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Short Communication

Characterization of Drug-Resistance Mutations in HIV Type 1 Isolates from Drug-Naive and ARV-Treated Patients in Bulgaria

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Abstract

Little information is available about the prevalence of resistance mutations to reverse transcriptase (RT) and protease (PR) inhibitors of HIV-1, after the introduction of antiretroviral treatment in Bulgaria. To fill this gap, we analyzed 80 plasma samples from HIV-1-infected Bulgarian patients, 22 naive at antiretroviral treatment (ARV) and 58 ARV experienced. The subtypes B and A resulted in the two most prevalent (41 patients and 18 patients, respectively). The proportion of subtype B among naive and treated patients was similar in each group (57% vs. 47%, $p = 0.62$), while a major proportion of subtypes A was present in drug-naive patients rather than in treated patients [8/22 (36.4%) vs. 10/58 (17.2%), $p = 0.08$]. Two (9.1%) naive patients and 40 (70.1%) drug-experienced patients had viruses carrying at least one mutation conferring resistance to ARV drugs. Of 57 patients having experience with nucleoside reverse transcriptase inhibitors (NRTI), 32 (56.1%) had NRTI resistance mutations; 8/14 (57.2%) patients having experience with non-NRTI (NNRTI) had viruses carrying NNRTI resistance mutations; and 21/46 (45.7%) patients having experience with protease inhibitors (PI) had PI resistance mutations. The commonest resistance mutations resulted in the NRTI mutation M184V (42.1%) and the PI mutation L90M (24.1%). In conclusion, due to the detection of the substantial transmission of resistant variants to newly infected individuals, continuous surveillance is required, since greater access to highly active antiretroviral therapy (HAART) will be expected in Bulgaria. Furthermore, surveillance of PR and RT sequences is also convenient to monitor the introduction of nonsubtype B HIV-1 strains in Bulgaria.

In recent years significant progress has been made in the management of HIV infection. Central to these advances has been the development and clinical use of drugs for HIV-1 treatment. Highly active antiretroviral therapy (HAART) has achieved sustained suppression of HIV replication and reduced morbidity and mortality rates in patients with advanced HIV infection,[1] but the success of the treatment is frequently limited by low drug potency, poor adherence to treatment regimens, and, as an important cause, the appearance of HIV drug resistance.[2] Following the end of the cold war a decade ago, most Balkan countries have undergone significant social and political changes, and are currently going through a delicate transition to a market economy and democratic governance. These changes in social and cultural norms may result in an increase in factors enabling the spread of sexual transmitted infection (STI)/HIV/AIDS. Moreover, the central geographic location of Bulgaria (at the cross point between Western Europe, Eastern Europe, and the Middle East) makes it important to define both the resistance prevalence and evolution of HIV-1. In 2003 access to antiretroviral treatment was only partial and was available only in the capital city of Bulgaria (Sofia); a

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process of decentralization was successfully initiated in 2005 and, currently, three infectious diseases clinical centers in hospitals provide antiretroviral treatment in different Bulgarian cities.[3]

To date, no certain information is available on the prevalence of resistance to antiretroviral drugs among HIV-1-infected persons in Bulgaria. Since antiretroviral drugs will become more widely available in the very near future, data on drug resistance will be of the outmost importance. Therefore, the aim of the present study was to investigate the prevalence of mutations in the protease (PR) and reverse transcriptase (RT) associated with resistance to antiretroviral drugs in HIV-1-infected patients living in Bulgaria.

The study included HIV-1-infected patients under monitoring in different Bulgarian hospitals, with a first HIV^+ determination between 1986 and 2006; 22 patients were drug naive while 58 were ARV treatment experienced. At the time of the genotypic resistance test (performed between the years 2002 and 2006), all patients have failed at least one nucleoside reverse transcriptase inhibitor (NRTI)-containing regimen, 14 at least one NNRTI-containing regimen, and 46 at least one protease (PI)-containing regimen (30 failing at ritonavir-unboosted PIs and 16 at ritonavir-boosted PIs). Sequencing of PR and RT of the HIV-1 *pol* gene was performed in the National HIV Confirmatory Laboratory in Sofia, Bulgaria, by the Applied Biosystems Viroseq HIV-1 Genotyping System (Abbott, Wiesbaden, Germany) following the manufacturer's instructions. Drug resistance mutations in both genes (PR ad RT), as well as polymorphic changes compared with an HIV-1 subtype B consensus reference strain,[4] were analyzed for each patient's plasma sample. HIV-1 subtypes were determined by phylogenetic analysis of *pol* region sequences.[5] The nucleotide sequences obtained in this study have been submitted to GenBank under accession numbers EF517409–EF517489.

