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Sharp increase in rates of HIV transmitted drug resistance at antenatal clinics in Botswana demonstrates the need for routine surveillance

C. F. Rowley[1,2]*, I. J. MacLeod[2], D. Maruapula[3], B. Lekoko[3], S. Gaseitsiwe[3], M. Mine[4] and M. Essex[2,3]

[1]*Division of Infectious Diseases, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA;* [2]*Harvard School of Public Health, Boston, MA, USA;* [3]*Botswana Harvard AIDS Institute, Gaborone, Botswana;* [4]*Botswana Ministry of Health, Gaborone, Botswana*

*Corresponding author. Tel: +1-617-632-7706; E-mail: crowley1@bidmc.harvard.edu

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Objectives: The aim of the study was to evaluate for the presence of drug resistance to HIV medications in treatment-naive individuals in Botswana.

Methods: Two different populations were evaluated for evidence of HIV drug resistance at three different geographical locations in Botswana. In the first study population, consisting of pregnant females diagnosed with HIV during pregnancy, participants were enrolled at the time of their HIV diagnosis. The second population included pre-ART enrollees at Infectious Diseases Care Clinics (IDCCs) who had a CD4 T cell count >350 cells/μL.

Results: A total of 422 genotypes were determined: 234 for samples from antenatal clinic (ANC) participants and 188 for samples from IDCC participants. Between 2012 and 2014, 6 of 172 (3.5%) genotypes from ANC participants exhibited transmitted drug resistance (TDR), with 3 (1.7%) showing resistance to first-line ART. In a subset of samples from Gaborone, Botswana's capital and largest city, the TDR rate was 3 in 105 (2.9%), but only 1 in 105 (1.0%) showed first-line ART resistance. Between December 2014 and April 2015, the rate of resistance to any ART in Gaborone was 6 in 62 (9.7%), with 5 (8.1%) exhibiting first-line ART resistance.

Conclusions: These data demonstrate that TDR rates for HIV differ geographically and temporally in Botswana, with significant increases in TDR observed at ANCs in Gaborone between 2012 and 2015. These findings stress the importance of continued testing for TDR, particularly as access to HIV treatment increases and guidelines recommend treatment at the time of HIV diagnosis.

Introduction

The global expansion of ART has allowed almost 15 million HIV-positive people to initiate lifesaving treatment.[1] The greatest increase in ART use has been in sub-Saharan Africa, where ~120 000 people start ART each month.[2] An expected consequence of the global increase in ART access is a corresponding increase in HIV drug resistance,[3] which can render one or more antiretroviral medications in an ART regimen completely ineffective, a particularly relevant issue as most national programmes use a single ART regimen for first-line therapy. The recent recommendations to initiate ART in all individuals at the time of diagnosis,[4] based on the evidence conclusively demonstrating that treatment at the time of HIV-infection diagnosis is beneficial regardless of CD4 T cell count,[5] will have profound implications on issues related to HIV drug resistance. Millions more are now eligible for HIV treatment by the revised guidelines, and surveillance to ensure the long-term viability of HIV-treatment programmes through surveillance for HIV drug resistance, particularly in resource-limited settings, will be crucial.

Transmitted drug resistance (TDR; resistance in treatment-naive individuals) may serve as an 'early warning indicator' of HIV-treatment programmatic failure.[6–9] The WHO considers drug-resistance monitoring a critical component of ART rollout surveillance,[10] with numerous studies following the WHO protocol for monitoring TDR having demonstrated varying global resistance patterns,[11] thus alerting national programmes of threats to the effectiveness of their first-line HIV drug regimens.

In 2011, one large study that looked at over 2400 samples estimated the overall rate of TDR at 5.6% in sub-Saharan Africa, with an expected overall 1% increase in the TDR rate each year.[12] This rise in TDR is mostly driven by increases in NNRTI resistance,[13] with national programmes that have been in existence longer demonstrating higher rates of resistance.[13,14] In Tanzania, the results of one study analysing samples from individuals not eligible for TDR monitoring according to the WHO protocol (older individuals, including males with unknown duration of infection) were of particular concern, as it revealed resistance rates that were much higher than expected (14.8% in treatment-naive individuals).[15]

This study suggested the need to consider broadening the criteria for resistance testing to possibly include everyone presenting for ART initiation. All of these data support the need for ongoing monitoring to ensure the stability and effectiveness of national ART programmes.

