Observed HIV drug resistance associated mutations amongst naïve immunocompetent children in Yaoundé, Cameroon

George Mondinde Ikomey[1,*], Marie Claire Okomo Assoumou[2], Josiah Otwoma Gichana[3], Duncan Njenda[4], Sello Given Mikasi[5], Martha Mesembe[6], Emilia Lyonga[7], Graeme Brendon Jacobs[8,*]

Abstract

Introduction The emergence of drug resistance mutations (DRMs) has been a major threat for successful lifelong combination antiretroviral therapy (cART), especially for HIV-vertically infected children within the context of the prevention of mother-to-child transmission (PMTCT). This study aimed to evaluate DRMs amongst immune competent treatment-naïve children in Cameroon.

Methods A cross-sectional study was conducted between 2015 and 2016 amongst 55 proxy consented HIV-1 positive children, aged 9 months to 6 years. They were all immune competent, cART naïve and with unknown history of PMTCT. CD4 cell counts and genotypic drug resistance testing were performed using standard methods.

Results Levels of DRMs to protease (PR) inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-NRTIs were 27.6%, 3.7% and 40.7%, respectively. Only minor DRMs were observed for PR. The observed mutations for NRTI were K65R, T215I and K219E (33.0% each) and for NNRTI: V106M, Y181C and Y188H (6.0% each). Only minor accessory mutations were found in the integrase (IN) region.

Conclusion Despite widely available cART we still observe naïve HIV children, especially from the rural communities. We observe that a proportion of study participants had HIV-1 drug resistance associated mutations (RAMs). Data generated could help strengthen the current PMTCT programmes within the country. There is a need to upscale approaches for drug resistance testing for children in Cameroon and many other resource-limited settings.

Keywords HIV, resistance, prevention of mother-to-child-transmission (PMTCT), infants, Cameroon, treatment-naïve, immune competent

Introduction

HIV-1 is still a major public health problem with approximately 36.7 million people living with HIV/AIDS globally.[1] Sub-Saharan Africa remains the worst affected as the number of HIV patients continues to rise.[2] In Cameroon, HIV remains a huge health burden with approximately

600,000 to 720,000 infected adults and 53,000 to 63,000 infected children of 0-14 years.[3,4] The introduction of combination antiretroviral therapy (cART) has seen a considerable reduction of the pandemic globally. In 2016, 19.5 million people were able to access cART (UNAIDS, 2017) – approximately a 19% increase from 15.8 million in June 2015, attaining the global treatment goal of 15 million people on cART by 2015.[3] More coverage is ongoing, especially with the rolling-out of programmes like prevention of mother-to-child transmission (PMTCT) and the 90-90-90 concept by the United Nations (UN) program on HIV/AIDS that started in 2013 focusing on the "Sustainable Development Goals" of ending the AIDS pandemic by 2030.[3] Despite these commendable efforts by the global community, the emergence of resistance to cART is a serious threat for the overall success of HIV treatment and this requires continued surveillance. In Cameroon, the HIV-1 drug resistance rates stand at approximately 3.6% for children as reported by the Cameroon national AIDS committee (2016).[5] The implementation of cART, especially in pregnant women and young infected children, has shown a notable reduction in the disease.[5] Unfortunately, epidemiological data generated on the pediatric HIV population on cART in Cameroon are limited. Cameroon follows the WHO recommendation for cART, with the first-line treatment regimen of two nucleoside reverse transcriptase inhibitors (NRTI), mainly tenofovir (TDF) or zidovudine (AZT) and lamivudine (3TC), and one non-nucleoside reverse transcriptase inhibitor (NNRTI), either efavirenz (EFV) or nevirapine (NVP). At the time of cART initiation the following recommendations were given for children under the age of 3 and under 10 kg: either AZT, stavudine (d4T) or abacavir (ABC), with either EFV or NVP as NNRTI. From 2006, the threshold of CD4 cell percentage levels for severe immunodeficiency was less than 25% for infants 11 months or less, less than 20% for

children aged 12 to 35 months, and less than 15% for children 3 years or older. Pre-treatment viral load was not mandated prior to cART initiation.

