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## Oligonucleotide Ligation Assay Detects HIV Drug Resistance Associated with Virologic Failure among Antiretroviral-Naïve Adults in Kenya

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### Abstract

**Background**—Transmitted drug resistance (TDR) is increasing in some areas of Africa.

Detection of TDR may predict virologic failure of first-line non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based antiretroviral therapy (ART). We evaluated the utility of a relatively inexpensive oligonucleotide ligation assay (OLA) to detect clinically relevant TDR at time of ART initiation.

**Methods**—Pre-ART plasmas from ART-naïve Kenyans initiating an NNRTI-based fixed-dose combination ART in a randomized adherence trial conducted in 2006 were retrospectively

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Conflicts of Interest

None of the authors has a conflict of interest in this study.

analyzed by OLA for mutations conferring resistance to NNRTI (K103N, Y181C, and G190A) and lamivudine (M184V). Post-ART plasmas were analyzed for virologic failure ( < 1,000 copies/mL) at 6 month intervals over 18-month follow-up. Pre-ART plasmas of those with virologic failure were evaluated for drug resistance by consensus and 454-pyrosequencing.

**Results**—Among 386 participants, TDR was detected by OLA in 3.89% [95% Confidence Interval (CI), 2.19-6.33], and was associated with a 10-fold higher rate of virologic failure [Hazard Ratio (HR), 10.39; 95% CI, 3.23-32.41;  $p < 0.001$ ] compared to those without TDR. OLA detected 24 TDR mutations (K103N,  $n=13$ ; Y181C,  $n=5$ ; G190A,  $n=3$ ; M184V,  $n=3$ ) in 15 subjects (NNRTI,  $n=15$ ; 3TC,  $n=3$ ). Among 51 participants who developed virologic failure, consensus sequencing did not detect additional TDR mutations conferring high-level resistance, and pyrosequencing only detected additional mutations at frequencies <2%. Mutant frequencies <2% at ART initiation were significantly less likely to be found at the time of virologic failure compared to frequencies  $\geq 2\%$  (22% vs. 63%;  $p < 0.001$ ).

**Conclusions**—Detection of TDR by a point mutation assay may prevent use of sub-optimal ART.

### Keywords

transmitted drug resistance; oligonucleotide ligation assay; HIV; Kenya; antiretroviral therapy

### Introduction

Access to antiretroviral therapy (ART) has substantially increased in many areas of sub-Saharan Africa over the last decade,<sup>1,2</sup> yet patients are failing ART and HIV resistance to some antiretroviral medications is increasing.<sup>3,4</sup> As a result, ART-naïve individuals are becoming infected with transmitted drug resistant virus (TDR) and failing first-line ART based on non-nucleoside reverse transcriptase inhibitors (NNRTI).<sup>5</sup> Public health officials in Africa will need to decide when resistance testing prior to ART initiation is warranted to prevent early treatment failure.<sup>6</sup>

HIV resistance testing by consensus sequencing or newer ultra-deep sequencing methods are costly and require equipment and technical capacity beyond what is accessible at many African HIV clinics. An oligonucleotide ligation assay (OLA), which is relatively inexpensive compared to conventional sequencing, has been shown to detect HIV resistance selected by single-dose nevirapine that is associated with virologic failure.<sup>7</sup> It is not known whether the implementation of OLA prior to ART initiation might decrease virologic failure in Africa.

We designed a retrospective study to describe the prevalence of TDR in Nairobi, Kenya in 2006 and to determine whether OLA-detected TDR was associated with virologic failure. In addition, we examined whether low-frequency mutations or mutations at codons not evaluated by OLA contributed to virologic failure.

## Methods

### Setting

A retrospective analysis of TDR using data and specimens collected from participants in a randomized controlled trial on adherence in Nairobi, Kenya was conducted. The design and methods of the adherence study are described elsewhere.<sup>8</sup> In brief, 400 ART-naïve individuals eligible to start ART at the Coptic Hope Center for Infectious Diseases were enrolled in 2006 and equally randomized to: adherence counseling; alarm device; combined adherence counseling and alarm device; or a control group that received neither intervention.<sup>8,9</sup> First-line ART regimen was initiated with a fixed-dose combination of nevirapine (NVP), lamivudine (3TC) and stavudine (d4T). The trial demonstrated that adherence counseling significantly increased adherence and reduced virologic failure while alarm device had no effect.<sup>8</sup>

### Study design

Sociodemographic information, adherence data, and blood samples from the adherence trial were used in this analysis. After initiating ART, participants had monthly pill counts and ART refills. Plasma HIV RNA levels (Gen-Probe, San Diego, CA, USA) and CD4 counts (FACScan, Becton Dickinson, Franklin Lanes, NJ, USA) were determined at baseline prior to ART initiation, and at 6, 12, and 18 months of ART. Virologic failure was defined as plasma HIV RNA copies > 1,000 copies/mL at least 4 months after ART initiation.<sup>10</sup>

To measure TDR, plasma collected at baseline from all participants was evaluated for NNRTI and 3TC drug resistance mutations by OLA. Participants who experienced virologic failure were evaluated for additional mutations at baseline by consensus sequencing and 454- pyrosequencing of their pre-ART plasma specimen. Plasma samples at the time of virologic failure were analyzed by OLA and consensus sequencing.

