Use of Amplification Refractory Mutation System PCR assay as a simple and effective tool to detect HIV-1 drug resistance mutations

Running title: Use ARMS-PCR to detect HIV drug resistance mutations

Aubin J. Nanfack1,2,3, Lucy Agyingi4,5, Jean Jacques N. Noubiap5,6, Johnson N. Ngai5, Vittorio Colizzi2,7 and Phillipe N. Nyambi1,8#.

1. Department of Pathology, New York University School of Medicine, New York, NY, United States
2. Department of Immunology and Applied Biotechnology, University of Rome “Tor Vergata”, Rome, Italy
3. “Chantal Biya” International Reference Center, Sequencing Unit, Yaounde, Cameroon
4. Faculty of Sciences, University of Dschang, Dschang, Cameroon
5. Serology Unit, Medical Diagnostic Center, Yaounde, Cameroon
6. Internal Medicine Unit, Edéa Regional Hospital, Edéa, Cameroon
7. UNESCO Chair of Interdisciplinary Biotechnology, University of Rome “Tor Vergata”, Rome, Italy

# Corresponding author:
Phillipe N. Nyambi
Department of Pathology, New York University School of Medicine, c/o V.A. Medical Center, 423 East 23rd Street, Room 18124N, New York, NY 10010, United States.
Tel.: +1 212 263 6769; fax: +1 212 951 6321; E-mail: phillipe.nyambi@nyumc.org
Access to genotyping assays to determine successful antiretroviral treatment (ART) is limited in resource-constrained settings by high cost, suggesting the need for a cost effective and simplified method to identify HIV-1 drug resistance (HIVDR) mutations. In this study, the Amplification Refractory Mutation System (ARMS)-PCR assay was developed and used to investigate the most frequent HIVDR mutations affecting first line ART in settings that apply WHO ART guidelines. Seventy-five HIV+ samples from Cameroon were used to assess the performance of this assay. Sequencing of HIV-1 reverse transcriptase was simultaneously performed for comparison and discordant samples were tested with Trugene HIV-1 genotyping kit. ARMS-PCR assay was able to detect M184V, T215Y/F, K103N, and Y181C mutations with sensitivity of 96.8%, 85.7%, 91.3%, 70%, respectively, and specificity of 90.6%, 95%, 100%, 96.9%, respectively, when compared with data on sequencing. The results indicated the highest positive predictive value for K103N (100%) and the highest negative predictive value for M184V (97.5%). ARMS-PCR’s limit of detection for mutations M184V, T215Y/F, K103N and Y181C were <75 copies/ml, 143 copies/ml, 143 copies/ml and 836 copies/ml, respectively. ARMS-PCR efficiently identified mutations in individuals with different HIV-1 clades (CRF02_AG and non-CRF02_AG). More so, this approach was more cost-effective than other genotyping assays. Given the high throughput, the cost-effectiveness, and simplicity of the ARMS-PCR assay makes it a suitable tool to monitor HIVDR patterns in resource-constrained settings with broad HIV-1 genetic diversity.

Key words: HIV drug resistance mutation, Amplification Refractory Mutation System PCR (ARMS-PCR), sequencing, Resource-constrained settings.
Introduction:

In recent years, significant progress has been made in the treatment of HIV infected patients. Gains in antiretroviral treatment have been achieved in low and middle income countries with more than 9.7 million people living with HIV/AIDS of whom 7.2 million live in Sub-Saharan Africa as of end of 2012 (WHO, 2013). This scaling up of antiretroviral therapy in resource-constrained settings where the logistics of medication disbursement and patient follow-up are not always optimal, will result in the emergence and transmission of HIV drug resistant variants thus, providing obstacles to successful ART programs (3). The monitoring of HIV drug resistant mutations in developed countries is an important aspect of clinical management of HIV infection. In these developed countries, sequencing-based drug resistance assays are used to select the most appropriate regimens when initiating or switching ART (18, 24). In resource-constrained settings, due to its high cost, only a limited number of HIV drug resistance mutations tests are performed for patient management. In such countries, the majority of tests are used for research purposes or surveillance of HIVDR mutations recommended by WHO to update antiretroviral therapy policies (5, 6).

Resource-constrained settings use the WHO public health approach for the treatment of HIV infected patients. This approach recommends the standardized and simplified treatment protocol consisting of two nucleoside reverse transcriptase inhibitors (NRTI) plus one non-NNRTI (NNRTI) for first line therapy. Treatment initiation or switch is based on clinical parameters and CD4 count because viral load assessment is not feasible for the majority of patients (14, 19). In addition to these challenges, the low genetic barrier of commonly used antiretrovirals (ARV) for first line treatment further increases the risks of HIVDR mutations. The most prevalent mutations that cause intermediate to high-level resistance to this first line regimen in countries such as those in the Central African region including Cameroon, Central African Republic, Gabon, etc. that use WHO approach, are M184V (37-90%), T215Y/F (11-47.2%), K103N (14-44%) and Y181C (9.4-19.8%) (2, 9, 10, 27, 29, 37). These can restrict
treatment options and increase cost by requiring new and more expensive ARV regimens (WHO, 2012).

