Transmitted HIV Resistance to First-Line Antiretroviral Therapy in Lima, Peru

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Abstract

Transmission of drug-resistant HIV (TDR) has been associated with virologic failure of “first-line,” non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy (ART). A national ART program began in Peru in 2004. We evaluated the prevalence of TDR in individuals initiating ART and their virologic outcome during 2 years of ART. HIV-infected, ARV-naïve subjects who met criteria to start ART in Lima, Peru were enrolled in a longitudinal observational study between July 2007 and February 2009. Blood plasma and cells obtained prior to ART initiation were assessed for antiretroviral (ARV) resistance by an oligonucleotide ligation assay (OLA) sensitive to 2% mutant at reverse transcriptase (RT) codons K103N, Y181C, G190A, and M184V and a subset by consensus sequencing. A total of 112 participants were enrolled; the mean CD4 was 134 ± 89 cells/ μ l and the median plasma HIV RNA was 93,556 copies/ml (IQR 62,776–291,364). Drug resistance mutations conferring high-level resistance to ARV were rare, detected in one of 96 (1%) evaluable participants. This subject had the Y181C mutation detected in both plasma and peripheral blood mononuclear cells (PBMCs) at a concentration of 100% by OLA and consensus sequencing; nevertheless nevirapine-ART suppressed her viral replication. Consensus sequencing of 37 (19%) participants revealed multiple polymorphisms that occasionally have been associated with low-level reductions in ARV susceptibility. A low prevalence of TDR was detected among Peruvians initiating ART. Given the increasing availability of ART, continuing surveillance is needed to determine if TDR increases and the mutant codons associated with virologic failure.

Introduction

AS ACCESS TO ANTIRETROVIRAL therapy (ART) has increased, the transmission of drug-resistant HIV has become more prevalent.^{1–9} The acquisition of drug-resistant HIV has been associated with an increased risk of failure with first-line therapy in some^{10–14} but not all studies.^{15–17} In Peru, ART has been part of the national strategy since 2004,¹⁸ through a government-supported treatment program based on World Health Organization guidelines.^{19,20} In 2009, the estimated number of persons living with HIV in Peru was 75,000 (estimation range 58,000–100,000),²¹ the majority with subtype B. As of July 2010, 15,216 Peruvians were receiving ART, of whom 12,193 were receiving the first-line combination of zidovudine (ZDV) or stavudine (d4T), lamivudine (3TC), and efavirenz (EFV) or nevirapine (NVP) (J. Arevalo, personal communication). The current policy is to initiate

ART without testing for drug resistance. Protease inhibitor-based therapy is a second-line treatment and is available. However, additional ART options are very limited. Consequently, surveillance for transmitted drug-resistant (TDR) HIV is important for guiding strategic decisions to maximize benefits of the national ART program.

The most common method used to detect drug resistance is consensus sequencing,²² which detects resistant viruses that comprise more than 20–50% of the viral population.^{23,24} Thus, when used for detection of TDR, consensus sequencing can detect pure populations of mutant virus, or mixed populations where the fitness of the mutant virus is similar to that of the wild-type virus. Since wild-type HIV is often more fit than drug-resistant virus, an acquired resistant viral strain may persist at low concentrations in two scenarios: (1) when wild-type virus evolves from drug-resistant HIV in the absence of ARV pressure,²⁵ or (2) when both mutant and wild-type virus

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were transmitted and over time the wild-type virus outgrows the mutant.²⁶ In these instances, the majority of the circulating virus would be wild-type, with resistant virus archived in peripheral blood mononuclear cells (PBMCs) at concentrations that may not be detected by consensus sequencing.^{22,27} More sensitive assays, such as the oligonucleotide ligation assay (OLA), can detect low concentrations of drug-resistant virus to a substantially greater degree compared to consensus sequencing.^{28–36}

In this study we evaluated the prevalence of TDR using a sensitive OLA assay in a population of ARV-naïve Peruvian patients. Four drug-resistant codons were selected to screen for transmitted drug resistance based on the drug regimen being used as first-line ART in Lima, Peru, and the prevalence of transmitted resistance mutations observed in ARV-naïve populations living in communities that primarily use non-nucleoside reverse transcriptase inhibitor (NNRTI)-based ART.^{37,38} TDR was correlated to plasma HIV RNA over 2 years of treatment to determine whether mutations were associated with virologic failure of ART.