The prevalence of all drug-resistant mutations reported by the International AIDS Society (IAS)-USA[6] and HIV Drug Resistance Database[4] was calculated in drug-naive and drug-treated patients. The genotype results were interpreted for each drug using Stanford algorithm 1.4.4,[4] for which levels of resistance are ranked as susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance. In particular, to estimate the prevalence of resistance strains, we focused our attention on the following mutations in the PR gene: L23I, L24I, D30N, V32I, L33F, K43T, M46I/L, I47A/V, G48M/V, I50L/V, I54A/L/M/S/T/V, G73A/C/S/T, L76V, V82A/F/L/M/T/S, I84A/C/V, N88D/S, and L90M.[4,6] In the RT gene, we analyzed the NNRTI mutations A98G, L100I, K101E/P, K103N/S, V106A/M, V108I, V179D/E, Y181C/I/V, Y188C/H/L, G190A/C/E/S/Q, P225H, F227L, M230L, P236L, and K238N/T and the NRTI mutations M41L, A62V, K65R, D67N/G, D67del, T69D, T69ins, K70E/R, L74V, V75A/M/T, Y115F, Q151M, M184I/V, L210W, T215Y/F, and K219E/Q/R; this list also includes the mutations 215C//D/E/I/S/V that are considered revertant forms of 215F/Y.[7] The NRTI mutations (E44D, F116Y, and V118I) in the RT gene and the PI mutations (I13V, G16E, K20I/M/R/T/V, L33I/V, E34Q, E35G, M36I/L/T/V, F53L/Y, Q58E, D60E, I62V, L63P, I64L/M/V, H69K, A71I/L/T/V, T74A/P/S, V77I, V82I, N83D, I85V, N88T, L89V, and I93L/M) in the PR gene were not counted in calculating the prevalence of resistance because they confer resistance only when they occur in combination with other NRTI and PI resistance mutations, respectively. The analyzed plasma samples were 79 for RT sequences and 80 for PR sequences.

For quantitative measurements, data sets with non-normal distributions were compared nonparametrically using the Mann–Whitney *U* test. Categorical data were analyzed by using the Fisher exact test. A false discovery rate of 0.05 was used to determine statistical significance. The statistical program used was JAVA stat (http://stapages.org).

The phylogenetic analysis[5] revealed that subtype B was the prevalent one [41/80 (51.2%), distributed in 13 drug-naive patients and 28 treated patients], followed by subtype A [18/80 (22.5%), distributed in 8 drug-naive patients and 10 treated patients]. Of the other 21 *pol* gene sequences, 6 (7.5%) were classified as subtype C, 3 (3.7%) as subtype F, 2 (2.5%) as subtype G, and 2 (2.5%) as subtype H; 8 sequences (10.0%) were classified as putative CRFs [5 (6.2%) as 01_AE, 1 (1.2%) as 02_AG, and 2 (2.5%) as 05_DF]. Among these 21 sequences, 20 were obtained from treated patients, while one (subtype F) was from a drug-naive patient.

The proportion of subtype B among naive and treated patients was similar in each group [13/23 (57%) vs. 28/58 (47%), $p = 0.62$], while a major proportion of subtype A was present in drug-naive patients rather than in treated patients [8/22 (36.4%) vs. 10/58 (17.2%), $p = 0.08$].

