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MARVEL-minimising the emergence and dissemination of HIV-1 drug resistance in Portuguese-speaking African Countries (PALOP): low-cost portable NGS platform for HIV-1 surveillance in Africa

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Abstract

Background HIV-1 infections remain a global public health concern. Scaled-up antiretroviral treatment (ART) is crucial for reducing morbidity and mortality related to HIV/AIDS. The emergence of drug-resistance mutations (DRMs) compromises viral suppression and contributes to the continued HIV-1 transmission. Several reports indicate a recent increase in acquired (ADR) and transmitted (TDR) drug resistance in Africa, probably linked to the lack of implementation of HIV drug resistance (HIVDR) testing and suboptimal treatment adherence. Herein, we will develop a low-cost protocol using third-generation sequencing (Oxford Nanopore Technology) for HIV-1 surveillance in Portuguese-speaking African Countries - PALOP [Angola (AO), Cape Verde (CV), Mozambique (MZ), and Sao Tome & Principe (STP)].

Methods This is a multicentric cross-sectional study that includes around 600 adult patients newly diagnosed with HIV-1 in the PALOP. An epidemiological questionnaire previously validated by our research team will be used to collect sociodemographic and clinical data. Also, whole blood samples will be collected and the plasma samples will be subjected to drug resistance testing using an *in-house* low-cost NGS protocol. Data analysis will involve bioinformatics, biostatistics and machine learning techniques to generate accurate and up-to-date information about HIV-1 genetic diversity, ADR and TDR.

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Discussion The implementation of this low-cost NGS platform for HIV-1 surveillance in the PALOP will allow: (i) to increase DRM surveillance capacity in resource-limited settings; (ii) to understand the pattern and determinants of dissemination of resistant HIV-1 strains; and (iii) to promote the development of technical and scientific skills of African researchers for genomic surveillance of viral pathogens and bioinformatics analysis. These objectives will contribute to reinforcing the capacity to combat HIV infection in Africa by optimizing the selection of ART regimens, improving viral suppression, and reducing ADR or TDR prevalence in PALOPs, with relevant implications for public health.

Keywords HIV-1, Drug resistance, Machine learning, PALOP, Africa

Background

HIV-1 infections remain a global public health concern. Maintaining high levels of HIV viral load (VL) suppression in the community is crucial to achieving the World Health Organization (WHO) 95-95-95 target of ending AIDS as a public health threat by 2030 [1, 2]. The global response to HIV has been threatened by the worldwide emergence of HIV drug resistance (HIVDR), mainly in sub-Saharan African countries [3]. HIVDR testing is recommended for all HIV-positive patients newly diagnosed before starting antiretroviral therapy (ART) in many resource-rich settings, but this approach is not always viable, especially in resource-limited African countries, such as the Portuguese-speaking African Countries (PALOP) community [4]. Drug-resistant HIV strains selected in the context of treatment failure (Acquired Drug Resistance - ADR) have the potential to limit the response to subsequent treatment and constitute a reservoir for onward transmission to newly infected individuals (Transmitted Drug Resistance - TDR) [3].

The use of antiretroviral (ARV) drugs has proven remarkably effective in controlling the progression of HIV infection and prolonging survival [5]. However, there are concerns about increasing levels of TDR in Africa, given the rapid scale-up in access to ART and the persistently high incidence of HIV-1 infections [6]. The use of ARV as prevention, including Pre-Exposure Prophylaxis (PrEP), has also been largely used and implemented in Africa. However, major concerns about the implementation of these strategies are the reportedly suboptimal treatment adherence combined with the lack of access to VL measurements and HIVDR testing for the follow-up of HIV-positive patients whether ART-naïve or experienced [7–9]. Once TDR exceeds 10%, modelling suggests that in Africa, HIVDR strains could account for almost half a million new infections, and additional treatment costs will reach $6.5 billion by 2030, when more people will need second and third-line ART regimens [10].