### Nucleic acid extraction and PCR amplification of HIV pol

RNA was extracted from 0.5-1mL of pre-ART plasma (NucliSENS miniMAG, bioMérieux, Durham, NC, USA). After reverse transcription (Superscript III, Invitrogen, Grand Island, NY, USA) with random hexamers, HIV *pol* spanning codons 1-239 of RT was amplified (MyTaq DNA Polymerase, Bionline USA Inc, Taunton, MA, USA) by nested polymerase chain reaction (PCR) (1<sup>st</sup> round primers; forward: GARAGACAGGCTAATTTTTAGGGA, and reverse: AAYTTCTGTATATCATTGACAGTCCA; 2<sup>nd</sup> round primers forward: CAAATCACTCTTTGGCARGACC and reverse: CAYTTGTCAGGATGGAGTTCATA).

### Oligonucleotide ligation assay (OLA)

Amplicons from baseline and virologic failure were tested by an OLA for point mutations conferring resistance to NNRTI (K103N, Y181C and G190A) and 3TC (M184V).<sup>11,12</sup> The OLA used probes optimized for subtypes A, D and C prevalent in Kenya. Standards of plasmids mixed to contain 0%, 2%, 5%, 10%, 25%, 50%, 75% or 100% mutant in a wild-type background were included in each assay plate, and comparisons of the optical densities

of each specimen to those of the standards was used to quantify the proportion of mutant in the HIV population.<sup>13</sup>

### Dideoxy-nucleotide consensus sequencing and parallel 454-pyrosequencing

Amplicons from individuals with virologic failure underwent dideoxy-nucleotide consensus sequencing to identify mutations both at baseline and at the first time point when virologic failure was detected (GenBank sequence accession numbers: KF544089-KF544288, KJ395348 and KJ395349).<sup>14,15</sup> Massive parallel 454-pyrosequencing was performed on baseline plasma samples of individuals with virologic failure and/or OLA-detected mutations. After reverse transcription of plasma HIV RNA as described above, the cDNA was quantified by HIV LTR real-time PCR.<sup>16</sup> Approximately 1,000 templates of amplifiable HIV were submitted to a median of three nested PCR (range, 1 to 20 reactions) with subsequent pooling of amplicon. First round PCR used the primers described above and FastStart High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). Subsequently, two regions of HIV reverse transcriptase were amplified in separate second round PCR with 454-barcoded-fusion primers (454 Life Sciences, Roche Diagnostics Corporation, Branford, CT, USA), with 14 discrete multiplex identifiers (MID) or barcodes:

Amplicon 1 (RT codons 21-134)

Forward: CGTATCGCCTCCCTCGCGCCATCAG-  
MIDGTTAAACAGTGGCCATTGACAGA

Reverse: CTATGCGCCTTGCCAGCCCGCTCAG-  
MIDACTAGGTATGGTGAATGCAGTATA

Amplicon 2 (RT codons 150-242)

Forward: CGTATCGCCTCCCTCGCGCCATCAG-  
MIDCACAGGGATGGAAAGGATCAC

Reverse: CTATGCGCCTTGCCAGCCCGCTCAG-  
MIDCTGGACTGTCCATYTGTCAGGATG

Amplicons were visualized on agarose gels, purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany), and quantified using a Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). Pools containing barcoded amplicons from 14 subjects, diluted to  $1 \times 10^7$  molecules/ $\mu\text{L}$ , underwent emulsion PCR and 454-sequencing using a 2-region gasket and the GS FLX Titanium System per manufacturer's instructions (454 Life Sciences, Roche Diagnostics Corporation, Branford, CT, USA).

The output was processed through a pipeline aligning sequences to a subtype-specific consensus reference.<sup>17</sup> Nucleotide frequencies at each position were calculated in forward, reverse and overall reads to determine the frequency of mutants at codons conferring resistance to NRTI and NNRTI as defined in the Stanford Database.<sup>14</sup>

Errors introduced by PCR and 454-sequencing were estimated by including amplicon of an HIV-1 subtype A plasmid in each 454-sequencing plate. A control for errors in reverse transcription was not included. The limit of mutant quantification was estimated based on the number of HIV templates from each specimen submitted to the assay and the error rate observed across 454-sequencing of the plasmid. Testing of a median of 1,000 (range 275-1,300) HIV templates per specimen by pyrosequencing and an average substitution error rate of 0.06  $\pm$  0.01% SD observed in the plasmid control set the limit of mutant detection at  $>0.1\%$ .

### Statistical methods

Wilcoxon rank sum and Fisher's exact tests were used to compare continuous and categorical characteristics between those with and without TDR. Among those who initiated ART, time to virologic failure was estimated using Kaplan-Meier (KM) analysis and groups with and without TDR were compared using a log-rank test. Participants were censored if they died or were lost to follow-up before the 18-month study endpoint.

