Point-of-Care Detection of Nonadherence to Antiretroviral Treatment for HIV-1 in Resource-Limited Settings Using Drug Level Testing for Efavirenz, Lopinavir, and Dolutegravir: A Validation and Pharmacokinetic Simulation Study

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Background: Virological failure during antiretroviral treatment (ART) may indicate the presence of drug resistance, but may also originate from nonadherence. Qualitative detection of ART components using drug level testing may be used to differentiate between these scenarios. We aimed to validate and implement qualitative point-of-care drug level tests for efavirenz (EFV), lopinavir (LPV), and dolutegravir (DTG) in rural South Africa.

Methods: Qualitative performance of immunoassays for EFV, LPV, and DTG was assessed by calculating limit of detection (LoD), region of uncertainty, and qualitative agreement with a reference test. Minimum duration of nonadherence resulting in a negative drug level test was assessed by simulation of treatment cessation using validated population pharmacokinetic models.

Results: LoD was 0.05 mg/L for EFV, 0.06 mg/L for LPV, and 0.02 mg/L for DTG. Region of uncertainty was 0.01–0.06 mg/L for EFV, 0.01–0.07 mg/L for LPV, and 0.01–0.02 mg/L for DTG. Qualitative agreement with reference testing at the LoD in patient samples was 95.2% (79/83) for EFV, 99.3% (140/141) for LPV, and 100% (118/118) for DTG. After simulated treatment cessation, median time to undetectability below LoD was 7 days [interquartile range (IQR) 4–13] for EFV, 30 hours (IQR 24–36) for LPV, and 6 days (IQR 4–7) for DTG.

Conclusions: We demonstrate that qualitative ART drug level testing using immunoassays is feasible in a rural resource-limited setting. Implementation of this technology enables reliable detection of recent nonadherence and may allow for rapid and cost-effective differentiation between patients in need for adherence counseling and patients who require drug resistance testing or alternative treatment.

Key Words: HIV, ART, drug level testing, EIA, EFV, LPV, DTG

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INTRODUCTION

Access to antiretroviral therapy (ART) is rapidly expanding to people living with HIV in low-income and middle-income countries (LMICs). Achieving high and durable virological suppression rates in this expanding patient population is a crucial component of efforts to control the global HIV epidemic.[1] World Health Organization (WHO) guidelines advise annual viral load monitoring for monitoring of the virological response to ART.[2] In case of virological failure, these guidelines advise switching from standardized first-line to second-line ART without additional testing to gain insight into resistance development or patient adherence to treatment. However, 13%–35% of patients with WHO-defined virological failure on first-line ART do not harbor drug-resistant HIV.[3–11] In surveys of cases of WHO-defined

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Study data cannot be made publicly available because of local ethical guidelines set by the University of Pretoria Faculty of Health Sciences Research Ethics Committee. Researchers interested in the data may contact the corresponding author. Requests will be reviewed on an individual basis and subjected to review by the University of Pretoria Faculty of Health Sciences Research Ethics Committee.

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failure on second-line ART, up to 80% of patients with failure harbor viral strains that are sensitive to second-line ART.[12] The lack of diagnostic ability to differentiate between viremia due to drug resistance and viremia due to nonadherence is one of the potential reasons for the profound delay in switching between lines of ART in clinical practice in LMICs.[13]

In case of virological failure without accompanying drug resistance, retrospective drug level testing frequently reveals undetectable antiretroviral drug levels, indicating that failure is likely due to profound nonadherence.[14–16] These findings suggest that in clinical practice, qualitative drug detection testing at failure could be used to identify patients with adherence problems who are at low risk of harboring drug-resistant HIV.[16] Such testing could form an entry point for targeted and intensified adherence counseling, aid in the interpretation of viral load results, and may determine eligibility for drug resistance testing, which comes at a significant cost. However, current methods for drug level testing require complex and costly laboratory procedures and significant expertise.

In recent years, rapid enzyme immunoassays (EIA) have been developed that enable detection and quantification of antiretroviral drugs at low cost and short turn-around times, using standard chemistry analyzers that are an integral part of basic laboratory infrastructure.[17,18] These assays have the potential to enable objective detection of nonadherence to ART in clinical practice in LMICs.

In this study, we validated qualitative drug level immunoassays for the detection of the major WHO-recommended antiretroviral drugs efavirenz (EFV), lopinavir (LPV), and dolutegravir (DTG). We used pharmacokinetic modeling to assess the minimum duration of nonadherence that is required to yield a negative drug level test result below the established thresholds to inform clinical interpretation of drug level testing results.

