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Short communication

Evaluation of GS Junior and MiSeq next-generation sequencing technologies as an alternative to Trugene population sequencing in the clinical HIV laboratory

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A B S T R A C T

Population HIV-1 sequencing is currently the method of choice for the identification and follow-up of HIV-1 antiretroviral drug resistance. It has limited sensitivity and results in a consensus sequence showing the most prevalent nucleotide per position. Moreover concomitant sequencing and interpretation of the results for several samples together is laborious and time consuming. In this study, the practical use of GS Junior and MiSeq bench-top next generation sequencing (NGS) platforms as an alternative to Trugene Sanger-based population sequencing in the clinical HIV laboratory was assessed. DeepChek®-HIV TherapyEdge software was used for processing all the protease and reverse transcriptase sequences and for resistance interpretation. Plasma samples from nine HIV-1 carriers, representing the major HIV-1 subtypes in Israel, were compared. The total number of amino acid substitutions identified in the nine samples by GS Junior (232 substitutions) and MiSeq (243 substitutions) was similar and higher than Trugene (181 substitutions), emphasizing the advantage of deep sequencing on population sequencing. More than 80% of the identified substitutions were identical between the GS Junior and MiSeq platforms, most of which (184 of 199) at similar frequency. Low abundance substitutions accounted for 20.9% of the MiSeq and 21.9% of the GS Junior output, the majority of which were not detected by Trugene. More drug resistance mutations were identified by both the NGS platforms, primarily, but not only, at low abundance. In conclusion, in combination with DeepChek, both GS Junior and MiSeq were found to be more sensitive than Trugene and adequate for HIV-1 resistance analysis in the clinical HIV laboratory.

1. Paper

Resistance to antiretroviral drugs is a major obstacle in effective and long-term treatment of human immunodeficiency virus 1 (HIV-1) infections. In treatment naïve individuals, minority variants of resistance may reduce anti-retroviral response altering the natural course of the disease (Gianella and Richman, 2010). In treated patients, incomplete adherence to treatment causing insufficient drug concentrations, might sustain such low frequency resistance mutations (Fisher et al., 2012).

Population sequencing suffers from limited sensitivity detecting mutations present in at least 20% of the viral population (Avidor et al., 2013; Le et al., 2009; Schuurman et al., 2002).

Abbreviations: NGS, next generation sequencing; DRMs, drug resistance mutations; GSJ, GS Junior.

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Table 1
Demographics, clinical information and NGS coverage.

Patient number	Sex	Current age (years)	Risk group [a]	Treatment	HIV sub-type	VL (copies/ml)	CD4 (counts)	GSJ Coverage per PR_RT (average) [b]	MiSeq Coverage per PR_RT (average) [b]
2333	M	28	MSM	–	B	120,000	483	1646	21,750
2334	F	NA	OGE-F	–	C	830,000	NA	1548	11,414
1188	M	30	MSM	–	B	24,000	437	1282	20,310
1939	F	39	OGE-IL	–	C	6500	436	922	21,389
2352	M	37	MSM	–	F	500,000	429	1875	19,883
2498	M	52	MSM	–	B	53,000	NA	867	22,705
2261	M	23	MSM	Truvada, Atazanavir	B	620	596	1130	25,945
2054	M	31	MSM	Truvada, Atazanavir	B	510	494	1015	18,876
2275	F	40	NA	Truvada, Atazanavir	A	980	NA	1362	17,959

[a] Risk group: MSM = men having sex with men; OGE-IL/F = originating in country with generalized HIV epidemic – Israeli/foreigner. VL = viral load; NA = not available.
[b] The DeepChek®-HIV TherapyEdge tool was used to assess PR-RT coverage by counting the average number of reads covering amino acids 4-99 and 40-247 in the PR and RT proteins.

Next generation sequencing (NGS) capable of detecting also the minor variants was suggested as a better alternative for surveillance of transmitted drug resistance mutations and for monitoring anti-HIV-1 therapy failures (Archer et al., 2012b; Armenia et al., 2012; D'Aquila et al., 2011; Fisher et al., 2012; Le et al., 2009). The low cost high coverage MiSeq (Illumina) as well as PGM IonTorrent (ABI) and the long reads GS Junior (GSJ, Roche) bench-top NGS machines, which are suitable for the limited space available in several clinical virology laboratories, are expected to increase the sensitivity of HIV-1 mutation detection (Fisher et al., 2012; Shendure and Lieberman Aiden, 2012). However these technologies generate huge amount of sequencing data requiring special bioinformatics and data mining tools and unlike Trugene Open Gene system (Trugene, Siemens Healthcare, Malvern, USA), an FDA approved HIV-1 resistance testing population sequencing technology, do not produce an integrated clinically relevant solution for monitoring HIV drug resistance. DeepChek®-HIV (ABL, Luxemburg), a CE marked downstream analysis software which allows an automated sequencing analysis for both next generation and population sequencing, and includes interpretation and clinical reporting of results which is regularly updated with the most recent drug resistance information may be a useful software in laboratories which lack in-house tools for large data analysis (Paredes et al., 2012).

