

HIV/AIDS

Antiretroviral Newsletter



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The aim of this biannual newsletter is to provide health workers in the Region with a brief, up-to-date summary of the latest developments in antiretroviral therapies.

Drug Resistance: Part 2 Application to clinical practice

INTRODUCTION

Several factors contribute to antiretroviral drug failure. Viral resistance is an increasingly important factor. Randomized trials have demonstrated that patients who have virologically failed a treatment regimen may benefit from antiretroviral resistance testing¹. Two panels of experts have endorsed the use of resistance testing as an integral part of the clinical management of HIV-infected individuals^{2, 3}. They both concluded that testing should be recommended or strongly considered for patients in the following situations:

1. Patients on antiretroviral therapy who are virologically failing their regimen. Virological failure is defined as the repeated detection of virus after initial suppression of the plasma HIV RNA below the assay limits of detection or a three-fold or greater increase in plasma HIV RNA above the nadir RNA level;

2. Patients who have achieved a sub-optimal response after the initiation of antiretroviral therapy. Sub-optimal virologic response is defined as $<0.5-0.75 \log_{10}$ copies/ml reduction in plasma HIV RNA at four weeks following initiation of Highly Active Antiretroviral Therapy (HAART) or $<1 \log_{10}$ copies/ml reduction at eight weeks or failure to suppress plasma HIV RNA levels below assay detection by four to six months of therapy;
3. Patients with acute HIV infection;
4. Patients who are pregnant.

The EuroGuidelines Group for HIV Resistance also recommends resistance testing in the setting of post-exposure prophylaxis. Recommendations by different groups are summarized in Table 1.

Table 1 - Summary of Resistance Testing Recommendations

CLINICAL SITUATION	IAS-USA	US DHHS GUIDELINES	EURO-GUIDELINES	RATIONALE FOR TESTING AND REMARK
Primary HIV Infection	Recommend testing but do not delay therapy waiting for results	Consider testing	Consider testing. Test if resistance rate is high or transmission from treated individual suspected.	Detect transmission of drug resistant virus, do not delay therapy and modify, if necessary. Store plasma, if possible, for future reference.
First Regimen Failure Multiple Regimen Failure	Recommend testing Recommend testing	Recommend testing (particular regimen failure not specified).	Recommend testing where all therapy changes are considered due to virological failure.	Determine the role of resistance in therapy failure and optimize number of active drugs in the next regimen.
After discontinuation of drugs	Not specified	Testing not generally recommended.	Testing not generally recommended.	Mutant viruses may become minor species in the absence of drug pressure. Current assays may not detect minor species.
Post-exposure prophylaxis	Not specified	Not specified	Recommend testing.	Do not delay therapy, but if a sample from the index case is available test and modify treatment of recipient accordingly.
Pregnancy	Recommend testing	Not specified	Recommend testing if mother has detectable virus	Optimize maternal treatment and prophylaxis for the neonate

TYPES OF TESTS

There are two types of resistance assays:

- 1) **Genotyping:** Maps the genetic sequence of the virus.
- 2) **Phenotyping:** Measures the viral activity directly against antiretroviral drugs. A variation of this assay—virtual phenotyping—applies a computerized database to the interpretation of genotype results.

Genotypic and phenotypic tests usually give the same results, but not always (see Table 2). For patients failing a first antiretroviral regimen, there is similar benefit from genotypic and phenotypic resistance testing. For patients with multiple ARV treatment failures, there may be an advantage to phenotypic resistance testing, or the concurrent use of both tests, to optimize therapy. It is currently unlikely that patients who are on more than their third regimen will be able to achieve viral suppression below the level of detection. Therefore, careful selection of drugs used in the regimen is critical to maximize benefit. For these patients, consideration should be given to using phenotypic resistance testing, possibly combined with genotyping.