There has been substantial progress in the implementation of the simplified WHO approach, since the cART services have been decentralized.[6] According to reports gathered from stakeholders like The Joint United Nations Programme on HIV/AIDS (UNAIDS), the United States Centers for Disease Control and Prevention (CDC), together with the Cameroon government, there are about 585 PMTCT clinics across various regions in Cameroon.[3] Despite the huge success and availability of cART, it leads to an increased prevalence of HIV drug-resistant strains. It is often difficult to study the prevalence of HIV resistance associated mutations (RAMs) in resource limited settings, such as the conditions found in Cameroon. This is further fueled by insufficient patient adherence to treatment or incomplete suppression of viral replication during treatment, potentially associating the emergence of drug-resistant viruses.[6] There are limited centers in Cameroon performing HIV-1 resistance testing and these are found at the major research laboratories in the main cities of Yaoundé and Douala. Diagnostic resistance testing remains too expensive to implement despite government efforts to subsidize. The aim of this study was to investigate the HIV resistance patterns from an infant population from rural and urban surroundings of Yaoundé, Cameroon.

Methods
Ethics statement
Ethical clearances were obtained from the Cameroon National Ethical Committee and the Health Research Ethics Committee at Stellenbosch University with reference numbers 049/CNE/SE/2015 and N14/10/130, respectively. A formal study agreement has been signed by the two centers to conduct joint research.

Study design and procedures
In a cross-sectional study conducted between 2015-2016, 55 HIV-1 positive naïve and treated children were recruited from The Chantal Biya

Foundation Hospital and other health institutions around Yaoundé (Figure 1). Two main reasons hampered the enrollment process for this particular study. First, the majority of the babies were under critical clinical observation and most caregivers refused to participate in the study. Second, most infants admitted to the different hospitals had a recorded history of severe anemia and were excluded. For the patients who were included, a proxy consent form was obtained from the caregivers, which was generated using a standard designed questionnaire and included all the necessary demographic data. The majority of the caregivers lived in rural settings where health facilities are limited. We collected 3 mL of venous whole blood under standard conditions in EDTA anticoagulated tubes.

(CSCCD), University of Yaoundé I, Cameroon, and the Division of Medical Virology, Stellenbosch University, South Africa.

HIV screening

HIV serology status was confirmed based on the Cameroon national algorithms, which involves the use of two HIV rapid diagnostic tests and a confirmation with a p24 antigen/antibody detection ELISA kit. The Alere Determine™ HIV-1/2 (Abbott Laboratories, Abbott Park, Illinois, USA) and the SD Bioline HIV 1/2 version 3.0 (Standard Diagnostics, Inc., Kyonggi-do, Korea) were used with an ELISA antigen antibody kit (Integral Enzygnost ELISA kits, Siemens, Paris, France). All tests were performed following the manufacturer's guidelines.

Figure 1. A map of the different health districts around Yaoundé, Cameroon.
The samples were obtained from the 5 different health districts surrounding the CSCCD and the University of Yaoundé I, Cameroon. The map was created with scribble maps (www.scribblemaps.com).

Analysis sites

Samples were analyzed at the Immunovirology laboratory of the Center for the Study and Control of Communicable Diseases

HIV viral load determination

The RT-PCR Amplicor 1.5 commercial viral load kit was used to determine the patients' plasma HIV viral load (Roche Diagnostic

Systems, Pleasanton, California, USA). The assay was performed with a prepared master mix containing oligonucleotide primers specific to regions of the HIV-1 *gag* genes with upstream sense primers of Sk145 (5'-ACTGGGGGGACATCAACCACCCATGCA AT-3') and the downstream antisense of SKCC1B (5'-TACTAGTAGTTCCTGCTATGTCACTTCC-3') (HXB2 position 1359-1513). The minimum detection limit of the kits was 1.67 $\log_{10}$ or less than 40 copies/mL.

Determination of CD4+ cell counts and percentages

Fifty μL of whole blood collected in EDTA tubes were used for CD4 absolute and percentage counts. This was based on the principle of immunophenotyping using an automated Fluorescence Activated Cell Sorting (FACS) Count Analyzer with the BD FACSCount tri CD4/CD8/CD3 reagent kit (BD Biosciences, San Jose, California, USA). Samples, including quality controls, were analyzed based on the manufacturers' guidelines.

HIV resistance testing

HIV-1 resistance testing was done as previously described.[7,8] Briefly, viral RNA was extracted using the Qiagen Viral RNA extraction kit using the manufacturer's instructions (Qiagen, Hilden, Germany). A one-step RT-PCR reaction was performed with the SuperScript® III One-Step RT-PCR System with Platinum®Taq DNA Polymerase kit (Invitrogen, USA), followed by a second round PCR with GoTaq DNA polymerase (Promega, Wisconsin, USA). We amplified a 507 bp region of the HIV-1 protease (PR) (HXB2 position 2136-2650) and a 798 bp region of the reverse transcriptase (RT) (HXB2 position 2530-3334). We also amplified a small 250 bp fragment of the HIV integrase (IN) with the Unipol primers (HXB2 position 4025-4275). The primers were previously designed to ensure the PCR amplification and detection of the majority of HIV variants found in Cameroon.[9] All PCR products were sequenced using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI Prism 3130 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). Overlapping DNA fragments were assembled using Sequencer version 5.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) using the default parameter settings, with sequence quality above 75.0%. The sequences were characterized for RAMs according to the Stanford University HIV Genotypic Resistance Interpretation Algorithm version 8.3 (https://hivdb.stanford.edu/).

