

Evolving patterns of HIV-1 resistance to antiretroviral agents in newly infected individuals

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Objective: To assess temporal changes in prevalence of transmitted HIV-1 drug resistance in a homogeneous cohort of newly infected individuals.

Methods: Pretreatment genotypic and phenotypic drug resistance was tested in 154 subjects with primary HIV-1 infection identified between 1995 and 2001 (group A; n = 76) and 1999 and 2001 (group B; n = 78). Sequence analysis was assessed by population-based sequencing. Virus susceptibility to antiretroviral agents was determined by the PhenoSense assay (ViroLogic).

Results: The frequency of resistance-associated mutations in protease (PR) and reverse transcriptase (RT) genes increased from 13.2% (1995–1998) to 19.7% (1999–2001). Although the overall prevalence of viruses with phenotypic resistance did not vary (1995–1998, 10.0%; 1999–2001, 10.8%), the distribution of drug classes changed [nucleoside RT inhibitor (NRTI): 8.3% to 2.7%; non-NRTI: 5.0% to 8.1%; protease inhibitors (PI): 1.7% to 5.4%]. The decrease of phenotypic resistance to NRTI in 1999–2001 was caused by the absence of transmitted lamivudine-resistant variants. Phenotypically susceptible variants with aspartic acid or serine residues at position 215 of RT (5.3%; $P = 0.04$) instead emerged. Hypersusceptibility to PI decreased from 18.3% to 5.4% ($P = 0.02$) while the amino acid substitutions in PR increased over time: M36I (6.6% to 19.7%) and A71V/T (3.9% to 15.8%).

Conclusions: There was an increase in the number of HIV-1 variants with both genotypic and phenotypic resistance to non-NRTI and PI over time. Furthermore, viruses with altered genotypes compatible with thymidine analogue or PI exposure but susceptible phenotypes were seen in 1999–2001. The latter findings suggest transmission of viruses from subjects who have either changed or discontinued therapy.

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Introduction

Monitoring newly infected patients for HIV-1 resistance to antiretroviral agents is important for the assessment of optimal treatment for the individual as well as for the public health considerations. The

presence of drug-resistant viral variants poses a significant challenge to effective pharmacological intervention at any stage of infection, including acute and early HIV-1 infection. Early initiation of appropriate treatment in the setting of acute HIV-1 infection provides the best opportunity for effective antiretroviral therapy

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[1] and for restoration of the immune responses [2,3]. Drug-resistant HIV-1 in acutely infected individuals considerably diminishes the likelihood of long-term viral suppression [4] and, therefore, reduces the potential benefits of early treatment intervention.

Data from Europe and North America have suggested that the prevalence of transmitted drug-resistant HIV-1 variants in recently infected individuals ranges from 10 to 20% [5–8]. However, there is no single consensus definition of ‘drug resistance’. Frequencies of transmitted viral resistance reported are dependent on the detection method used (genotyping or phenotyping), the cohort studied, and the definition of resistance with respect to associated mutations and the ‘fold’ reduced susceptibility to specific antiretroviral drugs.

It has been suggested that the epidemic of drug-resistant HIV-1 is mainly generated by the selection of drug-resistant variants during therapy [9]. The fact that an increase in frequency of drug resistance in newly infected individuals is observed in both the United Kingdom [10] and North America [4] implies that the transmission of resistant HIV-1 is likely because of the growing number of patients in whom current antiretroviral regimens are non-suppressive. In the British cohort, the risk of being infected with a viral variant containing resistance-associated mutations increased over time (adjusted relative risk per year: 1.74) [10]. Similarly, the frequency of isolates with phenotypic resistance increased in subjects with recent HIV-1 infection identified in North America (16.5% in 1999–2000 versus 4.6% in previous years) [4]. We can, therefore, speculate that changes in the accessibility and quality of pharmacological treatment have influenced and will continue to influence the patterns of transmitted drug-resistant HIV-1 viral variants.

Based on our previously reported findings [6] that transmission of HIV-1 variants with resistance-associated mutations occurred in a sizeable proportion of acutely infected subjects (16%), the present study examines and characterizes further the evolving resistance patterns in newly HIV-1 infected individuals identified between 1995 and 2001.

Methods

Study population

The temporal changes in prevalence of transmitted drug-resistant viral variants was examined in a total of 154 individuals identified as having been newly infected with HIV-1 and referred to the clinical service of the Aaron Diamond AIDS Research Center (ADARC), New York City, between July 1, 1995 and January 31, 2001. The patients were divided into two

groups of similar sample size according to the year of identification [group A ($n = 76$), 1995–1998; group B ($n = 78$), 1999–2001]. All subjects were naive to antiretroviral therapy at study entry, and resistance testing was performed on samples obtained before initiation of treatment. Resistance data regarding 80 individuals enrolled between 1 July 1995 and 8 April 1999 have been previously reported [6].

Criteria used to confirm newly acquired HIV-1 infection were that HIV-1 RNA was detectable in plasma by either a branched DNA assay (Quantiplex, Bayer Diagnostics, Emeryville, California, USA) or by a reverse transcriptase (RT) polymerase chain reaction (PCR) assay (HIV Amplicor Monitor, Roche Diagnostics, Alameda, California, USA); Western blot analysis indicated absent or evolving humoral immune response to HIV-1; clinical history was consistent with acute retroviral syndrome within 90 days of presentation; and negative HIV-1 serology was documented within 120 days of screening. Written informed consent was obtained from all subjects and the human experimentation guidelines of the Rockefeller University Institutional Review Board were followed.

