Evaluation of a Benchtop HIV Ultradeep Pyrosequencing Drug Resistance Assay in the Clinical Laboratory

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Detection of low-abundance drug resistance mutations (DRMs) of HIV-1 is an evolving approach in clinical practice. Ultradeep pyrosequencing has shown to be effective in detecting such mutations. The lack of a standardized commercially based assay limits the wide use of this method in clinical settings. 454 Life Sciences (Roche) is developing an HIV ultradeep pyrosequencing assay for their benchtop sequencer. We assessed the prototype plate in the clinical laboratory. Plasma samples genotyped by the standardized TruGene kit were retrospectively tested by this assay. Drug-treated subjects failing therapy and drug-naïve patients were included. DRM analysis was based on the International AIDS Society USA DRM list and the Stanford algorithm. The prototype assay detected all of the DRMs detected by TruGene and additional 50 low-abundance DRMs. Several patients had low-abundance D67N, K70R, and M184V reverse transcriptase inhibitor mutations that persisted long after discontinuation of the drug that elicited these mutations. Additional patient harbored low-abundance V32I major protease inhibitor mutation, which under darunavir selection evolved later to be detected by TruGene. Stanford analysis suggested that some of the low-abundance DRMs were likely to affect the resistance burden in these subjects. The prototype assay performs at least as well as TruGene and has the advantage of detecting low-abundance drug resistance mutations undetected by TruGene. Its ease of use and lab-scale platform will likely facilitate its use in the clinical laboratory. The extent to which the detection of low-abundance DRMs will affect patient management is still unknown, but it is hoped that use of such an assay in clinical practice will help resolve this important question.

HIV drug resistance genotyping is considered standard of care for patient management (1). This test has the clinical value of detection of antiretroviral (ARV) resistance and allows the selection of a new ARV regimen in patients who have experienced failure of their current ARV therapy regimen. Furthermore, use of genotypic resistance testing prior to the start of a treatment regimen, increases the likelihood of virological response to this regimen. Thus, most current HIV treatment guidelines recommend drug resistance genotyping in case of a failure therapy, as well as in untreated drug-naïve patients prior therapy (www.aidsinfo.nih.gov/guidelines).

Various drug resistance genotyping tests have been developed, several of which are clinically approved and widely used proprietary systems, such as TruGene (TG), which is associated with Siemen's FDA-approved HIV protease (PR) and reverse transcriptase (RT) sequencing kit, and ViroSeq which is associated with Celera's U.S. Food and Drug Administration (FDA)-approved PR and RT kit, as well as an integrase (IN) sequencing kit. Additional commercial testing services are also available, including VircoType HIV-1 drug resistance testing (Virco Laboratories) and the GeneSeq system, which is available at Monogram Biosciences. Most of these tests involve the generation of a "bulk" RT-PCR product derived from multiple viral genomes extracted from plasma of patients, followed by PCR amplification of the PR, RT, and IN gene regions, as well as recently added the V3 loop of the env sequence. This is followed by sequencing of the amplified "bulk" amplicons by the standard Sanger technique (referred to as "bulk" sequencing, population sequencing, or direct sequencing). The resulting sequences are checked against one or more drug-resistant mutation (DRM) databases and interpretation systems, which have been

developed by academic, commercial, or professional expert groups. Most commonly used publicly are the International AIDS Society USA (IAS-USA) DRM list, Stanford HIV database (HIVdb), ANRS (Agence Nationale de Recherches sur le Sida), Rega Institute, Antriretroscan system (Italian Antiretroviral Resistant Cohort), and the Geno2pheno German National Reference Center (2).

Most of the currently used genotypic assays generally fail to detect the presence of low-frequency drug-resistant mutations (also referred to as minority variants) if they account for <20% of the viral quasiespecies present in plasma of patients (3, 4). Nevertheless, more sensitive methods which have been developed, including point mutation assays and clonal sequencing (5), have shown that preexistent minor drug-resistant variants that are undetected by "bulk" sequencing can contribute to subsequent treatment failure in drug naïve, as well as in treated experienced patients (4, 6–8). These highly sensitive methodologies, however, suffer from major drawbacks. The point mutation assays, including allele-specific PCR (4, 9) and ligation amplification (10) assays can only detect a few drug-resistant mutations at a time, making these techniques impracticable for clinical settings, where simul-