The goal of this study was to assess the practical use of such bench-top platforms together with DeepChek in the clinical HIV laboratory. The sequencing results created by GSJ and MiSeq and obtained using the recommended protocols for each system, were compared to Trugene population sequencing and the pattern of HIV-1 resistance mutations was assessed using the DeepChek-HIV version 1.1 software. Residual plasma samples collected from nine HIV-1 carriers were included. 1 ml plasma aliquots were used for HIV-1 RNA extraction using NucliSENS Easy MAG total RNA extraction system (Biomerieux, Marcy l'Etoile, France), which allows high extraction efficiency of viral RNA compared to other systems (Dundas et al., 2008; Shulman et al., 2012). Roche GSJ platform samples were processed using a prototype of the 454 HIV-1 library preparation kit, providing 4 partially overlapping amplicons spanning the protease (PR) and reverse transcriptase (RT), according to the manufacturer's protocol (454, Roche, Branford, CT). An HIV-1 PR and RT fragment (1.8 kb) was amplified according to published protocols (Snoeck et al., 2005) and used for preparation of paired end indexed libraries for MiSeq, using Nextera DNA sample preparation kit (Illumina, San Diego, CA). All multiplexed libraries were spiked with PhiX to improve cluster detection by increasing base complexity.

GSJ sequences were preprocessed with amplicon variant analyzer software version 2.6 (454 Life Sciences, Roche). MiSeq sequences were preprocessed using sickle (Joshi and Fass, 2011) and Burrows-Wheeler Aligner, BWA, (Li and Durbin, 2010) and converted to a sorted bam file (Li et al., 2009). Mapping was done using HIV-1 HXB2 K03455 as reference. Only reads included in all sequencing platforms (amino acids 4-99 in the PR and 40-247 in the RT) were selected for further analysis. The resulting alignment files from GSJ, MiSeq and the fasta files from Trugene were all analyzed with DeepChek-HIV version 1.1 (ABL, Luxemburg) using the integrated Stanford HIV resistance interpretation tool (Shafer, 2006). When Trugene sequences were analyzed, an identical set of amino acid substitutions was identified by both Trugene Open Gene system and the DeepChek tool (data not shown), suggesting that DeepChek is capable of correctly interpreting population sequencing data. A cutoff of 3% of the sequences was selected for mutation detection, which is higher than the reported calculated 0.1–1% sensitivity of various NGS platforms (Glenn, 2011), to further increase the validity of the results especially in the low viral load samples. Paired Student *T* test (two tailed) was used to compare the total number of amino acid variants detected by the systems. Bland–Altman analysis was used to assess the level of agreement between the NGS platforms by plotting the percent variant differences between MiSeq and GSJ against the average of the two measurements. More information on library preparation and sequencing analysis is provided in supplement 1.

Samples from six naïve and three drug treated patients that represent the main risk groups and the various viral subtypes typical for Israel were included in this study (Table 1). As expected (Beerenwinkel et al., 2012), MiSeq had 10 times more reads per any amino acid compared to GSJ. However, the minimum GSJ average coverage was in most cases above 1000 reads per PR and RT position (except a single GSJ sample of 867 reads), sufficient for correct HIV-1 mutation detection, even for low frequency variants (Gall et al., 2012).

The total number of amino acid substitutions identified in the nine samples by the NGS systems was similar and higher than Trugene (232 substitutions by GSJ, 243 by MiSeq and 181 by Trugene, Table 2). Bland–Altman analysis showed similar performance of both GSJ and MiSeq with no tendency for detection of higher or lower frequency variants by either methods (Fig. 1S).

The number of amino acid substitutions identified by the three platforms is shown in Table 2. 199 of all amino acid substitutions (85.7% of GSJ substitutions and 81.9% of MiSeq substitutions) were identified by both NGS platforms, 92.5% of which (184/199) were identified at similar frequency (delta difference <20% between the identified frequencies). 170 of these 199 substitutions were also identified by Trugene. Most of the substitutions detected by the NGS platforms were present at >20% of the sequences. However,

Table 2
Number of amino acid substitutions detected by GSJ or MiSeq (MIS), at above a threshold of 3%, and by Trugene (TG) ($n = 9$).

