Performance characteristics of Allele-Specific PCR (ASPCR) in detecting drug resistance mutations among non-B HIV-1 Variants

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**Abstract**

Allele-Specific Polymerase Chain Reaction (ASPCR) is an affordable point-mutation assay whose validation could improve the detection of HIV-1 drug resistance mutations (DRMs) in resource-limited settings (RLS). We assessed the performance of ASPCR onforty-four non-B HIV-1 plasma samples from patients who were ARV treated in failure in N’Djamena-Chad. Viral RNA was reverse-transcribed and amplified using LightCycler® FastStart DNA MasterPLUS SYBR Green I. Detection of six major DRMs (K70R, K103N, Y181C, M184V, T215F, T215Y) was evaluated on Roche LightCycler®480 automated system (with dilutions 0.01-100%). ASPCR-results were compared to Sanger-sequencing (gold-standard). Correlations of mutation curves were excellent (R2 *>* 0.97); all DRMs were detected with desirable mutant/wild-type threshold differences (ΔCt≥9) except K70R(ΔCtK70R=6; ΔCtK103N=13; ΔCtM184V=9; ΔCtT215F=12; ΔCtT215Y=12; ΔCtY181C=9) and positive controls were below required thresholds. Also, ASPCR reproducibility on DRMs was assessed by using dilutions of intra-assay and inter-assay coefficient of variations respectively with a threshold of less than 50(i.e. *<* 0.50 variation) which are;: K70R (0.02-0.28 vs. 0.12-0.37), K103N (0.08-0.42 vs. 0.12-0.37), Y181C (0.12-0.39 vs. 0.31-0.37), M184V (0.13-0.39 vs. 0.23-0.42), T215F (0.05-0.43 vs. 0.04-0.45) and T215Y (0.13-0.41 vs. 0.19-0.41). DRM detection-rate by ASPCR vs Sanger was respectively: M184V (63.6% vs. 38.6%); T215F (18.1% vs. 9.1%); T215Y (6.8% vs. 2.3%); K70R (4.5% vs. 2.3%). K103N (22.7% vs. 13.6%); Y181C (13.6% vs. 11.4%). Correlations of mutation curves were excellent (R2 *>*0.97); all DRMs were detected with desirable mutant/wild-type threshold differences (ΔCt≥9) except K70R(ΔCtK70R=6; ΔCtK103N=13; ΔCtM184V=9; ΔCtT215F=12; ΔCtT215Y=12; ΔCtY181C=9) and positive controls were below required thresholds. Also, ASPCR reproducibility on DRMs was assessed by using dilutions of intra-assay and inter-assay coefficient of variations respectively with a threshold of less than 50(i.e. *<* 0.50 variation) which are;: K70R (0.02-0.28 vs. 0.12-0.37), K103N (0.08-0.42 vs. 0.12-0.37), Y181C (0.12-0.39 vs. 0.31-0.37), M184V (0.13-0.39 vs. 0.23-0.42), T215F (0.05-0.43 vs. 0.04-0.45) and T215Y (0.13-0.41 vs. 0.19-0.41). DRM detection-rate by ASPCR vs Sanger was respectively: M184V (63.6% vs. 38.6%); T215F (18.1% vs. 9.1%); T215Y (6.8% vs. 2.3%); K70R (4.5% vs. 2.3%). K103N (22.7% vs. 13.6%); Y181C (13.6% vs. 11.4%). ASPCR appears more efficient for detecting DRMs on diverse HIV-1 non-B circulating in RLS like Chad.

# 1. Introduction

A major risk in scaling-up antiretroviral therapy (ART) is the selection and transmission of HIV drug resistance (HIVDR) variants due to poor monitoring and surveillance systems in place (Damond and Descamps, 1998; Grant et al., 2002). As HIVDR represents a major threat in ensuring treatment success (Roquebert et al., 2009), implementation of HIVDR surveillance and testing in clinical practice should be recommended to support the long-term effectiveness of ART, especially in sub-Saharan Africa (SSA) where therapeutic options are limited, antiretrovirals with poor genetic to resistance are widely used, and several viral clades have been identified (Roquebert et al., 2009). Unlike in the western world, standard methods (i.e. Sanger-sequencing) for detecting drug resistance mutations (DRMs) are not yet routinely implemented in several resource-limited settings (RLS) like in SSA, due to several pitfalls: sequencing is financially unaffordable, its procedure is cumbersome, it requires high-level staff for processing/interpretation, and the maintenance/procurement systems are not available locally (Larder et al., 1991; Brun**-**Vézinet et al., 2004; Grant et al., 2003). Thus, implementing simple and affordable approaches for HIVDR testing, such as point mutation assays (PMA), might be convenient for RLS scaling-up ART.

Among PMAs, Allele-Specific Polymerase Chain Reaction (ASPCR) is one of the promising techniques for implementation in RLS. Of note, ASPCR was first reported on HIV clinical isolates for detecting DRMs down to ≥ 1% of minority variants (Larder et al., 1991). In addition to the aforementioned pitfalls, standard sequencing methods only detects variants present at levels ≥ 20% of the overall viral population (Brun**-**Vézinet et al., 2004; Grant et al., 2003; Halvas et al., 2006; Palmer et al., 2005). With the advent of Real-Time PCR, the diagnostic performance of ASPCR has been improved for possible use in clinical practice (Halvas et al., 2006; Metzner et al., 2005, 2003; Palmer et al., 2005).

