Pitfalls of antiretroviral drug resistance genotyping of HIV-1 Group M and Group N from Cameroon by sequenced-based assays

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INTRODUCTION

Treatment of HIV-1 infection with highly active antiretroviral therapy in the past decades has remarkably reduced HIV/AIDS-related mortality and morbidity. However, the emergence of drug resistance in persons on antiretroviral therapy and the transmission of drug-resistant HIV strains to newly infected persons are a major threat to the global success on HIV prevention and treatment effort.¹ High rate of virological failure was recently reported in patients

ABSTRACT

Background: HIV-1 genotyping for antiretroviral drug resistance mutations (DRMs) were developed based basically on subtype B HIV-1 Group M, which represents only 10% of HIV strains worldwide. In sub-Saharan Africa, non-B subtypes HIV-1 largely predominate and HIV-1 genetic diversity could affect the performance of drug resistance genotyping assays. We compared prospectively the performance of the ViroSeq® and Trugene® genotyping assays to detect DRM in HIV-1-infected adult patients living in Douala, Cameroon. Materials and Methods: DRM in protease (P) and reverse transcriptase (RT) genes were assessed in parallel using both ViroSeq® and Trugene® assays in plasma samples from 45 first-line antiretroviral treatment-experienced patients in Douala, Cameroon. Results: Trugene HIV-1 Genotyping Assay® (Siemens Health Care Diagnostics, NY, USA) and ViroSeq HIV-1 Genotyping System® (Celera Diagnostics, CA, USA) assessed equivalently antiretroviral DRMs in P and RT genes from non-B HIV-1 Group M in 44 Cameroonian adults in virological failure; Trugene® was slightly more sensitive than ViroSeq® (100% vs. 91%). One patient infected by HIV-1 Group N was successfully amplified only by the Trugene HIV-1 Genotyping assay®, while ViroSeq HIV-1 Genotyping System v2.0 assay could not. Conclusion: Results showed the higher performance of the Trugene® system to detected and amplify P and RT genes targeting DRM to the principal antiretroviral drugs used in sub-Saharan Africa. Discrepancies between the results of HIV viral load assays and molecular tests should alert clinicians and virologists to the possibility of infection by an atypical variant virus, especially in Central Africa where very broad HIV-1 genetic diversity exists.

Key words: Cameroon, Central Africa, HIV-1 Group N, non-B subtypes HIV-1 Group M, Trugene® HIV-1 Genotyping assay, ViroSeq® HIV-1 Genotyping System v2.0

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followed up by the private sector of Douala, the economic capital of Cameroon, and treated according to the World Health Organization recommendations, and some patients presented with complex genotypic profiles diagnosed only by genotypic resistance tests, highlighting the absolute need for carefully monitoring therapeutic failure in resource-limited settings.\(^2\)

Population sequencing-based genotyping methods are widely used and the most informative and affordable genotyping methods for monitoring patients on antiretroviral treatment in clinical practice. HIV-1 genotyping commercial assays for antiretroviral drug resistance mutations (DRMs) were developed basically on subtype B HIV-1 Group M,\(^3\) which represents, however, only 10% of HIV strains worldwide.\(^4\) In sub-Saharan Africa, non-B subtypes HIV-1 largely predominate.\(^5\) As a consequence, HIV-1 genetic diversity could affect the performance of drug resistance genotyping assays.\(^6,7\)

The ViroSeq HIV-1 Genotyping System v2.0\(^®\) (Celera Diagnostics, CA, USA) and Trugene HIV-1 Genotyping Assay\(^®\) (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) are the two Food and Drug Administration (FDA)-approved commercially available assays for detecting DRM.\(^8\) The ViroSeq HIV-1 Genotyping System v2.0\(^®\) needs external sequencer and was developed using primers mostly associated with subtype B HIV-1.\(^9\) The Trugene\(^®\) is a sequence-based assay targeted at the protease (P) gene region (codons 1–99) and reverse transcriptase (RT) region (codon 40–247) of the HIV-1 genome, using its own automated DNA sequencer which is charged by a gel toaster polymerization unit.\(^10,11\) The assay was primarily designed for HIV-1 Group M subtype B viruses,\(^12\) and improvement in primers design for non-B subtypes HIV-1 was further proposed.\(^13\) The Trugene\(^®\) system is useful for intermediate level laboratories, as frequently encountered in sub-Saharan Africa since it does not necessitate external sequencer and is particularly adapted to limited series.