METHODS

Materials and Assays

Enzyme Immunoassay

Immunoassay drug level testing procedures were implemented at Ndlovu Medical Centre, a rural HIV care provider based in Elandsdoorn, Limpopo, South Africa, with approximately 3600 patients in care receiving ART. Immunoassay kits for the detection of EFV, LPV, and DTG were obtained from ARK Diagnostics, Inc. (Fremont, CA). This homogeneous EIA is based on competition in antibody binding between drug in the sample and drug labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PD). The concentration of the drug is directly proportional to G6PDH activity, which can be monitored as the conversion of nicotinamide adenine dinucleotide (NAD+) to NADH at a wavelength of 340 nm. For each assay, sample was incubated with reagent containing antibody and NAD+ (R1) for 90 seconds, before mixing with a reagent containing analyte-labeled G6PD (R2). For LPV and EFV, input volumes were 150 μL of R1, 75 μL of R2, and 4 μL of sample. For DTG, input volumes were 100 μL of R1, 50 μL of R2, and 5 μL of sample. The experiments were performed on a standard-issue benchtop chemistry analyzer (Indiko Plus chemistry system, Thermo-Scientific, Waltham, MA). Calibrator and control samples for immunoassay testing were used as supplied by the manufacturer.

Reference Bioanalytical Method

External validation of the immunoassay was performed using 3 reference bioanalytical methods at the Department of Pharmacy of the Radboud University Medical Center (Nijmegen, the Netherlands). These assays were validated in accordance with the EMA guideline for bioanalytical methods validation.[19] Quantification of EFV in EDTA plasma was performed by means of ultra-performance liquid chromatography coupled with ultraviolet detection (UPLC-UV). In short, 200 μL of acetonitrile was added to 100 μL of plasma for purposes of protein precipitation. After centrifugation, 7 μL of the clear supernatant was injected onto a reversed-phase column, and the analytes were eluted from the column using a mixture of phosphate buffer in water (0.05 mol/L, pH = 6) and acetonitrile. Ultraviolet detection was performed at a wavelength of 251 nm. The lower limit of quantitation of this assay was 0.050 mg/L. LPV was also quantified using a UPLC-UV method. Sample preparation consisted of a liquid–liquid extraction of the analytes from 0.5 mL of plasma with 5 mL of tert-butyl methyl ether under basic conditions. A volume of 7.5 μL was injected onto the reversed-phase column, and the analytes were eluted from the column using a gradient of acetonitrile in phosphate buffer (0.05 mol/L, pH = 5.6). Detection was performed at a wavelength of 215 nm. The lower limit of quantitation was 0.100 mg/L. Finally, DTG was assayed, as previously described, using ultra-performance chromatography coupled with tandem mass spectrometric detection (UPLC-MS/MS).[20] The lower limit of quantitation of this assay for dolutegravir was 0.010 mg/L. Together, these assays will be referred to as the reference assays throughout the study.

Bioanalytical Validation

Internal Validation

The manufacturer-reported limit of blank (LoB) and limit of detection (LoD) for the EIAs were verified in accordance with the Clinical Laboratory Standards Institute guideline EP17-A using the approach described by Armbruster and Pry.[21] In brief, 60 aliquots of a sample without analyte were assayed to measure the mean and SD and calculate the LoB using the formula $mean_{blank} + 1.645\ (SD_{blank})$. Twenty replicates of a spiked sample with a low analyte concentration (0.010 mg/L) were then assayed to measure the mean and SD around the low-concentration sample. Using the formula $mean_{blank} + SD\ (1.645_{low\text{-}concentration\ sample}) = LoD$, the LoD of the assay was calculated. The LoD for each assay was determined based on the additional rule that the LoD had to be above the highest observed test result for a blank value, rounded to 2 decimals. All concentrations were reported in mg/L up to 3 decimal figures and summarized as mean values with SD.

Based on the calculated LoB and LoD, additional experiments were performed to assess the unreliability region around the LoD for each assay according to methods described by Trullols et al.[22] In brief, 20 replicates of spiked samples from blank with 0.010 mg/L increments to above the calculated LoD were measured. The results were reported as percentage of samples reported as detected above the LoD for each concentration and were visualized using performance characteristic curves.