Statistical analyses

Data for the study were collected in an Excel sheet and analyzed using the Graph Pad Prism 5.0 software programme. The CD4 percentages were evaluated using Fisher's exact test. For the baseline characteristics and variables of gender and sex we applied the Chi-squared test.

Results

Demographic information

Of the 55 children recruited, 32 (58.0%) were females and 23 (42.0%) were males. Their ages ranged from 9 months to 6 years, with the mean age of 2.5 years old. Most mothers (34; 62.0%) were naïve to cART, while 21 (38.0%) were exposed to cART during their pregnancy. The demographic data are summarized in Table 1.

Table 1. Clinical and demographic information

Number of patients (n=55)	Female: 32 (58%) Male: 23 (42%)
Age	Youngest: 9 months Eldest: 6 years Mean: 2.5 years
Settings	Rural: 41 (74%) Urban: 14 (26%)
Treatment	cART naïve mothers: 34 (62%) cART exposed pregnant mothers: 21 (38%)
CD4 cell count	Lowest: 15% (500 cells/cmm) Highest: 44% (2000 cells/cmm) Mean: 958 cells/cmm
Viral load	Lowest: 4.6 $\log_{10}$ Highest: 5.87 $\log_{10}$ Mean: 4.40 $\log_{10}$

CD4 cell count and viral load

CD4 percentages and absolute counts ranged between 30% and 44.0% (median 33.0%, 500-2000 cells/cmm with a mean of 957), respectively. All patients were considered immunocompetent based on the CDC immune category classification system. The HIV-1 plasma RNA viral loads ranged between 4.6 and 5.87 $\log_{10}$ or (23,000 to 150,000) copies per milliliter with a median of 4.96.

PCR amplification and sequencing

We amplified and successfully sequenced 37/55 (67.3%) of our cohort samples for at least one of the HIV fragments analyzed. For the PR we could sequence 29 (52.7%) fragments, for the RT 27 (49.1%) and for the integrase (IN) 28 (50.9%). This was most likely due to the high HIV genetic variability found in the region, as all the major HIV groups and subtypes are found in the region.

HIV-1 resistance associated mutations

The observed numbers of RAMs against PI, NRTI and NNRTI are listed in Table 2.

Table 2. Observed RAMs found in the study

	Number of samples analyzed	Number of samples with RAMS	Percentage
Major NRTI	27	1	3.7%
Major NNRTI	27	11	40.7%
Minor PI	29	8	27.6%

NRTI – nucleoside reverse transcriptase inhibitors; **NNRTI** – non-nucleoside reverse transcriptase inhibitors; **PI** – protease inhibitors; **RAM** – resistance associated mutations.

We noticed no major PR RAMs in the 29 sequences examined. We detected minor PI RAMs in 8 sequences. L10I, observed in 4/29

sequences (20.7%), is a polymorphic accessory mutation, while K20I, observed in 20/29 sequences (62.1%) is the consensus amino acid in subtype G and CRF02_AG. Other minor mutations observed include: L10V (n=2; 6.9%), K20M (n=1; 3.5%) and G48R (n=1; 3.5%). For the 27 RT sequences evaluated we observed NNRTI resistance associated mutations in 11 of the 27 sequences (40.7%). These include the mutations V106M, Y181C and Y188H. The V106M mutation indicates high-level resistance to NVP and EFV, Y181C indicates reduced susceptibility to NVP, ETR, RPV and EFV, while Y188H indicates reduced susceptibility to NVP and EFV. One patient had multiple major NNRT and NRTI RAMs (Table 3). The NRTI mutations observed for this patient were K65R, T215I and K219E. The K65R mutation indicates high-level resistance to TDF, DDI and D4T, but low to intermediate-level resistance to 3TC and FTC. The T215I mutation indicates intermediate to high-level resistance to AZT and D4T, with low-level resistance to ABC, DDI and TDF. The K219E mutation, with accessory thymidine analogues mutations (TAMs), is associated with reduced susceptibility to AZT and possibly to D4T. These mutations indicate that this strain is highly resistant to all current classes of NRTI and NNRTI drugs.

