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Monitoring the Emergence of Antiretroviral Resistance

**Report of a WHO Consultation organized in collaboration
with Istituto di Sanità and the International AIDS Society**

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Executive Summary

The World Health Organization (WHO) has played a leading role in developing strategies for the surveillance and containment of antimicrobial resistance in bacterial and parasitic diseases. The goal has been to optimize patient care and to minimize the emergence and spread of antimicrobial drug resistance.

Just as for bacterial and parasitic diseases, a global resistance monitoring programme is also needed for HIV/AIDS. In the developed world the remarkable reduction of HIV-related morbidity and mortality produced by potent antiretroviral therapy has been accompanied by an increase in the prevalence of drug-resistant viruses unresponsive to available therapies. In the developing world, as access to antiretroviral agents increases, drug resistance may be enhanced by inappropriate treatment and lack of adherence to treatment regimens.

The need to develop a global antiretroviral resistance monitoring programme was addressed at the consultation organized by WHO in collaboration with the International AIDS Society (IAS) and the Istituto Superiore di Sanità (ISS) and held in Rome, October 2000.

It was proposed that WHO, in collaboration with IAS, develop a detailed plan of action involving partnerships with existing antiretroviral (ARV) resistance monitoring centres and networks. The plan will be based on the following priorities agreed upon by the participants at the consultation:

- to identify sites that are currently involved in HIV-1 drug resistance monitoring activities and to catalogue these activities
- to develop uniform criteria for the collection and reporting of HIV-1 drug resistance
- to develop and maintain a surveillance system that determines HIV-1 drug resistance among:
 - previously untreated patients
 - targeted ARV-experienced populations (e.g. those who have a history of ARV therapy; those who are receiving active therapy; or those who have received therapy through perinatal transmission prevention programmes)
- to monitor simultaneously the subtype of circulating HIV-1 strains by using protease and/or reverse transcripts sequences
- to determine trends in the prevalence of drug resistance in different geographical areas in relation to the introduction of ARV therapy
- to establish linkages between surveillance sites and quality controlled laboratories and to promote technology transfer of drug resistance testing methodologies to sites in the developing world
- to promote education about strategies that reduce the selection of antiretroviral resistance.

Section 1

The Challenge

The HIV epidemic continues to evolve

Almost 20 years after the first cases were detected, the HIV/AIDS epidemic continues to progress, with an estimated 36.1 million adults and children infected worldwide.¹ It has become increasingly apparent that the epidemic does not follow the same course in all societies. The vast majority of HIV infections are concentrated in developing countries and infection rates continue to increase in countries with poor health systems and limited resources for prevention and care, with a profound impact on life expectancy and economic growth.

Fortunately, this is not without solution: education, prevention and effective treatment and care can dramatically modify this scenario, and HIV/AIDS is increasingly seen as a manageable disease. Where effective prevention programmes aimed at reducing risk behaviours have been undertaken, rates of new infections have declined. However, prevention alone is insufficient: health care infrastructure, together with a greater access to potent antiretroviral (ARV) combination therapies must be established worldwide to achieve the same impressive decline in HIV related mortality and morbidity that most industrialized countries have experienced over the last four years.

The major benefit of effective ARV therapy is achieved when it is begun before the immune system is compromised. The majority of HIV infections in the world occur however, in resource-limited countries, where infected people (often belonging to low-income groups) usually have advanced-stage disease at diagnosis when treatment (where available) is initiated. This late initiation of therapy, together with the use of sub-optimal regimens and the practice of uncontrolled treatment interruptions due to financial constraints, mean that even where drugs are partially accessible, a high proportion of people with HIV do not benefit fully from life-saving therapeutic strategies.

The threat of antiretroviral drug resistance

Despite the continuing introduction of potent ARV drugs, HIV resistance to all categories of existing drugs continues to develop, limiting the successful treatment of many patients with HIV infection. Data published over the last two years from clinical trials and observational cohort studies show that an important proportion of patients fails to achieve a complete and durable response to current treatment regimens.

Poor adherence to schedules of therapy (due to the complexity of regimens or toxicity) and use of sub-optimal or inappropriate therapies, together with the ability of HIV to mutate under the selective pressure of ARV agents, all favour the emergence of resistance. Resistance is an undesired but inevitable consequence of any antimicrobial treatment, and might be a future major obstacle to the control and containment of the

HIV epidemic. If the number of patients harbouring resistant strains of HIV increases, transmission of these viruses is also expected to increase. The resultant increase in the prevalence of resistance implies that treatment outcomes will be further compromised.

Current data addressing the problem of resistance are limited and not entirely consistent. However, all studies report a significant transmission of resistant strains with some new infections caused by HIV variants with reduced susceptibility to one or more ARV agents. This will increase the likelihood of a poor response to initial ARV treatment. Different patterns of drug-resistant HIV transmission are developing in Europe and North America. The highest prevalence is observed in studies among untreated HIV infected subjects in some North American cities such as New York, Seattle and Montreal, while there are declining rates in San Francisco and also in Switzerland.

Several factors may be responsible for these different results. Although these are likely to include geographical and genetic issues, differences in data collection may also account for the discrepancies. Random variation due to the small number of individuals in each study may also play a role. It is important to note that the detection of resistance in a given individual may be a function of time from primary infection. This means that years after infection the major circulating virus population in an individual subject may not reflect the pool of “archived” viruses. The reversal of mutations in virus from plasma may indeed occur after the transmission in the absence of antiretroviral therapy. Therefore, comparing resistance data collected from HIV infected untreated subjects at different times after the primary infection may be misleading.

Based on these considerations, it appears that resistance in individuals not yet exposed to ARV agents represents an increasing public health concern. There is a need for ARV drug resistance surveillance systems similar to those existing for other diseases of public health importance such as tuberculosis, malaria and some bacterial infections.

Monitoring an evolving virus

Phylogenetic studies from different geographical areas have revealed a remarkable genetic diversity among HIV strains, within several distinct lineages.² Most strains in the global epidemic belong to HIV-1 group M, which includes several subtypes, or clades, designated by the letters A to K. In addition, nearly 20% of group M isolates are recombinants, with genetic material originating from different subtypes. Overall, the predominant viral clades are A and C, which account for one-half of the infections, and B, the major subtype in North America and Europe. However, a remarkable prevalence of non-clade B subtypes has been reported recently in some European countries, being 35, 15.5 and 7.6% in Switzerland, France and Italy, respectively. The greatest genetic diversity in HIV-1 is in Africa, where all subtypes and groups are found. Subtype E is predominant in South-East Asia and the Philippines (recent evidence suggests it is actually a recombinant A/E strain). Subtype C is predominant in India. Due to global travel and migration, a wider geographic dissemination and mixing of all HIV-1 subtypes is expected in the near future, as well as the emergence of an increasing number of recombinant variants.

Most information on ARV drug efficacy, and most existing data on resistance have been collected for group M, subtype B virus, which is the most prevalent type in North America and Europe.

Transmission of drug-resistant viruses

The first case of HIV-1 transmission with reduced susceptibility to an ARV drug, zidovudine, was reported in 1989.³ Subsequently, several reports have documented the transmission of HIV-1 with reduced susceptibility to other reverse transcriptase inhibitors (RTIs) or protease inhibitors (PIs) in patients with acute and early HIV infection. Observations vary widely, with countries with ready access to ARV drugs reporting figures up to 10% resistance for zidovudine and other nucleoside reverse transcriptase inhibitors (NRTIs), 8% for non-nucleoside reverse transcriptase inhibitors (NNRTIs), and 6% for PIs. Shortly following the introduction of potent combination regimens in 1996, transmission of multidrug resistant HIV-1, with reduced susceptibility to several ARV drugs in different pharmacological groups, was reported.^{4 5 6 7 8 9}

A specific issue of concern is the potential selection of drug-resistant virus following short course ARV treatment to prevent mother to child transmission (MTCT) of HIV-1. Up to 20% of women who received a single dose of nevirapine in the "HIVNET" regimen¹⁰ developed resistance. This regimen, which is about 50% effective in reducing MTCT, represented a breakthrough for countries with limited resources, in which longer-term regimens are not affordable.¹¹ However, the lack of correlation of resistance with vertical transmission of HIV-1, together with the disappearance of the relevant mutation in subsequent maternal samples suggest that nevirapine-based short-term prophylactic regimens remain appropriate. The benefits currently outweigh concerns related to the development of drug resistance, including reduced response to future treatment and circulation of resistant strains of HIV-1.

Transmission of resistant viruses has also been described in rare cases of infection acquired after a needle-stick injury, leading to failure of post-exposure prophylactic treatment.¹²

Public health implications of antiretroviral drug resistance

From a public health perspective, wider access to ARV therapy and an increasing number of patients who fail combination therapy will lead to transmission of drug-resistant HIV-1 in the next generation of patients, both from treatment-experienced and treatment-inexperienced individuals.¹³ Any increase in transmission of drug-resistant HIV-1 will invariably have an impact on prevention programmes and treatment guidelines. The problem is global, but it is likely that more resistance will occur where sub-optimal ARV regimens and uncontrolled treatment interruptions are common.