Currently, the strategies for controlling the emergence of resistant HIV-1 strains include HIVDR testing and surveillance, as well as guaranteeing optimal adherence and clinical follow-up of HIV patients [11–13]. Since in most resource-limited countries VL and HIVDR testing are not available for patients who have never received treatment or ART, conducting surveillance studies is important to understand HIV transmission patterns in order to inform ART guidelines and strengthen local HIV patient management strategies. Conventional HIVDR genotyping qualitatively detects DRM using Sanger sequencing approaches [12]. However, this method outputs only a single consensus sequence per sample, which does not represent the quasispecies of HIV-1 strains present at the intrapatient level and that can include minority resistance variants (MRVs) presenting ARV drug-resistant mutations [12]. The increased sequencing capacity of the newest high-throughput sequencing instruments offers greater depth, allowing more sensitive detection of MRV than conventional HIVDR genotyping [11]. There is increasing evidence showing that the presence of MRVs may be clinically relevant since they are associated with an increase in the risk of treatment failure in patients with MRVs [11–13]. The use of artificial intelligence models, such as machine learning (ML) approaches capture patterns from the vast amount of data on HIV drug resistance mutations around the world and can help to better understand and predict the emergence of MRV.

In countries where routine resistance testing is not feasible, it is recommended to conduct regular surveys to monitor TDR and ADR, especially in adults starting or restarting first-line ART regimens. This project aims to collect demographic, clinical, and genomic data from Portuguese-speaking African countries (PALOP), where scarce genomic data from HIV patients is available. Specifically, we will implement a low-cost next-generation sequencing (NGS) platform for HIV-1 surveillance combined with the application of epidemiological questionnaires. We will then combine this with other database-available HIV genomic information. This integrated data will then be used to develop ML models that will help to understand and predict the emergence of DRM, whether TDR or ADR. With such an approach, we expect to contribute to minimise the emergence and dissemination of resistant HIV-1 strains in the Portuguese-speaking African Countries, such as Angola (AO), Cape Verde (CV), Mozambique (MZ), and Sao Tome & Principe (STP). This HIV-1 surveillance platform adapted to PALOP resource constraints will result in an increased response capacity in low-resource settings and

strengthen ongoing HIV-1 surveillance efforts in Africa as a global effort towards HIV/AIDS eradication until 2030.

Methods/design

MARVEL project

The MARVEL project is a collaborative effort between several groups of international researchers affiliated with health research centres or institutes in Portugal and PALOP such as Angola (Centro de Investigação em Saúde de Angola-CISA|Instituto Nacional de Investigação em Saúde-INIS), Mozambique (Centro de Investigação em Saúde de Manhiça - CISM), Cape Verde (Universidade de Cabo Verde and Instituto de Saúde Pública), and Sao Tome & Principe (Centro de Endemias de São Tome & Principe and Laboratório Central de Tuberculose e HIV de São Tome & Principe). The project will increase the response capacity against the HIV/AIDS pandemic in Africa, particularly at the level of PALOP communities, being in line with global efforts to end HIV/AIDS as a global public health concern by 2030. The project brings a multidisciplinary team of biomedical scientists, epidemiologists, virologists, bioinformatics, statisticians, and artificial intelligence experts. The project aims to investigate various aspects of the HIV/AIDS pandemic and the emergence of DRM in resource-limited settings in Africa, including identifying the epidemiological determinants that drive the dissemination of resistant HIV-1 strains in PALOP communities.

The aim of the study

The MARVEL project aims to develop and implement a low-cost NGS platform for HIV-1 surveillance in Africa, to minimise the emergence and dissemination of resistant HIV-1 strains in Angola, Cabo Verde, Mozambique, and Sao Tome & Principe, which will result in increased response capacity and strengthen ongoing HIV-1 surveillance efforts in Africa.

