HIV-1 GENOTYPIC DRUG RESISTANCE TESTING: DIGGING DEEP, REACHING WIDE?

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Abstract
For many years, population-based Sanger sequencing has been the golden standard for drug resistance testing within the routine follow-up of HIV-1 infected patients in resource-rich settings. Often, the data generated within this framework were subsequently used for research and surveillance purposes: to understand therapy response and to gain insights into epidemiological processes. Sanger sequencing was however ill suited for diagnostic and prognostic use in resource-limited settings (RLS) and therefore not broadly implemented. Next-generation sequencing (NGS) technologies provide high-throughput approaches by the rapid acquisition of thousands to millions of short nucleotide sequences. Depending on the experimental design, the roll-out of NGS drug resistance testing at a larger scale is feasible, providing better characterization and understanding of the evolving population of viral variants within a patient and potentially improving the prognostic value of drug resistance testing. Whether the same will become true for RLS will largely depend on affordability and sample logistics, and this may affect other mutation-specific approaches.

Introduction

Combination antiretroviral therapy (cART) has significantly improved survival rates and quality of life of patients infected with the Human Immunodeficiency Virus type 1 (HIV-1). However, lifelong treatment and medical follow-up are required as a therapeutic cure remains elusive, while host-, virus- and drug-related suboptimal treatment and adherence are accompanied with emerging drug resistance and subsequent transmission of drug-resistant strains which jeopardize the advancements in HIV-1 care [1].

In particular, the high evolutionary rate of HIV-1, driven by the high mutation rate during reverse transcription, results into a heterogeneous viral population within HIV-1 patients [2]. When virus replication cannot be shut down during treatment, the competitive advantage of a minority variant with higher fitness under drug selective pressure will result in an increase of its frequency ultimately allowing resistance mutations to accumulate and treatment to fail.

Since many years, drug resistance testing has enabled personalized strategies for the treatment of HIV-1 infected patients in resource-rich settings (RRS) (Table 1) [3]. At diagnosis and entry into care, such testing is to uncover transmitted drug resistance (TDR) while at virological failure, causality assessment of failure is the goal. In both instances, the detected mutation profile guides the clinician in the subsequent selection of a potent cART, although current methods have limitations in explaining and predicting therapeutic outcome [4]. In the absence of simple and affordable resistance tests, resource-limited settings (RLS) opted for a public health approach with the implementation of drug resistance surveillance at sentinel sites to guide the decisions on country-wide cART programs [5].

Recent technical innovations may enable the roll-out of new and powerful genotypic testing in both RSS and RLS and contribute to a more comprehensive assessment of the emergence and spread of drug resistance and how to maintain long-term suppressive cART. Implementation of the various strategies should however be preceded by an evaluation of their performance in the context of a changing HIV epidemic.

Population-based Sanger sequencing as current golden standard for drug resistance testing in clinical virology

For many years, routine monitoring of drug resistance is predominantly done using population-based Sanger sequencing (PBSS) due to its superior characteristics compared to phenotypic resistance assays and mutation-specific assays. Although the interpretation of complex mutational patterns into clinically relevant cut-offs is not straightforward, genotyping is characterized with shorter turnaround times, lower cost, greater accessibility and an earlier detection of emerging drug resistance than phenotyping (Table 2) [6]. In comparison to Line Probe Assay (LiPA), a technology that displayed a higher analytical sensitivity for minority variants and enabled the simultaneous investigation of several mutations, PBSS provided more complete information on drug resistance and proved to be more reliable [7].

Next-generation sequencing technologies

Several next-generation sequencing (NGS) technologies are currently being explored for HIV drug resistance testing [8]. The 454-pyrosequencing method (Roche) used to be the preferred technology because of its longer read lengths (400-700bp), but uncompetitive prices and frequency of indel errors (~1%) recently resulted in its discontinuation. These errors were especially detrimental for HIV-1 drug resistance testing as they mainly occur in homo-polymer stretches which are commonly found within or adjacent to HIV-1 codons associated with drug resistance [9]. Although suffering from similar problems inherent to the pyrosequencing methodology, Ion Torrent partially circumvents the problem through template barcoding and bioinformatics pipelines and is still clinging to the market because of its highly competitive pricing and shorter run times. The main player in the field is currently Illumina, given its simple work flow and lower rate of systematic errors (~0.1%, substitutions), although long run times and shorter read lengths (125-250bp) are still disadvantages. While local reconstruction of minority variants can identify the co-occurrence of resistance

mutations in a single variant when their genomic distance is lower than the average read length, inferring larger sequence fragments of minority variants is challenged by the short read lengths and high error rates [10,11]. The latest innovative technologies that could be a major improvement in this respect are Pacific Biosciences and Oxford Nanopore Technology that both enable single molecule, real-time DNA sequencing of extensive read length (≥1000bp). Their breakthrough is still pending due to their high error rates (≥12% at single runs) and costs.