Discussion

In this study we assessed the HIV resistance patterns from a cohort of 55 treatment naïve infected infants from Yaoundé, Cameroon. The majority of the study participants resided in rural areas, where much less attention is given to HIV/AIDS infections and healthcare. Most of the children had CD4% and absolute values within the therapeutic normal range, namely >30% and >500 copies/mL.[3] The value confers their immune-competent status as outlined in the WHO and CDC treatment guideline for HIV immunological staging.

In this study, levels of DRMs to PIs, NRTIs and NNRTIs were 27.6%, 3.7% and 40.7%, respectively. This includes one infant who had RAMs against multiple drugs. In a previous study in Cameroon Fokam et al. (2011) showed low

Table 3. Observed resistance associated mutations for one patient in the study (multiple resistance associated mutations against NRTIs and NNRTIs).

NRTI	Comments
K65R	High-level resistance to TDF, ddI, ABC and d4T. Low to intermediate-level resistance to 3TC and FTC.
T215I	Intermediate to high-level resistance to AZT and d4T. Low-level resistance to ABC, ddI and TDF.
K219E	K219Q/E are accessory TAMs associated with reduced susceptibility to AZT and possibly d4T.
NNRTI	
V106M	High-level resistance to NVP and EFV.
Y181C	Reduces susceptibility to NVP, ETR, RPV, and EFV.
Y188H	Causes reduced susceptibility to NVP and EFV.

ABC – abacavir; **AZT** – zidovudine; **d4t** – stavudine; **ddI** – didanosine; **EFV** – efavirenz; **FTC** – emtricitabine; **NRTI** – nucleoside reverse transcriptase inhibitors; **NNRTI** – non-nucleoside reverse transcriptase inhibitors; **NVP** – nevirapine; **PI** – protease inhibitors; **RAM** – resistance associated mutation; **RPV** – rilpivirine; **TAMs** – thymidine analog mutations; **TDF** – tenofovir disoproxil fumarate; **3TC** – lamivudine.

levels of HIV drug resistance in therapy naïve pediatric patients (4.9%), but high levels in patients failing first-line cART (90%).[10] Similarly, Ceccarelli et al. (2012) showed a RAM prevalence rate of 8.2% in therapy naïve children from Yaoundé, Cameroon.[11] In other previous studies done in Cameroon on therapy naïve adult patients a prevalence between 4% and 8% was observed for both NRTI and NNRTIs.[12,13,14] These studies suggest that resistance testing should become part of routine HIV diagnostics. There is a need to standardize resistance testing protocols, especially where there is a high endemic genetic diversity of HIV. Access to genotyping and resistance testing has been hampered by high cost and absence of technology, especially in the rural regions. The assays and data gained can help manage HIV patients failing cART by providing baseline knowledge on the RAMs.[15] We recommend that an HIV resistance test be given before initiating cART and that patients, especially infants have routine clinical testing to ensure the maximum efficiency of cART. Implementing resistance testing will also help avoid extra costs that may incur. Second-line therapy is already considered more expensive, and ineffective treatment can lead to additional morbidity with opportunistic infections, which may also become expensive to treat.[16,17]

Another major concern is the high number of infants still being born HIV positive from exposed mothers, even though widespread PMTCT programmes have been implemented in the country, as recommended by the WHO (2015).[1] To minimize HIV transmission, PMTCT programmes need to be improved. Access to clinics and healthcare facilities remain difficult and many HIV positive patients are too easily lost to follow-up.[5,18] Thus, infrastructure and access to facilities in rural Cameroon need to be improved. As yet, there are no core facilities to perform basic viral load testing, which is only done at major centers. The current estimates show that over a million children will still require cART by 2020.[19] The WHO programmes should be robustly implemented, as they look to support regimens that are more simplified, less toxic, have higher genetic barriers and would ultimately require less clinical monitoring for each patient, while maintaining therapeutic efficacy.[19]

The study limitations include the relatively small size of the study cohort, which was especially due to the diagnosis and treatment approach. The lack of follow-up and treatment and adherence data from the mothers is also a notable concern.

Conclusions

The observed drug RAMs in treatment naïve infants in our study raise concerns on the availability and effectiveness of the current treatment programmes. There is a need to have improved, cost effective HIV diagnostic and resistance assays for the region. The data generated from this study would help to strengthen HIV monitoring and resistance

testing in Cameroon and many other sub-Saharan African Countries.