The detection of HIVDR mutations essentially depends on genotyping assays. However, their use is limited in resource-constrained settings because these assays require sophisticated equipment such as genetic analyzer, costly reagents, and highly sophisticated manpower. Some laboratories in developing countries have developed and validated highly sensitive in-house HIV-1 genotyping assays comparable to FDA-approved assays to reduce the cost and to overcome subtype specificities (3, 12, 31). Despite these efforts, these in-house genotyping assays still remain unaffordable to the majority of patients in need.

Point mutation assays have been developed as alternatives for genotyping assays. These assays are allele-specific and include the mutant-allele-specific amplification (MASA), PCR amplification of specific alleles (PASA), and the amplification refractory mutation system (ARMS)-PCR (17, 28-30, 32). These methods utilize the difference in extension efficiency between primers with matched and mismatched 3' bases. Product detection and identification are the most variable steps that contribute to the throughput capacity of each particular method. The drawback with some of these assays is the detection method that includes fluorescence resonance energy transfer, fluorescence polarization, luminescence or absorbance (4, 13, 26), requiring expensive equipment and reagents; which still have limited use in resource-constrained settings. Thus, we have developed and evaluated the use of a simplified ARMS-PCR, to detect the most frequent HIV-1 drug resistance mutations in individuals infected with strains of broad HIV-1 genetic diversity.

**Material and methods**

**Ethical Considerations**
This study was approved by the Institutional Ethical Review Board of New York University School of Medicine, New York, USA and by the Institutional Review Board of Cameroon’s Ministry of Public Health. Written informed consent was obtained from all the study participants.

**Characteristics of the study patients**

The samples analyzed in this study are part of an ongoing project in Cameroon aimed at analyzing viral evolution and humoral immune response to dual HIV-1 infection. Plasma samples from 75 HIV-1 infected patients were included in the study whose demographics and clinical characteristics are described in Table 1. Forty-eight (64%) of them were women and the median age was 38 years [IQR 33.5-45]. Thirty-three (44%) patients were ART naïve, thirty-nine (52%) were receiving 1st line ART and three (4%) were receiving 2nd line ART. The median time on ART was 15 months [IQR 11-26.5] for subjects on first line ART and 32 months [IQR 26-38] for patients receiving 2nd line ART. The median viral load (VL) was 9,373 copies/ml for naïve [IQR 2,878-18,762], 17,314 [IQR 7,031-34,927] for patients on first line ART and 14,535 [IQR 8,890.5-16,394] for patients on second line ART. Among patients receiving first line ART, AZT+3TC+NVP/EFV, TDF+3TC+NVP/EFV, d4T+3TC+NVP, and 3TC+NVP combinations were taken by 28%, 18.6%, 2.6% and 2.6%, respectively. Among patients receiving 2nd line ART, TDF+3TC+LPV/r and TDF+3TC+ATV were taken by 2.6% and 1.3%, respectively.

**Viral load determination**

HIV-1 plasma viral load was determined by the Versant HIV RNA 3.0 Assay (bDNA; Siemens, IL) as recommended by the manufacturer using the same plasma specimen used for ARMS-PCR and the sequencing of reverse transcriptase.
Selection of HIV drug resistance mutations for ARMS-PCR assay

HIV drug resistance mutations were selected based on their prevalence, their effect on first line regimen recommended by WHO for resource-constrained settings and the Stanford HIV drug resistance data base mutation scoring system (http://hivdb.stanford.edu/DR/asi/releaseNotes/index.html). Using the aforementioned criteria, M184V and T215Y/F were selected as nucleoside reverse transcriptase inhibitor (NRTI) resistance mutations, while K103N and Y181C were selected as non-NRTI (NNRTI). M184V is the most common NRTI resistance mutations (37-90%) causing high level resistance to lamivudine (3TC) and Emtricitabine (FTC). T215Y/F is the most common thymidine analog mutation (TAM) (11-47.2%) that causes intermediate level resistance to zidovudine (AZT) and stavudine (d4T), and with the potentials of causing high level resistance to AZT and d4T when occurring with other TAMs (M41L, D67N, K70R, L210W, K219QE). K103N is one of the most common NNRTIs resistance mutations (14-44%) causing high-level resistance to nevirapine (NVP) and efavirenz (EFV). Y181C is a common NNRTIs resistance mutation (9.4-19.8%) that reduces >50 fold the susceptibility to NVP (http://hivdb.stanford.edu/). The prevalence of these mutations varies from 8.2% in naïve patients to up to 90% in patients failing a first-line ART (2, 7, 8). Drugs affected (FTC, 3TC, AZT, ABC, EFV and NVP) are those which are among the most commonly used regimen as recommended by WHO guidelines for first line treatment in resource-constrained countries, especially in sub-Saharan Africa (14).

RNA Extraction and Reverse Transcription PCR (RT-PCR)

Virus in 500 µl of plasma was concentrated by ultracentrifugation at 14,000 x g for 1 hour at 4°C prior to RNA extraction. After removal of 360 µl of supernatant, the virus pellet was resuspended by vortexing and viral RNA was extracted from plasma using the QIAamp Viral
RNA Mini kit (Qiagen, Inc., Valencia, CA) according to manufacturer's instructions. A total of 2.5 µl of RNA extract was used for the amplification of 1,750-bp of the pol region using the Superscript one step RT-PCR system with platinum Taq (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. For RT-PCR, the forward primer RTPOL1F (5'—GTATTAGTAGACCTACCTGTC-3'; HXB2 location 2475–2498) and the reverse primer RTPOL1R (5'—ACCTTCCTGATTCCATTACTGAC-3'; HXB2 location 4203–4225) were used. The cycling conditions includes an initial cDNA synthesis step for 30 min at 50°C, followed by denaturation for 2 min at 94°C, then 35 cycles at 94, 50, and 72°C for 60, 60, 90 sec respectively, and a final extension step at 72°C for 7 min.