External Validation

External validation was performed using stored plasma samples from adult patients receiving EFV (n = 80), ritonavir-boosted LPV (n = 76), and DTG (n = 50). For LPV and DTG, patients with no LPV and DTG exposure were used as additional negative controls. Patients receiving EFV and LPV were attending clinical care at Ndlovu Medical Centre, Limpopo, South Africa, and were sampled in May and June 2017. Patients receiving DTG were attending routine clinical care at the Radboud University Medical Center, the Netherlands, and were sampled between August and December 2017. The samples consisted of surplus EDTA-derived venous blood plasma. Surplus plasma was aliquoted and stored at −80°C. For external validation, we compared patient plasma EIA results with the results of the reference assays. Agreement between EIA and reference test was reported as sensitivity and specificity, false-negative rate, and false-positive rate, with their respective 95% confidence intervals (CIs).

Evaluation of Drug Washout

To translate the obtained limits of detection to clinically useful information on adherence, we assessed the theoretical drug washout from plasma after cessation of treatment. We performed Monte Carlo simulations with validated population pharmacokinetic models for EFV 600 mg once daily,[23] ritonavir-boosted LPV 400/100 mg twice daily,[24] and dolutegravir 50 mg once daily[25] using the software package Nonmem V.7.4.3. For each drug, we simulated plasma washout after steady-state pharmacokinetics, assuming perfect adherence in 2000 virtual individuals. The results were displayed using R version 3.3.0 (The R Foundation for Statistical Computing, 2016) and the ggplot2 graphics package version 3.2.1 software.

Ethics Statement

This work was conducted in the framework of the ITREMA randomized controlled trial (RCT) (ClinicalTrials.gov registration number: NCT03357588) and received ethical approval from the University of Pretoria Faculty of Health Sciences Research Ethics Committee (Ref No 69/2015). This study was performed in accordance with the ICH GCP Guidelines and principles of the Declaration of Helsinki.

Samples were obtained from participants in the ITREMA RCT and from patients attending routine clinical care. Samples were collected as a part of routine clinical monitoring during ART and for additional research purposes in case of participants in the ITREMA RCT. No dedicated or additional blood draws were performed for the purpose of this study. Researchers were blinded to clinical and laboratory data of patients other than the patients' currently prescribed ART regimen. Participants in the RCT provided written consent for drug level testing. Patients in routine clinical care provided written consent for the storage of surplus plasma for research purposes.

RESULTS

Bioanalytical Validation

Internal Validation

Replicate EIA testing of blank samples demonstrated a mean blank result of 0.011 mg/L (sd: 0.006 mg/L) for EFV, 0.009 mg/L (sd: 0.013 mg/L) for LPV, and 0.001 mg/L (sd: 0.002 mg/L) for DTG, resulting in a calculated LoB of 0.020 mg/L for EFV, 0.030 mg/L for LPV, and 0.005 mg/L for DTG. The highest result observed for a blank sample was 0.039 mg/L for EFV, 0.053 mg/L for LPV, and 0.007 mg/L for DTG.

Replicate EIA testing of samples with a low concentration of analyte (0.010 mg/L) showed a mean result of 0.035 mg/L (sd: 0.005 mg/L) for EFV, 0.010 mg/L (sd: 0.015 mg/L) for LPV, and 0.008 mg/L (sd: 0.003 mg/L) for DTG. The calculated LoD was 0.029 mg/L for EFV, 0.054 mg/L for LPV, and 0.010 mg/L for DTG. Based on the calculated LoD and highest blank result, the LoD was established at 0.05 mg/L for EFV, 0.06 mg/L for LPV, and 0.02 mg/L for DTG (Table 1).

Analysis of spiked samples at incremental concentrations around the LoD using EIA demonstrated good qualitative performance for all assays. Sample concentrations of 0.000

TABLE 1. Qualitative Test Performance—Internal Validation

	EFV	LPV	DTG
Mean *(SD)*—blank sample	0.011 mg/L (*0.006*)	0.009 mg/L (*0.012*)	0.001 mg/L (*0.002*)
Highest result—blank sample	0.039 mg/L	0.053 mg/L	0.007 mg/L
Calculated LoB	0.020 mg/L	0.029 mg/L	0.005 mg/L
Mean (SD)—0.01 mg/L sample	0.035 mg/L (*0.005*)	0.010 mg/L (*0.015*)	0.008 mg/L (*0.003*)
Calculated LoD (selected LoD)	0.029 mg/L (*0.05* mg/L)*	0.054 mg/L (*0.06* mg/L)*	0.010 mg/L (*0.02* mg/L)*

