Molecular-based Laboratory Testing and Monitoring for Human Immunodeficiency Virus Infections

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Applications of laboratory testing for human immunodeficiency virus type 1 (HIV-1) infection have made significant impact on clinical care of HIV-infected patients globally. As these technologies continue to evolve and new technologies emerge, unique and highly sensitive nucleic acid-based testing methods will offer more and better means for us to guide physicians in anti-retroviral treatment strategies and clinical management of HIV infected patients. In this review we discuss a variety of current molecular-based methods that are available for HIV testing including diagnosis, monitoring disease progression, and detection of drug resistance to anti-retroviral therapy. Newer approaches that could be used in future HIV testing are also introduced.

ABBREVIATIONS: bDNA = branched DNA; IPCR = immuno-PCR; NASBA = nucleic acid sequence based amplification; NAT = nucleic acid tests; PCR = polymerase chain reaction.

INDEX TERMS: diagnosis; disease progression; drug resistance; HIV infection; monitoring.

Clin Lab Sci 2005;18(4):263

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LEARNING OBJECTIVES

1. Describe the uses of molecular-based assays in addressing issues related to HIV infection.
2. List three specific molecular methods commonly used to quantify HIV viral load and describe how they differ in principle.
3. Describe molecular methods that can be used to determine viral resistance to anti-retroviral drugs.

Methods for detection of viral nucleic acids (RNA or DNA) using nucleic acid tests (NATs) grew from a mere research tool to a clinically useful tool in the mid 1990s. Within a few years, molecular tests were being used to detect viral RNA for screening of blood and blood products.[1] Subsequently they were implemented in the clinical laboratories for use in the management of infected individuals and prognosis of disease outcome. More recently, NATs have been further modified to allow genotyping of viral types, groups, and clades. They can be additionally used to determine gene mutations associated with viral resistance to anti-retroviral therapy. They are now becoming indispensable tools in the clinical setting for assisting physicians and practitioners in their successful management of persons with HIV/AIDS. For example, the development of highly active antiretroviral therapy (HAART) has heralded marked advances in understanding the dynamic equilibrium of HIV-1 replication, cell destruction, and cell replenishment. The rapid reduction in virus levels a few weeks after initiation of HAART has led to dramatic improvements in the clinical management of HIV-infected patients. However, many problems remain mainly because of the emergence of viral resistance to drug therapy, the realization of the existence of viral genomes

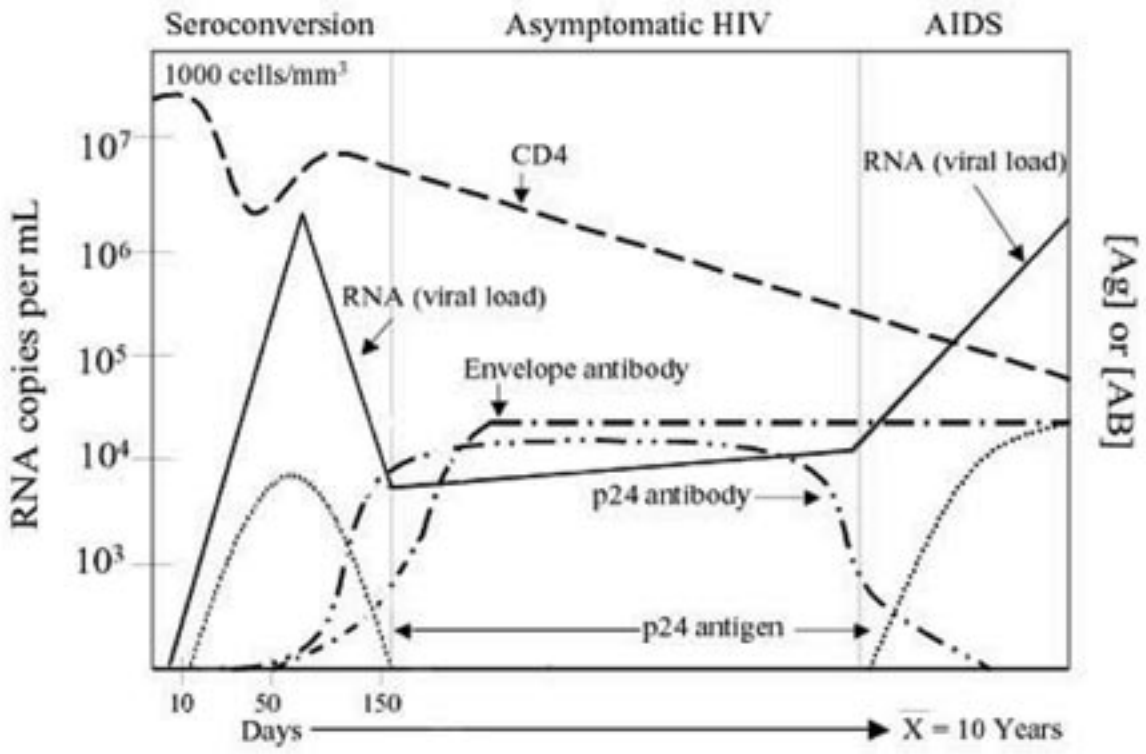
integrated in chromosomes (viral latency), and an understanding of the distribution of virus in other cellular compartments, e.g., lymphatic tissue. The use of NATs for quantification of viral burden offers a powerful means for us to: 1) measure the viral load baseline prior to initiation of HAART, 2) assess the efficacy of initiation of antiviral drug treatment, 3) detect the onset of drug resistance both after initial therapy and after prolonged therapy, and 4) predict the development of AIDS-defining opportunistic infections and disease progression. Thus, molecular diagnostics has dramatically changed the way HIV related medicine is conducted, and has not only helped to prevent the spread of infection but has resulted in an extension of the length and quality of life. This brief review provides an update on some of the major NATs that are available and their appropriate usage for addressing HIV infection.

