Minority HIV-1 Drug Resistance Mutations Are Present in Antiretroviral Treatment–Naïve Populations and Associate with Reduced Treatment Efficacy

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Abbreviations: 3TC, lamivudine; ABC, abacavir; ART, antiretroviral therapy; CI, confidence interval; EFV, efavirenz; GSK, GlaxoSmithKline; NNRTI, non-nucleotide reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; nt, nucleotide; PR, protease; RT, reverse transcriptase; SE, standard error; TAM, thymidine analog mutation; VL, viral load

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ABSTRACT

Background

Transmitted HIV-1 drug resistance can compromise initial antiretroviral therapy (ART); therefore, its detection is important for patient management. The absence of drug-associated selection pressure in treatment-naïve persons can cause drug-resistant viruses to decline to levels undetectable by conventional bulk sequencing (minority drug-resistant variants). We used sensitive and simple tests to investigate evidence of transmitted drug resistance in antiretroviral drug-naïve persons and assess the clinical implications of minority drug-resistant variants.

Methods and Findings

We performed a cross-sectional analysis of transmitted HIV-1 drug resistance and a case-control study of the impact of minority drug resistance on treatment response. For the cross-sectional analysis, we examined viral RNA from newly diagnosed ART-naïve persons in the US and Canada who had no detectable (wild type, $n = 205$) or one or more resistance-related mutations ($n = 303$) by conventional sequencing. Eight validated real-time PCR-based assays were used to test for minority drug resistance mutations (protease L90M and reverse transcriptase M41L, K70R, K103N, Y181C, M184V, and T215F/Y) above naturally occurring frequencies. The sensitive real-time PCR testing identified one to three minority drug resistance mutation(s) in 34/205 (17%) newly diagnosed persons who had wild-type virus by conventional genotyping; four (2%) individuals had mutations associated with resistance to two drug classes. Among 30/303 (10%) samples with bulk genotype resistance mutations we found at least one minority variant with a different drug resistance mutation. For the case-control study, we assessed the impact of three treatment-relevant drug resistance mutations at baseline from a separate group of 316 previously ART-naïve persons with no evidence of drug resistance on bulk genotype testing who were placed on efavirenz-based regimens. We found that 7/95 (7%) persons who experienced virologic failure had minority drug resistance mutations at baseline; however, minority resistance was found in only 2/221 (0.9%) treatment successes (Fisher exact test, $p = 0.0038$).

Conclusions

These data suggest that a considerable proportion of transmitted HIV-1 drug resistance is undetected by conventional genotyping and that minority mutations can have clinical consequences. With no treatment history to help guide therapies for drug-naïve persons, the findings suggest an important role for sensitive baseline drug resistance testing.

The Editors' Summary of this article follows the references.

Introduction

Antiretroviral drugs have been remarkably successful in suppressing HIV-1 infection; however, transmitted drug resistance can reduce the efficacy of first-line regimens. Surveys using conventional bulk sequencing in North America and Europe, where the history of ART is extensive, have shown that transmitted or primary HIV-1 drug resistance is present in 8%–20% of ART-naïve persons [1–7]. As antiretroviral drug use expands to cope with the pandemic, adequately assessing the prevalence and transmission of drug resistance mutations will be increasingly important for optimizing treatment efficacy.

Early in HIV infection, the founding virus inoculum saturates the majority of target cells, and transmitted drug-resistant viruses become well established in long-term reservoirs that allow drug resistance to persist [8–10]. Because many resistance mutations reduce replicative fitness, drug-resistant viruses not under ART drug pressure can reverse resistance mutations to become more fit [11]. Over time, revertants outcompete the drug-resistant viruses to become the predominant viruses in circulation; however, the rates of mutant virus decay can vary substantially [12–14] because of differences in fitness cost. Moreover, the cost imparted by a drug resistance mutation to viral fitness can be modulated by other resistance mutations in the virus, and thereby accelerate or retard decay of the mutation [15].

HIV-1 drug resistance mutations are conventionally detected by bulk sequence analysis of the virus sample. Bulk genotyping may detect certain mutations in clinical samples at frequencies as low as 10%; however, the detection limitation does not allow for reliable identification of variants that constitute less than 20%–30% of the virus population in a sample [16]. This detection limitation is a concern because most newly diagnosed persons have been infected for several months to years, providing time for drug-resistant viruses to decay to levels that conventional testing is not able to detect.

Drug resistance mutations at frequencies detectable by conventional genotyping are known to reduce the efficacy of ART; however, there is increasing interest in the clinical consequences of these minority drug-resistant variants not detected by conventional genotyping. Earlier observations have suggested that persisting minority drug resistance in persons previously exposed to non-nucleoside reverse transcriptase inhibitors (NNRTIs) could lead to poor virologic outcomes under a subsequent NNRTI-containing regimen [17,18]. However, the clinical significance of minority mutations in drug-naïve persons is poorly understood.

We previously developed sensitive real-time PCR assays to examine minority drug-resistant variants in HIV-1 clinical samples [19]. These tests were validated on pre-ART wild-type HIV samples from the early 1980s to establish cutoffs that are above the mutation frequencies appearing with natural quasispecies variation. At these cutoffs the PCR assays are 10- to 67-fold more sensitive than conventional genotyping. The clinical application of this methodology is further supported by the examination of HIV-1 from infected women provided nevirapine to prevent mother-to-child HIV-1 transmission. This testing did not detect nevirapine resistance in antiretroviral drug–naïve women prior to receiving nevirapine and identified a greater emergence of resistance mutations in these women after nevirapine exposure compared to bulk sequencing [20].

