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Protocols

Hybrid next-generation sequencing protocol for testing HIV-2 drug resistance

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ABSTRACT

HIV-2 affects over 1 million people globally and can lead to AIDS if untreated. Treating people living with HIV-2 (PLHIV-2) is challenging because the virus is inherently resistant to some drugs. Effective treatment monitoring, particularly drug resistance testing, is critical for managing therapeutic failure. Without commercial tests to identify drug resistance mutations (DRM), laboratories have felt the need to develop in-house methods. NGS provides improved sensitivity for detecting minority DRM, which is crucial for effectively treating individuals, especially with limited therapeutic options. This study aimed to evaluate the effectiveness of a hybrid NGS Ion Torrent protocol for the detection of DRM in PLHIV-2 and its use in clinical practice. One hundred samples from PLHIV-2 collected from hospitals across Portugal were analyzed using a hybrid NGS protocol. Of these, 48 samples were also subjected to Sanger sequencing for comparative purposes. NGS successfully amplified 92 % of protease, 91 % of reverse transcriptase, and 49 % of integrase regions. The two sequencing methods agreed on the majority of DRM identified, with the only difference in two samples for the reverse transcriptase, which NGS identified as K70E and M184V, while Sanger did not. Hybrid NGS was able to identify DRM, demonstrating strong statistical agreement. In conclusion, hybrid NGS detected all DRM identified by Sanger, with the added ability to detect minority variants. The implementation of NGS-based protocol can provide clinicians with more comprehensive data, allowing for adjustments to ART regimens, and ultimately improving patient outcomes and quality of care for PLHIV-2.

1. Background

Human immunodeficiency virus type 2 (HIV-2), one of the pathogens of acquired immunodeficiency syndrome (AIDS) (Ferreira et al., 1991), is endemic in West Africa (Barré-Sinoussi et al., 1983; Clavel et al., 1986), with 1–2 million people living with HIV-2 (PLHIV-2) worldwide (Gottlieb et al., 2018). In Europe, Portugal is the country with the highest number of PLHIV-2 due to its colonial links (Gottlieb et al., 2018; Carvalho et al., 2012; Visseaux et al., 2016) accounting for an estimated 2135 cases in 2022 (Instituto Nacional de Saúde, 2023). Although HIV-2 infection progresses more slowly compared to human immunodeficiency virus type 1 (HIV-1), it can still lead to AIDS and death in the absence of antiretroviral therapy (ART) (Sharp and Hahn, 2011; Gottlieb et al., 2006; Peeters et al., 2013; Thiébaut et al., 2011). Development of treatment strategies for HIV-2 is of the utmost importance, yet, available protocols are largely based on those developed for the treatment of HIV-1 (Desbois et al., 2008; APECS, 2023; Moranguinho et al., 2023). To assist clinicians in monitoring PLHIV-2, to effectively treat individuals in

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therapeutic failure, caused by acquired drug resistance (ADR) or transmitted drug resistance (TDR), laboratories have implemented in-house methods for sequencing the regions of the virus targeted by ART, namely, protease (PR), reverse transcriptase (RT), and integrase (IN) (Roquebert et al., 2008; Ntemgwa et al., 2007; Cardoso et al., 2021). WHO recommends drug resistance mutations (DRM) testing for PLHIV-2 in countries with access to ART (WHO, 2023).

To date, the Sanger sequencing has been the primary approach to test for HIV drug resistance (HIVDR). However, the advent of next-generation sequencing (NGS) offers enhanced sensitivity and the ability to detect minority viral populations, it enables the detection of low-frequency antiretroviral resistance mutations by providing millions of reads of the viral genome per run (Stella-Ascariz et al., 2017), which may be important for optimizing ART (Cardoso et al., 2021; WHO, 2023; Stella-Ascariz et al., 2017; Bonifacio et al., 2022; Brandin et al., 2003; Appliedbiosystems, 2020; Lapointe et al., 2015). While Sanger sequencing can identify minority populations above 15–20 %, NGS can detect these populations at levels as low as 1–5 % (Dessilly et al., 2018). A comparative study between Sanger sequencing and NGS demonstrated a high success rate, with NGS detecting a greater proportion of minority variants and exhibiting strong repeatability and reproducibility (Bonifacio et al., 2022).