Changing scenes in antiviral therapy and drug resistance

Since a few years, the prevalence of virological treatment failure and acquired drug resistance have been declining significantly in RRS [12]. These recent successes are mainly attributed to the introduction of new drug classes and to more potent, tolerable and convenient drugs within existing classes, as well as to resistance-guided therapy.

Despite this decline in acquired resistance, levels of TDR have remained relatively stable over 1999-2013 although TDR rates and trends varied by geographical region, risk group and drug class [13]. An in-depth analysis demonstrated that TDR slightly increased during times lacking access to new drugs and only sharply decreased upon the introduction of a new potent drug class, such as boosted protease and integrase inhibitors, keeping the overall TDR trend stable [14]. This is a potential cause of concern when drug pipelines dry up. Therefore, TDR will remain a problem to be monitored.

By contrast, drug stock-outs and insufficient laboratory monitoring still challenge the long-term success of cART in RLS. Several studies have shown increasing levels of transmitted and acquired drug resistance in these regions [15-16]. As non-B subtypes prevail and other drug combinations than the ones used in RRS have been used, drug-selected amino acid substitution patterns might be different and their impact on therapy outcome unclear [17,18]. The need for drug resistance testing is thus increasing in RLS.

Ultra-wide next-generation sequencing for genotypic drug resistance testing

With Trugene HIV-1 Genotyping Assay (Siemens) abandoned, ViroSeq HIV-1 Genotyping System (Abbott Molecular) is the only FDA-cleared and CE marked propriety system for in vitro diagnostic use of drug resistance testing. As a result, many virology laboratories have switched to in-house methods to address the need for simultaneous drug resistance testing against all six drug classes. They require more extensive optimization, standardization and validation efforts than commercial assays (Table 2) [19]. Furthermore, with an ever larger number of genetic regions becoming clinically relevant, PBSS costs and turnaround times are rising steadily.

NGS may bridge this gap, as it could enable the reliable sequencing of more extended and even problematic (by PBSS methods) genetic regions (Figure 1) (Table 2) [20,21]. Ultra-wide sequencing of the full-length viral genome, generating additional information on amino acid substitutions in Gag, Pol and Env, could enhance therapeutic outcome predictions [22-27].

For the required template enrichment, the current full-genome protocols rely on amplification of large genomic regions with subtype-wide primers and therefore perform less well at viral loads below 1000-3000 copies/ml, making these assays currently more appropriate for surveillance efforts than for monitoring of individual patients [28,29]. However, several studies have already shown that experienced laboratories can achieve accurate NGS results at frequencies as low as 1% [30-33]. Although sequencing reagents cost per sample drops significantly when multiplexing is considered, this up-scaling requires more experimental steps and extensive in-silico analyses which increase direct and indirect costs, risk for human error and turn-around times (Table 2).

Far-reaching innovations: mutation-specific assays versus next-generation sequencing for surveillance in resource-limited settings

In view of the paucity of PBSS for routine monitoring in RLS, mutation-specific assays have been developed to meet the need for large-scale drug resistance surveillance at a reasonable cost [34,35]. These assays are based upon selective amplification, hybridization or ligation at only a limited

number of pre-defined sentinel codons, but with the advantage of detecting minority variants below the threshold of PBSS (Table 2) [36].
Although mutation-specific assays are considered to be easy to perform and therefore suitable for implementation at local sites, experience with the specific methodology were important factors for achieving valid results [37]. In addition, primers and probes needed to be modified to the locally circulating viral variant of a subtype or, more extremely, to the patient-specific consensus sequence to decrease the number of indeterminate and inaccurate results [37-39]. It seems the problems formerly experienced by LiPA have not all been solved.
Therefore, other research teams opted for the implementation of more complex NGS methods in centralized national or regional laboratories for the surveillance of drug resistance and the investigation of transmission dynamics [40].