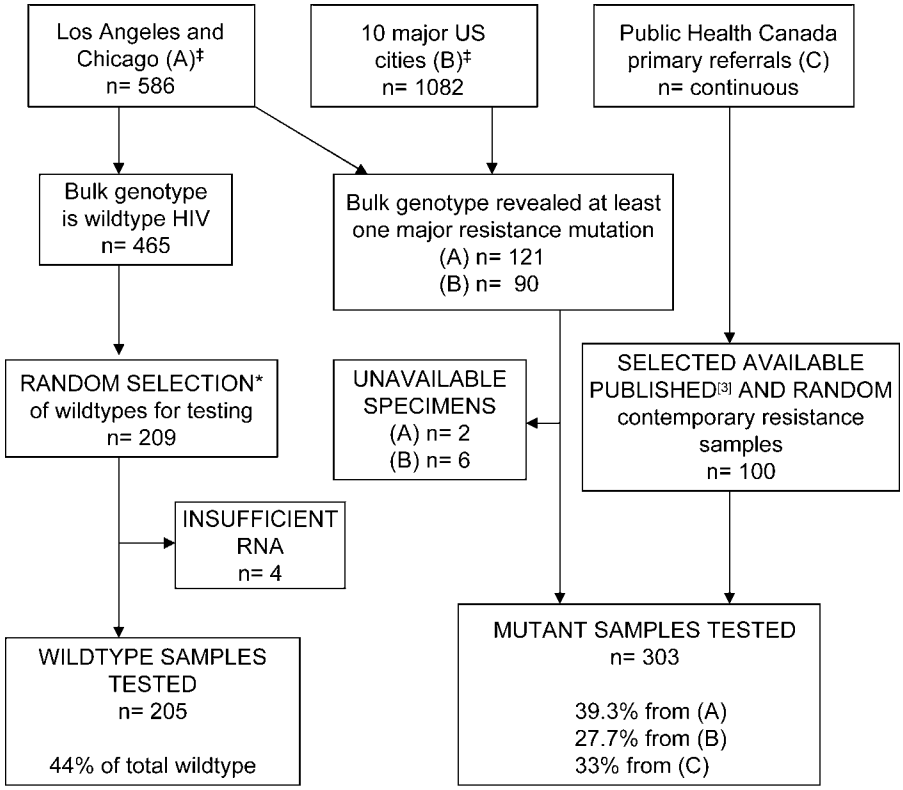
Here we report two studies based on sensitive real-time PCR. First, we performed a cross-sectional study to identify transmitted resistance mutations at low levels (0.4%–19%) in newly diagnosed individuals infected with subtype B HIV-1. For this, we examined samples from individuals who, by conventional genotyping, appeared to have wild-type infections and another group of persons who had at least one resistance-associated mutation. Second, we performed a retrospective case-control study on another group of participants to assess the impact of baseline minority treatment-relevant resistance mutations on the ability of ART to suppress virus replication. These findings shed light on both the prevalence of transmitted drug resistance and the clinical consequences of minority drug resistance mutations in the ART-naïve.

Materials and Methods

Study Populations

Cross-sectional analysis of transmitted drug resistance mutations. The populations for the cross-sectional study consisted of two groups of newly diagnosed HIV-1 subtype B-infected ART-naïve individuals (Figure 1; Table 1). The first group included 205 persons from Los Angeles and Chicago diagnosed with HIV-1 within 6 mo prior to enrollment into a CDC behavioral characterization study and who were determined to have wild-type infections by conventional sequencing (wild-type group). The wild-type samples were randomly selected to achieve the target sample size of greater than 1.5 times the number of resistance samples in the parent cohort. The parent cohort consisted of 586 individuals examined in 2003–2005 who self-reported by questionnaire their clinical history and ART-naïve status [21]. The overall prevalence of drug resistance in the parent cohort was 20.7% by conventional genotyping. The second cross-sectional study group consisted of 303 individuals who had at least one drug resistance mutation detected by conventional genotyping (Figure 1; Table 1, mutant group). Because only a minor proportion of transmitted HIV has bulk sequence–detectable resistance mutations, we tested for hidden low-frequency mutations in all available majority resistance samples. Of these specimens, 119 were from the same Los Angeles–Chicago parent cohort as the wild-type group, and 84 were collected in 1997–2001 from ten major urban areas in the US [1,21], representing nearly all (> 94%) of the resistance samples in each parent cohort. The remaining 100 samples were collected from individuals in Canada in 2000–2001 [3].

The ART-naïve status of individuals with sequence-detectable resistance was determined by personal interview and medical chart review, if available, or physician reporting as previously described [1,3]. All samples were collected as part of HIV surveillance studies under institutional review board approval (CDC IRB protocol nos. 1774, 3621, and 3910) and were provided without personal identifiers. The patients consented to storage of their blood samples for drug resistance testing. More than half (52%) of all the US samples were from MSM (men who have sex with men), heterosexual exposure was reported for 34% of the population, and 14% of the cohort were non-MSM participants who engaged in intravenous drug use.



‡Targeted all bulk genotype resistance samples in cohort

*Target sample number was >1.5-times the number of mutants in parent cohort

[3]Jayaraman, et al. (2006)

Figure 1. STROBE-Type Flow Diagram for the Retrospective Cross-Sectional (Newly Diagnosed Surveillance) Study Illustrating Drug Resistance in the Parent Cohorts and the Sample Selection for Real-Time PCR Resistance Testing

Additional details are provided in Table 1.

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Case-control study evaluating the impact of minority drug resistance. To evaluate whether minority variants with resistance mutations influence the virologic response to ART, we performed a retrospective case-control study using real-time PCR to test baseline pre-treatment samples from ART-naïve persons in the US who had participated in treatment studies with efavirenz/lamivudine (EFV/3TC) plus abacavir (ABC) or zidovudine (ZDV) (GlaxoSmithKline trials CNA 30021 and CNA 30024) during 2000–2003 (Figure 2; Table 1, case-control group) [22,23]. We analyzed blinded plasma-virus samples, all wild-type by conventional genotyping, from participants who experienced virologic treatment failure and a control group of participants who suppressed virus replication during the 48-wk study period. Because we expected very few cases with minority resistance using the assay cutoffs we established, all 95 participants who failed treatment and had wild-type baseline genotypes were included. The total percentage of participants experiencing virologic failure for the two GSK studies was 9% for CNA 30021 and 5% for CNA 30024 (Figure 2). Virologic failure was defined as (1) rebound of two consecutive plasma HIV-1 RNA levels to $>$ 50 copies/ml after achieving $\leq$ 50 copies/ml during randomized treatment, or (2) plasma HIV-1 RNA levels never achieving suppression of $\leq$ 50 copies/ml with or without the discontinuation of treatment due to insufficient viral load response.