Genotypic drug resistance testing

Protease (PR) and RT genes were analyzed using population sequencing-based methods. Viral RNA was isolated from frozen pretreatment plasma samples using QIAamp viral extraction kit (Qiagen, Valencia, California, USA). Subsequently, an in-house assay consisting of a RT reaction followed by nested PCR; direct sequencing of the entire PR gene and the first 220 amino acid residues of RT was performed on an automated ABI sequencer [6]. Beginning in August 2000, a commercially manufactured kit-based assay [TRUGENE HIV-1 Genotyping Kit (PR, residues 4–98; RT, residues 38–245); Visible Genetics, Toronto, Canada] was used in conjunction with the OpenGene automated DNA sequencing system (Visible Genetics).

The sequences generated were compared with the HIV-1 clade B consensus sequence (Los Alamos HIV database) using Lasergene software. All amino acid substitutions were recorded and subsequently classified as resistance-associated mutations (21 amino acid substitutions in PR and 28 in RT) based on recently published consensus guidelines [11,12]. The subtype of the generated *pol* sequences was determined using the Stanford Resistance Database [13].

Phenotypic drug resistance testing

Viral susceptibility testing was performed by ViroLogic, Inc. (San Francisco, California, USA) on frozen plasma samples using a recombinant virus assay (PhenoSense).

Reduced susceptibility to the currently approved antiretroviral drugs was defined as any IC_{50} value (inhibi-

tory concentration of 50%) greater than 2.5 times that of the drug-susceptible reference strain (NL4.3) based on assay validation studies [14]. Recently amended clinical cut-off values for phenotypic resistance to abacavir (4.5-fold), didanosine (1.7-fold) and stavudine (1.7-fold) were adopted [15].

Increased susceptibility to any antiretroviral agents, also termed hypersusceptibility/hypersensitivity, was defined as an IC₅₀ value of less than 0.4-fold compared with the reference virus isolate NL4.3 [16].

Cloning of reverse transcriptase viral variants with serine or asparagine residues at position 215

Viral variants carrying serine (S) or asparagine (D) residues at codon 215 of RT were identified by population-based sequencing. In order to determine the existence of viral quasispecies, a separate RT-PCR followed by cloning of the 1.5 kb long PCR fragments was performed. After an initial RT reaction, a first round PCR using the oligonucleotides 5'-TAAGTGTTC AAYTGTGGCAAAGAAGGRCA-3' [nucleotide (nt) position 1959–1988 HXB2] and 5'-CYTGYTTCTGTATTTCTGCTAYTAAGTCTTTTG-3' (nt position 3514–3547 HXB2) was performed. The reaction was initiated with an incubation of 3 min at 94°C, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 1.5 min and a final elongation step of 10 min at 72°C. PCR products from the first round were amplified with the nested primer 5'-AATTGCAGGGCCCCTAGGAAAAAGGGCTGTT-3' (nt position 1999–2029 HXB2) and 5'-CACTC CATGTACCGTTCTTTTAGAAT-3' (nt position 3474–3500 HXB2) using the same conditions as for the first-round PCR, but with an annealing temperature of 54°C. The 1501 bp fragments were cloned in a TOPO XL TA vector (Invitrogen, Frederick, Maryland, USA) according to the manufacturer's recommendations. Plasmid DNA of ten positive clones was prepared according to the manufacturer's instructions (Qiagen) and sequenced as previously described [6].

Statistical analysis

Changes in frequencies of resistance-associated mutations in the two different groups were analyzed using the χ^2 test. The strength of association was tested with odds ratio (OR) and 95% confidence intervals (CI). Mann–Whitney test was used to compare the numbers of secondary mutations in PR per viral variant in groups A and B. All *P* values are two sided, and significance was established at *P* < 0.05. All statistical analyses were performed with the Prism software (version 3.0, GraphPad Software Inc.).

Results

Baseline characteristics of patients

Changes in the prevalence of transmitted drug resistance were analyzed based on groups A (*n* = 76) and B (*n* = 78), which consisted of newly infected individuals identified in 1995–1998 and 1999–2001, respectively (1995: 7; 1996: 28; 1997: 23; 1998: 18; 1999: 33; 2000: 38; 2001: 7). The two groups were comparable with respect to sex, ethnic background and risk factors (group A: 96% male, 76% Caucasian, 12% Hispanic, 9% African-American, 93% men who have sex with men; group B: 97% male, 74% Caucasian, 12% Hispanic, 10% African-American, 97% men who have sex with men). In contrast, median plasma HIV-1 RNA viral load values were higher in individuals identified in 1999–2001 (group A: median 5.13 log₁₀ copies/ml; group B: median 5.49 log₁₀ copies/ml; *P* = 0.007). Furthermore, the duration between onset of clinical symptoms compatible with acute retroviral syndrome (126/154 patients, 81.8%) and resistance testing decreased (group A: median 53 days; group B: median 31 days; *P* < 0.001). In summary, the cohort studied remained homogeneous with respect to the demographic distribution, although newly infected individuals in 1999–2001 were identified earlier during primary HIV-1 syndrome.