To optimize use of available therapeutic options, programmes to monitor the occurrence of drug-resistant viruses (i.e. the prevalence of drug-resistant HIV-1 strains among newly-infected people) in different risk groups are essential. A global surveillance system will describe the magnitude of the problem and allow development of

appropriate strategies to limit the spread of drug-resistant virus. In addition to surveillance, a better understanding of the mechanisms that influence transmission of both drug-sensitive and drug-resistant virus will permit more focused intervention strategies.

There are two major points that need to be addressed prior to designing any surveillance system: 1) uniform criteria to define the cohorts of newly-infected subjects and 2) consensus on nomenclature and reporting of resistance results.

The Purpose of the WHO Consultation

WHO is actively engaged in the development and implementation of a Global Strategy for Containment of Antimicrobial Resistance.¹⁴ The first steps of this strategy deal with the identification of gaps in knowledge and interventions most likely to be effective. In HIV/AIDS, the emergence of resistance to ARV drugs is a complex problem driven by multiple factors. Available data are currently too limited to define to what extent genetic factors, clinical practice, and life-style affect the observed patterns of transmission of drug-resistant HIV-1.

Within the framework of the WHO Global Strategy for Containment of Antimicrobial Resistance, the first Consultation on Monitoring the Emergence of Antiretroviral Resistance was organized by WHO, in collaboration with the Istituto Superiore di Sanità (ISS) and the International AIDS Society (IAS) in Rome, in October 2000. The aim of the consultation was to assemble virologists, clinicians and epidemiologists involved in ARV resistance clinical and basic research, to:

- review the most recent data on the prevalence of ARV resistance in newly-infected and treatment naïve patients
- identify methods and tools for detecting and evaluating the transmission of ARV drug resistance in the context of a global monitoring programme
- consider the development of monitoring of ARV resistance in order to provide data to guide intervention messages
- produce a document targeted to public health decision-makers, covering technical aspects of HIV-1 resistance detection and operational steps for setting up a surveillance network.

The meeting was chaired by Dr Stefano Vella. Dr Charles Boucher, Dr Veronica Miller and Dr Gayatri Jayaraman were Rapporteurs for the working groups. The full list of participants is given in Annex I. The agenda of the consultation is given in Annex II.

A summary of the current knowledge in ARV resistance and the outputs from the reviews of data, methods and tools, as discussed in the working groups, are presented in Section 2.

The Response to the Challenge

There is now substantial evidence that strains of HIV-1 resistant to one or more ARV drugs can be transmitted and that this can result in therapeutic failure in newly-infected patients. In this context, experience with the global spread of multidrug-resistant strains of other pathogens, such as *Mycobacterium tuberculosis*, is a warning that ARV-resistant HIV-1 could become a much larger problem.

During the consultation, issues considered of major importance for the surveillance and containment of ARV resistance were discussed, and interventions and research needs which would be components of a comprehensive programme were identified. The approaches proposed during the consultation are summarized below.

Monitoring HIV-1 resistance to antiretroviral drugs

The technical challenges of a global HIV-1 resistance monitoring system include the selection of the population to be monitored, methods for measuring resistance and issues related to data handling in developing a global network.

Selection of the population

The ability to obtain appropriate specimens from a representative sample of the population under surveillance will vary among countries and settings. The choice of samples for ARV drug resistance monitoring needs to balance convenience and representativeness (Table 1).

Table 1. HIV-1 infected target populations for a surveillance programme

Population group	Advantages	Disadvantages
Newly infected	Best information for choosing ARV therapy Trends over time	Difficult to identify and enrol Poorly representative of all HIV- infected
Newly diagnosed	Easy to enrol More representative	Possible change in resistance pattern with duration of infection
Facility-based	Easy to enrol Trends over time	Less representative
Random sample from general population	Easiest to enrol	Difficult to interpret results
Pregnant women	Easy to enrol Critical data for MTCT prevention	Less representative

Because of the strong selective pressure that drug therapy exerts on HIV-1, random sampling of HIV-1 positive individuals whose clinical and treatment history is not completely known is not suitable for epidemiological purposes. The best population therefore would be the “HIV-seroconverters” and/or those with primary symptomatic HIV-1 infection. This treatment-naïve (i.e. untreated) population may provide reliable data on the circulation of resistant strains and the most valid estimate of trends in resistance. It is also appropriate for clade/subtype circulation information. However, it is a population difficult to identify due to the non-specific symptomatology (or absence of symptoms) of acute HIV-1 infection.

Sentinel surveillance of selected well-characterized populations, such as newly-diagnosed untreated patients with infection of unknown duration, or pregnant women involved in MTCT prevention, could also be undertaken. These populations are easy to access and the data will provide important baseline susceptibility information and, possibly, indicators of primary drug resistance transmission. However, the sample must be clearly defined, and it assumes no change in prevalence of drug-resistant strains with duration of infection. A different population is represented by patients failing ARV treatment. Resistance data in these individuals should also be collected, together with clinical data and drug usage history. Targeting this patient population, as ARV therapy is introduced into new areas, will capture the emergence of drug-resistant virus.

Methods for measuring resistance

The utility of ARV resistance testing, both genotyping and phenotyping, in the clinical management of HIV-1 infection is becoming clear, and more will be learned through ongoing and future trials. However, currently no specific type of assay is recommended and several technical issues of standardization and clinical validation have to be resolved.

Standardization will be the major problem in applying resistance testing to global surveillance of HIV-1 resistance. Relevant considerations include availability, cost, complexity, and level of expertise required (Table 2). However, phenotyping is unlikely to be an effective means of monitoring resistance. Full-length sequencing, possibly supported by phenotyping when needed, may represent the best current approach. Point mutation assays, eventually supported by sequencing, could be an alternative in some settings (details of these methods are described in Section 2). For all methods, analysis and the need for expert interpretation of the data may be a challenge. Resistance reporting should also be standardized, since both the results and how they are reported are crucial to make comparisons and to monitor trends over time.

Table 2. Characteristics of phenotypic and genotypic resistance assays

Characteristic	Phenotypic assays	Genotypic assays
Relatively simple to perform		✓
Readily available		✓
Acceptable rapid turn-around time		✓
Detection of sentinel mutations		✓
“Net” effect of mutations	✓	
Detection of cross resistance	✓	✓*
Cost saving		✓
More familiar and easier interpretation of results	✓	
Early detection of resistance		✓

* if mutation(s) related to cross-resistance are already characterized

The recommended sample source is plasma. It is easy to collect and likely to be more representative of actively replicating virus. The volume of plasma required, sample preparation, transport, and storage conditions should be simplified and defined. The potential use of serum samples and virus from circulating infected cells should also be explored.

The development of a global network

Several steps are needed for the development of a worldwide surveillance network (see Section on Action Plan for further details). First, a survey should be conducted in order to catalogue ongoing activities both in developed and developing countries. A second

issue refers to the infrastructure needed for effective global monitoring. The experts in this consultation suggested a review of the existing regional and national networks to identify sentinel sites and reference laboratories to participate in ARV drug resistance surveillance. Where these are not available, and particularly in resource-limited settings, it may be more appropriate to begin with the standardized collection of samples to be analysed and sequenced at one of the participating reference laboratories in a network. Each participating laboratory will be expected to establish, or have in place, a quality assurance programme for HIV genotyping. Finally all the information should be collected in a single global database including appropriate clinical, laboratory and epidemiological data.

Containment strategy

In addition to the establishment of a global surveillance network, the following areas were identified as of special interest:

- availability and appropriate use of ARV regimens
- education of health care providers on the mechanisms and impact of HIV-1 drug resistance
- research

Availability of drugs and appropriate use of antiretroviral regimens

Under-treatment (or no treatment at all) is a reality in resource-limited countries, where only a minority of patients have access to drugs through participation in clinical trials, in funded or donor-supported projects, or purchasing drugs at subsidized cost.

Maximally-suppressive therapies, which reduce the likelihood of mutations in the viral genome, are the best tool to minimize the occurrence of resistance. Sub-optimal regimens, including only one or two agents, and poor adherence, are major factors determining the development of resistance. Priority should be given to developing simpler, well-tolerated, effective therapies, as well as developing interventions to support patients' adherence.

Short regimens for the prevention of MTCT should be carefully monitored for the potential induction of resistance that might compromise effectiveness in subsequent pregnancies, and promote the circulation of resistant HIV-1.

A global initiative is underway to improve availability of life-saving treatments in resource-limited countries. It is clear that there should not be different standards of care between developed and developing countries. It is equally clear that a reliable system for drug supply requires an established programme for monitoring resistance.

