Comparison of ultra-deep versus Sanger sequencing detection of minority mutations on the HIV-1 drug resistance interpretations after virological failure

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Objective: Drug-resistance mutations are routinely detected using standard Sanger sequencing, which does not detect minor variants with a frequency below 20%. The impact of detecting minor variants generated by ultra-deep sequencing (UDS) on HIV drug-resistance interpretations has not yet been studied.

Design: Fifty HIV-1 patients who experienced virological failure were included in this retrospective study.

Methods: The HIV-1 UDS protocol allowed the detection and quantification of HIV-1 protease and reverse transcriptase variants related to genotypes A, B, C, F and G. DeepChek-HIV simplified drug-resistance interpretation software was used to compare Sanger sequencing and UDS.

Results: The total time required for the UDS protocol was found to be approximately three times longer than Sanger sequencing with equivalent reagent costs. UDS detected all of the mutations found by population sequencing and identified additional resistance variants in all patients. An analysis of drug resistance revealed a total of 643 and 224 clinically relevant mutations by UDS and Sanger sequencing, respectively. Three resistance mutations with more than 20% prevalence were detected solely by UDS: A98S (23%), E138A (21%) and V179I (25%). A significant difference in the drug-resistance interpretations for 19 antiretroviral drugs was observed between the UDS and Sanger sequencing methods. Y181C and T215Y were the most frequent mutations associated with interpretation differences.

Conclusion: A combination of UDS and DeepChek software for the interpretation of drug resistance results would help clinicians provide suitable treatments. A cut-off of 1% allowed a better characterization of the viral population by identifying additional resistance mutations and improving the drug-resistance interpretation.

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Introduction

Current genotypic drug-resistance tests utilize the Sanger sequencing method. Although this clinical method has been validated for genotypic resistance determination, it is generally limited to the detection of variants with greater than 20% prevalence [1–3]. Several studies have clearly demonstrated that patients with mutation rates

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between 1 and 20%, particularly for nonnucleoside reverse transcriptase inhibitors (NNRTIs), are more likely to fail therapy [4–9]. It is therefore important to detect minor variants that occur below 20% frequency using a robust and sensitive method available at an affordable price and providing an easier interpretation for HIV drug-resistance monitoring.

Minority variant detection and drug-resistance monitoring using UDS were successfully performed within the population of HIV-1 [10–12]. The introduction of new antiretroviral treatments has improved patient prognosis. However, treatment failure of the new drugs has been reported with a low genetic barrier both in randomized trials and in clinical settings [12–14]. Several genotypic and drug-resistance interpretation algorithms are available online and have been demonstrated to significantly predict the virological response in retrospective analyses; the French National Agency for AIDS Research (ANRS), Stanford HIV RT and Protease Sequence Database (HIVdb), Rega institute (Rega) are the most highly used algorithms [15,16]. Because different interpretation algorithms use different rules to predict drug susceptibility, results may differ between these methods [17].

The first aim of this study was to evaluate UDS, performed using the GS Junior (Roche 454 Life Sciences Branford, Connecticut, USA), with a new protocol designed to detect and quantify minor and major variants of protease (Pro) and reverse transcriptase of HIV-1 genotypes A, B, C, F and G. The second aim was to compare the three most frequently used publicly available algorithms (ANRS, HIVdb and Rega). Finally, UDS resistance data generated with DeepChek-HIV software, which allows a simplified interpretation of drug resistance, using two sensitivity levels ($\geq$1 and $\geq$20%), were compared. This study focused on NNRTI mutations, but the data were also used to search for NRTI and protease inhibitor mutations.

Materials and methods

Patients

In this retrospective study, during 2007 until 2012, 58 patients with virological failure were selected according to the analysis of our viral load measurements database. Samples were collected from patients attending the Alphabio laboratory (Marseille, France) for HIV infection diagnosis or monitoring. The patients were diagnosed with HIV-1 infections. Patients received a triple therapy with a first-line treatment of tenofovir combined with efavirenz, nevirapine or etravirine. The patients were followed for a median of 46.1 months. Virological failure was defined as a viral load of more than 1000 copies/ml on two consecutive viral load measurements after 3 months adequate viral suppression. In accordance with WHO guidelines, viral suppression is defined as a viral load of less than 400 copies/ml [18]. According to Article L1121-1 of the French Public Health law, noninterventional studies are not subject to a legal framework. Noninterventional studies are defined as actions that are routinely performed without any additional procedure or unusual diagnostics or monitoring. Patients were informed that the samples could be used for research purposes and were free to refuse. The samples were used anonymously, ensuring medical confidentiality. Viral load was determined using commercial method COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 (Roche Diagnostics, GmbH, Germany). The viral RNA was extracted from 200 µl of plasma (previously stored at −80°C) using the Nuclisens RNA extraction kit (Macherey-Nagel, Germany). The RNA was eluted in 50 µl of elution buffer according to the recommendations of the manufacturer. Quality of Pro and reverse transcriptase sequences of each patient was checked using the sequence coverage of the amplicons. Finally, good quality of sequence coverage was obtained for 86% (50/58) of patients analysed.