Table 2. Genotyping vs. Phenotyping Resistance Testing

Genotyping	Phenotyping
Detects actual viral mutations associated with drug resistance	Measures virus susceptibility to a particular drug <i>in vitro</i> , not <i>in vivo</i>
Determines all currently known mutations responsible for resistance	Examines the amount of drug needed to stop the replication of the virus
May utilize software to analyse mutations and mutation interactions (cross resistance) as they relate to drug resistance to generate resistance interpretation by drug	Reports results in terms of IC ₅₀ values and fold changes.
Software can report effect of multiple mutations on resistance to one drug or combination of drugs	Tests for the effect of one drug at a time. Current commercially available tests do not report effect of drug combinations
May not detect minority species of virus present at levels less than 10%-20 %	May not detect minority species of virus present at levels less than 10%-20 %
Genotypic changes which predict <i>in vivo</i> virologic failure are reliably detected even when <i>in vitro</i> phenotypic assays fail to show significant shifts in IC ₅₀ values	No consensus on appropriate cut off values for each drug
Can be performed in any 'PCR (polymerase chain reaction) capable' diagnostics laboratory with standardized kit/system	Labor intensive, complex, 'home brew' procedure that can only be done in a centralized laboratory facility
Robust and fast technology (takes less than two days to perform the test in the laboratory)	Difficult and time-consuming procedure (can take over a month to get results)
Costs US\$350 to \$500	Costs US\$750 to \$900

GENOTYPIC TESTING

HIV drug resistance genotyping by sequencing is a complex technology that presents a challenge for analysis, interpretation and reporting. Most commonly, genotypic testing is done using a machine that reads the gene sequence of the protease and reverse transcriptase genes. The results are compared to an original, or not mutated, HIV gene sequence. Any mutations are checked against a list of changes known to cause drug resistance. Most drugs follow a set pattern of resistance mutations. For example, high-level resistance to lamivudine is always conferred by a single mutation at the 184 codon. Protease inhibitors (PI) have much more

varied mutation patterns and interpretation of genotypic resistance test results is more complicated.

Genotypic testing detects only mutations that make up 20%-50% of the total viral population. It will not detect very low levels of resistant virus.

Costs of genotypic tests

In the United States, genotypic testing with full sequencing for nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI) and PI mutations costs US\$ 350 to US\$ 500 per test. In Thailand, the same test costs Baht 5000 (US\$ 125). The

cost of sequencing for NRTI and NNRTI, without PI sequencing, is Baht 3000 (US\$ 75)

Types of genotypic tests

There are two types of genotypic tests:

1. Kit-based tests contain the necessary reagents to prepare the HIV genetic material, extract it from a patient's plasma, and perform gene sequencing. The kits are sold by the manufacturer for use in independent laboratories. Currently, one kit-based test is licensed by the US Food and Drug Administration (FDA) for use in clinical practice.
2. Non-kit-based tests, or proprietary genotypic tests, are developed by individual commercial or academic laboratories. These assays, also referred to as in-house assays, are provided commercially by the laboratory for research use only.

Comparing kit-based tests

Only one kit-based assay is currently approved by the FDA for marketing (TRUGENE™ *HIV-1* Visible Genetics). Another kit-based assay would be available soon (HIV-1 Genotypic systems: ViroSeq™). A comparison of genotypes for 34 non-subtype B HIV-1 isolates (subtypes A-H) run with both kits showed only minor differences in sequence results.⁴

Several studies have compared the relative performance of these two kits in B or non-B subtypes⁵. One study (Erali) addressed the relative technical issues and training required to perform each assay. Future studies need to address the capital and per-assay costs to establish and run these two systems in order to assist laboratories in deciding which system to implement. The ease of use of the test and the reliability of the interpretation tools associated with each kit, will be critical factors in deciding which system and which kit to use.

Equipment requirements for kit-based testing



1. Automated DNA sequencer
2. Gel cassette fixer
3. Computer and software
4. Pipette, gel cassette, and reagents

To run a genotypic test using a kit-based assay, plasma is separated from the cells and the HIV genetic material (RNA) is extracted from the virus in the plasma. A DNA copy of the HIV RNA is made. This DNA is multiplied to produce enough material to run the test. This is done only for the part of the virus' genetic code that affects drug resistance.

Genetic information is written in a language using an alphabet of four letters (A, C, T and G). In the next steps, a process called 'sequencing' occurs. Four reactions are run, one for each letter of the genetic alphabet. In each reaction, multiple partial copies of the patient's HIV genetic code are made. Each copy is a different length, stopping at the letter for which the reaction is looking. The reagents required for these reactions are included in the kit.

Next, partial copies are arranged in order from longest to shortest. They are then inserted into a gel filled cassette to which an electrical voltage is applied, causing the genetic material to travel through the gel with the strings of letters travelling at different rates according to their size ('electrophoresis'). Lasers track this process and software calculates the full sequence of 'letters' in the virus' original genetic code. These sequences are compared to the code of a standard reference virus, which contains no mutations or errors, enabling identification of mutations in the genetic code of the patient's virus.