A discrete time Cox proportional hazards regression model assessed differences in rates of virologic failure. A preliminary model included TDR and randomization assignment. Adjusted models also included baseline characteristics associated with TDR and virologic failure, as well as age, employment status, baseline viral load, randomization assignment, and adherence. Adherence was examined as a time-varying covariate as average adherence in the prior 6 months.

Mutant frequency ( $<2\%$  versus  $\geq 2\%$ ) detected by pyrosequencing at baseline was assessed for subsequent detection at viral failure by consensus sequencing using generalized estimating equations (GEE) models with binomially-distributed errors, log link, and exchangeable correlation matrix with robust errors. Among the subjects with virologic failure, linear regression was used to assess time to viral failure between no (0%), "very low" ( $>0\%$  to  $<2\%$  frequency), and "low-high" ( $\geq 2\%$ ) levels of mutations by pyrosequencing. Stata SE v12 (Statacorp, College Station, TX, USA) was used in all analyses; significance tests were two-sided, with  $\alpha=0.05$ .

### Ethical review

The study protocol was reviewed and approved by the institutional review boards at the University of Washington (Seattle, WA, USA), Seattle Children's Hospital (Seattle, WA, USA), and Kenyatta National Hospital (Nairobi, Kenya).

## Results

### Study population

Of 1,096 adults eligible to initiate ART at the Hope Center in 2006, 437 qualified for the study and 400 were enrolled (Figure 1). Of the 400 enrollees, 386 had baseline pre-ART plasma samples tested by OLA and 356 participants initiated ART and were assessed for virologic failure. Prior to initiating ART, 22 participants were lost to follow-up and 8 died.

A total of 307 participants (86%) completed 18 months of study follow-up. Between ART initiation and the 18-month endpoint, 21 participants were lost to follow-up and 28 died.

### Transmitted drug resistance and virologic failure

Among the 386 participants evaluated at baseline, 15 had one or more mutations detected by OLA for a TDR prevalence of 3.89% [95% Confidence Interval (CI), 2.19-6.33]. The median age among those with TDR was 37 years [Interquartile Range (IQR), 32-43], 60% were female, 47% were married, and the median CD4 count was 100 cells/mm<sup>3</sup> (IQR, 23-155) (Table 1). Compared to those without TDR, those with TDR were significantly more likely to have had more lifetime sexual partners (median, 7 vs. 4;  $p=0.004$ ), never attended school (13% vs. 2%;  $p=0.044$ ), and been casual laborers (33% vs. 13%;  $p=0.045$ ). CD4 count, plasma HIV-1 RNA, and randomization assignment did not differ between those with and without TDR.

Among the 356 participants who initiated ART, 13 (3.65%) had TDR detected by OLA and 51 (14.3%) experienced virologic failure. Overall, the cumulative probability of virologic failure at 18 months was 0.16 (95% CI, 0.12-0.21). The cumulative probability of virologic failure among those with TDR was significantly higher (0.70; 95% CI, 0.42-0.93) compared to those without TDR (0.14; 95% CI, 0.11-0.19) (Figure 2). Characteristics associated with TDR in this subset were similar to those in the overall cohort. Those with TDR experienced virologic failure at a rate 7.99 times (95% CI, 2.95-21.64;  $p<0.001$ ) higher than those who did not have TDR. In a model adjusting for age, employment status, baseline viral load, adherence, and randomization assignment, the risk of virologic failure associated with TDR was 10.39 (95% CI, 3.23-32.41;  $p<0.001$ ).

### Detection of baseline mutations by OLA

OLA, sensitive to a mutant frequency of 2%, detected 24 mutations in 15 participants at baseline. These 15 had mutations associated with resistance to NNRTI and 3 also had mutations associated with resistance to 3TC (Figure 3). The median mutant frequency in each subject's viral population was 67% (range, 2%-100%).

Of the 15 participants who had mutations detected by OLA at baseline, virologic outcome could not be ascertained for 5 participants; 3 died and 2 were lost before viral load was measured. Of these 5 participants, 3 participants had a single mutation (K103N), 1 participant had 2 mutations (K013N and M184V), and 1 participant had 3 mutations (K103N, Y181C, and G190A). Among the 10 with virologic outcome, 7 experienced virologic failure: 3 of these participants had single mutations (K103N,  $n=2$ ; Y181C,  $n=1$ ); 2 participants had 2 mutations (K103N and G190A,  $n=1$ ; Y181C and G190A,  $n=1$ ); and 2 participants had 3 mutations (K103N, M184V, and Y181C). The median mutant viral load in the pre-ART specimen of those who failed was 536,167 copies/mL (range, 12,976 – 2,128,896 copies/mL). Three participants with mutations detected by OLA at baseline did not experience virologic failure after 18 months on ART and each had only a single mutation (K103N); their mutant viral loads were 112,316, 642,240, and 585,469 copies/mL.<sup>18</sup>

Among the 7 participants who had mutations detected by OLA at baseline and experienced virologic failure, all failed by month 6 (the first time point evaluated after ART initiation) and 6 of the 7 had adherence  $\geq 95\%$ .