Education of health care providers

There is a need to educate people living with AIDS, health care providers and policy-makers on the mechanisms and potential consequences of HIV-1 drug resistance. Without this, there is both the short-term risk for the individual patient, and the long-term risk of losing the benefits of ARV therapy in a population. However, the form of the messages is critical: it is important to state clearly that ARV resistance must not be used as an excuse to delay access to life-saving ARV therapy.

Educational programmes must take into account the diversity of social, political and economic settings. The use of clinical practice guidelines is important. The consequences of ARV resistance on treatment outcome and public health should always be emphasized. Education and active involvement of people living with HIV/AIDS is desirable in education programmes, to enhance patients' commitment and encourage adherence.

Research

In many developing countries, health-care systems may be unable to support currently recommended therapeutic approaches. Less intensive approaches, such as intermittent therapy may be more practical, as well as delaying the initiation of ARV therapy until the risk of clinical progression becomes high. Fundamental research programmes, which evaluate cost-benefit in terms of efficacy of alternative therapeutic regimens and their consequences with regard to ARV resistance in these populations, are urgently needed.

Joint efforts and closer linkages between academia and industry should be encouraged to study issues relevant to resistance. Some partnerships have already led to important progress, such as the development of simplified regimens and directly-observed ARV therapy to improve compliance. There are still, however, many unsolved questions that require continued, aggressive research activity.

The consultation identified the following research questions as important:

- ◆ identifying and controlling factors leading to the emergence of resistance in countries with full access to ARV drugs
- ◆ assessing the impact of available guidelines for ARV treatment with respect to the emergence of resistance at both the individual and population level
- ◆ defining procedures to support expanded access to ARV drugs in developing countries while avoiding the dissemination of HIV-1 resistance
- ◆ assessing the cost-benefit of different potential approaches to prevent and contain resistance
- ◆ evaluating the emergence and spread of ARV resistance in different geographical areas, in relationship to viral subtype
- ◆ evaluating the impact that innovative strategies, such as “on/off” intermittent therapy, may have on drug resistance.

Action Plan

WHO has played a leading role in efforts to develop strategies for the surveillance and containment of antimicrobial resistance in bacterial and parasitic diseases in order to optimize patient care while attempting to minimize the emergence and spread of drug resistance. A global resistance monitoring programme is also needed for HIV/AIDS.

This will track drug susceptibility of HIV-1 in a rapidly evolving epidemic, providing information to develop effective treatment strategies and to assist in targeting and implementing drug resistance containment and prevention strategies.

The participants at the WHO consultation agreed that a plan to monitor resistance to ARV drugs should have the following priorities:

- ◆ to identify sites that are currently involved in HIV-1 drug resistance monitoring activities and to catalogue these activities
- ◆ to develop uniform criteria for the collection and reporting of HIV-1 drug resistance
- ◆ to develop and maintain a surveillance system that determines HIV-1 drug resistance among:
 - previously untreated patients
 - targeted ARV-experienced population (e.g. those who have a history of ARV therapy; those who are receiving active therapy; or those who have received therapy through perinatal transmission prevention programmes)
- ◆ to monitor at the same time the subtype of circulating HIV-1 strains by using protease and/or reverse transcriptase sequences
- ◆ to determine trends in the prevalence of drug resistance in different geographical areas in relation to the introduction of ARV therapy
- ◆ to establish linkages between surveillance sites and quality-controlled reference laboratories and to promote technology transfer of drug resistance testing methodologies to sites in the developing world
- ◆ to promote education about strategies that reduce the selection of antiretroviral resistance.

Implementation

The consultation proposed that WHO, in collaboration with IAS, develop a detailed plan of action involving partnerships with existing ARV resistance monitoring centres and networks.

Section 2

Antiretroviral resistance: background

Current antiretroviral therapy of HIV-1 infection

Antiretroviral drugs

There are now 15 (FDA-approved) ARV agents available for the treatment of HIV-1 infection, together with a number of new drugs in the pipeline. All the approved agents and most of those under development belong to two major pharmacological classes:

- ◆ REVERSE TRANSCRIPTASE INHIBITORS (RTIs) further divided into two groups:

Nucleoside Reverse Transcriptase Inhibitors (NRTIs): zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir.

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs): nevirapine, delavirdine, efavirenz.

- ◆ PROTEASE INHIBITORS (PIs) : saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir.

A comprehensive list of compounds in these classes is provided in Table 3.

The antiviral effect is based on the inactivation of two key enzymes in the life cycle of HIV-1: 1) reverse transcriptase (RT), which acts in an early phase of virus replication and results in the retrotranscription of virus RNA in DNA, and 2) protease (P), which acts in a later phase and is essential for the assembly of virus structural proteins.

NRTIs, also referred to as nucleoside analogues, act by competing with natural deoxynucleoside triphosphates for the binding to RT and for the incorporation into newly synthesized viral DNA. Zidovudine and stavudine, of the thymidine subgroup, are preferentially active against HIV-1 in activated CD4 cells; the non-thymidine group may have equivalent activity in resting and activated CD4 cells. These different mechanisms provide the rationale for avoiding combinations of agents acting on the same cell target. NRTIs were introduced sequentially, starting with zidovudine in 1986, the only FDA-approved ARV agent until 1991, and were routinely employed as monotherapy to 1994. This strategy rapidly resulted in the emergence of resistant HIV-1 strains, and since 1994 dual therapy has been used. This resulted in a more marked and durable suppression of HIV-1 replication. Dual NRTIs combinations still represent the backbone of all currently available regimens administered as initial ARV therapy.

The target of NNRTIs is the same as NRTIs. i.e. viral reverse transcriptase. However, they act by binding in a reversible and non-competitive manner to the RT enzyme, which is therefore inactivated. NNRTIs must always be administered as part of a maximally-suppressive ARV regimen; otherwise, there is a high chance of selective

mutations occurring in the HIV-1 genome leading to reduced susceptibility to all drugs in this pharmacological class. The combination of a NNRTI and dual NRTIs is one of the currently recommended regimens for initial ARV therapy.

PIs prevent infectious virus production by blocking the HIV-1 protease. In the presence of a PI, only defective viral particles, unable to infect new cells, are produced. In combination with dual NRTIs, PIs represent one of the recommended regimens for initial ARV therapy, and are also included in many second- and third-line regimens.

Table 3. Currently available antiretroviral drugs

	Name	Trade name	FDA approval (year)
Nucleoside Reverse Transcriptase Inhibitors	zidovudine ZDV,AZT	Retrovir (Glaxo-Wellcome)	1987
	didanosine ddl	Videx (Bristol-Myers Squibb)	1991
	zalcitabine ddC	HIVID (Hoffman-LaRoche)	1992
	stavudine d4t	Zerit (Bristol-Myers Squibb)	1994
	lamivudine 3TC	Epivir (Glaxo Wellcome)	1995
	abacavir ABC	Ziagen	1998
	zidovudine/lamivudine ZDV/3TC	Combivir (Glaxo Wellcome)	1997
	zidovudine/lamivudine / abacavir ZDV/3TC/ABC	Trizivir (Glaxo Wellcome)	2000
	Non-Nucleoside Reverse Transcriptase Inhibitors	nevirapine NVP	Viramune (Boehringer Ingelheim)
delavirdine DLV		Rescriptor (Agouron)	1997
efavirenz EFZ		Sustiva (Merck Sharp & Dohme)	1998
Protease Inhibitors	saquinavir* SQV	Invirase (Roche)	1995
	Ritonavir RTV	Norvir (Abbot Laboratories)	1996
	indinavir IDV	Crixivan (Merck Sharp & Dohme)	1996
	nelfinavir NFV	Viracept (Agouron)	1997
	amprenavir APV	Agenerase (Glaxo Wellcome)	1998
	lopinavir LPV/r	Kaletra (Abbot Laboratories)	2000

* Saquinavir is available in two formulations (Invirase and Fortovase)

Principles of antiretroviral therapy

The years since 1996-97 have been identified as “the HAART era”. HAART (highly active antiretroviral therapy) combination regimens containing at least three ARV drugs, is the current gold standard for therapy. These potent regimens result in a rapid and marked decrease in viremia. Most patients achieve undetectable levels of circulating HIV-1 RNA (<20-50 copies/ml) within three or four months of treatment, associated with an increase in CD4 cell count which correlates directly with pre-therapy baseline values. HAART rapidly became standard care in many developed countries, where it has resulted in an impressive decline in the rates of opportunistic infections and deaths due to HIV-1 infection.

Currently, all national and international guidelines recommend the use of potent regimens, including at least 3 agents, acting with different or similar mechanisms, for starting ARV therapy: 2 NRTIs + 1 PI; 2 NRTIs + 1 NNRTI; 2 NRTIs + 2 PIs; 3 NRTIs. No conclusive data exist to establish which regimen represents the best choice, and all have advantages and disadvantages.¹⁵

When to start antiretroviral therapy

The best time to initiate antiretroviral therapy (ART) and the best initial regimen remain controversial. Ideally, to prevent progressive immune damage, treatment should be initiated as early as possible in the course of the disease.

