An Overview of the Human Immunodeficiency Virus Featuring Laboratory Testing for Drug Resistance

EMIL SCOSYREV

The human immunodeficiency virus (HIV) pandemic is unique in human history in its rapid spread, its persistence, and the depth of its impact. The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that approximately 65 million people have been infected with HIV since the beginning of the epidemic. During this time, approximately 25 million people have died from acquired immune deficiency syndrome AIDS.[1]

HIV-associated morbidity and mortality was substantially reduced during the last decade following the introduction of highly active antiretroviral therapy (HAART). In spite of the striking success of HAART in treating HIV infection, many patients experience treatment failure as genetic changes emerge in the virus leading to drug resistance.[2]

Laboratory testing for drug resistance in HIV strains is now used in combination with other methods to guide antiretroviral therapy. The purpose of this report is to review the background information on HIV with the focus on the problem of drug resistance and to describe the laboratory methods of testing for drug resistance in HIV strains.

ABBREVIATIONS: ABC = abacavir; AIDS = acquired immune deficiency syndrome; AZT = zidovudine; ddC = zalcitabine; ddI = didanosine; dNTP = deoxynucleotide triphosphate; ddNTP = dideoxynucleotide triphosphate chain terminator; d4T = stavudine; FDA = Food and Drug Administration; FTC = emtricitabine; HIV = human immunodeficiency virus; HAART = highly active antiretroviral therapy; LTR = long terminal repeats; NNRTI = non-nucleoside reverse transcriptase inhibitor; NRTI = nucleoside analogue reverse transcriptase inhibitor; PI = protease inhibitor; PR = protease; RT = reverse transcriptase; TAM = thymidine analogue mutations; TDF = tenofovir; 3TC = lamivudine.

INDEX TERMS: genotypic resistance testing; HIV drug resistance; phenotypic resistance testing

Clin Lab Sci 2006;19(4):231

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***Emil Scosyrev MS** is the **Focus: Human Immunodeficiency Virus** guest editor.*

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LEARNING OBJECTIVES

1. Describe the main genetic properties of the human immunodeficiency virus (HIV).
2. Describe the major events in the life cycle of HIV.
3. Identify the primary functions of each of the following viral proteins: gp120, gp41, reverse transcriptase, integrase, protease.
4. List the three major stages in the natural course of the HIV infection.
5. Describe the changes in the viral loads and the CD4 counts during the natural course of the HIV disease.
6. List the four FDA-approved classes of antiretroviral drugs and identify the molecular targets of therapy for each class.
7. Describe benefits and limitations of antiretroviral therapy.

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8. Describe the mechanisms of resistance in each of the four FDA-approved classes of antiretroviral drugs.
9. List the two fundamental approaches to HIV drug resistance testing.
10. Describe the principles of phenotypic resistance testing and list the main steps of the testing process.
11. Define IC50 and calculate the X-fold reduction in susceptibility using the IC50 values.
12. Describe the principles of sequencing-based genotypic resistance testing and list the main steps of the testing process.
13. Describe the principles of dideoxynucleotide sequencing.
14. Describe the principles and the limitations of hybridization-based resistance assays.
15. Discuss the clinical utility of HIV drug resistance testing.

BIOLOGICAL CHARACTERISTICS OF HIV

Overview

This section contains information on biological characteristics of HIV such as taxonomy, genetic properties, structural components, life cycle, pathogenesis, and the virulence factors.

Taxonomy

Human immunodeficiency virus type 1 (HIV-1) is assigned to genus Lentivirus, subfamily Orthoretrovirinae, family Retroviridae.[3] Other human pathogens included in this family are HIV-2 (genus Lentivirus), HTLV-1, and HTLV-2 (genus Deltaretrovirus). Most cases of HIV infection worldwide are caused by HIV-1. The HIV-2 is endemic in West Africa, but cases are also reported in other parts of the world.[4]

Three major groups of HIV-1 are M (main), N (new), and O (outlier). Among M group viruses, which account for the overwhelming majority of HIV infections worldwide, there are several subtypes (clades), designated by the letters A-H, J and K, as well as many recombinant forms. Clade B, the most prevalent subtype in the United States (US) and western Europe, differs considerably from the subtypes found in Africa and Asia, where the majority of HIV infected individuals reside.[5]

Genetic properties and structural components of HIV-1

The genetic material of HIV-1 is linear, single-stranded, positive-sense RNA, 9.2 kilobases in length. The genome is dimeric (two identical strands of RNA), the 5'-end has a methylated nucleotide cap, the 3'-end has a poly(A) tail, and both ends are flanked by long terminal repeats (LTR).[3]

The genome includes nine genes that encode various functional proteins of HIV.[6] The HIV-1 genes and gene products are summarized in Table 1. The main structural components of HIV virions are shown in Figure 1.

The virions are spherical, icosahedral, and enveloped particles, approximately 100 nm in diameter.[3] The envelope is composed of glycoproteins embedded in a lipid membrane, which is derived from the host cells. The two viral envelope proteins, gp120 and gp41, form 72 trimeric functional units each consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane.[7,8] These functional units appear as knob-like structures on the surface of viral particles.