Botswana's national ART programme began in January 2002, making it one of the oldest in Africa, with over 200 000 individuals currently on treatment. From the outset, routine viral-load testing was performed to monitor patients upon treatment initiation. Surveillance for TDR performed on samples obtained in 2007 demonstrated no significant resistance to ART drugs.[16] This report includes the results of genotyping analyses of samples from participants who would be included in the WHO protocol for resistance monitoring as well as individuals who present for care but have not met national guidelines for treatment (CD4 T cell count >350 cells/μL) in order to gauge the extent of HIV resistance in treatment-naive individuals in Botswana.

Methods

Participants were recruited at antenatal clinics (ANCs) and Infectious Diseases Care Clinics (IDCCs) in three different locations in Botswana: Gaborone (urban area, capital of Botswana), Molepolole (large village), and Mochudi (traditional village). Initially, women enrolled at the ANCs were in their first pregnancy and were between 18 and 25 years of age, had never previously tested positive for HIV or taken HIV medications, were newly discovered to be HIV-positive, and had no clinical signs/symptoms to suggest advanced HIV infection. Enrolment age range was increased after year 1 of the study to ensure attainment of adequate sample size, as first pregnancies were occurring at older ages than anticipated, and the inclusion criterion no longer required the pregnancy to be the first pregnancy. Any individual who tested positive for HIV at the ANCs had the national programme database checked for any prior positive HIV tests or CD4 T cell counts to confirm this was the first positive test. Those invited to participate at the IDCCs included HIV-infected men and women 18–65 years old and with a CD4 T cell count >350 cells/μL (i.e. indicating that ART was not imminent, allowing for resistance results to be returned to participants/providers if important resistance was discovered). Enrolment took place between April 2012 and April 2015. Recruiting began initially in Gaborone, followed by Molepolole and Mochudi, before returning to Gaborone in December 2014.

Each consenting individual had 10 mL of blood drawn during their visit. Samples were processed at the laboratory of the Botswana Harvard AIDS Institute in Gaborone. RNA was isolated using an EZ1 Virus Mini Kit v2.0 (Qiagen, Valencia, CA, USA) and an EZ1 Advanced XL (Qiagen) automated instrument.

PCR amplification

PCR amplification and sequencing were performed using a modified version of an in-house genotyping protocol developed in South Africa for HIV-1 subtype C.[17] A 20 μL aliquot of each RNA sample served as the template for the initial round of PCR amplification with the Transcriptor One-Step RT–PCR protocol (Roche, Indianapolis, IN, USA). The primers CWR1 and CWF1 were modified with locked nucleic acids [as denoted by the plus symbol (+) preceding the nucleic acid symbol in the sequences shown below] and used for the initial PCR amplification. For each reaction, primers CWF1-LNA2 (5′-+GAA+G+GACACCAAATGAAAGAYTG-3′) and CWR1-LNA3 (5′-G+CA+TAC+TTYCCTGTTTTCAG-3′) were added at a final concentration of 100 nM each to 10 μL of 5× reaction buffer, 1 μL of enzyme and a 20 μL RNA sample, and made up to a final volume of 50 μL with distilled water (dH_20). The cycling parameters for the first-round PCR were reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 7 min, and then 10 cycles of 94°C for 10 s, 55°C for 30 s and 68°C for 2 min, followed by 35 cycles of 94°C for 10 s, 55.5°C for 30 s and 68°C for 2 min, increasing each cycle by 10 s, with a final elongation step of 5 min at 68°C.