Although all cases of virologic failure were chosen for evaluation, we analyzed only a subset the 964 total success samples potentially introducing a sampling bias. To minimize this bias, we made a conservative assumption that only 2% of the samples would have minority resistance. Using this expected proportion, a minimum sample size ($n = 209$) was determined (nQuery Advisor, Statistical Solutions) so that a 95% confidence interval (CI) for minority resistance in the treatment successes would not cover zero (95% CI 0.001–0.039). Our final sample size of 221 success samples exceeded this minimum. The first 70 success samples were simply the first enrolled who met the success criteria; the remaining 151 were selected randomly. The success samples tested represented 23% of all treatment successes from the two GSK studies. Fifty-seven percent of the success samples tested were from CNA 30021, and 43% were from CNA 30024, which was similarly proportional to the contribution of participants remaining at the completion of both studies (CNA 30021 $n =$

Table 1. Characteristics and Conventional Drug Resistance Genotypes of Newly Diagnosed Drug-Naïve Populations Sampled for Sensitive Real-Time PCR Drug Resistance Testing in the Cross-Sectional and Case-Control Studies

Category	Group	Cross-Sectional Studies			Case-Control Study
		Los Angeles and Chicago (2003–2005), $n = 586$ [21]	Sentinel Surveillance of Ten US Cities (1997–2001), $n = 1,082$ [1]	Primary Care Reported to Public Health Canada (2000–2001) [3]	GSK Trials CNA 30021 and CNA 30024 (2000–2003), $n = 1,419$ [22,23]
Gender	Male	90%	81%	83%	81%
	Female	10%	19%	17%	19%
Ethnicity	White	32%	44%	76%	53%
	Black/African	30%	31%	0[a]	24%
	Hispanic	30%	22%	2%	20%
	Native/aboriginal	1%	0[a]	17%	0[a]
	Asian/other	7%	3%	5%	31%
Infection duration[b]		24% recent, 76% chronic	27% recent, 73% chronic	31% recent, 69% chronic	NT
Drug resistance prevalence (bulk)		20.7%	8.3%	8.1%	8.4%[c]
Subset(s) analyzed[d]	Wild-type samples	205 (wild-type group)[e]	—	—	316 (case-control group)[f]
	Mutant samples	119 (mutant group)[e]	84 (mutant group)[e]	100 (mutant group)[e]	—

[a]None reported.
[b]Recency of infection was determined using the Vironostika HIV-1 LS EIA (bioMerieux), recent = < 4–6 mo.
[c]Baseline drug resistance prevalence estimated from genotyping 21% of cohort.
[d]Wild-type samples have no drug resistance mutations, and mutant samples have ≥ 1 major drug resistance mutation by conventional bulk sequencing.
[e]Minority resistance in these populations was assessed by testing with a panel of eight key resistance mutations.
[f]Samples were tested for the three most common resistance mutations relevant to the treatment regimens.
NT, not tested.
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583, 54%; CNA 30024 $n = 490$, 46%). We tested these samples for three treatment-relevant reverse transcriptase (RT) mutations, K103N, Y181C, and M184V. Testing was performed on archived specimens which, after review of participants' consent and sample masking, the CDC IRB determined did not involve research on identifiable individuals. All p-values for the (exact) Wilcoxon rank sum tests were computed using StatXact version 6.2 (Cytel Software). The p-value for the Fisher exact test and the logistic model for treatment success/failure were obtained from SAS version 9.1 (SAS Institute).

Primary Virus Template Amplification

HIV-1 genomic RNA from the US samples was extracted (Qiagen UltraSens RNA kit or Roche Amplicor) from 200 μl of patient plasma or serum. The samples sent from the Public Health Agency of Canada's HIV/Retrovirology Laboratory were provided as purified RNA. A region of the HIV-1 template that included nucleotide (nt) 1 of protease (PR) to nt 777 of RT was RT-PCR amplified as previously described [19]. This RT-PCR template was used in the real-time PCR testing for drug resistance mutations.

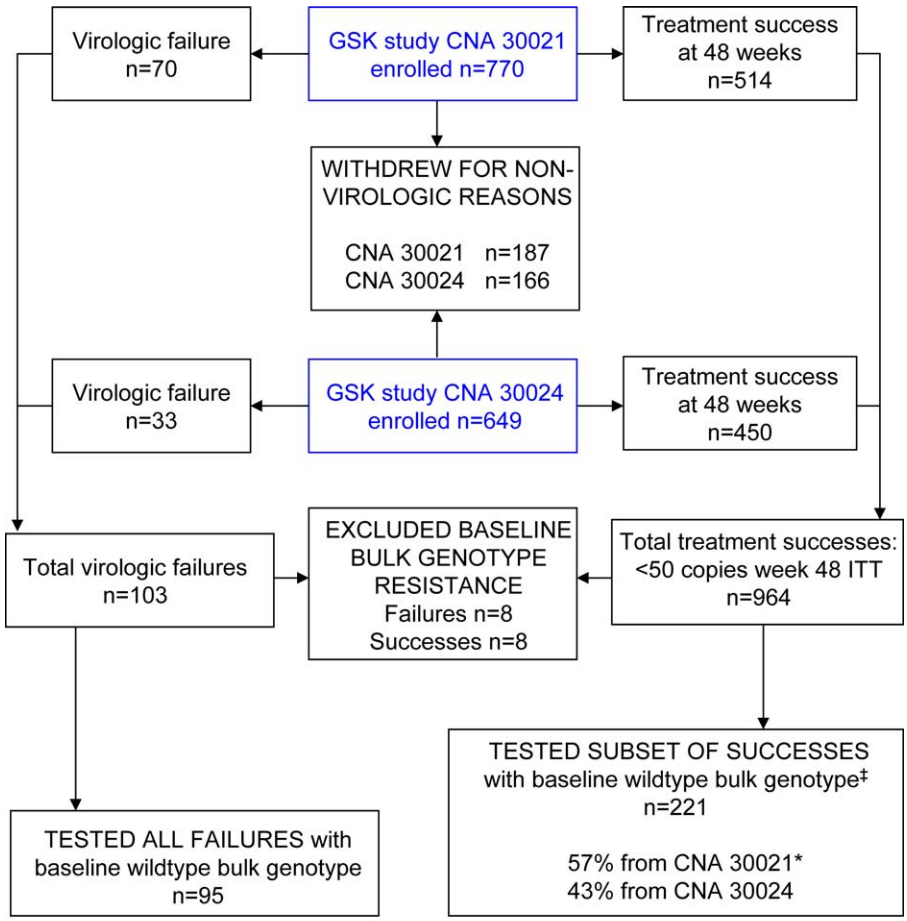
Sensitive Real-Time PCR Drug Resistance Testing