Calculated LoB = $\text{mean}_{\text{blank}} + 1.645 \times \text{SD}_{\text{blank}}$. Calculated LoD = LoB + $1.645 \times \text{SD}_{0.01\text{mg/L}}$.
*LoD was selected so that rounding up of the highest ever recorded blank result would not lead to a false-positive result.

mg/L or 0.010 mg/L uniformly yielded test results below the LoD for all assays, indicating good discrimination of negative samples. The unreliability region, the range of sample concentrations for which the probability of a test result at or above the LoD was between 10% and 90%, was relatively narrow, ranging from 0.020 mg/L to 0.040 mg/L for EFV and from 0.020 mg/L to 0.080 mg/L for LPV. For DTG, unreliability was only present at the LoD concentration of 0.020 mg/L itself but not at other measured concentrations. At the LoD concentration, probability of a test result at or above the LoD was 95% for EFV, 30% for LPV, and 30% for DTG (Figs. 1A–C).

External Validation

Agreement at the LoD in parallel testing using EIA and the reference assays revealed 95.2% (79/83) correct classification for EFV, 98.6% (139/141) correct classification for LPV, and 100% (118/118) correct classification for DTG (Table 2). In the 2 cases of a positive EIA test result for LPV above the 0.060 mg/L LoD and a negative UPLC-UV result, the reported EIA test results were below the LoD of the UPLC-UV, which was 0.100 mg/L. False-negative EIA results did not occur for any of the assays, resulting in a sensitivity of 100% (95% CI: 94% to 100%) for EFV, 100% (95% CI: 92% to 100%) for LPV, and 100% (95% CI: 92% to 100%) for DTG. False-positive EIA results occurred in 4 of the 21 (19.0%) cases for EFV, 4 of the 99 (4.0%) cases for LPV, and in 0 of the 71 (0.0%) cases for DTG. Specificity was 81% (95% CI: 58% to 95%) for EFV, 98% (95% CI: 93% to 100%) for LPV, and 100% (95% CI: 95% to 100%) for DTG.

Evaluation of Drug Washout

In the simulated data, we observed median steady-state plasma drug concentrations before simulated therapy cessation of 1.39 mg/L for EFV [interquartile range (IQR): 0.77–2.28], 7.17 mg/L for LPV (IQR: 5.97–8.76), and 1.35 mg/L for DTG (IQR: 0.94–1.90). After simulated cessation of EFV, median time to undetectability below the assay LoD was 7 days (IQR: 4–13) after the last drug intake. For LPV and DTG, time from cessation to undetectability below the assay LoD was 30 hours (IQR: 24–36) and 6 days (IQR: 4–7) after the last drug intake, respectively. (Figs. 2A–C).

DISCUSSION

This study shows that qualitative detection of anchor drugs of WHO-recommended ART regimens is reliable and can be implemented on standard chemistry analyzers in rural settings in LMICs, creating the potential for rapid feedback on treatment adherence to clinicians. Implementation of this assay resulted in reliable identification of patients with undetectable drug levels. We showed that an undetectable drug level signifies no medication intake for at least 1 day for LPV and approximately a week for EFV and DTG, assuming perfect adherence before cessation.

Previous research of antiretroviral drug level testing mainly focused on potential implementation as therapeutic drug monitoring, in which drug trough levels were assessed quantitatively to monitor toxicity and adherence and to guide dosing. Clinical trial data do not support the routine clinical use of therapeutic drug monitoring for monitoring of currently recommended ART regimens.[26] The current WHO guidelines recommend viral load monitoring as the primary method of monitoring of treatment response and define virological failure as a confirmed viral load above 1000 copies/mL. In case of virological failure on first-line ART, these guidelines advise to perform an empirical switch from first-line to second-line ART.[2]

However, surveys have repeatedly shown that a subset of patients with virological failure do not harbor drug-resistant viral strains warranting a switch of treatment, indicating that failure is merely due to nonadherence.[27,28] A recent analysis demonstrated that patients experiencing failure are left on a failing regimen for an average period of almost 1.5 years before a switch to second-line ART is finally made, allowing for disease progression and onward transmission of HIV.[13] Although drug resistance testing would be able to identify these patients, the high costs and required laboratory infrastructure for drug resistance testing limit its availability in LMICs.