Received 4 October 2012 Returned for modification 13 November 2012

Accepted 21 December 2012

Published ahead of print 2 January 2013

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doi:10.1128/JCM.02652-12

TABLE 1 Characteristics of study patient population^a

Patient	Age (yr)	Gender	Risk group	Country of origin	ART status	Plasma sample date (day.mo.yr)	Viral subtype	Viral load (copies/ml)	CD4 count (cells/ μ l)
1	30	F	HP	FSU	Treated	22.6.09	A/AE	3,280	39
2	55	M	MSM	Africa	Treated	13.5.08	B	1,850	376
3	52	F	HP	FSU	Treated	8.1.08	A/AE	1,830	546
4	76	M	EC	Africa	Treated	3.6.08	C	4,250	368
5	45	M	MSM	Europe	Treated	16.3.08	B	1,150	399
6	36	M	EC	South America	Treated	21.11.10	B	6,472	10
7	29	F	HP	South America	Treated	21.4.09	B	100,000	65
7a	30	F	HP	South America	Treated	31.05.10	B	17,490	182
8	39	F	HP	FSU	Treated	22.11.09	AE/B	2,347	648
9	35	M	HP	FSU	Treated	13.06.11	B	13,708	336
10	28	M	MSM	Israel	Naive	16.9.07	B	3,130	276
11	47	M	MSM	Israel	Naive	31.05.09	B	100,000	150
12	40	F	EC	Africa	Naive	25.09.11	C	7,669	462
13	54	M	MSM	USA	Naive	14.6.11	B	84,490	276
14	29	M	IVDU	FSA	Naive	15.5.11	A/AE	5,405	621
15	23	M	MSM	Israel	Naive	22.3.11	B	32,816	416
16	38	M	EC	NK	Naive	27.2.11	B	3,068	420
17	44	M	NK	FSU	Naive	9.12.10	AE	37,370	529
18	40	M	MSM	Israel	Naive	14.11.11	B	42,711	676
19	27	M	MSM	Israel	Naive	16.8.11	B	28,725	414
20	47	M	MSM	Israel	Naive	11.09.11	B	149,835	255

^a Abbreviations: M, male; F, female; MSM, men who have sex with men; IVDU, intravenous drug user; HP, heterosexual partner of an IVDU; NK, not known; EC, country where HIV is endemic; FSU, former Soviet Union; ART, antiretroviral treatment.

taneously detection of a large array of HIV DRMs is required. Furthermore, the clonal sequencing methodology, which includes limiting dilution and single genome sequencing (3), is a highly tedious process, unsuited for testing a large number of clinical samples. The recent advances in high-throughput next-generation sequencing (NGS) have revolutionized HIV drug resistance testing. This type of technology produces an enormous number of sequences, enabling the detection of multiple DRMs with ultradeep coverage. The first such system to be introduced was from 454 Life Sciences (Roche) and used emulsion PCR coupled with massive parallel sequencing. A later platform, the gene sequencer (GS) FLX pyrosequencing system is capable of producing 400 to 600 million bases per 10-h run (11). This technology also has a relatively long sequencing read length of 400 to 600 bp, which has the added advantage over the point mutation assays of being able to detect mutations in their sequence context and not just at a single locus (12).

Studies using this technology have shown its ability to detect low-frequency HIV DRMs undetectable by direct sequencing (13, 14). Furthermore, it was also demonstrated that these low-abundance mutations can have a significant impact on virological failure, mostly for non-nucleoside analog RT inhibitor (NNRTI) mutations (6, 15–18). However, despite its high accuracy and effectiveness, the GS FLX platform exceeds the capacity of small- and medium-scale projects, limiting its application for routine testing. Recently, Roche introduced a benchtop version of this sequencing platform, the GS Junior (GSJ) System, which is scaled to accommodate fewer samples. Currently, in-house HIV genotypic tests have been developed for this platform (16, 17, 19); however, the lack of a standardized commercial kit limits the wide-use application of this system in clinical settings.

Roche is currently developing an HIV next-generation ultradeep pyrosequencing (UDPS) assay for the GSJ system. This

prototype assay, however, has not been launched yet, but has been distributed to various locations for critical clinical evaluation and expert input for further kit refinement. We present here a study in which we assessed the performance of this assay in the clinical laboratory. The goal of the present study was to verify the accuracy of the new test to detect DRMs which are readily detected by TG and to investigate whether it has an added value in detecting low-abundance drug resistance mutations undetected by the TG system.