### **Detection of baseline mutations among virologic failures by consensus and pyrosequencing**

Of the 51 participants who experienced virologic failure, consensus sequencing detected 23 mutations in 10 participants and pyrosequencing detected 56 mutations in 24 participants at baseline (Figure 3). Consensus sequencing confirmed baseline mutations in 6 of the 7 participants identified by OLA, and detected baseline mutations at sites associated with “potential low-level resistance” to didanosine and abacavir or “low-level resistance” to zidovudine and stavudine in 4 additional participants at codons not evaluated by OLA (T215T/I, n=1; T69S, n=1; T69T/N, n=2) (hivdb.stanford.edu).

In addition to the 7 participants who had mutations identified by OLA, pyrosequencing detected 17 additional participants with either very low (<2%) frequency mutations at the codons tested by OLA (M184V, K103N, Y181C, G190A), or mutations (frequencies 0.1%-100%) in other codons conferring drug-resistance. Eleven individuals had mutations at the OLA codons, all at frequencies <2%. The remaining 6 individuals had mutations at non-OLA codons, all at frequencies <2%, except for 3 individuals: one with L210W detected at a frequency of 5.9%, and two with T69N detected; one at a frequency of 22.5% and the second at 25.0% (Figure 3).

### **Detection of baseline mutations at virologic failure**

Of the 13 mutations detected in 7 participants by OLA at baseline, OLA detected 11 (85%) and consensus sequencing detected 10 (77%) at the time of virologic failure. Two mutations were detected by OLA at baseline but not at failure. In one participant, K103N at a frequency of 5% co-existed with G190A; only the latter was detected at failure along with Y181C and M184V. In another, Y181C was detected at baseline with additional mutations, and at failure it had shifted to Y181V (Figure 3). Of the mutations classified as “potential low-level resistance” or “low-level resistance” in the Stanford HIV Drug Resistance Database (hivdb.stanford.edu), none were detected by consensus sequencing at virologic failure.

Mutant variants detected in the pre-ART plasma at a frequency of <2% by pyrosequencing were significantly less likely than those >2% frequency to be found by consensus sequencing at the time of virologic failure. Of the 32 mutations detected by pyrosequencing at baseline at frequency <2%, 7 (22%) were detected by consensus sequencing at the time of virologic failure. Of the 24 mutations at frequency  $\geq 2\%$ , significantly more, 15 (63%), were found at virologic failure (63% vs. 22%;  $p<0.001$ ). This was also true when restricted to codons evaluated by OLA (K103N, Y181C, G190A, and M184V). Among the 13 mutations detected in “OLA codons” by pyrosequencing (and OLA) at frequency  $\geq 2\%$ , 10 (77%) were detected by consensus sequencing at virologic failure compared to 5/14 (36%) at baseline frequencies <2% (77% vs. 36%;  $p=0.049$ ).

### Baseline mutant frequency and time to virologic failure

When examining OLA codons (K103N, Y181C, G190A, and M184V), the mean time to virologic failure was significantly earlier among participants with baseline mutation frequencies  $\geq 2\%$  by pyrosequencing compared to those with minority mutations ( $>0\%$  to  $<2\%$ ) ( $p=0.002$ ) or no mutations ( $0\%$ ) ( $p=0.008$ ) (Figure 4). In an analysis adjusting for age, employment status, baseline viral load, adherence, and randomization assignment, participants with mutant frequencies  $\geq 2\%$  experienced virologic failure 9.6 months earlier compared to those with mutational frequencies between  $0-2\%$  ( $p=0.001$ ) and 7.0 months earlier compared to those with no mutations ( $p=0.004$ ). There was no significant difference in time to virologic failure between participants with resistance mutations present at frequencies  $<2\%$  compared to those with no mutations detected ( $p=0.194$ ).

### Discussion

In this analysis of 386 ART-naïve adults initiating ART in Nairobi, Kenya in 2006, we found a TDR prevalence of 3.9%. TDR was determined using OLA that detected mutations conferring high-level resistance to NNRTI and 3TC and was significantly associated with virologic failure in this study. Those with TDR detected by OLA at one or multiple codons (M184V, K103N, Y181C, and G190A) were 10 times more likely to experience virologic failure than those without TDR. This evidence suggests that OLA performed prior to ART initiation may guide antiretroviral prescribing practices to reduce rates of virologic failure.