It has been reported that genotyping sensitivity with both FDA-approved systems using non-B subtypes varies.\(^13,14\) Some studies indicated that these two systems performed well for B and non-B subtypes\(^3,9,15,16\) while others demonstrated that they were less sensitive to non-B subtypes and circulating recombinant forms (CRFs);\(^2,17-21\) For instance, only 52% of serum samples were genotyped in an Ethiopian threshold survey using ViroSeq\(^®\) and Trugene\(^®\) assays sequentially.\(^22\) To our knowledge, the Trugene\(^®\) system was never evaluated in the field for non-B subtypes HIV-1 from Central Africa.

We herein compared prospectively the performance of the ViroSeq\(^®\) and Trugene\(^®\) genotyping assays to detect DRM in HIV-1-infected adult patients living in Douala, Cameroon, a country of broad genetic diversity.\(^7,21\)

**MATERIALS AND METHODS**

DRM in P and RT genes were thus assessed in parallel using both assays in plasma samples obtained from 45 first-line antiretroviral treatment-experienced patients living in Douala, Cameroon, and suffering from a virological failure according to the 2013-revised WHO (e.g., viral load >3.0 log copies/ml).\(^23\) Informed written consent was obtained from all patients participating in the study. The resulting pol sequences were aligned using ViroSeq\(^®\) HIV-1 Genotyping System Software v2.6 (Celera Diagnostics). HIV-1 subtype was assessed with the Genotyping software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi), as described previously.\(^24\) DRM interpretation was carried out according to the algorithm of the Agence Nationale de Recherches sur le SIDA et les hépatites virales (September 2013, www.hivfrenchresistance.org). GenBank accession numbers were for pol sequences by Trugene\(^®\), KF735850 - KF735877, and by ViroSeq\(^®\), KF735813 - KF735849. GenBank accession numbers for patient # MVE were for pol sequences by Trugene\(^®\) HIV-1 Genotyping assay, LM994715 and LM994716.

**RESULTS**

All but one HIV-1 belong to HIV-1 Group M with broad HIV-1 genetic diversity as assessed using pol sequences by Trugene\(^®\) (CRF02_AG [60%], D [8%], F2 [6%], CRF01_AE [4%], A1 [4%], G [6%], K [2%], CRF11 [2%], and CRF09_cpx [2%]).

One patient #MVE was infected by HIV-1 Group N: The Trugene HIV-1 Genotyping Assay\(^®\) succeed to amplify a sequence of 925 nucleotides, whereas none sequence could be obtained by the ViroSeq assay. Figure 1 depicts the >MVE amino acid translated sequence by the Trugene

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**Figure 1:** Genotyping in protease and reverse transcriptase pol genes by two commercially available assays (Trugene HIV-1 Genotyping Assay\(^®\) (Siemens Health Care Diagnostics, Tarrytown, NY, USA) and ViroSeq HIV-1 Genotyping System v2.0\(^®\) (Celera Diagnostics, CA, USA)), of the plasma HIV-1 from the Cameroonesne patient #MVE in virological failure under the first-line WHO antiretroviral regimen
HIV-1 Genotyping assay\textsuperscript{a}, in which a poly-N stretch segment of 15 positions could not be identified. We carried out the genetic analysis of the >MVE sequence in parallel by the software Genotyping (Rozanov et al., 2004)\textsuperscript{b} and the software HIV BLAST (Los Alamos National Laboratory; http://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html).\textsuperscript{c}

Surprisingly, discordant results were obtained according to both genotyping softwares regarding the conservation or deletion of the poly-N stretch segment. Thus, by genotyping, both >MVE sequence and >poly N-deleted MVE sequence were identified as HIV-1 Group N. Furthermore, the sequences before and after the poly-N stretch, harbored 87% and 91% homology, respectively, with the HIV-1 Group N isolate 02CM-SJGddd (GenBank #GQ324959.1). By HIV BLAST, the full >MVE sequence was identified as HIV-1 Group M subtype D (76% homology), whereas the >poly N-deleted MVE sequence was identified as HIV-1 Group N with 85% homology with the HIV-1 Group N isolate 02CM-SJGddd.