Materials and Methods

Study design and population

HIV-infected, ARV-naïve subjects referred to the Hospital Dos de Mayo in Lima, Peru who met criteria to start ART according to Peruvian national guidelines (<200 CD4 cells/ml or clinical AIDS) were eligible to enroll in a longitudinal observational study of HIV genital shedding between July 2007 and February 2009, as approved by the Seattle Children's Hospital Institutional Review Board in Seattle and the Hospital Nacional Dos de Mayo Comité de Ética in Lima. Participants had to be HIV infected, planning to receive care at the clinic for the subsequent 2 years, and willing to provide genital fluid samples (semen for men, cervicovaginal fluid for women) at quarterly examinations. After signing informed consent participants' blood was collected and subjects were prescribed ZDV or d4T, 3TC, and EFV or NVP. Subjects were seen quarterly for 2 years with blood collected at each visit. Plasma HIV RNA was determined quarterly using the COBAS Taqman system and CD4 cells counts biannually using standard flow cytometry as part of routine monitoring for the Peruvian national ART program. Virologic failure was defined as plasma HIV RNA concentration >1000 copies/ml after 3 months of ART, confirmed by testing a subsequent specimen.

Specimen processing

At each study visit 14 ml of blood collected into EDTA tubes was separated into plasma and PBMCs using Ficoll (GE Healthcare, Piscataway, NJ) and frozen at -80°C . Frozen specimens were shipped to Seattle, Washington on a quarterly basis. Nucleic acids were extracted from 500 μl of plasma³⁹ and HIV RNA was quantified.⁴⁰ DNA was extracted from the PBMCs using the PureGene Cell and Tissue Kit (Gentra Systems Inc., Minneapolis, MN) following the manufacturer's instructions.

Oligonucleotide ligation assay

The HIV *pol* gene was amplified from plasma cDNA and PBMC DNA.^{29,31} A minimum of 1000 copies of HIV RNA

from plasma and 4 μg of PBMC DNA was submitted for polymerase chain reaction (PCR). The PCR product from each sample was subsequently assessed by OLA for mutations in RT codons K103N, Y181C, G190A, and M184V.^{29–31} Briefly, PCR amplicons were added to a ligation reaction containing three oligonucleotide probes; two of these are complementary to either the mutant or wild-type codon at the 3' end and labeled at the 5' end with either fluorescein (mutant specific) or digoxigenin (wild-type). The third (common) oligonucleotide probe is biotinylated at the 3' end and anneals adjacent to the base of interest. Ligation occurs only if the two bases abutting the ligation site are complementary to the amplicon, which provides specificity to the assay. Depending on the subject's viral sequence either the mutant or wild-type probe is ligated to the common probe. An ELISA, with alkaline phosphatase (AP) and peroxidase-labeled antibodies to fluorescein and digoxigenin, indicates the mutant and wild-type codons, respectively.⁴¹ The enzymes are developed using a Yellow substrate (Sigma, St. Louis, MO) for the mutant or tetramethylbenzidine (TMB) One solution (Fisher, Pittsburgh, PA) for the wild-type codon.

All participant's specimens and assay controls were analyzed in duplicate. The presence and quantity of resistant virus were assessed using standards with mixtures of mutant and wild-type plasmids (0%, 2%, 5%, 10%, and 100% mutant) on each OLA plate. As little as 2% mutant can be detected when at least 100–200 copies of HIV are submitted to the assay.^{29,31} Participants' samples with optical densities (OD) exceeding that of the 2% mutant control were considered positive for mutant at codons 103, 181, and 190, and those exceeding the 5% mutant control were considered positive for mutant at codon 184. Reactions negative for mutant and with a wild-type OD less than 50% of the 100% wild-type control were considered indeterminate. In preliminary studies, sequencing of indeterminates identified patterns of polymorphisms in the Peruvian specimens in the regions of our standard subtype B probes for codons 103 and 181. Modified K103N and Y181C wild-type probes were combined with the standard probes, creating mixtures of -AAA/-AAG at the 3' end of K103N and -CTA/-TTA at the 3' end of Y181C, which resolved most indeterminate reactions.^{29,41} The amplicons that produced indeterminate OLA reactions from the plasma or PBMCs underwent consensus sequencing to identify mutations near the ligation site that interfered with annealing of one or both probes, and specimens with a resistant codon by OLA were sequenced to look for additional mutations, as we hypothesized based on our experience that individuals with one mutant would be more likely to harbor additional drug-resistant mutants.

Consensus sequencing

Consensus sequencing spanned *pol* that encoded RT amino acids 2596–3243 based on reference sequence HXB2. Amplicons that either produced indeterminate OLA results or detected drug resistance mutations underwent conventional dideoxynucleotide consensus sequencing.^{29,31} The Stanford HIVseq Sequence Analysis Program, version 3.2^{42,43} (<http://sierra2.stanford.edu/sierra/servlet/JSierra>) was used to identify polymorphisms and mutations within the sequences. As part of routine quality assurance, sequences were aligned using Clustal W and all sequences were compared against other *pol*

sequences derived in the laboratory within the past 3–6 months to ensure there was no cross-contamination with amplicons from controls or other subjects' specimens.