Thus, developing NGS-based assays for HIV-2 is expected to improve the monitoring of, providing clinicians with more detailed genotyping information to optimize ART regimens. Although NGS sequencing may have higher costs compared to Sanger sequencing in some cases, it proves to be more effective in detecting minority variants, demonstrating its recognized value in the detection of DRM (Bonifacio et al., 2022). In this study, we developed an ART resistance test for HIV-2 by NGS using the Ion Torrent approach. Specifically, we compared the hybrid NGS sequencing with the Sanger sequencing, the gold standard for implementing this analysis in routine clinical laboratory practice.

2. Study design

2.1. Study population and sample collection

One hundred plasma samples from PLHIV-2, from 16 hospitals in Portugal were genotyped using hybrid NGS-Ion Torrent. To compare methodologies, 48 were also sequenced by Sanger. All samples were collected during routine laboratory monitoring of PLHIV-2 with a positive HIV-2 viral load (VL) or those experiencing ART failure.

In the PCR reactions, some of the primers used were previously described by Brandin et al. (2003) and Roquebert et al. (2008), and some were designed by our research team (Supplementary Table 1).

2.2. PCR and sanger sequencing conditions

A total of 48 samples were tested using Sanger sequencing. Samples were extracted from 1 mL of plasma using the EMAG®-BIOMERIEUX automated extractor.

The PR/RT region was amplified by RT-PCR, using the Promega-Access RT-PCR System master mix, to which was added 10 mM of each nucleotide (dATP, dCTP, dGTP, dTTP), 10 μM of primers JA218 and JA221 (Supplementary Table 1), 25 mM of MgSO4 and 5U/μL of the enzymes Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and Thermostable DNA polymerase (Tlf DNA Polymerase) with 5 μL of extracted RNA for a final volume of 50 μL. For cDNA synthesis the conditions used were 48ºC for 45 min; initial denaturation 94ºC for 2 min; 40 amplification cycles (94ºC for 15 s, 54ºC for 30 s, and 72ºC for 30 s), and a final elongation at 72ºC for 5 min. 5 μL of the amplified product was used as a template in the nested PCR using the master mix AmpliTaq GoldTM 360 MasterMix 2x reaction mixture, 10 μM of primers JA219 and JA220 modified (Supplementary Table 1), for a final volume of 50 μL. The amplification conditions were initial denaturation at 94ºC for 5 min; 50 amplification cycles (94ºC for 30 s, 54ºC for 30 s, and 72ºC for 1,30 s), and a final elongation at 72ºC for 7 min.

The IN region was amplified by RT-PCR, using the QIAGEN OneStep RT PCR Kit reaction mixture with 10 mM of nucleotide mixture (dATP, dCTP, dGTP, dTTP), 32.4 μM 1SA NOVO primer, 44.2 μM 1S NOVO primer (Supplementary Table 1), and 8 μL of extracted RNA for a final volume of 50 μL. The amplification conditions were cDNA synthesis 50ºC for 30 min; initial denaturation 95ºC for 3 min; 15 amplification cycles (94ºC for 1 min, 68–54ºC for 1 min, and 72ºC for 1 min), followed by 30 amplification cycles (94ºC for 1 min, 54ºC for 1 min, and 72ºC for 1 min) and final elongation at 72ºC for 7 min. 2 μL of PCR product was used as a template for two nested PCR. For one of the PCR, the mix used was: Taq DNA polymerase recombinant (5 U/μL) Thermo Scientific™ for a final volume of 50 μL, magnesium-free buffer used, with 1.5 mM magnesium and 100 mM of each nucleotide (dATP, dCTP, dGTP, dTTP), 10 μM of the primers FW and ISF (Supplementary Table 1), and 5U/l of Taq DNA polymerase enzyme. The PCR profile consisted of denaturation at 95ºC for 3 min; 45 amplification cycles (95ºC for 45 s, 46ºC for 30 s, and 72ºC for 1,30 s), and final elongation at 72ºC for 7 min. For the other nested PCR, it was used the same PCR mix, and the 1S NOVO (44.2 μM) and IF (37.9 μM) primers (Supplementary Table 1), 2.5 mM magnesium with 2 μL of the first PCR. The PCR profile consisted of denaturation at 95ºC for 3 min; 45 amplification cycles (95ºC for 45 s, 54ºC for 30 s, and 72ºC for 1,30 s), and final elongation at 72ºC for 7 min.

To verify the amplified products, electrophoresis was performed on a 1 % agarose gel. The products were purified using the PCR Clean-up Kit (Appliedbiosystems) (Appliedbiosystems, 2020) and then sequenced.