The detection of TDR by this study in Nairobi, Kenya is consistent with increased prevalence of antiretroviral resistance found in many resource-limited settings since the widespread rollout of ART.<sup>3</sup> To our knowledge, this study is the largest single site cohort study of TDR detected among ART-naïve patients in East Africa. While the prevalence of 3.9% is less than 13.2% found among 68 Kenyans in Mombasa in 2009-10,<sup>19</sup> 7.5% among 53 in Nairobi in 2005,<sup>20</sup> and 4.5% among 200 in Nairobi in 2008-9,<sup>21</sup> it is greater than 1.1% found among 182 Kenyans in Kilifi in 2008-10 and 3.1% among 76 across Kilifi, Nairobi, and Mtwapa in 2006-9.<sup>22,23</sup> In relation to these figures, the study's TDR prevalence is low given the clinic's urban setting where ART coverage is relatively high. This may be due to enrollment of only ART-naïve adults, examination of antiretroviral resistance at only 4 codons, and testing of specimens from 2006. It is expected that TDR prevalence will continue to increase paralleling expansion of ART and evolving recommendations for earlier initiation of treatment.<sup>24</sup>

The association between the detection of TDR and virologic failure is not new and has been noted in both resource-rich and constrained settings.<sup>25-27</sup> However, detection of TDR by OLA, a point mutation resistance test, is novel. Inexpensive antiretroviral resistance testing is needed in resource-limited settings, where most HIV patients cannot afford resistance testing by consensus sequencing. OLA may be as much as 10-20 times less expensive than consensus sequencing, and has been successfully implemented by laboratories in resource-limited countries.<sup>12,28</sup>

Consensus sequencing and pyrosequencing at baseline did not appear to contribute more information than OLA. Among those who experienced virologic failure, consensus



sequencing did not detect additional subjects with pre-ART mutations associated with “high-level resistance” beyond those identified by OLA testing (hivdb.stanford.edu). In addition, the baseline mutants detected by consensus sequencing were not selected at virologic failure, and suggests that these mutations may not have been clinically relevant in these subjects.

These data suggest that the codons tested by OLA were appropriate for this setting and time period. Baseline mutations detected by pyrosequencing but not by OLA were primarily at low frequencies (<2%) in participants’ viral populations, and were significantly less likely to be selected at the time of virologic failure compared to those at a frequency 2%, which were detectable by OLA. Given this finding as well as the shorter time to virologic failure among those with mutant frequencies 2%, it appears that baseline mutations at low frequencies <2% did not significantly contribute to virologic failure.

Weaknesses in this study include the limited number of samples tested by consensus sequencing and pyrosequencing due to cost constraints. Although consensus sequencing was not performed on all samples, detection of TDR by consensus sequencing probably would have been just as predictive of virologic failure as OLA. Although the prevalence of TDR was reported, the study is likely to have underestimated the actual prevalence, as a comprehensive survey of all drug resistance codons was not performed since our primary focus was to evaluate the utility of the OLA to predict virologic failure. Based on consensus sequencing and pyrosequencing, OLA did not appear to miss the detection of mutations associated with virologic failure. However, additional codons will be needed to screen for TDR by OLA when antiretroviral regimens change within a community. Setting the limit of mutant detection by pyrosequencing at >0.1% may be considered too low. While the 0.1% estimate accounts for errors introduced by PCR and pyrosequencing, it does not account for those introduced by reverse transcription. We chose to report the detection of mutations detected above the error rate of our plasmid controls to allow the reader to compare detection of mutations around the cutoff of OLA. Samples collected at 6-month intervals may not have captured the exact time of virologic failure, and among participants with poor adherence to antiretrovirals, the absence of selective pressure may have allowed resistance mutations to be outcompeted by wild type viruses with greater replication capacity. Additionally, our testing of pre-ART plasma may not have accurately measured the rate of TDR in this cohort. It is possible that HIV infection was founded by drug-resistant variants in some participants, but in the time interval between acute infection and collection of the pre-ART specimen these mutants could have reverted to wild type variants.

The increasing rate of TDR observed in Africa could threaten efficacy of first-line ART. Cost-effective strategies to contain drug-resistant HIV are needed for resource-poor countries. This study suggests that pre-ART testing for HIV drug resistance with a point mutation assay, which is less expensive than conventional sequencing, may be a viable strategy to help preserve the efficacy of first-line ART. Among those with >95% adherence, detection of TDR by OLA at ART initiation and subsequent regimen modification would have averted 6/13 (46%) virologic failures at 6 months follow-up (data not shown). Further development of simple, inexpensive assays to test for multiple drug-resistance mutations appears warranted, as do feasibility and cost-effectiveness studies of these assays in low-resource communities.

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M Chung designed and implemented the study, supervised the on-site data management, interpreted the data, and wrote the paper. L Frenkel led the laboratory analysis, designed the study, interpreted the data, and wrote the paper. I Beck conducted the laboratory analysis, interpreted the data, and helped write the paper. S Dross conducted the laboratory analysis, interpreted the data, and helped write the paper. K Tapia analyzed the data and helped interpret the results. J Kiarie helped implement the study, oversee data collection, and helped write the paper. B Richardson analyzed the data and interpreted the results. J Overbaugh helped conduct and analyze the laboratory data and write the paper. S Sakr helped implement and design the study. G John-Stewart helped interpret the data and write the paper.