Control

The specificity of the ARMS-PCR assay was assessed using an HIV-1 subtype B molecular clone containing several NRTIs and NNRTIs associated mutations. This clone (AIDS reagent program Cat # 12229; GenBank accession number # AF324493) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr Robert Shafer.

ARMS-PCR procedure

HIV-1 specific primers were manually designed after compiling different HIV-1 pure and recombinant subtypes that are most prevalent in Central Africa from the HIV sequence database (http://www.hiv.lanl.gov). For each mutation tested, two reverse primers were designed so that their 3' terminus nucleotide ends where both sequences, wild type (Wt) and mutant (Mut) variants, differ. Additional deliberate mismatches were introduced within the last four nucleotides at the 3' terminus to increase the specificity of the assay using principles established for ARMS-PCR primer design (22, 28). The forward primers were designed following the classical rules and so as to obtain a short length amplification product (200 to 600
An amplified pol gene fragment from RT-PCR was used as the template to detect wild
type and mutant sequences at different codons (M184V, T215Y/F, K103N and Y181C) by
ARMS-PCR. Two reactions (wild type and mutant) were performed for each mutation with the
respective specific primers using platinum PCR SuperMix High fidelity (Invitrogen, Carlsbad,
CA). The cycling conditions include an initial denaturation of 2 min at 94°C, followed by 35
cycles at 94, 55 and 72°C, respectively. Ten microliters of ARMS-PCR products were visualized
on a 2% agarose gel after ethidium bromide staining and picture was taken with inverted gel
image using the ChemiDoc MP system from Biorad. The two amplified products from each
sample were run on the 2% agarose gel one next to the other. The ARMS-PCR result was
recorded as Wt or Mut depending on the amplicon band detected on the agarose gel picture.
For patients harboring wild type variant, only one band was expected while two bands of the
same molecular size were expected for patients harboring a mutant variant due to the presence
of mixed viral populations (wild type and mutant). ARMS-PCR results were compared to the
sequencing data, the latter being the gold standard. The specificity of the ARMS-PCR primers to
detect M184V, T215Y/F, K103N and Y181C mutations was assessed using the subtype B clone
obtained from NIH. Only mutant amplicons bands for mutations M184V, T215Y/F, K103N and
wild type amplicon band for mutation Y181C were expected on the agarose gel.

Sequencing

Two microliters of RT-PCR product was used for nested PCR using RTPOL2F as
forward primer (5′-TAAAGCCAGGAATGGATGGCCC-3′; HXB2 location 2584–2605) and
RTPOL2R as reverse primer (5′-CCTCCAATCCCTTTGTGTGCTG-3′; HXB2 location 4159–
4180). The PCR cycling conditions were an initial denaturation for 2 min at 94°C, followed by 35
cycles at 94, 50, and 72°C for 60, 60, 90 sec, respectively, and a final extension step of 7 min at 72°C. Direct sequencing of the 5’ and 3’ ends of the purified PCR amplicons were performed with the two nested primers RTPOLF2 and RTPOLR2 by Microgen (New York, NY), using the ABI Big Dye Terminator cycle sequencing chemistry on an ABI 3730XL DNA analyzer.

Phylogenetic Analysis and Drug-Resistance Genotyping

All sequences were automatically aligned with reference sequences of all known HIV-1 group M subtypes and circulating recombinant forms (CRFs) from the Los Alamos HIV sequence database using CLUSTAL X with minor manual adjustments. Phylogenetic analyses were conducted using the MEGA version 3.1 software package (20) with pairwise evolutionary distances estimated by using Kimura's two-parameter method. Phylogenetic trees were constructed by the neighbor-joining method and the reliability of the topologies was estimated by performing bootstrap analysis. Clustering of sequences with bootstrap value of more than 70% was considered significant for subtyping.

The reverse transcriptase DNA sequences amplified from patients were analyzed for potential drug-resistance mutations using the Stanford University HIV database genotypic-resistance interpretation algorithm (http://hivdb.stanford.edu/index.html). Mutations in the study sequences were defined as differences from the consensus B reference sequence and were further characterized as NRTI-resistance mutations or NNRTI-resistance mutations.

Trugene® HIV-1 genotyping assay

Ten patients with concordant results and 10 patients with discordant results between sequencing and ARMS-PCR, and with varying viral load and different HIV-1 clades were genotyped using Trugene HIV-1 genotyping kit (Siemens, IL), an FDA-approved assay for the detection of HIV-1 drug resistance; the test was performed as recommended by the manufacturer.
ARMS PCR performance analysis

Using sequencing as gold standard, the sensitivity, specificity, positive predictive and negative predictive values were calculated from the following formulas: Sensitivity = true positives/(true positives + false negatives) \times 100; true positives being the number of samples positive on ARMS-PCR and Sequencing (gold standard), and false negatives being the number of samples negative on ARMS-PCR but positive on sequencing. Specificity = true negatives/(true negatives + false positives) \times 100; with true negatives being the number of samples negative on ARMS-PCR and sequencing (gold standard), and false positives being the number of samples positive on ARMS-PCR and negative on sequencing (gold standard). Positive predictive value (PPV) = true positive / (true positive + false positive) \times 100. Negative predictive value (NPV) = true negative / (true negative + false negative) \times 100.