Mutation-specific assays versus next-generation sequencing for sensitive detection of drug resistance

Advances in resistance genotyping for monitoring individual patients are expected to come from a better knowledge of the impact of minority resistant variants. Studies have demonstrated that the use of mutation-specific assays and ultra-deep sequencing results in higher drug resistance levels in therapy-naïve and –experienced patients compared to PBSS [41-49].
However, this effect was strongly depended on the number of mutations evaluated, the experimental set-up (detection cut-off used), the clinical context (drug exposure [50,51], time elapse since infection or drug exposure [52,53]), and the specific drugs or mutations evaluated [54,55].
The transmission dynamics within a population and the fitness cost of a particular mutation affects the relevance of more sensitive testing. In RRS, the rarely observed M184V in recently-infected patients is most often transmitted from patients failing virologically, even though therapy-naïve patients seem to be the major source of new infections and therefore of TDR [56]. M184V has a high fitness cost, and thus its reversal and gradually decay in a drug-free environment prevents its onward transmission, in contrast to low fitness cost drug resistance mutations, such as M41L, T215 revertants and K103N [57]. This might explain why sensitive testing does not always result in higher TDR rates in chronically infected patients [58].

Predictive value of ultra-deep next-generation sequencing for therapeutic response

In recent years, studies have suggested that drug resistant variants not detected by PBSS may influence the response to first-line cART [59]. Consequently, mutation-specific assays and NGS methods have been used to determine the impact of drug-resistant minority variants on therapeutic outcome in several retrospective observational studies.
The relevance of testing for minority variants has been shown primarily for maraviroc and non-nucleoside reverse transcriptase inhibitors (NNRTI). When retrospectively reanalyzing patients treated with maraviroc-containing cART, prediction of treatment outcome was improved when using assays with enhanced sensitivity for CXCR4-using variants [60-62]. Although the presence of CXCR4-using variants was associated with an increased risk of therapeutic failure, yet, the superiority of ultra-deep sequencing over PBSS has not been shown.
HIV-1 patients with NNRTI-resistant variants that could only be detected as minority variants show a more than 2-fold higher risk of virologically failing an NNRTI-containing first-line regimen. In some studies, a dose effect of the frequency or mutational load was shown [63,64]. The clinical relevance of NNRTI resistant minority variants has not consistently been confirmed, and has not been demonstrated for other first-line regimens, such as those containing integrase or boosted protease inhibitors, therefore guidance on the use of deep sequencing is still lacking.
In the context of virological failure, an improved predictive performance for success of salvage regimen was shown by Pou *et al.* in a retrospective analysis, while Charpentier *et al.* failed to demonstrate a similar effect [54,55,65]. An added complexity is that current NGS methods need viral loads that are higher than usually found at treatment failure to reliable detect minority variants.

Although sensitive methods possess a great potential in improving the prognostic value of drug resistance testing, to date, technical improvements and a better understanding of the impact of minority resistant variants are required before proceeding to clinical studies, sufficiently powered for the assessment of ultra-deep sequencing as a prognostic tool.

Digging deep and reaching wide

In contrast to mutation-specific assays that are limited to the detection and quantification of particular drug resistance mutations, NGS has the potential to reconstruct the viral population by identifying the set of individual virus variants with their respective frequency or mutational load (ultra-deep and ultra-wide sequencing). Information on the co-occurrence of drug resistance and accessory mutations at the level of individual viral variants could lead to a better understanding of viral escape dynamics from therapeutic pressure. By distinguishing minority variants with single drug resistance mutations that were merely the result of random genetic variation from variants that have subsequently further adapted to therapeutic pressure, NGS could potentially lead to more accurate predictions of therapeutic response [51].

Conclusion

The decision whether new technologies are fit for HIV-1 drug resistance testing and can transit from research purposes to clinical and epidemiological settings will largely depend on the strength of findings from large studies. High-throughput technologies can clearly contribute significantly to more extensive surveillance of drug resistance globally. Although, consistent results that could support the prognostic use of ultra-deep sequencing for individual patients are lacking to date, routine follow-up by ultra-wide sequencing with a reporting limit set at depths similar to PBSS might be a pragmatic approach and readily implemented (Figure 1). From such efforts, continuous growth of data and knowledge will speed up the development of standardized drug resistance interpretation systems and guidance documents for the clinical implementation of NGS, as has been done for PBSS [3,66]. An eventual introduction into clinical practice will ultimately depend on specifications and performance characteristics (Table 2). The biggest challenges relate to wet-lab automation and a bioinformatics pipeline [11], which are not straightforward for many virology laboratories. These hurdles could be partly surpassed by centralizing HIV drug resistance testing or partnering with genomics core centers, and the development of user-friendly software enabling a standardized approach for the reconstruction of minority variants, the detection of drug resistance and the prediction of therapeutic outcome.