In September 2017 a breakthrough pricing agreement was reached to provide generic dolutegravir for HIV treatment in low- and middle-income countries (LMICs). Combined with tenofovir disoproxil fumarate and lamivudine, this single pill, fixed-dose combination will cost approximately USD 75 per person per year and is likely to be cost effective when compared to current non-nucleoside reverse transcriptase inhibitor (NNRTI) first-line regimens (Jienchi et al. Lancet HIV. 2018 Jul). So, in March 2019, the Chadian Ministry of Health approved the induction of dolutegravir (DTG) based regimens as per WHO recommendations in their settings where the first packs of dolutegravir arrived in 2020 (Abderrazzack et al., 2021). But in clinical practice, the impact of minority DRMs (i.e. variants present at levels below 20% of the overall viral population) of human immunodeficiency virus type 1 (HIV-1) could influence the virological response to treatment based on non-nucleoside reverse transcriptase inhibitors (NNRTIs) not rilpivirine in which data on minority rilpivirine resistant variants are scarce. (Raymond S et al., 2018). In this context, ASPCR might be useful for detecting HIVDR to first-line ART in RLS consisting mainly of NNRTI- based regimens (Eshleman et al., 2001; Jackson et al., 2000). However, HIVDR testing using ASPCR has not been substantially ascertained on non-B viral populations, especially within the SSA context (Gutsche et al.*,* 2004; Lecossier et al., 2005).

Following the *“proof of principle”* of PMA, the footprint in a successful implementation of ASPCR relies on knowledge of emerging DRMs (Grant et al., 2002). In several SSA countries, NRTI and NNRTI are the most commonly used antiretrovirals. Regarding NRTIs, cytidine analogues are found in almost all drug combinations and select for M184V mutation, thus causing high-level resistance to lamivudine (3TC) and emtricitabine (FTC) while decreasing the viral replicative fitness (Wei et al., 2002, 2003). Thymidine analogue mutations (TAMs) are class of variants selected by two NRTIs, zidovudine (AZT) and stavudine (d4T), and subcategorised as either TAMs-1 (M41L, L210W and T215Y) or TAMs-2 (D67N, K70R, T215F and K219Q/E), each having distinct effects on resistance to AZT and D4T (Bennett et al., 2005; Yeni, 2008). Furthermore, TAMs might also cause cross-resistance to other NRTIs (abacavir [ABC], didanosine [ddI], tenofovir [TDF]), especially when selected cumulatively (Calvez et al., 2002; Johnson et al., 2010; Kellam et al., 1992; Kuritzkes et al., 2004; Larder and Kemp, 1989; Whitcomb et al., 2003). Regarding NNRTIs, commonly selected mutations are K103N and Y181C causing high-level resistance tonevirapine (NVP) and efavirenz (EFV), drugs with low genetic barrier to resistance and present in most first-line ART regimens within SSA countries (Hirsch et al., 2008; Richman et al., 1994; Torti et al., 2001; Yang et al*.,* 2004).

Considering the most used ARV drugs, major DRMs and viral clades found within the Chadian national ART program (Adawaye et al., 2017), we sought to ascertain the performance of ASPCR in detecting HIVDR mutations targeting NNRTIs (K103N, Y181C) and NRTIs (K70R, M184V, T215Y/F) across diverse non-B HIV-1 clades circulating in Chad.

# 2. Materials and methods

## 2.1. Study design and population

A comparative assessment on the performance of ASPCR (investigative tool) vs. Sanger-sequencing (gold-standard) was conducted on 44 plasma samples from consenting HIV-1 infected patients with virological failure receiving ART at the National Reference General Hospital of N’Djamena, Chad. ASPCR and Sanger-sequencing were performed at the AIDS reference laboratory of the University Health Centre (*Laboratoire de Référence Sida du CHU de Liège*) of Liège in Belgium.

## 2.2. Principle of the ASPCR

ASPCR is performed in two different tubes among which: in one tube, the forward primer A1 is highly specific to the wild-type allele and the reverse primer A3 recognises both alleles (wild-type and mutant); in the other tube, forward primer A2 is highly specific to the mutant allele and the reverse primer A3 recognises both alleles (wild-type and mutant). Amplification and revelation are performed in real-time using the Roche LightCycler®480 System (Ameziane et al., 2006).

Based on evidence from our previous reports (Adawaye et al*.,* 2017), the six major DRMs (with corresponding codons) were selected for ASPCR performance evaluation (K70R [AGA]; K103N [AAC]; Y181C [TGT]; M184V [GTG]; T215Y [TAC]; T215F [TTC]).

### 2.2.1. Procedure of Reverse Transcription and amplification

RNA was extracted from 140 µl of plasma using the QIAamp Viral RNA mini kit, as per the manufacturers’ instructions. RNA extract was collected using 60 µl of elution buffer, and aliquots of 10 µl were stored at - 70 °C.