There is, however, an increasing tendency to defer initiation of ART until immune deficiency is measurable and the risk of disease progression is high.

This approach follows from several observations:

- ◆ the risk of disease progression is low until substantial CD4 cell loss has occurred
- ◆ significant immune recovery occurs even with delayed therapy
- ◆ many patients achieve only an incomplete or transient control of viral replication with therapy, resulting in the selection of resistant strains of HIV-1 and limiting future therapeutic options
- ◆ all regimens have associated toxicity, some of which is serious and has a significant negative impact on quality of life
- ◆ costs of ART are very high.

These considerations have led to a recent revision of international and national therapy guidelines on “when to start”. The goal of therapy is to maintain the patient in a healthy state and to avoid opportunistic infections. To achieve this goal, it is now considered appropriate to delay therapy in the asymptomatic individual until the CD4 count decreases to a level where there is an appreciable risk of serious opportunistic infections (i.e. when CD4 lymphocytes fall below 350 cells/mm³). An earlier initiation of treatment may expose the patient to unnecessary medication-related risks, ranging from

adherence problems and negative impact on quality of life, to the potential for early development of resistance to ARV agents, and the possibility of serious metabolic complications. Irrespective of CD4 cell count, high values of plasma HIV RNA (above 50 000 copies/ml) or the presence of clinical symptoms should prompt initiation of therapy.

Once therapy is initiated, the ultimate goal is the maximal suppression of HIV-1 replication because of the major short-term risk of persisting viral replication (even at low levels) in the presence of ARV therapy leading to the emergence of drug resistance. This goal is particularly desirable for persons being treated for the first time. To achieve this, regimens composed of three or four drugs, usually with a backbone of two NRTIs, plus one (or two) PIs or one NNRTI are used. Regimens including three NRTIs are also being evaluated.

There is at present no clear superiority of any one of these acceptably potent initial regimens; recommendations for any specific combination of individual drugs cannot be made. However, since future options for therapy may be trained by an initial regimen, the choice of a particular schedule should be individualized. It should consider the strength of data supporting the agents, the potential for adverse effects, the likelihood of important drug interactions, likelihood of adherence, and the potential for subsequent treatment options should the regimen fail.

Unfortunately, clinical trials report that only 20 to 40% of previously untreated patients achieve complete virus suppression (defined as plasma HIV-1 RNA below the limits of detection), even with the currently-available potent regimens. Evidence from several laboratories using sensitive molecular assays suggests persistent virus replication in lymphoid tissues of at least some of these patients. Such persistent replication may be responsible for the occasional “blips” in plasma HIV-1 RNA that are observed in some patients.¹⁶

Intermittent non-adherence, inter-individual variation in pharmacokinetics, drug interactions, and inadequate potency of current regimens, may all contribute to persistent virus replication in the face of what should be optimal therapy.

With the high rate of HIV-1 replication, the concern is that any residual turnover of virus could rapidly repopulate the HIV-1 quasispecies. Recently, much interest has arisen around the feasibility of intermittent antiretroviral treatment. This follows anecdotal observations in subjects who discontinued HAART for different reasons but showed a persistent control of viremia, associated with a vigorous anti-HIV-1 immunological response. The presence of circulating viral antigens might serve as stimulus to the maintenance or re-induction of specific cytotoxic T lymphocytes and anti-HIV-1 helper activity. In addition, reducing the total amount of drugs would decrease costs and therapy-related toxicity, possibly improving patients' quality of life.

The approach of intermittent therapy - periods of standard treatment separated by intervals of “off-therapy” (Structured Treatment Interruptions) - is being tested in several clinical trials in HIV-1 infected patients at different stages of disease. These studies will determine the long-term safety and effectiveness of this approach. Pending

the results of these studies, intermittent therapy in clinical practice should be discouraged.

Treatment failure and its management

After a variable time period, nearly all HAART treated subjects undergo virological failure, with a rebound in HIV-1 RNA plasma levels. Virologic escape is usually followed by immunologic and, eventually, clinical deterioration. The time lag between HIV-1 RNA rebound and clinical failure varies from patient to patient. It has become clear that the CD4 cell count may remain high even in the presence of a clear rebound in HIV-1 RNA. This “disconnection” between immunological and virological response is observed in 20-30% of treated subjects, in whom acceptable values of CD4 cell count may be maintained in spite of high levels of plasma viremia. The current trend is to consider these patients as “partial” responders rather than as “failures”, and to continue monitoring them, usually without changing the regimen if the clinical condition remains stable. However, if resistance mutations related to the currently administered drugs are detected, changing the antiretroviral treatment should be considered.

Treatment failure in HIV-1 infection is multifactorial: drug factors (limited potency, low genetic barrier, sub-inhibitory plasma levels, pharmacological interactions) interconnect with host factors (poor compliance, limited recovery capacity of the immune system) and viral-related factors (presence of resistant variants). The appearance of mutations conferring resistance in the viral genome is not always the primary or main cause of failure, but it invariably represents the final result of all the possible causes that lead to persistent virus replication.

Before changing an ARV regimen, it is important to determine why the current regimen is failing, so an appropriate response can be made. In particular, poor adherence to the prescribed regimen is a frequent cause which requires specific counselling and use of “easy to take” regimens. Once possible causes are eliminated, if evidence of treatment failure remains, continuing with the same regimen will eventually lead to the development of high-level drug resistance. This diminishes the likelihood that salvage regimens will be successful. Thus, if clear treatment options exist, early switching could maximize the chances of therapeutic success of the next treatment regimen and preserve future options.

The situation is different for patients who are highly treatment-experienced, and for whom fewer options remain. In such cases, a more conservative approach may be warranted. Despite the general principle that the new regimen should consist entirely of drugs not previously taken, available therapeutic options may be limited, especially for patients experiencing their second or third failure, and given the high degree of cross-resistance among all ARV drug classes. In this highly treatment-experienced population “recycling” drugs or simply “intensifying” a failing regimen may be the only alternative. However, observational and prospective studies have shown disappointing results for most salvage strategies.

Antiretroviral drug resistance

HIV-1, like other RNA viruses, shows a high degree of genetic variability. Studies have demonstrated that viral turnover in an infected individual is extremely high: approximately 10^{10} viral particles per day. This means that more than 99% of the virus present at any time is produced by cells infected in the previous two weeks. This reproductive mechanism is error-prone, and an average of one error per RNA genome is made at each replication cycle. Thus HIV-1 exists as quasispecies in an infected individual where the continued replication of virus results in the production of all possible single base-pair mutations across the genome. Although the vast majority of mutations are either lethal or neutral (“viral polymorphisms”, i.e. naturally occurring mutants with unknown implications for resistance) this quasispecies diversity imparts a plasticity to HIV-1 which may allow the proliferation of mutations that impart increased fitness, particularly in response to strong selective pressures. ARV drugs that target the virally-encoded reverse transcriptase or protease enzymes will select drug resistance mutations that impart a drug-resistant phenotype.

Resistance may be broadly defined as any change, relative to a “wild-type” (i.e. a variant with a normal genetic constitution) virus, which is detected in the presence of an ARV agent and results in an improved replicative capacity. Resistance is not a “all-or-none” event, since it may be overcome, *in vitro*, by increasing drug concentration; therefore, the term “reduced susceptibility” seems more appropriate.

Resistance is mainly caused by changes in the HIV-1 genome, which can be point mutations, insertions and/or deletions and therapy must be maximally suppressive to avoid the overgrowth of such variants.

Nevertheless, even in patients receiving the most potent ARV regimens, a persistence of minimal residual replication has been found. Thus, the pressure exerted by the drugs eventually leads to the selection of mutants with reduced susceptibility.

It is of interest that if therapy is stopped in an individual with virologic failure and a high level of resistance, a “reversion” to wild type may be observed in plasma. In fact, in the absence of pharmacological pressure, there is “compartmentalization” of the resistant variants in cellular archives, from which they can re-emerge and re-expand if the same therapy is reintroduced.

The likelihood that an individual HIV-1 strain will lose susceptibility to an ARV agent depends on several factors; a key drug-related factor is the genetic barrier to resistance. This is defined as the number of mutations required before the antiviral activity of the drug is knocked out by resistance. If multiple mutations are necessary to achieve a marked reduction in susceptibility, the agent is said to have a high genetic barrier to resistance. On the other hand, when a high level of resistance develops with a single mutation, the agent has a low barrier to resistance. NNRTIs are an example of drugs with a low barrier, since a single mutation (K103N) may result in high-level resistance across the entire class of drugs. The NRTI lamivudine is another example of a drug with a low genetic barrier. Most of the other antiretroviral agents are in an intermediate position. Few drugs may be said to have a high genetic barrier; among these are two

NRTIs (ddI and ddC) and the recently commercialized co-formulation of PIs, lopinavir. A time frame of weeks/months is sufficient for resistance to develop when the genetic barrier is low; resistance may take years with compounds with high genetic barrier.