MATERIALS AND METHODS

Study sample population. A total of 21 Plasma samples of 20 HIV-1-infected patients were tested by the GSJ UDPS assay. Samples were retrieved from archived plasma samples of HIV patients which were collected between 2007 and 2011 and stored at -80°C . Nine of these subjects were drug-treated patients who had viral failure (1,150 to 100,000 copies/ml) and were previously diagnosed by TruGene as failing antiretroviral (ARV) drug treatment due to the presence of DRMs for at least one of the drugs included in the ARV regimen at the time of testing. An additional 11 patients were treatment naive, previously genotyped by TruGene at the time of HIV diagnosis. The characteristics of these patients is summarized in Table 1. The GSJ UDPS assay was performed retrospectively so the results had no impact on clinical decision-making. This study was approved by the institute ethical committee of Tel-Aviv Sourasky Medical Center.

HIV RNA extraction. HIV RNA was extracted and purified from plasma samples with the NucliSENS easyMag total nucleic acid extraction system (bioMérieux, Marcy l'Etoile, France). For the TG tests, HIV RNA was extracted from 0.5 ml of plasma samples and eluted into a 50- μ l final volume. For the GS Junior UDPS test, HIV RNA was extracted from 1 ml of plasma and eluted into 40 μ l. The eluted RNA was then mixed with 10 ng of MS2 phage RNA carrier/ μ l and stored at -80°C until used.

HIV drug resistance testing by TG. Testing was carried out routinely as part of standard of care of HIV patient management. The TG HIV-1 genotyping kit (Siemens, Berkeley, CA) amplifies a 918-bp fragment en-

compassing codons 4 to 99 of the HIV PR gene and codons 38 to 247 of the RT gene. The generated consensus sequences are aligned with the LAV-1 wild-type HIV virus by the commercially included OpenGene software and checked against internal algorithm guidelines rules to identify HIV DRMs. Consensus sequences were also checked against the Stanford database (<http://hivdb.stanford.edu>) to determine viral subtype and drug resistance score weight.

HIV drug resistance testing by the UDPS prototype assay. The assay was essentially performed according to the provided instructions, with only a few modifications. Reverse transcription was performed simultaneously on 10 samples in a microtiter plate with dried down proprietary specific primers targeted to the PR and RT gene regions. Two cDNA products were generated for each sample and subsequently PCR amplified in a microtiter plate with dried-down primer pairs, targeted to four overlapping gene regions, encompassing an ~1-kb sequence of the HIV PR and RT genes. Each PCR primers consisted of a 454 sequencing adaptor (either for forward or reverse sequencing), sample specific multiplex identifier (MID) sequence, and a specific viral sequence for PCR priming (20). A total of four amplicons were amplified for each sample. Each amplicon was purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Beverly, MA) and quantified by an Quant-iT PicoGreen dsDNA assay kit (Invitrogen Carlsbad, CA), using a NanoDrop fluorospectrometer ND3300 (Thermo Fisher Scientific, Wilmington, DE). Amplicons at low concentrations (<5 ng/μl) were analyzed by using an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) to verify their quality and length (an amplicon with a high primer/dimmer ratio was not further processed for pyrosequencing). All four amplicons from each individual sample were pooled in equimolar concentrations and were further used to prepare an equimolar mixture containing the total amplicons of all 10 samples. This pool was clonally amplified on capture beads by emulsion PCR. A total of 500,000 enriched DNA beads were deposited into wells of a PicoTiter plate device and pyrosequenced in both forward and reverse directions using the GS Junior system. After a 10-h run, the sequence reads were analyzed with GS amplicon variant analyzer (AVA) software (Roche), which assigns each read to the proper amplicon and patient sample using the MID. This software also aligns the generated sequence reads with the HIV wild-type HXB2 sequence and identifies quantitatively drug resistance mutations (variants) according to a pre-defined DRM list.

