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Routine drug resistance testing in HIV-1 proviral DNA, using an automated next- generation sequencing assay

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ABSTRACT

Background: HIV-1 DNA genotypic drug resistance testing is increasingly performed to guide treatment switching or simplification in controlled patients. The Sentosa NGS platform is a fully automated system marketed for drug resistance testing on HIV-1 RNA samples.
Objectives: The aim of this study was to evaluate this automated NGS solution for routine resistance genotypic resistance testing in proviral HIV-1 DNA.
Study design: Sanger sequencing (SS) of the reverse transcriptase (RT), protease (PR) and integrase (IN) genes was performed using the French ANRS protocol. NGS was performed retrospectively on frozen samples, using the Sentosa platform combined with the Sentosa SQ HIV genotyping Assay.
Results: A total of 77 samples were run once using NGS. A successful sequencing of the three HIV-1 genes (RT, PR, IN) was obtained for 45 samples. The number of cumulated RAMs was 179, 185 and 219 with SS, NGS 20% and NGS 10% respectively; however most of them were minor mutations in the PR region. The mutation detection rate was similar between SS and NGS 20%. Several discordances were observed between both methods in the RT and PR regions, mainly due to the use of different DNA extracts, and hypermutation.
Conclusions: HIV-1 DNA genotypic resistance testing can be performed with the Sentosa platform. Few technical optimizations are still needed to include the extraction step and to improve the sequencing efficiency.

1. Background

HIV-1 drug resistance testing is increasingly performed by sequencing of HIV-1 DNA to guide clinicians for treatment switching or simplification in controlled patients without available previous plasma resistance tests, or in patients who experienced virological failure with very low viral load and unsuccessful HIV-1 RNA sequencing [1–4]. However, this approach was reported to be less sensitive as compared to historical plasma HIV RNA genotypes in treated patients [5,6], and is sometimes biased by interference of defective proviruses [7,8]. Sanger bulk sequencing is the most common method used for HIV genotypic drug resistance testing in clinical settings. This approach can detect variants higher than 20%, and is not able to determine whether resistance associated mutations (RAMs) are harbored by defective proviruses [9,10]. Next generation sequencing (NGS) methods with appropriate bioinformatic tools could overcome these issues. In most of clinical laboratories, the SS is performed “manually”, using published or national protocols. Even if technical teams master this method, it can be time-consuming and error-prone, with the need of thorough optimization for laboratory accreditation purpose.

The Sentosa NGS platform combined with the Sentosa SQ HIV genotyping Assay (Vela Diagnostics) is a fully automated system marketed for HIV-1 resistance testing on plasma samples (http://www.veladx.com/HIV.html). The aim of this study was to evaluate this automated NGS solution for routine genotypic resistance testing in proviral HIV-1 DNA.

2. Materials and methods

2.1. Patients and samples

This study included 77 HIV-1 infected patients followed at the Infectious Diseases University Department of Tourcoing Hospital (North of France). All subjects have signed the ethics board-approved informed consent form of the HIV Nadis® electronic patient record database (www.nadis.fr). Patients with undetectable plasma VL, who underwent

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proviral DNA genotypic resistance testing, with a whole blood sample available (between 2013 and 2017), were included in the study. Resistance analysis through DNA Sanger sequencing of the RT, PR and IN genes was performed as part of routine laboratory monitoring of these patients. DNA next-generation sequencing was performed retrospectively on frozen samples. Demographics and clinical data of patients were retrospectively collected from the Nadis® database.

2.2. Nucleic acid extraction

Total DNA was extracted from 400 µl of whole blood using Nucleospin Blood kit (Macherey Nagel, Hoerdt, France).

2.3. Viral load (VL) quantification

HIV-1 RNA VL determination was done in routine with the Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 (Roche Diagnostics) with a limit of detection/quantification of 20 copies/mL.