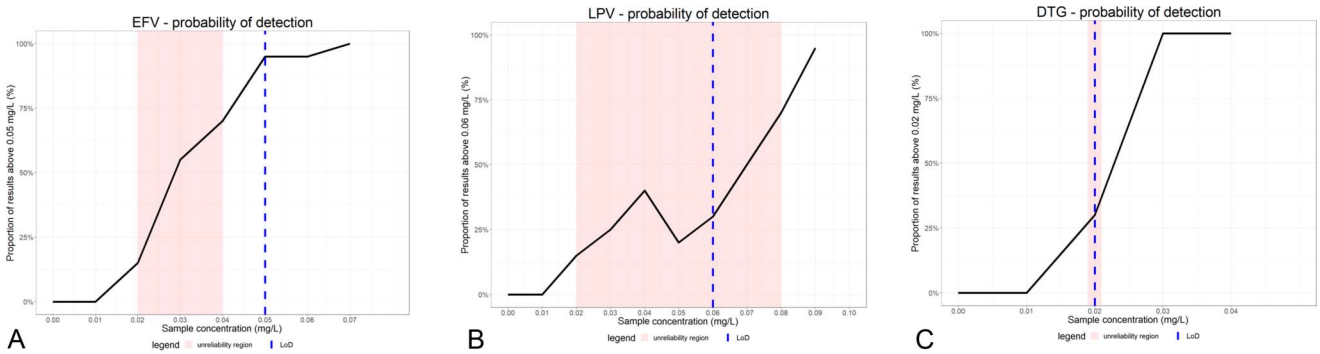

FIGURE 1. A–C, Probability of detection for EFV, LPV, and DTG assays. Note: Probability of detection analysis of replicates (n = 20 per concentration) at incremental concentrations (step = 0.01 mg/L) ranging from 0.00 to above the calculated assay limit of detection. Red band indicates region of uncertainty, defined as the region in which the positivity rate is 10%–90%. Blue line indicates the established LoD. full color online

TABLE 2

A. EFV	EIA Positive (≥0.05 mg/L)	EIA Negative (≥0.05 mg/L)	Total
UPLC-UV positive (≥*0.05* mg/L)	74.7% (62)	0.0% (0)	62
UPLC-UV negative (<*0.05* mg/L)	4.8% (4)	20.5% (17)	21
Total	66	17	83
B. LPV	**EIA Positive (≥0.06 mg/L)**	**EIA Negative (<0.06 mg/L)**	**Total**
UPLC-UV positive (≥*0.10* mg/L)	29.8% (42)	0.0% (0)	42
UPLC-UV negative (<*0.10* mg/L)	1.4% (2)*	68.8% (97)	99
Total	46	95	141
C. DTG	**EIA Positive (≥0.02 mg/L)**	**EIA Negative (<0.02 mg/L)**	**Total**
UPLC-UV positive (≥*0.01* mg/L)	39.8% (47)	0.0% (0)	47
UPLC-UV positive (<*0.01* mg/L)	0.0% (0)	60.2% (71)	71
Total	47	71	118

Note: Qualitative agreement for lopinavir assay.
*The 2 cases of a positive EIA result and negative UPLC-UV result had reported EIA concentrations below the LoD of the UPLC-UV assay (0.10 mg/L).

We show that drug exposure testing using enzyme immunoassays can be readily implemented in resource-limited environments and is able to reliably and rapidly detect nonadherence to antiretroviral compounds. Low costs and widespread availability of chemistry analyzers are important advantages of enzyme immunoassays that would enable point-of-care implementation, enabling direct feedback of results to patients. Although the use of enzyme immunoassays for detection of individual ART components has been previously reported,[29] this is the first report of successful implementation of a panel of tests that covers all available anchor drugs as a qualitative assay.

Several limitations must be mentioned. False-positive EIA results were sporadically encountered in the case of the LPV and EFV assays. In the case of the LPV assay, the selected LoD for the EIA was below the LoD for our LPV UPLC-UV reference test, and the quantitative EIA result was in between these 2 values in 2 cases. We are, therefore, unable to establish whether these cases are false-negative or correctly classified by the EIA. For the EFV assay, 4 false-positive cases were encountered, suggesting measurement inaccuracy in the lower concentration range. Importantly, false-negative EIA results did not occur for any of the tested assays. As such, although these cases resulted in a slight reduction in the specificity for the EFV assay, the sensitivity of the assay was not affected. This study did not evaluate assays for detection of atazanavir and nevirapine, which are in use in selected patient populations in some LMICs. However, these assays are available and could be implemented tailored to local ART availability. The assays reported in this study require basic laboratory infrastructure that is in widespread use. However, in some severely resource-constrained settings, such infrastructure may not be available. The recent introduction of tenofovir urine testing using dipstick technology is an exciting technological advancement that may enable drug level testing even in these settings.[30,31]

