HAART Therapy—Antiretroviral Resistance Genotyping

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INTRODUCTION

Although the introduction of highly active antiretroviral therapy (HAART) in the mid-1990s has resulted in a decrease of the morbidity and mortality in the HIV-1 patient population that has access to treatment, therapy failure still occurs. The surrogate markers for therapy failure, which ultimately results into clinical progression, are CD4 cell count and viral load. As changes in viral load mostly precedes changes in CD4 count, the determination of the viral load is the most relevant laboratory procedure to monitor the short-term in vivo activity of a therapy given to an individual patient. Lack of adherence, lack of therapy potency, pharmacological reasons, drug–drug interactions, and preexisting drug resistance can be factors resulting into therapy failure and rising viral loads.

The evolution of resistance against HIV-1 inhibitors within a patient depends on the generation of genetic variation, reflected by substitutions, insertions, deletions, and recombination events, and on the selection of drug-resistant variants during antiretroviral therapy. The high HIV-1 genetic variability is caused by the error-prone nature of HIV-1 reverse transcriptase, by the absence of any enzymatic proofreading activity, and by the huge rate of HIV-1 replication in vivo. Some of the created genetic variants will result in alterations to the structure and function of the molecules targeted by the antiviral drugs (currently reverse transcriptase, protease, and glycoprotein gp41). These alterations could confer changes in susceptibility to one or more of these drugs. In the presence of therapy, the variants with some level of resistance will gradually overgrow the wild-type variants. Under the continuous selective pressure of inhibitors and with the presence of residual replication as a result of insufficient potency of the treatment, additional mutations will accumulate.

Thus, the main goal of HAART is to reduce the viral load as much as possible to prevent further clinical progression. However, its immediate goal is to reduce the viral load to prevent the formation of variants that result in antiviral resistance to the current therapy and that, because of the phenomenon of cross-resistance within drug classes, ultimately might lead to a limitation or even lack of future treatment options.

METHODOLOGY

Antiviral drug resistance can be determined at the genotypic level by the detection of resistance mutations. Alternatively, it can be determined at the phenotypic level by measuring the ability of an HIV-1 isolate to grow in the presence of a drug, or by measuring the HIV-1 reverse transcriptase or protease enzyme activity in the presence of an inhibitor.

Sequencing is the most commonly used genotypic assay for the monitoring of resistance mutations. It provides information on all nucleotides of the sequenced region. The target sequence is amplified via polymerase chain reaction, and the sequence is determined based on the incorporation of dideoxynucleotides. At the moment, most clinical laboratories perform home-brew sequencing or they use one of the two commercially available sequencing assays [ViroSeq HIV-1 Genotyping System (Abbott) and Trugene HIV-1 Genotyping Kit (Bayer)]. Another option is to send samples to service laboratories (e.g., ViroLogic and Virco).

Presently, only three companies (ViroLogic, Virco, and VIRalliance) and a few national reference laboratories routinely perform replication-based phenotypic assays. These assays start with the amplification of the patient-derived HIV-1 target sequence via polymerase chain reaction. The amplification product is subsequently incorporated into a proviral laboratory clone via ligation or homologous recombination. This generates a stock of chimeric viruses. The viruses are tested for their ability to grow in the presence of different concentrations of inhibitors. The principle of chimeric viruses for the determination of the susceptibility profile is preferred over other phenotypic assays to obtain better reproducibility and automation possibilities.^[1,2]

Resistance testing has to be performed on plasma samples before starting, stopping, or changing therapy. It is highly important to monitor the active replicating viral population responsible for the therapy failure, because it is the resistance pattern of this population that can be useful in understanding the observed therapy failure.

An important limitation of current available assays is their need for plasma samples with an HIV-1 viral load exceeding 1000 copies/mL because of the lower

sensitivity of the cDNA synthesis and amplification procedures of long templates. This is required to achieve reliable results. Otherwise, nonrepresentative variants could be picked up because of stochastic events during the experimental procedures. However, switching therapy early after viral failure, when the HIV-1 viral load is detectable but still very low, results in better chance of response to the salvage therapy. There are only few data that provide the optimal point at which therapy should be changed in terms of long-term clinical outcome, but the short-term risk of any viral replication in the presence of inhibitors is the development of resistance.

Results of the resistance assays represent only the genotypic or phenotypic profile of the majority of quasi-species present in vivo because they have difficulties in detecting minor variants that reflect less than 10–50% of the total viral population.^[3] It remains uncertain what the contribution of minor viral variants is to therapy failure.