The general principle of the real-time PCR testing for subtype B clinical specimens has been described in detail [19]. Briefly, the protocol compares the difference in the threshold amplification cycles (ΔCT) between mutation-specific reactions and the total virus copy reaction for the sample. A qualitative determination of whether the sample had detectable resistance mutations is based on previously validated ΔCT cutoffs for each mutation. We have previously reported that the real-time PCR assays are able to detect mutant sequences at levels as low as 0.001%–0.2% when testing prepared mixtures of cloned virus sequences [19]. However, with the quasispecies diversity in clinical samples, we found it was necessary to use more conservative cutoff values to gain confidence in our ability to specifically detect transmitted drug resistance. Thus, for each mutation, we had established assay cut-offs above the background reactivity observed when testing wild-type virus samples from the early 1980s pre-antiretroviral era (Table 2) [19].

All real-time PCRs were performed in duplicate, and mean ΔCTs were used for interpreting the results. The reactions were performed in a total volume of 50 μl/well in 96-well PCR plates using iCycler real-time PCR thermocyclers with optical units (Bio-Rad) and AmpliTaq Gold polymerase (2.5 U/reaction; Applied Biosystems). Final reagent concentrations were 320 nM for the forward and reverse primers, 160 nM probe(s), and 400 mM dNTPs. Wild-type virus samples were tested for eight mutations: L90M in PR; and M41L, K70R, K103N, Y181C, M184V, and T215Y/F in RT. The 303 samples in the mutant group were tested for minority mutations not detected by conventional genotyping, which included 222 for L90M, 101 for M41L, 251 for K70R, 202 for K103N, 200 for Y181C, 260 for M184V, and 209 for 215Y/F mutations.

Cloning and Sequence Verification

To verify newly detected minority resistance mutations, a 709 bp PR-RT region (nt 8 of PR to nt 420 of RT) or a 651 bp region of RT (nt 57–708 of RT) was amplified from the primary RT-PCR of the specimen and cloned (pCR2.1 vector with Top 10F' *E. coli*, Invitrogen). Typically, 126 white colonies (ranging from 84 to 168 in batches of 42 clones) were screened as previously described [20], using the same real-time PCR test that had identified the low-frequency mutant in the sample. Colonies testing positive underwent double-strand chain-termination sequencing (Big Dye kit v1.1, Applied Biosystems).



‡Expected 2% of samples had minority resistance; power calculated minimum sample size = 209

*For comparison, the % of total participants from each cohort was 54% CNA30021 and 46% CNA30024

Figure 2. STROBE-Type Flow Diagram for the Retrospective Case-Control Treatment Study Illustrating the Parent Cohort (Blue) Outcomes and Sample Selection for Real-Time PCR Resistance Testing

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Results

Minority Drug Resistance Mutations in the Cross-Sectional Wild-Type Virus Group

Table 3 describes the real-time PCR test results for the 205 drug-naïve individuals with wild-type genotypes by conventional genotyping. The eight mutations tested were detected at least once as a minority variant in 34 (17%) samples (Figure 3). We found that the K70R thymidine analog mutation (TAM) [24] was the most prevalent minority mutation (10/205, 5%), followed by the M41L TAM, which was found in nine samples (4.5%). One-half of the K70R mutations (5/10) were in samples that also had M41L. The remaining resistance mutation assays identified eight (4%) K103N, seven (3.5%) L90M, three (1.5%) Y181C, two (1.5%) M184V, one (0.5%) T215Y, and four (2%) T215F samples. Four samples (2% overall) had the following mutations to two drug classes: L90M+M184V, L90M+Y181C, Y181C+T215F, and L90M+M41L+K70R.

Minority Drug Resistance Mutations in the Cross-Sectional Mutant Virus Group

Figure 3 and Table 4 show the real-time PCR test results for the 303 specimens known to have at least one drug resistance mutation by conventional genotyping. We identified minority mutations in a total of 30 (10%) samples, which added 6/222 (3%) samples with L90M, 9/101 (9%) with M41L, 14/251 (6%) with K70R, 2/202 (1%) with K103N, 8/200 (4%) with Y181C, 6/260 (2%) with M184V, 10/209 (5%) with T215Y, and 13/209 (6%) with T215F. This again showed that all resistance mutations are also present as minor variants. As was seen with the wild-type virus group, the TAMs M41L and K70R were the most common low-frequency mutations. Minority (between 1% and 19%) NNRTI resistance mutations were found only in samples collected after 2002.

Multi-Drug-Class Resistance Mutations in Cross-Sectional Analysis of Transmitted Viruses

We also examined whether the newly detected minority mutations increased the prevalence of multi-drug class

Table 2. Real-Time PCR Resistance Mutation Assay Cutoffs and Relative Sensitivities for Clinical Sample Testing Established Using Drug-Resistant and Pre-ART Wild-Type Clinical Samples [19]

Assay	ΔCT Cutoff (# Cycles)	Cutoff Mean % Mutant Equivalence
L90M	10.5	0.4
M41L	10.0	0.8
K70R	7.0	2.0
K103N	10.0	0.9
Y181C	10.0	1.0
M184V	8.5	0.5
T215Y[a]	10.5	1.0
T215F[b]	10.5	0.7

[a]Also detects 215H, N, and D revertants.
[b]Also detects 215L, I, and V revertants.
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resistance in the cross-sectional study. We found that some of the newly identified minority mutations within the mutant virus group samples were associated with resistance to drugs from classes for which resistance was not detected by conventional genotyping. Minority mutations to drugs in other classes were identified in 21/303 (7%) mutant virus samples. The majority of the class increases were in samples that had mutations to only one drug class by conventional genotyping, resulting in an increased prevalence of dual-class mutations from 14% to 18% (a 27% increase). The prevalence of resistance mutations to three drug classes doubled from the 2% detectable by conventional genotyping to 4%. Additionally, in 11 (44%) of 25 samples that had both majority nucleoside reverse transcriptase inhibitor (NRTI) mutations and minority variants, resistance was expanded to impact another drug within the NRTI class. Combining the minority mutations detected in the wild-type and mutant virus groups we found a cumulative increase in transmitted $\geq$ 2-class resistance mutations from 20% to 27% (a 35% increase).