The software then uses a set of rules that relate individual or combinations of genetic mutations to the degree of resistance to each drug. These rules were developed and are regularly updated by an international panel of leading scientists and physicians with expertise in HIV drug resistance.

Finally, a resistance report is generated which indicates the likelihood of the patient's virus responding to each of the available drugs. It also lists the individual genetic mutations in that patient's virus.

This complex combination of hardware, software and chemistry is integrated into a system about the size of two desktop computers, which can be operated by trained and certified technicians.

Alternative techniques for genotyping

Line probe assay (LiPA) uses a specific probe (something that looks for specific mutations) to detect resistant mutations. There is a probe for each of the mutations known to lead to drug resistance. This technique can detect mutations that make up as little as 2%-5% of the total virus population.

GeneChip uses a chip that has many markers built onto it. A blood sample is put onto the chip and it is passed through a scanner. The results are compiled by a computer, which shows any mutations in the genes.

PHENOTYPIC TESTING

Phenotypic testing measures the amount of drug needed to suppress the growth of HIV in a laboratory setting. Known levels of drug stop reproduction of non-resistant HIV. Resistant HIV, however, requires higher levels of the same drug to stop reproduction if at all possible. In the test, the amount of the drug is increased until it is enough to stop virus reproduction.

Phenotypic tests may pick up resistance not seen in genotypic tests if there are only low levels of resistant virus. Also, the mutations that lead to resistance are not yet well understood, particularly for newer drugs, and thus may not be included in genotypic tests.

INTERPRETING RESISTANCE REPORTS

GENOTYPIC TESTING

In addition to listing each mutation detected, the genotypic test report lists the interpreted effects of that set of mutations on each drug, using the following algorithm:

- 1) **No evidence of resistance:** No known mutations were detected, or reduced susceptibility has not been associated with the mutations detected in this assay.
- 2) **Possible resistance:** Mutations detected in this assay have been associated with diminished virologic response in some, but not all, patients. This description is also used if the detected mutations have been associated with an intermediate decrease in antiretroviral susceptibility *in vitro* in viral isolates.
- 3) **Resistance:** Mutations detected in this assay have been associated with a maximum reduction in susceptibility.
- 4) **Insufficient evidence:** There is inadequate direct or indirect evidence to determine susceptibility.

PHENOTYPIC TESTING

Resistance is usually reported as the level of drug needed to reduce viral replication by 50% (called inhibitory concentration 50 or IC₅₀) or 90% (IC₉₀). The level of resistance is graded by comparing this value for an individual's HIV with the levels for non-resistant (commonly called wild-type) virus.

There are three levels of resistance:

- 1) **Low-level:** 2- to 4-fold increase in the amount of drug needed to stop HIV reproduction
- 2) **Moderate-level:** 4- to 10-fold increase
- 3) **High-level:** 10-fold or greater increase

With most drugs, high-level resistance means that drugs are no longer able to block viral replication. Moderate-level resistance might be overcome by achieving higher drug levels in the blood. This may be accomplished by using novel combinations of some drugs. However, increasing the dose of drugs can increase the risk of side effects.

The protease inhibitor ritonavir, for example, can increase the blood levels of many other drugs, including other protease inhibitors. In some cases, using ritonavir may be helpful to increase the potency of other anti-HIV drugs and overcome some resistance. Drugs that have low- or moderate-level resistance may still work as a part of combination therapy. As with genotypic testing, results can be difficult to interpret. Experienced physicians are needed to analyse phenotypic resistance assay results.

LABORATORY QUALITY ASSURANCE

Quality assurance is required not only for the assay, but also for the laboratory performing it. All laboratories in the United States that perform commercial genotyping or phenotyping must have certification, according to the Clinical Laboratory Improvement Act (CLIA) 1988, indicating review of the laboratory's performance standards. Although the clinical scientist in charge of the local laboratory may be the decision-maker on this issue, the treating clinician should inquire about the quality of the assay and the particular reference laboratory being used. Clinicians should also provide feedback about the readability and interpretability of the report forms to the local laboratory director who decides which reference laboratory conducts their genotyping and phenotyping.

CASE STUDY 1

Patient Profile

The patient is a 39-year-old man who was diagnosed with HIV infection in 1997 after presenting with pneumonia. At diagnosis, he had an HIV RNA viral load of 15 000 copies/ml and a CD4 count of 250/mm³.

