

Threshold survey evaluating transmitted HIV drug resistance among public antenatal clinic clients in Addis Ababa, Ethiopia

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Background: Expanded access to HIV therapy in the developing world raises serious concerns regarding the potential emergence and transmission of drug-resistant HIV strains. Although HIV drug resistance surveillance is recommended to track transmitted HIV drug resistance among newly infected individuals, the financial constraints in resource-limited countries prohibit such surveillance on a regular basis. The World Health Organization (WHO) recently introduced guidelines to address this issue.

Methods: A survey was conducted in Ethiopia following the WHO guidelines to assess transmitted HIV drug resistance among recently HIV-infected individuals in Addis Ababa. Antiretroviral drug usage started 3 years earlier than commencement of the current expanded access to antiretroviral therapy in Ethiopia.

Results: Of 75 eligible samples, 39 (52%) were successfully sequenced and genotyped in the protease and reverse transcriptase region, using both the ViroSeq[®] and TrueGene[®] genotyping systems, and analysed for drug resistance mutations using an algorithm from the Stanford HIV Reverse Transcriptase and Protease Database. The analysis revealed that transmitted HIV drug resistance in Addis Ababa is below the 5% threshold level for all three classes of antiretrovirals.

Conclusions: The current first-line antiretroviral therapy strategy can be used with confidence in Ethiopia at this time; however, Ethiopia should conduct similar periodic surveys that include the capitals of Ethiopia's larger regional states to ensure early detection of any changes in the country's HIV drug resistance trend.

Introduction

Since its identification in the early 1980s [1,2], the HIV pandemic has been mounting an alarming global attack. According to reports from the Joint United Nations Programme on HIV/AIDS (UNAIDS) [3], the global burden of HIV at the end of 2005 was estimated at 38.6 million individuals, of whom nearly two-thirds (about 24.5 million) lived in sub-Saharan Africa.

Ethiopia has suffered some of the world's most devastating human losses due to HIV. Of the nearly 73 million Ethiopian people, about 1,300,000 are estimated to be living with HIV/AIDS [4]. AIDS deaths in adults and children were estimated at between 38,000 and 130,000 in 2005 alone [3]. In 2005, antenatal care (ANC) surveillance data revealed that the adjusted

national HIV prevalence for the country was 3.5% (urban 10.5% and rural 1.9%) [4]. The lower estimate of overall prevalence in 2005 compared with that found in 2003 (4.4%; urban 12.6% and rural 4.6) is attributed to expansion of surveillance into rural areas where prevalence is known to be lower [3,4].

The availability and widespread use of highly active antiretroviral therapy (HAART) in developed countries has shown that HIV/AIDS-related mortality and morbidity can be reduced significantly [5-7]. Unfortunately, widespread access to antiretrovirals (ARVs) in Ethiopia remained unaffordable until very recently. Although ARVs were introduced into the country in 2000 by a few people who could purchase

drugs on the black market [8], formal delivery of anti-retroviral therapy (ART) in Ethiopia did not begin until 2003. With the launch of the ART programme in 2003, five ARVs were made available to the public: the three nucleoside analogue reverse transcriptase inhibitors (NRTIs) zidovudine, lamivudine and stavudine; and two non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine and efavirenz. The drugs were distributed to a few selected pharmacies, most of which were in Addis Ababa, and were available for patients by prescription from ART-certified physicians. The drugs were dispensed from selected Urban Dwellers Association Pharmacies owned by local administrations and Ethiopian Red Cross Pharmacies. A total of 5,000 patients started ART, of whom 90% were paying US\$30–90 a month while the remaining 10% were receiving treatment free of charge [8]. Free treatment was available for employees of international non-governmental organizations, embassies and private companies. Patients who could produce written evidence from the local administration (Kebele) also received free treatment from public health institutions.