The inner surface of the lipid membrane is covered by the matrix protein (p17). Viral capsid, located beneath the matrix protein layer, contains two copies of HIV RNA associated

Table 1. HIV-1 genes and gene products

Genes	Gene products
Gag	p55 precursor is cleaved to form p17 (matrix protein), p24 (capsid protein), and p15 precursor which is further processed into p7 (nucleocapsid protein), p6 (accessory protein), p1 and p2
Pol	p11 (protease, also referred to as p10), p32 (integrase, also referred to as p31), p51/p66 (reverse transcriptase)
Env	gp160 precursor is cleaved to form gp120 (envelope protein) and gp41 (transmembrane protein)
Tat	p14 (transcriptional transactivator)
Rev	p19 (regulator of viral gene expression)
Vif	p23 (viral infectivity factor)
Nef	p27 (negative effector)
Vpr	p15 (viral protein R)
Vpu	p16 (viral protein U)

with nucleocapsid protein (p7), reverse transcriptase (p66/51), protease (p11) and integrase (p32). The outer layer of the capsid is formed by p24 (the capsid protein).[6]

The HIV is characterized by extreme genetic variability. Retroviral replication is a very unstable process with a high error rate (approximately 0.0001 per base per replication cycle) and rapid turnover of the virus.[9,12] As a result of extensive viral replication with high mutation rates, each HIV-infected individual accumulates a large number of viral variants referred to as "quasispecies". Genetic mutations occurring in HIV over time result in the production of altered antigens to which prior immune responses are ineffective. This property of HIV complicates the process of vaccine development. Genetic variability of HIV also results in the emergence of drug resistant strains.[13]

Life cycle and pathogenesis

The first step in the reproductive cycle of HIV is attachment of the virus to a susceptible CD4 positive host cell. The CD4 antigen serves as a receptor for the virus by binding the gp120 molecule on the outer surface of the HIV envelope. The T helper lymphocytes are the main target for HIV infection because they express high numbers of CD4 antigens. Other CD4 positive cells (macrophages, monocytes, dendritic cells, Langerhans cells, and microglial cells of the brain) may also be infected.[14]

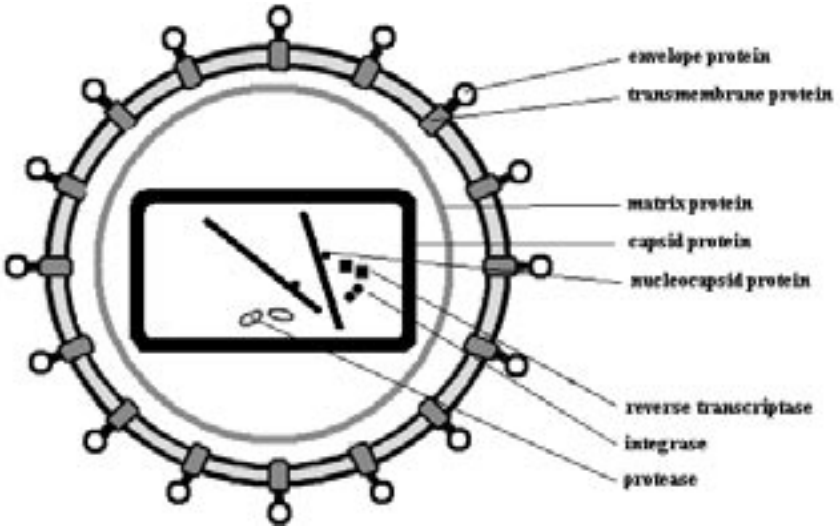
Entry of HIV into the host cells requires an additional binding step involving a specific subset of chemokine receptors on the cell surface (CCR5 or CXCR4). Binding of the chemokine receptors allows for entry of HIV by inducing a conformational change in the gp41 glycoprotein, which mediates fusion of the virus to the cell membrane.[14-16]

Following membrane fusion, the viral particle is taken into the cell, and uncoating of the particle exposes the viral genome. Action of reverse transcriptase produces complementary DNA from the viral RNA. This cDNA is integrated into the host's genome by the action of integrase. The insertion of viral DNA (provirus) into the host genome may be followed by a period of transcriptional latency, which explains the inability of potent antiviral therapies to eradicate the virus from the body. The period of transcriptional latency also makes it difficult for the immune system to recognize and eliminate the HIV infected cells.[13,17]

After the provirus is integrated into the host genome, the transcription of HIV genes and the formation of new viral particles may begin. The HIV transcription process is initiated by the host proteins. Certain sequences in HIV's 5'LTR region provide the binding sites for transcription activators that are normally present in the host cells. The 5' LTR is similar to eukaryotic transcriptional units. It contains downstream and upstream promoter elements, which include the initiator (Inr), TATA-box, and three Sp1 sites.[18] These regions help position the RNA polymerase II at the site of initiation of transcription. Slightly upstream of the promoter is the enhancer domain with binding sites for nuclear factor kappa-B, nuclear factor of activated T cells, and Ets family members. These proteins activate the transcription of HIV genes.[13,18,19]

The initial transcription results in the synthesis of the regulatory proteins tat and rev. These proteins regulate the formation of other HIV gene products.[13] Some of these products are translated as large precursor molecules that are later cleaved by the protease (p10) to form functional HIV subunits. The virions are assembled and released from the cell with the help of vif, nef, and vpu.[13,20,21]

Figure 1. Structural components of the HIV virions



The pathogenesis of HIV infection is primarily associated with the destruction of T helper lymphocytes. These cells play a central role in the immune system by regulating the activities of B and T lymphocytes and their destruction results in decreased effectiveness of both antibody- and cell-mediated immune responses.[14] Severe immunosuppression leads to opportunistic infections, malignancies, and other complications of advanced HIV disease.

The exact mechanism of T helper lymphocyte destruction in HIV infected individuals remains unclear.[22] Various factors, such as direct cytopathic effect of HIV, defects in the process of lymphocyte proliferation and replacement, chronic immune activation with high lymphocyte turnover, and the role of HIV induced apoptosis were investigated.[22-27]

Summary

The HIV is a retrovirus with high affinity for CD4 positive (T-helper) lymphocytes. The destruction of these cells leads to severe immunosuppression with emerging opportunistic infections, malignancies, and other complications of advanced HIV disease.

Once the infection is established, HIV cannot be eliminated by natural or vaccine induced immune responses, drugs, or any other forms of therapy. The persistence of infection results from a number of unique biological properties of HIV, such as its extreme genetic variability with short generation time and high mutation rate, the phenomenon of transcriptional latency, and the incompletely understood mechanism of CD4 cell depletion.