RT-PCR and ASPCR were performed using a solution of 10X Mix of random hexamers (RocheDiagnostics, ref# 11 277 081001), dNTP (Roche Diagnostics, ref# 1127704901), SuperScript II RT 10,000 U containingdNTPs and 5Xfirst Strand Buffer (Invitrogen,ref# 18064-014), RNA Ribonuclease Inhibitor 10 000 U (Promega, ref# N2515. Ultra Pure), 1 M Tris-HCL (Life Technology, ref# 15568-025).

The MasterMix 1 (MMix1, sample mix) contains8pl H2O, 2pl random hexamers 10X, and 10pl RNA, for a final volume of 20pl for MMix1. The following cycling conditions were used on 9700 thermocycler for annealing primer: 70 °C 10 min; 25 °C 10 min; 4°Cforever. MasterMix 2

(MMix2, enzyme mix) contains pour 8μl 5Xfirst strand Buffer, 4μl DTT, 2μl dNTP (10 mM), 1μl RNAse inhibitor (40 U), 1μl SuperScript II and 4μl H2O, for a final volume of 20μl for MMix2.

On 9700 thermocycler, 20μl MMix2 were added to each tube containing MMixl (following first reaction), for a total volume of 40µl. Cycling conditions were as follows: 25°C 10 min; 37°C 45 min; 42°C 45 min; 70°C 15 min; 4°C forever. Generated cADN was then used for testing the selected PMA as aforementioned.

### 2.2.2. Procedure for outer PCR

Outer PCR was performed using theLightCyclerFastStart DNA Master Plus SYBR Green I, as per the manufacturer’s instructions (Roche, Mannheim, Germany). Briefly, HIV-1 *pol* region of ~700 base pair (bp) was amplified from HXB2 2613-2635 bp (forward) to 3234-3256 (reverse).

## 2.3. Procedure for ASPCR

A volume of 14μl enzyme (1a) was added to the reaction mix of tube 1b and mixed thoroughly, for a final volume of 20μl (containing 2μl undiluted CDNA from outer PCR, 0.5μl of both outer forward and outer reverse primers (20μM each). Cycling conditions on 9700 Applied Biosystems thermocycler were as follows:95°C (incubation) 10 min;94°C (denaturation) 10 min;50 cycles (94°C 15 s; 62°C 20 s; 72°C 1 min), and a final step of 72°C 7 min and 4°C forever.

Melting points (from 60°C to 95 °C) of PCR products were analysed to ensure optimal specificity of cDNA, and only samples with melting temperature of 78°C were considered for ASPCR. Outer PCR products were diluted to 1/200 in 5 mM TrisHCl Buffer (pH 8). Using 2μl diluted outer PCR cDNA, two reactions were performed separately for each mutation: one for the wild-type allele and the other for the mutant allele, with 0.5μl of each specific primer (20μM forward and reverse) for a final volume of 20μl on the LightCyclerFastStart DNA Master Plus SYBR Green I (Roche, Mannheim, Germany), using the following conditions for each point mutation:

* K70R (AGA): 95 °C 5 min; 45 cycles (95 °C 10 s, 48 °C 10 s, 72 °C 10 s);
* K103N (AAC): 95 °C 5 min; 45 cycles (95°C10 sec, 62° C10 sec, 72°C10 sec);
* Y181C (TGT): 95 °C 5 min; 45 cycles (95 °C 10 s, 60°C10 sec,72°C10 sec);
* M184V (GTG): 95 °C 5 min; 45 cycles (95°C10 sec, 56°C10 sec, 72°C10 sec);
* T215Y (TAC) and T215F (TTC): 95 °C 5 min; 45 cycles (95 °C 10 s, 56°C10 sec,72°C10 sec).

For each point mutation, amplification was performed in two different tubes using the Roche LightCycler480, one containing primers for the wild-type allele and the other containing primers for the mutant allele. The absolute value of nucleic acid in an unknown sample was determined based on a standard curve generated by serial dilutions of control samples with defined concentration. Each control sample with a well characterized subtype and mutation was diluted (1/5) and tested with the wild-type and mutant primers separately. A wild-type HIV-1 non-B sample, used as negative control, was also diluted and included as a separate sample in each run.

## 2.4. Reporting of results

Revelation was done in the form of curves appearing in real time, and interpretation was based on the comparison of the difference in threshold cycles (ΔCt) or crossing points (Cp) between the mutant and the wild-type virus for each of the study mutation. Each of the five serial dilutions (from 0.01% to 100%) had two reactions with the wild type and with the mutant allele (K70R, K103N, Y181C, M184V, T215F, T215Y). To obtain the wild-mutated mixture, wild-type virus was put in dilutions of the mutated sample. The threshold cycle was determined by the average Ct value ± 2 (standard deviation).

## 2.5. Result validation

To estimate the precision of ASPCR, intra-assay and inter-assay reproducibility was measured by using the coefficient of variation (CV) or relative standard deviation, with CV being calculated as the ratio of the standard deviation to the mean. The higher the CV value, the greater the distribution around the mean; meanwhile the lower the CV value, the more precise the estimate. Intra-assay and inter-assay reproducibility was performed with triplicates of serial dilutions (1%-100%) in the same series. Values obtained for mean and standard deviation were then used to calculate the CV for each series. A good correlation for ASPCR standards was defined as R2 *>* 0.97.

