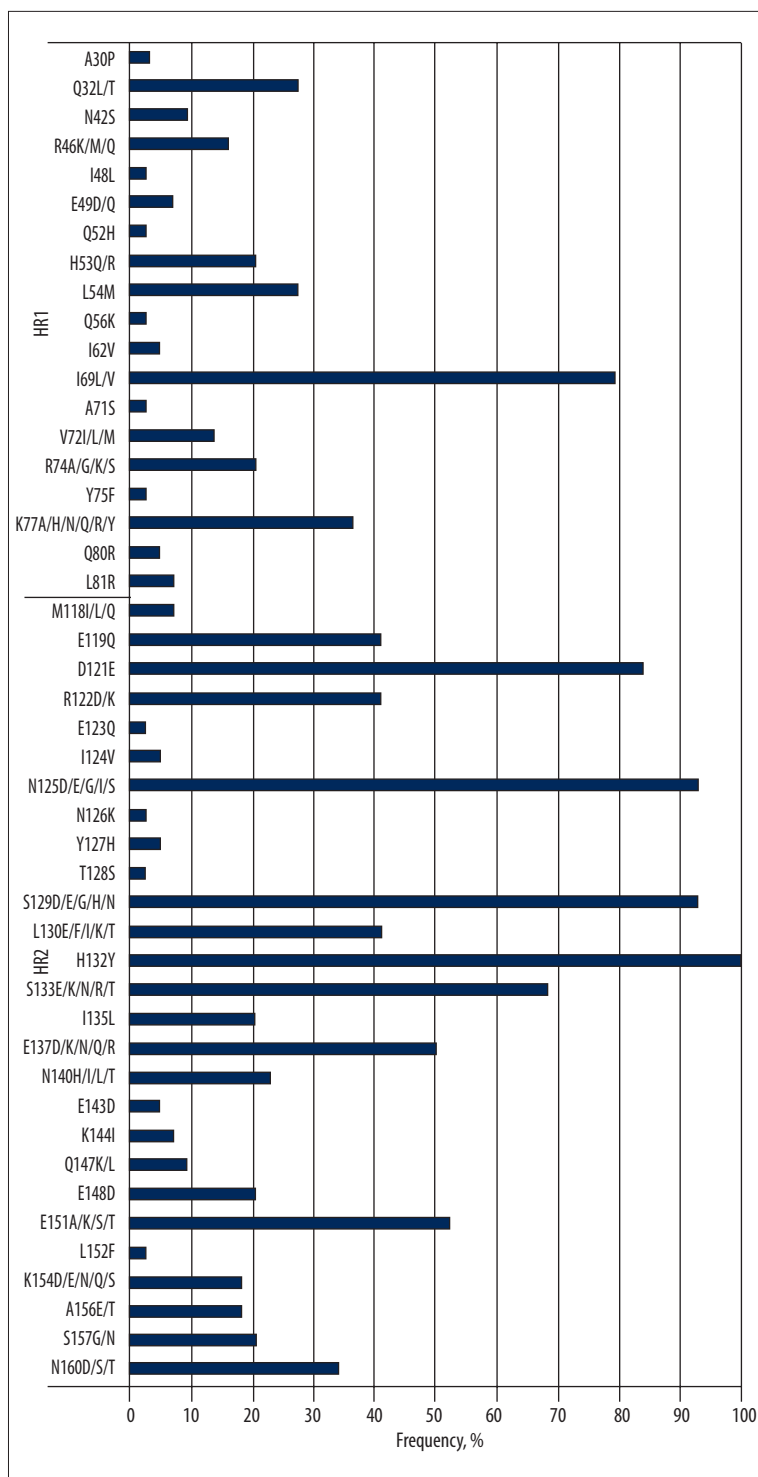
Figure 1. Representation of resistance-associated mutations and polymorphisms in the reverse transcriptase, protease, and gp41, listed in the IAS-USA guidelines (2014 update) and/or in Stanford University HIV Drug Resistance Database *http://hivdb.stanford.edu/DR/* (2015 update, or 2008 for fusion inhibitors resistance-related mutations) among patients with recent HIV-1 infection diagnosed in the years 2008–2014. NRTI – nucleoside reverse transcriptase inhibitors, NNRTI – non-nucleoside reverse transcriptase inhibitors, PI – protease inhibitors, FI – fusion inhibitors.

Figure 2. Number of protease sequences with different number of minor protease inhibitors resistance mutations.