NATURAL HISTORY OF HIV INFECTION

Overview

The course of HIV infection has been observed to progress through three clinical stages, which coincide with the level of viral replication and the amount of immune destruction: primary infection, clinical latency, and acquired immune deficiency syndrome (AIDS).[14]

Primary HIV infection

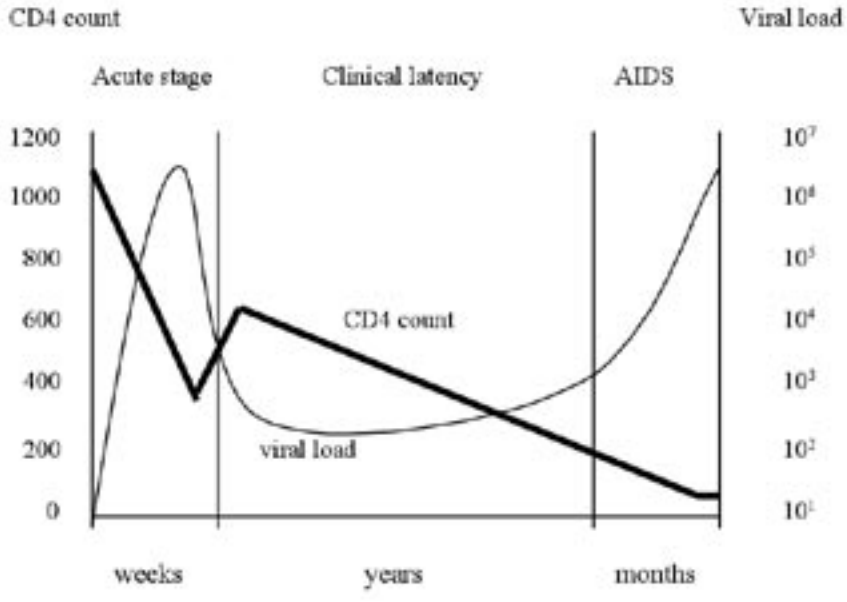
The primary stage, also known as acute HIV infection, is characterized by a rapid burst of viral replication prior to the development of HIV-specific immune responses. During this stage plasma viral load often reaches very high levels in the range of millions of RNA copies/mL and HIV begins to disseminate to lymphoid organs.[14,28,29]

Over the following weeks, viremia declines before reaching a viral setpoint. The magnitude of this setpoint is a strong predictor of long term disease progression rates. The initial reduction of viremia is associated with massive, oligoclonal expansion of HIV-specific cytotoxic T lymphocytes.[30]

The CD4 counts and CD4 function may decline during acute HIV infection, occasionally to levels that allow opportunistic infections to develop. Even though the CD4 count rebounds with the resolution of the primary infection, it rarely returns to the pre-infection levels in the absence of antiviral therapy.[30]

Approximately 50 percent to 70 percent of patients with acute HIV infection develop flu-like symptoms, such as fever, sore throat, arthralgia, myalgia, fatigue, and lymphodenopathy. These symptoms, collectively known as acute retroviral syndrome usually appear three to six weeks after initial infection and resolve within a few days to a few weeks.[14]

Figure 2. Viral loads and CD4 counts during the natural course of the HIV disease



Clinical latency

As HIV-specific immune responses develop, they begin to suppress the replication of the virus, and patients enter the period of clinical latency. This stage is characterized by a decrease in viremia and the absence of clinical symptoms.[14]

Despite the lack of clinical symptoms at this stage, viral replication and CD4 cell turnover remain active.[10] The CD4 cell counts are gradually decreasing at an estimated rate of 50-90 cells/ microliter per year.[31] The length of clinical latency can vary widely in individual patients, but typically lasts for several years.

AIDS

Most untreated individuals will ultimately progress to AIDS, which is characterized by extremely low CD4 counts, resurgence of viremia and severe immunosuppression. The relationship between viral load and CD4 counts during the natural course of HIV disease is summarized in Figure 2.

According to the Centers for Disease Control and Prevention criteria, AIDS is diagnosed when the CD4+ T-lymphocyte count drops below 200 cells/microliter, or below 14 percent of total lymphocyte count, or when the patient develops one of the AIDS-defining conditions.[32] Most complications of advanced HIV disease are associated with opportunistic infections and malignancies. Individuals with AIDS are susceptible to a variety of bacterial, viral, parasitic, and fungal infections, including *Pneumocystis jiroveci* pneumonia, cerebral toxoplasmosis, CMV retinitis, and infections with *Mycobacterium avium* complex. Most of these conditions do not occur in immunocompetent individuals at all or cause mild self-limiting illness. Malignancies commonly seen in AIDS patients are Kaposi's sarcoma, non-Hodgkin's lymphomas, and invasive cervical cancer. These conditions are included in the list of AIDS-defining illnesses.[33]

Summary

The course of the HIV infection has been observed to progress through three clinical stages which coincide with the level of viral replication and the amount of immune destruction: acute infection, clinical latency, and AIDS. The acute stage is characterized by a rapid burst of viral replication prior to the development of HIV-specific immune responses. The stage of clinical latency is marked by decrease in viremia as the virus is cleared from the circulation, and the absence of clinical symptoms. The AIDS stage is characterized by extremely low CD4 counts, resurgence of viremia and severe immunosuppression with emerging opportunistic infections and malignancies.

ANTIRETROVIRAL THERAPY

Overview

The HIV-associated morbidity and mortality has been substantially reduced during the last decade following the introduction of effective antiretroviral therapy.

There are currently four Food and Drug Administration (FDA)-approved classes of antiretroviral drugs in general use: nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors.