The first round of PCR generated a 1569 bp amplicon comprising nucleotides 2044–3619 (HXB2 numbering), which was purified by electrophoresis on a 1% agarose gel. If a single specific band was observed, the first-round amplicon was then sequenced. If the band was faint or undetectable, a second round of PCR was performed as follows. For each reaction, primers CWF1-LNA2 (5′-+GAA+G+GACACCAAATGAAAGAYTG-3′) and RT-20C (5′-CTGCCAATTCTAATTCTGCTTC-3′) were added at a final concentration of 100 nM each to 10 μL of Phusion Flash high-fidelity PCR master mix (Thermo Scientific, Waltham, MA, USA) and 5 μL of the first-round amplicon, and made up to a final volume of 20 μL with dH_20. The cycling parameters for the second-round PCR were 98°C for 10 s, then 40 cycles of 98°C for 1 s, 55°C for 5 s and 72°C for 20 s, and then hold at 4°C. This round resulted in a 1418 bp amplicon (HXB2 numbering 2044–3462).

Sequencing

Standard Big Dye chemistry was used for sequencing on an ABI 3100 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) using the following primers: CWF1 (5′-GAAGGACACCAAATGAAAGAYTG-3′), CWCS2 (5′-AGAACTCAAGA CTTTTGGG-3′), CWCS3 (5′-TGCTGGGTGCGGTATTC-3′), CWCS5 (5′-TGGTAAA TTTGATATGTCCAT-3′), Seq2.1-F2 (5′-GGCCAGGGAATTTTCTTCAGAGC-3′), Seq6 (5′-CCATCCCTGTGGAAGCACATTA-3′) and RT-20C (5′-CTGCCAATTCTAATTCTG CTTC-3′). Sequences were manually edited and aligned using Geneious version 6.1.8 (Biomatters, New Zealand).

Genotype analysis

The generated consensus sequences were entered into the Stanford HIV database (http://hivdb.stanford.edu/) for determination of resistance and subtype identification, which were calculated on 5 June 2015. Using Geneious, all sequences were aligned to a consensus HIV-1 subtype-C oligonucleotide spanning the protease and reverse transcriptase genes. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 6,[18] with the maximum-likelihood method, based on the model by Tamura and Nei.[19]

Determination of TDR

HIV resistance was assessed using the Calculated Population Resistance program (http://cpr.stanford.edu/cpr.cgi) from Stanford.[20] Sequences analysed by the program are evaluated for surveillance drug-resistance mutations (SDRMs), mutations selected for epidemiological surveillance of HIV TDR as outlined in the 2009 WHO drug-resistance mutations guideline.[21] Likely susceptibility to initial HIV drug regimens was calculated, and resistance mutations deemed significant were those that exhibited intermediate-level resistance or above (a score of ≥30), according to the Stanford HIV database (http://sierra2.stanford.edu/sierra/servlet/JSierra).

Positive results showing resistance

Genotyping results were given to providers at the time results were available (median time to genotype result was 23 days), along with an interpretation of the resistance profile. Changes were made to regimens by the primary caregivers, with those changes being in accordance with the Botswana National Treatment programme in order to maximize the likelihood that pregnant participants would be able to suppress virologically. In all cases, this involved changing from an NNRTI-based regimen to a PI-based regimen.

Statistical analyses

All statistical analyses were performed using SigmaPlot 11.0 (Systat, San Jose, CA, USA).

Human subjects review

The study protocol was approved by the human subjects committees of both the Harvard T.H. Chan School of Public Health (protocol # 20770) and the Human Resource Development Council (protocol # HRDC 0638) in Gaborone, Botswana.

Results

Demographic characteristics of the individuals in both study groups are shown in Table 1. CD4 T cell counts were performed by the Botswana national programme or as part of this study if data were not available at the time of enrolment. All HIV-infected pregnant women in Botswana are advised to commence treatment for HIV with a fixed-dose regimen of emtricitabine/tenofovir disoproxil fumarate/efavirenz, regardless of their CD4 T cell count or pregnancy stage. Individuals presenting to an IDCC were started on HIV medications if they had a CD4 T cell count <350 cells/μL or an AIDS-defining illness.