Sixty-two out of 80 (77.5%) patients had their first HIV-1 determination before age 40 years, but there was no obvious evidence about different distributions of subtypes by age. Male gender was significantly prevalent in patients infected with subtype B (33/41, 80.5%) in comparison with the patients infected with subtype A, who were predominantly females (11/18, 61.1%) ($p = 0.002$), and the patients infected with other subtypes (male gender: 12/21, 57.1%) ($p = 0.072$).

Sixty-seven (83.7%) patients were presumably infected in Bulgaria. The commonest transmission route was sexual contact (70/80, 87.5%); in particular, 65/70 (92.9%) patients were infected through heterosexual contact, while 5/70 (7.1%) were infected through homosexual contact (all 5 infected with subtype B). No statistically significant differences in viremia and CD4 cell count values at the time of the genotypic resistance test were found between drug-naive and drug-experienced patients harboring the same subtype virus (A subtype or B subtype; data not shown).

Two out of 22 (9.1%) naive patients harbored viruses carrying at least one resistance mutation; in particular, 2 NRTI mutations (M41L and V75A) and the NNRTI mutation Y188H were observed in one patient, while in the other one only the NNRTI mutation V179D was observed. No primary mutations related to resistance to PI were observed, while all 22 patients carried viruses with PI minor mutations considered as natural polymorphisms.

Among drug-experienced patients, 40 (70.1%) patients showed viral strains with at least one mutation conferring resistance to ARV drugs. Detailed resistance levels to each antiretroviral administered to the patients analyzed and cross-resistance to all other drugs used in clinical practice are summarized in Table 1.

Of 57 NRTI-experienced patients (one sample was excluded from this analysis, for the availability of the only PR sequence), 32 (56.1%) had NRTI resistance mutations. Twenty-six patients (45.6%) were infected with an HIV-1

Table 1. Frequency of Samples with High or Intermediate or Low Resistance Levels among 58 Patients Treated at the Moment of Genotypic Resistance Test[a,b]

Antiretrovirals used in clinical practice	*Samples from experienced patients (N)*	*Levels of resistance*		
		High/Intermediate n (%/N)	*Potential low/Low* n (%/N)	*Susceptible* n (%/N)
NRTIs[c]	57	26 (45.6)	6 (10.5)	25 (43.9)
3TC	45	24 (42.1)	2 (3.05)	31 (54.4)
AZT	37	17 (29.8)	6 (10.5)	34 (59.6)
D4T	27	17 (29.8)	6 (10.5)	34 (59.6)
DDI	25	14 (24.6)	8 (14.0)	35 (61.4)
ABC	16	15 (26.3)	15 (26.3)	27 (47.4)
FTC	0	24 (42.1)	2 (3.05)	31 (54.4)
TDF	0	5 (8.8)	15 (26.3)	37 (64.9)
NNRTIs[d]	14	6 (42.9)	2 (14.3)	6 (42.3)
EFV	14	6 (42.9)	1 (7.1)	7 (50.0)
NVP	1	6 (42.9)	2 (14.3)	6 (42.3)
PIs[e]	46	21 (45.7)	0 (0.0)	25 (54.3)
SQV	25	16 (34.8)	3 (6.5)	27 (58.7)
IDV	24	13 (28.3)	5 (10.9)	28 (60.9)
NFV	9	21 (45.7)	0 (0.0)	25 (54.3)
ATV	0	13 (28.3)	6 (13.0)	27 (58.7)
DRV	0	3 (6.5)	10 (21.7)	33 (71.7)
FAPV	0	10 (21.7)	9 (19.6)	27 (58.7)
LPV	0	8 (17.4)	11 (23.9)	27 (58.7)
TPV	0	9 (19.6)	5 (10.9)	32 (69.6)

[a]The analysis was performed on 57 samples. The genotype results were interpreted for each drug using the Stanford algorithm 1.4.4. (http://hivdb.stanford.edu), for which levels of resistance are ranked as susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance.

[b]NRTIs, nucleoside reverse transcriptase inhibitors; 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; DDI, didanosine; ABC, abacavir; FTC, emtricitabine; TDF, tenofovir. NNRTIs, non-NRTIs; EFV, efavirenz; NVP, nevirapine. PIs, protease inhibitors; SQV, saquinavir; IDV, indinavir; NFV, nelfinavir; ATV, atazanavir; DRV, darunavir; FAPV, fosamprenavir; LPV, lopinavir; TPV, tipranavir.