Selection of the at least 1 and at least 20% thresholds

Two thresholds ($\geq$1 and $\geq$20%) were selected on the basis of the current consensus. The at least 20% threshold was selected as a reference for comparison with the Sanger sequencing method [9]. Mutations were considered significant at a frequency at least 1% among the total number of reads if they were present in both sequence directions. This threshold was selected on the basis of previous results [19].

Sanger sequencing

Antiretroviral resistance mutations were genotyped using the TruGene-HIV Genotyping Kit Version 1.0 (Siemens Diagnostics, Tarrytown, New York, USA) according to the manufacturer's instructions. This assay combines the cross-linking immunoprecipitation high-throughput sequencing technology with automatic analysis. The related nucleotide sequences were analysed to identify the HIV genotypes and the drug-resistant mutants through ViroScore-HIV.

Ultra-deep sequencing

Two cDNAs per patient were generated using Transcriptor Reverse Transcriptase (Roche Diagnostics GmbH, Mannheim, Germany). Primers were used to amplify four amplicons (Pro1, Pro2, RT1 and RT2) for each sample in the entire protease gene and reverse transcriptase gene using the Fast Start HiFi PCR system (Roche Diagnostics GmbH, Germany). Ten fusion primers were designed with amplicon adaptor sequences, multiplex identifier (MID) tags on both the forward and reverse primers and the sequence-specific primer. PCR products were then purified with Agencourt AMPure XP magnetic beads (Agencourt, Beckman Coulter, Beverly, Massachusetts, USA), followed by quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Molecular probe, Eugene, USA). Amplicons quantified

below 5 ng/μl and the PCR controls were verified using the 2100 Bioanalyzer (Agilent Technologies, Waldbroon, Germany). After amplicon mixing and equimolar pooling to 2×10^6 molecules/μl, emulsion-PCR was performed at a ratio of two molecules per bead. To evaluate the amount of enriched DNA beads, the GS Junior bead counter was used. To ensure an optimal picotiter plate loading, 500 000 enriched DNA beads were used. Amplicons were then sequenced from both ends (forward and reverse).

Data analysis

The amino acid substitutions called with a frequency ranging from 1 to 20% were classified as minor variants. The GS Amplicon Variants Analyzer software (AVA) (Roche 454 Life Sciences, Branford, Connecticut, USA) was used for read alignment mapping using the HIV-1 reference strain HXB2, variant calling and to demultiplex the 10 pooled patients' data using the MID sequences. The data generated from AVA were analysed with the DeepChek-HIV software (ABL SA, Luxembourg, Luxembourg), and drug-resistance interpretations were assessed for 19 antiretroviral drugs using the ANRS, HIVdb and Rega algorithms. The following compounds, all currently approved by the Food and Drug Administration (FDA) (http://www.fda.gov/oashi/aids/virals.html) and European Medicines Agency (EMEA) (http://www.emea.europa.eu), were considered: NNRTIs with abacavir (ABC), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), rilpivirine (RPV); nucleotide/side reverse transcriptase inhibitors (NRTIs) with didanosine (DDI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir (TDF), zidovudine (AZT); protease inhibitors with atazanavir/ritonavir (ATV/r), darunavir/r (DRV/r), fosamprenavir/r (fAPV/r), indinavir/r (IDV/r), lopinavir/r (LPV/r), nelfinavir (NFV), saquinavir/r (SQV/r), tripanavir/r (TPV/r).

Drug-resistance interpretation

The prevalence of drug resistance was defined according to the list of mutations for TDR surveillance as recommended by the International AIDS Society USA (IAS-USA) 2011. Resistance was classified into three groups defined by the Stanford Sierra database [susceptible (S), intermediate (I) and resistant (R)]. Genotypic resistance of the 50 Pro and reverse transcriptase sequences harbouring mutations obtained by UDS was tested using DeepChek-HIV (ABL SA, Luxembourg) at different user-defined sensitivity levels ($\geq$1 and $\geq$20%). DeepChek uses ANRS, HIVdb, Rega, Centre Hospitalier de Luxembourg (CHL), Genotypic Resistance-Algorithm Deutschland (Grade), the Brazilian Network for HIV-1 Genotyping (RenaGeno) and Red de Investigación en SIDA (RIS) algorithms to predict drug susceptibility. The ANRS algorithm (22 - 2012–09), Rega algorithm (v8.0.2 – 16/06/2009) and Stanford HIVdb algorithm (v6.2.0 – 29/05/2012) were used. These are the three most frequently used publicly available algorithms.

The HIV subtypes of the Sanger sequences were identified using Viroscore. UDS subtyping was determined by DeepChek-HIV based on the existence of at least 20% sequence homology between the consensus sequence generated from all reads and the updated set of reference sequences.