## 2.6. Data analysis

The mean Ct value of each dilution was used to evaluate the performance of ASPCR for each point mutation with reference to Sanger- sequencing (goal-standard). Considering the wide HIV-1 genetic diversity in Chad, we also evaluated the performance of ASPCR in detecting DRMs according to viral strains revealed by Sanger- sequencing.

# 3. Results

## 3.1. Demographic and clinical characteristics of the study population

The entire 44 plasma samples included in the study were from HIV- infected individuals, with a female to male ratio of 5/2 and a median [IQR] age of 40 [26-82] years; 9individuals were reported to be at the WHO clinical stage of 3 or 4; the median [IQR] CD4 was 308 [110-654] cells/mm3 and the median plasma viral load was118,826 [50-966,000] copies/mL.

According to treatment history, all were receiving a first-line ART consisting of two NRTI and one NNRTI. The median [IQR] duration on ART of 1.5 [1.0-7.0] years, and 32 were reported to be non-adherent to ART (defined as less than 95% drug intake during the past 30 days). Drug regimens received by the patients were 40.9% (18/44) Duovir-N (AZT+3TC+NVP), 38.6% (17/44) Triomune (d4T+3TC+NVP), 15.9% (7/44) TLE (TDF+3TC+EFV), 2.3% (1/44) ABC+ 3TC+NVP and2.3% (1/44) ABC+ 3TC+EFV.

## 3.2. HIV strains and DRMs detected from the study population

Following Sanger-sequencing and **interpretation using the Stanford HIV database (HIVdb) algorithm**, the 44 sequences were all reported as HIV type 1 group M subtypes, with CRF02\_AG 29.5% (13/44) being the **most predominant subtype.**. DRMs found among the sequences were predominantly:

* For mutations targeting NRTIs: 38.6% (17/44)M184V, 9.1% (4/ 44) T215F, 2.3% (1/44)T215Y, 2.3% (1/44) K70R;.
* For mutations targeting NNRTIs: 13.6% (6/44)K103N and11.4% (5/44)Y181C.

## 3.3. Validation of standards for ASPCR

For each mutation, the maximum detection threshold set at 35 cycles was achieved, above which the sample was considered negative or wild type. The difference in detection thresholds (ΔCt) was evaluated. A positive control (selected sample with a mutational load of 105 log copies/mL) and a negative control (a wild type sample) selected from non-B infected patients and used for technical validation yielded excellent results. Furthermore, dilution ranges of control samples (from 0.01% to 100%) were tested with specific primers for each mutation. Following results of the standard curve generated after serial dilution (0.01%, 0.1%, 1%, 10% and 100%), each mutation had a good correlation: K70R (R2 =0.989), K103N (R2 =0.977), Y181C (R2 =0.991), M184V (R2 =0.965), T215F (R2 =0.988), T215Y (R2 =0.981), as detailed in Fig. 1.

Comparison of the standard curve for each mutation against their corresponding wild type virus also revealed good correlations for both the mutants (R2 *>* 0.97) and the wild-type viruses (R2 *>* 0.96), as shown in Fig. 2**.**

For operationalization, the performance in detecting each mutation was evaluated by calculating the differences in the Ct (ΔCt) between the serial dilutions of the mutant and wild type viruses. Ct value was set for each mutation and was used as reference for assessing the presence of the respective mutation within clinical sample. The ΔCt obtained as standards for result validation were: ΔCt = 13 for K103N mutation; ΔCt= 9 for M184V mutation; ΔCt = 12 for T215F mutation; ΔCt = 12 for T215Y mutation; ΔCt = 9 for Y181C mutation and ΔCt = 6 for K70R mutation. All positive controls (i.e. mutants) appeared effectively below the threshold values, thus supporting a good sequence matching of the designed primers. Fig. 3 provides a snapshot of the real-time amplification curve from K103N versus its wild type virus.

## 3.4. Performance evaluation of ASPCR on clinical samples

All Ct values following serial dilution (0.01-100%) were below the reference threshold value of the respective mutation, indicating that DRMs can be detected even at levels *<* 1% of the overall viral population present in a clinical specimen.