DRM analysis. The analysis of DRMs in the present study was carried out by using the 2011 edition of the IAS-USA DRM list updates (21) and the Stanford database. The PR, nucleoside analog RT, and non-nucleoside analog RT inhibitor mutations (PI, NRTI, and NNRTI, respectively), which are included in both of these databases, are essentially similar, except for several differences. The PI mutations I13V, G16E, K20R/T/V, M36I, D60E, I62V, L63P, V77I, I85V, L89M, and I93L, which are defined by the IAS-USA DRM list as minor PI mutations, are defined by Stanford as “other” PI mutations with 0 or low-scoring weight. The I54V mutation identified as a minor PI mutation in the IAS-USA list is defined by Stanford as a major PI mutation. The H69K mutation included in the IAS-USA list as a minor PI mutation is excluded from Stanford. The NRTI mutations T69I/N, V75A, and T215E and the NNRTI mutations K103E/T are included in the Stanford database but are excluded from the IAS-USA DRM list. The NNRTI mutations V90I, V106I, and E138G/Q are included in the IAS-USA list but are defined by Stanford as “other” NNRTI mutations, having a low-scoring weight.

Definitions of low-abundance and high-abundance drug resistance mutations. For the purpose of discussion here, low-abundance DRMs were defined as mutations detected at <20% of the viral quasiespecies, whereas high-abundance DRMs were detected at ≥20%.

RESULTS

Comparison of DRM detection by the UDPS prototype assay and the TG system. Altogether, a total of 179 DRMs were detected by both methods, of which 129 (72%) were found by both TG and

UDPS. An additional 50 DRMs (28%) were detected only by the UDPS. The detailed results are presented in Table 2. All of the DRMs detected by TG were also detected by UDPS at a level ranging from 29.7 to 99.9% of the total viral quasiespecies sequenced. We defined these mutations in our study as high-abundance DRMs. The DRMs detected by UDPS at a level of <20% were undetected by TG. These mutations were defined as low-abundance DRMs. The difference in the numbers of DRMs detected by the two methods remained significant also when mutations were classified into PI, NRTI, and NNRTI. A mean of 3.5 (ranging from 2 to 6) additional mutations per patient were detected by UDPS in the drug-treated subjects compared to TG, whereas a mean of 1.5 (ranging from 1 to 4) mutations were additionally detected by UDPS in the drug-naive patients. Only three of the drug-naive patients had no detectable low-abundance DRMs.

Low-abundance DRMs. Low-abundance DRMs were detected in all of the 9 drug-treated individuals (patients 1 to 9, in Table 2) and in 6 of 11 (55%) of the drug-naive patients (patients 10, 11, 13 to 16, 19, and 20 in Table 2). Of the total 50 low-abundance DRMs detected by UDPS, 26 (52%) were detected at levels 1 to 4% and 24 (48%) at levels of 5 to 19% (Table 2). The majority (66%) of these mutations were in the drug-treated patients. The low-abundance DRMs were detected in all three antiretroviral classes. Three of 20 (15%) patients harbored low-abundance DRMs that predicted resistance to three antiretroviral class (patients 2, 4, and 5), eight (40%) to two classes (patients 1, 5, 6, 7, 8, 9, 13, and 15), and six (30%) to only one class (patients 3, 10, 11, 16, 19, and 20). Patient 7 harbored DRMs to two antiretroviral classes but had a second sample, a year later, with low-abundance DRMs to only one antiretroviral class (samples 7 and 7a, respectively). Two (10%) patients, both untreated, had no detectable low-abundance DRMs.

The low-abundance PI mutations comprised 53% of the total low-abundance mutations and occurred in 14/20 (70%) patients. The majority of these mutations were minor IAS-USA PI mutations. However, there were 3 major PI mutations—V32I, M46I, and I47V—which were detected at levels of 12.77, 1.3, and 1.47%, in patients 7a, 5, and 3, respectively (Table 2). The low-abundance NRTI mutations occurred in 7 of 9 drug-treated patients and in only 1 of the drug-naive patients. The most common low-abundance NRTI mutations were the thymidine analog-associated mutations (TAMs) D67N, K70E/R, T215F, and K219E/N, which comprised 55% of the total low-abundance NRTI mutations. Low-abundance NNRTI mutations occurred in 7/9 drug-treated patients and in 3/11 of drug-naive patients.