Treatment with multiple drugs is more effective than treatment with a single drug. Multidrug regimens involving drugs from two or three of the classes mentioned above are now a standard of treatment known as highly active antiretroviral therapy or HAART.[34]

NRTIs

NRTIs are false building blocks competing with physiological nucleosides. The incorporation of a nucleoside analog into a growing DNA chain aborts DNA synthesis, as phosphodiester bridges can no longer be built to stabilize the double strand.[34] The FDA has approved seven nucleoside and one nucleotide analog.[35] Tenofovir (TDF) is the nucleotide (adenosine monophosphate) analog. Zidovudine (AZT) and stavudine (d4T) are thymidine analogs. Lamivudine (3TC), zalcitabine (ddC), and emtricitabine (FTC) are cytidine analogs. Abacavir (ABC) is a guanosine analog. Didanosine (ddI) is an inosine analog, which is converted to dideoxyadenosine.[34]

NNRTIs

The NNRTIs inhibit viral replication by binding directly to reverse transcriptase at a position in close proximity to the substrate binding site.[34] There are three FDA approved NNRTIs: nevirapine, delavirdine, and efavirenz.[35]

Protease inhibitors

Protease inhibitors interfere with the post-translational modification of viral proteins by directly binding to the active site of the HIV protease. Drug levels achieved during PI therapy can vary greatly among individuals, often dropping below the optimal therapeutic range. This has led to the practice of administering small doses of ritonavir, a P450 enzyme inhibitor, in combination with other PIs to increase drug concentrations by decreasing the clearance rate – a practice known as PI boosting.[34,36] There are nine FDA-approved PIs: ritonavir, amprenavir, fosamprenavir, indinavir, lopinavir

(manufactured in combination with ritonavir), nelfinavir, saquinavir, atazanavir, and tipranavir.[35]

Fusion inhibitors
In 2003 T-20, the first drug of this class, was approved by the FDA. The T-20, also known as the Fuzeon, is a relatively large peptide composed of 36 amino acids. Unlike other antiretroviral agents, it needs to be administered by subcutaneous injection. Fuzeon inhibits fusion by binding to gp41.[34]

Recommended combinations
Common initial regimens consist of two nucleoside analogs, combined with either a PI, possibly boosted with ritonavir, or an NNRTI. Virological treatment success is usually understood as the suppression of viral load to below the level of detection (< 50 copies/ml). The best time for initiation of therapy remains the subject of controversial debate. The risk of AIDS must be weighed against the risks of long-term toxicity and viral resistance.[34]

Benefits and limitations of HAART
Antiretroviral therapy reduces HIV-associated morbidity and mortality by reversing the natural course of the HIV disease. Patients receiving HAART usually have lower viral loads (virologic response), higher CD4 counts (immunologic response), and remain free of opportunistic infections and other AIDS-related conditions (clinical response). The progression to AIDS may be reversed or delayed by many years.

Nevertheless, HAART has limitations that may eventually lead to treatment failure. The following factors limit the efficacy of antiretroviral therapy in HIV patients:

- HAART does not result in cure. HIV infection, therefore, must be managed as a chronic condition with the problem of poor patient adherence to therapy, high cost of treatment, and limited availability of HAART in resource-poor settings.
- Antiretroviral therapy is associated with serious side effects, including hepatotoxicity, myelotoxicity, CNS problems, pancreatitis, lactic acidosis, nephrotoxicity and other complications.[37]
- HIV may become resistant to all currently available antiretroviral agents, including those used in salvage regimens. The problem of drug resistance is discussed separately in the next section.

Summary
During the last decade, with the introduction of HAART, AIDS was transformed from a rapidly fatal illness to a manageable chronic condition. However, HAART does not result in cure, it produces serious side effects, and viral resistance is recognized quickly following the introduction of new drugs. There are currently four FDA-approved classes of antiretroviral drugs in general use: NRTIs, NNRTIs, PIs, and fusion inhibitors. Treatment with multiple drugs is more effective than treatment with a single drug. Multidrug regimens involving drugs from two or three of the classes mentioned above are now a standard of treatment.

THE PROBLEM OF DRUG RESISTANCE

Overview
This section will review the prevalence of drug resistant strains of HIV in the US, the origins and the mechanisms of resistance, and the relationship between drug resistance and clinical progression.

Prevalence and origins of resistance
In the US, as many as 50% of patients receiving antiretroviral therapy are infected with viruses resistant to at least one of the available antiretroviral drugs.[12] The average prevalence of resistance in treatment-naïve patients is approximately 14 percent, and may be as high as 23 percent in some areas.[38-40]

The origins of HIV drug resistance are strongly associated with the high mutation rate in the HIV genome, which is one of the key biological characteristics of the virus. Most antiretroviral drugs target viral proteins. The inhibitory effect of the drug may be reduced when the structure of the target protein is altered in a certain way. These structural changes result from alterations in the corresponding HIV genes. The rate of resistance-conferring structural changes in target proteins is proportional to the mutation rate in the HIV genome.[12]

The genomic mutation rate is determined by two factors: 1) the number of mistakes per genome per replication cycle, which is very high in HIV because reverse transcriptase has no proof reading ability, and 2) by the number of replication cycles per unit of time.[12,41] The magnitude of the second factor is reflected in the viral loads. High viral loads are markers of active replication. It is clear that incomplete suppression of viral replication during HAART (VL > 50 copies/mL) is a risk factor for the emergence of drug resistance. This risk is proportional to the viral load.[42]

Incomplete suppression of replication with emerging drug resistance may result from the pharmacokinetic factors that decrease the drug levels and from treatment interruptions due to the dose-limiting side effects, poor patient adherence to therapy, or limited availability of HAART in resource-poor settings. The emergence of resistance in patients with stable virologic response to HAART (VL < 50 copies/mL) is less likely. However, as was stated earlier, resistant strains may be transmitted between individuals and appear in treatment-naïve patients.