Major and minor discrepancies in drug-resistance interpretations

AVA (v2.7) (Roche 454 Life Sciences) read alignments and Sanger sequences were processed using DeepChek-HIV software (ABL, SA and TherapyEdge, USA). The resistance interpretation was performed using the ANRS, HIVdb and Rega algorithms. A discrepancy was defined as a discordant drug-resistance interpretation between the ANRS, HIVdb and Rega algorithms using the three levels of susceptibility predictions. Minor and major discrepancies in drug-resistance interpretations were defined according to previous published results [17,20,21]. A major discrepancy was defined as a susceptible result from one algorithm and a resistant result from another algorithm. A minor discrepancy corresponded to an intermediate interpretation in one system, with a susceptible or resistant result in another.

Statistical analysis

Minor and major discrepancies between Sanger and UDS 1% on one side, and Sanger and UDS 20% on the other side were compared using a Chi-square test for a proportion comparison. All *P* values below the α equal to 0.05 criterion were considered statistically significant. All calculations were performed using SAS V9.1 software (SAS Institute Inc., Cary, North Carolina, USA).

Results

Genotyping concordance

HIV genotyping was successfully performed with 100% concordance between the UDS protocol and Sanger sequencing. Among the 50 patients selected, we found 35 B subtypes, three A subtypes, eight A/G recombinants, one B/C recombinant, two B/F recombinants and one A/E recombinant.

Ultra-deep sequencing amplicon analysis

We obtained 50 samples from HIV-1 infected patients. At time of viral load, viral load median was 27 424 HIV-1 RNA copies/ml of plasma (range 1250–1 251 106 copies/ml). The limit of detection was 1000 copies/ml. Patient samples were completely sequenced in five independent GS Junior runs; the average sequence length that aligned to the reference sequence was 318 bp, and the mean number of sequence reads was 96 136. The quality of Pro and reverse transcriptase sequences from each patient was

checked using the sequence coverage of the four amplicons.

A comparison of ultra-deep sequencing and Sanger sequencing

To process one sample by Sanger sequencing, the time required for waiting, sample preparation and overall time to result were 7.75, 1.5 and 9.55 h, respectively. The reagents cost $80 per patient. One HIV-Plate for UDS was designed to process 10 samples per run. To process one sample by UDS, the time required for waiting, sample preparation and overall time to result were 22, 7 and 29.03 h, respectively. The reagents cost $100 per patient. The full workflow of database processing, analysis and reporting using DeepChek-HIV was less than 5 min per sample.

Drug-resistance interpretation

The interpretation of drug resistance, based on a mutation interest list from the guidelines of the International AIDS Society USA (IAS-USA) 2011 for routine clinical samples, from the UDS and Sanger sequencing results revealed a total number of 7428 and 2588 mutations, respectively. Clinically relevant mutations associated with I and R to several drugs obtained from both sequencing assays were also analysed: 643 and 224 clinically relevant mutations were found with UDS and Sanger sequencing, respectively.

Mutations associated with resistance to NNRTIs

Eleven of the 50 patients (22%) harboured the mutation V179I. The K101R, K103R and Y181C mutations, which are associated with resistance to the NNRTI drug class according to the ANRS, HIVdb and Rega algorithms, were detected in 4, 8 and 9% of the patients, respectively. Among all patients, the following mutations were detected: V90I (4%), A98S (4%), V106I (2%), E138A (6%), G190E (4%), Y188H (2%) and M230I (4%) (Fig. 1a). In addition, 30% (15/50) minority resistance mutations with 20% or less prevalence were detected by UDS and not by Sanger sequencing: K101Q (4%), K103N (1.8, 2.7 and 3.2%) V106I (1.4%), V179I (1.8 and 2.2%), Y181C (7% and 5.2%), G190E (2.6 and 2.7%), G190 V (1.1%), Y188H (2.2%) and M230I (2.6 and 7.5%). Three resistance mutations with at least 20% prevalence were detected by UDS and not by Sanger sequencing: A98S (23%), E138A (21%) and V179I (25%).

Mutations associated with resistance to nucleoside reverse transcriptase inhibitors

At positions associated with resistance to NRTI, the D67G, T69N, M184 V, L210W and T215Y mutations occurred in 8, 6, 12, 10 and 10% of the 50 patients, respectively (Fig. 1b). In addition, 32% (11/34) minority resistance mutations with 20% or less prevalence were detected by UDS only: D67E (1%), D67G (1.1, 1.3 and 3.5%), T69N (1.1 and 3.5%), V75I (4.7%), L210W (2.1%), T215F (8%), T215H (3.5%) and T215S (2.3%).

Mutations associated with resistance to protease inhibitors

The following resistance mutations were found in more than 10% of the 50 patients: L10 V (12%), L10I (26%), I15 (32%), G16I (20%), K20I (16%), M36I (44%), I62 V (28%), L63P (44%), I64 V (26%), H69K (22%), K70R (20%), A71 V (14%), A71T (12%), V77I (18%) and L89 M (24%). Other resistance mutations were also observed at lower frequencies: L10F (2%), K20R (6%), K20 M (2%), K20T (2%), K43R (8%), M46I (4%), M46L (2%), H69R (2%), H69Y (2%), K70N (2%) and K70T (2%) (Fig. 1c). In addition, 16% (37/233) minority resistance mutations with 20% or less prevalence were detected by UDS only: L10I (10.5, 2.9, 5.7 and 6.7%); I15 V (1, 6.3, 6.7 and 10.8%); G16E (2.7%); K20R (2.4%); M36I (1.6, 2.5, 3.8 and 4%); K43R (1.2 and 4.4%); M46I (7%); M46L (1.5%); I62 V (3.3%); L63P (1.5, 4.2, 4.7, 10.6 and 12.6%), I64 V (3.8%); H69Y (1.4%); K70N (7.1%), K70R (5.8%); K70T (2%); A71T (3.1, 4.2 and 13.8%); V77A (1.4%); V77I (1.1, 1.5 and 3.4%) and L89 M (18.5%).