The specific objectives of this study are:

(i) To identify the clinical and socio-behavioural determinants that are associated with HIV-1 TDR and ADR emergence in Angola, Cabo Verde, Mozambique, and Sao Tome & Principe;
(ii) To identify and quantify minority resistance variants and its impact on antiretroviral treatment response;
(iii) To develop a machine learning approach to understand clinical and genomic determinants of the emergence of DRM and classify genomic signatures of previous treatment experience;
(iv) To develop an online framework to provide an evidence base for real-time clinical decision-making to clinicians who follow HIV patients in PALOP settings.

Study design, population, and setting

The methodological description is shown in Fig. 1. This is a multicentric cross-sectional study that will include around 600 adult patients with an estimate of around 150 participants in each country, all newly diagnosed with HIV-1 in testing and monitoring centres for HIV-positive

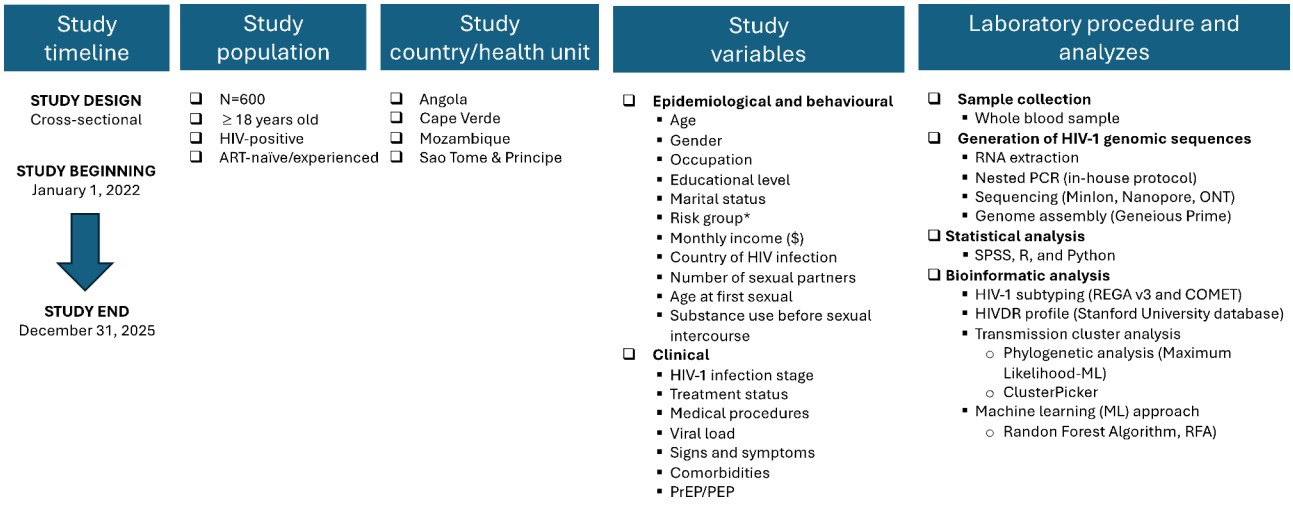

Fig. 1 Methodological description of the study. The study begins in January 2022 and ends in December 2025. About 600 patients diagnosed with HIV (naïve or treated) in health units in Angola, Cape Verde, Mozambique and Sao Tome and Principe are invited to take part in the study. Epidemiological, behavioural, and clinical data, as well as blood samples, are being collected for sequencing using the Minlon platform, a third-generation sequencing from Oxford Nanopore Technology. Statistical programs such as SPSS, R, and Python are used to explore qualitative and quantitative data. REGA, SCHUEAL and HIVDB will be used for subtyping and to identify the pattern of resistance to NNRTI, NRTI, PI and INSTI. *Men who have sex with men, people deprived of liberty, people who inject drugs, sex workers and transgender people