[c]The level of resistance for each NRTI used in clinical practice was calculated on $N = 57$ samples from NRTI-experienced patients.

[d]The level of resistance for each NNRTI used in clinical practice was calculated on $N = 14$ samples from NNRTI-experienced patients.

[e]The level of resistance for each PI used in clinical practice was calculated on $N = 46$ samples from PI-experienced patients.

virus resistant to lamivudine and emtricitabine, the last one never administered in our group of patients analyzed; 20 patients, naive to tenofovir, infected with the HIV-1 virus, carried tenofovir-resistant mutations. The M184V was the commonest NRTI resistance mutation, observed in 24 (42.1%) NRTI-experienced patients. Mutations associated with thymidine analogues (TAMs: M41L, D67N, K70R, L210W, T215F/Y, and K219Q/F/C) were present in 22 (38.6%) ARV-experienced patients (Table 2).[8]

Eight out of 14 (57.2%) NNRTI-experienced patients had viruses carrying NNRTI resistance mutations; in particular, 7 patients carried efavirenz-resistant strains and 8 carried nevirapine-resistant strains (Table 1). Of these, 4 (28.6%) patients carried HIV-1 strains mutated at the 190 position of RT, while 2 (14.3%) patients carried HIV-1 strains with K103N (Table 2).

Regarding PIs, of 46 PI-experienced patients, 21 (45.7%) had PI resistance mutations. The patients analyzed had experience only with indinavir, nelfinavir, and saquinavir, even if resistance for all available PIs drugs used in the clinical practice was detected (Table 1). L90M was the prevalent PI-resistant mutation (13/46 patients, 28.3%), followed by I84V (6/46 patients, 13.0%) (Table 2).

Although drug resistance was widely described in clade B infections in North America and Europe both in drug-naive[9,10] and in drug-treated patients,[2,11] most areas remained without effective prevalence data. To our knowledge, this is the first study evaluating ARV resistance of HIV-1 in both drug-naive and drug-treated patients from Bulgaria.

Regarding the subtype distribution, even if B, predominant in neighboring countries,[11] remains the prevailing one, an increase of subtype A, which is highly prevalent in Ukraine, Yugoslavia, and Albania,[13–15] is being recording in Bulgaria. In this regard, subtype A tends to be more commonly detected in women, suggesting its entry through sex workers in East Europe. However, the presence of 9 different subtypes, including 3 recombinant forms, highlights the needs for further investigations to assess a rational therapy for all different HIV-1 subtypes.

With regard to antiretroviral drug resistance, we observed a high level of resistance detected in drug-experienced patients; in particular, the presence of revertant forms (I, S) at the 215 position suggests suboptimal therapeutic regimens, unable to prevent the development of resistance[16] and limiting anti-HIV-1 treatment options. We also found a high prevalence of PI drug-resistant mutations, probably due to the lack of pharmacological boosting for many antiretroviral therapy. The widespread use of boosted regimens in other countries has decreased the likelihood of PI resistance and

Table 2. Prevalence of Mutations Associated with Resistance in the Different Subtypes Harbored in Bulgarian Patients Treated at the Moment of GRT[a,b]