Mechanisms of resistance

Antiviral drug resistance may be defined as the reduction in the susceptibility of mutated viruses to specific antiviral drugs. Fully susceptible viral strains not exposed to the selective pressure of antiviral drugs are known as the wild-type strains. Antiretroviral resistance is usually mediated by changes in the molecular target of therapy as a result of point mutations in the HIV genome.[12]

There is a standard numbering system for HIV-1 protease, reverse transcriptase, and gp41 which is based on the amino acid sequences of these peptides. Mutations are described using a shorthand notation in which a letter indicating the wild-type amino acid is followed by the amino acid position number and a letter indicating the mutation. For example, PR: I54V means that isoleucine (I) has been replaced by valine (V) in position 54 of the protease. If there is a mixture of more than one amino acid at a certain position, the components of the mixture are written after the position, separated by a slash.[36] For example, RT: M184M/V means that the sequence has a mixture of the wild-type residue methionine (M) and the mutant residue valine (V) at position 184 of reverse transcriptase.

It has become customary to label some drug resistance mutations as "primary" and other mutations as "secondary". Primary mutations are those that reduce drug susceptibility by themselves whereas secondary mutations reduce drug susceptibility in combination with primary mutations.[36] The following discussion will focus on the main mechanisms of resistance in the four FDA-approved classes of antiretroviral drugs.

NRTIs

The NRTIs are chain terminators that block the extension of proviral DNA during reverse transcription. Reverse transcriptase is a heterodimer consisting of p66 and p51 subunits. The p51 peptide is composed of the first 440 amino acids translated from the pol gene. This subunit has no enzymatic activity and functions as a supporting structure for the enzymatically active p66 subunit. The p66 peptide is composed of all 560 amino acids of the pol gene. This subunit contains the DNA-binding groove and the active polymerization site. The polymerase domain of p66 subunit has sub-domains referred to as "fingers", "palm", and "thumb". The remainder of the p66 subunit contains an RNaseH sub-domain and a connection sub-domain. Most RT inhibitor resistance mutations are in the 5' polymerase coding regions, particularly in the "fingers" and "palm" subdomains.[36] There are two main biochemical mechanisms that lead to NRTI resistance: sterical inhibition and primer unblocking.

Sterical inhibition is caused by mutations enabling the reverse transcriptase to recognize structural differences between NRTIs and the naturally occurring dNTPs. Incorporation of NRTIs is then prevented in favor of dNTPs. Examples of mutations associated with this mechanism are: M184V, Q151M, L74V, and K65R.[39]

Primer unblocking is caused by phosphorylysis via ATP or pyrophosphate leading to the removal of the NRTIs already incorporated in the growing DNA chain. This is the case with the following mutations: M41L, D67N, K70R, L210W, T215Y and K219Q. These substitutions are historically known as thymidine analog mutations (TAMs) because they were initially observed with zidovudine therapy.[39] Mutations associated with primer unblocking mechanism are also referred to as NEMs (nucleotide excision mutations).[36] Individual mutations and their effects on susceptibility to different antiretroviral agents are summarized in Table 2.

NNRTIs

The NNRTIs inhibit reverse transcriptase by binding to a hydrophobic pocket in the p66 subunit.[43] Unfortunately, a single point mutation (most often K103N) can lead to a 20 to 30-fold resistance to all available NNRTIs.[39]

Residue 103 is located on the outer rim of the NNRTI-binding pocket. Structural studies of HIV-1 RT with K103N have shown that this mutation creates a network of hydrogen bonds which is not present in the wild-type enzyme. These changes appear to stabilize the closed pocket form of reverse transcriptase and interfere with the ability of inhibitors to bind to the enzyme.[43]

HIV-1 group O and HIV-2 are intrinsically resistant to most NNRTIs.[36] Other mutations associated with NNRTI resistance are listed in Table 2.

Protease inhibitors

The HIV-1 protease is a homodimeric protein composed of two chemically identical subunits each consisting of 99 amino acids. The enzyme contains a hydrophobic substrate cleft which recognizes and cleaves different peptide sequences to produce functional HIV proteins and a flexible flap region that closes down on the active site upon substrate binding.[36,44]

Table 2. FDA-approved antiretroviral drugs and selected resistance mutations

FDA-approved antiretroviral drugs	Examples of mutations associated with drug resistance
NRTIs	
Zidovudine	M41L, D67N, K70R, Q151M, L210W, T215Y, K219Q, T69SSX
Stavudine	M41L, D67N, K70R, Q151M, L210W, T215Y, K219Q, T69SSX
Didanosine	K65R, L74V, Q151M, T69SSX
Zalcitabine	K65R, L74V, Q151M, M184V, T69SSX
Emtricitabine	K65R, M184V/I, T69SSX
Lamivudine	K65R, M184V/I, T69SSX
Abacavir	K65R, L74V, Y115F, Q151M, M184V, T69SSX
Tenofovir	K65R, T69SSX
NNRTIs	
Efavirenz	L100I, K101E, K103N, V106M, V108I, Y181C/I, G190S/A
Nevirapine	L100I, K101E, K103N, V106M, V108I, Y181C/I, G190A
Delavirdine	L100I, K101E, K103N, V106M, Y181C, P236L
PIs	
Indinavir	M46I/L, I54V, V82A, I84V, L90M, L10I/V, K20M/R, L24I
Ritonavir	M46I/L, I54V, V82A, I84V, L90M, L10I/V, K20M/R, L24I
Nelfinavir	D30N, M46I/L, V82A, I84V, L90M, L10I/V
Saquinavir	>3 of the following: L10I, G48V, I54V/L, A71V/T, V77I, L90M
Tipranavir	>2 of the following: L10I/V, M46I, I54V, V82A, I84V, L90M
Lopinavir (with ritonavir)	>7 of the following: L10F/I/R/V, K20M/R, L24I, V32I, L33F, M46I/L, I47V/A, I50V, F53L, I54V/T/L, L63P, A71V/T, G73S, V82A/F/T, I84V, L90M
Amprenavir (with ritonavir) or Fosamprenavir (with ritonavir)	>5 of the following: L10F/I/V, V32I, E35D, G73S, I54/V/L/M, I84V, L90M
Atazanavir (with ritonavir)	>5 of the following: L10I/V/F, K20R, L24I, L33I, M36I/L, M46I/L, I50L, L71V, G73C/S/T/A, V82A/F, I84V, L90M
Fusion inhibitors	
T-20	G36D/E/S/V, N42T/D/S, N43D/K/H/S