Figure 3. Trends in the number of minor mutations related to protease inhibitors resistance over time.

K20R and L33V, each present in 2 specimens, as well as L10V, L33I, H69K, G73S, and T74S, which occurred in a single protease sequence each. The number of minor mutations related to PIs resistance in a separate protease sequence ranged from 0 to 6 (Figure 2). The most abundant were sequences with 3 and 4 PIs resistance-related mutations (10 and 15

protease sequences, respectively), accounting for over 50% of samples. On the other hand, samples containing 0, 1, and 6 of such mutations were the least frequent, and together constituted 11% of the protease sequences. The remaining 33% of sequences harbored 2 or 5 PIs accessory resistance-related

Figure 4. Representation of mutations and polymorphisms within the heptad repeat 1 and 2 (HR1, HR2) domains of gp41 coding region among patients with recent HIV-1 infection diagnosed in the years 2008–2014.

mutations. At least 1 accessory resistance-associated mutation was present in 96% of protease samples. The number of minor mutations related to PIs resistance displayed an increasing trend over the study period (Figure 3), but this trend was not statistically significant.

For 2 protease sequences (4%), resistance to PIs was recognized according to the analysis with the HIVdb: Genotypic Resistance Interpretation Algorithm. In a single subtype B sample from 2010, protease mutations L10I and G73S were responsible for low-level resistance to nelfinavir (NFV) and saquinavir (SQV), and potential low-level resistance to atazanavir (ATV) and

indinavir (IDV). The second sequence from 2013 represented subtype F and contained mutations L10V and T74S related to the low level of resistance to NFV.

Mutations in the gp41 coding region according to IAS-USA guidelines and/or Stanford University HIV Drug Resistance Database

In the amplified region of gp41 sequences there were 201 mutations in 90 positions. No mutation related to fusion inhibitor (FI) resistance within the HR1 domain of gp41 coding region (codons 36-45, crucial for ENF resistance) was found. The only change found in this region was N42S, which is a common polymorphism not associated with a reduced enfuvirtide susceptibility [13]. This polymorphism was present in 4 (9%) gp41 sequences. In turn, screening of the HR2 domain revealed 2 compensatory mutations (Figure 1); these were N126K, detected in a single specimen, and E137K observed in 25% of the gp41 sequences. Mutations N126K and E137K did not occur simultaneously in any sequence, but a single subtype A sample contained E137K and N42S together. Strains with compensatory mutations in gp41 HR2 constituted 27% of samples, and no significant trend in their occurrence over time was observed.

Frequencies of all mutations and polymorphisms found within the entire HR1 (codons 29–82) and HR2 (codons 117–162) domains of gp41 coding region are presented in Figure 4. Among these, I69V, D121E, N125D, and H132Y were the most frequent, being present in more than 50% of specimens. In 3 additional HR2 positions (129, 133, 151) the frequency of overall nonsynonymous changes, but no single mutation exceeded 50%.

Association between number of accessory mutations in gp41 HR2 and reverse transcriptase/protease coding regions

We found an association between the mean number of drug resistance-associated mutations in the reverse transcriptase and protease sequences together and the presence of compensatory mutation in the HR2 domain of gp41 coding region. HIV-1 strains with N126K and E137K polymorphisms in the gp41 HR2 region had significantly fewer accessory mutations in the reverse transcriptase and protease sequences than those with no HR2 compensatory mutations (2.8±1.2 vs. 3.7±1.3; p=0.031). This correlation was also found for accessory resistance mutations in protease sequences alone; among HIV-1 strains with N126K and E137K polymorphisms, the mean number of accessory mutations in protease was estimated to be 2.7±1.2, and 3.7±1.4 in viruses with no compensatory mutations in the HR2 domain (p=0.025). The presence of N126K and E137K was not linked to the number of all mutations detected in the reverse transcriptase and protease sequences, neither combined

(p=0.573) nor separately (p=0.631 for reverse transcriptase, and p=0.351 for protease sequences).

Discussion

Our study demonstrates a low rate of TDR among persons with likely recent HIV-1 infection diagnosed in southern Poland. Only 1 sample (2%) contained recognized TDR mutation. However, variants exhibiting from potential-low to intermediate level resistance to antiretroviral drugs (NNRTIs or PIs), according to HIVdb Genotypic Resistance Algorithm, were as frequent as 9%. There were no strains with major NRTIs, NNRTIs, PIs, or FI resistance mutations, but viruses with at least 1 accessory mutation in any region of the viral genome (reverse transcriptase, protease or gp41) were found in 98% of patients.