USES OF MOLECULAR METHODS FOR HIV DIAGNOSIS

NATs can be used to resolve the infection status of individuals with indeterminate serological results. They are especially valuable in identifying HIV infection in newborns from seropositive mothers. NATs such as DNA or RNA PCR now allow us to diagnose HIV infection soon after birth instead of waiting at least three to seven months for a final diagnosis, thus enabling physicians to treat only those babies with infections. Unlike adults, serological testing of children under the age of 18 months has little diagnostic value because of the presence of maternal antibody in the newborn.[2]

In most individuals, the HIV seronegative window period of about three weeks following initial infection can be shortened to about 12 days by using molecular DNA or RNA tests.[3] Viral RNA is the earliest marker appearing after infection and follows a predictable course with RNA levels 'ramping up' during the first five to six weeks to reach a peak of nearly one million copies/mL within two to six months; it subsequently falls (without treatment) 2 to 3 logs to a 'set point' of about 10,000 copies/mL.[4] The ramping up period represents the doubling rates of HIV (21.5 hours), and about one billion virions are produced each day, regardless of the stage of infection. From

Figure 1. Laboratory markers during the course of HIV infection, showing peak viral load levels early during infection, establishment of the set point (near 150 days), and the gradual increase during infection



the set point, viral load remains at fairly constant levels (low to moderate) for up to ten years before rising to high levels when AIDS occurs. It is the immune response that combats the viremia and keeps the viral levels in the blood fairly constant. The set point appears to dictate the future course of the disease, with higher set points signaling a poor prognosis for the patients. For example, infected persons whose HIV RNA viral load levels exceed 100,000 copies/mL at six months after infection are 10-fold more likely to progress to AIDS within five years than those with levels less than 100,000 copies/mL.[5] Figure 1 depicts the relationship of RNA levels to other laboratory markers during the course of HIV infection.

MONITORING RESPONSE TO THERAPY AND DISEASE PROGRESSION

Monitoring viral burden has become essential to determine when to institute drug therapy and when therapy may need to be changed. The use of NATs has proven to be the most important means to assist in the management of patients because NATs yield insight into the degree of the viral burden. Experts suggest to measure viral load twice during a two-week interval to determine the viral baseline. The same tests should be followed by a single test every several months thereafter to determine disease progression.[6] Typical response of a drug naïve patient after initiation of antiviral treatment should be expected to be 0.7 to 0.8 log viral reduction using reverse transcriptase inhibitors or 2-3 logs viral reduction with protease inhibitors. Combined use of RT and protease inhibitors often results in significant reduction in viral load to a non-detectable level by the current molecular methods, i.e., less than 50 viral RNA copies/mL.[7] Clinical data further suggest that early treatment in acutely infected patients may provide additional benefit.[8,9]