The quantification of total HIV-1 DNA was performed in whole blood using a real-time PCR assay (Biocentric, Bandol, France), as previously described [11]. DNA extracts from whole blood were tested in triplicate. The real-time PCR results were expressed as copies/10^6 leucocytes and then converted in copies/10^6 PBMCs, using the white blood cell differential count.

2.4. Sanger sequencing

Sanger bulk sequencing was performed using primers and protocols provided by the French National Agency for AIDS Research (ANRS, http://www.hivfrenchresistance.org) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific). The sequences were purified with the BigDye XTerminator Purification Kit (Thermo Fischer Scientific). Sequencing products were analyzed on a 3500Dx genetic analyser (Thermo Fischer Scientific).

Detection of hypermutation was performed with the Hypermut 2.0 tool using default settings (https://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html).

2.5. Automated next generation HIV-1 DNA sequencing

The integrated workflow includes the automated Sentosa NGS platform (robotic systems and instruments for sequencing); the Sentosa SQ HIV genotyping assay comprising all kits for the sequencing of HIV-1 protease (PR), reverse transcriptase (RT), and integrase (IN) regions; and the software (SQ reporter) for data analysis and reporting.

The assay processes simultaneously 15 samples and a positive control in each run. In the initial workflow, the first step of the assay is the extraction of RNA from plasma samples and RT–PCR preparation on the Sentosa SX 101 instrument. This step was slightly modified for DNA samples. Briefly 60 µL of DNA extracts were loaded in sample tubes, and a special DNA program allowed skipping the extraction step and directly distributes the extracts into the PCR plate. The following steps were conducted similarly to RNA samples. After UDG treatment, PR/RT and IN coding regions were amplified on a Veriti thermal cycler using 4 primer pools. The 1500 bp-length PR/RT amplicon includes PR codons 1–99 and RT codons 1–411, while the IN amplicon covers codons 1–280 (~1000 bp). A 200-nucleotide fragment library was prepared on a second dedicated Sentosa SX 101 instrument (normalization, shearing, purification and ligation). Thereafter, emulsion PCR and enrichment were done on SX 401i and SX 401e instruments respectively. Sequencing was performed using the Sentosa SQ 301 system (Ion PGM DX) with Sentosa Sequencing kit (200bp) and Ion 318 Chip. Ion semiconductor sequencing is based on the detection of hydrogen ions that are released during the polymerization of DNA. A primary data analysis was done on the SQ 301 Server followed by a secondary analysis with the SQ reporter (quality control and alignment). In this study, the mutation analysis was not performed with the SQ reporter because it did not allow choosing the cut-off for variant calling, and the ANRS algorithm was not updated.

2.6. Mutation analysis and identification of resistance associated mutations

Sanger electropherograms were transferred to the cloud-based SmartGene IDNS® HIV-1 Module version 3.8 (SmartGene, Zug, Switzerland) for editing and mutation analysis (via pair-wise alignment against HXB2). For NGS data analysis, FASTQ files were extracted from the Vela System and transferred to the SmartGene ASP-IDNS®-5 pipeline for NGS data using the "Provirus Pipeline" version 2.0.5_HIV1_v1.4, which works as follows: briefly, the original FASTQ files produced by the sequencer were uploaded, automatically filtered for low quality stretches, which cannot be mapped. Remaining read sections were mapped against HIV-1 provirus profiles for PR, RT and IN. Relevant events, such as mutations, stop-codons and changes of reading-frame were detected and reported in a quantitative manner (% of reads aligned). The RAMs in the three HIV-1 regions were identified using the ANRS algorithm (version 27-2017-09) embedded on the platform, at an interpretation cut-off of 10 and 20 % with a minimum coverage of 50 reads.

2.7. Statistical analysis

Patients' characteristics were presented as median with interquartiles (IQR), mean with standard deviations or as percentages. Comparisons between continuous data were performed using t-test. $p < 0.05$ was considered significant. All calculations were performed with GraphPad Prism v6 software (La Jolla, CA, USA).