Drug resistance has been detected for all ARV drugs introduced into clinical practice and the most common patterns of resistance mutation have been identified.¹⁷ Using a generally accepted terminology based upon the magnitude of the effect on drug resistance *in vitro* and *in vivo*, primary and secondary resistance mutations are defined¹⁸. Primary mutations have high specificity for one compound, and significantly compromise the susceptibility of the virus to that agent. They may be selected early in the resistance process. The role of secondary mutations is less clear. They tend to occur later and to accumulate in a viral genome, apparently without producing a detectable level of phenotypic resistance. However, they may enhance viral replication by increasing viral fitness.

Resistance to NRTIs

Resistance to zidovudine was first documented in 1989, two years after the agent became widely available, in subjects treated with monotherapy for 6 or more months. These individuals experienced viral rebound to pre-therapy levels. Viral isolates were 100-fold less susceptible to zidovudine than isolated pre-treatment strains. A set of mutations at six codons in the RT, usually appearing in sequence, was responsible for resistance development. Codon 215 mutations are found in nearly all viral isolates exhibiting high-level zidovudine resistance.¹⁹ The linked codon mutations 41 and 215 are commonly associated with high levels of resistance and appear to confer selective growth advantage. These mutations may also appear in patients who are receiving prolonged ddI monotherapy.

Resistance to lamivudine, a drug with a low genetic barrier, occurs through a single mutation, the M184V. Much interest has arisen recently around the so-called TAMS (thymidine associated mutations) changes associated with resistance to zidovudine, didanosine and stavudine. It has become evident that stavudine selects for genotypic changes previously considered exclusive to zidovudine. Thus, the presence of these mutations compromises the effectiveness of stavudine. Multiple mutations are required for high level resistance to abacavir.

Perhaps due to the frequent use of NRTIs in combination, strains of HIV-1 resistant to multiple NRTIs have been isolated. The principal site involved in NRTI cross-resistance (defined as resistance to two or more agents within the same pharmacological class) is codon 151. In fact, the Q151M mutation alone confers high level resistance to all NRTIs but, in addition, impairs viral replication, so further changes occur as compensatory mutations restoring replicative capacity to the highly resistant virus. Furthermore, an insertion of 6 basepairs at RT position 69 has been shown to be involved in HIV-1 resistance to all NRTIs but d4T.

Mutations selected by NRTIs often result in structurally modified reverse transcriptase; this results in a reduction of the enzyme's affinity for the drug and a reduced ability to compete with the cell natural nucleotides for catalytic binding to HIV-1 RT.

Resistance to NNRTIs

The use of NNRTIs as monotherapy results in rapid emergence of high level resistance, due to the mutations around and within the drug-binding site. A low genetic barrier characterizes NNRTIs and a single amino acid substitution at codons 103 or 181 can result in high level resistance. In addition, significant cross-resistance exists among all the three approved NNRTIs (nevirapine, efavirenz, and delavirdine), so that sequential use of NNRTIs in antiretroviral therapy is usually not feasible.

Resistance to PIs

Resistance to PIs has been attributed to mutations in the active site of the HIV-1 protease and elsewhere. Changes in at least 25 different codons have been implicated; some mutations appear to be specific for one agent in this class, but resistance to most of the approved PIs shares the same mutational pattern, so there is a significant degree of cross-resistance among them. A number of secondary mutations also develop under PIs therapy; their role is not completely understood, but some of them are compensatory or may influence viral fitness.

Resistance reversal

Resistance reversal occurs when mutations resulting in resistance to one drug reverse the effect of resistance to another drug, thereby restoring virus susceptibility to it. The classic example of resistance reversal is the suppressive effect of mutation M184V (selected by lamivudine) on the zidovudine resistance mutation at codon 215. Resistance reversal *in vivo*, however, may not be straightforward or durable.

Multi-drug resistance

Multi-drug resistance refers to resistance to drugs of more than one class or mechanism of action. It is an inevitable and predictable consequence of the widespread use of ARV drugs combinations, as well as the sequential use of these drugs. Multidrug resistant HIV-1 strains have been isolated from a number of ARV-experienced individuals, as well as from a small number of untreated HIV-1 infected individuals.

Resistance in the era of combination therapies

Advances in the knowledge of viral resistance mechanisms led to the widespread acceptance of ARV combination therapy as the standard of care for HIV-1 infection. However, even with maximally suppressive regimens, resistance occurs and limits the success of subsequent salvage treatments. While pharmacological research focuses on the development of new agents with more favourable profiles, efforts should be made

to maximize the effectiveness of therapy and delay the emergence of resistant strains. General recommendations for this include:

- 1) Using potent first-line therapies to maximize the chances of long-term suppression;
- 2) Avoiding uncontrolled and irregular interruptions of therapy which allow viral replication in the presence of suboptimal drug exposure;
- 3) Rational planning of a sequence of second- and third-line therapeutic options to overcome resistance if the initial therapy fails.

Methods for antiretroviral resistance testing

The replication of HIV-1 depends on viral reverse transcriptase and cellular RNA polymerase, a reproductive mechanism that is error-prone and lacks the proof-reading function of eukaryotic DNA replication. Spontaneous mutation without a repair mechanism in the nucleotide sequence of the viral genome has been demonstrated to occur in 10^5 nucleotides, or one error on average per RNA genome. Reverse transcriptase or protease enzymes will select drug resistance mutations that impart a drug resistant phenotype.

Drug resistance of HIV-1 is assessed in plasma virions that are the progeny of recent, active infection and represent the form of the virus with best replicative capacity. Tests for drug resistance depend either on genotype or phenotype of the virus. Genotypic tests identify specific mutations in the consensus sequence of circulating RNA from viral genomes associated with drug resistance, and the relative drug resistance of virus is deduced from the mutations present in circulating virus. Phenotypic tests directly measure the enzymatic activity of viral gene products or the replication of the virus in the presence of increasing concentrations of drug.

Genotypic testing

Genotypic testing of HIV-1 drug resistance is an application of the recent advances in DNA sequencing and data analysis techniques. Briefly, viral RNA is extracted and reverse transcribed; the DNA is amplified by polymerase chain reaction; the sequence of nucleic acids is determined and translated to the amino acid structure of the encoded protein. The presence or absence of drug resistance is estimated based on databases of viral sequences and evolving algorithms to identify specific mutations and combinations of mutations associated with drug resistance. Many sequencing methods produce a consensus sequence for most of the genes of interest. Some methods of genotyping are limited to the identification of mutations only at selected nucleic acid residues through differential hybridization to determine whether a specific codon is “wild-type” or “mutant”. These methods, Line Probe immuno assay (LiPA) and Oligonucleotide Ligation Assay (OLA), are more sensitive than sequencing for detecting resistant variants which are present in a low proportion in mixed populations of resistant and wild type viruses.

Visible Genetics®, Applied Biosystems® and Affymetrix® have each developed specific primers, sequencing, and data analysis methods with the capacity to obtain viral sequences within days from the extraction of HIV-1 RNA from plasma where greater than 1,000 copies per ml of RNA are measured. An improved and sensitive assay to obtain sufficient amplified material in most plasma samples can be useful for detecting genotypic resistance mutations in patients in which viral load is lower than 400 copies per ml.²⁰

Rigorous comparison of methods has not been performed. However, international quality control studies suggest that greater than 99% concordance is attained in the correct identification of nucleic acid residues between laboratories and techniques. Quality assurance programmes for laboratories undertaking these tests have been developed to assess reproducibility and accuracy of genomic results.

Phenotypic testing

Phenotypic tests of ARV drug susceptibility were initially developed using cultured virus isolates. However, molecular methods to create recombinant viruses or vectors which contain genes amplified from plasma HIV-1 RNA have largely supplanted direct assays of virus cultures. Phenotypic tests which examine the susceptibility to the 15 currently licensed ARV drugs are provided by two proprietary methods; ViroLogics® and Virco® have each developed methods to rapidly assess the activity of drugs on virus replication *in vitro*. These methods derive the concentration of drug, *in vitro*, which results in a 50% inhibition of a patient's virus compared to a standard, wildtype or drug-susceptible control. The results are reported as a "fold-change" in susceptibility for each drug, based on the direct comparison of the inhibition of replication or enzymatic activity of patient and control virus. Fold-changes in individual drug susceptibility are used in the interpretation of phenotypic testing to assess whether the drug is likely to be active against the virus tested. A key issue is the "cutoffs" used, i.e. the value of the fold increase in IC₅₀ at which a virus is considered resistant. So far, these have been the same for each drug and have been calculated by the assay variability seen on repetitive testing of a single reference virus. To define new, biologically relevant, cut-off values, Virco® has recently used its Antivirogram assay to determine the susceptibility to all antiretroviral agents of approximately 1000 HIV-1 positive samples from untreated subjects of different geographical areas. Assuming that results obtained in this population reflected the natural variation in infected population, the new cut-offs have been established considering as normal range the values within 2 SD of the mean values obtained for each drug. Consequently, patients' isolates are now divided in two categories: within normal range (susceptible) and outside normal range (resistant). It is noteworthy that, with this new approach, normal ranges vary for different ARV agents, being 3- to 4.5-fold for the NRTIs, 6- to 10-fold for NNRTIs and 2.4-to 4-fold for PIs.