Overall, CD4 T cell counts of ANC participants and IDCC participants reflected relatively preserved immune function with a median ANC CD4 T cell count of 407 cells/μL (IQR 269.5, 549.5) and a median IDCC CD4 T cell count of 431.5 cells/μL (IQR 395, 487). The viral load in pregnant female patients was lower in the pregnant female patients than in the IDCC participants, median $\log_{10}$ RNA copies/mL 4.02 (IQR 3.44, 4.53) versus 4.32 (IQR 3.68, 4.79), ($P<0.001$ Mann–Whitney rank sum test). The ANC participants from Gaborone enrolled in 2014–15 are statistically different from the 2012 participants in Gaborone, as older women were included in this cohort given our expansion of the enrolment criteria to account for later timing of first pregnancies. The median age in 2012 was 23 years (IQR 21, 24) compared with 25 years (IQR 23, 31) in 2014–15 ($P<0.001$ Mann–Whitney) and CD4 T cell counts were higher in 2012 at 454 cells/μL (IQR 319, 555) compared with 320 cells/μL (IQR 209, 525) in 2014–15 ($P=0.008$ Mann–Whitney), possibly reflecting longer duration of HIV infection.

Table 2 shows the results of resistance testing at each site while Table 3 reflects all the SDRMs seen in this study. Of the 443 individuals enrolled, 422 genotype results were available. With respect to the 21 patients for whom genotype results were unavailable, 12 missing results were due to difficulty amplifying because of low HIV load, which ranged from <40 copies/mL to 500 copies/mL. Of the 422 genotypes performed, 234 were on samples from ANC participants and 188 were on samples from IDCC participants. In the 172 genotypes from ANC participants performed between 2012 and 2014 at the three different geographical locations, 6 (3.5%) exhibited evidence of TDR, with 3 (1.7%) showing resistance to first-line ART (emtricitabine/tenofovir disoproxil fumarate/efavirenz). Between December 2014 and April 2015, 62 genotypes were performed at the ANCs in Gaborone, and 6 of these (9.7%) showed TDR, with 5 (8.1%) exhibiting resistance to first-line ART. This finding represented a significant increase in TDR in Gaborone, as the previous survey encountered resistance to first-line ART in only 1 of 105 samples tested. No mutations that would have impacted first-line ART were detected in the 188 samples taken from the IDCC participants over the 3 years of the study, with only 4 of 188 (2.1%) showing any evidence of TDR. The overall prevalence of individuals with resistance to NRTIs, NNRTIs and PIs for the entire study was 3.8% (16 participants out of 422).

NNRTI-associated mutations that could impact the national programme's first-line ART included K103N, G190A and Y181C, each of which had a Stanford database score of ≥30, consistent with intermediate- or high-level resistance. Because the K101E mutation is not associated with intermediate-level resistance, no recommendation was made to change the HIV regimen in the individuals with this mutation. In addition, primary PI mutations, though potentially significant to a PI-containing regimen, were detected but did not prompt a change in the NNRTI-containing HIV regimen.

Phylogenetic trees were constructed for all samples to ensure that each sequence represented a unique sample and to evaluate potential resistance clustering. The phylogenetic tree for the ANC samples from Gaborone in particular was of interest as these samples represented those exhibiting a sharp increase in TDR. Two individuals with resistance were found to harbour closely related viruses, but the remainder of the TDR was not linked.

Table 1. Clinical characteristics of patients enrolled at ANCs and IDCCs

Year	ANCs/IDCCs	Age (years), median (IQR)	CD4 T cell count (cells/μL), median (IQR)	Viral load ($\log_{10}$ RNA copies/mL), median (IQR)
	ANCs			
2012	Gaborone ($n=114$)	23 (21, 24)	454 (319, 555)	4.11 (3.53, 4.57)
2013–14	Molepolole ($n=36$)	24 (19, 39)	418 (284.5, 545.5)	3.99 (3.20, 4.82)
2013–14	Mochudi ($n=36$)	25 (23, 31.5)	392 (295.5, 552)	3.98 (3.47, 4.56)
2014–15	Gaborone ($n=62$)	25 (23, 31)	320 (209, 525)	3.86 (3.27, 4.43)
	total ($n=248$)	24 (22, 26.8)	407 (269.5, 549.5)	4.02 (3.44, 4.53)
	IDCCs			
2012	Gaborone ($n=117$)	31.5 (27, 37)	419 (392, 465)	4.42 (3.69, 4.83)
2013–14	Molepolole ($n=51$)	33 (29.25, 38)	444 (396, 531.5)	4.22 (3.68, 4.53)
2013–14	Mochudi ($n=27$)	35 (30.5, 37)	495 (422.5, 577.5)	4.42 (3.32, 4.83)
	total ($n=195$)	33 (29, 37)	431.5 (395, 487)	4.32 (3.68, 4.79)