patients in the PALOP (Angola, Cabo Verde, Moçambique, and São Tomé e Príncipe). The study began on January 1, 2022, and is scheduled to end on December 31, 2025. The inclusion criteria will be HIV-positive patients aged 18 years old and older. The study protocol was reviewed and approved by the healthcare institutions in Angola (protocol number 39/C.E./2021, dated December 1st, 2021), Cape Verde (protocol number CNEPS CP47, dated February 16th 2023), Mozambique (protocol number 368/CNBS/23, dated July 10th 2023), and Sao Tome & Principe (protocol number CESIC 021_2022, dated February, 8th 2023). Data (questionnaires and blood samples) will be made available by collaborator institutions to the study investigators using an appropriate blinded code to guarantee the protection of personal data and address all ethical and legal requirements. The researchers team will additionally ensure data protection according to the Helsinki Declaration. The study participants will receive written information, including the description and the goals of the study and information about data privacy procedures, including the voluntary nature of the participation, the right to withdraw from the study at any moment without any penalties and with the destruction of all data collected, confidentiality assurance and the right to request information from the principal investigator will be explained. Informed signed consent will be requested from all participants before they are enrolled in the study. If the participant is illiterate, oral consent, in the presence of a witness who signs the form, will be accepted. When interpreters are needed, they will commit to confidentiality in written form.

Study centres

The project leader is allocated at the Hygiene and Tropical Medicine Institute (IHMT) in Lisbon, Portugal (https://www.ihmt.unl.pt/o-ihmt/). In Angola, the study is coordinated by the Angola Health Research Center (CISA)|National Institute for Health Research (INIS), located in Luanda, the capital city of Angola. In Cape Verde, the study will be coordinated by the University of Cape Verde and the National Institute of Public Health, located in Praia, Cape Verde. In Mozambique, the study is coordinated by the Manhiça Health Research Center (CISM), located in Maputo, Mozambique. In Sao Tome & Principe, the study is coordinated by the National Endemic Diseases Center, located in the capital of this archipelago.

Data and sample collection

The variables in the present study protocol were defined considering the objectives of the study, as well as the availability and structure of data in the participating health centres in each country. A questionnaire containing epidemiological/behavioural (age, gender, occupation, educational level, marital status, risk group, Monthly income ($), country of HIV infection, number of sexual partners, age at first sexual, and substance use before sexual intercourse), and clinical (HIV-1 infection stage, treatment status, viral load, signs and symptoms, comorbidities, PrEP and Post Exposure Prophylaxis - PEP) information will be applied to the participants. The variables defined for the present study are crucial for addressing the study objectives as well as the proposed outcomes. Therefore, all the participants will be invited to answer the questionnaire with the support of a member of our research team. The online platform Google Forms will be used to directly collect the demographic, behavioural and clinical information of each enrolled participant. In addition to this demographic and clinical information, an estimated 10 mL of whole blood samples will be collected from each participant and stored in tubes containing ethylenediaminetetraacetic acid (EDTA). The blood plasma samples will be then separated by centrifugation and frozen at -80 °C until further laboratory investigation. All the data and blood sample collection will be done by an experienced health professional who will avoid causing discomfort to the participants.

Generation of HIV-1 genomic sequences

HIV-1 genomic sequences of these HIV-positive patients will be generated through Next Generation Sequencing. This task will be developed in close collaboration between the IHMT and the local focal points in PALOP. Peripheral Blood Mononuclear Cells (PBMC) samples from PALOP HIV patients will be collected. The plasma samples will be submitted to manual RNA extraction, using the QIAmp Viral RNA kit (QIAGEN or similar) according to the manufacturer's instructions. After generation of cDNA and conventional nested-PCR, using 4 pools of primers for amplification of the protease (PR), partial transcriptase reverse (RT) and Integrase (IN) fragment, all located in HIV-1 *pol* genes.