3. Results

3.1. Patients and sequences

A total of 77 samples were run once using NGS. A complete or partial sequencing failure (insufficient sequence length, low coverage for one or several regions) was observed in 32 samples. An optimal sequencing for the three HIV-1 regions (RT, PR, IN) with both Sanger and next-generation sequencing methods was obtained in 45 samples. The median HIV-1 DNA VL was 3.3 log copies /10^6 PBMCs (range from 2.80 to 3.96 log copies /10^6 PBMCs) in these samples, and was similar to that in samples with sequencing failure (median: 3.29 log, range from 2.85 to 4.10 log copies /10^6 PBMCs) ($p = 0.84$). Only the 45 samples are included in the following analysis. The patients' characteristics are described in Table 1. The median age was 49 years old and patients were mainly male (68.9%). They were mainly infected by a subtype B virus (66.7%) since 14.8 years and treated for a median period of 10.8 years. At the time of sampling, CD4 count and CD4 nadir were 643 and 177 cells/mm^3 respectively.

Table 1
Patient characteristics.

Patient characteristics	Median (IQR)	Percentage
Age (years)	49 (41.1 – 56.4)	
Sex (% of male)		68.9
HIV-1 subtype (% of subtype B)		66.7
Time since HIV-1 diagnosis (years)	14.8 (7.9 – 20.5)	
Time since HIV-1 ART initiation (years)	10.8 (5.6 – 17.9)	
Time since undetectable plasma HIV-1 RNA (months)	13.6 (0 – 45.2)	
HIV-1 DNA VL (log10 copies/10^6 PBMCs)	3.3 (3.1– 3.4)	
CD4 cell count (cells/mL)	643 (481 – 818.5)	
CD4 nadir (cells/mL)	177 (108 – 293.5)	

Table 2
Comparison of RAMs detection rate between SS, and NGS 20 and 10%.

	Total number of RAMs (mean +/- SD per sample)			p value	
	SS	NGS 20%	NGS 10%	SS vs NGS 20%	SS vs NGS 10%
RT (n = 45)	41 (0.91 +/- 1.26)	43 (0.96 +/- 1.38)	63 (1.40 +/- 2.00)	0.76	0.08
PR (n = 45)	131 (2.91 +/- 1.59)	136 (3.00 +/- 1.81)	146 (3.24 +/- 1.89)	0.62	0.10
IN (n = 45)	7 (0.16 +/- 0.42)	7 (0.16 +/- 0.42)	10 (0.22 +/- 0.56)	1	0.18

RAMs: Resistance associated mutations; SS: Sanger sequencing; NGS: Next-generation sequencing; SD: standard deviation.

A good sequencing quality and coverage was obtained for Sanger sequences. Five RT sequences were hypermutated. No hypermutation was detected in PR and IN sequences. For NGS, the median total number of reads was 12,821 per sample, and the average length of reads was 156. The median number of reads per sample and per nucleotide position was 1683 and 3048 for PR/RT and IN regions respectively.

3.2. Detection of RAMs by SS and NGS

The number of cumulated RAMs was 179, 185 and 219 with SS, NGS 20% and NGS 10% respectively. The mean number of RAMs detected in each region is summarized in Table 2.

3.2.1. Mutations associated with resistance to reverse transcriptase inhibitors (RTIs)

The total number of RAMs in the RT region was 41, 43 and 63 with SS, NGS 20% and NGS 10% respectively. At least one RT mutation was detected in 23 patients with SS and NGS 20%, and 25 patients with NGS 10%. The detail of RAMs is shown in Fig. 1. The most common RAMs (detected in 2 patients whatever the method used) include positions 41, 70, 184, 210 and 215 for nucleoside/nucleotide RTIs (NRTIs), and positions 90, 98, 103, 138, 179 and 230 for non-nucleoside/nucleotide RTIs (NNRTIs).