Results

Use of ARMS-PCR to detect the M184V, T215Y/F, K103N and Y181C mutations in plasma samples.

In these studies, we first insured that the ARMS-PCR was able to correctly detect the different HIVDR mutations in the subtype B molecular clone which we obtained from the NIH reagent program. These mutations included: K103N, M184V, Y181C, and T215Y/F and were correctly detected as shown in the gel picture presented in Figure S1. Representative gel pictures revealing the detection of the different HIVDR mutations among eight HIV-1 patients infected with different HIV-1 subtypes, on different treatment regimens, and varying viral loads studied are shown in Figure 1, to illustrate that this assay is able to detect the various HIVDR mutations studied. These HIVDR mutations as well as the wild type amino acids identified by
ARMS-PCR shown in the gel pictures in Figure 1 were also confirmed by sequencing as shown in Figure 2.

We also evaluated the ARMS-PCR assay to detect HIVDR mutations in samples with varying viral loads as well as with different HIV-1 subtype variants. Thus, we tested the limit of detection of HIVDR mutations using a plasma sample from a patient with high viral load (18,253 copies/ml) infected with a CRF02_AG variant. The plasma sample was serially diluted at 1/5, 1/25, 1/125 and 1/625 in RPMI and the viral load measured and corresponded to 4,977, 836, 143, and <75 copies/ml, respectively. All the four HIVDR mutations, M184V, T215Y/F, K103N and Y181C were detected in samples corresponding to a viral load of ≥836 copies/ml. Furthermore, T215Y/F and K103N could also be detected at 143 copies/ml; and M184V was also detected at a viral load as low as <75 copies/ml (Figure 3). This analysis confirms detections of HIVDR mutations with low viral loads, assuring the high sensitivity of ARMS-PCR assay.

The 75 HIV-1 infected patients studied harbored viral loads that ranged between <75 to 400,927 Copies/ml. We noted that ARMS-PCR was able to detect mutations in patients with low and in those with high viral loads. For example, in patient 06ARC001 with a low viral load of 3,135 copies/ml the M184V and K103N mutations were detected, in patient 06ARC036 with moderate viral load of 8,221 copies/ml mutations M184V, T215Y/F, K103N and Y181C were detected, and in patient NYU6525-10 with a high viral load of 17,876 copies/ml mutation M184V was detected (Figure 1). These results suggest that these mutations can be detected in plasma samples with low to high viral loads.

While the CRF02_AG recombinant variant is the predominant strain infecting individuals in Cameroon, several other subtypes and recombinants also cause infections. Thus, we examined whether the ARMS-PCR assay was able to detect mutations in CRF02_AG variants as well as in non-CRF02_AG variants. As shown in Figure 1 with the eight patient samples, infections due to CRF02_AG were present in patient 06ARC001, 06ARC010, 06ARC036 and
MDC020-5; and non-CRF02_AG infections were present in patient MDC029-1 (subtype D), MDC179-3 (subtype F2), NYU6525-10 (subtype CRF11_cpx), and 128-01 (subtype CRF22_01A1). Using the samples from these patients, we were able to detect mutations in these different subtype infections. For example, M184V mutation was detected in patient 06ARC001 (CRF02_AG), NYU6525-10 (CRF11_cpx), and 128-01 (CRF22_01A1); Mutation T215Y/F was detected in patient 06ARC036 (CRF02_AG), and 128-01 (CRF22_01A1), and mutation Y181C was detected in patient 06ARC036 (CRF02_AG) and 128-01 (CRF22_01A1).

The results of these analyses were confirmed by sequencing of the reverse transcriptase gene (Figure 2). Overall, HIV-1 drug resistance mutations were found in patients infected with CRF02_AG subtypes as well as in those with non-CRF02_AG infections of which the M184V mutation was the most prevalent in both groups and none of the mutation was found exclusively in one of the groups (see Table S2).

Detection of M184V/T215Y/F (NRTIs) and K103N/Y181C (NNRTIs) drug resistance mutations using ARMS-PCR assay.

After establishing that the ARMS-PCR could correctly detect HIVDR mutations, we then used the assay to study the mutations in the 75 study subjects including those who were ART-naïve (n=33) and those on ART (n=42). The combinations of HIVDR mutations are shown in Table 2.

Detection of HIVDR mutations in ART-Naive patients: Of the 33 ART naïve patients studied 6 (18.1%) harbored viruses with M184V or T215Y/F mutations, of which one (3%) concomitantly had both mutations. Four (12%) patients had M184V mutation whereas 3 (9%) patients had T215Y/F mutation. Five (15.1%) ART-naïve patients had K103N mutation and none of them had Y181C mutation.