Correlation of low-abundance DRMs with previous ARV treatment history of patients. Current failing ARV regimens as well as previous ARV exposure history were known for all of the nine drug-experienced patients (Table 2). In three of these subjects the UDPS assay detected low-abundance DRMs that persisted long after discontinuation of the drug that elicited these mutations. One of the patients (i.e., patient 1) had low-abundance TAMs, D67N and K70R, which were detected at levels of 1.68 and 4.67%, respectively. This patient had history of a zidovudine (AZT)-containing regimen but had been off the drug for ~10 months. Additional patient (patient 7) had a low-abundance TAM mutation, K70E, detected at a level of 5.47%. He was previously exposed to AZT but had been off the drug for ~7 months. The third patient (patient 2) had a low-abundance M184V mutation, detected at a level of 7.04%, despite the fact that he was not treated with a lamivudine (3TC)- or emtricitabine (FTC)-con-

TABLE 2 Detection of HIV drug-resistant mutation by the UDPs assay compared to TG^a

ARV treatment			DRMs detected by both TG and UDPs (% abundance by UDPs)			
Patient	Failing ARV regimen (date)	Previous ARV exposure (date)	PI	NR1T1	NRK1T1	DRMs detected only by UDPs (% abundance by UDPs)
1	FTC, TDF, LPV/r r (22.6,09)	3TC, AZT, LPV/r (26.8,08)	G16E (4.3), M36I (99.68), L89M (99.16), I93L (98.66)	M184V (99.51), A62V (98.81)	K101Q (54.82), E138G (87.25)	None
2	ABC, AZT, DDI, EFV, IDV/r (13.5,08)	3TC, AZT, D4T, DDI, EFV, IDV/r (26.2,01)	L33F (98.68), M46I (89.8), I62V (98.54), I63P (98.68), I84V (34.61), N88S (66.08), L90M (99.77), I93L (98.61), L10I (98.59), A71T (99.39)	M41L (98.9), D67N (94.55), L74V (99.48), L210W (99.48), T215Y (98.39)	K101P (94.37), K103N (94.55), E138Q (99.53)	M36I (15.66)
3	FTC, TDF, FPV/r (8.1,08)	3TC, AZT, LPV/r, EFV (17.10,06)	L10I (99.23), M36I (99.23), M46I (96.43), L89M (99.63), I93L (99.33)	M184V (99.78)	V108I (79.73)	I33F (17.15), I47V (1.15)
4	3TC, ABC, AZT, FPV/r (3.6,08)	3TC, D4T, IDV/r (20.12,05)	L10I (97.66), L33F (70.36), G16E (97.66), K20I (70.36), M36I (99.77), I54V (99.46), H69K (ND), V82A (99.88), L89M (99.63), L90M (99.49), I93L (99.33)	K65R (29.68), M184V (99.66)	None	K20V (6.68)
5	FTC, TDF, EFV	None	D60E (99.76), I63P (99.65)	K65R (82.79), M184V (99.88)	K103N (97.11)	M36I (3.43), M46I (1.3), L89M (1.98)
6	3TC, AZT, EFV	None	L10V (97.7), D60E (99.12), I62V (99.1), I63P (99.66)	V75I (98.93), M184V (99.12), T215Y (77.74)	K103N (96.7), P225H (99.68)	I33V (4.76), M36I (4.36), A71T (2.84)
7	FTC, TDF, FPV/r r (21.4,09)	3TC, ABC, AZT, LPV/r	M36I (92.86), M46I (92.86), I47V (98.3), I50V (99.32), I62V (99.32), I63P (99.73), I85V (80.66)	M184V (99.65)	None	I33F (19.39), V82I (2.2)
7a	FTC, TDF, DRV/r (31.5,10)	3TC, ABC, AZT, EFV, FPV/r	L10I (72.65), I33F (97.89), M36I (99.92), M46I (97.1), I47V (99.37), I50V (99.92), I62V (72.65), I63P (99.84), V82I (99.84), I85V (97.04), L89M (97.86)	K70E (39.98), M184V (84.18)	None	K20R (7.52), V32I (12.77)
8	3TC, AZT, LPV/r r (22.11,09)	FTC, TDF, LPV/r (3.7,07)	M36I (99.74), L89M (99.88)	M184V (99.94)	None	None
9	3TC, TDF, EFV	None	M36I (99.36)	None	None	None
10	None	None	I62V (96.96), V77I (100), A71T (98.62)	None	None	None
11	None	None	L10V (96.94), I63P (97.47), V77I (99.48), L90M (99.72)	None	None	None
12	None	None	K20R (99.39), M36I (99.8), I62V (99.64), V82I (99.73), L89M (99.54), I93L (99.86)	None	V90I (97.5)	None
13	None	None	I62V (28.62), I63P (97.47)	None	None	None
14	None	None	M36I (99.83), I63P (46.13), V77I (39.52), L89M (99.6), I93L (97.03)	A62V (98.57)	None	None
15	None	None	I62V (99.06), A71T (99.39), V77I (99.75)	None	None	None
16	None	None	L33V (98.47), M36I (99.92), M46L (58.02), A71V (99.61), I63P (99.84), I93L (95.5)	None	None	None
17	None	None	M36I (99.23), I63P (99.64), L89M (99.23), I93L (99.64)	None	None	None
18	None	None	I62V (98.97), A71T (98.68), V77I (99.77)	None	None	None
19	None	None	I62V (98.34), A71T (97.89), V77I (99.86)	None	None	None
20	None	None	L10I (98), I62V (99.19), A71T (99.19), V77I (92.70)	None	None	None