Analysis of discrepant results using different rules-based algorithms

Genotypes were grouped into three groups (S, I and R) to determine the percentage of discrepant interpretation results between Sanger sequencing and UDS (at $\geq$1 and $\geq$20% of sensitivity) using the ANRS, HIVdb and Rega algorithms. For NNRTI, NRTI and protease inhibitors, the differences between interpretations were observed not only between the two methods but also between the algorithms. UDS detected each mutation found by population sequencing and identified additional resistance mutations in all patients primarily with a 1% of sensitivity (Fig. 2). The major interpretation differences between the algorithms are presented in Table 1. The differences were minor for most of the NNRTIs except for EFV, ETR and RPV. The Y181C mutation reduced the susceptibility to EFV by approximately two-fold using the HIVdb algorithm, and the Y181C mutation was responsible for drug resistance as determined by the ANRS and Rega algorithms. The HIVdb classified the G190E mutation as a mutation intermediate to the high level of resistant against ETR. A combination of mutations was necessary for the other two algorithms. The Y181C mutation was classified as a mutation associated with drug resistance by ANRS algorithm and reduced the susceptibility to RPV by approximately three-fold as determined by the HIVdb. Genotypic HIV-1 resistance was not found for RPV in the Rega algorithm.

The differences were more pronounced for all of the NRTI drugs (DDI, d4T, TDF and ZDV). Only the K65R mutation was classified as an intermediate resistance to DDI by the ANRS algorithm. In the HIVdb algorithm, the T215Y mutation with other NRTI-resistance mutations reduced the susceptibility to DDI, and the K65N/R, K70E/G, L74I/V and V75T mutations or combination of mutations were classified as