Minority Drug-Resistant Variant Confirmation by Clonal Sequencing

Clonal sequencing confirmed minority mutations in ten of ten randomly selected positive samples from the wild-type and mutant virus groups. For the three mutant virus samples positive for M184V, clonal analysis found the mutation to be at frequencies of 5.5%, 1.4%, and 0.6% (ΔCTs of 7.8, 7.5, and 8.4 cycles, respectively) (sequences submitted to GenBank [http://www.ncbi.nlm.nih.gov/Genbank/index.html], accession numbers EU439613–EU439615). The bulk genotypes of these three specimens revealed only one resistance-associated mutation for each: K70R, K219Q, and K103S, respectively. The 184V clones from the first two samples showed linkage to TAMs.

Association of Minority Drug-Resistant Variants with Virologic Failure in the Case-Control Group

Real-time PCR testing for three relevant mutations showed that nine of the 316 baseline case-control samples had one or two minority mutations (Figure 4; Table 5). Unmasking after test completion revealed that seven (7.4%) of the minority resistance samples were among the 95 participants who experienced virologic failure, and two (0.9%) with minority resistance were among the 221 treatment successes ($p = 0.0038$, two-sided Fisher exact test) (Table 6). Hence, seven (78%) of the nine participants with minority resistance mutations experienced virologic failure. One participant who experienced failure within 2 mo had both the K103N and the M184V mutations, which confer resistance to two drugs in the regimen, EFV and 3TC, respectively. Four of five available virologic failure bulk genotypes had majority levels of the same resistance mutations we identified as being at minority levels in their baseline samples. One participant who had detectable Y181C at baseline experienced virologic rebound with wild-type virus by week 12. Of the two individuals who had resistance mutations at baseline and maintained virus suppression during the 48-wk course of the study, one person had K103N and the other Y181C; both had been treated with the ABC+3TC+EFV regimen.

Table 3. Minority Drug Resistance Mutations Detected in the Wild-Type Group Cross-Sectional HIV-1 Samples Collected in the US between 2003 and 2005

Sample ID	Minority Mutations	Recent Infection[a]
2	L90M, Y181C[b]	No
5	L90M	No
6	L90M	No
9	M41L	No
14	M41L	Yes
23	Y181C	Yes
28	L90M	No
29	L90M	No
55	T215F	No
56	M41L, K70R	Yes
59	T215F	No
74	M41L	Ind
83	K70R	No
85	K70R	No
88	L90M, M184V[b]	Yes
89	Y181C, T215F[b]	No
90	M184V	No
92	L90M, M41L, K70R[b]	No
98	M41L, K70R	Yes
102	K70R	Yes
104	K103N	Yes
109	M41L, K70R	Yes
110	K70R	Yes
122	K103N	Ind
130	K103N	Yes
132	M41L, K70R	Yes
134	T215Y	Yes
150	K103N	Yes
167	K103N	No
173	K103N	No
177	K70R	No
178	M41L	No
181	K103N	No
196	K103N	No

[a]By Vironostika HIV-1 LS EIA and in-house avidity index (see Text S1).
[b]Two drug-class mutations.
Ind, indeterminate (two incidence tests not concordant).
doi:10.1371/journal.pmed.0050158.t003

Statistical Analysis of Clinical Markers and Outcome in the Case-Control Group

We assessed the impact of baseline virus load (VL) and $CD4^+$ T cell count (CD4 count) on virologic success and

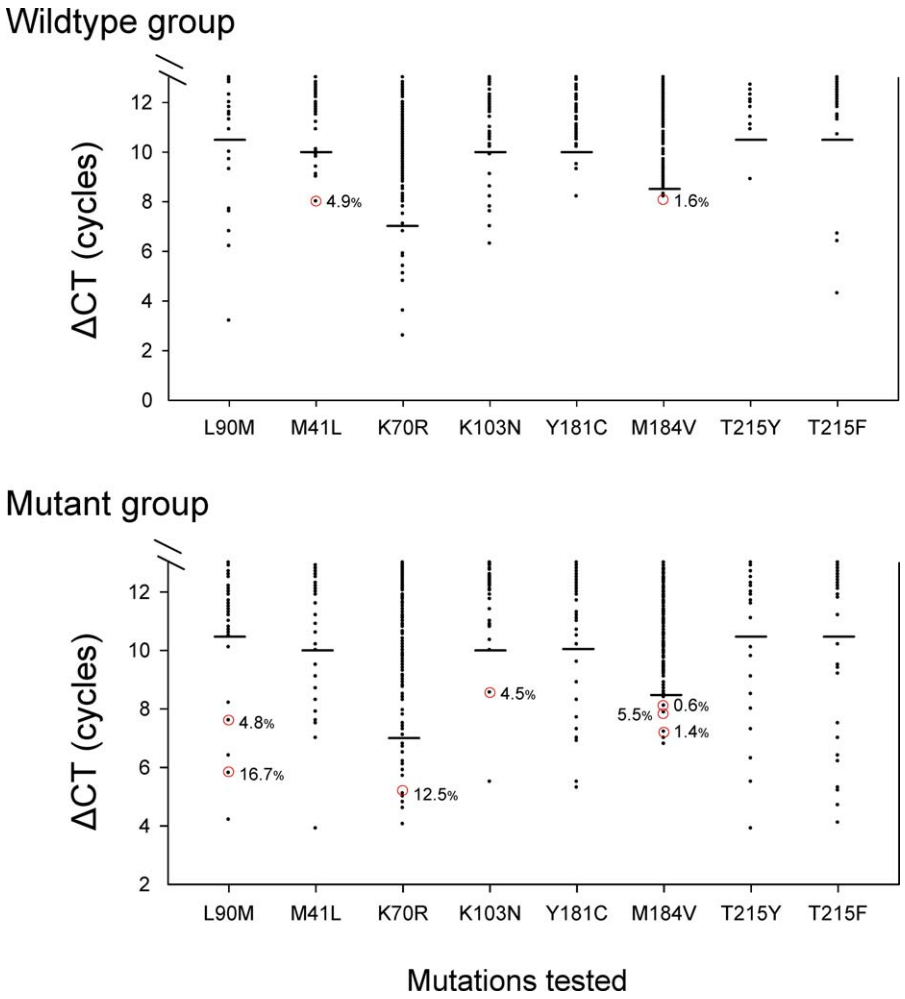

Figure 3. Real-Time PCR Mutation Test Results (ΔCT) for the Cross-Sectional Group

The samples positive for minority resistance are emphasized (ΔCT results above 13 cycles are not shown). The wild-type and mutant groups were tested for eight key resistance mutations. Horizontal bar (—) denotes the assay cutoff for the mutation. Samples falling below this bar are positive for the mutation. The clonal frequencies are shown for encircled data points.