Relief for the majority of AIDS patients came more recently when Ethiopia became one of the beneficiary countries of ART roll-out programmes, including the Global Fund for Tuberculosis, AIDS and Malaria (GFATM), and the US President's Emergency Plan for AIDS Relief (PEPFAR). However, of the 277,800 AIDS patients requiring ART in 2005, only 13,100 had started on ART and this figure rose to less than 46,000 at 132 facilities by the end of July 2006; of these just over 35,000 are currently on treatment [4]. Of the 46,000 patients who started ART by 2006, and of the 35,000 who are currently on ART, about 39% (18,012) and 38% (13,441), respectively, reside in Addis Ababa [4]. However, there has been a steady improvement in ART uptake over the past 2 years. The number of patients receiving ART with PEPFAR funds rose from 9,500 at the end of September 2004 to 26,800 by the end of March 2006 [9]. PEPFAR and the Government of Ethiopia have set a target of placing 210,000 patients on ART by September 2009 [9].

To achieve this goal the Federal Ministry of Health (MOH) of Ethiopia and other relevant bodies have prepared policies and guidelines for the appropriate use of ART [10]. Currently, four first-line NNRTI-based regimens are in use for adults and adolescents: stavudine, lamivudine and nevirapine; zidovudine, lamivudine and nevirapine; stavudine, lamivudine and efavirenz; or zidovudine, lamivudine and efavirenz. Second-line regimens include abacavir, tenofovir disoproxil fumarate or zidovudine (if not used previously), plus didanosine and one of three low-dose ritonavir-boosted protease inhibitors: lopinavir, saquinavir or indinavir. There are also separate regimens recommended for pregnant

women and children. Both generic and brand drugs are used in the country. The major drug providers include Bristol-Myers Squibb (stavudine, didanosine, efavirenz), GlaxoSmithKline (lamivudine, abacavir, zidovudine), Gilead Sciences (tenofovir disoproxil fumarate), Boehringer Ingelheim (nevirapine), Abbott Laboratories (lopinavir), Roche (saquinavir), and Merck (indinavir).

Expanded access to ART in developing countries has raised serious concerns about potential barriers to the long-term success of ART programmes [11–12]. The most prominent of these fears is the potential emergence of drug-resistant HIV strains. Experience from the use of HAART in the developed world has indicated that ARV-associated resistance is common among HIV-1-infected individuals who are naive to ARVs [13–18]. Such transmitted drug resistance can reach 15% or more in populations in which ARVs have been in use for longer periods of time [11]. Several studies have shown that the presence of drug resistance mutations prior to starting treatment is associated with poorer virological response [19,20].

Monitoring the prevalence of transmitted drug resistance in areas where ART is introduced extensively is, therefore, important to the success of treatment programs. Recently, the World Health Organization (WHO) designed a novel approach of tracking the emergence of HIV drug resistance and transmission of drug-resistant strains of HIV in countries scaling up ART, the so-called HIV Drug Resistance Threshold Survey (HIVDR-TS), to circumvent the need for wide-scale surveillance [21,22]. The principles and procedures of this approach are described elsewhere in this issue.

This study was initiated to assess the frequency of transmitted HIV drug resistance in Ethiopia using the WHO's approach. The study was conducted in Addis Ababa where drug-resistant HIV transmission is thought to be more likely, because ARV usage started in Addis Ababa 3 years prior to ART scale-up.

Methods

Study area, population and ethical issues

This threshold survey was designed and implemented according to the recommendations of the Centers for Disease Control and Prevention (CDC) and the WHO [21] for conducting HIV drug resistance surveillance in resource-limited countries. The population for this ARV resistance threshold survey consisted of women who participated in the 2005 ANC-based HIV surveillance programme at seven sentinel sites in Addis Ababa, where ARVs were in use prior to the scale-up of ART in Ethiopia. In these seven sites (see Table 1), specimens are collected for syphilis screening (rapid plasma reagin [RPR] testing) as part of routine ante-

natal care; remnant specimens are tested for HIV. Remnant HIV-positive specimens from eligible pregnant women attending the seven ANC sentinel sites during the working days from 6 April to 8 August 2005 were included in the HIVDR-TS. Eligibility criteria were age <25 years, first pregnancy, tested for syphilis, and residence in Addis Ababa. Demographic data collected for the HIV surveillance (age, gravidity and residence) were also used for the threshold survey.

National HIV Sentinel Surveillance Guidelines [23] were followed in selecting specimens. ANC-based HIV surveillance is unlinked and anonymous; therefore, consent is not sought nor results delivered. However, women were informed about the availability of voluntary HIV counselling and testing (VCT) and prevention of mother-to-child HIV transmission (PMTCT) services available in their area.