Table 1 describes the primer set to be used for the present study designed by Primer3Plus (https://www.primer3plus.com/index.html). The amino acid positions 1 to 99 in PR (HXB2 position: 2253 to 2549), 1 to 415 in RT (HXB2 2550 to 3793) and 1 to 289 in the INT (HXB2 4230 to 5096) will be amplified in a 20μL final PCR reaction mixture containing Taq Platinum High Fidelity enzyme and primers. RNA will be amplified using the nested one-step reverse transcription polymerase chain reaction (RT-PCR) method, optimized by Coelho et al. [14]. Briefly, the amplification of the partial HIV-1 *pol* gene will be performed with 5μL of RNA input, Superscript III (200U/μL), and Taq Platinum High Fidelity 0.625U (Life Technologies). The one-step RT-PCR cycling conditions will be as follows: (i) 30 min at 50 °C

Table 1 Description of primers according to HXB2 positions

Nome	Gene	Direction	Sequence (5´→ 3´)	Fragment size	Position (HXB2)
VPolF1	PR + RT	Forward (PCR)	CAGGGCCCCTAGGAAAAAGG	1889	2004 → 2023
VPolR1	PR + RT	Reverse (PCR)	CTGCTAGCTGCCCCATCTAC		3873 ← 3892
VPolF2	PR + RT	Forward (*nested*-PCR)	GCAGGAGCCGATAGACAAGG	1580	2214 → 2233
VPolR2	PR + RT	Reverse (*nested*-PCR)	TCCCACTCAGGAATCCAGGT		3774 ← 3793
VINF1	IN	Forward (PCR)	GGAGCAGAAACCTTCTATGTAGATGG	1833	3855 → 3880
VINR1	IN	Reverse (PCR)	CCCTAAGCCATGGAGCCAAA		5668 ← 5687
VINF2	IN	Forward (*nested*-PCR)	AAGCTTTGCAGGATTCGGGA	1299	4000 → 4019
VINR2	IN	Reverse (*nested*-PCR)	GGAGACTCCCTGACCCAGAT		5279 ← 5298

and 5 min at 94ºC for cDNA synthesis; (ii) 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 54 °C), and elongation (2.5 min at 68 °C), and (iii) a final elongation step for 10 min at 68 °C. The amplified DNA fragments will be analyzed through 1% agarose gel electrophoresis with SYBR Safe DNA Gel Stain (Invitrogen). The second PCR will be done using 5µL of RT-PCR products with Go Taq Green Master Mix (Promega, USA). The *nested*-PCR cycling conditions will be as follows: (i) 3 min at 94 °C; (ii) 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (1.5 min at 72 °C), and (iii) a final elongation step for 10 min at 72 °C. This *in-house* RT-PCR protocol will be optimized using plasma samples of HIV-positive patients from Angola, Cape Verde, Mozambique and Sao Tome & Principe. To estimate the sensitivity of this in-house protocol, we established serial dilutions of RNA extracted from individuals with previously known HIV viral load. The amplification rate of PR, RT and IN fragments has been verified even in samples with viral loads of up to 500 copies/mL.

The generated amplicons will be purified using 1xAMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified with Qubit™ 4 Fluorometer and the sample concentrations will be normalized to an initial input of 80ng for each sample including PR, RT, and IN fragments. DNA library preparation will be conducted with the Ligation Sequencing kit (SQK-NBD114.24, ONT) and Native Barcoding Expansion 1–24 kit (R10.4.1, ONT), following the reaction conditions previously described by Quick et al. The Mk1C Sequencing Device will be used to obtain the nucleotide sequences. The reference assembly of new sequences will be carried out using the Geneious Prime 2024.0.5 and the assembled FAST or FASTQ files exported for bioinformatic analysis.

HIV-1 subtyping and drug resistance mutation analysis

Subtype assignments will be done based on the REGA v3 [15] and COMET [16] subtyping tools. To evaluate HIVDR, the nucleotide sequences will be submitted to the Calibrated Population Resistance (CPR) tool (https://hivdb.stanford.edu/cpr/) considering the WHO 2009 update list and HIVdb Program which is available online in the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/).