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failure with regard the presence or absence of minority resistance. The 307 participants with no evidence of resistance had a median CD4 count and VL of 240 cells/mm^3 (range 50–650 cells/mm^3) and $10^{5.07}$ copies/ml (range= $10^{3.08}$–$10^{5.90}$ copies/ml), respectively. These values are similar to the median CD4 count of 230 cells/mm^3 (range 45–560 cells/mm^3) and VL of $10^{4.95}$ copies/ml (range $10^{3.70}$–$10^{5.52}$ copies/ml) for the nine participants with minority resistance (CD4 count $p = 0.416$, VL $p = 0.493$, Wilcoxon rank sum test). No significant difference was found in VL ($p = 0.43$) or CD4 count ($p = 0.316$) between those with baseline minority resistance who experienced failure and all treatment successes (Wilcoxon rank sum test). However, overall, VL was significantly higher when all failures (cases) were grouped ($10^{5.26}$ copies/ml) as compared to all successes (controls) ($10^{4.95}$ copies/ml) ($p < 0.0001$); and, conversely, CD4 count was significantly lower in the treatment failure group (227 versus 254 cells/mm^3, $p = 0.023$) (Wilcoxon rank sum test). The two individuals with minority K103N and Y181C who successfully suppressed virus had baseline VLs of $10^{4.11}$ and $10^{3.70}$ copies/ml, respectively. These VLs are well below the medians but within the ranges observed for both the failures and successes with no resistance.

In a logistic model for the probability of treatment success, with $\log_{10}$ VL and minority resistance as independent variables, persons with low-frequency resistance mutations at baseline had 11.2 times the odds of experiencing treatment failure versus those in whom minority resistance was undetectable ($p = 0.004$, 95% CI 2.2–58.8, coefficient estimate = –2.414, standard error [SE] = 0.839). For $\log_{10}$ VL as the variable, the coefficient estimate was –0.794 ($p = 0.0002$, SE = 0.214). The coefficient at intercept was 4.919 ($p < 0.0001$, SE = 1.108). Although the number of people with low-frequency resistance was small for both virologic failure and treatment success, seven and two respectively, model diagnostics supported the fit of a logistic model to the data.

Discussion

Using assays validated for drug resistance mutations in HIV-1 clinical samples, we identified, in ART-naïve persons from the US and Canada, a substantial number of minority mutant viruses at levels above the natural quasispecies

Table 4. Minority Resistance Mutation Prevalence in 303 Mutant Virus Group Samples in the Cross-Sectional Study of Individuals from the US (1997–2005) and Canada (2000–2001), and the Observed Increase in Prevalence for Each Mutation when Minority Variants Are Included

Mutation Tested (Minority Prevalence)	Change in Mutation Prevalence[a]	Percentage Change in Mutation Prevalence[b]
L90M (3%)	8% → 11%	+25%
M41L (10%)	9% → 17%	+70%
K70R (6%)	9% → 14%	+60%
K103N (1%)	19% → 20%	+5%
Y181C (4%)	3% → 6%	+100%
M184V (2%)	8% → 10%	+23%
T215Y (3%)	17% → 20%	+20%
T215F (5%)	2% → 7%	+340%

[a]Conventional mutation prevalence → new mutation prevalence with minority variants added (rounded to nearest percent).
[b]Reflects change in observed (nonrounded) mutation prevalence; 30 /303 (10%) of the mutant group samples had evidence of minority drug resistance mutations.
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frequency of each mutation. The preponderance of minority resistance mutations implies that a considerable proportion of transmitted drug resistance decayed to low levels by the time of HIV-1 diagnosis. The detection of minority resistance mutations in 17% of the wild-type virus group, part of a US cohort of newly diagnosed HIV-1–infected persons that had 20% bulk sequence-detectable primary drug resistance, suggests that bulk sequencing missed 40% of the resistance samples in this population. The frequent occurrence of drug resistance likely reflects a high prevalence of ART in these locations and suggests that transmission of HIV-1 from antiretroviral drug-experienced persons expressing virus is not uncommon.

Minority HIV-1 variants increased the observed frequency of transmitted multi-drug resistance genotypes in the cross-sectional study group by one-third, emphasizing that drug-naïve individuals may harbor hidden resistance to drugs from different classes. The several cases of low-frequency drug resistance mutations that we identified is likely an underestimation of minority resistance prevalence, because only eight of the nearly 40 codons in PR and RT that are associated with drug resistance were evaluated [25]. Clonal sequencing and resistance mutation linkage analysis (see Figure S1 and Text S2) have shown that multiple minority drug resistance mutations can be present and that these mutations may coexist unlinked from majority mutations in ART-naïve persons.

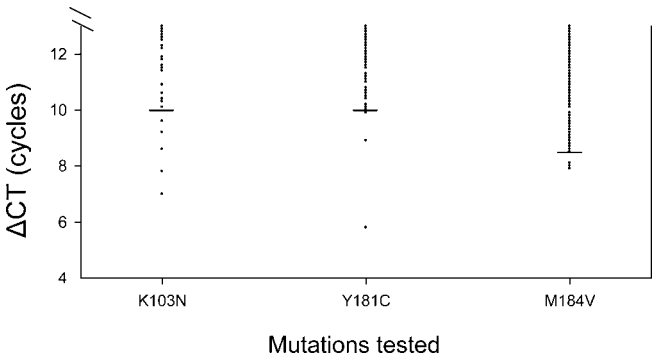

Figure 4. Real-Time PCR Mutation Test Results (ΔCT) for Three Treatment-Relevant Mutations in the Case-Control Study Group

Horizontal bar (—) denotes the assay cutoff for the mutation. Samples falling below this bar are positive for the mutation.
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The presence of unlinked mutations in multidrug-resistant ART-naïve persons might reflect mutations that were once linked in oligoclonal populations and then independently reverted as a result of mutation (fitness) modulations or immune selection [15,26,27]. Alternatively, some dissociated mutations could have been a result of infections with more than one drug-resistant variant, possibly through needle sharing or, less frequently, by sexually acquired superinfection [28]. Of note, we found that in the wild-type virus group minority mutations were equally prevalent in recently infected persons and in those infected for longer duration (17% of both groups). This might suggest that a considerable proportion of transmitted drug resistance decays very rapidly. A better understanding of drug resistance transmission and mutation decay requires sensitive testing of longitudinal samples from a large number of acutely infected persons with dissimilar drug resistance mutation genotypes.