3.2.2. Mutations associated with resistance to protease inhibitors (PRIs)

In the PR region, the number of RAMs was 131, 135 and 146 with SS, NGS 20% and NGS 10% respectively. Most of mutations were minor mutations. At least one PR mutation was detected in almost all patients (43 patients). The mutations detected in at least 5 patients whatever the method, include positions 10, 15, 16, 20, 36, 63, 69, 71, 77 and 89 as detailed in Fig. 1.

3.2.3. Mutations associated with resistance to integrase inhibitors (INIs)

Fewer mutations were detected in the IN region (7, 7 and 10 with SS, NGS 20% and NGS 10% respectively). IN RAMs were detected in only 5 patients with SS and NGS 20%, and were observed only at positions 74 and 97. NGS 10% allowed detection of two additional mutations (positions 138 and 263) in a sixth patient.

3.3. Analysis of discordances between SS and NGS 20%

It's generally admitted that the variant detection limit of SS is around 20%; we then compared the RAMs found with SS and NGS 20%. The mutation detection rate is similar in the 3 regions (see Table 2). However, several discordances regarding the detection of RAMs were found between SS and NGS. A plausibility check was performed via pair-wise or multiple alignments between the sequences obtained with SS and NGS using the "search similar sequences" tool of the SmartGene HIV-1 Module.

Overall, NGS 20% detected 33 of the 41 (80.5%) mutations found by SS in the RT region. However, the 8 mutations reported by SS and not by NGS 20% were harbored by hypermutated sequences and included 2 E138 K, 3 M184I and 3 M230I mutations. Four of these mutations (1E138 K, 2 M184I and 1 M230I) were found by NGS at prevalence lower than 10%. An example of discordant result is shown in Fig. 2. After hypermutation cleaning, NGS 20% detected all the 28 mutations (100%) reported by SS. NGS 20% allowed detection of 10 additional mutations, not reported by SS, at positions 41, 44, 69, 101, 103 and 215.

In the PR region, 120 of the 131 mutations (91.6%) reported by Sanger were detected by NGS 20%. Other 15 additional mutations were

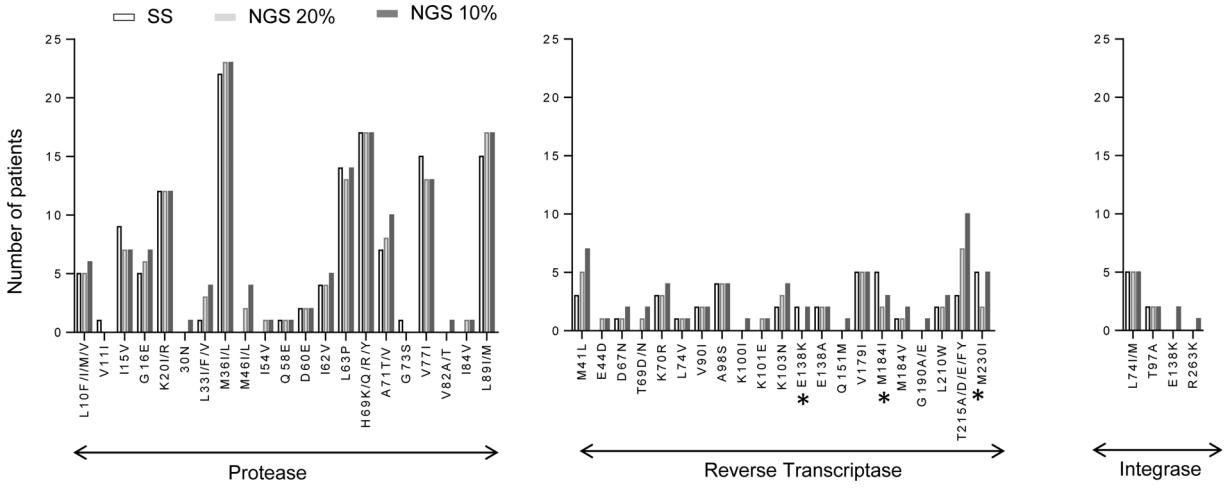

Fig. 1. List of resistance associated mutations detected by SS, NGS 20% and NGS 10%.
The resistance associated mutations detected in patients according to the ANRS list (2017) were presented. The number of patients with the different methods is shown for each mutation associated with resistance to protease inhibitors, reverse transcriptase inhibitors and integrase inhibitors. SS: Sanger sequencing; NGS: Next-generation sequencing
* Mutations associated with hypermutated DNA sequences