Table 2. Summary of HIV TDR at both ANCs and IDCCs in Botswana

	NRTIs	NNRTIs	PIs	Samples with resistance, % (*n*)	Resistance to first-line therapy[a], % (*n*)
ANCs					
Gaborone					
2012 (*n*=105)	—	K101E, G190A	L90M	2.9 (3)	1.0 (1)[b]
2014–15 (*n*=62)	—	K103N (4), G190A	L90M	9.7 (6)	8.1 (5)[b]
Molepolole (*n*=34)	—	—	—	—	—
Mochudi (*n*=33)	M184V[c]	K103N, Y181C[c]	M46I	9.1 (3)	6.1 (2)
total (*n*=234)				5.1 (12)	3.4 (8)
IDCCs					
Gaborone (*n*=115)	T215S	K101E	M46I, V82L	3.5 (4)	—
Molepolole (*n*=49)	—	—	—	—	—
Mochudi (*n*=24)	—	—	—	—	—
total (*n*=188)				2.1 (4)	—

[a]Resistance to first-line therapy is defined as a mutation that confers intermediate- or high-level resistance to emtricitabine/tenofovir disoproxil fumarate/efavirenz.
[b]$P=0.027$ by Fisher's exact test for differences in resistance at ANCs in Gaborone in 2012 and 2014–15.
[c]Same individual.

Table 3. Summary of SDRMs observed among all participants

NRTI mutations	**M184V**, T215S
NNRTI mutations	K101E (2), **K103N** (5), **Y181C**, **G190A** (2)
PI mutations	M46I (2), V82L, L90M (2)

Mutations highlighted in bold are those with intermediate- or high-level resistance to first-line ART in Botswana. In parentheses are the number of occurrences of each mutation.

Discussion

Monitoring for the emergence of HIV TDR in low- to middle-income settings is essential to preserve the future of national ART programmes. Routine resistance testing at the time of HIV diagnosis or pretreatment is commonplace in the USA and Europe, but, currently, there is no evidence supporting this type of individualized approach to genotyping in resource-limited settings with the rollout of ART. Instead, the WHO has recommended monitoring for evidence of TDR through testing of individuals newly diagnosed with HIV presenting at ANCs, as this population likely represents those who are recently infected and thus could provide early warning of declining first-line therapy efficacy.

Botswana's national programme was the largest of its kind when it began in 2002, and it has grown to now treating ∼240 000 individuals, or 20% of the adult population. The move to a fixed-dose combination of emtricitabine/tenofovir disoproxil fumarate/efavirenz in 2009 was consistent with best practices globally at that time and remains the first-line ART regimen today. The data presented here show a significant increase in TDR over a short period of time, with HIV mutations being detected that would be expected to impact NNRTI-containing first-line ART.

The increase in HIV resistance to first-line ART in pregnant female patients in Gaborone from <1% in 2012 to >8% in <3 years is of great concern and quite unexpected. The 2012 data were in line with the 2007 national surveillance, which demonstrated no significant HIV resistance. In Mochudi, a village ∼30 miles from Gaborone, there was also evidence of significant resistance (6.1%) at the ANCs, although recruitment for this part of the study was very slow, with a low number of ANC participants. No resistance to first-line therapy was detected in samples from the IDCCs at any of the three sites, suggesting that: (i) a proportion of these participants were infected with a resistant virus that had reverted to WT; (ii) resistance has yet to extend into the broader, general population; or (iii) new samples from IDCCs, if collected now, may start to reflect the increasing resistance seen at the ANCs.