Further progress in improving clinical relevance of phenotyping results has been made by ViroLogic®, who recently introduced the concept of "clinical cut-offs" for abacavir (NRTI class) and lopinavir (PI class). To this purpose, clinical trial data were used to assess the impact of changes in drug susceptibility on treatment outcome: reduced

susceptibility to abacavir appeared to be clinically significant (i.e. correlated with clinical response) at 4.5-fold, while for lopinavir the cut-off value has been established at 10-fold. Future studies will hopefully yield clinically relevant cut-offs for all ARV drugs.

Performing and interpreting resistance tests

Both genotypic and phenotypic assays are technically complex, requiring expert personnel working under strict standard operating procedures. The interpretation is also complex. Expertise is required to assess the implications of genotypic results in the context of the patient's clinical and pharmacological history, and the significance of complex mutational patterns including multiple mutations. Naturally-occurring polymorphisms may be a further confounding factor. Several algorithms have been proposed to assist in applying laboratory information to patient management, but none of these yet meets all the criteria for widespread use by practitioners. Although phenotyping apparently provides more straightforward results, expert opinion is still necessary to interpret their implications for treatment decisions.

One approach to maximize genotypic information, reinforced with data on phenotype, is in using a "virtual phenotype" (commercially available through Virco®). In this computerized system, the patient's viral genotype is matched with similar genotypes in a large relational database of thousands of clinical samples analysed by both genotype and phenotype. A virtual phenotype is therefore generated which is a probabilistic estimate of a genotype-derived phenotype. Published data suggest that virtual phenotype is well correlated with actual phenotype. It is less clear if it is equally accurate in predicting clinical outcome. A general consideration common to both types of assays which should be kept in mind when applying the results of resistance assays is that, to date, they have been more useful in identifying drugs likely to be ineffective, than in pinpointing which agent will produce a significant response *in vivo*.

Resistance testing has been developed and applied mainly to the HIV-1 B subtype; however, published data suggest it may be utilised for non-B subtypes as well.^{21 22}

Drug resistance testing and patient management

Clinical guidelines on the use of resistance testing have been published by International and European Collaborative Groups.^{23 24} Both report critical caveats in the use of drug resistance testing for patient management.

Both phenotype and genotype testing currently measure only the major replicating virus obtained from a patient at the time of testing. The relative susceptibility to a drug or drug class, assessed by either assay, has been significantly associated with antiviral effectiveness as measured by relatively short-term inhibition of virus replication. However, drug resistance testing only provides evidence for or against the susceptibility

of a patient's predominant and currently replicating virus. Resistance mutations or phenotypic resistance selected against drugs taken in the past, while invariably present in the "archive" of latent proviruses, is often not detected by either test. Finally, drug resistance is often accompanied by a decrease in fitness, the replicative capacity of the virus, and pathogenic potential. Several studies have demonstrated that certain mutations which impart drug resistance also decrease the replicative capacity of the virus. One could say that the "price" paid by viruses selected by drug resistance is diminished enzymatic efficiency.

It is not entirely clear whether the continued clinical and immunologic improvement seen in the majority of patients despite the presence of resistant virus is the result of diminished fitness. However, it is likely that the decrease in morbidity and mortality which has coincided with the introduction of triple drug regimens is also the result of diminished viral pathogenicity, which in turn results from decreased fitness of the virus due to drug pressure.

Resistance testing has been evaluated in prospective, randomized, clinical trials with virologic suppression as an outcome (GART²⁵, VIRADAPT²⁶, HAVANNA²⁷, NARVAL²⁸, VIRA 3001²⁹). These studies compared virologic outcomes between patients assigned to drug regimens on the basis of genotypic or phenotypic testing with those patients for whom the new regimen was chosen empirically without reference to resistance testing. Overall, resistance testing (compared to not testing) has led to a superior virologic outcome among patients who have failed at least one ARV regimen. However, the results of these complex clinical trials must be interpreted in light of the evolving interpretation of resistance testing, differences in the numbers of drugs provided in each study arm and on the variable response of physicians and patients to testing and "expert recommendations" with respect to the drugs actually taken.

Resistance testing is currently recommended in several settings, and should be considered in many others. Among patients initiating therapy who demonstrate an inadequate response, resistance testing can determine whether (and which) alternative drugs or adherence strategies should be considered. Similarly, in patients failing treatment (as evidenced by the sustained rebound of the virus after prolonged treatment) resistance testing is recommended before switching to alternative drugs or attempting to "intensify" a regimen.

In untreated patients initiating therapy, many experts agree that the local epidemiological circumstances and exposure history of the patient should guide the decision about testing for resistance before initiating treatment. There are instances of phenotypically-resistant virus with the expected genotype, i.e. resistance mutations demonstrated, in newly-diagnosed or infected patients, and it is clear that the acquisition of resistant virus diminishes the virologic effectiveness of subsequent therapy. With an increasing frequency of resistant viruses isolated from untreated patients, resistance testing may be warranted in untreated patients, particularly if they have been exposed to viruses from treated individuals.

Finally, where occupational or sexual exposure leads to post-exposure prophylaxis, resistance testing of virus from the source patient is warranted to minimize exposure of the uninfected "recipient" to potentially toxic drugs. In general, the US Centers for

Disease Control and Prevention (CDC) recommend the use of a protease inhibitor and ZDV+3TC as occupational post-exposure prophylaxis when the source patient has received multiple therapies. This regimen could be altered or augmented, based on resistance testing from the donor, although clinical trials evaluating this approach are required.

Using resistance tests for epidemiological purposes

Several points should be considered when using resistance tests for epidemiological purposes, i.e. to assess the circulation of drug-resistant HIV-1 variants in selected patient populations. First, it must be recalled that, within each individual patient, HIV-1 is present as a mixture of genetic variants (quasispecies) in a dynamic equilibrium and that a resistance assay measures only the dominant species, i.e. that representing more than 20% of the total, at the time the test is performed. Second, multiple mutations may interfere with viral fitness, as shown by the reduced replicative capacity of viruses carrying multiple mutations. This may also result in a disadvantage for transmission. A further issue is that to collect reliable data for epidemiological purposes, resistance tests should be performed on virus from subjects with a known pharmacological history. In fact, samples from patients with poor compliance or those having stopped therapy may yield misleading results since when drug pressure is removed resistant species become a minority, and are not detectable. This may lead to an underestimation of the resistance rate, especially in subjects with heavy drug-experience.

What is the most suitable methodology for epidemiological purposes? Phenotyping, at least with the currently available techniques, seems to be too laborious and expensive. Genotypic assays based on either hybridization or direct sequencing are appealing, being more rapid, less technically cumbersome for decentralized laboratories, and less expensive. Point mutation assays, despite their intrinsic limitations, might nevertheless be utilized when epidemiological interest is focused on specific codons of the HIV-1 genome.

Simultaneous detection of drug resistance and HIV-1 subtype

It is noteworthy that protease and reverse transcriptase gene sequences can also be used to determine the genetic subtypes and inter-subtype recombinants. This allows a correlation of resistance patterns with genetic subtype and monitoring of the prevalence of different subtypes in a given geographic region. This information could prove to be of value for vaccine development and testing.³⁰

In addition to serological procedures, HIV-1 subtype assignment is currently achieved by heteroduplex mobility assay or sequencing of portions of *envelope* and *gag* structural genes and methods to infer the phylogenetic relationships between them. Genotyping of HIV-1 *pol* reverse transcriptase (RT) and protease (PR) regions generates long stretches of nucleotides (approximately 300 and 900 bp, respectively). These sequences may be used to define the clade clustering. Inter-subtype recombination of non-clade B strains may also be assessed. Therefore, *pol* sequencing is useful in tracing

epidemiological trends in HIV-1 populations and the use of antiviral drug resistance genotyping for clinical purposes may allow the concurrent surveillance of circulating HIV-1 subtypes on a large scale.