Resistance to protease inhibitors is mediated by the appearance of protease amino acid substitutions, at positions either in direct contact with the inhibitor or at distant sites. These substitutions reduce the binding affinity between the inhibitor and the mutant protease enzyme.[45] Primary resistance mutations are frequently seen in the substrate cleft (V82A/T/F/S, I84V, D30N) and in the flap (I54V) regions. Protease mutations may also occur at other conserved residues, for example, L90M contributes to the development of resistance to each of the nine approved PIs.[36,39,46] It is interesting that some protease inhibitors, especially when boosted with ritonavir, have a high genetic barrier to resistance meaning that several mutations must first accumulate before the resistance can develop. Resistance to boosted Lopinavir, for example requires the accumulation of at least eight different mutations.[39] Protease resistance mutations are summarized in Table 2.

Fusion inhibitors

In the process of fusion, two heptad repeat domains (HR1 and HR2) of gp41 form a helical bundle containing trimers of each domain. The first FDA-approved inhibitor of viral entry (T-20) is a synthetic peptide designed to inhibit the interaction of HR1 and HR2 by mimicking part of HR2, residues 127 to 162.[12,36,39]

The entire gp41 coding region consists of 351 codons. The T-20 resistance is generally accompanied by the appearance of mutations at positions 36 to 45 in the HR1 (e.g., G36/D/E/S, N42T/D/S).[39] See Table 2 for a list of mutations associated with T-20 resistance.

Drug resistance and response to therapy

The relationship between resistance mutations and response to therapy is very complex. Each resistance mutation may be characterized by two factors: 1) level of associated phenotypic resistance, measured as an X-fold reduction in susceptibility compared to the wild-type virus, and 2) specificity of resistance mutation to one or more drugs. For example, D30N is a protease substrate cleft mutation that confers five to 20 fold resistance to nelfinavir, but not to other PIs.[36,39] This mutation is relatively specific for one drug. On the other hand, K103N is an RT mutation that causes 20 to 30-fold resistance to all available NNRTIs (cross-resistance).[39]

Specificity and the X-fold reduction in susceptibility associated with individual mutations may be influenced by the presence of other mutations. For example, resistance to zidovudine increases with increasing number of TAMs. Two TAMs result in a 5-fold reduction in susceptibility, three TAMs confer approximately 30-fold resistance, and four or more TAMs result in 100-fold resistance.[39]

The complexity of relationship between genotypic resistance and phenotypic response may be demonstrated in the following example. M184V is a common RT mutation conferring resistance to certain NRTIs by the sterical inhibition mechanism. M184V by itself causes high-level (>100-fold) resistance to lamivudine and emtricitabine. In the presence of TAMs, M184V also decreases susceptibility to didanosine, zalcitabine, and abacavir, but increases susceptibility to zidovudine, stavudine, and tenofovir (increasing susceptibility is only possible if no more than three TAMs are present).[34,36,39] Another interesting feature of M184V mutation is that it impairs viral fitness. For this reason, lamivudine may sometimes be included in the combination ARV therapy despite proven resistance in order to conserve the M184V mutation and thus reduce the replicative capacity of HIV.[34]

Because antiretroviral resistance depends on specific combinations of primary and secondary mutations and the relationship between individual mutations is very complex, special algorithms were developed for interpretation of drug resistance data. These algorithms will be reviewed in the next section.

Summary

Drug resistance is one of the main limitations of HAART. The prevalence of drug resistant strains of HIV in the US is at least 50% in patients receiving ARV therapy, and may be as high as 23% in treatment-naïve patients. The origins of resistance are very diverse and include biological and epidemiological factors, such as extreme genetic variability of HIV with high mutation rate and short generation time, incomplete suppression of replication during treatment due to pharmacokinetic factors, poor patient adherence to therapy, dose-limiting side effects, and limited availability of HAART in resource-poor settings. The mechanisms of resistance include various structural changes in the target proteins as a result of point mutations in the HIV genome. The relationship between individual mutations and response to therapy is very complex. The interpretation of drug resistance data requires special rules and guidelines.

LABORATORY TESTING FOR DRUG RESISTANCE IN HIV STRAINS

Overview

HIV drug resistance can be measured using either genotypic or phenotypic assays. Two genotypic assays, TruGene and

ViroSeq, have been approved by the FDA. Clinical utility of resistance testing has been demonstrated in a number of randomized prospective studies. HIV-resistance testing is now recommended by various national and international treatment guidelines.[36,39]

Principles of phenotypic resistance testing

Phenotypic resistance testing is based on the quantitative assessment of viral replication in cell cultures under the selective pressure of increasing concentrations of specific antiretroviral drugs. Drug concentration that results in 50% inhibition of viral growth is termed IC50. Phenotypic resistance is reported as an X-fold reduction in susceptibility, which is calculated by dividing the IC50 of the patient's isolate by the IC50 of the wild-type virus. For example, if the wild-type virus requires 0.5 mg of zidovudine to reduce viral growth by 50% and the patient's isolate requires five mg, then the phenotypic resistance to zidovudine will be reported as ten-fold reduction in susceptibility.[2]

To interpret the phenotypic resistance data (an X-fold reduction in susceptibility), it is important to know the reproducibility of the assay for a given drug (technical cutoff), the variation in IC50 required to inhibit wild-type viruses (biological cutoff), and the clinical significance associated with different levels of reduced drug susceptibility (clinical cutoff). The clinical cutoffs indicate up to which levels of reduced drug suscepibility virological success can still be expected. For example, if a phenotypic assay reported a 15-fold resistance for a particular drug and the clinical cutoff for this drug/assay combination is 10-fold resistance, then the patient will probably no longer benefit from the drug. For protease inhibitors, one has to know whether the respective clinical cutoffs have been determined for unboosted or boosted PIs. Higher drug concentrations achieved with ritonavir boosting may overcome certain levels of resistance.[39]