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Table 1: HIV-1 drug resistance testing in clinical and epidemiological virology

Purpose of test:	
Diagnostics	To detect transmitted drug resistance at diagnosis
	To detect (naturally occurring) drug resistance before therapy initiation [a]
	To detect selected drug resistance at therapeutic failure
Prognostics	To improve therapeutic outcome

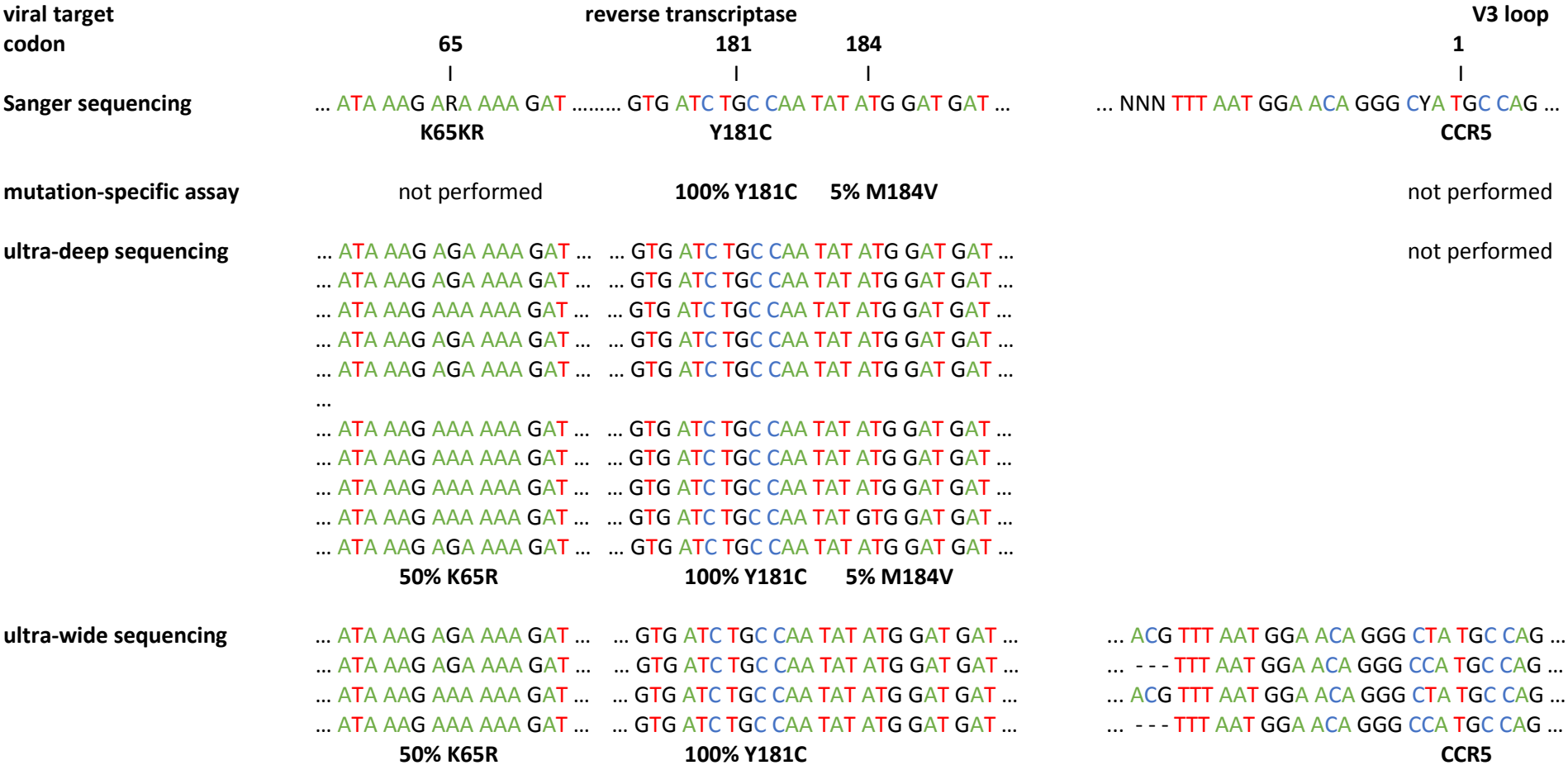
[a] Naturally occurring drug resistance defined as the presence of polymorphisms or viral variants that could negatively impact activity of antiviral drugs (e.g. A98GS or E138A in reverse transcriptase, E157Q in integrase, CXCR4-using variants, …) [17,18,67,68].