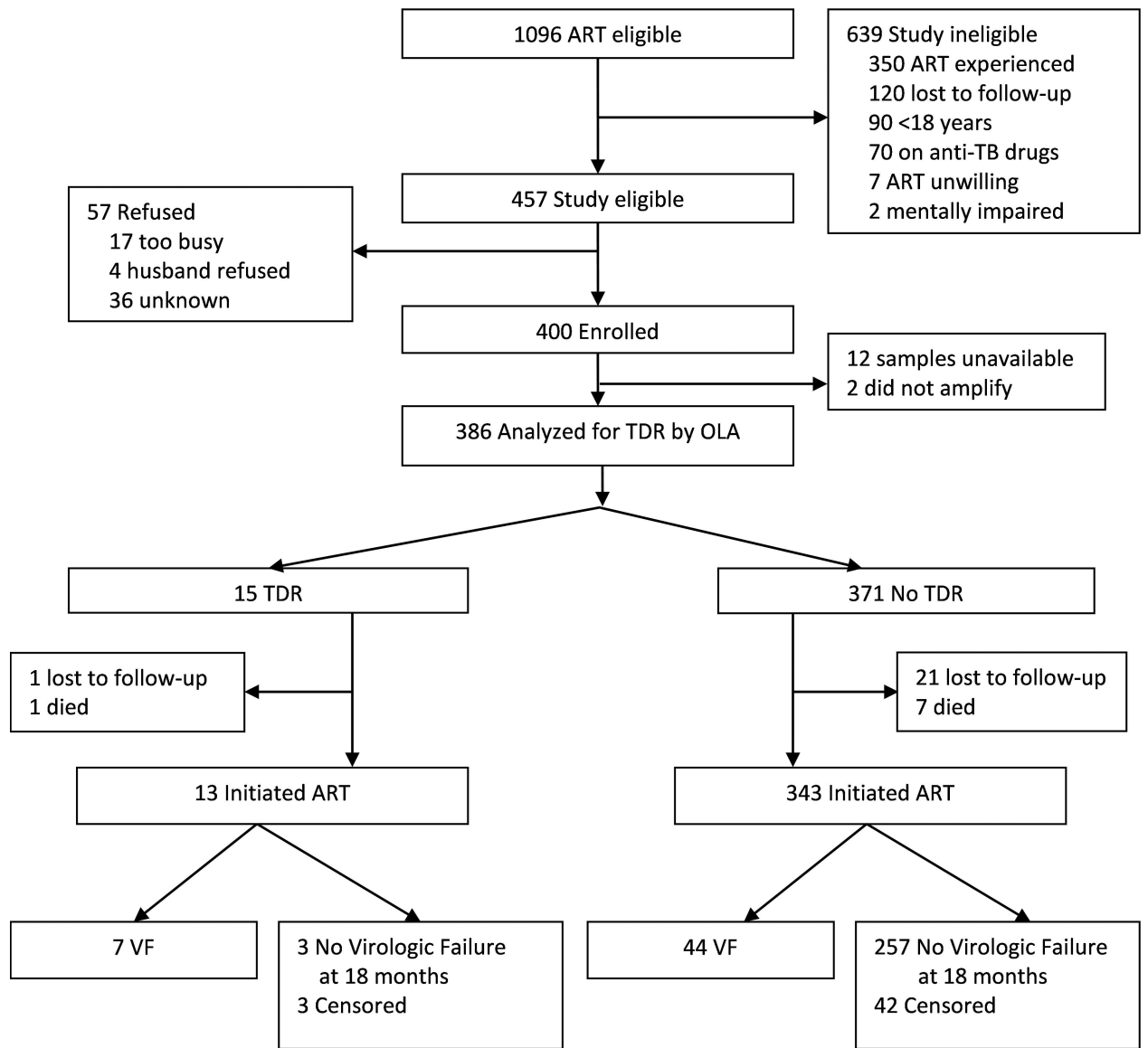
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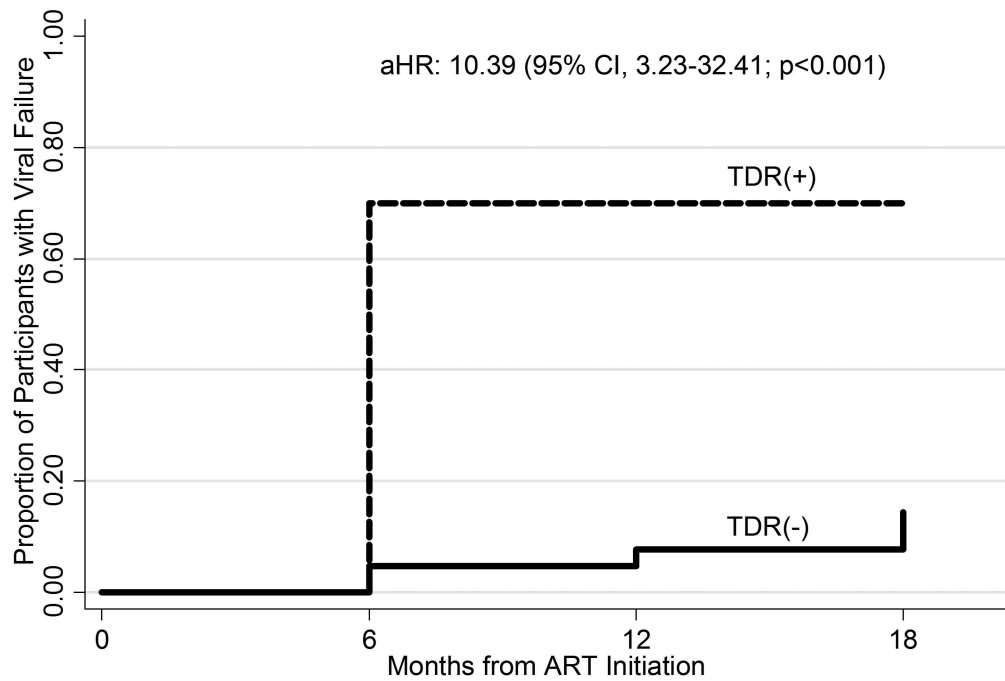
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**Figure 1.**  
Trial Profile