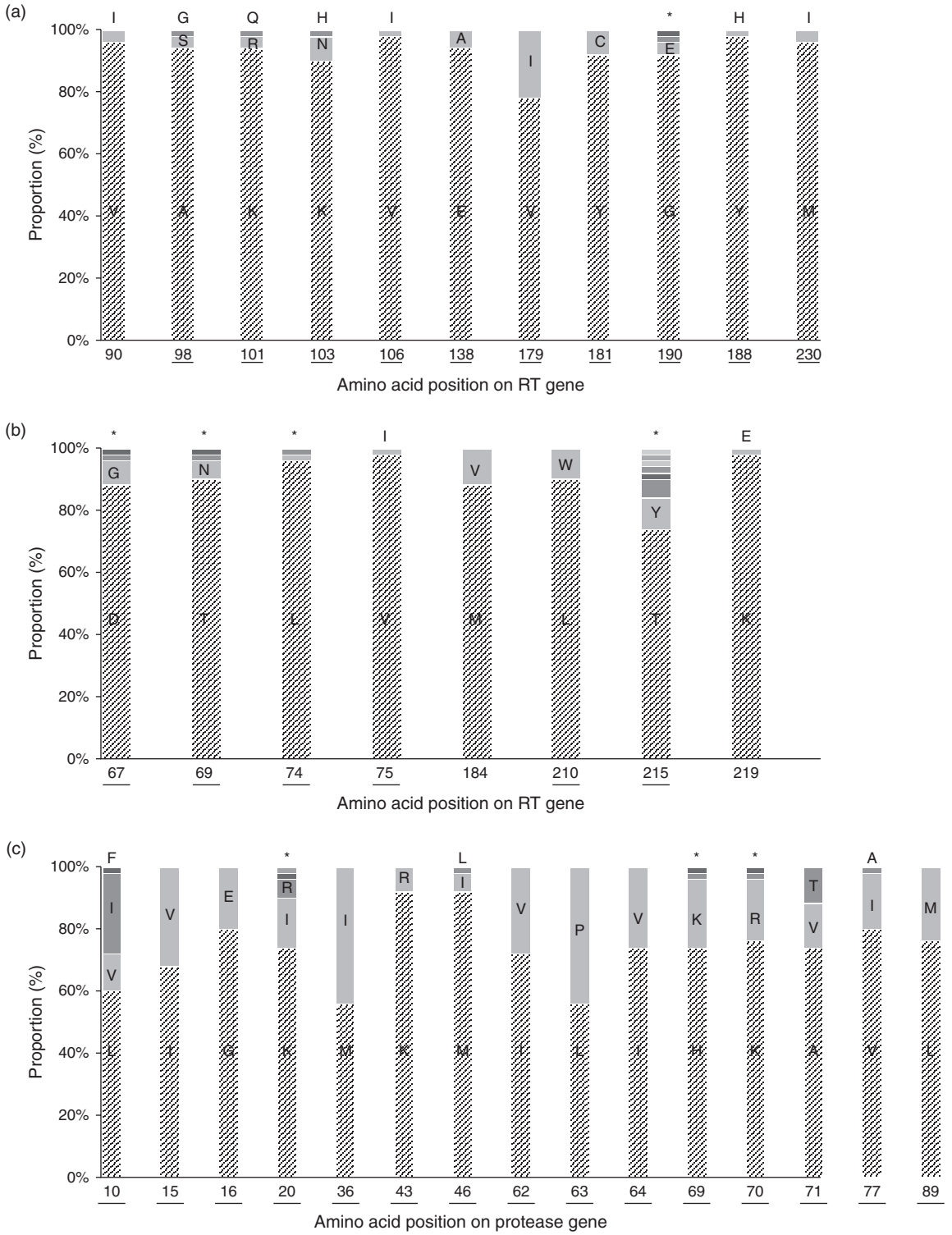

Fig. 1. The proportions of amino acids found at resistance-associated positions in 50 HIV-1 infected patients using the mutation interest list from IAS-USA 2011 for NNRTIs (a), NRTIs (b) and PIs (c). The wild-type amino acid is indicated by dashed lines. *More than one amino acid was detected at the following position: codon 190 of the RT gene: A (2%), V (2%); codon 67 of the RT gene: E (2%), N (2%); codon 69 of the RT gene: D (2%), S (2%); codon 74 of the RT gene: I (2%), V (2%); codon 215 of the RT gene: C (10%), D (6%), F (2%),H (2%), L (2%), S (2%); codon 20 of the protease gene: M (2%), T (2%); codon 69 of the protease gene: R (2%), Y (2%); codon 70 of the protease gene: N (2%), T (2%). The underlined amino acid positions represent minority mutations detected by UDS only. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