Genotypic drug resistance

From the 154 newly infected individuals, 154 PR and 152 RT gene sequences were available. Because of sample insufficiency, complete RT sequence data were not available for two patients in group B and they are, therefore, omitted from the overall sequence analysis (complete genotypic results for 76 in each group). The proportion of newly infected individuals with viral variants carrying mutations associated with resistance to protease inhibitors (PI) and/or reverse transcriptase inhibitors (RTI) increased from 13.2% (10/76) in group A to 19.7% (15/76) in group B (*P* = 0.389, Fig. 1a).

Genetic analysis of the *pol* sequences obtained suggested that all isolates were closest to HIV-1 subtype B clade (data not shown).

Genetic analysis of the protease gene

The frequency of primary PR resistance-conferring mutations (M46I, V82A, L90M) increased from 1/76 (1.3%) in group A to 4/76 (5.3%) in group B (*P* = 0.17; Table 1). In all but one viral variant, these primary mutations were associated with resistance-conferring amino acid substitutions in the RT gene. Primary PI mutations other than the above mentioned ones were not detected.

Secondary mutations in PR have a less direct effect on inhibitor binding *in vitro* than primary mutations but

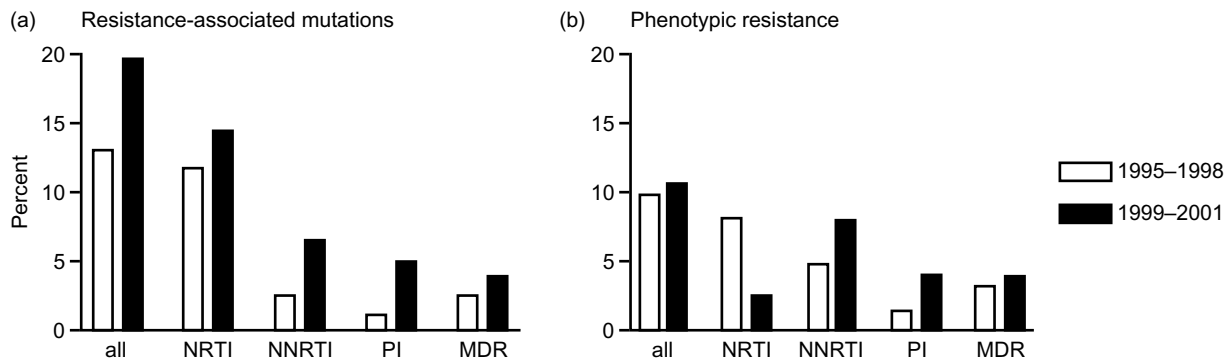


Fig. 1. Comparison of temporal changes in frequency of resistance-associated mutations and phenotypic resistance to all drugs, to each class of drug and to multiple drug classes for the years 1995–1998 and 1999–2001. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; MDR, multiple drug resistance.

Table 1. Frequency of resistance-associated mutations in protease and reverse transcriptase genes in group A (1995–1998) and B (1999–2001).

Mutations ^a	No. individuals ^b (%)		<i>P</i> value
	Group A	Group B	
RTI total	10 (13.2)	14 (18.4)	0.374
NRTI			
Total	9 (11.8)	11 (14.5)	0.631
M41L	2 (2.7)	4 (5.3)	0.405
E44D	1 (1.3)	1 (1.3)	1.000
T69D	0	3 (3.9)	0.080
K70R	3 (3.9)	3 (3.9)	1.000
V118I	2 (2.7)	2 (2.7)	1.000
M184V*	4 (5.3)	0	0.043
L210W	1 (1.3)	4 (5.3)	0.172
T215Y	1 (1.3)	2 (2.7)	0.550
T215D/S*	0	4 (5.3)	0.043
K219Q	0	3 (3.9)	0.080
NNRTI			
Total	2 (2.6)	5 (6.6)	0.246
K103N	1 (1.3)	2 (2.7)	0.550
V179E/D	1 (1.3)	3 (3.9)	0.311
Y181C	0	1 (1.3)	0.316
Y188L	0	1 (1.3)	0.316
Primary PI			
Total mutations	1 (1.3)	4 (5.1)	0.172
M46I	0	1 (1.3)	0.316
V82A	1 (1.3)	1 (1.3)	1.000
L90M	1 (1.3)	3 (3.9)	0.311
Secondary PI			
Total mutations	58 (76.3)	61 (80.2)	0.555
L10V/I/F	11 (14.5)	8 (10.5)	0.462
K20M/R	3 (3.9)	3 (3.9)	1.000
M36I*	5 (6.6)	15 (19.7)	0.016
I54V	1 (1.3)	1 (1.3)	1.000
L63X	42 (55.3)	50 (65.8)	0.184
A71V/T*	3 (3.9)	12 (15.8)	0.014
G73S/A	1 (1.3)	2 (2.6)	0.550
V77I	13 (17.1)	20 (26.3)	0.168
I93L	25 (32.9)	18 (23.7)	0.207

RTI, reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

^aMajor mutations not listed were not detected. L63X denotes any amino acid substitution detected at codon 63.

^bTotal number 76.

*Mutations for which the difference was statistically significant.

can contribute to drug resistance [11]. Although the proportion of individuals with viral variants carrying secondary PI mutations was comparable (group A: 76.3%; group B: 80.2%), the mean (\pm SE) number of secondary mutations per variant (Table 1) increased from 1.34 ± 0.12 in group A to 1.71 ± 0.14 in group B ($P = 0.059$).