Detection of HIVDR mutations in patients on 1st line ART: Among the 39 patients on first line ART, 29 (74.3%) harbored viruses with M184V or T215Y/F mutations, of which 9 (23%)
concomitantly had both mutations. Twenty-nine (74.3%) patients had M184V mutation whereas 9 (23%) had T215Y/F mutation. Nineteen (48.7%) patients harbored viruses with K103N or Y181C mutations of which 4 (10.2%) concomitantly had both mutations. Sixteen (41%) patients had K103N mutation whereas 9 (23%) patients had Y181C mutation.

**Detection of HIVDR mutations in patients on 2nd line ART:** All the three patients harbored viruses with the two NRTI (M184V and T215Y/F) mutations tested. Of the NNRTI mutations, one patient harbored virus with the K103N mutation and another patient had virus with the Y181C mutation.

**Detection of M184V/T215Y/F (NRTIs) and K103N/Y181C (NNRTIs) drug resistance mutations using sequencing.** The HIVDR mutations identified by sequence analysis are shown in Table 2. The results reveal that among the 33 ART naïve patients studied, 3 (9%) harbored viruses with M184V or T215Y/F mutation, of which 1 (3%) concomitantly had both mutations. Three patients (9%) had M184V mutation whereas 1 (3%) had T215Y/F mutation. Five (15.1%) patients had K103N mutation and no ART naïve patient was identified with Y181C mutation.

Among the 39 patients on first line ART, 26 (66.6%) harbored viruses with M184V or T215Y/F of which 10 (25.6%) concomitantly had both mutations. Twenty-six (66.6%) patients had M184V mutation whereas 10 (25.6%) had T215Y/F mutation. Twenty-two (56.4%) patients harbored viruses with K103N or Y181C of which 5 (12.8%) concomitantly had both mutations. Seventeen (43.5%) patients had K103N mutation whereas 9 (23%) had Y181C mutation.

The results of sequence analysis of all the three patients on 2nd line ART were identical to those of ARMS-PCR in detecting the two NRTI (M184V and T215Y/F) mutations tested. Furthermore, the detection of the NNRTI mutation in only one patient who harbored virus with...
the K103N mutation and another patient that had virus with the Y181C mutation was also similarly revealed by the ARMS-PCR.

**Performance of ARMS-PCR assay vs sequencing and Trugene HIV-1 genotyping kit.**

The mutation rates obtained with ARMS-PCR and sequencing was comparable. Sixty three out of 75 patients had similar results for all the mutations tested using ARMS-PCR and sequencing. The highest number of false positives (n=4) was obtained for M184V mutation whereas the highest number of false negatives (n=3) was obtained for Y181C mutation as shown in Table 3.

Ten patients with concordant results and 10 patients with discordant results between sequencing and ARMS-PCR were tested with Trugene HIV-1 genotyping assay and the results obtained were 100% concordant with the sequencing results. The sensitivity, specificity, PPV and NPV of ARMS-PCR assay were calculated for each mutation and results are presented in Table 3. The highest sensitivity (96.8%) and the highest NPV (97.5%) was for the detection of the M184V mutation, while the highest specificity (100%) and the highest PPV (100%) was for the detection of K103N mutation.

**Concordance of prevalence rates of mutations based on ARMS-PCR and sequencing**

The overall prevalence using ARMS-PCR was 48%, 20%, 29.3% and 12% for M184V, T215Y/F, K103N and Y181C mutations, respectively whereas the prevalence rates using sequencing were 42.6%, 18.6%, 30.6% and 13.3% for M184V, T215Y/F, K103N and Y181C mutations, respectively (see Figure S2). The ability of the ARMS-PCR to detect the various HIVDR mutations at the individual level and at the population level when all the 75 samples are analyzed are comparable to those of sequence analysis (Figure S2).

**HIV-1 subtypes infecting study subjects**
Phylogenetic analysis of the reverse transcriptase gene of studied patients revealed a predominance of CRF02_AG and several other variants. These HIV-1 subtypes included: CRF02_AG (70.6%), CRF11_cpx (9.3%), CRF22_01A1 (8%), F2 (4%), D (4%), A1 (1.3%), G (1.3%) and CRF18_cpx (1.3%). The distribution of the infecting subtypes was similar among treatment naïve patients versus those on treatment (data not shown).

Discussion

The scaling-up of antiretroviral treatment (ART) in resource-constrained settings without adequate virological follow up raises concerns on ART program sustainability. Sequencing technology is not available for all patients in need, and where available, it is not affordable because of financial constraints, therefore providing an alternative cost effective and less laborious assay to identify HIVDR mutations will be a great asset. In this study, ARMS-PCR assay was developed and used to investigate the presence of the most common HIVDR mutations (M184V, T215Y/F, K103N and Y181C) affecting first line ARV regimen. The results of the ARMS-PCR was highly concordant to those of sequencing in identifying all four mutations studied when comparing the results of each sample in both assays for each mutation (Figure S2a) or when all the samples are compared at the population level (Figure S2b). In particular, the assay was most sensitive in identifying the M184V and K103N mutations with sensitivities of 96.8% and 91.3%, respectively. The specificities of these mutations, M184V and K103N were also high, 88.5% and 100%, respectively. HIVDR drug resistance mutations were found in patients infected with CRF02_AG subtypes and non-CRF02_AG at comparable rates and the M184V mutation was the most prevalent in both groups.