With the knowledge that minority drug resistance can be considerable in ART-naïve populations, we then assessed the impact of low-frequency resistance mutations on treatment responses in a case-control study of persons with no treatment-relevant mutations detected by conventional genotyping. In the baseline samples of this previously ART-naïve group we were again able to identify minority treatment-relevant mutations. The minority mutations were significantly associated with virologic failure using the Fisher exact test ($p = 0.0038$) and in a logistic model. These results suggest that minority transmitted resistance mutations can be clinically important.

We observed in the case-control study what appeared to be a stronger association with treatment failure for participants with minority resistance than that seen for resistance-associated mutations detected by bulk genotyping [22,23]. A virologic explanation for these seemingly disparate outcomes is currently not evident, and the finding could be biased by only a few samples having any of the three relevant resistance mutations. Notably, a similar observation was recently made with low-level RT-simian–human immunodeficiency virus (SHIV) mutants in a macaque infection model. In that study, minority NNRTI mutations were associated with virologic failure, whereas viruses with resistance mutations at higher

Table 5. Baseline Samples with Detectable Minority Mutations and Treatment Outcomes for Persons Who Participated in the NNRTI-Based Treatment Studies (Case-Control Group)

Sample ID	Baseline Minority Mutations	Regimen	Outcome	Treatment Week	Bulk Genotype Mutations at Failure
11	M184V	ABC+3TC+EFV	Failure	48	Unk
25	M184V	ABC+3TC+EFV	Failure	12	M184V
31	K103N	ABC+3TC+EFV	Failure	8	K103N, M184V
41	K103N, M184V	ABC+3TC+EFV	Failure	8	K103N, M184V
44	K103N	ZDV+3TC+EFV	Failure	24	K103N
63	K103N	ZDV+3TC+EFV	Failure	48	Unk
67	Y181C	ABC+3TC+EFV	Failure	12	WT
193	Y181C	ABC+3TC+EFV	Success	—	NA
251	K103N	ABC+3TC+EFV	Success	—	NA

Unk, genotype not known; WT, wild-type virus; NA, not applicable; ZDV, zidovudine.
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levels detectable by sequencing were successfully suppressed by the NNRTI regimen [29]. It is possible that some minor variants have higher phenotypic resistance than the dominant mutants, presumably due to additional mutations. These minor variants could be less fit than majority mutants in the absence of drug, but more fit under drug pressure, leading to virologic failure. Additional studies are required to examine these possibilities.

While we report a significant association between poor virologic suppression and minority levels of three mutations relevant to two EFV-based regimens, the impact of a particular minority resistance mutation on other drug regimens is not entirely understood. Further investigation with sensitive tests will help define the clinical significance of individual mutations in the context of specific drug (or drug class) combinations. Data from those studies could assist with the selection of regimens that are most active for persons infected with drug-resistant viruses.

A limitation of our study is that, as with all self-reported histories, some participants with resistance mutations may not have disclosed previous exposure to antiretroviral drugs and, therefore, did not represent cases of primary drug resistance. Additionally, the small number of participants with detectable minority resistance mutations in the case-control study may have allowed for an artifactual association with virologic failure. It may be possible that the higher median viral load in the virologic failures versus the treatment successes allowed for increased opportunities to detect minority mutations in failures; however, this was not evident from the viral loads for the failures with resistance, which were comparable to the successes. A possible explanation for the low number of detectable minority variants is that NNRTI resistance mutations were not as prevalent during the period of the GSK studies as they are today, as seen in the populations sampled for our cross-sectional analysis. Additionally, the participants in the case-control study may have had infections of comparatively longer duration with substantially greater decay of resistance to undetectable levels. Nevertheless, our findings are in agreement with three other studies that reported a significant association between treatment failure and minority NNRTI resistance mutations [18,30,31].

The prevalence of primary drug resistance in geographic areas where ART is common suggests that initial therapies can be significantly impacted; therefore, efforts to prevent transmission from ART-experienced persons are of great importance. The association between minority resistance and poor virologic suppression suggests a need for practical and sensitive testing to identify drug-resistant variants before treatment.

In conclusion, the data from drug-naïve persons demonstrate that sensitive testing improves detection of HIV-1 drug resistance mutations and, therefore, could be valuable not only to HIV-1 surveillance but also to ART management, particularly when treatment history is unavailable.

Table 6. Fraction of Treatment Success or Failure Versus the Presence of Detectable Minority Drug Resistance Mutations for the 316 Treatment Study Participants Evaluated (Fisher exact test, $p = 0.0038$)

Mutation Status	Treatment Success ($n = 221$)	Treatment Failure ($n = 95$)
No detectable drug resistance mutation	219 (99.1%)	88 (92.6%)
Minority drug resistance mutation	2 (0.9%)	7 (7.4%)

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Supporting Information

Figure S1. Evidence of Minority Drug Resistance Mutation Linkage and Dissociation by Mutation-Specific Amplicon Sequencing

A transmitted mutant group virus sample that had only K103N by bulk genotyping also had an unlinked minority M184V+T215N variant that was detected by the 215 PCR test. "...", nucleotides from the contiguous sequence not shown.

Found at doi:10.1371/journal.pmed.0050158.sg001 (77 KB PDF).

Text S1. Protocol: Avidity Index Testing

Found at doi:10.1371/journal.pmed.0050158.sd001 (24 KB DOC).

Text S2. Supplemental Mutation Linkage Data

Found at doi:10.1371/journal.pmed.0050158.sd002 (34 KB DOC).