Results

Study population

A total of 112 participants were enrolled, 46 women and 66 men with a mean age of 36 years; the mean CD4 count was 134 ± 89 cells/ μ l and the median plasma viral load of 93,556 copies/ml (interquartile range 62,776–291,364). All participants (112/112) had detectable viral loads (range 5400–749,395 copies/ml) but four subjects had insufficient sample for the plasma OLA assay and nine subjects had insufficient PBMC sample for the OLA assay and were excluded from analyses. Of the 99 (41 women, 58 men) subjects remaining, three had previously taken short-term ART and were excluded, leaving 96 for analysis of transmitted drug-resistant variants.

Resistance mutations by OLA and consensus sequencing

Drug-resistant mutations were rarely detected in PBMCs or plasma: K103N, G190A, and M184V were detected in 0/96 [0%; 97.5% confidence interval (CI): 0–4%] and Y181C in 1/96 (1%; 95% CI: 0–6%). The single participant with mutations detected had Y181C in both plasma and PBMCs at a concentration of 100% by OLA and consensus sequencing.

Initial OLA testing of 96 specimens using reagents developed for subtype B in the United States resulted in indeterminate results across each of the four codons in 3–10% of subjects evaluated. The rate of indeterminate assays was highest for codon G190A in both plasma and PBMCs (10/96; 10%), followed by K103N (7/96; 7% of PBMCs and 9/96; 9% of plasma), Y181C (9/96; 9% of PBMCs and 7/96; 7% of plasma), and M184V (3/96; 3% of PBMCs and 8/96; 8% of plasma). Viral sequences obtained by consensus sequencing were subtype B, and in most cases the indeterminate specimens were found to have polymorphisms in regions of the oligonucleotide probes. This occurred in seven subjects at K103N, five subjects at Y181C, and seven subjects at G190A. Utilization of alternate OLA probes designed to anneal to regional variants resolved indeterminate K103N and G190A in seven/seven and five/five subjects, respectively. No new probes were designed for G190A as polymorphisms were not consistent across subjects, as we have noted in select codons in some non-B subtypes.²⁸

Consensus sequences were derived from 21 PBMC and 32 plasma samples from 37 subjects. Polymorphisms that can contribute to HIV drug resistance but were not evaluated by the OLA were detected in 13/21 (62%) of subjects' PBMCs and in 16/32 (50%) of subjects' plasma by consensus sequencing (Table 1). The V179D polymorphism was detected most frequently: 7/37 had the mutation detected in either PBMCs or plasma. Of the subjects with V179D polymorphism, sequences derived from the plasma of 2/7 (29%) had additional minor resistance-associated mutations,⁴⁴ including V90I, K103R, and E138A.

Outcome of ART in participants with drug resistance mutations

The subject with 100% Y181C in her pre-ART plasma and PBMCs had rapid suppression of viral replication upon initiation of ART composed of 3TC, ZDV, and NVP. Her plasma HIV RNA at enrollment pre-ART was 301,000 copies/ml (5.48 log₁₀), and her viral replication was suppressed (<30 copies/ml) within 3 months. Her plasma HIV RNA remained undetectable over 15 months of effective ART until her death from lymphoma. She reported one lifetime sexual partner and no prior use of ARV. Her partner's ARV history is not known.

Of the seven people with V179D detected, six have been followed in the study for 18–24 months, with viral replication suppressed continuously to <30 copies/ml. One discontinued the study early due to relocating outside of Peru.

Discussion

Among ARV-naïve HIV-infected people who initiated ART between July 2007 and February 2009 in Lima, Peru the prevalence of transmitted resistance mutations to 3TC and NVP, both included in first-line ART in Peru, was 1% (95% CI 0–6%). The rate of TDR in our study was lower than rates in U.S., Brazilian, and Mexican cohorts that ranged from 6.1% to 26%.^{37,45–48} However, the prevalence we detected is similar to a separate study of ARV-naïve Peruvian men who have sex with men [12 of 359 (3.3%)] sampled between October 2002 and March 2003,⁴⁹ as well as to that found in treatment-naïve patients in a Chilean cohort (2/79; 2.5%),⁵⁰ another country with more recent implementation of universal access to ART. The subjects in our study most likely became infected with HIV 8–10 years prior to the study when ARVs were available only to individuals with private insurance or the resources to purchase ARV. Therefore, our study likely underestimates the current incidence of transmitted resistance.