Taken together all the mutations studied, a few samples (n=9) revealed discordant results for the different mutations whereby some were identified by ARMS-PCR as positive whereas they were negative on sequence analysis. Also, a few (n= 8) were negative on ARMS-PCR but were positive on sequence analysis (Table 3). The few false positives identified could
be due to the fact that like many point mutation assays, the ARMS-PCR is more sensitive than sequencing in detecting mutations in mixed HIV-1 genotypic populations (33). The few results that were false negatives could be due to unusual polymorphisms. These false negatives and positives raise the need to further study the variables that account for them to improve the assay. These also raise the need to randomly select specimens and quality control the results in other reference labs especially when conducting population based surveillance in public health programs prior to introduction of antiretroviral drugs. Nonetheless, the ARMS-PCR still revealed a high performance in identifying HIVDR when compared with sequence analysis and has several advantages over other alternate approaches that have been developed. For example, most of the point mutation assays use mass spectrometry, fluorescence, or luminescence to identify the allele-specific PCR products (5,17), this implies the use of expensive equipment and reagents. Furthermore these assays are designed to be performed in 96-well plates requiring many samples to perform the test while ARMS-PCR can be performed even with only one sample. The use of ARMS-PCR reduces the time to results taking an average of 8 hours. The high throughput of this assay also reduces the cost of supplies, and reagents. These advantages make the ARMS-PCR cost effective and affordable for resource-constrained settings.

The results of our newly developed ARMS-PCR assay which is confirmed by sequence analysis and the Trugene HIV-1 genotyping kit now allows us to describe the HIVDR mutations identified in our study subjects. The samples from the study subjects were obtained between the period 2006 to 2014. This study revealed a high rate of the first line drug resistance mutations studied in ART naïve (33) and patients on ART (42). Among the ART naïve patients, 18% and 15% harbored viruses with NRTI (M184V, T215Y/F) and NNRTI (K103N, Y181C) associated mutations, respectively. This prevalence is higher when compare to earlier studies in Cameroon that reported transmitted drug resistance in Cameroon ranging from 1.9 to 12.3% (3, 8, 27) in ART naïve patients. This increasing rate of transmitted HIVDR mutation is consistent with the
increase availability of ARV in Cameroon over time. A meta-analysis of data from many low and middle income countries including African countries with ART programs similar to Cameroon, have also reported an increase of transmitted drug resistance mutations (16). Similarly, reports in developed countries with long ART experience have shown an increase in transmitted drug resistance mutations to up to 25% (9, 15, 25). The most prevalent mutation was K103N with 15%. This could be explained by the wide use of Nevirapine in first line combinations and prophylaxis for prevention of mother-to-child transmission of HIV-1, therefore K103N mutation is more likely to develop and spread in the population (21).

Treated patients exhibited high rates of HIVDR mutations tested: M184V (76.1%), T215Y/F (28.5%), K103N (40.4%) and Y181C (21.4%). These findings are consistent with previous studies that have reported similar rates of acquired drug resistance mutations in Cameroon (2, 8) and other African countries with similar ART programs (1, 10, 34). The most prevalent mutations identified were M184V and K103N. The high frequency of these mutations in patient on ART is related to the wide use of 3TC and NVP/EFV based first line combinations representing 94.8% (37/39) of drug used by patients on first line ART in our study, therefore these mutations are more likely to develop in this population. This high rate of HIVDR mutations is the result of a median time of only 15 months for the patients that were on first line ART and 32 months for patients on second line ART.

The mutations identified in this study confer intermediate to high level resistance to all the ARVs (3TC, FTC, AZT, NVP and EFV) used in resource-constrained settings for first line treatment except TDF. Patients with these mutations require a switch to second line treatment only after a median time of 15 months on first line in settings where the drug options for therapeutic switch are already limited, thus, requiring new and more expensive ARV regimens (WHO, 2012). The high rate of transmitted and acquired HIVDR mutations observed in this study underlines the necessity to improve efforts for closer monitoring of viral load in HIV
infected patients and the necessity to make drug resistance testing assays more accessible and more affordable. This will guide physicians in the choice of the most appropriate therapy for treatment initiation and a therapy switch in a timely manner to sustain ART programs in resource-constrained settings. It should be noted that better outcomes are expected in patients who failed first line only when they switch ART within 8 weeks of failure (23).

Because of financial constraints, evaluations of ARV treatment programs in poor settings are usually done in a limited number of patients and only in a few sites (6, 11). The ARMS-PCR assay would be an advantageous tool to evaluate current antiretroviral programs in larger scale populations and in more sites, to better assess the need for the introduction of new ARVs. In fact, ARMS-PCR assay requires only $10 to cover the reagent cost of one mutation ($40 for four mutations) compared to sequencing and commercial genotyping assays that cost $120-280 in resource-constrained settings (15). The use of this assay will facilitate surveillance studies and will improve ART programs, thereby reducing the risk of transmitted and acquired drug resistance mutations and improve the monitoring and care of HIV-1 infected patients.