As genotypic and phenotypic assays start with an amplification procedure, the sensitivity of both tests depends on the efficient hybridization of primers, and this is strongly influenced by the variability at the primer sites. Most of the assays have originally been optimized by using subtype B HIV-1 strains because such strains were responsible for the epidemic in developed countries.

However, in recent years, the prevalence of non-B subtypes has been increasing in Europe, and access to therapy has been extended to the developing world where non-B HIV-1 subtypes constitute the largest part of the epidemic.^[4] Effort has been made to improve the performance of the assays on non-B HIV-1 subtypes by developing primers at more conserved sites or by the inclusion of backup primers, but no assay can guarantee to reproduce adequate results for all strains.

Both genotypic and phenotypic resistance assays have respective advantages and disadvantages (Table 1). Resistance assays have to be performed in highly specialized laboratory facilities. This particularly holds true for the phenotypic resistance assay because it requires biosafety level 2 or 3 facilities, whereas a genotypic resistance assay can be performed in a dedicated molecular biology laboratory. Because both types of assays require sophisticated technology and knowledge, a strict adherence to current laboratory standards is important; well-trained and experienced laboratory technicians are also essential.

Another practical consideration in the choice of resistance testing is the cost of the procedure. Phenotypic assays are still more expensive than genotypic assays.

Genotypic resistance assays can deliver results within a few days, whereas longer time is required to obtain

Table 1 Advantages and disadvantages of genotypic and phenotypic resistance assays

Advantages	Disadvantages
<p><i>Genotypic resistance assays</i></p> <p>Have a wider availability, requiring biosafety level 1 facilities</p> <p>Are less expensive</p> <p>Display a shorter turnaround time (typically 1–2 weeks)</p> <p>Detect sentinel mutations that are linked to reduced clinical response but not necessarily to reduced phenotypic susceptibility</p> <p><i>Phenotypic resistance assays</i></p> <p>Measure the effective susceptibility to all (also new) drugs</p> <p>Measure effects of mutational interactions</p>	<p>Require plasma samples in general with viral load >1000 RNA copies/mL</p> <p>Exhibit difficulties in detecting minor variants</p> <p>Exhibit a sensitivity that is strongly influenced by virus genetic variability</p> <p>The relevance of some mutations remains still unclear</p> <p>Some (complex) mutation patterns may be difficult to interpret</p> <p>Require highly specialized laboratory facilities, typically biosafety level 2 or 3</p> <p>Require plasma samples in general with viral load >1000 RNA copies/mL</p> <p>Exhibit difficulties in detecting minor variants</p> <p>Exhibit a sensitivity that is strongly influenced by virus genetic variability</p> <p>Are more expensive (in general twice as much as genotyping)</p> <p>Have longer turnaround time (typically 2–4 weeks)</p> <p>The reproducibility range of the assay extends for some drugs above clinically relevant cut-offs</p> <p>The clinical relevance of drug susceptibility levels is not always clear</p>

phenotypic resistance results. This turnaround time can be an important factor in some clinical situations, where immediate results are needed, e.g., postexposure prophylaxis and primary HIV-1 infection.

Sequencing has the advantage of detecting all known resistance-related mutations—mutations considered as markers for developing resistance patterns (e.g., K70R or Q151M in the RT gene)—or substitutions that are considered to be associated with a lower genetic barrier to resistance development (e.g., the reversal mutations T215A/C/D/S in the RT gene). Phenotypic assays measure the effective susceptibility, resulting from known, but also unknown, resistance-related mutations. However, they do not detect the presence of the latter two sets of mutations. Although these mutations are associated with reduced clinical response, they are not always linked with reduced phenotypic susceptibility.

In addition, the current phenotypic assays have difficulties in detecting resistance to some nucleoside reverse transcriptase inhibitors. These drugs compete with the natural deoxynucleoside triphosphates (dNTPs) for binding to the reverse transcriptase and incorporating into the growing DNA chain. The phenotypic assays use activated cells with high intracellular dNTP pools that do not always reflect the relevant *in vivo* cell conditions. The high intracellular dNTP pools lead to resistance levels that cannot be reliably measured because they are within the reproducibility range of the assays.

Another issue in the interpretation of genotypic results is whether the mutations constituting a part of a set of multiple mutations exist on a single clone or are spread across multiple clones of variants. Phenotypic resistance assays resulting in low-level resistance may be caused by a small change in susceptibility of the whole virus population or a mixture of resistant and susceptible virus. The linkage of mutations could have important clinical implications, but at the moment this issue cannot be routinely elucidated with the current resistance assays.