The increase was most pronounced for the residues M36I [5/76 (6.6%) in group A and 15/76 (19.7%) in group B; $P = 0.016$; OR, 3.5; CI, 1.2–10.2] and A71V/T [3/76 (3.9%) in group A and 12/76 (15.8%) in group B; $P = 0.014$; OR, 4.6; CI, 1.2–17.0]. The only two amino acid substitutions that were observed less frequently in group B were I93L [decrease from 25/76 (32.9%) in group A to 18/76 (23.7%) in group B; $P = ns$] and L10V/I/F [decrease from 11/76 (14.5%) in group A to 8/76 (10.5%) in group B; $P = ns$].

Although the frequency of both primary and secondary mutations in PR increased over time, the difference was more marked for the secondary mutations M36I and A71V/T.

Genetic analysis of the reverse transcriptase gene

The frequency of resistance-associated mutations in the RT gene (M41L, T69D, K70R, K103N, V118I, V179E/D, M184V, Y181I, Y188L, L210W, T215Y, T215D/D, K219Q) increased from 10/76 (13.2%) in group A to 14/76 (18.4%) in group B (Table 1). Other mutations associated with nucleoside reverse transcriptase inhibitor (NRTI) (L74V, V75T, Y115F, F116Y, Q151M) or non-nucleoside reverse transcriptase inhibitor (NNRTI) (L100I, V106A, V108I, G190A) drug resistance were not detected.

While an overall increase of 5% was observed in RT gene mutations from group A to group B, specific mutations varied greatly, ranging from previously ab-

sent to newly appearing and vice versa. NNRTI resistance-associated mutations (K103N, 179D/E) and thymidine analogue-related mutations (M41L, L210W, T215Y) were detected in twice as many individuals in group B as in group A (Table 1). In contrast, the primary lamivudine mutation M184V was only detected in group A [4/76 (5.3%); $P = 0.043$; OR, 0.1; CI, 0.006–2.00].

The amino acid substitutions T69D, T215S/D and K219Q were exclusively detected in viral variants of group B: T69D (3/76, 3.9%), T215S/D (4/76; 5.3%; $P = 0.043$; OR, 9.5; CI, 0.5–180) and K219Q (3/76, 3.9%). Of note, none of these mutations translated into reduced drug susceptibility to zalcitabine (T69D) or thymidine analogues (T215S/D, K219Q), as measured by phenotypic resistance testing. In contrast, amino acid substitutions M184V (lamivudine), K103N (delavirdine, efavirenz and nevirapine), V179D/E (delavirdine) and T215Y (zidovudine) were uniformly associated with phenotypic resistance.

Nucleotide sequence accession numbers

Nucleotide sequences obtained from the patients in group A (AD 1–76) and group B (AD 77–154) have been submitted to GenBank (accession numbers AF459101 to AF459284).

Phenotypic drug resistance

For 134 of the 154 newly infected individuals (60/76 in group A and 74/78 in group B), phenotypic data were available for analysis (Fig. 1b). Phenotypic resistance testing was not successful for 20 patients because of technical reasons and/or sample insufficiency (e.g. low viral load, presence of internal restriction sites, insufficient frozen plasma).

Reduced viral drug susceptibility, as determined by the PhenoSense assay, was categorized into low-level [> 2.5 -fold for all drugs except didanosine (> 1.7 -fold), d4T (> 1.7 -fold) and abacavir (> 4.5 -fold)] and moderate/high-level [> 5 -fold for all drugs except ddI (> 3.4 -fold), d4T (> 3.4 -fold) and abacavir (> 9 -fold)] groups. In the absence of well-defined clinical cut-off values for most of the antiretroviral compounds, the moderate/high-level category was termed phenotypic resistance. The frequency of low-level reduced susceptibility as well as phenotypic resistance per compound and group are shown in Fig. 2a.

Although the relative frequency of phenotypic resistance was similar in both groups [6/60 (10.0%) in group A and 8/74 (10.8%) in group B], the patterns detected differed (Fig. 1b). While phenotypic resistance to PI [1/60 (1.7%) in group A and 3/74 (5.4%) in group B] and NNRTI [3/60 (5.0%) in group A and 6/74 (8.1%) in group B] increased, resistance to NRTI decreased from 5/60 (8.3%) in group A to 2/74 (2.7%)

in group B. Of note, phenotypic resistance to NRTI in group B was exclusively detected in the context of multidrug-resistant viral variants.

In all but two viral variants, phenotypic resistance correlated with the detection of the expected resistance-associated mutations. In the first discordant case, where phenotypic lamivudine resistance was found, a minor population of M184V was detected by limiting dilution PCR but not consensus sequencing [6]. In the second patient, a 9.7-fold reduced susceptibility towards delavirdine was detected (residues 1 to 305 of RT, PhenoSense assay), whereas the corresponding RT sequence (residues 37–245 of RT, genotyping performed with TRUGENE HIV-1 assay) did not reveal any known NNRTI resistance-related mutations.

Phenotypic hypersusceptibility

Viral variants with a hypersusceptible phenotype to any drug in a drug class were observed, although the overall frequency varied (overall 1/134 for NRTI, 8/134 for NNRTI and 15/134 for PI; Fig. 2b). The PI-associated hypersusceptibility decreased significantly from 11/60 (18.3%) in group A to 4/74 (5.4%) in group B ($P = 0.02$; OR, 0.26; CI, 0.078–0.86) whereas the NNRTI-associated hypersusceptibility increased slightly over the years [3/60 (5%) in group A to 5/74 (6.8%) in group B]. All eight NNRTI hypersusceptible variants were fully susceptible to NRTI, although two had either a T215S or V118I substitution in RT.