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References


FIGURE LEGENDS

Figure 1: HIV-1 drug resistance mutations detected by ARMS-PCR in plasma samples from HIV-1 infected individuals. These pictures represent the 2% agarose gel stained with ethidium bromide showing the results of eight individuals infected with different HIV-1 subtypes, on different ART and harboring varying viral loads. M, molecular marker (Low range DNA ladder, Invitrogen). WT, wild type; MUT, mutant; N Ctrl, Negative control. The presence of a band in the MUT lane indicates the presence of the mutation in the sample. Pictures were taken with inverted gel image using the ChemiDoc imaging system, Biorad. The viral load in each sample...
were 06ARC001 (3,135 copies/ml), 06ARC010 (14,715 copies/ml), 06ARC036 (8,221 copies/ml), MDC020-5 (9,572 copies/ml), MDC029-1 (36,988 copies/ml), MDC179-3 (11,272 copies/ml), 128-01 (3,246 copies/ml) and NYU6525-10 (17,876 copies/ml). The infecting subtypes were: CRF02_AG (06ARC001, 06ARC010, 06ARC036, MDC020-5), D (MDC029-1), F2 (MDC179-3), CRF11_cpx (NYU6525-10) and CRF2201A1 (128-01).

**Figure 2:** Reverse transcriptase sequences of HIV-1 in plasma samples from eight patients indicating wild type or HIV drug resistance mutations. These same samples were also analyzed by ARMS PCR and their results are in Figure 1. The positions of the amino acids are indicated above the sequences in boxes. HIV drug resistance mutations analyzed include M184V, K103N, Y181C, and T215Y/F. The presence of a wild type amino acid is underlined and the presence of a mutant amino acid is bolded.

**Figure 3:** Detection of HIV-1 drug resistance mutations by ARMS-PCR in samples with varying viral load. Sample 2298-09 (viral load: 18,253 copies/ml) was serially diluted 1/5, 1/25, 1/125 and 1/625 corresponding to 4,977, 836, 143 and <75 copies/ml, respectively. The four HIVDR mutations, M184V, T215Y/F, K103N and Y181C were detected in samples corresponding to a viral load of >836 copies/ml. Furthermore, T215Y/F and K103N were detected at 143 copies/ml; and M184V was detected at a viral load of <75 copies/ml.
### Table 1: Demographics and characteristics of the HIV-1 infected individuals studied.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>48 (64%)</td>
<td>27 (36%)</td>
<td>75 (100%)</td>
</tr>
<tr>
<td>Mean age [IQR]</td>
<td>37.5 [33.25-45]</td>
<td>38 [34-45]</td>
<td>38 [33.5-45]</td>
</tr>
<tr>
<td><strong>Viral load (VL)</strong> (copies/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;75</td>
<td>4 (8.3%)</td>
<td>1 (3.7%)</td>
<td>5 (6.6%)</td>
</tr>
<tr>
<td>75&lt;VL&lt;5,000</td>
<td>17 (35.4%)</td>
<td>5 (18.5%)</td>
<td>22 (29.3%)</td>
</tr>
<tr>
<td>&gt;5,000</td>
<td>27 (56.2%)</td>
<td>21 (77.7%)</td>
<td>48 (64%)</td>
</tr>
<tr>
<td><strong>ART</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>22 (45.8%)</td>
<td>11 (40.7%)</td>
<td>33 (44%)</td>
</tr>
<tr>
<td>1st Line therapy</td>
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<td></td>
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</tr>
<tr>
<td>AZT+3TC+NVP/EFV</td>
<td>13 (27%)</td>
<td>8 (29.6%)</td>
<td>21 (28%)</td>
</tr>
<tr>
<td>TDF+3TC+NVP/EFV</td>
<td>9 (18.7%)</td>
<td>5 (18.5%)</td>
<td>14 (18.6%)</td>
</tr>
<tr>
<td>3TC+NVP</td>
<td>1 (2%)</td>
<td>1 (3.7%)</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td>d4T+NVP</td>
<td>1 (2%)</td>
<td>1 (3.7%)</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td>2nd Line therapy</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TDF+3TC+ATV</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>TDF+3TC+LPV/r</td>
<td>1 (2%)</td>
<td>1 (3.7%)</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td><strong>Duration on ART (months [IQR])</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ART, Antiretroviral treatment; IQR, Interquartile; AZT, Zidovudine; 3TC, Lamivudine; TDF, Tenofovir; d4T, Stavudine; NVP, Nevirapine; EFV, Efavirenz; ATV, Atazanavir; LPV/r, Lopinavir boosted with Ritonavir. Viral load (VL) <75 indicates a successful ART, 75<VL<5,000 indicates ART failure and can still be managed with increased adherence, VL>5,000 necessitates therapy change.
Table 2: Drug resistance mutations among HIV-1 infected individuals determined by sequencing and ARMS-PCR.