^a IDRMs that were scored by the Stanford algorithm are indicated in boldface. Abbreviations: ARV, antiretroviral; DRMs, drug resistance mutations; TG, TruGene; GSJ, GS Junior; PI, protease inhibitor; NR1T1, nucleoside analog reverse transcriptase inhibitor; NNRTI, non-nucleoside analog reverse transcriptase inhibitor.

taining regimen at the time of viral failure. However, he had previously been exposed to a 3TC-containing regimen and had been off this drug for ~7 years. Of special interest is the case of the low-abundance major PI mutation, V32I, which was detected in sample 7a at a level of 12.77% (Table 2). This mutation imparts resistance to darunavir, which was included in the failing ARV regimen of this patient. The mutation, however, was detected only 19 months later, in the course of routine resistance testing by TG (data not presented).

Impact of low-abundance DRMs on the resistance burden.

The Stanford HIV drug resistance scoring algorithm was used to evaluate the extent to which low-abundance DRMs detected by UDPS, added to the resistance burden in each patient. The DRMs that were scored are indicated in boldface in Table 2. Four of 9 (44%) treated patients (patients 2, 3, 6, and 7, including sample 7a) had additional low-abundance DRMs, which affected the resistant burden to at least one of the drugs in the current failing regimen. One patient (patient 3) had PI mutations, L33F and I47V, detected at levels of 17.15 and 1.15%, respectively, which increased resistance to FPV/r from low level to high level. Another patient (patient 7) had an NRTI mutation, K70E, at a level of 5.47% that imparted low-level resistance to tenofovir (TDF), which otherwise was not revealed by the standard TG testing. The same patient had additional sample (sample 7a) that harbored a low-abundance PI mutation, V32I, detected at a level of 12.77%, which significantly increased resistance to ritonavir-boosted darunavir (DRV/r) treatment. An additional drug-treated patient (patient 2) had a low-abundance NRTI mutation, M184V, detected at a level of 7.04%, which decreased the resistance to TDF, from a high level to an intermediate level. The low-abundance DRMs, including the PI minor mutations L10I and L33I and the NNRTI K103E mutation, that were detected in 5/11 drug-naïve patients (patients 12, 13, 14, 15, and 19) have low significance to the resistance burden of potential ARV treatment. Nevertheless, one of the drug-naïve subjects (patient 13) had a low-abundance TAM mutation, D67N, detected at a level of 2.5%, which might imply a low level of resistance to potential AZT and stavudine (D4T) treatment.

DISCUSSION

The present study was undertaken to assess the performance of a Roche prototype HIV UDPS assay in the clinical laboratory. Currently, the “bulk” sequencing technique is still considered the golden standard for drug resistance testing in the clinical laboratory. We routinely use the TruGene (TG) drug resistance genotyping system. It was therefore important for us to verify that the prototype assay could detect all DRMs that were detected by TG. Our results show that indeed all DRMs detected by TG were also detected by the Roche prototype assay and, as predicted, were at a level of 20% and above of the viral quasispecies present in the patient’s sample. Furthermore, no DRMs detected by the Roche prototype assay at levels of 20% and above were undetected by TG, suggesting that the new assay had no “false-positive” results compared to TG. Of note, however, are reports of others that showed the detection of DRMs at high frequency (>20%) that were undetected by standard direct sequencing technology. Le et al. used the GS FLX platform (Roche), which detected two NRTI DRMs, V118I and A98G, at levels of 30.5 and 23.9%, respectively, that were not detected by direct sequencing (22). Similarly, Fisher et al., using the GS-FLX method, detected low-abundance drug re-

sistance mutations (PIs, K20R and M36I; NNRTI, K103R) at levels of 28.1 to 44.7%, which were undetected by direct sequencing (23). It is likely that these mutations were missed by the standard sequencing techniques due to poor results; however, it cannot be ruled out that these were “false-positive” results compared to TG. This issue should be further investigated.