Phylogenetic analysis of HIV-1 transmission patterns and drug resistance mutations clustering in PALOP

All samples included in this study will be subtyped using REGA v3 [15], as described above. Using BLAST, global control sequences will be selected from the Los Alamos database (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html). For each subtype, the sequences will be aligned with the global background dataset chosen as a control, using Virulign [17] to ensure the reliability of the alignments. The positions of the nucleotide sequence (2251–3251) of the *pol* gene of HIV-1 with access number K03455.1 (HXB2), will be included in the alignment as a reference and edited manually using AliView software [18], resulting in a final sequence alignment of approximately 1,000 base pairs. Codons associated with DRMs for surveillance will be removed from the sequences. Maximum likelihood (ML) phylogenies will be constructed in FastTree [19] using the best-fit model, as determined using ModelTest. The statistical support of the clusters will be assessed using the Shimodaira-Hasegawa-like test (SH-test). Potential transmission clusters will be identified using ClusterPicker v1.332 and defined as clades with (1) high branching support (≥0.90 SH-test) and (2) a maximum pairwise genetic distance of less than 3.5% between all clades.

Machine learning approach to predict the emergence of drug resistance mutations

Each of the HIV genes (PR, RT and IN) will be translated separately into amino acid (aa) sequences, which will then be concatenated into a single sequence for each genome (about 800 aa). The residues will be numbered and reported in relation to the HXB2 prototype as a reference sequence, following the convention in the field. Different machine learning algorithms will be applied to the concatenated HIV sequences, using the known resistance phenotype to antiretrovirals (resistant or susceptible variant) of each sequence as the classification variable and the aa sites (removing the sites known in the literature as resistance codons) as predictive variables.

This will be done in search of the codons that maximize the prediction of the sample phenotype (resistant or susceptible). The method will thus identify potential sites related to resistance. To ensure the robustness of predictions, cross-validation techniques will be employed, allowing the evaluation of the predictive capacity of the selected sites in different subsets of the data. Additionally, covariance analyses will be conducted to investigate possible interactions between the aa sites, using mutual information-based approaches. These analyses will help understand how the selected sites contribute to definition of the resistance phenotype. Throughout the study, the data will be continuously monitored and adjusted as necessary to improve the accuracy and reliability of the results.

Data analysis

To analyze the demographic, clinical and/or genomic data, we will use SPSS v29, R, and Python. We will check the normality of the data distribution with the Shapiro-Wilk test and examine the skewness and kurtosis of the data. Mean and standard deviation (SD) will be presented for the data found to be normally distributed, while medians and interquartile ranges (IQR) will be presented for variables with skewed distributions. After descriptive data analysis, we will apply parametric and non-parametric tests for hypothesis testing, as appropriate. We will use the Chi-square (X^2), univariate and multivariate logistic regression to identify factors associated with transmission clusters, ADR, and/or TDR. We will report the odds ratio (OR) or adjusted odds ratio (AOR) along with their 95% confidence interval (CI). All reported p-values will be two-tailed and judged statistically significant when $p<0.05$.

Discussion

This study protocol adapted to PALOP resource constraints will increase the clinical and scientific response capacity of African HIV researchers in the efforts towards HIV/AIDS eradication by 2030. To the best of our knowledge, this is one of the biggest cohort studies involving the PALOP communities that will provide genomic surveillance and an advanced response to the ongoing HIV-1 pandemic. The focus of the MARVEL project is to minimize the dissemination of HIV-1 viral strains with DRM capable of affecting the effectiveness of ARVs, whether for NNRTI, NRTI, PI or INSTI. Monitoring drug resistance in African countries with limited resources in terms of surveillance or individual management of HIV patients is crucial in the fight against HIV-1 dissemination in the PALOP community.