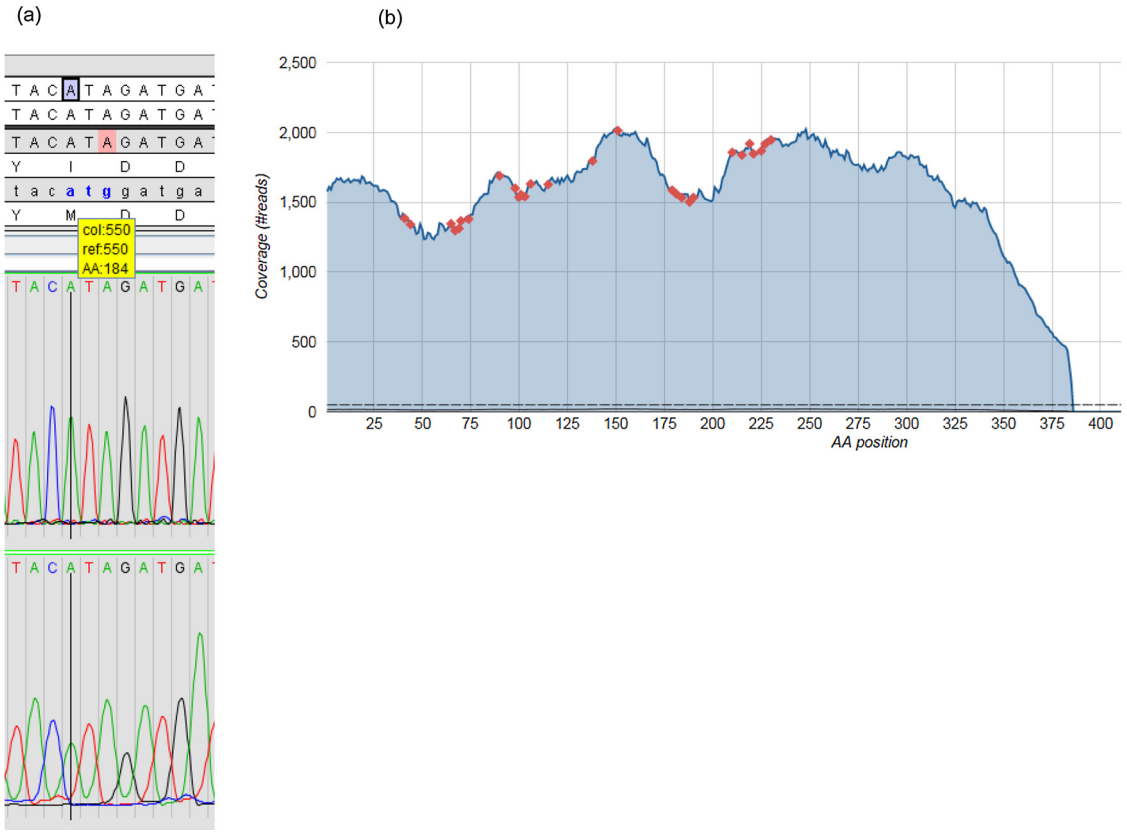

Fig. 2. Example of discordant SS and NGS results.
This is an example of RT sequence with a discordance at position 184.
(a) SS electrophoregram showing a M184I mutation; (b) NGS data with critical positions shown in red. No mutation observed at AA position 184.

found by NGS 20%.

No discordance was observed between SS and NG 20% regarding the IN region

4. Discussion

We assessed in this study the feasibility in routine of a proviral HIV-1 DNA genotypic resistance testing using the commercial and automated Sentosa NGS system.