	PR			RT			PR and RT		
	GSJ	MIS	TG	GSJ	MIS	TG	GSJ	MIS	TG
Identified by GSJ & MIS & TG	77	77	77	93	93	93	170	170	170
Identified by GSJ & MIS only	13	13	0	16	16	0	29	29	0
Identified by GSI & TG only	0	0	0	1	0	1	1	0	1
Identified by MIS & TG only	0	1	1	0	6	6	0	7	7
Identified by GSJ only[a]	13	0	0	19	0	0	32	0	0
Identified by MIS only[a]	0	14	0	0	23	0	0	37	0
Identified by TG only	0	0	1	0	0	2	0	0	3
Total number of substitutions[b]	103	105	79	129	138	102	232	243	181

[a] The median frequency of these substitutions was 8.7% and 6.5% for GSJ and MiSeq, respectively.
[b] The total number of substitutions >3% frequency was significantly different between MiSeq and Trugene ($p < 0.005$, 95% CI: 2.91–10.87) and between GSJ and Trugene ($p < 0.009$, 95% CI: 1.92–9.41). No significant difference in the total number of such substitutions was found between GSJ and MiSeq.

21.9% (51/232 in GSJ) and 20.9% (51/243 in MiSeq) were present in less than 20% of the sequences of which more than 85% were not identified by Trugene. While 21 of these GSJ minority variants and 18 of the MiSeq minority variants were also identified by MiSeq or GSJ respectively, 58.8% (30/51) and 64.7% (33/51) were GSJ or MiSeq specific. Few amino acid substitutions present at a frequency above 20% in at least one or both NGS outputs (20 in MiSeq and 13 in GSJ) were missed by Trugene. When reexamining the Trugene chromatograms of all these major substitutions, most (e.g. RT-V90I of patient 2334, PR-I13V for patient 1939, PR-I15V for patient 2352, supplement 2) were identified as a very minor mixed peak which was not reported by Trugene. Taken together, these results show that though the majority of the substitutions were identical between the three platforms, at least some of them were platform specific. Also, the frequency of several of the identified substitution varied between the NGS technologies. It is difficult to determine which of these different variations is real and what is the correct frequency of each substitution without validation with a different technology like allele specific PCR. Analysis of sequence variations between different samples from a single patient should better be performed by a single method to increase consistency and enable better long term comparison of the results.

Most of these identified substitutions are not known to be of clinical relevance but some do cause HIV-1 drug resistance (Shafer, 2006). When the three platforms were compared, additional drug resistance mutations (DRMs) were identified by the GSJ and MiSeq platforms (Table 3), rendering more anti-retroviral drugs to be ineffective. DRMs were identified in samples from the drug-naïve patients 2333 and 2334 and in samples from patients 2261 and 2275, for which DRMs were identified by NGS platforms only. Indeed, using similar technologies, Avidor et al. (2013) and Stelzl et al. (2011) also identified additional low abundance DRMs by GSJ compared to population sequencing. Though most DRMs identified (e.g. RT-V90I, RT-Y181C and RT-T69AN for patient 2334) were detected by both NGS platforms, some, like the RT-D67G and the RT-K65R mutations (for patients 2333 and 2334 respectively) were identified by only one platform. These mutations are located adjacent to homopolymers and therefore could be regarded as artifacts (Archer et al., 2012a). However, in these cases they were observed as a single stranded very minor variant in Trugene chromatograms (data not shown) suggesting that they are real. A single mutation was Trugene specific. At position RT-T215 in sample from patient 2333, the NGS platforms identified T215L substitution in >95% of the sequences, while substitutions to both phenylalanine and the leucine were found by Trugene. All other Trugene DRMs were identified by both NGS platforms. Recently a multicenter study comparing HIV-1 drug resistance testing by 454 Life Sciences/Roche GS FLX platform showed that deep sequencing results were highly consistent between and within different laboratories and concluded that 454 is accurate and highly reproducible method for HIV-1 drug resistance testing (Simen et al., 2014). In the future it will be beneficial to perform similar multicenter comparison utilizing DeepChek and each of these MiSeq and GSJ newer NGS technologies.

The present study has several limitations. First, the amplification and library preparation protocols were different between the platforms possibly increasing the variability between the sequencing results. Also, the number of HIV genomic templates added into each of the systems following PCR reaction was not calculated (it was normalized based on the extracted RNA 260/280 ratio). On the other hand, the HIV copy number input per sample was known and was similar between the NGS systems. Also, similar sequence outputs were obtained by the various systems in samples with different HIV-1 subtypes suggesting that system specific sequencing errors were not common. Indeed, many identical amino acid substitutions, including low abundance mutations, were identified

Table 3
DRMs identified by MiSeq, GSJ and Trugene.