For PR/RT Sanger sequencing reaction 5 μL of RNase and DNase-free water was added to 2 μL of BigDye™ Terminator v2. 1 Cycle Sequencing Kit (Thermo Fisher Scientific), 1 μL of each 10 μM JA219, JA220, JA222, JA223, HIV-2 FW and HIV-2 RV primers (Supplementary Table 1) and 2 μL of purified and diluted PCR product, the amplification profile was initial denaturation at 95ºC for 5 min; and 35 amplification cycles (95ºC for 10 s, 50ºC for 5 s, and 60ºC for 4 min).

For IN Sanger sequencing, the following reaction was used: 1.5 μL of 5x Buffer, 6 μL of RNase and DNase free water, with 1 μL of BigDyeTM v2. 1 Cycle Sequencing Kit, 1 μL of purified PCR product and 0.5 μL of each primer (10 μM) (Supplementary Table 1). Simultaneously, two amplification reactions were done. One reaction with initial denaturation at 95ºC for 5 min and 35 amplification cycles (95ºC for 10 s, 46ºC for 5 s, and 60ºC for 4 min) using FW, 2SA NOVO, and ISF primers. For the other reaction using IF, 1S Novo, Backup FW, and Backup RV primers (Supplementary Table 1), the amplification profile was initial denaturation at 95ºC for 5 min and 35 amplification cycles (95ºC for 10 s, 54ºC for 5 s, and 60ºC for 4 min). Amplification reactions were carried out in a GeneAmp* PCR System 9700 thermal cycler.

Sanger sequencing took place in the 3130xl Genetic Analyser (Applied Biosystems®). The electropherograms obtained were edited in the ChromasPro v.2.1.10 software and FASTA consensus sequences were submitted at http://www.hiv-grade.de/HIV2EU/deployed/grade.pl?program=hivalg).

2.3. PCR and hybrid NGS (Ion Torrent) conditions

For the hybrid NGS (Ion Torrent) protocol, samples were extracted from 730 μL of plasma using the Sentosa® platform, as indicated by the manufacturer (VELA Diagnostics®).

For the amplification reaction, the following primers pools were used: 10 μM JA218, JA221 (pool 1) JA219, JA220 modified (pool 2), 1S NOVO, 1SA NOVO (pool 3), Backup FW, and Backup RV (pool 4) (Supplementary Table 1). PCR protocol was cDNA synthesis at 50ºC for 30 min; initial denaturation at 98ºC for 2 min; 40 amplification cycles (98ºC for 15 s, 55ºC for 30 s, and 72ºC for 1 min), and final elongation at 72ºC for 2 min. The amplification was carried out in a Veriti 96 well thermal cycler - Applied Biosystems, resulting in DNA fragments of 1300 bp.

The library was generated using this amplification product and a

restriction enzyme that cleaves the 1300 pb fragments into 200 bp fragments. Unique molecular barcode adapters were then attached to each sample. The library containing all samples was pooled and the adapters served as primers during emulsion PCR. In the presence of microspheres with complementary sequences, millions of copies of each of these fragments were generated. For sequencing, the microspheres containing the DNA fragments were placed into a chip, with each microsphere occupying a microwell. During the sequencing process, nucleotides were added one by one, and upon binding, a hydrogen ion was released, causing a pH change detected by a sensor. The sequencing generated an average of 142,178 reads per sample. This high read depth contributes to the robustness of the sequencing process, allowing for reliable detection of minority variants. The Ion Reporter™ Software 5.6 then generated a BAM file, which was analyzed using bioinformatics tools such as Samtools (available at http://www.htslib.org/doc/samtools-fasta.html) and Genome Detective (available at https://www.genomedetective.com/), a FASTA consensus sequence was obtained and then interpreted in the HIV2EU Grade software (available at http://www.hiv-grade.de/HIV2EU/deployed/grade.pl?program=hivalg).

2.4. Statistical analysis

SPSS v27 statistical software was used to analyse the data. Descriptive statistics were presented as absolute numbers (N), relative frequencies (%), and central tendencies (median and IQR). The Shapiro-Wilk test was used to test the normal distribution of data, hypotheses were tested using the chi-squared test (X^2) for variables with normal distribution, Fisher's exact test when chi-squared assumptions were not met, and the Mann-Whitney *U* test for variables without normal distribution. Cohen's kappa coefficient of the agreement was used to compare the agreement between DRM in Sanger and hybrid NGS. For statistical analysis, we considered a 5 % significance level.

3. Results

The study included 65 women and 35 men. Of these, 65 were treated with ART and 35 had never used ART. The median age at the time of resistance testing was 58 years, IQR (Arteche-López et al., 2021, Storto et al., 2018, Novitsky et al., 2023, Novitsky et al., 2022), all individuals had HIV-2 viral RNA > 40 copies/mL (>1.60 log10), the median VL was 3.47 log10 (IQR 2.66–4.30). 34/100 samples had a VL< 1000 copies/mL (<3.00 log10). All were HIV-2 group A.