Multidrug resistance

Viral variants were recorded as multidrug resistant if primary resistance-associated mutations and phenotypic resistance to at least two drug classes were detected. The frequency of transmitted multidrug-resistant viral variants increased only slightly, from 2/76 (2.6%) in group A to 3/76 (4%) in group B. The resistance patterns of the multidrug-resistant variants are summarized in Table 2.

Analysis of viral quasispecies in variants containing the T215S/D substitution in the reverse transcriptase

Mutation of amino acid 215 of RT from threonine to tyrosine (T215Y) confers resistance to zidovudine [17]. In contrast, cysteine (C), asparagine (N), serine (S) or aspartic acid (D) residues at this position do not translate into phenotypic resistance to zidovudine but rather reflect viral evolution from the 215Y substitution in a drug-free environment [18,19]. T215S/D substitutions were detected by population-based sequencing in 5.3% of individuals in group B [two subjects with T215S (patients 1 and 2) and two with T215D (patients 3 and 4); Fig. 3]. All four viral variants were susceptible to NRTI. Clones were analyzed by sequencing in order to assess the spectrum of viral quasispecies present and

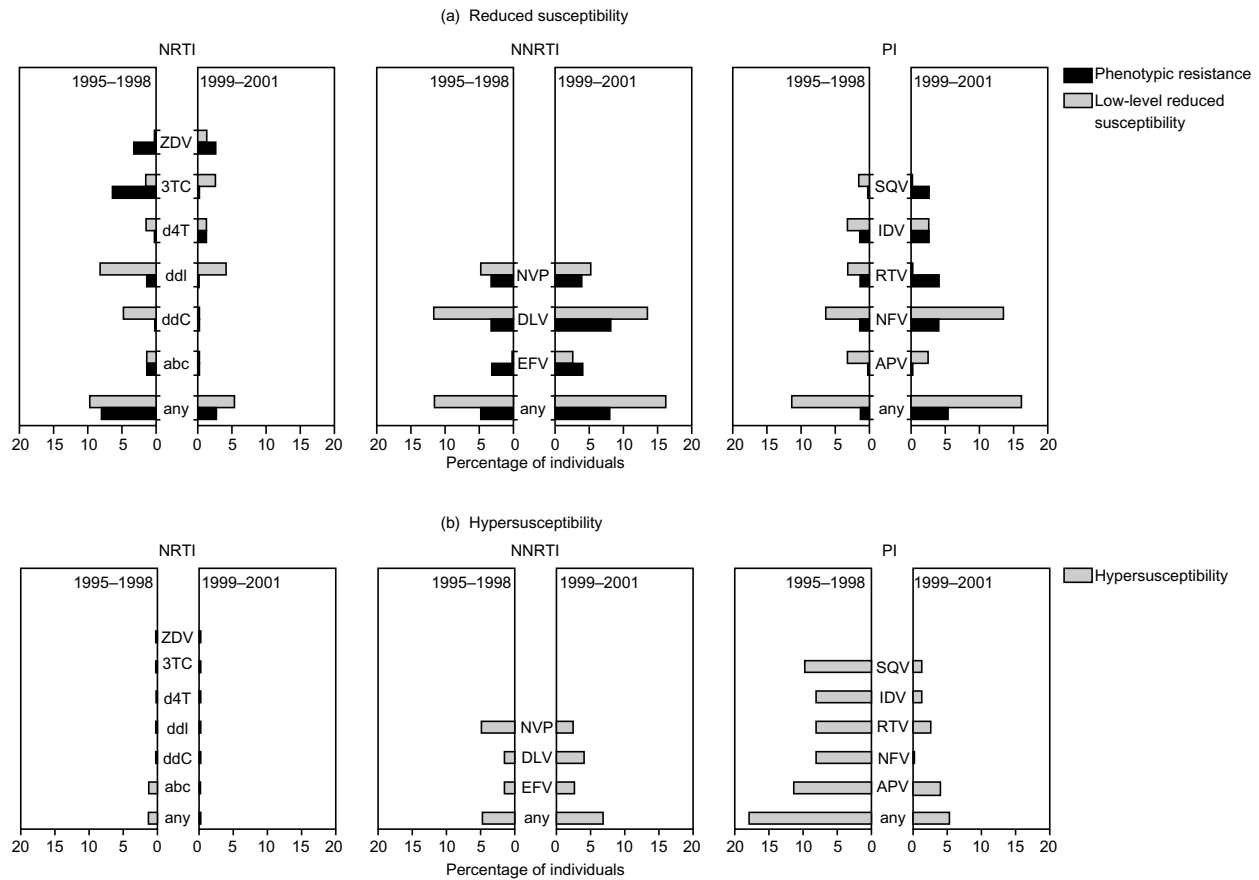


Fig. 2. Frequency of changed viral susceptibility, reduced (top) and increased (bottom), to the different antiretroviral drugs in group A (1995–1998) and B (1999–2001). Reduced viral susceptibility was categorized as phenotypic resistance and low-level reduced susceptibility according to the respective cut-off values. PI, protease inhibitor; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; APV, amprenavir; NRTI, nucleoside reverse transcriptase inhibitor; ZDV, zidovudine; 3TC, lamivudine; d4T, stavudine; ddl, didanosine; ddC, zalcitabine; abc, abacavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz.