Table 2: Characteristics to assess clinical relevance of HIV-1 drug resistance assays

Specifications to assess whether test is fit for purpose: accurate, timely, accessible, understandable, comparable, coherent, complete and reasonably priced		
Advantages and disadvantages of the different technologies		
Population-based Sanger sequencing	advantages: turnaround time of a few days, easily implemented into molecular biology lab, reasonable cost ($40-80)[a] disadvantages: low analytical sensitivity for minority variants, no genetic linkage, no consensus sequence for regions displaying mixtures of indels (insertions and deletions)	
Mutation-specific technology	advantages: high analytical sensitivity for minority variants, shortest turnaround time, easiest to be implemented into molecular biology lab, easy interpretation, qualitative and quantitative results, relative inexpensive ($40)[a], suitable for large batches disadvantages: only sentinel positions, lower analytical specificity, no genetic linkage	
Next-generation sequencing	advantages: high analytical sensitivity for minority variants, genome-wide approach, potential of genetic linkage, qualitative and quantitative results, low reagent costs ($100)[a], suitable for large batches disadvantages: sequencing errors, turnaround time, need for multidisciplinary team (wet-lab and in-silico), high indirect costs (equipment, labor)	
Common	disadvantages: errors during template enrichment by RT-PCR (mutation, recombination), false negative results due to genetic variability at annealing/hybridization sites, resampling and stochastic amplification at low template concentrations	
Evaluation of performance characteristics:		
	Analytical characteristics	*Diagnostic characteristics*
Approved commercial assay [b]	Measurement range, precision, trueness, linearity [d]	Reference range [d]
In-house assay [c]	Detection limit, quantification limit [d], robustness, analytical sensitivity and specificity, selectivity	Diagnostic sensitivity and specificity

[a] Reagent cost for monitoring protease and reverse transcriptase with in-house methods [35,69]. [b] Characteristics that should be evaluated for the verification of an approved commercially available assay. [c] Characteristics that additionally should be evaluated for the validation of an in-house assay. [d] Characteristics that should additionally be evaluated for quantitative assays.

Figure 1: Consequences of applying various methods for genotypic drug resistance testing.
The consensus sequence obtained with population-based Sanger sequencing from a patient infected with HIV-1 CRF02_AG and failing a combination of tenofovir (TDF), lamivudine (3TC) and nevirapine (NVP), displays a mixture at reverse transcriptase (RT) position 65 (associated with TDF resistance) and a pure mutation at RT position 181 (associated with NVP resistance). An *env* consensus sequence cannot be obtained for the region immediately upstream of the V3 loop due to mixtures of viral variants with and without a deletion.
Mutation-specific assays do not monitor the rare K65R position in RT and the V3 loop, but viral variants displaying the 3TC mutation M184V are detected at a low frequency (5%). Ultra-deep sequencing gives in-depth information on the resistance profile within the genetic region covered, quantifying 50% K65R, 100% Y181C and 5% M184V. In an ultra-wide approach of next generation sequencing, more regions are covered, but at a lower sensitivity and therefore missing the M184V-containting variants.


viral target
reverse transcriptase
V3 loop
codon
65
181
184
1
Sanger sequencing
… ATA AAG ARA AAA GAT …… GTG ATC TGC CAA TAT ATG GAT GAT …
… NNN TTT AAT GGA ACA GGG CYA TGC CAG …
K65KR
Y181C
CCR5
mutation-specific assay
not performed
100% Y181C
5% M184V
not performed
ultra-deep sequencing
… ATA AAG AGA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT …
… ATA AAG AGA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT …
… ATA AAG AAA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT …
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… ATA AAG AAA AAA GAT … … GTG ATC TGC CAA TAT GTG GAT GAT …
… ATA AAG AGA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT …
not performed
50% K65R
100% Y181C
5% M184V
ultra-wide sequencing
… ATA AAG AGA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT … … ACG TTT AAT GGA ACA GGG CTA TGC CAG …
… ATA AAG AGA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT … … - - - TTT AAT GGA ACA GGG CCA TGC CAG …
… ATA AAG AAA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT … … ACG TTT AAT GGA ACA GGG CTA TGC CAG …
… ATA AAG AAA AAA GAT … … GTG ATC TGC CAA TAT ATG GAT GAT … … - - - TTT AAT GGA ACA GGG CCA TGC CAG …
50% K65R
100% Y181C
CCR5