There are three commercial test kits that are FDA-approved for the measurement of HIV-1 RNA (viral load) in plasma: 1)

Table 1. Comparison of three commonly used commercial methods in determination of HIV-1 viral load

METHOD Test name (Manufacturer)	**RT-PCR** Amplicor (Roche)	**NASBA** NucliSens (Organon Teknika)	**bDNA** Versant (Bayer)
Lower detection limit	400 copies/mL (standard) 50 copies/mL (ultrasensitive)	40-400 copies/mL 40 (but not consistently)	500 copies/mL (2.0) 50 copies/mL (3.0)
Linear detection range (copies/mL)	400 – 750,000 (standard) 50 – 100,000 (ultrasensitive)	40 – 10,000,000	500 – 1,600,000 (2.0) 50 – 500,000 (3.0)
Volume of plasma required	0.2 mL (standard) 0.5 mL (ultrasensitive)	0.01 to 2.0 mL	1.0 mL
Coeffieient of variation (%)	<30	≤29	≤20
Estimated time to perform test	9 hours (standard) 10 hours (ultrasensitive)	5 hours	36 hours
Specimen type	Plasma (EDTA or ACD)	PBMC, whole blood or any body fluid	Plasma (EDTA or ACD)
Maximum number of tests per run	21 to 45	20	80 to 120
Estimated cost of capital equipment	$20,000	$10,000	$40,000 to $45,000

two versions of the Amplicor Monitor™ (Roche Diagnostics Systems) which is a RT-PCR method, 2) the Versant™ HIV-1 RNA 3.0 kit (Bayer Inc) which is known as bDNA, and 3) the NucliSen™ HIV-1 RNA QT System (bioMerieux Inc), which is also known as nucleic acid sequence based amplification (NASBA). The Amplicor Monitor RT-PCR based testing system is a target-based amplification assay based on the principle of RT-PCR, i.e., the viral RNA is first reverse transcribed by reverse transcriptase into cDNA and the DNA is then amplified by PCR. Similarly, the NucliSen HIV-1 RNA QT assay is a target-based amplification process, but it directly amplifies the viral RNA using a method mimicking natural replication of retroviruses.[10] In contrast, the Versant HIV RNA 3.0, formally known as the Quantiplex™ or bDNA assay (Chiron), uses a signal-based amplification principle.[11] All three commercial methods, described in more detail below, achieve very similar results for the quantification of HIV-1 RNA (Table 1).[12]

Amplicor Monitor

The Amplicor Monitor assay utilizes a template-based RT-PCR amplification process to convert the target viral RNA template to a DNA form that can be amplified and detected, i.e., the method combines reverse transcription and DNA amplification reactions. The enzyme reverse transcriptase (RT) converts viral RNA into cDNA which is amplified using PCR. By incorporating a specific RT (*rTth* polymerase isolated from a thermophilic bacterium, *Thermus thermophilus*), a one step assay is possible because *rTth* polymerase can carry out both reverse transcription and DNA amplification.

The RT-PCR reaction is carried out in an instrument called a thermocycler that allows for the rapid changes in temperature required for cycling through extension and denaturation steps. Oligonucleotide primers that are biotin-labeled, initiate the replication of DNA and the DNA sequence products (amplicons) become biotinylated. These products are subsequently captured by complementary oligonucleotides immobilized on a microtiter plate. The products are detected by an avidin-horseradish peroxidase conjugate that binds to the biotinylated captured amplicons; a substrate is subsequently added to form color, and a microtiter plate spectrophotometer is used to measure the level of signal (color) generated by the amplified products.

An automated system called the COBAS Ampliscreen is also available for HIV RNA testing and is approved by the FDA; it offers full automation for the RT-PCR method. Technically, the RT-PCR assay is moderately difficult to learn and requires a skilled user; a relatively slow learning curve should be expected.

Cross contamination by amplicons is a potential problem for PCR-based assay. To prevent cross contamination, an enzyme, uracil-N-glycosylase (UNG, commercially known as AmpErase®), is included within the Amplicor reaction to eliminate previously amplified amplicons, in which deoxyuridine triphosphates (dUTP) are intentionally incorporated into DNA. Furthermore, a uni-directional work flow is strongly recommended in order to maintain a low risk of cross contamination when performing PCR.