elucidate whether viral evolution occurred before or after transmission. Patients 1 and 2 revealed a predominance of viral variants with T215S residues (9/10 clones 215S). For patient 3, the analysis of clones revealed a mixed viral population (6/10 clones 215D; 4/10 215S). In patient 4, a homogenous T215D viral population (10/10 clones T215D) was detected. Interestingly, in both patients that showed T215D substitutions, additional resistance-associated mutations were present (patient 3: M41L and T69D in 9/10 clones and L210W in 10/10 clones; patient 4: M41L and L210W in 10/10 clones). Of note, 215D (GAC) and 215N (AAC) require only a single nucleotide change to reach the resistance-conferring 215Y (TAC) substitution. 215S (AGC), in contrast, requires, like wild-type 215T (ACN), the exchange of two nucleotides. The stepwise evolution as well as the viral quasiespecies detected in these four individuals is shown in Fig. 3.

Discussion

While several studies have described the prevalence of HIV-1 drug resistance in newly infected individuals [5–8], only limited data have been available concerning the temporal changes in transmitted HIV-1 drug resistance in demographically homogeneous cohorts.

The findings of our study not only summarize the resistance data of 154 newly infected individuals recruited at a single American clinical site between 1995 and 2001 but also permit an assessment of changing resistance patterns. We believe that the observed altered pattern of transmitted drug resistance is not a consequence of any shift in the patient population studied since the cohort remained demographically consistent over the years. It should be noted, however, that the time between clinical symptoms compatible with acute

Table 2. Genotypic and phenotypic findings of the five transmitted multiple drug resistance (MDR) viral variants.

	Year	Genotype		Phenotype
		PR	RT	
MDR1	1996	L63P; V77I	K103N; M184V	PI susceptible NRTI 3TC > 300.0 NNRTI NVP 79.2; DLV 93; EFV 32.5
MDR2	1998	L10I; I54V; L63P; V77I; V82A; L90M	M41L; E44D; V118I; L210W; T215Y	PI SQV 5.0; IDV 12.4; RTV 54.7; NFV 18.9; APV 3.0 NRTI ZDV > 1000.0; 3TC 4.9; d4T 3.6; ddC 2.7; abc 9.0 NNRTI NVP 5.7
MDR3	1999	L10V; K20I; M36I; L63P; A71V; G73S; L90M	M41L; T69D; K70R; V179D; Y188L; T215F; K219Q	PI SQV 16.6; IDV 5.4; RTV 8.9; NFV 33.2 NRTI ZDV 30.3; 3TC 5.0; d4T 3.1; ddl 2.7 NNRTI NVP > 700; DLV 36.5; EFV 162.9
MDR4	1999	K20I; M36I; L63Q; L90M	K103N; Y181C	PI NFV 6.0 NRTI susceptible NNRTI NVP > 700.0; DLV > 200.0; EFV 79.0
MDR5	2000	K20I; M36I; M46I; L63P; G73S; L90M; I93L	M41L; E44D; D67N/S; T69D; L210W; T215Y	PI SQV 14.8; IDV 47.1; RTV 38.3; NFV 131.8; APV 4.4 NRTI ZDV 49.6; 3TC 4.3; d4T 3.5; ddl 1.8 NNRTI susceptible

PI, protease inhibitor; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; APV, amprenavir; NRTI, nucleoside reverse transcriptase inhibitor; ZDV, zidovudine; 3TC, lamivudine; d4T, stavudine; ddl, didanosine; ddC, zalcitabine; abc, abacavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz.