Treatment history

Six months after diagnosis, he started his first regimen of zidovudine, lamivudine, and nelfinavir, which was well tolerated. The patient's CD4 count increased to 550 cells/mm³. His viral load remained at <50 copies/ml until November 2000, and then increased to 150 copies/ml. He remained on the same regimen over the next few months as the viral load increased. In February 2001, his viral load was 2100 copies/ml.

Interpreting the resistance report

A genotypic resistance test was conducted to ascertain if more than one of the components of the regimen was failing. If only one ARV in the regimen was failing, only that drug would need to be replaced. The resistance report showed that the patient's virus had the M184V mutation, which confers high-level resistance to lamivudine. Lamivudine was replaced with didanosine and the next viral load was undetectable.

CASE STUDY 2

Patient Profile

The patient is a 26-year-old man living in Bangkok, Thailand. He was diagnosed with HIV infection at routine screening when he joined the army.

Treatment history

This patient was treated with dual nucleoside therapy (stavudine plus didanosine) from 1999 to 2002. This scenario is common in many developing countries because dual therapy was promoted during this time. At the beginning of therapy, his CD4 count was 190 cells/mm³ and viral load was not available. In June 2002, his viral load was 2550 copies/ml and CD4 count was 220 cells/mm³. Genotypic resistance testing was ordered to plan the second-line regimen.

Interpreting the resistance report

The genotypic resistance assay found mutations at codons 41, 67, 151 and 219. These mutations are associated with high-level resistance to all nucleoside analogue reverse transcriptase inhibitors (NRTIs) except lamivudine. There were no protease inhibitor or non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations as would be expected since the patient was naïve to these two drug classes. The presence of three or more nucleoside analogue mutations (NAMS), as detected in this case means that none of the NRTIs,

except lamivudine, can be used in the second line regime. The patient was switched to a combination of indinavir, ritonavir, lamivudine and efavirenz. After eight weeks, his viral load was undetectable and his CD4 count had increased to 300 cells/mm³.

CASE STUDY 3

Patient Profile

The patient is a 46-year-old woman who was diagnosed with HIV infection in 1997 when she was admitted with bacterial pneumonia. She is failing her current ARV regimen due to incomplete adherence, probably caused by side effects of the regimen. Her CD4 count now is 390 cells/mm³ and her HIV-RNA viral load is 3000 copies/ml.

Treatment History

The patient started her first ARV regimen in 1997 after being discharged following the pneumonia. She stayed on the zidovudine, lamivudine, and indinavir regimen for one year and was then switched to a simpler regimen due to poor adherence. The second regimen (abacavir, stavudine and efavirenz) was also not well tolerated. The side effects of sedation and dizziness interfered with her work and resulted in her missing doses regularly.

Interpreting the resistance report

The resistance report showed that the patient's virus differed from wild-type at a single RT codon, K103N, which confers high-level resistance to all