References

1. World Health Organization (WHO). Cameroon country report. 2015. Accessed on: 17 March 2017. Available at: http://www.who.int/countries/cmr/en/.
2. Budambula V, Musumba FO, Webale MK, et al. HIV-1 protease inhibitor drug resistance in Kenyan antiretroviral treatment-naive and -experienced injection drug users and non-drug users. AIDS Res Ther 2015; 12:27. [Crossref] [PubMed] [FullText]
3. UNAIDS/WHO working group on global HIV/AIDS & STD surveillance. 2013/2015. AIDS epidemic update. Geneva, Switzerland: UNAIDS; 2015.
4. Takow SE, Atashili J, Enow-Tanjong R, et al. Time for Option B+? Prevalence and characteristics of HIV infection among attendees of 2 antenatal clinics in Buea, Cameroon. J Int Assoc Provid AIDS Care 2015;14:77-81. [Crossref] [PubMed]
5. Billong SC, Fokam J, Aghokeng AF, et al. Population-based monitoring of emerging HIV-1 drug resistance on antiretroviral therapy and associated factors in a sentinel site in Cameroon: low levels of resistance but poor programmatic performance PLoS One 2013;8:e72680. [Crossref] [PubMed] [FullText]
6. Aghokeng AF, Kouanfack C, Eymard-Duvernay S, et al. Virological outcome and patterns of HIV-1 drug resistance in patients with 36 months' antiretroviral therapy experience in Cameroon. J Int AIDS Soc 2013;16:18004. [Crossref] [PubMed] [FullText]
7. Jacobs GB, Loxton AG, Laten A, Robson B, van Rensburg EJ, Engelbrecht S. Emergence and diversity of different HIV-1 subtypes in South Africa, 2000-2001. J Med Virol 2009;81:1852-9. [Crossref] [PubMed]
8. Plantier JC, Dachraoui R, Lemée V, et al. HIV-1 resistance genotyping on dried serum spots. AIDS 2005;19:391-7. [Crossref] [PubMed]
9. Miura T, Sakuragi JI, Kawamura M, et al. Establishment of a phylogenetic survey system for AIDS-related lentiviruses and demonstration of a new HIV-2 subgroup. AIDS 1990;4:1257-61. [Crossref] [PubMed]
10. Fokam J, Salpini R, Santoro MM, et al. Drug resistance among drug-naive and first-line antiretroviral treatment-failing children in Cameroon. Pediatr Infect Dis J 2011;30:1062-8. [Crossref] [PubMed]
11. Ceccarelli L, Salpini R, Moudourou S, et al. Characterization of drug resistance mutations in naïve and ART-treated patients infected with HIV-1 in Yaounde, Cameroon. J Med Virol 2012;84:721-7. [Crossref] [PubMed]
12. Ndembi N, Abraha A, Pilch H, et al. Molecular characterization of human immunodeficiency virus type 1 (HIV-1) and HIV-2 in Yaounde, Cameroon: evidence of major drug resistance mutations in newly diagnosed patients infected with subtypes other than subtype B. J Clin Microbiol 2008;46:177-84. [Crossref] [PubMed] [FullText]
13. Aghokeng AF, Vergne L, Mpoudi-Ngole E, et al. Evaluation of transmitted HIV drug resistance among recently-infected antenatal clinic attendees in four Central African countries. Antivir Ther 2009;14:401-11. [PubMed]
14. Agyingi L, Mayr LM, Kinge T, et al. The evolution of HIV-1 group M genetic variability in Southern Cameroon is characterized by several emerging recombinant forms of CRF02_AG and viruses with drug resistance mutations. J Med Virol 2014;86:385-93. [Crossref] [PubMed] [FullText]
15. Fokam J, Billong SC, Bissek AC, et al. Declining trends in early warning indicators for HIV drug resistance in Cameroon from 2008-2010: lessons and challenges for low-resource settings. BMC Public Health 2013;13:308. [Crossref] [PubMed] [FullText]
16. Lessells RJ, Avalos A, de Oliveira T. Implementing HIV-1 genotypic resistance testing in antiretroviral therapy programs in Africa: needs, opportunities, and challenges. AIDS Rev 2013;15:221-9. [PubMed] [FullText]
17. Levison JH, Wood R, Scott CA, et al. The clinical and economic impact of genotype testing at first-line antiretroviral therapy failure for HIV-infected patients in South Africa. Clin Infect Dis 2013;56:587-97. [Crossref] [PubMed] [FullText]
18. Fokam J, Billong SC, Bissek AC, et al. Declining trends in early warning indicators for HIV drug resistance in Cameroon from 2008-2010: lessons and challenges for low-resource settings. BMC Public Health 2013;13:308. [Crossref] [PubMed] [FullText]

19. Penazzato M, Dominguez K, Cotton M, Barlow-Mosha L, Ford N. Choice of antiretroviral drugs for postexposure prophylaxis for children: a systematic review. Clin Infect Dis 2015;60 Suppl 3:S177-81. [Crossref] [PubMed]