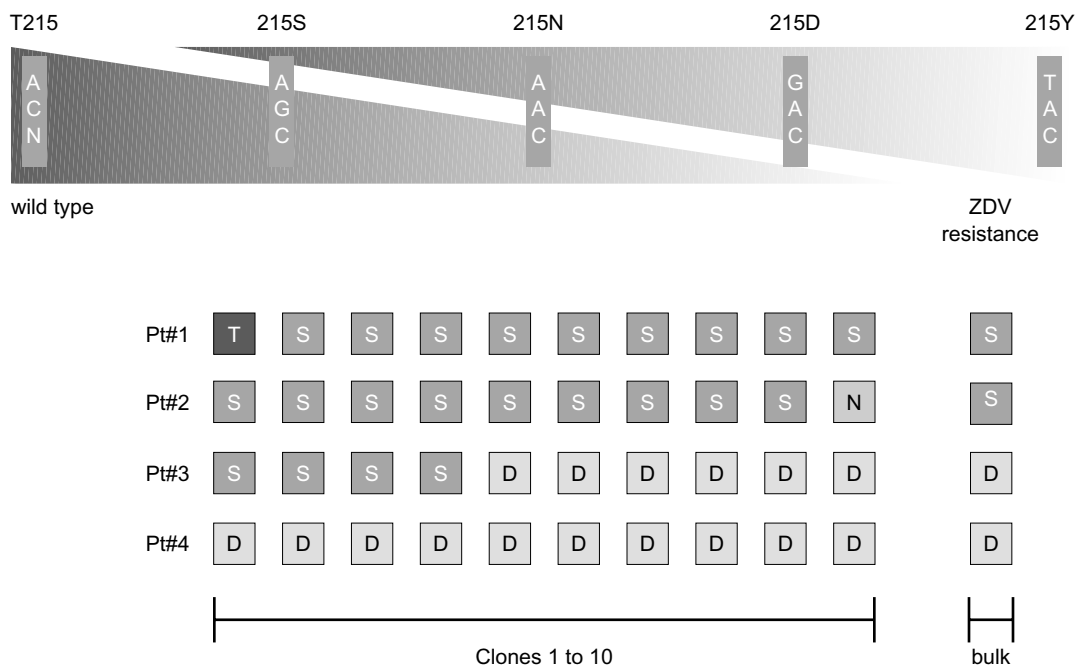


Fig. 3. Schematic pathway of stepwise mutations for the reversion of the zidovudine resistance conferring 215Y substitution in the reverse transcriptase. 'Bulk' denotes the amino acid substitutions detected by population-based sequencing in contrast to the analysis of ten individual clones. Amino acids threonine (T), serine (S), asparagine (N) and aspartic acid (D) were uniformly coded by the triplets shown.

retroviral syndrome and resistance testing was shorter for individuals identified in 1999–2001. Similarly, plasma viremia at the time of resistance testing was significantly higher in this group. Taken together, these observations suggest an overall earlier identification of individuals with primary HIV-1 infection in group B.

Consistent with other studies [4,10], we observed an overall increase of resistance-associated mutations from 13% in 1995–1998 to 20% in 1999–2001. More specifically, our data demonstrate an increase in PI- and NNRTI-related genotypic and phenotypic resistance while there was a decrease in transmission of viral

variants with mutations conferring phenotypic resistance to the NRTI class of drug. Of note, lamivudine-resistant variants were only detected in individuals of group A. One possible explanation is that viruses containing the M184V substitution in addition to multiple PI resistance-conferring mutations display reduced fitness and transmissibility. In the later observation period, phenotypic NRTI resistance was restricted to zidovudine and was strictly detected in the context of multidrug-resistant isolates. All eight viral variants with isolated NRTI-associated mutations in group B had fully susceptible phenotypes.

Fifty percent of the genotypically altered but fully susceptible viruses found in subjects identified in 1999–2001 had 215D/S substitutions in RT. These substitutions, however, were not detected in the earlier observation period ($P = 0.042$). Detailed analyses of the viral quasispecies in the four individuals with 215D/S variants revealed almost homogenous populations consisting of either serine or aspartic acid substitutions in three out of the four patients. In one subject, a mixture of 60% 215D and 40% 215S variants was detected. Notably, the variants with 215D substitutions were found to have additional resistance-associated mutations (e.g., M41L, L210W). The gain in fitness conferred by 215D, but not 215S, in a RT background carrying additional thymidine analogue mutations could explain these two patterns [18]. These differences may be of clinical relevance since 215D (GAC) has an increased ability to evolve towards 215Y (TAC) whereas 215S (AGC) could be considered a stable, new wild-type variant. Nevertheless, clinical data regarding the effects of these variants on treatment responses to thymidine analogue-containing regimens are lacking. Since no variants with 215Y substitution were detected in any of these patients with primary HIV-1 infection, these findings suggest that, although viral evolution continues to a certain extent in the new host, the 215S/D variants were themselves transmitted. It appears, therefore, that the transmitted viruses originated in individuals previously treated with thymidine analogues.

Another observation that supports the hypothesis that viral variants originating in previously treated patients were more frequently transmitted in group B is the increase of secondary mutations in PR per viral variant. In HIV-1 clade B isolates, the secondary mutations M36I and A71V/T have been described in the context of PI resistance [11,20]. By themselves they do not confer measurable reductions in viral susceptibility to these compounds but rather restore the viral replication capacity [21,22]. Both substitutions, M36I and A71V/T, were detected more frequently in group B ($P < 0.05$). Furthermore, we observed a decrease of hypersusceptibility toward PI from 19% (1995–1998) to 5% (1999–2001). In contrast to NNRTI hypersus-

ceptibility, which has been associated with an improved virological response when treatment with NNRTI is initiated [23], the clinical significance and etiology of hypersusceptibility to PI is not well understood. Recently, polymorphisms in p6 have been associated to it and a correlation between PI hypersusceptibility and decreased viral replication capacity has been demonstrated [24]. However, it is unclear if this observation is relevant *in vivo* as these viruses have successfully been transmitted and subsequently replicated to high levels. Our results suggest that increased frequency of viral variants with secondary mutations in PR is associated with the shift from hypersusceptibility to normal range phenotypic susceptibility to PI.

While the demographic homogeneity of our cohort over the years studied was crucial for accurate analysis of temporal changes, it can also be seen as a limitation of the study. Because our cohort consists almost entirely of homosexual men, the findings presented here may not be applicable to other risk groups. It may be that the described trends towards changing resistance in newly infected individuals are more pronounced in our highly selective cohort. Nonetheless, the overall frequency of drug resistance observed (genotypic 16%, phenotypic 10%) is of the same order of magnitude as that reported by other groups [5,25,26].

In summary, we detected an increased frequency of resistance towards NNRTI and PI, while transmission of lamivudine-resistant viruses only occurred between 1995 and 1998. In addition, we discovered phenotypically wild-type HIV-1 variants with aspartic acid and serine residues at position 215 of the RT in 5% of individuals recruited between 1999 and 2001. Furthermore, the increased frequency of secondary mutations in PR coincided with a significant decrease of PI-associated hypersusceptibility during the same time period. These data suggest that there is increased transmission of variants that evolved in patients after they discontinued antiretroviral therapy. This permitted the evolution of drug-resistant HIV-1 in a drug-free environment, resulting in viral variants with alternative genotypes but susceptible phenotypes.