The survey protocol was approved both by the National Ethical Review Committee of Ethiopian Science and Technology Agency and the CDC.

Specimen collection, processing and handling

About 10 ml blood is drawn for syphilis screening using anticoagulant-free vacutainer tubes. Serum was separated from the clot at the blood collection site between 30 and 120 min after blood draw by centrifugation at 400–800 g for 10 min. Approximately 4 ml of the separated serum was taken by the ANC clinic and site laboratories (Addis Ababa Regional Laboratory, Federal Armed Forces Hospital, and Federal Police Hospital) for RPR test and HIV surveillance, respectively. For HIVDR-TS specimens, the remaining 1 ml left-over serum was transferred into a cryotube aseptically, and stored refrigerated until transported to the Ethiopian Health and Nutrition

Research Institute (EHNRI) laboratory. More than 90% of the specimens were transported to EHNRI and stored at -80°C on the same day the blood was drawn. The remaining <10% were transported within 96 h of the blood draw. Upon arrival at EHNRI and before storing at -80°C, each specimen was divided into two cryotubes aseptically. All specimen-containing cryotubes transported to EHNRI were registered in a log book, and the demographic data for each subject were recorded and stored electronically.

HIV serostatus of the samples was determined at site laboratories using Vironostika (HIV antigen/antibody enzyme immunosorbent assay (EIA) (BioMérieux bv, Boxtel, The Netherlands). All specimens that tested positive and 10% of those that tested negative by site laboratories were further tested at EHNRI by Enzygnost (Dede Behring Marburg GmbH, Germany) for confirmation. The Murex Antibody test (Abbott Murex, UK) was used as a tie breaker. All confirmed HIV-positive specimens were checked for eligibility to the threshold survey using gravidity (first pregnancy), age (<25 years), and residence in Addis Ababa as criteria. The eligible specimens were arranged consecutively according to date of blood draw and transported frozen in dry ice for genotyping to Central Virology Laboratory (Tel-Hashomer), a collaborating laboratory in Israel that is well-experienced in drug resistance genotyping of Ethiopian HIV-1 subtype C.

Antiretroviral drug resistance testing

Viral RNA was extracted using automated BioMeriex extractor (easyMAG 00102, firmware 1.0.8; BioMérieux, Lyon, France). Viral load was first determined using in-house real-time one-step reverse transcriptase PCR (RT-PCR) Master Mix

Table 1. Number of individuals recruited and HIV-positive eligible samples amplified and genotyped for the HIVDR-TS from the 2005 Addis Ababa ANC-based sentinel HIV survey

Sample sites	NSC	HIV-positive*	Eligible for HIVDR-TS [†]	RNA copies/ml				Attempted amplification [‡]	Genotyped samples [§]
				>2,000	1,000–2,000	100–1,000	Undetectable		
Teklehaimanot	326	35	6	3	1	1	1	5	4
Kazanchis	349	66	20	5	2	6	7	13	8
Kefitegna 23 Clinic	443	47	13	4	0	6	3	10	4
Gulele Clinic	393	56	18	9	2	6	1	17	13
Akaki Clinic	435	40	14	9	1	3	1	13	10
Armed Forces Hospital	236	30	0	NA	NA	NA	NA	NA	NA
Federal Police Hospital	250	56	4	1	0	2	1	3	0
Total	2,432	330	75	31	6	24	14	61	39

*Number of confirmed HIV-positive samples. [†]Number of eligible samples for the HIV Drug Resistance Threshold Survey (HIVDR-TS) according to WHO criteria.

[‡]Number of samples on which amplification was attempted. [§]Number of successfully amplified and genotyped samples. ANC, antenatal care; HIVDR-TS, HIV Drug Resistance Threshold Survey; NA, not applicable; NSC, number of samples collected.