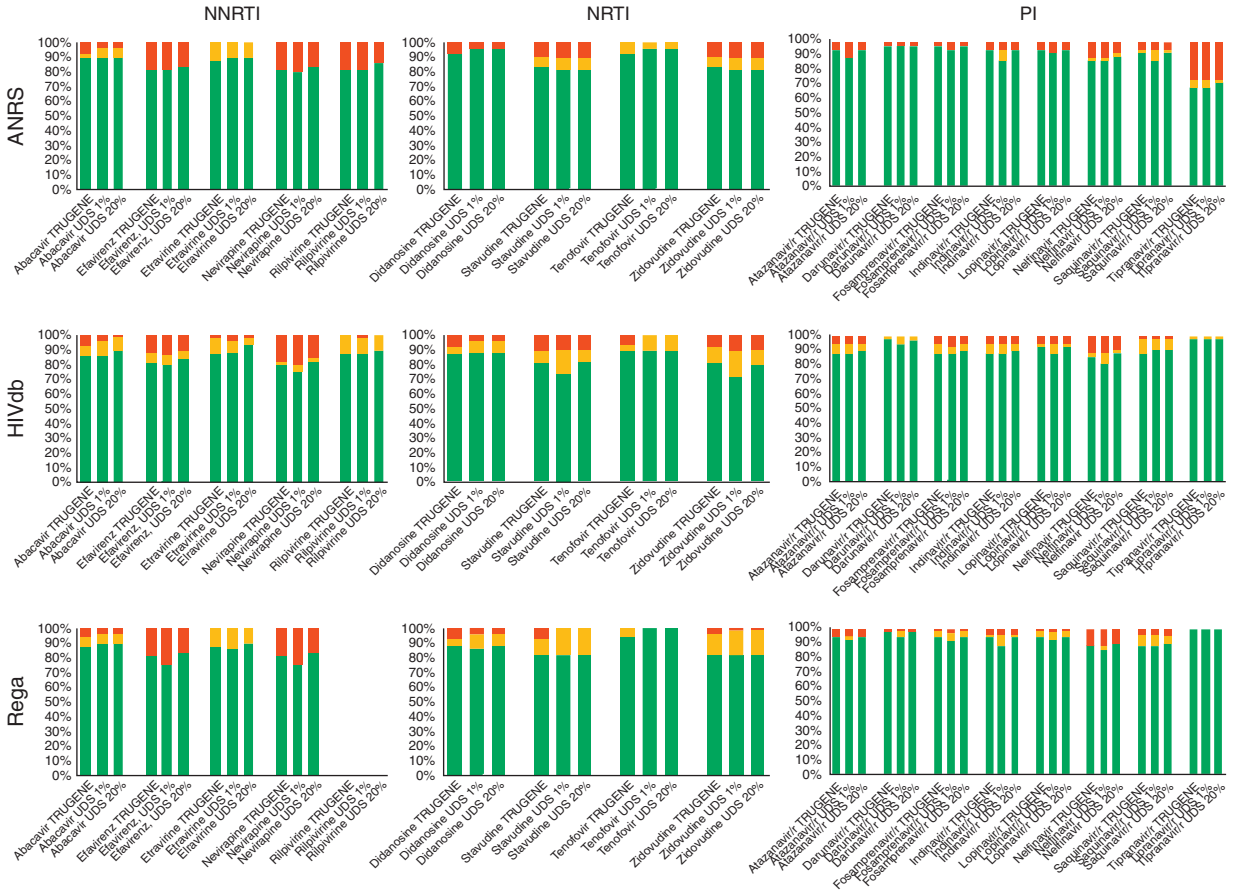

Fig. 2. A comparison of the mutation resistance rates for each antiretroviral between the Sanger, UDS 1% and UDS 20% data using the ANRS (22 - 2012-09), HIVdb Stanford (v6.2.0 – 29/05/2012) and Rega institute (v8.0.2 – 16/06/2009) algorithms on 50 HIV-infected patients. Green (S), yellow (I) and red (R).

an intermediate interpretation by the Rega algorithm. The T215Y mutation was associated with resistant to d4T by the ANRS and HIVdb algorithms. For the Rega algorithm, resistance to D4T required a combination of three or four mutations. Intermediate resistance to TDF was observed using the HIVdb algorithm, due to the T215Y mutation. The K70EG mutation or combination of mutations was necessary for the Rega or ANRS algorithms. Using ANRS and HIVdb, the T215Y mutation was associated with resistance to ZDV. Using the Rega algorithm, Q151 M or a combination of three or four mutations was necessary for resistance to ZDV.

The differences were minor for protease inhibitors, with the exception of DRV and TPV/r. Using the HIVdb algorithm, none of the mutations were associated with high levels of resistance to DRV. A combination of four mutations and a genotypic susceptibility score (GSS) of 3.5 were necessary for ANRS and Rega, respectively. Using the HIVdb algorithm, the M46IL mutation in combination with other resistance mutations reduced the susceptibility to TPV/r. A GSS of 3 and 3.5 were necessary for the ANRS and Rega algorithms, respectively.

Major and minor discrepancies in drug-resistance interpretations

The number of major and minor discrepant results using the three levels of interpretation for each drug is illustrated in Fig. 3. The number of discrepant results ranged from zero to six. NVP demonstrated the highest number of major discrepancies for the three algorithms using the UDS 1% data. There were no minor or major discrepancies for FTC or 3TC between the three algorithms using the UDS data at any threshold. Table 2 shows the rates of minor and major discrepancies between the Sanger versus UDS 1% and Sanger versus UDS 20% comparisons. The rate of major discrepancies between the Sanger and UDS methods for the ANRS algorithm was significantly higher at the 1% UDS threshold than the 20% threshold (1.79 versus 0.84%, respectively; $P = 0.035$). With the HIVdb algorithm, the rate of minor discrepancies between the two methods was significantly higher at the 1% UDS threshold than the 20% threshold (4.32 versus 2.11%, respectively; $P = 0.003$). For the HIVdb algorithm, the rate of major discrepancies had a strong tendency to be higher at the 1% UDS threshold than at the 20% threshold (0.84 versus 0.32%, respectively; $P = 0.068$). With the Rega algorithm, the rate of major discrepancies between the Sanger and UDS methods was

Table 1. An analysis of the major discrepant results using the ANRS, HIVdb and Rega rules-based algorithms.

Drug	Algorithms	Mutations (rules)[a]	Resistance interpretation
Efavirenz	ANRS	Y181C	Resistance
	HIVdb	Y181C	two-fold ↘ susceptibility
	Rega	Y181C, M230I, G190E	Resistance
Etravirine	ANRS	Combination of four mutations	Resistance
	HIVdb	G190E	Intermediate-to-high level resistance
	Rega	Combination of three mutations	Resistance
Rilpivirine	ANRS	Y181C	Associated with resistance
	HIVdb	Y181C	Three-fold ↘ susceptibility
	Rega	No mutation	No interpretation
Didanosine	ANRS	K65R	Intermediate
	HIVdb	T215Y	Low-level resistance
	Rega	At least one mutation of 65NR, 70EG, 74IV, 75T or a combination of two or three mutations	Intermediate
Stavudine	ANRS	T215Y	Resistance
	HIVdb	T215Y	Resistance
	Rega[b]	Combination of three or four mutations	Resistance
Tenovofir	ANRS	Combination of three, four or five mutations	Intermediate
	HIVdb	T215Y	Low-level resistance
	Rega	K70EG or combination of two, three or four mutations	Intermediate
Zidovudine	ANRS	T215Y	Resistance
	HIVdb	T215Y	Resistance
	Rega	Q151M or combination of three or four mutations	Resistance
Darunavir	ANRS	At least four mutations	Resistance
	HIVdb	No mutations are associated with the highest levels	–
	Rega	GSS >3.5	Resistance
Tipranavir/r	ANRS	GSS >3 : M36I/L/V –F53L/W/Y + Q58E + H69I/K/N/Q/R/Y + L89I/M/R/T/V	Resistance
	HIVdb	M46IL	↘ Susceptibility with other resistance mutations
	Rega	Combination to obtain score at least 3.5	Resistance

GSS, genotypic susceptibility score; ↘, reduced. Mutations in bold were responsible for the DR interpretation differences.
[a]Mutation or combination of mutations. Lists of mutation combinations associated with resistance for the ANRS, HIVdb and Rega algorithms are accessible online through the Stanford database website (http://hivdb.stanford.edu/).
[b]The T215 mutation has been identified as a key mutation in a pathway leading to high-level resistance.

significantly higher at the 1% UDS threshold than at the 20% threshold (1.81 versus 0.48%, respectively; $P = 0.002$). Similarly, the rate of minor discrepancies was also higher at the 1% threshold than at the 20% threshold (2.57 versus 1.33%, respectively; $P = 0.020$).