TDR(-): 343	320 (15)	288 (9)	277(20)
TDR(+): 13	10 (7)	3 (0)	3 (0)

**Figure 2.** Kaplan-Meier curve of time to virologic failure between those with presumed transmitted drug resistance (TDR) detected in codons M184V, K103N, Y181C, and/or G190A by oligonucleotide ligation assay (OLA) (dashed line) and those without TDR by OLA (solid line).

Note: The number of participants at risk at each time point is listed and the number experiencing virologic failure is in parentheses.

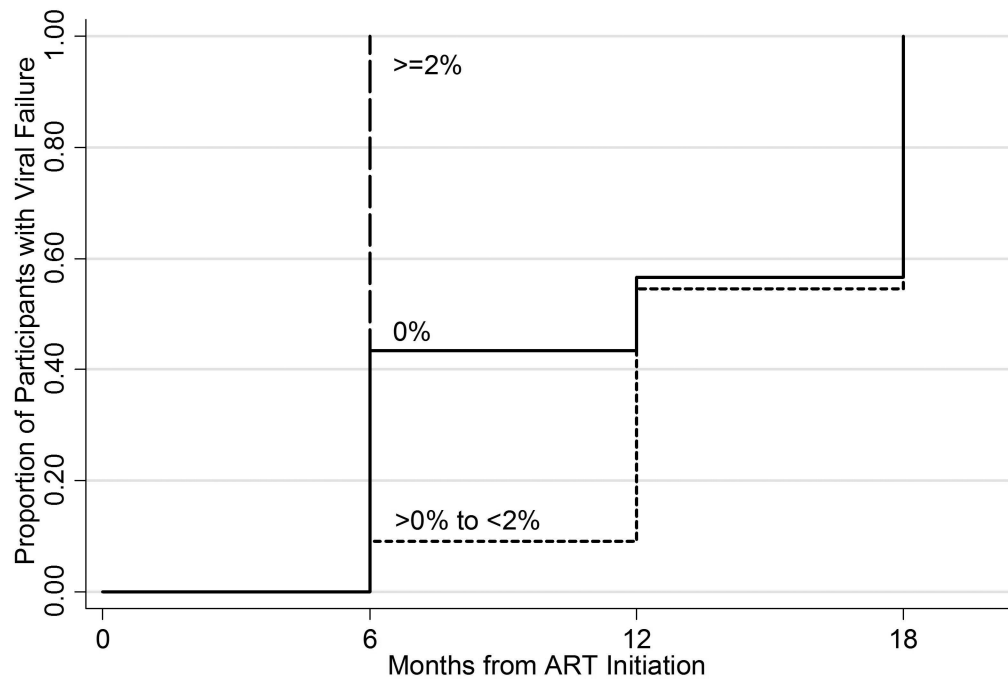
PID	Time to VF	Mutations assayed by OLA, pyrosequencing and CS										Mutations detected by pyrosequencing and/or CS at codons not evaluated by OLA																
		M184V		K103N		Y181C		G190A		M41L		K65R		D67N		K70R		L74V		L210W		T215Y/F		K219E/Q		Infrequent Mutants		
		Pre-ART O-P-CS	VF O-CS	Pre-ART O-P-CS	VF O-CS	Pre-ART O-P-CS	VF O-CS	Pre-ART O-P-CS	VF O-CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	Pre-ART P-CS	VF CS	
Subjects with high proportion of high-level resistance mutations Pre-ART; with virologic failure on ART																												
113	6	54	100	8	6	52	100	1.3																			22 V106A	
199	6	97	100	99	100	98	ind		49	M41L			96.3	D67N			39.7			98	L210W	100	T215Y					Y181V
401	6					43	86	41	5																		53.8 K101E	H221Y
184	6		100	5					15	50	83																	
228	6		100	16	100																							
402	6			100	100																							
Subjects with low proportions of high- and low-level resistance mutations Pre-ART; with virologic failure on ART																												
169	6		92			9	100																				1.8 T69N	T69N
253	6		41			100															5.9							P225H
168	6		99				42	12				0.35																
375	6		100	0.23	100																							
234	6		87				100						K65KR														22.5 T69N	
267	12	0.13				79	0.13										0.14											
492	12	0.27	100			100										0.15												
325	12	0.17				78										0.13												
404	12		100	1				100																				
499	12		100	0.54	100											0.11												
371	18	0.28	100			100	0.14								0.23													
342	18			0.11	98																							
276	18						14																				1.2 V108I	V108IV
298	18	0.26			21			3				0.11															25 T69N	
315	18	0.25																									T69S	
283	18	0.93														0.23												
344	18								0.16					0.17														
155	18															0.11												
Subjects with no resistance mutations Pre-ART; with virologic failure on ART																												
450	6						100	97					K65R															
225	6		99			90	ind																					
441	6					7		44																				
128	12		100				98																					
224	12		100			100																						
158	12					14		85										L74V									T215IT	
495	18		99				100																					K101E
248	18		100				100																					L100LQ
391	18					99																						
451	18					32		17	5																			
466	18						3																					
Subjects with high proportion of high-level resistance mutations Pre-ART; without virologic failure >1000 HIV copies/mL																												
360	no VF			79	100		ind	99.5	M41L																		41 T69N	G190E
292	no VF	NT		90	NT		NT																			0.17		NT
264	no VF	NT		86^	NT		NT																					NT