Wild-type codon (consensus B) NRTI mutations	*Mutation*	*Samples from experienced patients* n *(%/57)*	*Subtype*								
			A (n = *10)*	*AE* (n = *5)*	*AG* (n = *1)*	*B* (n = *28)*	*C* (n = *5)*	*DF* (n = *2)*	*F* (n = *2)*	*G* (n = *2)*	*H* (n = *2)*
M41	L	8 (14.0)	—	—	—	7	1	—	—	—	—
E44	A	1 (1.7)	1	—	—	—	—	—	—	—	—
	D	3 (5.3)	—	—	—	2	1	—	—	—	—
D67	G	2 (3.5)	1	—	—	1	—	—	—	—	—
	N	12 (21.0)	1	2	—	7	2	—	—	—	—
T69	D	1 (1.7)	—	—	—	1	—	—	—	—	—
K70	E	1 (1.7)	—	—	—	1	—	—	—	—	—
	R	9 (15.8)	1	2	—	3	2	1[c]	—	—	—
L74	V	3 (5.3)	1	—	—	2	—	—	—	—	—
V75	M	6 (10.5)	4	—	—	2	—	—	—	—	—
V118	I	5 (8.8)	—	—	—	3	—	—	2	—	—
Q151	M	1 (1.7)	1	—	—	—	—	—	—	—	—
M184	V[d]	24 (42.1)	4	1	1	10	4	1	1	1	1
L210	W	7 (12.3)	1	—	—	6	—	—	—	—	—
T215	F	6 (10.5)	1	1	—	2	2	—	—	—	—
	I	3 (5.3)	—	1	—	1	1	—	—	—	—
	Y	8 (14.0)	1	—	—	6	—	—	—	1	—
	S	1 (1.7)	—	—	—	—	1	—	—	—	—
K219	E	3 (5.3)	—	1	—	2	—	—	—	—	—
	Q	7 (12.3)	—	1	—	3	2	1	—	—	—
	N	3 (5.3)	—	—	—	3	—	—	—	—	—
NNRTI mutations		n *(%/14)*	*A* (n = *4)*	*AE* (n = *1)*	*AG* (n = *0)*	*B* (n = *5)*	*C* (n = *1)*	*DF* (n = *2)*	*F* (n = *1)*	*G* (n = *1)*	*H* (n = *0)*
A98	G	1 (7.1)	—	—	—	1	—	—	—	—	—
K103	N	2 (14.3)	—	—	—	—	—	1	1	—	—
V106	M	1 (7.1)	—	1	—	—	—	—	—	—	—
V179	D	1 (7.1)	1	—	—	—	—	—	—	—	—
G190	A	1 (7.1)	—	1	—	—	—	—	—	—	—
	Q	2 (14.3)	1	—	—	1	—	—	—	—	—
	S	1 (7.1)	—	—	—	—	—	1	—	—	—
P225	H	1 (7.1)	—	—	—	—	—	1	—	—	—
PI mutations[e]		n *(%/46)*	*A* (n = *7)*	*AE* (n = *4)*	*AG* (n = *1)*	*B* (n = *23)*	*C* (n = *5)*	*DF* (n = *2)*	*F* (n = *0)*	*G* (n = *1)*	*H* (n = *2)*
L24	*I*	*1 (2.2)*	—	—	—	1	—	—	—	—	—
D30	N	2 (4.3)	—	—	—	1	1	—	—	—	—
L33	F	1 (2.2)	—	1	—	—	—	—	—	—	—
M46	I	4 (8.7)	—	—	—	3	—	1	—	—	—
	L	1 (2.2)	—	—	—	1	—	—	—	—	—
G48	V	2 (4.3)	—	—	—	1	1	—	—	—	—
I50	V	1 (2.2)	—	—	—	—	1	—	—	—	—

V82	A	2 (4.3)	—	1	—	1	—	—	—	—	—
	F	2 (4.3)	—	—	—	1	—	1	—	—	—
	T	1 (2.2)	—	—	—	1	—	—	—	—	—
I84	V	6 (13.0)	—	—	—	5	—	1	—	—	—
L90	M	13 (28.3)	—	—	—	10	1	1	—	—	1