Discussion

To the best of our knowledge, this is the first study to compare the drug-resistance detection accuracy of UDS and Sanger sequencing using several validated algorithms. The UDS method detected all of the mutations found by population sequencing and identified additional resistance variants in all patients, primarily by using a 1% threshold. Two thresholds ($\geq$1 and $\geq$20%) were chosen according to previous studies performed with more sensitive assays (UDS and pyrosequencing) [9,19]. We previously demonstrated that a pyrosequencing assay allowed the detection of 5% of the minority mutants, and a baseline drug-resistance assessment can predict therapy failure [9].

Beerenwinkel and Zagordi [22] developed a statistical method for error correction and reported that the UDS/ROCHE error rate was approximately 0.1–0.5%. Moreover, Mitsuya *et al.* [23] proposed that it was unlikely for variants occurring at a frequency above 1.0% to be the result of sequencing error rates. Because a 1% mutation frequency was selected in this study, it was not necessary to calculate the intrinsic error rate.

The total time required for the UDS protocol was found to be approximately three times longer that the Sanger sequencing protocol (29.03 versus 9.25 h) and five times longer for the handling preparation (1.5 versus 7 h). In this study, we present an improved protocol that reduces the time to result in comparison with the recent work of Stelzl *et al.* [24], which was approximately 37 h, although the authors only sequenced the HIV-1 genotype B. In a recent study, Dudley *et al.* [25] used the UDS method to process 48 patient samples per sequencing run, which was four times more than the current genotyping method, at three to five times less than the cost of Sanger-based tests. The cost of HIV genotyping using in-house Sanger sequencing was $80 per sample. In comparison, the total cost for the pooled UDS-based surveillance of TDR in Pro and RT was $100 per sample [26]. The costs were comparable, and UDS may be useful for global drug-resistance surveillance implemented at specialized HIV DR laboratories [27].

A comparison between the UDS and Sanger sequencing methods for HIV-1 drug-resistance interpretations has not been previously studied. A comparison has only been