reagents (P/N 4309 169; Applied Biosystems, Roche Branchburg, New Jersey, USA) according to Burgard *et al.* [24] on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, a division of Perkin-Elmer). The quality of the extraction was evaluated in terms of the DNA values of a single-copy gene (RNaseP) (TaqMan RNase P Control reagents, P/N 4316844, Applied Biosystems) in the serum using a validated in-house real-time PCR assay. Only samples containing the equivalent of >100 copies/ml RNA as estimated by the in-house real-time RT-PCR were subjected to genotyping. For the purpose of genotyping, RT-PCR was performed first using the RT-PCR primers and reagents of ViroSeq[®] HIV-1 genotyping system v2.0 (Celera Diagnostics LLC; Alameda, CA 94502, USA) following the manufacturer's instructions. In the event of ViroSeq[®] system failure, in-house primers were used according to Snoeck *et al.* [25] with the one-step RT-PCR kit from QIAGEN (cycling conditions: 50°C for 30 min, 95°C for 15 min; 39 cycles of 94°C for 30 s, 50°C for 30 s and 68°C for 3 min; 68°C for 10 min and 4°C hold). Sequencing reactions were performed on samples that gave amplified products using principally the ViroSeq[®] genotyping system v2.0 sequencing kit. For those samples that failed the sequencing reaction by ViroSeq[®] on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems), the TrueGene[®] system of Bayer (Bayer Health Care LLC subsidiary of Bayer Corporation, Tarrytown, NY, USA) was used. Protease and reverse transcriptase sequences and their respective reports were generated using both ViroSeq[®] and TrueGene[®] software. The sequences were then manually edited and saved in a FASTA format. A drug resistance report was produced by entering these FASTA-formatted sequences into the Stanford HIV Reverse Transcriptase and Protease Sequence Database [26]. The resulting list of drug resistance mutations was then compared with those on the WHO surveillance list, which includes 31 protease mutations for protease inhibitor resistance, 32 reverse transcriptase mutations for NRTI resistance, and 18 reverse transcriptase mutations for NNRTI resistance [27].

Results

A total of 2,432 samples collected for the national ANC-based HIV surveillance were considered for this HIVDR-TS during the study period (Table 1). Of these samples, 330 (13.6%) were found to be HIV-positive by both site laboratories and EHNRI. However, only 75 (22.7%) of the 330 HIV-positive samples were identified as eligible for HIVDR-TS (age <25, first pregnancy, and residence in Addis Ababa) [21].

The viral load of all the specimens was determined using in-house real-time RT-PCR before amplification

was attempted. Table 1 summarizes the laboratory results of amplification and genotyping conducted for the survey. Of the 75 eligible samples, 14 (18.7%) did not have detectable RNA (>100 copies/ml), and only 37 (49.3%) had a viral load >1,000 copies/ml.

Initially, RT-PCR was performed using the ViroSeq[®] RT-PCR system on samples from 26 of the 61 persons with detectable RNA, of which only six (23%) gave good products. Consequently, RT-PCR on the remaining 35 samples was performed using the in-house one-step RT-PCR kit from QIAGEN. Of these, 32 (94.1%) gave products, making the total number of samples with amplification products to be 38. Of these, 30 (78.9%) were sequenced using the ViroSeq[®] primers. Ten additional samples with detectable RNA were re-amplified from the duplicate aliquots stored at EHNRI and were transported to the same genotyping laboratory for analysis using the in-house method and for genotyping using the TrueGene[®] system. Nine of these repeated samples were amplified and sequenced successfully. Hence, the total number of successfully genotyped samples was 39 (52.0%).

After visual inspection of the chromatograms and manual editing of the raw data produced by the two genotyping methods, the sequences were saved in FASTA format. Altogether, 78 sequences (39 each of the protease and reverse transcriptase) were produced and used in this threshold survey. All important positions in the WHO's list for drug resistance mutations (codons 24–90 for the protease and codons 41–236 for reverse transcriptase) were successfully sequenced (Table 2). Each of the 78 sequences generated and stored in the FASTA format were analysed using the Stanford HIV Reverse Transcriptase and Protease Sequence Database [26] for the presence of the relevant drug resistance mutations and subtype distribution. Results from this analysis showed that none of the HIV drug resistance mutations searched for was detected in any of the 39 genotyped samples. The Stanford database identified 38 of the samples (97.4%) as subtype C in both the protease and reverse transcriptase. The remaining sample belonged to the recombinant CRF02_AG in both the protease (96.6%) and reverse transcriptase (88.4%).