Pharmacokinetic simulations used in this study yield novel insights into the clinical significance of undetectable drug level results. Median time to undetectable levels was approximately 1 week for EFV and DTG and 30 hours for ritonavir-boosted LPV. Of note, although individual variation in time to undetectable levels was fairly limited for DTG and LPV, there was substantial variation for EFV in the model population, which may be attributable to genetic polymorphisms of the gene encoding the cytochrome P450 2B6 enzyme involved in EFV metabolism.[32] The model used for the EFV simulations was derived from the 2NN-study, which incorporated mainly patients in LMICs, and was, therefore, believed to be representative of the target population. Because we assumed perfect adherence followed by an episode of treatment interruption, time to undetectability is expected to be shorter in patients who were already not fully adherent before interrupting treatment. In addition, time to undetectability may also be subject to drug–drug interactions and medical conditions affecting drug metabolism. Nevertheless, simulation of drug washout illustrated that these drugs are amenable to qualitative drug detection in case of suspicion of nonadherence.

Targeted qualitative drug exposure testing in patients with virological failure of ART is an alternative approach to

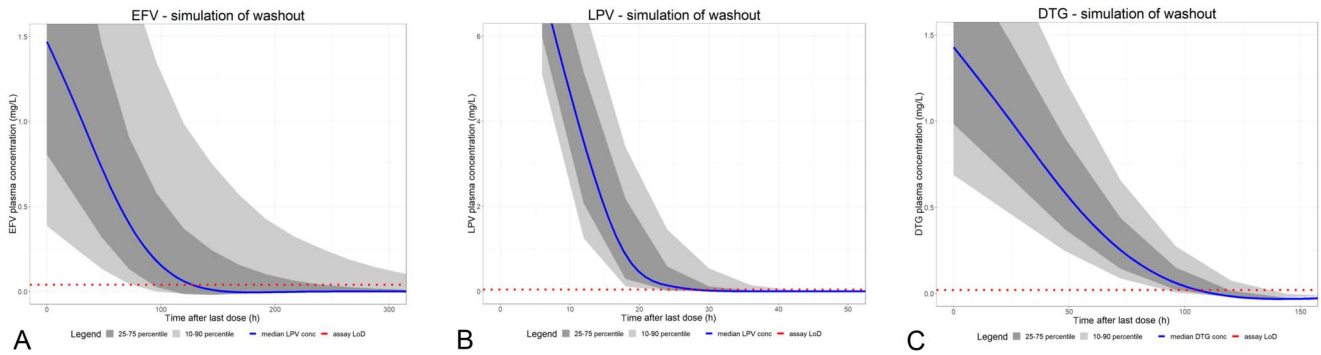

FIGURE 2. A–C, Pharmacokinetic modeling of washout (EFV, LPV, and DTG). Note: Pharmacokinetic modeling of washout of EFV, LPV, and DTG. full color online

treatment monitoring that may create valuable insight into the underlying reason for failure. Recent work has demonstrated the potential clinical utility of this approach in patients with virological failure of LPV/r-based second-line ART, showing that undetectability of drug during failure accurately identifies patients who do not harbor detectable drug-resistant HIV.[15,16] As such, qualitative drug level testing could be used as a screening test in case of an elevated viral load during ART to identify those patients in which viremia is likely to have occurred merely because of nonadherence. In this scenario, a negative drug level test would serve as an entry point to intensified adherence counseling, whereas a positive drug level test would be an indication for drug resistance testing. This approach is currently being studied prospectively in patients receiving EFV-based first-line ART in the ongoing ITREMA open-label RCT (NCT03357588). In this study, EIA-based testing will be performed as point-of-care during a clinical visit, with a turn-around time of 45 minutes, to allow for rapid clinical decision-making.

CONCLUSIONS

Nonadherence is an important threat to long-term successful treatment of people living with HIV. High rates of adherence are required to gain control of the HIV epidemic. This study demonstrates that qualitative drug level testing of EFV, LPV, and DTG can be reliably performed in resource-limited settings using standard laboratory infrastructure and low-cost technology. Drug level testing enables clinicians to accurately identify patients who did not take treatment for several days to weeks, depending on the drug tested. Implementation of this technology may allow for more rapid and cost-effective differentiation between patients in need for adherence counseling and patients who require drug resistance testing or alternative treatment.

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