HIV-1 genotypic resistance testing is currently routinely performed in most clinical laboratories with SS, and results provided by this approach have been shown to be highly reproducible and interpretable, and sufficient in most of cases to guide the clinician's prescription. However the preparation steps in this method are mainly manual and automation is desirable to avoid potential errors and to reduce hands-on time.

The implementation of NGS technology in clinical HIV laboratories is surely not an urgent need because the impact of minority variants on treatment outcomes has only been shown for first generation NNRTI drugs (nevirapine and efavirenz) [12,13].

The Sentosa HIV-1 assay is a highly automated NGS system with a very attractive workflow, suitable for an easier laboratory accreditation process. In addition, it offers the possibility to have the information about mutations below the 20% cut-off for particular clinical situations. The Sentosa platform was initially designed and marketed (CE-IVD) for HIV-1 drug resistance testing on plasma samples (http://www.veladx.com/HIV.html). This platform is already implemented in routine in few clinical laboratories.

Since HIV-1 resistance testing is now also performed on proviral DNA, it's desirable for the routine sequencing method to be runnable on both RNA and DNA samples.

In this proviral HIV-1 DNA study, we obtained with the Sentosa platform, an optimal sequencing data for the 3 HIV-1 regions in 58.4% of samples, and then a retesting should be needed in routine conditions for more than 40% of samples. Sequencing failure was not found to be associated with HIV-1 DNA levels. This rate needs to be reduced, especially through optimization of the extraction and sequencing workflow. The reagent cost per sample remains too high to allow a high rate of retesting in routine.

In this report, the DNA extraction was performed manually and added to the "plasma RNA" workflow. The improvement should include an integrated and automated DNA extraction from whole blood samples.

We compared the results obtained by SS and NGS with the 20% cut-off, and found a similar RAM detection rate between SS and NGS 20%. However, several discordances were observed especially in RT and PR regions. The most important reason for these discordances is the fact that both sequencing methods were performed on different DNA extracts from the same sample. Indeed, the distribution of HIV-1 variants in cells is not homogenous, following a Poisson distribution [14,15].

In addition, the discordances in the RT region were mainly associated with hypermutations. The Sentosa NGS pipeline reported unexpectedly less hypermutation associated RAMs. This observation needs to be confirmed on a larger sample to know whether this underrepresentation is associated or not with the pipeline, primarily designed for RNA samples.

These discordances are thought to be related to the DNA sample. Prior evaluation performed in our laboratory on plasma RNA samples, showed a perfect agreement in RAM detection between SS and the Sentosa NGS 20%. Moreover two published studies, assessing the Sentosa platform reported excellent concordance with SS on plasma samples [16,17].

The Sentosa platform is currently used in routine in our laboratory for plasma samples with very good results. A sequencing failure of any region occurs in less than 10% of samples, mainly those with a viral load below 500 copies/mL, and retesting is usually performed by SS (personal data). We currently provide resistance testing results to the clinician with a cut-off of 20%, since no consensus is available for minority variants.

In conclusion, HIV-1 DNA genotypic resistance testing can be performed with the Sentosa solution. Few technical optimizations are still needed to include the extraction step and to improve the sequencing performance. This NGS solution could thus be used in routine for both RNA and DNA samples, taking advantage of the automation (reduced hands-on time). However this solution can currently only be cost-effective in clinical laboratories, with a large number of samples (around 1000 tests per year).

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CRediT authorship contribution statement

Enagnon Kazali Alidjinou: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Pauline Coulon:** Methodology, Writing - review & editing. **Christophe Hallaert:** Methodology, Writing - review & editing. **Olivier Robineau:** Investigation, Writing - review & editing. **Agnès Meybeck:** Investigation, Writing - review & editing. **Thomas Huleux:** Investigation, Writing - review & editing. **Faiza Ajana:** Investigation, Writing - review & editing. **Didier Hober:** Supervision, Writing - review & editing. **Laurence Bocket:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors have no conflict of interest regarding this study

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