All 44 (100%) and 40 (91%) samples were successfully amplified by Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®} assays, respectively (\(P = 0.04\) by \(\chi^2\)-test). The genotype of the HIV-1 Group M not detected by ViroSeq\textsuperscript{®} were D (\(n = 2\)), G (\(n = 1\)) subtypes and CRF01_AE (\(n = 1\)).

A total of 95% and 94% patients harbored DRM in P gene by Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®}, respectively (not significant [NS]); and 85% and 90% patients harbored DRM in RT gene by Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®}, respectively (NS). Interestingly, 6% and 5% patients in virological failure showed no resistance mutations in P as well as RT genes by Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®}, respectively (NS). By Trugene\textsuperscript{®}, main DRM in P gene were M36I (88%), H69K (80%), L89M (63%), K20I (60%), L63P (40%), L10V (25%), I62V (23%), G16E (23%), I15V (15%), M46I (13%), I54V (13%), and L76V (10%); the DRM in P gene by ViroSeq\textsuperscript{®} were M36I (97%), H69K (92%), M36I (97%), K20I (77%), L89M (72%), L63P (41%), G16E (28%), L10V (28%), I15V (23%), M46I (13%), I54V (13%), I62V (10%), and L76V (10%) (NS for all comparisons vs. Trugene\textsuperscript{®}) [Figure 2]. By Trugene\textsuperscript{®}, main DRM in RT gene were M184V (60%), K103N (48%), T215Y (33%), M41L (25%), Y181C (23%), T215F (23%), V90I (22%), D67N (20%), K70R (15%), T69D (10%), G190A (10%), L210W (13%), K219Q (10%), and V179I (5%) (NS for all comparisons vs. Trugene\textsuperscript{®}) [Figure 2]. By Trugene\textsuperscript{®}, the DRM in RT were M184V (69%), K103N (59%), T215Y (38%), M41L (31%), V90I (28%), Y181C (23%), D67N (23%), T215F (21%), L210W (15%), K70R (15%), T69D (10%), G190A (10%), K219Q (10%), and V179I (8%) (NS for all comparisons) [Figure 1]. Finally, the agreements of the genotype results between Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®} assays were 94.2%, 94.1%, and 94.3% for the DRM to protease inhibitors (PI), nucleoside RT inhibitors (NRTI), and non NRTI (NNRTI) therapeutic classes, respectively.

After DRM interpretation using algorithm for PI, 15%, 23%, 13%, 10%, and 0% of plasma samples detected by Trugene\textsuperscript{®}, and 18%, 25%, 15%, 8%, and 0% of plasma samples detected by ViroSeq\textsuperscript{®} were resistant to lopinavir, indinavir, saquinavir, atazanavir, and darunavir, respectively (NS for all comparisons). Interpretation for NRTI, 55%, 63%, 65%, 35%, and 8% of plasma samples detected by Trugene\textsuperscript{®}, and 64%, 69%, 69%, 33%, and 5% of plasma samples detected by ViroSeq\textsuperscript{®} were concluded, respectively, as harboring HIV-1 resistant to zidovudine, stavudine, lamivudine, abacavir, and tenofovir, respectively (NS). Finally, interpretation for NNRTI showed that 80%, 82%, 13%, and 50% of plasma samples detected by Trugene\textsuperscript{®}, and 87%, 92%, 8%, and 57% of plasma samples detected by ViroSeq\textsuperscript{®} harbored HIV-1 resistant to efavirenz, nevirapine, etravirine, and rilpivirine, respectively (NS).

By Trugene GuideLines™ Rules, numerous DRM conferring resistance to first-line WHO antiretroviral molecules, including lamivudine, tenofovir, nevirapine, and efavirenz, were evidenced; possible resistances to atazanavir and saquinavir were surprisingly detected although the patient #MVE never took PIs, as recommended in first-line WHO antiretroviral treatment (WHO, 2013). By ANRS algorithm, similar DRM pattern was observed with slight differences with the Trugene GuideLines™ Rules, including resistance to rilpivirine, a new second-generation nonnucleosidic RT inhibitor never introduced in Cameroon, and lack of resistance even possible to any PI.