Only 11 % of the PLHIV-2 in the study were originally from Portugal, with the majority coming from Guinea-Bissau (48 %) and Cape Verde (14 %), followed by Angola (3 %), Senegal and São Tomé & Principe (1 %). The geographical origin of 22 % of the individuals could not be determined.

Statistically, there were no significant differences between sex, age, geographical origin, and VL between treated and untreated PLHIV-2. Characterisation of the 100 individuals studied is shown in Supplementary Table 2.

According to Sanger, the amplification success rates were 92 % (44/48) for both PR and RT and 78 % (28/36) for the IN region. The DRM identified in the PR region included V47A (4,55 %, 1/22), I50V (18,18 %, 4/22), I54M (36,36 %, 8/22), I82F (9,09 %, 2/22), I84V (18,18 %, 4/22), L90M (27,73 %, 5/22). In the RT region, K65R (36,36 %, 8/22), Q151M (27,27 %, 6/22), M184V/I (36,36 %, 8/22), and V111I polymorphism (59,09 %, 13/22) were detected. For the IN region, the DRM E92Q (22,72 %, 5/22), T97A (36,36 %, 8/22), and N155H (31,81 %, 7/22) were identified. Overall, this method detected resistance mutations in 46 % (22/48) of the specimens.

In the population tested using hybrid NGS (n = 100), amplification success rates were 92 % (92/100) for the PR region, 91 % (91/100) for the RT region, and 49 % (49/100) for the IN region. Only one sample (1 %, 1/100) failed to amplify any of the *pol* gene regions. In this group, DRM was identified in 44 % (44/100) of samples, all of which belonged to PLHIV-2 on ART. In PLHIV-2 without ART, TDR was not detected. The DRM identified in the PR region were V47A (6,81 %, 3/44), I50V (38,63 %, 17/44), I54M (31,81 % 14/44), I82F (11,36 %, 5/44), I84V (11,36 %, 5/44), and L90M (29,54 %, 13/44). In the RT region, mutations were K65R (45,45 %, 20/44), K70E (2,27 %, 1/44), Q151M (25,00 %, 11/44), M184V/I (56,81 %, 25/44) and the V111I polymorphism appeared in 63,63 % (28/44). For the IN region E92A/Q (18,18 % 8/44), T97A (31,81 % 14/44), and N155H (18,18 %, 8/44) were detected. The RT region exhibited the highest proportion of DRM (44.4 %), followed by the PR region (33.3 %) and the IN region (22.3 %).

When comparing the amplification rates for the 48 samples tested simultaneously using both Sanger and hybrid NGS sequencing, the PR and RT regions had the same success rate of 85 % for both methods. However, Sanger showed a higher amplification rate in the IN region compared to NGS (58 % vs. 54 %).

The identification of DRM in the three *pol* gene regions was concordant between both methodologies, except for the RT region. In this region, the hybrid NGS (Ion Torrent) detected K70E and M184V mutations in two samples that were not identified by Sanger sequencing (Fig. 1). Statistical analysis of resistance mutations using Cohen's Kappa coefficient of agreement showed perfect agreement (Kappa = 1, $p < 0001$) for the PR and IN regions, and Kappa 0.946, $p < 0.001$ for the RT region.

4. Discussion

This study aimed to evaluate the effectiveness of a hybrid next-generation sequencing (NGS) protocol using Ion Torrent technology for detecting DRM in PLHIV-2 and assessing its applicability in clinical practice.

This study found no statistically significant differences between treated and untreated PLHIV-2 groups in terms of sex ($p = 0.323$), age ($p = 0.691$), geographical origin ($p = 0.300$), or VL ($p = 0.289$). However, a significant difference in DRM was observed between PLHIV-2 on ART (44 %) and those not on ART (0 %), with a $p < 0.001$.

In the context of public health viral surveillance, NGS has emerged as a powerful tool. Studying HIVDR through sequencing is crucial in optimizing HIV/AIDS treatment strategies. Sequencing of mutations in the RT, PR, and INT genes is recommended in various clinical scenarios (APECS, 2023). However, commercially drug-resistant HIV-2 tests are currently unavailable. Laboratories in countries like Portugal, which has the highest number of HIV-2 cases in Europe (2135) (Instituto Nacional de Saúde, 2023), have developed in-house methodologies to enhance the timely diagnosis and treatment for this often-neglected infection. Though HIV-2 has received less attention than HIV-1, it can still progress to AIDS and death.