It is obvious from our study that the prototype assay has the beneficial added value of detecting low-abundance DRMs (<20% of viral quasispecies). Altogether, a total of 50 additional DRMs were detected by the prototype assay and not detected by the TG system. Our results show that the prototype assay was able to detect minor DRM variants that persisted long after discontinuation of the drug that elicited these mutations. Two patients (patients 1 and 7) had NRTI thymidine analogue mutations (TAMs), which persisted up to 10 months after discontinuation of AZT, and an additional patient (patient 2) harbored the M184V mutation, long after stopping 3TC treatment. These findings are in accord with the previous reports of Le et al., who have shown, using the GS-FLX pyrosequencing platform, that low-abundance TAMs persisted in treated HIV-infected patients, up to 7 years after discontinuation of AZT. These researchers did not, however, demonstrate the presence of low-abundance M184V mutation in patients who were off lamivudine/emtricitabine treatment (22). Nevertheless, Fisher et al. have recently shown, as we did, that low-abundance M184V mutation persisted for several years in patients who have discontinued lamivudine treatment (23). These findings, however, are in contrast with previous reports that have shown that the M184V mutation has poor fitness and wanes quickly from plasma of patients in the absence of lamivudine or emtricitabine selection pressure (14, 22).

Our results demonstrated that the GS Junior prototype assay detected in a patient the major PI V32I DRM in a patient before it evolved into a major virus population which could be detected by TG. This mutation, which imparts resistance to darunavir (24), was detected at a level of 12.8% in patient 7 (sample 7a) (Table 2), who failed a darunavir treatment. Unfortunately, it was undetected by TG at the time of testing and therefore the patient continued treatment with darunavir until 19 months later, when he was tested again by TG (data not presented). The emergence of low-abundance major PI mutations as dominant strains in PI-treated patients is not a common phenomenon but has been previously reported for the major M46I and L90M PI mutations, in a single patient who failed nelfinavir treatment, followed by a failure to boosted amprenavir-based regimen (25). Others, however, have shown that NRTI and NNRTI drug resistance minority quasispecies, such as M184I/V, K103N, and Y181C mutations, which are detected in treatment-naïve patients or in patients failing therapy, can emerge as dominant strains under drug selection, and can cause treatment failure (8, 16). Of interest is the detection of the low-abundance major PI mutation, I47V, which was detected in patient 3 (Table 2) at a level of 1.2%. This mutation specifically imparts resistance to fosamprenavir (FPV), and it is likely that its presence represents early event in resistance selection to this drug. This patient experienced viral failure on an FTC/TDF-ritonavir-boosted FPV regimen but had no significant PI mutations detected by TG. Such a finding is in accord with previous reports of prevalence of PI minor mutations in patients who experienced virological failure on boosted-PI regimens but had no drug-resistant mutations detected by standard drug resistance genotyping (23, 26).

Our study had several limitations. We were unable to amplify samples that had viral loads below 1,000 copies/ml. The vendors' instructions for both the TG and GS junior assays are to utilize plasma samples with HIV viral loads of at least 1,000 copies/ml. Our experience with TG shows that when using the NucliSENS easyMag extraction system for samples with viral loads of 1,000 copies/ml and above, it is sufficient to purify the HIV RNA from 0.5 ml of plasma and elute it into a 50- μ l final volume (a 20-fold concentration of the original sample volume). By doing so, it enables us to achieve more aliquots of plasma for future testing. Nevertheless, to achieve optimal results with TG with samples that have <1,000 copies/ml, a 1-ml plasma sample volume is required, as well as elution into a 25- μ l final volume (a 40-fold concentration of the original sample volume). The plasma samples in the present study had viral loads above 1,000 copies/ml and were all successfully amplified by the GS junior, using 1 ml of plasma and extraction into a 40- μ l final volume (a 25-fold concentration of the original sample volume). We did not try to utilize smaller volume of samples, as with TG, but were rather interested in attempting to amplify samples with viral loads of <1,000 copies/ml. Experiments were therefore carried out with samples having viral loads ranging from 259 to 925 copies/ml; however, amplification was only partially achieved (data not presented). Failure was independent of viral subtype. Attempts to concentrate the extracted RNA from samples by using 1 ml of plasma and eluting into a 25- μ l final volume or by using a larger starting volume of plasma sample were unsuccessful. Furthermore, doubling the viral cDNA volume input into the PCR amplification reaction was also not helpful (data not presented). It should be concluded, though, that the >1,000 copies/ml viral load requirement that we report here is similar to that required by the commercially available FDA-approved TG and ViroSeq genotyping kits.