TABLE 1. POLYMORPHISMS IN HIV *pol* ASSOCIATED OCCASIONALLY WITH LOW-LEVEL ANTIRETROVIRAL RESISTANCE DETECTED BY CONSENSUS SEQUENCING

Specimen (# tested)	Polymorphism								Any polymorphism
	T69N/S/T	V90I	K101Q	K103R	V106I	V118I	E138A/K	V179D/E	
PBMC (<i>n</i> =21)	2 (10%) ^a	0 (0%)	2 (10%)	2 (10%)	2 (10%)	1 (10%)	2 (10%)	6 (29%)	13 (62%)
Plasma (<i>n</i> =32)	3 (9%)	2 (6%)	2 (6%)	5 (16%)	2 (6%)	3 (9%)	3 (9%)	7 (22%)	16 (50%)
Plasma and/or PBMC (<i>n</i> =37)	4 (11%)	2 (5%)	2 (5%)	5 (14%)	3 (8%)	3 (8%)	4 (11%)	7 (19%)	18 (49%)

^aNumber (%) of participants with polymorphisms. PBMC, peripheral blood mononuclear cells.

The pure population of HIV mutant in both the plasma and PBMCs of a female who did not report previous use of ARV is a pattern typical of transmitted resistance.¹⁷ The suppression of her virus replication by NVP ART for at least 15 months is not the norm with Y181C mutants.¹⁷ Occasionally, others have observed that the Y181C mutation does not preclude suppression of viral replication by NNRTI-based ART.^{10,13,51}

The V179D/E mutations detected in 19% of participants with specimens evaluated by consensus sequencing has been associated with resistance to etravirine.⁵² However, given that this ARV was not available in Peru and that these subjects' viral replication was suppressed by NVP ART, V179D is likely a polymorphism common in the Peruvian epidemic,³⁷ and not due to selection by NNRTIs.

Individuals infected with drug-resistant virus have a higher probability of virologic failure with first-line therapy,⁵³ which led to recommendations to evaluate drug resistance before initiating ART.²⁰ Resistance mutations at codons 118, 184, 215, and 103 of the reverse transcriptase gene were most prevalent in subjects with primary HIV infection in the United States,¹⁷ and have been noted to persist at high concentrations detectable by consensus genotyping over many years.⁵⁴ Our understanding of resistance mutations that persist at a low frequency in the viral population is developing. Several studies suggest that the detection of low frequency resistance mutations at select codons predicts treatment failure, but resistance at other codons appears inconsequential.^{10,13,15,16,51}

Mutations were selected for evaluation in our study based on a high prevalence among surveillance drug resistance mutations³⁸ and the ARV utilized in Peru. We acknowledge that changes in ARV use may result in a shift in the codons pertinent to surveillance of transmitted resistance. Clearly, a limitation of our study is that mutations selected by ZDV or d4T, which have been relatively prevalent in large databases,⁵⁵ were evaluated by consensus sequencing in only a third of our population. Recent changes in the Peruvian guidelines, to initiate ART when CD4 counts fall to <350 cells/ μ l, may result in HIV-infected individuals starting ART after a relatively shorter duration of infection compared to the cohort we studied. Given the growing use of ART in Peru, we expect that the prevalence of transmitted resistance among individuals qualifying for ART will as a consequence likely escalate in the near future.

In Peru and in other low-resource settings with a limited number of ART options, transmitted resistance may potentially undermine HIV treatment programs. The proportion of patients that requires the use of second-line drugs has been increasing in Peru and is currently about 10% of patients receiving ART (J. Arevalo, personal communication).

Our study is a convenience sample of patients enrolled in a large observational cohort study and may not be representative of the population as a whole. The parent study enrolled only patients who plan to receive care for 2 consecutive years in the same place, and who are willing to have multiple genital samples taken. These participants may be less likely to have high-risk partners or behaviors, and thus may have lower rates of transmitted drug resistance than the general population. However, the rates of resistance in our cohort are similar to other cohorts in which ART has only recently become widely available.

In summary, TDR was not prevalent among individuals initiating ART in Peru between 2007 and 2009, most of whom

had likely acquired HIV infection before the national ART program began. As HIV resistance to ARV is an important challenge to effective treatment of the individual, and may negatively impact the control of the epidemic within communities,⁵⁶ additional surveillance is needed to determine if transmitted resistance increases with the national rollout of ART in public health clinics. Importantly, we observed sustained suppression of a pure (100%) Y181C mutant viral population by NVP-based ART, emphasizing the need for more studies to define the specific mutations and threshold concentrations that interfere with ART suppression of viral replication.

Nucleotide Sequence Accession Numbers

The gene sequences determined in this study were deposited in GenBank under accession numbers JF690127–JF690180.

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Author Disclosure Statement

No competing financial interests exist.

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