Furthermore, inequalities in access to HIVDR tests in these regions are notable when compared to other countries in different regions of the world and this can significantly impact the profile of the HIV pandemic in these regions. Currently, there are different methodologies recommended by the WHO for resistance surveillance [20]. However, these strategies although commendable, remain difficult to implement in some countries as it is a resource-consuming exercise and many countries, such as the PALOP community, cannot regularly offer large-scale genomic surveillance for drug resistance testing on a routine basis. Consequently, WHO reports on HIVDR often report little or no sharing of genomic surveillance data in resource-limited countries [21]. In these regions, the main challenges continue to be the costs of reagents, investment in ongoing maintenance of equipment and training of local technical skills to maintain laboratory procedures and in-depth bioinformatics analyses.

Currently, there is little published information on HIV-1 transmission dynamics, ADR and TDR, especially in the new context of large-scale use of integrase inhibitors, such as Dolutegravir (DTV) for most countries in the PALOP community. To date, there is only one published study reporting the prevalence of resistance to integrase inhibitors in Angola [22], where previous studies revealed a high genetic diversity driven by non-B subtypes such as C and F1 with a TDR prevalence of around 20% [23–26]. The most recent study conducted in Cape Verde revealed that the HIV-1 epidemic is driven by CRF02_AG and subtype G, with high ADR prevalence observed in NNRTIs (67%), PI (7%), NRTI (55%), and integrase inhibitors (10%) [27]. In Mozambique, the subtype C predominates in all regions with a TDR prevalence of around 15% [28]. HIV-1 epidemic in Sao Tome & Principe has been characterized by the circulation of non-B subtypes, with a predominance of subtypes CRF02_AG, A and G, with scarce data on resistance to ARV drugs [29].

The creation of a Portuguese-speaking scientific network for the genomic monitoring of HIV represents an important collaborative effort to strengthen surveillance and response capacity to HIV-1 in the PALOP. This collaborative network will facilitate the implementation of evidence-based interventions supporting the global goal of ending AIDS as a global health concern by 2030. Furthermore, combining demographic, clinical and genomic information for statistical, bioinformatics and artificial intelligence analysis will enable local public health authorities to guide and optimise ART regimens, enabling patients to achieve viral suppression as well as improving the quality of life for people living with HIV in Africa.

In summary, important challenges remain to be faced to end the HIV/AIDS pandemic, even with universal access to ART that has guaranteed a high success rate in viral suppression at a global level. The emergence of

DRM in resource-limited African countries is a major barrier to achieving larger-scale viral suppression that could compromise the goal of ending HIV/AIDS by 2030. In the context of such resource-limited settings and weak infrastructures, it is important to implement optimized low-cost HIVDR tests, that should be considered in all low-income countries, including the PALOP communities.

Author contributions

Conceptualization, V.P., M.P., and A.B.A.; study design: A.B.A., V.P., and M.P; methodology, C.S.S., T.N., I.PA., S.L., E.B., K.T., and L.L. software, V.P., C.S.S., M.M., and M.P.; validation, V.P., C.S.S, and A.B.A; formal analysis, V.P., C.S.S, and M.R.O.M; investigation, V.P., C.S.S., T.N., P.G., I.PA., S.L., E.B., K.T., L.L., and A.B.A.; resources, V.P., C.S.S., A.B.A.; data curation, V.P., C.S.S., and M.P.; writing—original draft preparation, M.P., A.B.A., C.S.S. and V.P.; writing—review and editing, C.S.S., V.P., M.P., A.B.A.; visualization, V.P. and C.S.S.; supervision, M.P. and A.B.A.; project administration, A.B.A., M.P., V.P.; funding acquisition, V.P., M.P., C.S.S., and A.B.A. All authors have read and agreed to the published version of the manuscript.

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Data availability

Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate

The research team will ensure data protection according to the Helsinki Declaration. The study protocol was submitted to ethical committees of participating healthcare institutions in Angola, Cape Verde, Mozambique, and Sao Tome & Principe. The study participants will receive written information, including the description and the goals of the study, as well as, information about data privacy procedures. Moreover, informed signed consent will be requested from all participants before they are enrolled in the study. All samples stored will be analysed and destroyed no later than December 31, 2044, following current legal and ethical requirements.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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