Discussion

Our analysis of specimens collected during Ethiopia's 2005 ANC-based HIV surveillance found no mutations associated with HIV drug resistance; therefore, on the basis of the WHO protocol, transmitted HIV drug resistance for all drugs and for each of the three drug classes (NRTI, NNRTI and protease inhibitor) is classified as <5% among HIV-infected patients in Addis Ababa. As this is the Ethiopian city where

Table 2. Successfully genotyped codons of protease and reverse transcriptase from the HIV Drug Resistance Threshold Survey

Sequenced regions	Successfully genotyped codons	Successful/total genotyped samples, <i>n</i> (%)	Drug resistance relevant mutations, <i>n</i> (%)
Protease	1–99	33/39 (84.6)	39 (100%)
	4–99	6/39 (15.4)	
Reverse transcriptase	1–333	27/39 (69.2%)	39 (100%)
	38–247	7/39 (17.9)	
	41–333	1/39 (2.5%)	
	38–333	1/39 (2.5%)	
	1–303	1/39 (2.5%)	
	1–295	1/39 (2.5%)	
	1–299	1/39 (2.5%)	

HIVDR transmission is expected to be seen first, we believe that the first-line ART strategy currently in use in Ethiopia can be used confidently; however, care must be exercised to maintain this low level of transmitted HIVDR. This is essential because when drug-resistant HIV strains are acquired at the time of primary infection they fuel the cellular reservoir and persist for a longer period of time [13,14], making future treatment options narrower. Because HIV treatment requires life-long routine follow-up, the present efforts and commitments including monitoring sustained drug supply, good prescribing practices, and enhancing patient support to ensure adherence must be maintained to sustain the observed low rate of transmitted HIVDR. Moreover, although use of ART by itself reduces HIV transmission rate by diminishing viral burden in infected persons, if transmission occurs in resource-limited settings there is a greater danger of infected persons being left with limited treatment options. Therefore, social support and awareness campaigns designed to reduce the likelihood that HIV-infected persons will transmit HIV to others must continue and should also encompass people on ART.

Generalization of the survey results has several limitations, these include the relatively low proportion of specimens (49%) with a viral load of >1,000 copies/ml. Explanations include accidental thaws during handling or transport of specimens, use of serum rather than plasma specimens (as serum has the inherent property of reducing viral load by at least twofold), delayed separation of serum from clot, and inclusion of individuals being treated with ARVs, which would lower the viral load to <1,000 copies/ml. If the specimen handling caused the difficulty, there is no reason to believe that specimens with drug-resistant virus would be more likely to be affected than other specimens, but bias remains a possibility. If specimens from individuals on ARVs were included, it would be considered an advantage that these could not be amplified for genotyping, as any resistance mutations

seen would not be likely to result from transmitted resistance but rather from treatment. A change to a more stable specimen type, such as plasma rather than serum, and minimizing the number of times specimens are transferred from one site to another could help improve amplification in future surveys. Strategies to evaluate ARV experience might also be explored for future surveys.

An additional limitation of the current threshold survey was that it included only public health centres in Addis Ababa. As up to 40% of ANC services are supported by private sectors in Addis Ababa, future surveys should include private health centres. Moreover, as ART is scaled up in Ethiopia, future surveys should extend beyond Addis Ababa and include major regional cities and towns.

Acknowledgements

This survey was financially supported by the US CDC. The investigators would like to thank all people who directly or indirectly contributed to the successful completion of this study: women who participated in the 2005 ANC-based HIV surveillance; laboratory technicians at the seven sentinel sites who were involved in blood drawing and serum separation; technicians at the EHNRI who assisted in the proper handling and storage of the samples; the drivers at EHNRI responsible for the timely collection and delivery of the samples from the ANC sites to the EHNRI laboratory; technicians at Addis Ababa Regional Laboratory, the Armed Forces Hospital, and the Federal Police Hospital, who did the HIV test on the surveillance samples at their respective site laboratories; technicians at EHNRI who did the confirmatory HIV test; and technicians at Central Virology Laboratory, Tel-Hashomer, who participated in several stages of sequencing and genotyping. International Medical Press is also acknowledged for their support with publishing this manuscript. Some of

the authors are staff members of the WHO. The authors alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions or stated policy of the WHO.

Disclosure statement

The authors declare that they have no competing interests.

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