Nucleic acid based sequence amplification

Similar to the Amplicor Monitor assay, the NASBA assay is a target-based amplification method.[14] In essence, it parallels the natural replication of retroviral RNA in vitro. With the combined use of avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase, and RNase H in one tube, the NASBA test involves repetitive steps of reverse transcription from the viral RNA template with subsequent RNA amplification from cDNA. Approximately 40 copies of RNA can be made per cycle for each copy of the RNA target, and within a 90 minute reaction; approximately 1,000 RNA molecules can be made from 10 starting copies of purified RNA molecules. The amplification process results in the production of large quantities (billions) of the target sequence of single-stranded, anti-sense RNA that is complementary to the original target RNA.

The amplified single-stranded RNA product is detected by hybridization to complementary wild-type or calibrator-specific ruthenium-labeled oligonucleotide probes tagged with an electrochemiluminescence moiety that is subsequently coupled to paramagnetic beads. The amount of chemiluminescence is proportional to the quantity of the amplified product.

Three internal controls are built into this reaction to monitor the efficiency of amplification and to calculate the viral load. An advantage to this method is that all the reaction steps are conducted at constant (isothermal) temperature (40 °C to 41 °C). A disadvantage is that a semi-automated electrochemiluminescence (ECL) detection instrument is required to measure the amplified products. Another advantage of using the NASBA system is that non-plasma samples can be used. The method requires a separate RNA extraction step, making it more labor intensive, although new pre-amplification procedures (NucliSens extractor) using silica for extraction are rapid and efficient.

bDNA assay

Unlike the Amplicor and NASBA assays that amplify the target viral nucleic acid, the bDNA technique amplifies the signal from a captured viral RNA target by sequential oligonucleotide hybridization steps.[11] After disrupting the virion and denaturing the target, probes directed toward the *pol* gene hybridize to the target sequences. A mixture of capture probes attached to a solid surface of a microtiter plate bind the target. Once the viral target is captured, amplifier and preamplifier extender probes are used to hybridize to the target at an adjacent sequence region, resulting in a branched DNA complex. Alkaline phosphatase-labeled oligonucleotides attach to these branches with approximately 3,000 to 22,380 branch sites per target molecule. This branched tree type complex is detected using chemiluminescence. The technique has advantages in that the assay is simple to perform with a low inherent variability and limited carry-over contamination. Also, because of the many DNA probes, it recognizes a broad spectrum of HIV sequences, including those of viral variants, and does not require viral RNA purification (virions are concentrated by centrifugation). Although a larger number of assays can be performed per day, the turn around time for results is significantly longer than the other assays. This may be an important factor in situations where rapid turnaround times are required. The detection limit has recently been reduced to 50 copies/mL in the 3.0 version.

The requirement of larger volumes of plasma may be a concern when testing infants and newborns. A pediatric assay that requires only 50 µL of plasma is available through Chiron. However, the assay design was based on the first generation of bDNA method, thus the detection limit is too high.[15]

Other methods to measure viral load

In addition to these three commercial viral load assays, a number of other methods are used internationally or are under development. These include the ligase chain reaction (LCR, Abbott Laboratories), Qβ-replicase (Gene Track Inc.), strand displacement amplification (SDA, Becton Dickinson Co), hybrid capture system (Digene Co.), transcription mediated amplification (TMA, Gen-Probe Inc), and a quantitative method to measure the enzyme reverse transcriptase (ExaVir Load, Cavidi Tech). Each is based on different scientific principles, but they all have potential in providing similar and competitive assays for measuring HIV-1 viral load.

The Cavidi Tech RT assay is unique in that it is an ELISA-based assay. It incorporates determination of reverse transcriptase enzymatic activity and quantification of revised transcribed cDNA for viral load determination.[3] Similar to an EIA method, the method requires two to three days for completion. Our laboratory recently reported on the method of immuno-PCR for the detection of ultra low levels of HIV p24 antigen.[16] In this technique, a serologic antigen capture EIA method is coupled to real-time PCR where the detector antibody is indirectly labeled with a 500 base pair strand of DNA. The DNA is subsequently used as a template for amplification with the detection of amplicons using fluorescence-generating probes. This method was shown to detect lower levels of HIV virions than NAT methods. The sensitivity of this technology surpasses the high sensitivity of boosted EIA p24 methods and attains a level of sensitivity equal to or surpassing that of NAT.[17,18]