**DISCUSSION**

Our observations confirm the broad genetic diversity of HIV-1 strains circulating in Cameroon. The study also demonstrates high sensitivity of Trugene\textsuperscript{®} and ViroSeq\textsuperscript{®} assays in genotyping in the P and RT target pol genes.
multiple non-B HIV-1 Group M subtypes and CRFs from plasmas collected from Cameroonian patients, thus allowing diagnosis of DRM to the current antiretroviral drugs classes recommended by the WHO for use in resource-constrained countries. When P and RT sequences were obtained by both Trugene® and ViroSeq® assays, the ViroSeq® genotyping profiles allowed easier detection of DRM, although the difference was not statistically significant by comparison with the Trugene® sequences, and the final interpretations of resistance genotype were similar with both assays. High concordance (99%) between the two assays was previously reported on clinical plasmas samples from individuals living in Salt Lake City, Utah.10

In this series, the Trugene® assay appeared slightly more sensitive than the ViroSeq® assay. This finding is in keeping with previous reports demonstrating amplification rates of the ViroSeq® assay lower for non-B subtypes HIV-1 than for B subtype HIV-1.2,12,26 High failure rates of 4 out of 7 ViroSeq® sequencing primers were reported in Central Africa, including Cameroon, Central African Republic, Chad and Gabon21,27 as well as in West Africa (Senegal).26 In Senegal, the ViroSeq HIV-1 Genotyping System v2.0® primer amplification and sequencing success were 98% (147/150) and 96.6% (142/147), respectively. The Trugene® assay has proved to have consistently high degree of accuracy and reproducibility, and the primers pairs used in the CLIP sequencing reaction were designed to bind to diverse HIV-1 subtypes allowing testing non-B subtypes HIV-1.11,12 These findings could contribute to explain the observed high sensitivity of the Trugene® assay for detecting and amplify non-B subtypes HIV-1 Group M circulating in Central Africa, as previously reported in West Africa.16

Patient #MVE was infected by HIV-1 Group N. From the first discovery of HIV-1 Group N isolate in 1998,28 around 20 cases of HIV-1 Group N infections have been reported primarily in patients originating from Cameroon where the virus even appeared to be rare.28,29 Thus, in two different studies, a total of 7,146 HIV-infected specimens from Cameroun were collected and screened from 1998 through 1999, only six HIV-1 Group N isolates were identified.28,29 The seroprevalence of HIV-1 Group N infection appears furthermore very low (0.1%) in this region.28,29 To our knowledge, the only case of HIV-1 Group N infection diagnosed in 2001 Paris, France, was related to an index case partner originating from Togo.20 The scarcity of HIV-1 Group N infection makes its diagnosis particularly challenging. We were first surprised that we could not amplify the P and RT genes with the ViroSeq HIV-1 Genotyping System v2.0®, despite the high viral load, and successful amplification and genotyping by Trugene HIV-1 Genotyping assay®. Lack of amplification and genotyping of HIV-1 Group N isolate by ViroSeq HIV-1 Genotyping System v2.0® and also by ANRS genotyping assay was previously reported once.30 We were furthermore astonished by the discrepant results obtained two different genotyping softwares, in fact due to the artefactual poly-N stretch introduced by the CLIP sequencing reaction of the Trugene HIV-1 Genotyping assay®.12 Taken together, these observations pointed to the discrepancies observed by different genotyping methods and molecular analysis, when subjected to HIV-1 Group N isolate.

CONCLUSION

The study using Cameroonian patients’ samples showed the higher performance of the Trugene® system to detected and amplify P and RT pol genes targeting DRM to the principal antiretroviral drugs used in sub-Saharan Africa by comparison with the ViroSeq® assay. Our findings also point the urgent need of improving and updating the sequencing primers of the ViroSeq® system, as previously recommended four years ago by Aghokeng and colleagues.21 Indeed, after effective sequencing, both Trugene® and ViroSeq® assays give basically quite similar DRM profiles and final resistance genotype interpretations. These results suggest that both assays should be considered for monitoring the occurrence of drug resistance among HIV-1-infected patients receiving antiretroviral therapy in Central Africa, although the broad genetic, viral diversity may provide rarely sequencing difficulties. Finally, discrepancies between the results of HIV viral load assays and molecular tests should alert clinicians and virologists to the possibility of infection by an atypical variant virus, especially in Central Africa where very broad HIV-1 genetic diversity exists.

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Conflicts of interest
There are no conflicts of interest.

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