<table>
<thead>
<tr>
<th>ARV Class</th>
<th>Mutation</th>
<th>ART-naïve, n=33</th>
<th>1st Line ART, n=39</th>
<th>2nd Line ART, n=3</th>
<th>Overall, n=75</th>
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<tbody>
<tr>
<td></td>
<td>Sequencing (%)</td>
<td>ARMS-PCR (%)</td>
<td>Sequencing (%)</td>
<td>ARMS-PCR (%)</td>
<td>Sequencing (%)</td>
</tr>
<tr>
<td>NRTI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M184V</td>
<td>3 (9)</td>
<td>4 (12)</td>
<td>26 (66.6)</td>
<td>29 (74.3)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>T215Y/F</td>
<td>1 (3)</td>
<td>3 (9)</td>
<td>10 (25.6)</td>
<td>9 (23)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>M184V or T215Y/F</td>
<td>3 (9)</td>
<td>6 (18)</td>
<td>26 (66.6)</td>
<td>29 (74.3)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>M184V + T215Y/F</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>10 (25.6)</td>
<td>9 (23)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>NNRTI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K103N</td>
<td>5 (15)</td>
<td>5 (15)</td>
<td>17 (43.5)</td>
<td>16 (41)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Y181C</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9 (23)</td>
<td>8 (20.5)</td>
<td>1 (33.3)</td>
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<tr>
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<td>5 (15)</td>
<td>5 (15)</td>
<td>22 (56.4)</td>
<td>19 (48.7)</td>
<td>2 (66.6)</td>
</tr>
<tr>
<td>K103N + Y181C</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>5 (12.8)</td>
<td>4 (10.2)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

ARV, Antiretroviral; ART, Antiretroviral Treatment; NRTI, Nucleoside Reverse Transcriptase Inhibitor; NNRTI, Non-NRTI. Percentages were calculated in each column based on the number of individuals in the respective ART group (ART-naïve, 1st line ART, 2nd line ART).
Table 3: Detection of HIVDR mutations in corresponding plasma samples by ARMS-PCR compared with sequence analysis.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>POS ARMS-PCR</th>
<th>POS Sequencing</th>
<th>False POS</th>
<th>False NEG</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
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<tbody>
<tr>
<td>M184V</td>
<td>35</td>
<td>32</td>
<td>4</td>
<td>1</td>
<td>96.8</td>
<td>90.6</td>
<td>88.5</td>
<td>97.5</td>
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<tr>
<td>T215Y/F</td>
<td>15</td>
<td>14</td>
<td>3</td>
<td>2</td>
<td>85.7</td>
<td>95</td>
<td>80</td>
<td>96.6</td>
</tr>
<tr>
<td>K103N</td>
<td>21</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>91.3</td>
<td>100</td>
<td>100</td>
<td>96.2</td>
</tr>
<tr>
<td>Y181C</td>
<td>9</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>70</td>
<td>96.9</td>
<td>77.7</td>
<td>95.4</td>
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</table>

POS, Positive; NEG, Negative; PPV, positive predictive value; NPV; negative predictive value. Sensitivity, specificity, PPV and NPV of ARMS-PCR were calculated using sequencing as gold standard.
Mutation M184V (328 bp)

Mutation T215Y/F (421 bp)

Mutation K103N (300 bp)

Mutation Y181C (533 bp)
<table>
<thead>
<tr>
<th>HIV-1 Subtype</th>
<th>Sample ID</th>
<th>Partial Amino Acid Sequence of the HIV-1 Reverse Transcriptase region</th>
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<td>B (Reference)</td>
<td>B.FR.1983,HXB2</td>
<td>NKRTQDFWEV QLGIPHPAGL KKEKSVTVLD VGDAVFSPV1 DEDFRRYTAQ TIPSIMNETP GIRIQYNYLVP QGWKGSPAIF</td>
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<td>CRF02_AG</td>
<td>06CM06ARC001</td>
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<td>CRF02_AG</td>
<td>06CM06ARC010</td>
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<tr>
<td>CRF02_AG</td>
<td>06CM06ARC036</td>
<td>NKRTQDFWEV QLGIPHPAGL KKEKSVTVLD VGDAVFSPV1 DEDFRRYTAQ TIPSIMNETP GIRIQYNYLVP QGWKGSPAIF</td>
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<tr>
<td>CRF02_AG</td>
<td>MDC020-5</td>
<td>NKRTQDFWEV QLGIPHPAGL KKEKSVTVLD VGDAVFSPV1 DEDFRRYTAQ TIPSIMNETP GIRIQYNYLVP QGWKGSPAIF</td>
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<tr>
<td>D</td>
<td>MDC029-1</td>
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<td>MDC179-3</td>
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<td>128-01</td>
<td>NKRTQDFWEV QLGIPHPAGL KKEKSVTVLD VGDAVFSPV1 DEDFRRYTAQ TIPSIMNETP GIRIQYNYLVP QGWKGSPAIF</td>
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<td></td>
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<tr>
<td>CRF02_AG</td>
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</tr>
<tr>
<td>CRF02_AG</td>
<td>06CM06ARC010</td>
<td>QASMTKILEP FPKQNPDDIVI QYVDOLYVG SDLI1IQKQRA KEELEHKHL MARFIPDFDKK HQKEPPFLWLM GYELHPDPKX~</td>
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</tr>
<tr>
<td>F2</td>
<td>MDC179-3</td>
<td>QCSTMKILEP FPKQNPDDIVI QYVDOLYVG SDLI1IQKQRA KEELEHKHL MARFIPDFDKK HQKEPPFLWLM GYELHPDPKX~</td>
</tr>
<tr>
<td>CRF11_cpx</td>
<td>NYU6525-10</td>
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</tr>
<tr>
<td>CRF22_01A1</td>
<td>128-01</td>
<td>QCSTMKILEP FPKQNPDDIVI QYVDOLYVG SDLI1IQKQRA KEELEHKHL MARFIPDFDKK HQKEPPFLWLM GYELHPDPKX~</td>
</tr>
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</table>