Commercially available phenotypic assays include: Antivirogram (Virco), PhenoSense (Monogram), and Phenoscript (Viralliance). All phenotypic assays follow the same steps with minor variations in the testing process. The testing procedure begins with extraction of HIV RNA from patient's plasma followed by reverse transcription and amplification of reverse transcriptase (RT) and protease (PR) sequences by PCR. The amplified sequences are then inserted into an RT-PR deficient vector. A second recombinant vector is prepared with RT-PR sequences from the wild-type virus. The replication of both recombinant forms in cell culture is measured under different concentrations of antiretroviral drugs. The results are displayed as percent inhibition of viral growth versus log10 drug concentration. Fold resistance values are calculated by dividing IC50 for the recombinant virus from the patient by the IC50 for the recombinant wild-type virus.[47-48]

Different commercial assays follow the same procedural steps but use different methods and reagents for RNA extraction, reverse transcription, amplification, recombinant vector preparation, and viral replication assessment. Both negative and positive controls are included for each step of the testing procedure. Negative controls for susceptibility testing are represented by the wild-type susceptible strain. Positive controls are derived from mutated viruses that are chosen to represent different resistance patterns for each drug class.

The performance characteristics of commercially available phenotypic assays were evaluated by the manufacturers and by independent research groups in recently published studies.[49-52] The agreement between the assays varied with drug classes and was highest for protease inhibitors and lowest for NRTIs.[49-50] One recently published study concluded that the PhenoSense assay is more precise than the Antivirogram assay and superior at detecting resistance to certain NRTIs (abacavir, didanosine, and stavudine).[50] Another study reported poor correlation between PhenoSense and Antivirogram for samples with lower resistance values (values near the cutoffs) that affected the interpretation of results. Using drug-specific cutoff values for viruses classified as resistant by the Antivirogram or PhenoSense assays, respectively, only 71.4% (95% CI: 58.7%-82.1%) and 57.0% (95% CI: 45.3%-68.1%) of the samples were classified as resistant using the other assay.[51]

Phenotypic resistance testing is used less frequently than genotypic resistance testing due to the higher cost, greater complexity of the assays and longer turnaround times.[36,39,53] The clinical utility of genotypic resistance testing has been demonstrated in a larger number of clinical trials, the evidence for clinical utility of phenotypic assays was less convincing.[36,54-55]

Principles of genotypic resistance testing

Genotypic testing is based on identification of mutations associated with resistance to specific antiretroviral drugs. These mutations may be detected by the sequencing of the amplified segments of HIV genome or by specific hybridization techniques.[39]

Commercially available genotypic assays are sequencing-based methods: HIV-1 TruGene (Visible Genetics/Bayer Diagnostics), ViroSeq (Celera Diagnostics/Applied Biosys-

tems), VircoType HIV-1 (Virco), GeneSeq (Monogram), and GenoSure Plus (LabCorp).[39] Some laboratories use "home-brew" methods with reagents obtained from separate vendors. TruGene and ViroSeq have been approved by the FDA in 2002 and 2003 respectively.[56] This section reviews the general principles of genotypic resistance testing.

All sequencing-based genotypic assays follow the same steps with minor variations in the testing procedure. The testing process begins with HIV RNA extraction from patient's plasma, followed by reverse transcription and amplification of the RT and PR segments by PCR. The amplification products are then sequenced by dideoxynucleotide sequencing procedure.[36] Dideoxynucleotide sequencing is based on the synthesis of new DNA strands (complementary to the target sequence of interest) in the presence of primers, deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphate chain terminators (ddNTPs). The synthesis of each new strand starts with the primer and continues until a chain terminator is incorporated in place of the appropriate dNTP. This process creates a mixture of DNA strands each differing from one another by the length of one nucleotide. The strands are arranged in the order of increasing length by polyacrylamide gel electrophoresis, and the final nucleotide on each strand is read by an automated sequencer using fluorometric methods that depend upon labeling of the primer or the terminators. This process is illustrated in Figure 3. The resulting sequence is compared to the wild-type reference sequence and examined for mutations associated with drug resistance. Known sequences of HIV-1 are processed with patients' specimens for quality control.

Figure 3. DNA strands arranged in the order of increasing length by polyacrylamide gel electrophoresis

DNA strands	Detected sequence
Primer-**A**	A
Primer-A**C**	C
Primer-AC**T**	T
Primer-ACT**G**	G
Primer-ACTG**A**	A
Primer-ACTGA**T**	T
Primer-ACTGAT**C**	C

The last nucleotide on each strand is a chain terminator.

Because a separate fluorescent marker is used for each of the four bases of DNA, a typical sequencing electrophoretogram appears as a series of colored peaks. Each peak corresponds to a specific nucleotide position in the target segment of DNA. If a mixture of two nucleotides is present at a specific position, a double peak will be generated for this position on the electrophoretogram. In the sequencing-based genotypic assays, a nucleotide mixture can be detected when the least common nucleotide is present in at least 20% of the total virus population.[36,39] Once the nucleotide sequence is determined, it may be converted to the amino acid sequence and aligned with the reference wild-type sequence as shown in Figure 4.