The number of patients and samples tested here was relatively small. Nevertheless, as noted throughout this study, our goal here was primarily to assess the performance of the new prototype assay, compared to TG, rather than to evaluate its clinical use for patient management. Our main goals were therefore to verify the analytical accuracy of the new test in detecting DRMs, which are readily detected by TG, investigate whether this assay can detect low-abundance DRMs undetected by TG, determine the threshold of viral load that can be used, and assess the user-friendly and ease of performance of this test in the clinical laboratory. Altogether, we tested 179 DRMs, of which 129 were detected by both assays, and performed three separate runs with a total of 30 patients, of which one was devoted to test samples with viral loads of <1,000 copies/ml (results for the latter experiments are not presented). To this end, we gained sufficient information to address the goals set forth here. Additional studies, however, with larger number of patients are needed to evaluate the clinical use of this assay.

Despite these intriguing findings, there are several issues that should be acknowledged. The UDPS technique is highly sensitive and therefore can detect mutation artifacts, occurring at low frequency, and usually not detected by direct sequencing. Samples are subject to two rounds of PCR prior to sequencing, and thus PCR errors occurring during an early run of amplification may later on be amplified. The Pyrosequencing methodology is a synthesis process, typically, a "wash-and-scan" technique, where the DNA polymerase halts between read steps and may introduce mutation errors. Furthermore, It has been shown that mutation errors

in pyrosequencing reads tend to occur predominantly in homopolymeric regions (17, 27). In the present study we did not assess the error rate of the present assay; however, others have estimated the overall error rate of the pyrosequencing technique to be on the average of 0.3% (12, 28). It was also previously shown that the pyrosequencing error rate for detection of 17 known drug resistance mutations, located at homopolymer regions in the PR and RT genes of subtype B viruses, was <0.71% (17). Nevertheless, it was recently reported that the RT K65R mutation that is located at a homopolymer region of subtype C viruses had a pyrosequencing error rate of detection of up to 1.3% (27). Others also reported an error rate of up to 3.8% for this mutation in subtype C viruses (23). Our study was focused on drug resistance mutations at a level above 1%. Of the low-abundance DRMs, 52% were at levels of 1 to 4%, and 48% were at levels of 5 to 19%. We did not take into consideration potential error rates that might be derived from DRMs located at homopolymer regions of different HIV-1 subtypes, as demonstrated for the K65R mutation in subtype C viruses. This issue should be further investigated. Our pyrosequencing read coverage, however, was reliable. The average reads were 10,000 reads/patient (range, of 3,736 to 16,643 reads), which amounted to an average of 2,500 reads for each of the four amplicons. Such coverage was sufficient to detect variants at a 1% frequency with ~25-fold coverage depth. Even though the number of reads varied between amplicons of the same patient, the coverage depth for detecting 1% variants was never fewer than 10 reads. Nevertheless, given these reservations, one might consider in the future to include, in each run, a wild-type HIV plasmid in order to control for inherent errors. This, however, will require the use of prototype plasmids representative of the different HIV subtypes that are usually tested.

Finally, compared to other similar next-generation sequencing technologies, the GS Junior system has the lowest error rate and the longest read length on the average 400 bp (12). The lab-scale benchtop platform of this prototype assay can easily be adopted for use in the clinical laboratory. The HIV UDPS assay described here can be carried out by a single trained technician and be completed in 3 days, including analysis of the results. In conclusion, although the clinical relevancy of detecting HIV minor populations of mutated viruses is still open to debate, and the threshold above which these minor mutated viruses might significantly influence the virological response to ARV therapy still remains unclear, it is hoped that the wider application of such an assay in the HIV diagnostic lab will help resolve these important issues.

ACKNOWLEDGMENTS

R.F. and I.V.S. are employed by Dyn Diagnostics, which is an official representative of Roche Diagnostics in Israel. The other authors declare no competing financial interests.

Roche provided the HIV-drug resistance prototype plates for this study, free of charge. Dyn Diagnostics provided the ultradeep pyrosequencing support, reagents, and expertise needed for this study. Neither Roche nor Dyn Diagnostics had a role in study design and analysis of the resulting data.

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