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Author contributions. JAJ, ERL, and WH designed the experiments/the study. JAJ, JFL, XW, JL, DI, AS, DEB, PS, and ERL collected data or did experiments for the study. JAJ, JFL, JL, AS, MM, ERL, and WH analyzed the data. JFL, JL, and XW participated in testing, validating the samples, and modification of the method. AS participated in the description of project methods by which the specimens were collected; collection/analysis of conventional sequencing data and behavioral data; and tracking and deleting specimens that were subsequently determined to be ineligible for the study. JAJ wrote the first draft of the paper. JAJ, DI, CC, DEB, MM, PS, ERL, and WH contributed to writing the paper. DI participated in the generation and communication to the primary author of a subset of the data used for the analysis; in the identification, preparation, and shipment of samples used by the laboratory of the primary author (JAJ) for the case-control study; and in the review of the manuscript summarizing the analysis of the results. CC provided characterized samples for the study in addition to intellectual input and approval of the final version of the document. DEB and AS contributed many of the sequences used in the cross-sectional study. DEB contributed to the cross-sectional study design. PS enrolled patients and was responsible for the collection and initial drug resistance analysis of diagnostic specimens from a subset of drug-naïve individuals used in the cross-sectional study. ERL played a significant role in the design of the analysis of clinical samples/data from the efavirenz trials used for this study; provided samples and data; and assisted in the analysis and interpretation of the case-control data.

Competing Interests: JAJ and WH are named on a CDC patent application on the use of the real-time polymerase chain reaction assays presented in this article. CC and ERL were employees and stockholders in GlaxoSmithKline during the period of this study. The other authors declare no competing interests.

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Editors' Summary

Background Since the mid-1990s, several powerful antiretroviral drug combinations have been developed that have greatly improved the prognosis of HIV infection. All antiretroviral therapy (ART) regimens combine drugs that act against HIV in different ways (so-called different drug classes). Multiple drugs are necessary because HIV continually accumulates random changes (mutations) in its genetic material (genome). Some of these mutations make HIV resistant to individual antiretroviral drugs, so a mixture of drugs is needed to keep the virus in check. However, the efficacy of ART (which itself selects for drug-resistant variants by giving them a growth advantage over drug-sensitive variants) is substantially reduced when these variants account for more than about 20% of the viruses in an infected person. This level of variant virus can be detected in blood samples with a technique called bulk sequencing. In North America and Europe, where ART has been widely used for many years, around 20% of HIV-infected people who have taken ART themselves develop this level of drug-resistant virus, which can be transmitted by the same routes as nonresistant HIV (typically unprotected sexual intercourse or needle sharing). In such cases, the person acquiring drug-resistant HIV may experience treatment failure when drugs later fail to work against the resistant virus. In these countries, therefore, resistance testing by bulk sequencing is done routinely before ART is initiated to decide which antiviral drugs are likely to be effective.

Why Was This Study Done? Several years usually elapse between the time a person becomes infected with HIV and the time he or she starts ART. During this time, the absence of selection pressure from antiviral drugs means that transmitted drug-resistant variants tend to decline to levels undetectable by bulk sequencing. These "minority drug-resistant variants" can be detected using other more sensitive tests but it is not known what proportion of HIV-infected people who have never taken ART carry minority drug-resistant variants (the "prevalence" of these variants). It is also unknown whether the presence of minority drug-resistant variants reduces the success of ART. In this paper, the researchers first report a "cross-sectional" study in North America using a sensitive assay to determine the prevalence of minority drug-resistant viruses among HIV-infected people who had never received ART. They then investigate whether minority drug-resistant variants have any impact on the effectiveness of ART in a "case-control" study.

What Did the Researchers Do and Find? In their cross-sectional study, the researchers used a highly sensitive test for detecting mutations (called a real-time PCR-based assay) to look for low levels of viruses carrying any of eight major drug-resistance mutations in people with newly diagnosed HIV infection who reported no prior treatment with ART. Seventeen percent of the people who had only wild-type (nonmutated) virus by bulk sequencing (205 participants) were found, in fact, to carry low levels of virus variants with 1–3 drug-resistance mutations; 2% of them carried viruses resistant to two different drug classes (called multi-drug resistance). Among the people with resistance mutations detected by bulk sequencing (303 participants), 10% had at least one additional minority drug-resistant variant, often a viral variant that was resistant to a drug class different from that detected by bulk sequencing. In the case-control study, the researchers used their sensitive assays to measure the levels of viruses containing any of the three most common drug resistance mutations likely to affect viral responses to the antiretroviral drugs efavirenz and lamivudine in 316 people just before they started their first HIV treatment, which included these drugs. Of people for whom ART failed, 7% were infected with minority drug-resistant virus variants at baseline compared with only 0.9% of people for whom ART worked; this difference was statistically significant.

What Do These Findings Mean? The findings of the cross-sectional study indicate that conventional bulk sequencing fails to detect a large proportion of transmitted HIV drug resistance and suggest that the transmission of drug-resistant variants from infectious ART-experienced people to ART-naïve individuals might not be uncommon. The findings of the case-control study suggest that the minority drug-resistant HIV variants may have clinical consequences. That is, the presence of such variants in individuals who have not previously taken ART may reduce the efficacy of some ART regimens. However, the number of participants meeting the criteria for analysis in the cross-sectional study was limited, and the association between minority resistance and treatment failure may have been influenced by other factors. Taken together, these findings suggest that, to ensure that first-line ART is as effective as possible, greater efforts should be made to prevent HIV transmission, whether from ART-experienced or ART-naive people. However, because data on minority drug-resistant virus are limited, more studies—particularly with recent populations—are needed before testing for these variants can be considered appropriate in the clinical management of newly diagnosed HIV infection.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050158.

- This study is further discussed in a *PLoS Medicine* Perspective by Steven G. Deeks
- Information is available from the US National Institute of Allergy and Infectious Diseases on HIV infection and AIDS
- HIV InSite has comprehensive information on all aspects of HIV/AIDS, including links to fact sheets (in English, French, and Spanish) about antiretrovirals and information on genetic testing for HIV drug resistance
- NAM, a UK registered charity, provides information about all aspects of HIVand AIDS, including fact sheets on types of HIV drug, drug resistance, and resistance tests (in English, Spanish, French, Portuguese, and Russian)
- The US Centers for Disease Control and Prevention provides information on HIV/AIDS and on treatment (in English and Spanish)