Unresolved technical issues and needs

Studies to increase understanding on a number of issues are needed:

- ◆ Both genotypic and phenotypic tests are based on the amplification of nucleic acid in the clinical specimen. This process, which was validated for B clades of HIV-1, may be less efficient for non-B clades;
- ◆ The utility of commercially-available tests is limited for ARV drugs still under development and the thresholds used to define susceptibility in phenotypic assays are arbitrary;
- ◆ Genotypic assays with the high throughput generation of data, magnified by natural polymorphisms and mixtures are difficult to interpret. The presence of a given mutation may strongly suggest resistance but does not prove it. Changes at codons other than those specifically associated with resistance to a particular drug may influence the phenotype;
- ◆ Assays specifically directed at detecting minority species should be developed;
- ◆ Application of methods for extraction and amplification of HIV-1 RNA from plasma samples with low viral load should be performed;
- ◆ The meaning of resistant variants presented in the “archive” of latent proviruses and their evolution in changing regimens are not well known;
- ◆ Correlation between genotype and phenotype is not perfect;
- ◆ There is lack of consensus with regard to nomenclature and reporting;
- ◆ The practical issue for developing standards and controls must be addressed and indicators for assay performance and quality control guidelines at laboratory level should be introduced.

The circulation of HIV-1 drug-resistant strains

Antiretroviral drug resistance in treatment-experienced populations

As already discussed, a remarkable proportion of HIV-1 infected subjects receiving ART experience failure. Although the appearance of genomic mutations is not always the primary cause of failure, it invariably represents the final result of all the possible causes (poor compliance, drug toxicity, metabolic abnormalities, low potency of the regimen) resulting in incomplete suppression of virus replication. Nevertheless, resulting drug resistance does not necessarily affect all drugs in a treatment regimen.³¹

In a recent study of 12 000 US-based patients in treatment during 1999, 27% harboured virus that was resistant to all three classes of ARV drugs, 29% had virus resistant to two classes of drugs and 22% had virus resistant to a single class.³² Resistance is a

worldwide phenomenon, also occurring in areas where ART has only been introduced recently. In Uganda, at a mean of 3 months after starting therapy, 63% of 107 patients receiving 2 NRTIs or HAART regimens showed high phenotypic resistance to > 1 drug, intermediate resistance was observed in 11% and 7% of the patients had multidrug resistance.³³ In these settings, the use of low potency combinations (e.g. dual NRTI regimens) and the likelihood that drug consumption is irregular may favour the emergence of resistant strains.

Even with successful therapy, residual virus replication may occur with the selection of ARV resistant virus. In some cases, especially in heavily pre-treated patients, the absence of resistance may be misleading, reflecting a transient “reversion” to a wild type virus, due to unreported treatment interruptions. In these cases the study of proviral DNA in peripheral blood mononuclear cells, instead of plasma virus sequences, might detect the presence of “archived” mutations.

The presence of a high proportion of resistant HIV-1 strains in treated populations is of public health concern as it increases the circulation and potential transmission of resistant HIV-1 variants. This limits the efficacy of ARV therapy in newly infected patients.³⁴ Furthermore, superinfection with highly resistant strains in a person on therapy has also been documented.³⁵

Antiretroviral drug resistance in treatment-naïve populations

Recently, increased attention has been focused on transmission of resistant HIV strains as a result of studies showing a high prevalence of resistant virus in subjects studied during or early after primary infection (recent seroconverters). Active surveillance in different geographical areas has yielded conflicting results.

Before reviewing the reports, the major issues in this field should be noted:

- ◆ A key factor in studying the circulation of resistant strains is to define clearly the target population. It is known that resistant strains are common in acutely infected subjects, and the likelihood of detecting them is correlated inversely with the time from infection. This underscores the need for a strict definition of study populations: in principle up to one year after primary infection is considered as a time-limit for reliable prevalence data. Beyond this time, mutations are presumably lost because resistant variants are less fit and do not grow in the absence of drug pressure.
- ◆ Data reported are heterogeneous, both in the characteristics of study populations (ethnic group and time from primary infection), the methodology used (genotyping or phenotyping or both) and the different cut-offs for defining resistance.
- ◆ When genotyping is used, primary mutations only or both primary and secondary mutations have been reported. However, the interpretation of secondary mutations in treatment-naïve, recently-infected populations, is not straightforward, as they may represent naturally-occurring variants (polymorphisms).

- ◆ For phenotypic assays, variable cut-off for defining resistance has been adopted. Most reports refer to intermediate levels (> 2.5-4 fold < 10 fold) of reduced susceptibility, whose interpretation may be problematic.
- ◆ Travel and movement of patients must be considered. It is the prevalence in the area where the patient actually became infected that is important.
- ◆ The varying prevalence of different HIV-1 subtypes in the study populations may account for differences in the rate of detected resistance. Indeed, there are no extensive data showing that genotypic tests perform equally well in B and non-B clades.

Considering the above limitations, a review of published and unpublished data on the prevalence of known resistance-conferring genotypes and of reduced susceptibility to antiviral agents detected by phenotype is summarized below. Data refer to untreated infected individuals: seroconverters (SC), primary HIV infection (PHI), recent HIV infection (RHI), established HIV infection (EHI). Data are summarized alphabetically by the geographical area.

Australia

AIDS incidence in Australia has declined since 1995 following the introduction of HAART regimens.³⁶ It is estimated that 68% of patients attending selected clinical sites are receiving triple combination treatment. Available data from genotyping (G) in PHI subjects is grouped by year³⁷:

Clinical status	Year	No. of patients	Method	RTIs % Primary	PIs % Primary
PHI	1992	6	G	16.6	0
“	1993	17	“	17.6	0
“	1994	14	“	21.4	0
“	1995	10	“	40	0
“	1996	12	“	0	0
“	1997	16	“	0	0
“	1998	27	“	0	0
“	1999	25	“	8	0
“	2000	11	“	36.4	9

Canada

A substantial proportion of individuals who have been recently infected with HIV-1 carry virus that is resistant to most available ARV drugs. Transmission of multidrug resistance was confirmed by the demonstration of a similar HIV-1 genotype in the source partners. More than 92% of primary HIV-1 infections are caused by subtype B virus.

Clinical status	Year	No. of patients	Method	RTIs%	NRTIs %	NNRTIs %	PIs %	MDR %	Overall %
SC,PHI ³⁸	97-99	81	G	20	-	-	6	9.9	-
PHI ³⁹	99-00	61	G	-	-	-	6.5	3.2	26
EHI ⁴⁰	97-00	98	G	-	6.1	3.1	2	-	11.9

Côte d'Ivoire

94 specimens from recently infected patients (<1 year), most subtype A, were analysed by genotyping and phenotyping. Extensive polymorphism but full susceptibility to PI and NRTI were observed; 12% of patients had mutations responsible for reduced susceptibility to NVP.⁴¹

France

Latest data refer to a national study involving 108 patients who presented with primary infection during 1999. Although the B subtype is predominant (84.5%), all non-B subtypes can now be isolated in France.

Clinical status	Year	No. of patients	Method	RTIs%	NRTIs %	NNRTIs %	PIs %	MDR %
PHI ⁴²	95-98	48	G	16.6	-	-	2	-
PHI ⁴³	99	108	G	-	6.5	3.7	2.8	0.9

Italy

Data collected from both recently and chronically infected untreated subjects showed that the prevalence of non-clade B subtypes has increased from 1.9% prior to 1997, to 8.4% in recent years. The potential correlation between baseline mutations and virological outcome was evaluated in 130 of the patients who initiated PI-containing triple combination therapy. The presence of 1 minor protease mutation (codon 10 or 36) was strongly associated with treatment failure.⁴⁴

Clinical status	Year	No. of patients	Method	NRTIs %	NNRTIs %	PIs %	MDR %
SC ⁴⁵	96-98	68	G	10.3	5.9	5.9	4.4
EHI ⁴⁶	98-00	347	G	11.5	-	1.7	-

South America

Non-B subtypes are prevalent in South America: in Argentina 65.6% of HIV-1 strains are B/F recombinants and subtype F is present in Brazil and Venezuela. Resistance studies suggest a lower proportion of resistance to both RTIs and PIs than in Europe. In untreated patients, 3.6% carried strains resistant to RTIs, and resistance to PIs or MDR was not observed.⁴⁷ Data from 56 HIV-1 seropositive asymptomatic patients during 1998 in Brazil indicated an increase in the prevalence of minor resistance mutations to PIs when compared to samples analysed in 1990. On the other hand, the prevalence of secondary mutations in the RT gene seems to be declining.⁴⁸

Spain

Available data from untreated patients shows:

Clinical status	Year	No. of patients	Method	RTIs%	NRTIs %	NNRTIs %	PIs %	Non-B subtypes %
EHI ⁴⁹	99-00	147	G	6.2	5.4	0.7	4.8	5.1*
EHI ⁵⁰	98	52	G	-	-	-	17	-

* In 6.7% of untreated patients infected with non-B subtypes, primary mutations to NNRTIs were observed, and no primary mutations to PIs were detected.