The ANC population has long been recognized as important in providing data for resistance, as this population is likely closer to sexual debut and therefore likely closer to time of acquisition of HIV than the general population. Testing individuals for resistance soon after HIV acquisition increases the chance of detecting a resistant strain prior to it reverting to WT virus. In Botswana, the healthcare system tests all pregnant individuals for HIV; those individuals who tested positive were evaluated for HIV drug resistance in this study prior to their initiation of ART. The detection of resistance is of obvious importance for women newly diagnosed with HIV, as resistance can decrease their chance of successfully suppressing the virus, which can affect infant health if ART given during pregnancy is not fully suppressing the virus, thus increasing the possibility of vertical transmission. An increase in TDR is also ultimately significant for the national programme, as forward transmission with resistant viruses will likely ensue, leading to compromised efficacy of universal HIV-treatment strategies, as these medications will not necessarily work in those individuals harbouring drug-resistant HIV.

The reason for the relatively sudden increase in TDR is unclear. Botswana continues to monitor the viral load of HIV-infected individuals as it has from the initiation of the national programme, a strategy that allows for early detection of treatment failure or adherence issues. Low rates of resistance were observed as recently as 2012 in this study, and no fundamental changes in the national programme have occurred since then that might explain the increase in TDR, although over the past year there

has been a significant decrease in the frequency of genotyping at the national HIV reference laboratory in Botswana, secondary to technical challenges and logistical issues. In Botswana, resistance genotyping is typically recommended at the time of failure of second-line therapy, but that has not been routine, and, subsequently, more individuals for whom treatment is failing clinically do not have corresponding resistance data to assist with the change to a third-line suppressive regimen. Phylogenetic analysis of the 2014–15 Gaborone samples did not show evidence of clustering, and thus the results suggest no obvious individual source of the resistant viruses.

The recently revised WHO HIV-treatment guidelines that recommend a test-and-treat strategy for HIV-treatment initiation as well as greatly expanded access to HIV medications for pre-exposure prophylaxis will increase the need for standardized surveillance monitoring for HIV drug resistance. While testing of IDCC participants in this study did not demonstrate significant resistance, it is likely that resistance testing of newly diagnosed individuals, similar to the ANC participants in this study, would demonstrate more TDR than our current testing did in the IDCCs. As of now, there does not appear to be a need to test for resistance at the time of HIV diagnosis given overall low rates of resistance. However, this situation may change in the future as resistance rates increase as more people are initiated on treatment. The results in this study raise the spectre of the need for resistance testing in pregnant individuals in Botswana, and they may serve as a harbinger of strategies that will be needed in the future for all newly diagnosed individuals given the apparent increase in TDR.

This study has several limitations. Although it evaluated three different-sized communities where ART was introduced early in the national programme, they all are relatively close to the capital; thus, these results may not reflect the rest of Botswana. There were challenges in recruiting in Mochudi due to decentralized clinics as well as several protracted periods of stock outs of HIV-antibody testing kits, thus limiting a full assessment of resistance in this region where there seemed to be a trend suggesting it may be significant. In addition, although initial resistance testing at ANCs in Gaborone followed the WHO protocol, evaluation for acute or recent infection by detuned ELISA or western blotting was not performed, potentially leading to underestimation of the TDR rate, as HIV from participants exhibiting ART resistance could have reverted to WT, or resistance may have been present below the limit of detection of Sanger sequencing and thus been missed. Our study also increased the age of entry for pregnant female patients, as the trend in Botswana is for an older age of first pregnancy and also allowed multiparous individuals. This change may have resulted in enrolling ANC participants with well-established HIV infection, thus missing the opportunity to most effectively detect TDR, resulting in an underestimation of the prevalence of resistance.

This study, one of the largest to date evaluating TDR from a single country in sub-Saharan Africa, shows that Botswana is beginning to experience an uptrend in ART resistance in treatment-naive pregnant female patients, with a significant change in the prevalence of resistance in <3 years in Gaborone. As of now, there does not appear to be evidence of resistance in individuals presenting for care at the IDCCs, suggesting that resources should be targeted at those who are recently infected or newly diagnosed. These data suggest that more-frequent, widespread monitoring strategies should be employed to maximize the chances of the long-term success of the national treatment programmes. These findings will have further implications as the move to start ART in all HIV-positive individuals becomes a reality and the exponential increase in patients receiving ART will naturally lead to more HIV drug resistance.

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Transparency declarations

None to declare.

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