Monitoring HIV proviral DNA

Even though patients starting HAART regimens often achieve non-detectable HIV-1 viral RNA levels, questions remain as to whether or not the proviral DNA, either integrated or un-integrated, responds to HAART. It is not known if relative changes in proviral DNA levels precede changes of viral RNA levels post HAART introduction. Therefore, one of the current challenges in HIV disease management is to detect the presence of low level proviral DNA in latently-infected CD4 lymphocytes and other reservoirs, especially because these hidden reservoirs can replenish and revive viral infection upon activation.[19-21] Thus, a highly reproducible and accurate assay to quantify proviral DNA would enable a more in-depth evaluation of the efficacy of antiviral therapies. Unfortunately, there are no commercially available assays for quantification of HIV-1 proviral DNA levels.

Several research-based assays have been reported previously for quantification of HIV-1 proviral DNA, all of which were based on the principle of conventional PCR.[22-25] The potential limitation associated with the traditional quantitative PCR is that the DNA copy numbers are calculated based on the final amplified gene products. Because DNA is amplified exponentially during PCR, a small variation in amplification efficiency early in the thermocycling process could potentially lead to a large variation in the final quantification of amplified products. To circumvent this problem, we have developed and validated a new protocol for quantification of HIV-1 proviral DNA.[26] This assay is based on the principle of real-time PCR, also known as TaqMan™.[25] Because of the inherent advantage of real-time PCR over classic PCR, this new assay provides a highly accurate and reproducible detection method for HIV-1 proviral DNA with a broad linear range of detection.[26]

DETECTION OF HIV-1 DRUG RESISTANCE

Drug resistance testing by molecular methods now provides valuable information to guide therapy in HIV infected persons. As more information becomes available on the specific mutations that occur during drug treatment, healthcare workers are able to tailor therapy to be more effective in controlling infection and even predicting which drugs will be most efficacious. It is likely that drug resistance testing will gain even wider use as more mutations are detected and as new drugs become available. It is clear that identification of specific mutations provides information that can be translated to a more effective therapy that can extend an infected person's life.

In spite of the striking success of HAART in treating HIV-infected patients, most patients will eventually fail treatment as genetic changes emerge in the virus leading to drug resistance. The observed resistance is usually restricted to changes in those viral genes that serve as targets for the therapies that the patient has begun. For example, patients taking AZT as part of their therapy will typically develop mutations of codon 215 of the reverse transcriptase region of the HIV *pol* gene, thereby leading to a change in a single amino acid of the gene product and decreasing the effectiveness of AZT in the patient. Over time, additional mutations may occur in codons 41 and 219, again leading to different amino acid changes in the reverse transcriptase enzyme. The cumulative acquisition of new mutations is likely to lead to a loss of effectiveness of AZT.[28,29] The identification of mutations can be accomplished through genotyping and phenotyping methods that provide information on the virus's drug resistance or susceptibility. Viral resistance is a decrease in virus susceptibility to a drug as evidenced by an increase in the concentration of drug required to inhibit viral replication by 50%.

The viral genotype reflects the genetic composition of the organism, which generates the phenotypic properties. Genotyping is a method for determining the constitution of nucleotide sequence in the viral genome that encodes the amino acids, and is usually carried out by directly sequencing the DNA of the viral genes. Genotyping assays are used more commonly than the phenotypic assays because they are less expensive, rapid, and more accessible than the phenotypic assays. In addition, it may be able to detect some of the viral gene mutations before drug resistance manifests at the phenotypic level. One of the technical challenges of the genotyping assay is that the knowledge and experience of the analyst may influence the interpretation of results. Moreover, results generated from genotypic assays are not always consistent with the results derived from the phenotypic assays.

The viral phenotype refers to any characteristic of the virus that can be detected or observed and that is related to its appearance, structure, or some measurable property. Phenotypic assays are able to assess the total overall effect of one or multiple mutations and mutational interactions. They evaluate the concentration of different drugs necessary to inhibit HIV replication in vitro, and directly measure, under controlled laboratory conditions, the level of resistance of the HIV population in an individual patient to each of the anti-HIV drugs. The phenotypic assay involves insertion of the reverse transcriptase and protease genes from the HIV clinical isolate of the infected individual via recombinant DNA techniques into a laboratory-derived molecular clone containing standardized viral envelope and accessory genes (experimental test). The recombinant clones are then grown in viral culture in the presence of varying concentrations of the drug being evaluated, enabling an assessment of the phenotypic characteristics expressed by the inserted genes. In addition, a wild-type virus lacking the recombinant inserts is included as a control.