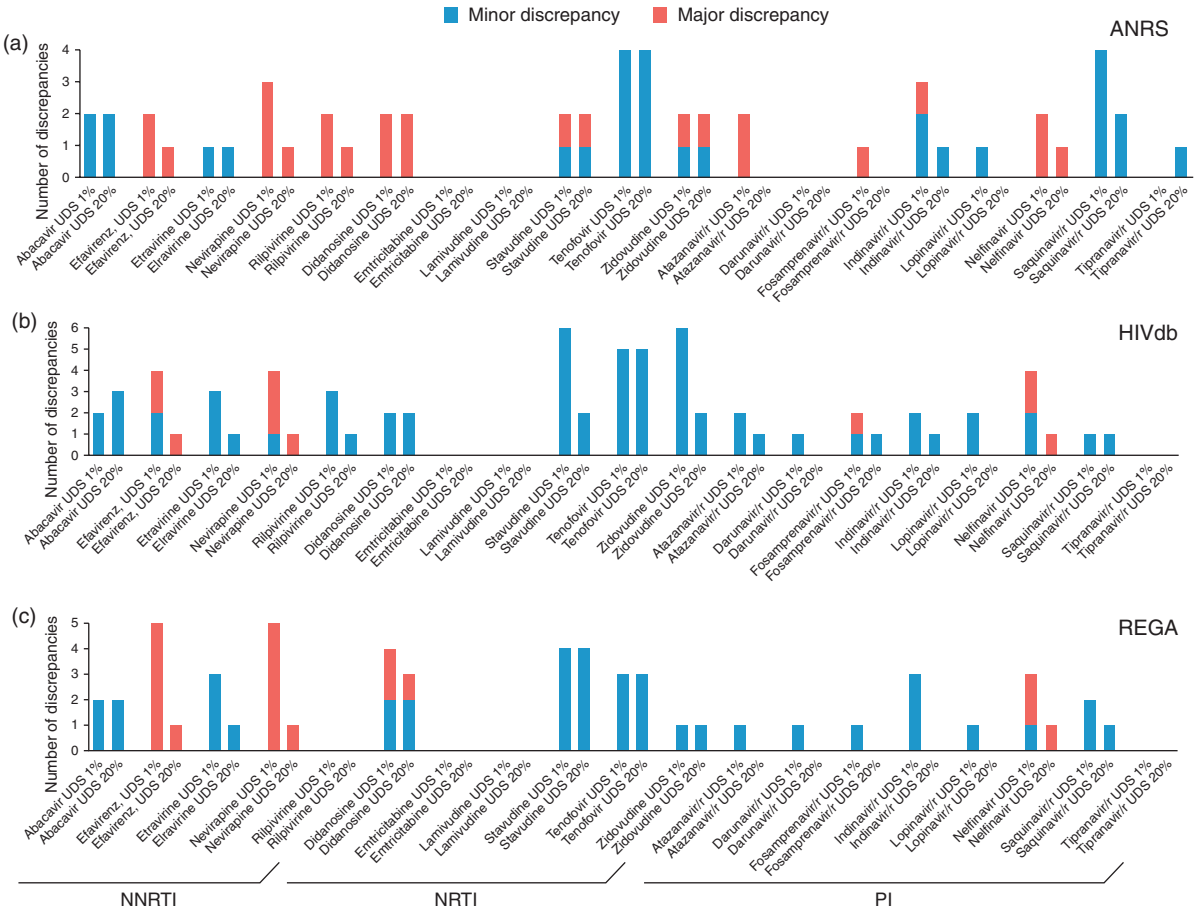

Fig. 3. The number of minor and major discrepancies for each antiretroviral between ultra-deep sequencing (>1 and ≥20%) and Sanger sequencing using the ANRS, HIVdb and Rega algorithms. (a) ANRS (22 - 2012-09), (b) Rega institute (v8.0.2 – 16/06/2009), (c) HIVdb Stanford (v6.2.0 – 29/05/2012).

made using Sanger sequencing [17]. Previous studies have demonstrated that differences between algorithm interpretations do exist with variable degrees of discordances [20,28,29]. In this study, we demonstrated that there were significant differences in drug-resistance interpretations when we analysed the UDS data at different thresholds of detection (≥1 and ≥20%). The relevant mutations identified by UDS based on the IAS-USA 2011 panel of mutations are associated with resistance to NNRTIs, NRTIs and protease inhibitors [30]. Among patients treated with efavirenz, the K103N mutation was the most frequently observed resistance mutation found in virological failure regardless of the baseline minority variants, and the Y181C minority mutation was associated

Table 2. A comparison of the major and minor discrepancies identified between the Sanger sequencing and ultra-deep sequencing methods according to the threshold selected.

	Algorithm	Minor discrepancy	Major discrepancy
Sanger versus UDS 1%	ANRS	1.68% (16/950[a])	1.79% (17/950)
Sanger versus UDS 20%		1.37% (13/950)	0.84% (8/950)
P		0.29	0.035
Sanger versus UDS 1%	HIVdb	4.32% (41/950)	0.84% (8/950)
Sanger versus UDS 20%		2.11% (20/950)	0.32% (3/950)
P		0.003	0.068
Sanger versus UDS 1%	REGA	2.57% (27/1050[b])	1.81% (19/1050)
Sanger versus UDS 20%		1.33% (14/1050)	0.48% (5/1050)
P		0.020	0.002

UDS, ultra-deep sequencing. *P* values were calculated using a Chi-square test performed with SAS V9.1 software (SAS Institute Inc., Cary, North Carolina, USA).
[a]950 is the number of calculation tests between the 19 ARV and 50 patients (ANRS and HIVdb do not give data for atazanavir and delarvirdine).
[b]1050 is the number of calculation tests between the 21 ARV and 50 patients (The Rega algorithm gave an interpretation of resistance for atazanavir and delarvirdine).

with a higher probability of Y181C detection after virological failure [31]. In this study, we observed the K103N and Y181C mutations with 20% of prevalence or less, and these variants were not detected by Sanger sequencing in 6 and 4% of patients, respectively. Due to the differences that may exist between the algorithms, DeepChek was chosen because it is likely the most ergonomic and efficient software available to optimize the prediction of virological failure or therapy success.

This study uniquely compared the Sanger sequencing and UDS methods at two thresholds of detection and also compared three algorithms in the same report for each patient with 19 antiretrovirals. We have clearly shown that the numbers of mutations detected using UDS 1%, UDS 20% and Sanger sequencing are significantly different. Moreover, the three-level comparisons showed that most of the discrepancies between the methods were minor. When compared with the Sanger method, the UDS 1% data showed more minor and major discrepancies than the UDS 20% data. Minor discrepancies may have less clinical importance than major discrepancies. In addition, our study confirmed that different drug-resistance interpretations can result from different algorithms [17,32].

ANRS, Stanford and Rega algorithms use the same definition of the level of resistance (S, I and R). From a clinical point of view, samples presenting intermediate resistance to a molecule are considered resistant (I = R). But in our study, we wanted to remain faithful to the interpretation nomenclature of these three algorithms. Moreover, this allows to clearly identify interpretation difference.

In conclusion, combining DeepChek software with UDS-generated data could allow for better data interpretations to ultimately help clinicians provide the appropriate and individualized treatment. Indeed, our data demonstrate that this combination allows drug-resistance status interpretation that is useful for HIV-1 ART monitoring. In addition, a 1% threshold of detection allows better characterization of the viral population thereby promoting the identification of additional resistance mutations and improving drug resistance interpretations.

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Conflicts of interest

None declared.

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