For the first time, we present a hybrid HIVDR test for HIV-2 using NGS (Ion Torrent). This method allowed us to successfully amplify the PR, RT, and IN regions with success rates of 92 %, 91 %, and 49 %, respectively. Our PR and RT data align with the work of Italian researchers, who achieved an 87 % success rate using a validated kit (Bonifacio et al., 2022). Some studies suggest that VL < 1000 copies/mL is linked to amplification success, and in this study, 34 % (34/100) of samples had VL < 1000 copies/mL (Lapointe et al., 2015).

The relatively lower amplification rate of the IN region points to the need for further protocol optimization, consistent with findings from other authors (Dessilly et al., 2018; Arteche-López et al., 2021). Moreover, when comparing Sanger sequencing and hybrid NGS, we identified the K70E and M184V mutations in two samples via hybrid NGS, which were not detected by Sanger. This was likely because NGS can detect minority variants as low as 5 %, whereas Sanger's detection threshold is 15–20 % (Storto et al., 2018; Novitsky et al., 2023, 2022). These findings in the RT region of the pol gene align with existing literature and may lead to more targeted and effective therapies for people living with HIV-2.

Fig. 1. A. Comparison of amplification success rates between Sanger and NGS. B. Detected mutations in the PR region by Sanger/NGS. C. Detected mutations in the RT region by Sanger/NGS. D. Detected mutations in the IN region by Sanger/NGS.

Our method has two main limitations, the difficulty in determining the percentages of minority populations and a lower amplification success rate for the integrase region. In our study, the integrase region showed a relatively low amplification success rate of 49 %, in contrast to the higher rates observed for the protease (92 %) and reverse transcriptase (91 %) regions. This notable discrepancy led to further investigation into the factors causing the lower success rate for the integrase region. In response, we developed and tested new primers to improve amplification efficiency, specifically targeting conserved regions of the integrase gene to address issues of primer binding and specificity. Despite these efforts, the amplification success rate for the integrase region remains below the desired threshold, highlighting the need for further optimisation.

In addition, a major limitation of our study is the inability to accurately quantify the relative frequencies of detected mutations within the viral population. Although the NGS protocol used in this study effectively identifies minority variants, our analysis did not extend to determining the exact proportion of the viral population harboring each mutation due to the limitations of current bioinformatics tools. We were limited to a 5 % cut-off. Future advances in bioinformatics will be critical in overcoming this limitation.

In our study, samples were collected from hospitals in our country and former Portuguese colonies in Africa, where HIV-2 Group A is predominant. We did not have access to samples from Group B, which reflects the epidemiological distribution of HIV-2 groups in these regions. Although Group A has a broader global distribution compared to other HIV-2 groups, there is a need for further validation of the protocol in more genetically diverse populations, particularly those with a higher prevalence of Group B. To ensure the broader applicability of this protocol, future studies should include samples from HIV-2 Group B.

Despite these limitations, hybrid NGS (Ion Torrent) was able to identify all DRM previously detected by Sanger sequencing, demonstrating strong statistical agreement between the two methodologies (Cohen's Kappa coefficient ranging from 0.946 to 1). This shows that hybrid NGS can be used for tracking and monitoring DRM in routine laboratories, although further improvements are still necessary.

In conclusion, hybrid NGS (Ion Torrent) sequencing successfully detected all DRM identified by Sanger sequencing, with the added capacity to detect minority variants. While this technological advancement shows promise, its direct impact on therapeutic decisions and clinical outcomes for individuals with HIV-2 remains to be further validated in clinical practice.

Ethics statement

The study was conducted by the Declaration: of Helsinki, and approved by the ethical committee of Centro Hospitalar de Lisboa Ocidental (CHLO) (60/CES-2020; 18 May 2020).

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CRediT authorship contribution statement

Costa Inês: Writing – review & editing. **Cabanas Joaquim:** Writing – review & editing. **Pimentel Victor:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Pingarilho Marta:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Gomes Perpétua:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gonçalves Fátima:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abecasis Ana:** Writing – review & editing. **Fernandes Sandra:** Writing – review & editing. **Ribeiro Marta:** Writing – review & editing, Writing – original draft, Validation, Data curation. **Sebastião Cruz S.:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Diogo Isabel:** Writing – review & editing. **Veloso Margarida:** Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest relevant to this work.

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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.2025.115112.

Data availability

Data will be made available on request.

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