The drug concentrations that result in 50%, 90%, and 95% inhibition of viral growth (in the experimental test) are termed IC_{50}, IC_{90}, or IC_{95}, respectively. Resistance is measured in terms of the IC_{50} and then compared with the IC_{50} for the fully sensitive, non-mutated wild-type virus. A 4-fold or higher shift between the IC_{50} of the recombinant clone and the wild-type virus indicates drug resistance. If the wild-type virus requires 0.5 μg of a drug to reduce viral growth by 50% and the sampled virus required 5 μg, this measurement would be referred to as a 10-fold resistance. Disadvantages of phenotypic assays are their high cost and a long turn around time for reporting (two to three weeks). In addition, the thresholds to define susceptibility are arbitrary, not standardized, and do not always reflect achievable drug concentrations.

The use of automated DNA cycle-sequencing based-technology is currently the most common way to conduct HIV-1 genotyping analysis. Currently, several types of genotypic and phenotypic assays exist for the analysis of HIV drug resistance. The genotyping methods include the FDA-approved TruGene™ HIV-1 Genotyping Kit and Open Gene DNA Sequencing Assay (Visible Genetics/Bayer Diagnostics), and the ViroSeq™ HIV-1 Genotyping System with the 3700 Genetic Analyzer (Celera Diagnostics). Both the ViroSeq HIV-1 Genotyping Systems and the Trugene HIV-1 test offer an integrated software algorithm to provide information to the physician about which gene mutations of RT and PR are

most likely to confer resistance to a specific drug regimen. Inter-laboratory comparison of various DNA sequencing-based genotypic methods indicates that there is a high concordance in determining RT and PR gene mutations.[30]

Currently, the two most popular phenotyping assays are the PhenoSense (ViroLogic, Inc., and the VircoTYPE HIV-1 (Virco). ViroLogic had developed a Therapy Guidance System (TGS) interactive database to help physicians guide patient therapy. The company has also introduced the PhenoSense GT™ system, which combines the PhenoSense HIV phenotyping assay, the GeneSeq HIV genotyping assay, replication capacity (RC), and HIV-1 subtyping to create a comprehensive HIV drug-resistance testing system. RC measures how well a patient's virus is able to replicate compared with a wild-type reference virus. The VircoTYPE HIV-1 testing service was introduced in July 2004 as a complete HIV-1 phenotyping and genotyping service to replace VirtualPhenotype. The VirtualPhenotype (Virco) is a computer software program that provides a phenotypic prediction based on a correlative database of more than 100,000 HIV phenotypes and genotypes (>18,000 paired genotypes and phenotypes).

Another method, the LiPA™ assay (Innogenetics) is a reverse hybridization method that uses easy to read strips for determination of specific viral gene codon changes that confer drug resistance; however, the test only identifies the gene mutations that have been targeted by certain oligonucleotides applied on the strip. Another method that uses the DNA repair enzyme Cleavase™ in the Invader™ assay (Third Wave) could potentially detect all of viral gene mutations, but it is unable to provide specific information on gene codon changes.

One of the most powerful genotypic ways to determine HIV-1 drug resistance is the use of microarray technology. Affimatrix Inc. has developed specific DNA chips that can be used to detect all of the possible gene mutations conferring drug resistance to either RT or PR inhibitors. However, this assay is limited by its high cost of chips and instrument setup.

CONCLUSIONS

The management of HIV infection has changed dramatically in the past several years because of advances in technology that provide more capability for the monitoring of infection and in finding the causes of viral resistance to drug regimens. These capabilities are essential for guiding therapy and to increase the quality and extension of life in those infected with HIV. In light of recent developments in new and improved therapies, we have entered a new era in HIV treatment and management. Nevertheless, drug resistance will continue and vigilance is necessary. Although we by no means have found a cure for HIV infection, we are making good progress and giving more hope for millions of persons infected throughout the world. As we strive to achieve full eradication of HIV infection, we must combine proper therapeutic designs with accurate viral load measurements and resistance testing to allow an assessment of our progress and for the maximum care of patients.

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