The only TDR mutation found in the current study was a minor G73S mutation related to PIs resistance, present in the subtype B sample. The prevalence of strains with TDR mutations in the group of patients with recent HIV-1 infection identified with the limiting antigen avidity EIA in the years 2008–2014 was 2%. A higher frequency of strains with TDR mutations (9%) was observed in the study performed among Polish antiretroviral-therapy-naïve patients, newly diagnosed with HIV-1 infection in the years 2008–2013, including both recent and long-standing infections [38]. Interestingly, a higher value (11.3%) was also established in our previous study assessing TDR among recently infected patients in 2008–2010, in which BED-EIA HIV-1 assay was used to classify infection as recent [39]. As the BED assay has higher false-recent ratio than the LAg-Avidity EIA assay used in the current study [28], the previous study likely also included individuals with long-standing infection. Although some authors found that the transmitted drug-resistant strains may persist for a considerable time during long-lasting infection among therapy-naïve persons [41–44], in the absence of antiretrovirals, HIV-1 strains with drug resistance mutations transmitted to a new host tend to revert to the wild-type or can be replaced by atypical variants with improved viral fitness over the time of infection [45–47]. Thus the ability to detect TDR mutations is likely compromised among persons with long-term infection, suggesting even higher level of TDR at the time of infection. Consequently, we hypothesize that our current results indicate a decreasing trend in TDR. Stable or decreasing trends in the frequency of transmitted drug-resistance mutations was also observed in other studies [38,48,49]. A possible explanation for the reduction of TDR prevalence may be the increasing proportion of patients achieving treatment success in terms of controlling viral load [50–52].

Although TDR mutations in the present study were uncommon, and there were no strains with major NRTIs, NNRTIs, PIs, or FI

resistance mutations, the search for mutations not included in the WHO TDR surveillance algorithm but present in the current IAS-USA list and/or at Stanford University HIV Drug Resistance Database revealed additional drug resistance-related mutations and polymorphisms in the reverse transcriptase, protease, and gp41 sequences. Such accessory mutations harbored by strains from persons with recent HIV-1 infection usually do not reflect the transmitted drug resistance, but may represent natural HIV-1 genetic variability with possible clinical implications [11,24,25]. In the current study, viruses with such accessory mutations were present in 98% of patients. Single accessory resistance mutations were present in over 10% (n=5) of reverse transcriptase sequences and over 25% (n=12) of gp41 samples. All but 2 protease sequences harbored at least 1 minor PIs resistance mutation, but over 50% (n=25) of protease samples contained 3 or 4 such mutations.

Among the reverse transcriptase sequences, there was 1 subtype B sample with NRTIs resistance-related accessory mutation, namely V118I, commonly accompanying thymidine analogue mutations type I (TAMs). The same strain harbored unusual mutation L210M, which was shown to be a marker of the presence of TDR minority species [53]. Coexistence of V118I and L210M, in the absence of TAMs, may suggest unrecognized transmission of drug-resistant virus either because of infection by minority drug-resistant variants or reversion of major drug resistance mutations (TAMs). Apart from L210M, another mutation predicting the presence of TDR minority species in patients with no evidence of drug resistance according to population sequencing, T69S [53], was detected in another subtype B sample. Another 2 reverse transcriptase subtype B sequences contained V106I mutation, reported as an accessory NNRTI-selected polymorphism, but when present without V179D, it probably has no effect on NNRTI susceptibility [36,54]. In another 2 reverse transcriptase samples of subtype B (4%), mutations in the codon 138 (E138A and E138K) were detected. The frequency of these mutations was similar to that observed in a recent survey performed in Poland on different population, in which samples containing E138A and E138K together accounted for 4.92% of the studied cases, and E138A alone was the most prevalent NNRTIs resistance mutation [38]. Although these mutations are considered as major changes associated with a second-generation NNRTIs (rilpivirine and etravirine) resistance [6,55], they are not included in the WHO TDR surveillance list, and thus were not taken into account in the calculation of TDR mutation prevalence. This suggests that the prevalence of strains with TDR mutations of clinical importance may in fact be higher than indicated by the WHO-proposed surveillance scheme.

Of note, in our former research, the only mutation linked to the NNRTIs resistance was K103N, responsible for the resistance to older drugs such as efavirenz and nevirapine, while mutations E138A/K, detected in the current study, impair the susceptibility to novel compounds from this class of antivirals, suggesting an important shift in mutation patterns influencing selection of first-line therapy.