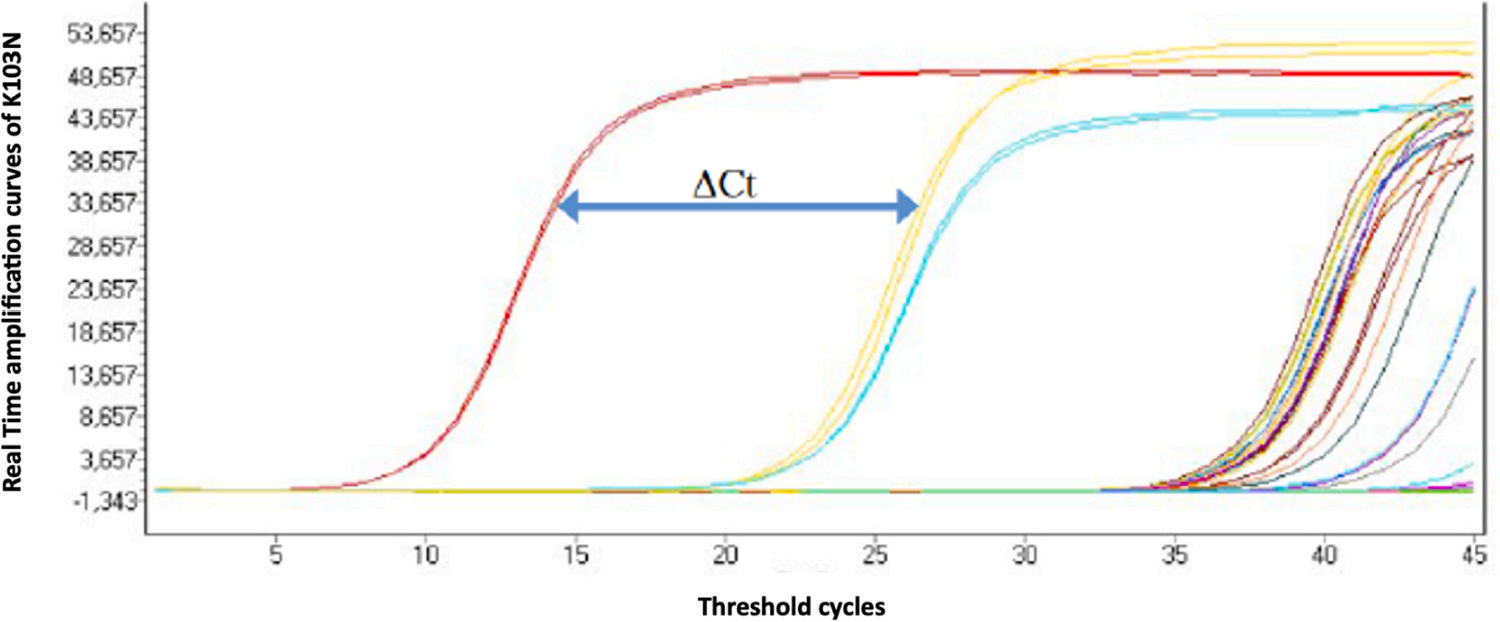
ASPCR had a better yield as compared to Sanger-sequencing for detecting DRM on clinical sample for both NRTI and NNRTI drug-classes (Table 1). This confirmed the capacity of ASPCR in detecting DRMs even at the level of minority viral populations.

Accuracy of the serial dilution (1-100%), defined as determine the intra-assay and inter-assay reproducibility of ASPCR, revealed the following CV for each type of mutation. The average intra-assay CVs (min-max) were 0.09 (0.02-0.13) for proportions of 100% wild-type virus (wt); 0.24(0.15-0.34) for proportions10% wt; and 0.38 (0.28-0.43) for proportions of 1% wt. The average inter-assay CVs (minmax) were higher as compared to intra-assays, ranging from 0.17 (0.12-0.23) for proportions of 100% wt; 0.30(0.24-0.37) for proportions of 10% wt and 0.40(0.37-0.45) for proportions of 1% wt. Overall, the inter assays and intra assays CVs were all *<* 0.46, indicating a high accuracy (i.e. *<* 0.50 variation) of the methodology (Table 2). **We also assessed results for each DRMs detected by AS-PCR for each patient individually with proportions as shown in** Table 3**.**