Patient Number	MiSeq			GSJ			Trugene		
	PI	NRTI	NNRTI	PI	NRTI	NNRTI	PI	NRTI	NNRTI
2333	L90M (97%)	T215L (98.7%)	A98G (97.9%) K103N (96.5%) E138A (98.3%)	L90M (99%)	D67G (9.7%) T215L (98.2%)	A98G (99.6%) K103N (99.5%) E138A (99.4%)	L90M	T215LF	A98G K103N E138A
2334	NONE	K65R (4.1%) T69AN (29.0%)	V90I (38.0%) E138A (14.9%) Y181C (62.0%)	NONE	T69AN (5.0%)	V90I (5.4%) E138A (33.8%) Y181C (7.5%)	NONE	NONE	E138A
1188	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
1939	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
2352	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
2498	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
2261	NONE	D67N (6.5%)	NONE	NONE	NONE	NONE	NONE	NONE	NONE
2054	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
2275	G73C (24.4%)	T215I (17.6%)	NONE	NONE	NONE	L100F (9.4%)	NONE	NONE	NONE

PI – protease inhibitors, NRTI – nucleoside reverse-transcriptase inhibitors; NNRTI – non-nucleoside reverse-transcriptase inhibitors.

by both the NGS systems. Secondly, because the calculated NGS error rate is below 1% (Jiang et al., 2012; Zagordi et al., 2010) in previous studies 1% cutoff was selected for identification of HIV-1 DRMs (Avidor et al., 2013; Buzon et al., 2011; Codoner et al., 2011). In this study a more stringent threshold of 3% was employed to compensate for the differences in viral loads and the NGS output sizes (Simen et al., 2009), therefore, very low frequency variants, some of which could have been clinically relevant may have been lost. The low-frequency and platform unique DRMs identified in this study, which could not be distinguished by population sequencing, require confirmation and should be assessed by other approaches. As comparative studies of genotypic assays for HIV-1 mutant detection have demonstrated the difference in performance of different assays, employing more than one assay, like allele specific RT-PCR and the Ty1HRT yeast system, may be beneficial (Halvas et al., 2006). Also, in cases with low viral load, extraction of RNA from larger volumes should be considered to increase the viral copy numbers and enable lower cutoff for mutation detection. This study did not attempt to assess the clinical relevance of the identified DRMs. To gain such information, follow-up of the studied patients is required. To this end, different patient's samples should be compared over time, especially as there is no clear evidence about how abundant a mutation must be to impact drug resistance (Messiaen et al., 2012).

Another limitation of this study is that it did not include samples with predefined mixtures of viruses. Though such samples would have assisted in evaluating the accuracy of the deep sequencing platforms, others have already shown that NGS systems produce valid results (Hedskog et al., 2010; Li et al., 2014; Pou et al., 2014). To enable correct counting of absolute number of viruses, tagging (Kivioja et al., 2011) or employment of non-PCR amplified samples could be considered for all platforms.

In conclusion, the comparison between MiSeq and GSJ to Trugene, which is considered to be a "gold standard" method for identification of HIV-1 drug resistance (Zhou et al., 2011) showed that these methodologies identified the high abundance HIV-1 resistance mutations detected by Trugene though unique mutations were detected by each system. Some of the NGS resistance mutations were not identified by Trugene. Though these require corroboration, they are representatives of the higher resolution of these NGS technologies. DeepChek-HIV software simplified the analysis of all sequencing results and provided a clinically meaningful report including interpretation of all the DRMs.

Both GSJ and MiSeq could provide practical solution for monitoring HIV-1 drug resistance. Selection of the optimal bench-top NGS platform for a clinical HIV laboratory should be based on availability, price, cost per sample (estimated in Israel to be 100 EU and 350 EU for MiSeq and GSJ respectively) and convenience. With the high cost (525 EU per sample), laborious nature and time required for each sample using Trugene analysis, both GSJ and MiSeq are a suitable alternative especially when many samples are processed simultaneously (Dudley et al., 2012). Moreover, if a software like DeepChek which is updated regularly according to the resistance guidelines and which costs 30–50$ per sample (depending on the volume of samples analyzed per year) is used for automated analysis and interpretation of the NGS data from more than 8 samples concomitantly (the number of samples recommended to be processed together by Trugene), a lower total cost per sample and a higher turnaround time is expected rendering these platforms superior to population sequencing technologies.

2. Funding

DYN LABS Ltd. provided the Roche library preparation kit and the NGS support for GSJ needed for this study, free of charge. ABL provided the DeepChek®-HIV TherapyEdge software and the relevant expertise free of charge.

3. Competing interests

DG is an employer of ABL, Therapy Edge. RF is an employer of Dyn Diagnostics, official representative of Roche Diagnostics in Israel. Roche, DYN LABS Ltd. and ABL TherapyEdge had no role in study design and analysis of the resulting data. All other declare not competing financial interests.

4. Ethical approval

The study was approved by the ethical committee of the Sheba Medical Center (8293-10-SMC).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.11.003.

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