Analysis of the protease sequences revealed that there were no major PIs resistance-related mutations, but over 70% of examined samples contained 3 to 6 minor PIs resistance mutations, and only in 2 specimens were such mutations absent. Minor mutations are described as PI-selected accessory or compensatory changes that improve HIV-1 replication fitness when present along with major PIs resistance mutations. Apart from being associated with therapy experience, they may also reflect natural variability of wild-type HIV-1 strains [6,11,36,56,57]. Higher number of these mutations was correlated with higher viral load, lower CD4+ T cell count, and improved estimated fitness in therapy-naïve individuals, suggesting the possibility of increased virulence [24]. Among 21 minor PIs resistance mutations detected in the study, 3 are known to be polymorphic or consensus in most non-B subtypes protease sequences [36,56,58]. These were M36I, T74S, and L89M, identified in at least 1 of non-B subtype sample. Indeed, 2 out of 12 M36I and 2 out of 3 L89M mutations were detected in subtype A and F sequences, while T74S was present exclusively in subtype F sample.

In the previous studies performed among ENF-naïve persons, the frequency of HIV-1 strains with fusion inhibitor resistance mutations located in the HR1 domain of gp41, after adjusting to current Stanford Database and/or IAS-USA guidelines, ranged from 1.8% to 16.7% [17,19,21,59,60]. In the current study, no enfuvirtide resistance mutation within the HR1 domain of gp41 coding region was detected. It can be expected that patients with multiple virological failure and coexisting resistance to other drug classes are the source of strains with ENF resistance mutations. Therefore, the lack of viruses carrying enfuvirtide resistance mutations in the HR1 region may indicate that such heavily therapy-experienced patients presumably are not a significant group of onward transmitters in Poland. This is in line with the observed low prevalence of viruses with TDR mutations, and the absence of strains with coincident resistance to 2 or more drug classes. The N42S polymorphism in HR1 region, previously associated with increased susceptibility to enfuvirtide, was identified in 9% of samples. The rate of detection of this polymorphism in other groups of ENF-naïve patients was greater (15–67%), reaching significantly higher values in groups with considerable proportion of infections with non-B subtypes [13,21,60–63]. In turn, the frequency of N126K and E137K compensatory mutations in the HR2 domain observed in the present report was slightly higher than in other studies (27% vs. 20–22%), and similarly to other studies, the frequency of E137K was higher than that of N126K mutation (25% vs. 2%) [60,62]. Polymorphisms in

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the HR2 region (N126K, E137K, and S138A) may increase viral fitness impaired due to the presence of major resistance mutations in HR1 domain by facilitating more stable interaction between HR2 and mutated HR1, which is necessary for fusion and viral entry into host cell [14,64,65]. For this reason, the high frequency of viruses with baseline N126K or E137K mutations observed in this study deserves attention, since such strains potentially will have shorter evolutionary pathways to develop ENF resistance without pronounced fitness loss.

Additionally, we found that a higher mean number of accessory resistance mutations in the reverse transcriptase and protease sequences was associated with the absence of compensatory mutations in the gp41 HR2 domain related to ENF resistance. This may suggest that accumulation of the accessory resistance mutations within the *pol* gene restricts the occurrence of compensatory mutations in the gp41 HR2, or an inverse association is possible. On the other hand, it was previously described that viral strains bearing multiple NRTIs, NNRTIs, and PIs resistance mutations demonstrated increased polymorphisms in the HR1 domain [66].

In our study we were limited by the small number of samples, which impacted the possibility of detecting trends in TDR. However, we purposefully included only individuals diagnosed with recent HIV infection, in order to be able to clearly identify the time of the transmission event. Next, it should be noted that although the LAg-Avidity EIA used here to recognize infection as recent has very low false-recent ratio [28], there is still the likelihood of false classification of individuals with long-standing HIV-1 infection as those with recent infection. Such a misclassification may impact the estimation of true TDR prevalence, but the size of this impact is supposed to be smaller than in studies performed among patients with new HIV-1 diagnosis.

Conclusions

Although the prevalence of strains with TDR mutations among persons with recent HIV-1 infection diagnosed in the years 2008–2014 was low, the occurrence of highly prevalent viruses with accessory resistance mutations may lead to more rapid development of drug-resistant strains with replicative fitness comparable to that of wild-type virus, and subsequently facilitated resistance development may compromise the long-term benefits of antiretroviral treatment.

Acknowledgments

Conflict of interest

Appendix

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