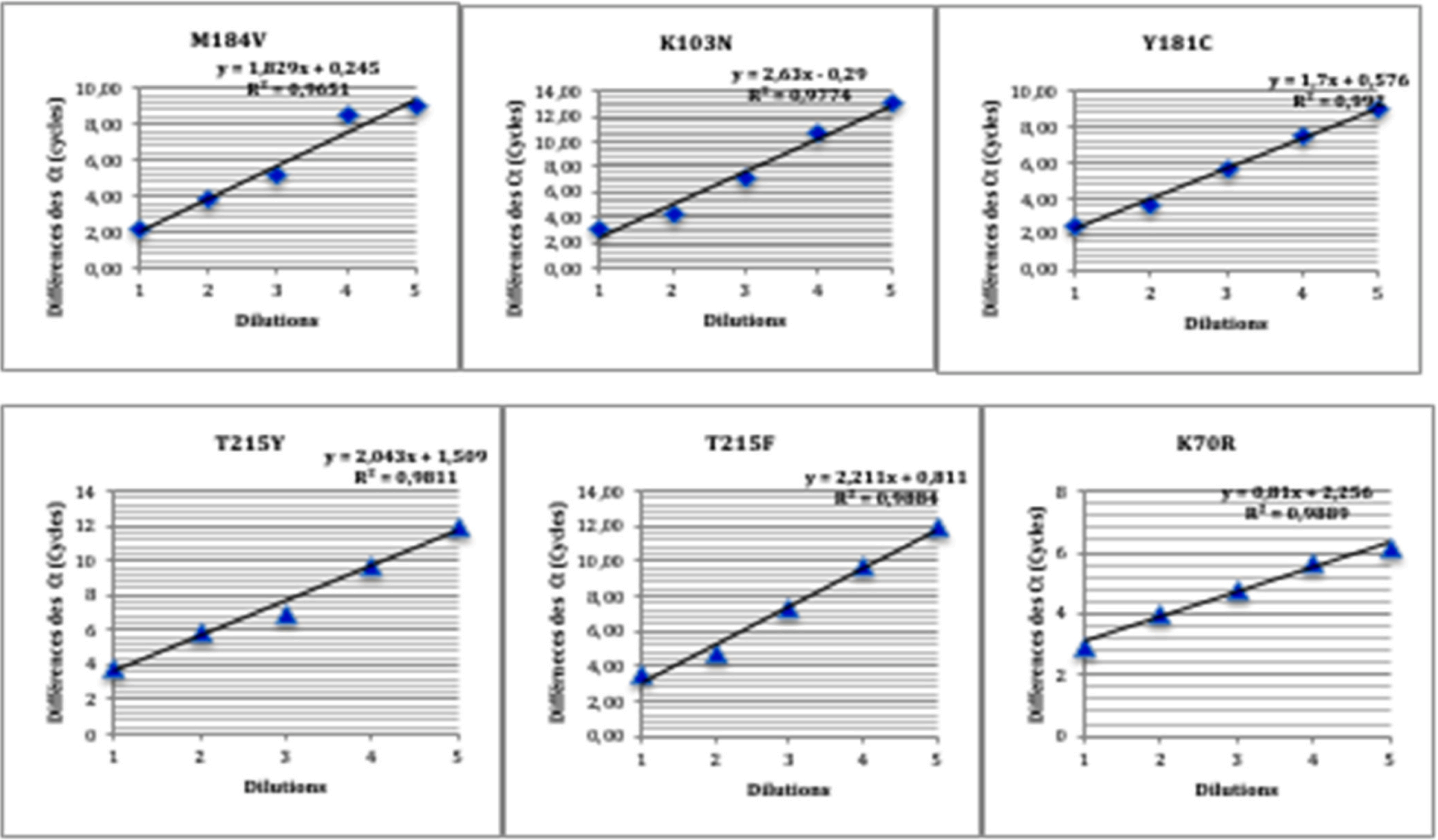
ASPCR detected DRMs across subtypes A, D, E, F, J and CRF02\_AG. This confirmed the high sensitivity of this assay on the wide range of HIV-1 clades, as detailed below:

* **CRF02\_AG:**0.0% (0/13) K70R, 15.4% (2/13) K103N, 15.4% (2/13) Y181C, 38.5% (5/13) M184V, 15.4% (2/13) T215F and 0.0% (0/13) T215Y.
* **Subtype A:** 0.0% (0/4) K70R, 25.0% (1/4) K103N, 25.0% (1/4) Y181C, 50.0% (2/4) M184V, 0.0% (0/4) T215F and 0.0% (0/4) T215Y.
* **Subtype D:**25.0% (1/4) K70R, 50.0% (2/4) K103N, 0.0% (0/4) Y181C, 75.0% (3/4) M184V, 0.0% (0/4) T215F and 25.0% (1/4) T215Y.
* **Subtype F:** 0.0% (0/2) K70R, 0.0% (0/2) K103N, 0.0% (0/2) Y181C, 100.0% (2/2) M184V, 0.0% (0/2) T215F and 0.0% (0/2) T215Y.
* **Subtype J:** 0.0% (0/13) K70R, 15.4% (2/13) K103N, 15.4% (2/13) Y181C, 38.5% (5/13) M184V, 15.4% (2/13) T215F and 0.0% (0/13) T215Y.