**Figure 3.**

Drug resistance mutations detected by OLA, pyrosequencing and consensus sequencing at baseline, and by OLA and consensus sequencing at virologic failure.

Note: Pre-ART specimens were tested by OLA (O), pyrosequencing (P), and consensus sequencing (CS); virologic failure (VF) specimens were tested by OLA and CS only.

Numbers indicate the % mutant virus quantified by OLA (bold font, limit of detection = 2%) or pyrosequencing (regular font, limit of detection 0.1%). Dark grey indicates mutation detected by all methods evaluated. Light grey indicates mutation not detected by consensus sequencing, except as indicated for subject #264. Numbers with no shading indicate mutations detected by pyrosequencing only. NT = not tested (due to undetectable HIV viral load). ind = indeterminate result by OLA due to the presence of different mutation. ^ = mutation detected by OLA and consensus sequencing but not by pyrosequencing due to sequence polymorphisms in the region of the pyrosequencing PCR primers.



0%:	30	30 (13)	17 (4)	13 (13)
>0% to <2%:	11	11 (1)	10 (5)	5 (5)
≥2%:	7	7 (7)	0 (0)	0 (0)

**Figure 4.**

Kaplan-Meier curve of time to virologic failure in participants grouped by whether or not M184V, K103N, Y181C, and/or G190A was detected in baseline (pre-ART) viral population, and if detected, the proportion of the mutant population: 0% (solid line), >0% to <2% (short dashed line), and ≥2% (long dash line).

Note: The number of participants at risk at each time point is listed and the number experiencing virologic failure is in parentheses.

**Table 1**

Baseline comparison between those with and without transmitted drug resistance (TDR) detected by oligonucleotide ligation assay (OLA) in pre-ART blood plasma.

Characteristic	TDR (N=15)	No TDR (N=371)	p-value
	n (%) or Median (IQR)	n (%) or Median (IQR)	
Age (years)	37 (32-43)	36 (30-42)	0.45
Female	9 (60)	246 (66)	0.59
Married or attached	7 (47)	192 (52)	0.80
Education (years)	11 (8-12)	12 (8-13)	0.19
Education			0.030 (overall)
None	2 (13)	7 (2)	0.04
Primary	4 (27)	124 (34)	0.78
Secondary	8 (53)	144 (39)	0.29
College	1 (7)	93 (25)	0.13
Employment			0.05 (overall)
Salaried job	1 (7)	117 (32)	0.045
Self-employed	4 (27)	82 (22)	0.75
Casual laborer	5 (33)	49 (13)	0.045
Unemployed	5 (33)	121 (33)	1.00
Rooms in house	2 (1-4)	2 (1-4)	0.76
Rent	2500 (570-7000)	2000 (1000-4500)	0.87
People in house	4 (1-6)	4 (2-5)	0.80
Flush toilet	8 (53)	173 (47)	0.79
Cost of travel to clinic US\$0.70	8 (53)	231 (62)	0.59
Distance to clinic (km)	9.5 (6.0-13.9)	10.3 (6.2-14.9)	0.64
Age at first sex (years)	17 (16-20)	18 (16-20)	0.76
Lifetime sexual partners	7 (4-10)	4 (2-6)	0.004
Money or favors for sex	3 (20)	35 (9)	0.18
Plasma HIV log <sup>10</sup> copies/ml	5.85 (5.12-6.20)	5.67 (5.24-6.07)	0.70
CD4 count, cells/ml	100 (23-155)	116 (61-183)	0.29
Randomization assignment			0.18 (overall)
Counseling	1 (7)	94 (25)	0.13
Alarm	7 (47)	91 (25)	0.07
Counseling and alarm	3 (20)	93 (25)	1.00
Control	4 (27)	93 (25)	1.00

Median (IQR) is presented unless otherwise specified