In light of these findings, systematic studies are needed to elucidate further the impact of transmitted drug-resistant viral variants on primary virological outcome. Additionally, we must evaluate the risk for early virological failure conferred by new HIV-1 genotypes bearing decreased genetic barriers to resistance.

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References

- Markowitz M, Vesanen M, Tenner-Racz K *et al.* The effect of commencing combination antiretroviral therapy soon after human immunodeficiency virus type 1 infection on viral replication and antiviral immune responses. *J Infect Dis* 1999, **179**: 527–537.
- Altfeld M, Rosenberg ES, Shankarappa R *et al.* Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J Exp Med* 2001, **193**:169–180.
- Rosenberg ES, Altfeld M, Poon SH *et al.* Immune control of HIV-1 after early treatment of acute infection. *Nature* 2000, **407**: 523–526.
- Little SJ, Holte S, Routy J-P *et al.* Antiretroviral resistance and response to initial therapy among recently HIV-infected subjects in North America. *Antiviral Ther* 2001, **6**:21.
- Yerly S, Kaiser L, Race E, Bru JP, Clavel F, Perrin L. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet* 1999, **354**:729–733.
- Boden D, Hurley A, Zhang L *et al.* HIV-1 drug resistance in newly infected individuals. *JAMA* 1999, **282**:1135–1141.
- Little SJ, Daar ES, D'Aquila RT *et al.* Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *JAMA* 1999, **282**:1142–1149.
- Salomon H, Wainberg MA, Brenner B *et al.* Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec Primary Infection Study. *AIDS* 2000, **14**:F17–F23.
- Blower SM, Aschenbach AN, Gershengorn HB, Kahn JO. Predicting the unpredictable: transmission of drug-resistant HIV. *Nat Med* 2001, **7**:1016–1020.
- UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. Analysis of prevalence of HIV-1 drug resistance in primary infections in the United Kingdom. *BMJ* 2001, **322**: 1087–1088.
- Hirsch MS, Brun-Vezinet F, D'Aquila RT *et al.* Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society–USA Panel. *JAMA* 2000, **283**: 2417–2426.
- Schinazi RF, Larder BA, Mellors JW. Mutations in retroviral genes associated with drug resistance: 1999–2000 update. *Int Antiviral News* 1999, **7**:46–69.
- Gonzales MJ, Machekano RN, Shafer RW. Human immunodeficiency virus type-1 reverse-transcriptase and protease subtypes: classification, amino acid mutation patterns, and prevalence in a northern California clinic-based population. *J Infect Dis* 2001, **184**:998–1006.
- Petropoulos CJ, Parkin NT, Limoli KL *et al.* A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob Agents Chemother* 2000, **44**:920–928.
- Johnson VA, Petropoulos CJ, Woods CR *et al.* Vertical transmission of multidrug-resistant human immunodeficiency virus type 1 (HIV-1) and continued evolution of drug resistance in an HIV-1-infected infant. *J Infect Dis* 2001, **183**:1688–1693.
- Whitcomb J, Deeks S, Huang W *et al.* Reduced susceptibility to NRTI is associated with NNRTI hypersensitivity in virus from HIV-1-infected patients. *Seventh Conference on Retroviruses and Opportunistic Infections*. San Francisco, January–February 2000 [abstract 234].
- Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* 1989, **243**:1731–1734.
- Garcia-Lerma JG, Nidtha S, Blumoff K, Weinstock H, Heneine W. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naïve persons. *Proc Natl Acad Sci USA* 2001, **98**:13907–13912.
- de Ronde A, van Dooren M, van Der Hoek L *et al.* Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J Virol* 2001, **75**:595–602.
- Race E, Gilbert SM, Sheldon JG *et al.* Correlation of response to treatment and HIV genotypic changes during phase III trials with saquinavir and reverse transcriptase inhibitor combination therapy. *AIDS* 1998, **12**:1465–1474.
- Mammano F, Trouplin V, Zennou V, Clavel F. Retracing the evolutionary pathways of human immunodeficiency virus type 1 resistance to protease inhibitors: virus fitness in the absence and in the presence of drug. *J Virol* 2000, **74**:8524–8531.
- Nijhuis M, Schuurman R, de Jong D *et al.* Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* 1999, **13**:2349–2359.
- Shulman N, Zolopa AR, Passaro D *et al.* Phenotypic hypersusceptibility to non-nucleoside reverse transcriptase inhibitors in treatment-experienced HIV-infected patients: impact on virological response to efavirenz-based therapy. *AIDS* 2001, **15**: 1125–1132.
- Whitehurst N, Chappey C, Petropoulos CJ, Gamarnik A. Polymorphisms in p6/p6* of HIV-1 Gag are associated with hypersusceptibility to protease inhibitors. *Antiviral Ther* 2001, **6**:40.
- Perez-Olmeda M, Del Romero J, Rubio A *et al.* Primary HIV-1 drug resistance in Spain before and after the introduction of protease inhibitors. *J Med Virol* 2001, **63**:85–87.
- Wegner SA, Brodine SK, Mascola JR *et al.* Prevalence of genotypic and phenotypic resistance to anti-retroviral drugs in a cohort of therapy-naïve HIV-1 infected US military personnel. *AIDS* 2000, **14**:1009–1015.