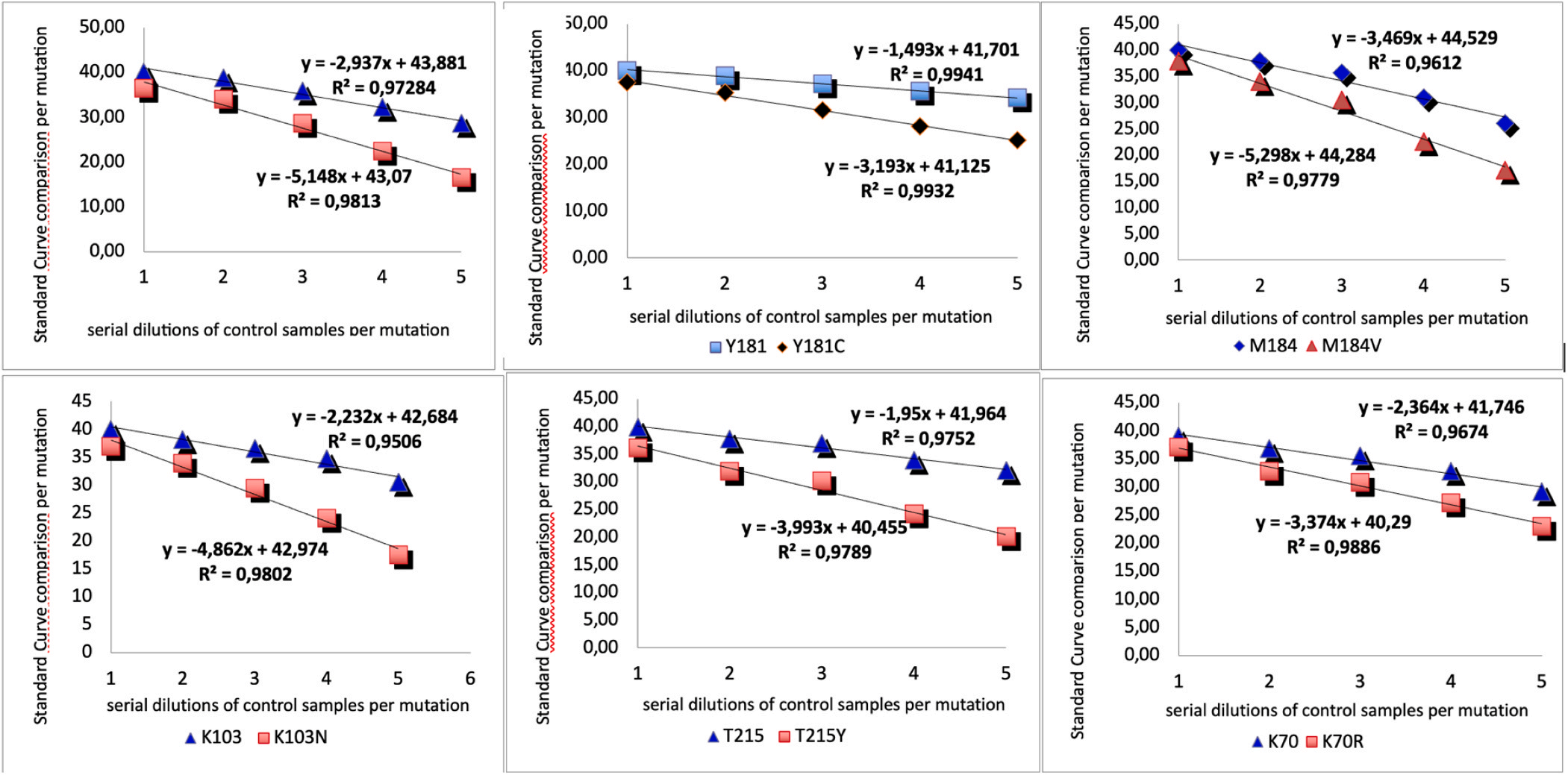
***Figure 1.*** *Standard curves of serial dilutions and* Δ*Ct (wild-mutant) for each mu.*



***Figure 2.*** *Correlation curves from different mutation.*



***Figure 3.*** *Amplification curves with different Ct for K103N.*



***Table 1*** *Detection rate of mutations between ASPCR and Sanger-sequencing.*

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| --- | --- | --- | --- |
| Antiretroviral drug class | Drug resistance mutation | Detection rate from ASPCR: % (n) | Detection rate from Sanger sequencing: % (n) |
| NRTI | K70R | 4.5 (2/44) | 2.3 (1/44) |
|  | M184V | 63.6 (28/44) | 38.6 (17/44) |
|  | T215F | 18.1 (8/44) | 9.1 (4/44) |
|  | T215Y | 6.8 (3/44) | 2.3 (1/44) |
| NNRTI | K103N | 22.7 (10/44) | 13.6 (6/44) |
|  | Y181C | 13.6 (6/44) | 11.4 (5/44) |

***Table 2*** *Intra and inter-assay variability of ASPCR.*

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| --- | --- | --- | --- | --- | --- | --- |
| Coefficients of intra-assays variation | | | | | | |
| Proportion of mutant (%) | M184V | Y181C | K103N | T215F | T215Y | K70R |
| 100 | 0.13 | 0.12 | 0.08 | 0.05 | 0.13 | 0.02 |
| 10 | 0.29 | 0.15 | 0.23 | 0.24 | 0.34 | 0.21 |
| 1 | 0.39 | 0.36 | 0.42 | 0.43 | 0.41 | 0.28 |
| Coefficients of inter-essays variation | | | | | | |
| Proportion of mutant (%) | M184V | Y181C | K103N | T215F | T215Y | K70R |
| 100 | 0.23 | 0.31 | 0.12 | 0.04 | 0.19 | 0.12 |
| 10 | 0.33 | 0.25 | 0.31 | 0.32 | 0.37 | 0.24 |
| 1 | 0.42 | 0.37 | 0.37 | 0.45 | 0.41 | 0.37 |