South Africa

Studies on HIV-1 drug resistance were recently initiated in women and children participating in clinical trials for the prevention of mother-to-child transmission. All patients were drug-naïve and were infected with HIV-1 subtype C viruses. No evidence of naturally-occurring resistance mutations to HIV-1 reverse transcriptase inhibitors was found.⁵¹

Switzerland

Transmission of HIV-1 drug-resistant variants was detected in the Swiss cohort study and reported according to the year of infection.⁵² Potential factors involved in the decrease of transmission are the increase in non-B subtypes from 23% in 1996 to 35% in 1999, and the wider use of treatment leading to undetectable viremia in an increased proportion of infected persons (from 10% to 53%).⁵³

Clinical status	Year	No. of patients	Method	RTIs%	NNRTIs %	PIs %	Overall %	Non-B subtypes %
PHI	96	36	G	5.6	-	3.2	8.6	23
“	97	40	“	10	-	8.6	14.6	-
“	98	62	“	7.1	-	2.0	8.8	-
“	99	59	“	3.4	-	2.0	5.0	35
“	96-99	48	P	-	4.1	-	-	-

United Kingdom

Data from a UK⁵⁴ cohort during years 1998-2000, showed a 90% prevalence of B subtypes, obtained by genotyping:

Clinical status	Year	No. of patients	NRTIs %		NNRTIs %		PIs %	
			Primary	Secondary	Primary	Secondary	Primary	Secondary
PHI	98-00	47	15	9	6	15	0	0
EHI	“	39	0	0	6	15	3	41

United States of America

In retrospective studies of phenotypic ARV drug susceptibility in acutely infected or recently seroconverted persons attending clinical centres in large urban areas, the prevalence of virus with a high level of resistance (>10-fold reduced susceptibility) to both NNRTI and PI drugs increased significantly between 1998 and 2000; from 1% to 7% (NNRTIs), and from 2% to 6% (PIs). The cohort includes 394 subjects (165 previously published, the remaining recently communicated) studied by recombinant phenotype.^{6,55} In the same cohort of patients, a correlation between resistance results and treatment outcome has also been found: time to virologic suppression among all patients initiating HAART was significantly longer in patients with > 10-fold reduction in susceptibility to one or more ARV drugs at baseline. Among patients achieving suppression, a trend toward earlier failure was observed in subjects with >2.5-fold reduction in susceptibility.

In an analysis of the most recent data from the CDC sentinel surveillance team monitoring the prevalence of genotypic mutations that promote drug resistance among untreated recently diagnosed HIV-infected people in 10 US cities, 437 persons were tested; 10% of them were recently infected (<6 months). It was found that untreated, chronically infected persons, (>6 months) were as likely to have mutations associated with decreased drug susceptibility as persons who were recently infected, suggesting the prevalence was not currently increasing.^{56, 57}

Clinical status	Year	No. of patients	NRTIs %	NNRTIs %	PIs % Primary	MDR %	Overall %
SC	93-98	99	6	2	1	5	7
RHI	97-99	437	8.5	2.5	0.7	1	10

In another study to determine the prevalence of mutations conferring to ARV agents in a cohort of newly infected individuals (average time from primary infection: 1.7 months) from New York and Los Angeles, an overall prevalence of 16 % was observed.

Clinical status	Year	No. of patients	Method	NRTIs %	NNRTIs %	PIs %	MDR %	Overall %
PHI ⁵⁸	95-99	80	G	12.5	7.5	2.5	3.8	16.3

The situation seems different in non-urban areas in south-eastern United States, where no primary mutations were detected in 20 sexually acquired PHI subjects between 1998 and 2000.

An active surveillance programme is being conducted currently among US military personnel. In 1997-98, a study was done of a cohort of therapy-naive subjects who had seroconverted in the previous three years, and prevalence of ARV resistance was investigated by genotyping and phenotyping. Resistance was found at a higher than the expected frequency.⁵⁹

Clinical status	Year	No. of patients	Method	NRTIs %	NNRTIs %	PIs %	MDR %	Overall %
SC	97-98	114	G	4	15	10	5	22
“	“	“	P	8	26	1	-	-

Conclusions

The previously mentioned limitations in these studies make it difficult to draw firm conclusions. However, some cautious observations can be made:

- ◆ Overall, the prevalence of HIV strains with resistance to at least one antiretroviral drug seems vary around 5-26% of all strains tested.
- ◆ The current situation appears to differ between Europe and the United States. In Europe, transmission of drug-resistant HIV-1 is not increasing. In fact, countries such as Switzerland reported a decrease, following the introduction of potent antiretroviral regimens. On the other hand, data from the US and Canada suggest a sharp increase in the prevalence of resistant strains in recently infected persons, from 3.5 % in 1995-98 to 14% in 1999-00. During the same period, there has been an increase in the transmission of multi-drug resistant strains.
- ◆ Controversy exists about NNRTI resistance. According to some experts, findings are overestimated as some mutations identified reflect natural polymorphisms, and the reported reductions in susceptibility are not clinically relevant.
- ◆ Secondary protease mutations at baseline are often present at a high level (25-75%): in some cases they appear to be predictive of subsequent virological failure.
- ◆ Data from the US show a correlation between drug resistance in the recently infected, untreated subject, and therapy outcome⁶⁰. Further studies are needed to confirm these data and identify which mutational patterns are most predictive of virologic success/failure.

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Istituto Superiore di Sanità

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Annex II: Agenda**Tuesday, 10 October 2000**

12:00-12:15	Welcome and introduction to the meeting	G. Benagiano R. Williams
12:15-12:30	Objectives of the meeting, programme and method of work	S. Vella
12:30-13:30	<i>Break</i>	

Presentations

13:30-13:50	Methods for the detection of antiretroviral resistance	C. Loveday
14:00-15:30	Antiretroviral resistance in North America Antiretroviral resistance in Europe	P. Sullivan V. Miller

Circulation of resistant strains in individual countries:

Canada	(G. Jaaraman, M. Wainberg)
Italy	(C. Balotta, CF. Perno)
Uganda	(C. Kityo)
Brazil	(A. Tanuri, R. Najera)
Spain	(B. Clotet, L. Ruiz)
Australia	(P. Cunningham)
South Africa	(L. Morris)
Russian Federation	(A. Rakhmanova)

General discussion

15:30-15:45	<i>Break</i>	
15:45-16:00	Working groups: objectives and composition	S. Vella
16:00-17:00	Meetings of the working groups	

1. Technical aspects of antiretroviral drug resistance monitoring
2. Options for setting up a global surveillance "network"
3. Containment of antiretroviral drug resistance

Wednesday, 11 October 2000

- 9:00-11:00 Meetings of the working groups
- 11:00-11:15 *Break*
- 11:15-11:30 Feedback of discussion from working group 1. Rapporteur: C Boucher
- 11.30-11.45 Feedback of discussion from working group 2. Rapporteur: G Jayaraman
- 11.45-12.00 Feedback of discussion from working group 3. Rapporteur: V Miller
- 12:00-13:00 General discussion on feedback
- 13:00-14:00 *Break*
- 14:00-16:00 Proposals for the global monitoring of antiretroviral resistance S. Vella
- 16:00 Close of the meeting

Annex III: Glossary

cross-resistance	Resistance selected by one drug which results in resistance to one or more drugs not included in the current treatment
drug resistance	Decreased susceptibility to a drug
drug-resistance mutation	An aminoacid change conferring reduced susceptibility, usually seen as a result of selection by drug treatment
fold resistance	Degree of resistance of a virus population in respect to a sensitive standard laboratory wild type virus
genetic barrier	Number of mutations required to reduce or loose the drug antiviral activity
genotype	Specific sequence of nucleotides that determine the genes of HIV-1
genotypic resistance	Presence of mutations that reduce the susceptibility to one or more drugs
genotyping/genotypic testing	Test conducted to determine the presence of mutation in the nucleotide sequence of the virus genome
multi drug resistance	Resistance to more than one drug either in a class or different ones
mutant virus	Viral variant with genetic change
mutation	Change in the genetic composition
natural polymorphism	Genetic variant that can circulate in wild-type population
phenotype	Characteristics and growth properties of a viral isolate
phenotypic resistance	Increase in IC 50
phenotyping/phenotypic testing	Assay used to determine the susceptibility of a virus to drug in a virus culture assay
point mutation	Single nucleotide substitution resulting in genetic change
primary resistance	Resistance detected in antiretroviral untreated patients
quasispecies	Distinct HIV variants that evolve from the initial virus inoculum
secondary resistance	Resistance recognised in antiretroviral experienced patients
viral polymorphisms	Viral variant with apparently equivalent fitness
virus fitness	The ability of a virus to replicate at best
wild-type virus	Strain of virus that has not been selected by drug treatment.