Hybridization techniques, as an alternative to complete sequencing, may be used to detect specific mutations associated with drug resistance. However, resistance testing by hybridization is challenging because HIV genome is very polymorphic. A recent study compared a hybridization-based method (LiPA reverse hybridization assay) to conventional sequencing-based methods (TruGene and home-brew sequencing assays). LiPA HIV-1 RT and PR resistance assays use reverse hybridization to detect wild-type and mutant codons at specific positions in the RT and PR segments. Codon-specific oligonucleotide probes are applied as discrete lines on a nitrocellulose membrane in a strip format. After denaturation, the amplified biotinylated DNA material hybridizes with the specific probes. A streptavidin conjugate labeled with alkaline phosphatase is then added to the mixture. The labeled conjugate attaches to the biotinylated DNA-probe hybridization products and a purple-brown color is formed after incubation with BCIP/NBT chromogen.[57]

The study reported the following concordance rates for LiPA versus conventional sequencing: for PR, 91.3% of the codon results were concordant, 3.0% were partially concordant, 4.5% were indeterminate by LiPA, and 1.3% were discordant. For RT, 88.0% of the codon results were concordant, 5.9% were partially concordant, 5.2% were indeterminate by LiPA, and 0.9% were discordant (partial concordance is reported if one method detected a mixture, while the other method detected one of the mixture's components). The authors concluded that the clinical utility of LiPA is limited by the high rate of indeterminate results.[57] Similar conclusions were made in the previous studies.[58]

Sequencing concordance as a measure of agreement between various sequencing-based methods was evaluated in a number of studies published between 2001 and 2006.[59-74] The two

FDA-approved assays were evaluated for reproducibility and compared to each other and to the reference sequences generated by other sequencing methods. The average sequencing concordance with B-subtype isolates ranged from 97.6% to 99.9%.[59,65-67,72-74] The performance of sequencing-based assays with non-B subtypes was less clear because multiple amplification and sequencing failures were reported in some studies, while other studies reported improved performance.[60-61,70]

The final step in the process of genotypic resistance testing is the analysis of generated sequences. The RT-PR sequences obtained from the patient are compared to the RT-PR sequences of the wild-type virus and examined for mutations at resistance sites. The sequences may be aligned using different software programs such as Bayer's OpenGene software (included in the TruGene kit), Applied Biosystems Sequencing Analysis Software (a component of the ViroSeq system), MegAlign program of Lasergene Navigator (DNASTAR), and others.[67,71]

When specific resistance-associated mutations are identified, an additional software program is used to generate the resistance report that relates phenotypic resistance to genotypic data. Both TruGene and ViroSeq include such programs.

The primary sequencing data may also be analyzed by an independent software program. The Stanford HIV data base (http://hivdb.stanford.edu) contains two programs: HIVseq and HIValg. The HIVseq program accepts user- submitted RT and PR sequences and compares them to a reference wild-type sequence. The HIValg program accepts user submitted RT and PR sequences or specific mutations and returns inferred levels of resistance to the FDA approved antiretroviral drugs using three different algorithms: HIVDB, ANRS, and Rega v6.4. A similar program called "Geno2Pheno" is located at http://www.geno2pheno.org/cgi-bin/geno2pheno.pl. The HIV sequence database is located at http://www.hiv.lanl.gov/content/index. A simulated genotypic resistance report is shown in Figure 5.

Clinical utility of HIV resistance testing

The clinical utility of HIV resistance testing has been evaluated in a number of studies. Most studies have demonstrated that patients with treatment failure, whose physicians had access to genotypic resistance data before the therapy was changed, usually had more significant decreases in the viral load than patients for whom treatment was changed without knowledge of the resistance profile.[36,75-78] Similar data was obtained for phenotypic testing. Some studies showed "no significant difference" in viral load reduction between resistance testing and physician guided therapy.[36,79] The benefit of phenotypic resistance testing was also questioned in some recently published reports.[54-55] Nevertheless, the clinical utility of resistance testing is now widely recognized, and resistance testing in patients with treatment failure is recommended by various expert panels, including the US Department of Health and Human Services.[36,39]

Several recently published reports also recommend resistance testing for treatment-naïve patients newly infected with HIV.[40,80-81] As was previously stated, the prevalence of primary resistance mutations in treatment naïve patients in the US may be as high as 23%. Resistance testing prior to the initiation of ARV therapy is cost effective and may improve the clinical outcomes.[80]

Summary

Figure 4. Simulated amino acid sequence of patient's isolate aligned with the reference wild-type sequence

Note that the patient's isolate has three mutations (deviations from the reference sequence) in this segment: L10I, K20M, and V32I. These mutations are associated with resistance to protease inhibitors (see Table 2). This diagram shows only one segment of protease, positions 1-40. Sequencing-based methods of HIV drug resistance testing usually sequence the entire protease (positions 1-99) and approximately 300 codons of reverse transcriptase.

Amino acid position:	1	10	20	32	40
Reference sequence:	PQVTLWQRPL	VTIKIGGQLK	EALLDTGADD	TVLEEMSLPG	
Patient's sequence:	……I……..	..M……	……I……		

Drug resistance of HIV can be measured using either genotypic or phenotypic assays. Genotypic assays detect mutations that cause drug resistance. Phenotypic assays measure the ability of the virus to grow in cell cultures with different concentrations of antiretroviral drugs. Genotypic testing is used more frequently than phenotypic testing because of lower cost, wider availability, shorter turnaround time, and more reliable evidence of clinical utility. The HIV drug resistance testing is now recommended by various national and international treatment guidelines.[36-39]

Figure 5. Simulated genotypic resistance report

Most algorithms report resistance data in three basic categories: "susceptible" (no evidence of resistance), "intermediate" (possible or emerging resistance), and "resistant".

Resistance Report

Patient ID:	Patient Name:
Sample ID:	Date Drawn:

Relevant RT mutations: M41L, K65R, L74V, D67N, K70R, Q151M, M184V, L210W, T215Y, K219Q, K103N, Y181C

NRTIs

Zidovudine	**resistant**
Stavudine	**resistant**
Didanosine	**resistant**
Zalcitabine	**resistant**
Emtricitabine	**resistant**
Lamivudine	**resistant**
Abacavir	**resistant**
Tenofovir	**resistant**

NNRTIs

Nevirapine	**resistant**
Delavirdine	**resistant**
Efavirenz	**resistant**

Relevant PR mutations: D30N

PIs

Indinavir	susceptible
Ritonavir	susceptible
Saquinavir	susceptible
Nelfinavir	**resistant**
Tipranavir	susceptible
Lopinavir/r	susceptible
Amprenavir/r	susceptible
Fosamprenavir/r	susceptible
Atazanavir	susceptible

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