***Table 3*** *DRMs with proportions detected by AS-PCR individually for each patient.*

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| MUTATIONS DETECTED BY AS-PCR | | | | | | | | | | | | | | |
| M184V | M184I | T215F | T215I | T215Y | T215S | Y181F | Y181C | Y181V | K103E | K103N | K103S | K70P | K70R | K70W |
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| Mutations detected by AS-PCR | | | | | | | | | | | | | Proportion (%) | |
| M184V |  |  |  |  |  |  |  |  |  |  |  |  | 56.8% | |
| M184I |  |  |  |  |  |  |  |  |  |  |  |  | 4.6% | |
| T215F |  |  |  |  |  |  |  |  |  |  |  |  | 18.2% | |
| T215I |  |  |  |  |  |  |  |  |  |  |  |  | 2.3% | |
| T215Y |  |  |  |  |  |  |  |  |  |  |  |  | 6.8% | |
| T215S |  |  |  |  |  |  |  |  |  |  |  |  | 4.6% | |
| Y181F |  |  |  |  |  |  |  |  |  |  |  |  | 2.3% | |
| Y181C |  |  |  |  |  |  |  |  |  |  |  |  | 13.6% | |
| Y181V |  |  |  |  |  |  |  |  |  |  |  |  | 9.1% | |
| K103E |  |  |  |  |  |  |  |  |  |  |  |  | 2.3% | |
| K103N |  |  |  |  |  |  |  |  |  |  |  |  | 22.7% | |
| K103S |  |  |  |  |  |  |  |  |  |  |  |  | 4.6% | |
| K70P |  |  |  |  |  |  |  |  |  |  |  |  | 2.3% | |
| K70R |  |  |  |  |  |  |  |  |  |  |  |  | 4.6% | |
| K70W |  |  |  |  |  |  |  |  |  |  |  |  | 2.3% | |

# 4. Discussion

In RLS, sequencing for HIVDR testing remains challenging and costly, thus limiting the selection of optimal ART regimens after treatment failure. Moreover, minority variants are not generally detected by conventional sequencing techniques (Gianella and Richman, 2010, Johnson et al., 2008; Metzner et al*.,* 2003). These raise interest onASPCR that appear as an easier and more efficient approach in detecting DRMs, including minority variants (Ugozzoli and Wallace, 1992; Wu et al., 1989). Of note, ASPCR appears to be a sensitive, accurate and user-friendlier technique (Guo e*t al.,* 2010, Paredes et al*.,* 2007).

In our dataset, the technical validation of ASPCR across different sample dilutions, based on excellent coefficient variations for each DRM, confirmed the high sensitivity of this approach for use in detecting mutations at low abundant viral populations in RLS like Chad. Similarly, sensitivity at *<* 1% threshold were reported for M184V/I (Paredes et al*.,* 2007);0.04% for K103N, 0.40% for M184V, 0.03% for T215F and 0.02% for T215Y (Guo et al*.,* 2010); 0.019% for K103N (AAC), 0.013% K103N (AAT) and 0.29% for Y181C (Hauser et al., 2009).

Across subtypes found (A, D, F, J and CRF02\_AG), DRMs were detected at higher proportions and at similar trends when compared to Sanger-sequencing. Thus varying rates of DRMs were simply related to the varying burden in different viral strains (Hauser et al., 2009).

Specificity of ASPCR, confirmed by ΔCt in this study (i.e. ΔCtM184V= 9) was similar to previous finding of 9.7 and 10 respectively (Guo et al., 2010; Paredes et al., 2007). The other values obtained in our study for K1013N, T215F, T215Y, Y181C and K70R were also similar to previous findings (Guo et al., 2010). Furthermore, the non-significant intra-test and inter-test variability *<* (0.46%) was concordant with a cross-test and intra-test CV (0.42%) reported previously (Guo et al*.*, 2010). Interestingly, all DRMs had higher inter-test coefficients compared to intra-test outcomes, even at proportion lower than 1% (Reed et al., 2002; Peuchant et al., 2008).

The capacity of ASPCR, in detecting major DRMs that affect the efficacy of drugs commonly found in RLS like Chad, is in support of implementing this point-mutation assay. This assay will also serve in preventing the emergence of DRMs that are still at minority level by anticipating on mutational threshold associated with clinical failure to ART. Of note, Y181C mutation was previously detected in minority quasi- species in the Ethiopian population at 6.5%, in Caucasians at 4.5% and within the East African communities at 1.8% (Ekici et al*.,* 2014). In the same study, K103N was detected it within the East African communitiesat the same percentage as Y181C (1.8%). Other DRMs were detected still at minority level at proportionsranging from 0.25% to 17.5% (Ekici et al*.,* 2014).

As the standard sequencing method (Sanger-sequencing) underestimates the real burden of DRMs in clinical settings, envisioning the implementation of ASPCR technique is being gradually supported for timely patient care in RLS (Cadranel and Giroux Leprieur, 2011; Gaudet et al., 2009). This assay is therefore highly encourage for both pre-treatment drug resistance surveillance strategies (Bucktonet al., 2011; Ekiciet al., 2014), as well as for the monitoring of acquired drug resistance among patients failing ART, even in a context of wide HIV-1 diversity, including B and non-B strains (Loubser et al., 2006).

Our study merits further assessment on its cost-effectiveness for implementation, as well as feasibility study with varying level of health professionals for scale-up in routine practice.

# 5. Conclusion

ASPCR is a high validity (low inter- and intra-assay variability), a high specificity (excellent correlation coefficient) and high sensitivity (detecting both minority and majority DRMs across viral clades). Performance goes beyond the standard sequencing that also requir- escumbersome facilities and high-level staff. ASPCR should be implemented for validation in routine practice of RLS having a broad viral diversity.

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### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114856.

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