INTERPRETATION

The information provided by genotypic resistance assays may be difficult to interpret. Current knowledge on the correlation between the genotypic and phenotypic characteristics of an HIV-1 isolate is mainly based on *in vitro* studies of laboratory and clinical HIV-1 strains. The clinical relevance of mutations is also determined from linking mutation patterns with previous therapy history or from linking them with subsequent therapy response. A decade ago, when monotherapy was the standard-of-care, the direct linkage between mutations, therapy experience,

and subsequently therapy response was more clear-cut than in the current HAART situation. The potency of new drugs is now investigated in “add-on” studies, where the new drug is added to the failing regimen. More recently, this strategy has encountered ethical problems because of the possibility that it could lead into fast resistance development against the new drug. An increasing number of efficiency studies are performed with combinations of the new drug and at least one other suspected active compound. This hampers the understanding of the impact of preexisting resistance and the subsequent viral response to the new drug.

Patients are currently treated with combination therapies that change in time and that result in complex patterns of mutations. The phenotypic effects of mutations and their interactions often cannot be reliably predicted. Some mutations that are developed under the selective pressure of a particular drug can cause resistance toward other drugs from the same class without any previous experience (cross-resistance), and others might reverse the phenotypic resistance level of certain resistance mutations. Phenotypic resistance assays give a direct measurement of susceptibility toward the tested inhibitors that includes the effect of all mutations and their interactions. However, the clinical relevance of phenotypic results can also be difficult to interpret because the threshold at which a specific inhibitor becomes ineffective *in vivo* has not been determined yet for all available drugs.

The investigation of the correlation between genotype and phenotype has resulted into the development of genotypic–phenotypic relational databases and rule-based algorithms.^[5] These systems can be helpful in the interpretation of *in vivo* resistance. Large datasets containing matched genotypes and phenotypes are already used to predict phenotype from genotype. Rule-based algorithms contain lists of mutations that are: 1) known to confer phenotypic resistance toward a particular drug; 2) known to be part of a mutation pathway toward high-level resistance; 3) and, most importantly, responsible for a reduced clinical response.^[6]

At the moment, several interpretation algorithms are available. They give concordant results in most cases. The observed discordances reflect the uncertainty that is still associated with particular set of mutations.^[7]

These algorithms should be subject to regular updates as soon as new correlations between genotype and phenotype are established and the clinical relevance of certain genotypes and phenotypes become evident.

From the time antiretroviral drugs were first used in the treatment of HIV-1 infection, it was observed that in many cases therapy failure was associated with the development of resistance mutations at the genetic level and decreases in drug susceptibility at the phenotypic level. Only a few

years ago, after the effect of resistance on subsequent therapy failure was investigated in retrospective studies, the idea to prospectively use the results from genotypic and phenotypic resistance assays in the management of HIV-1 infection was incorporated into the design of clinical trials. In these trials, viral response on therapy guided by genotypic and/or phenotypic resistance results was compared to that during standard-of-care. In some of these prospective trials, access to genotypic or phenotypic drug resistance results led into a short-term, modest viral benefit. In other studies, the effect of resistance testing was attenuated as a consequence of many factors.

The absence of resistance does not always result into therapy response because success is a result of many factors besides antiviral resistance, such as adherence, potency, and pharmacological reasons. It has also been shown that the interpretation of the raw resistance data plays an important role in the successful implementation of resistance testing.^[8] It is recommended that the interpretation should be left to experts. These experts can translate the data into recommendations that are useful for clinicians. Another limitation of the use of drug resistance assays in the clinical management of HIV-1 infection is the limited choice of drugs in the treatment of HIV-1 patients who have undergone extensive therapy with multiple failures.^[9] Because of the existing cross-resistance within drug classes, a badly managed combination therapy could result into broad cross-resistance toward all available drugs, even if these respective drugs had not been previously used. Whether or not the choice of subsequent therapy is then guided by resistance testing has no longer any relevance, because there remains only limited or no option at all.

Nevertheless, resistance testing has demonstrated its advantages and has been implemented into guidelines.^[10–12] It is recommended for primary and recent HIV-1 infection, before initiation of therapy in chronically infected patients if infected within the past 2 years, or in areas with high resistance prevalence, first or multiple therapy failure, and pregnancy.

CONCLUSION

Although resistance testing is widely used in the clinical management of HIV-1 treatment, questions remain. Up until now, the assays have shown a short-term modest viral benefit. Clinicians should value the results obtained with these assays but should, however, not overestimate their importance because there still exists some uncertainty concerning the clinical importance of certain genotypic mutations and phenotypic levels of susceptibility. The decision to use a specific combina-

tion of drugs should be made by the clinician, taking into account all clinical, immunological, viral, and pharmacological factors, as well as adherence issues and treatment history.

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