Wild-type codon (consensus B) Other PI mutations[f]	*Mutation*	*Overall* n *(%/46)*	*Subtype*								
			A (n = *7)*	*AE* (n = *4)*	*AG* (n = *1)*	*B* (n = *23)*	*C* (n = *5)*	*DF* (n = *2)*	*F* (n = *0)*	*G* (n = *1)*	*H* (n = *2)*
I13	V	20 (43.5)	7	3	1	4	1	1	—	1	2
G16	E	7 (15.5)	1	2	—	1	1	—	—	—	2
K20	I	2 (4.3)	—	—	1	1	—	—	—	—	—
	M	1 (2.2)	—	—	—	1	—	—	—	—	—
	R	3 (6.5)	—	1	—	1	—	—	—	—	1
	T	2 (4.3)	—	—	—	1	1	—	—	—	—
L33	V	4 (8.7)	—	—	—4	—	—	—	—	—	—
M36	I	21 (45.6)	6	4	1	3	4	—	—	1	2
	L	3 (6.5)	—	—	—	1	2	—	—	—	—
F53	L	1 (2.2)	—	—	—	1	—	—	—	—	—
	Y	1 (2.2)	—	—	—	—	1	—	—	—	—
Q58	E	3 (6.5)	—	1	—	1	1	—	—	—	—
D60	E	6 (13.0)	—	—	—	2	3	—	—	—	1
I62	V	18 (39.1)	2	—	—	14	1	—	—	—	1
L63	P	22 (47.8)	1	—	—	16	3	1	—	1	—
I64	L	2 (4.3)	—	—	—	2	—	—	—	—	—
	M	1 (2.2)	—	—	—	—	1	—	—	—	—
	V	4 (8.7)	—	—	—	4	—	—	—	—	—
H69	K	23 (50.0)	6	4	1	4	5	—	—	1	2
A71	I	1 (2.2)	—	—	—	1	—	—	—	—	—
	T	2 (4.3)	—	—	—	2	—	—	—	—	—
	V	5 (10.9)	—	—	—	4	—	1	—	—	—
T74	A	3 (6.5)	—	—	—	1	2	—	—	—	—
	P	1 (2.2)	—	—	—	1	—	—	—	—	—
	S	4 (8.7)	2	—	—	2	—	—	—	—	—
V77	I	5 (10.9)	—	—	—	4	—	1	—	—	—
V82	I	4 (8.7)	—	—	—	2	1	—	—	1	—
N83	D	1 (2.2)	—	1	—	—	—	—	—	—	—
L89	V	2 (4.3)	—	—	—	1	1	—	—	—	—
I93	L	10 (21.7)	—	1	—	5	3	1	—	—	—
	M	3 (6.5)	1	—	—	2	—	—	—	—	—

[a]The analysis was focused on the mutations associated with ARV resistance[8] (HIV Drug Resistance Database: http://hivdb.stanford.edu) and was performed on 58 sequences of the protease gene and 57 of the reverse transcriptase gene.

[b]NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI; PI, protease inhibitor.

[c]The amino acid R present at position 70 represents the wild-type codon in the DF subtype.

[d]The prevalence of M184V resulted in a significant (by Fisher exact test) difference between the HIV-1 A or B subtype compared to the C subtype (21% vs. 80%, $p = 0.025$ and 24% vs. 80%, $p = 0.028$, respectively).

[e]Mutations in the protease gene that confer high, intermediate, or low resistance to PIs.

[f]Mutations in the protease gene that only contribute to PI resistance.

the transmission of HIV-1 PI-resistant strains in drug-naive patients.

In this regard, a prevalence of HIV-resistant variants in drug-naive Bulgarian patients of 9.1% (4.5% to NRTIs, 9.1% to NNRTIs, and 0% to PIs) results in agreement with recent European and American reports evaluating the presence of resistance mutations in drug-naive individuals having ongoing access to antiretroviral medicines.[8,9] Once transmitted, HIV-1-resistant variants have a negative impact not only on the initial treatment response but also on the time to first virological failure.[17]

Some polymorphisms, having a compensatory role in drug resistance, occurred at higher rates in nonsubtype B viruses than in B subtypes in ARV-treated versus ARV-naive patients. An increasing number of communications have shown that these compensatory mutations can occur spontaneously in the genome of viruses belonging to subtypes other than B isolated from untreated patients.[18,19] It is not clear if the high frequency of these mutations may contribute to a more rapid crossing of the genetic resistance barrier; however, their existence in HIV-1 strains of naive patients might favor a more rapid evolution toward resistance when additional mutations are selected under therapy.

Because we detected a transmission of resistant variants to newly infected individuals, continuous surveillance is required, since greater access to HAART will be expected. Furthermore, surveillance of PR and RT sequences is also convenient to monitor the introduction of nonsubtype B HIV-1 strains.

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