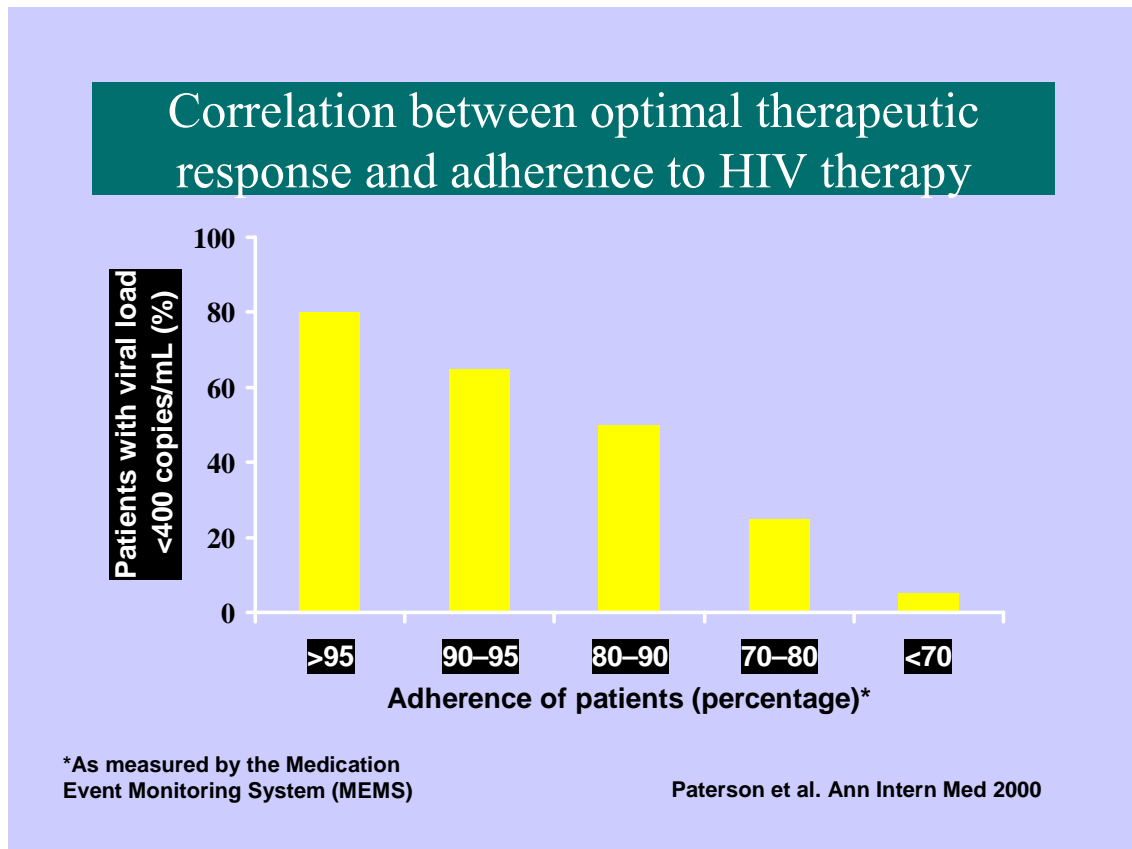
NNRTIs currently available. Any new regimen the patient is started on will need to be protease inhibitor-based, but NRTIs would be expected to remain active. In addition, the patient needs to be counseled strongly on the importance of adherence to ARV treatment.

Adherence to therapy and resistance

Adherence to therapy—taking drugs every day at the correct time—is essential if the treatment is to be successful and durable over time. Poor adherence leads to the development of drug resistance⁶ and virological failure and limits future therapeutic options. Second line and salvage regimens are often more complicated, involve a larger pill burden, have an increased likelihood of side effects and cost more.

Non-adherence is one of the strongest predictors of failure to achieve viral suppression below the level of detection, with the inevitable consequence of the development of resistance. A high degree of adherence is necessary for sustained virologic suppression. Several studies show that 90%–95% of doses must be taken for optimal suppression. Lesser degrees of adherence are associated with virologic failure (Figure 1).

Figure 1: The impact of adherence levels on the goal of undetectable viral load



EVOLVING PATTERNS OF RESISTANCE TO ANTI RETROVIRAL DRUGS

Interpretation of new data on emerging patterns of resistance to antiretroviral drugs is complex due to the selection of different target sample populations (e.g. patients on treatment or newly infected patients) and testing methods (genotyping and phenotyping).

In urban areas of the United States, there has been an increase in high-level phenotypic resistance from 3.5% in the period 1995-1998 to 14% in 1999-2000 and an increase in the transmission of multi-drug resistant virus from 0.4% to 5.4%.⁷

In the United Kingdom, the transmission of drug resistant viruses has increased substantially⁸; while in Switzerland, drug resistance levels have been declining since 1997. The widespread use of HAART with many patients having undetectable viral loads may explain this decrease in resistance. Increasing levels of resistance also have been documented in Uganda and the Ivory Coast.⁹

Several studies have documented increasing levels of resistance to antiretroviral drugs among newly infected

individuals. One study assessed pretreatment resistance in 154 individuals with primary HIV infection between 1995 and 2001¹⁰. The frequency of genotypic resistance mutations increased from 13.2% (1995-1998) to 19.7% (1999-2001). Overall, the prevalence of phenotypic resistance did not change over the seven-year period of the study.

THE GLOBAL HIV DRUG RESISTANCE SURVEILLANCE NETWORK

The data above suggest the possible emergence of a major public health problem: antiretroviral drugs are becoming less effective because of increasing resistance. The World Health Organization, in collaboration with the International AIDS Society-USA, is developing the Global HIV Drug Resistance Surveillance Network in order to track emerging resistance patterns in both developing and developed countries. Such surveillance is a critical adjunct to all country-level antiretroviral access programmes because it will help detect the circulation of resistance strains in the early stages of the implementation of the programme and will direct measures to preserve program effectiveness.

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