

REVIEW

Past, present and future molecular diagnosis and characterization of human immunodeficiency virus infections

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Substantive and significant advances have been made in the last two decades in the characterization of human immunodeficiency virus (HIV) infections using molecular techniques. These advances include the use of real-time measurements, isothermal amplification, the inclusion of internal quality assurance protocols, device miniaturization and the automation of specimen processing. The result has been a significant increase in the availability of results to a high level of accuracy and quality. Molecular assays are currently widely used for diagnostics, antiretroviral monitoring and drug resistance characterization in developed countries. Simple and cost-effective point-of-care versions are also being vigorously developed with the eventual goal of providing timely healthcare services to patients residing in remote areas and those in resource-constrained countries. In this review, we discuss the evolution of these molecular technologies, not only in the context of the virus, but also in the context of tests focused on human genomics and transcriptomics. *Emerging Microbes and Infections* (2012) 1, e19; doi:10.1038/emi.2012.15; published online 22 August 2012

Keywords: HIV; diagnosis

INTRODUCTION

Laboratory and clinical microbiologists perform various tests to determine the human immunodeficiency virus (HIV) infection status of a patient, evaluate the progression of disease, and monitor the effectiveness of antiretroviral therapy (ART). HIV infection can be diagnosed in five possible ways: (i) direct visualization of virions by electron microscopy; (ii) cultivation by lymphocyte culture; (iii) measurement of HIV-specific serologic responses; (iv) detection of viral antigens; and (v) detection of viral nucleic acids.^{1,2} Diagnosis can best be achieved by serologic testing but the monitoring of HIV disease progression is mostly accomplished by the quantitation of CD4 T cells and viral RNA. The detection of HIV nucleic acids in clinical specimens was first demonstrated in 1988 by amplifying HIV-1 genomic DNA from the peripheral blood mononuclear cells of HIV-1-infected individuals.³ Molecular methods have since been drastically improved and, along with serologic assays, have become a routine means to characterize HIV-1 infection and disease progression.

The acute HIV infection phase, or the window period, represents the first few days after the entry of HIV into the CD4 cells of a new host. During this phase, the host has yet to build up an immune response and thus HIV viruses rapidly replicate. The number of viral RNA molecules can increase to several million copies per ml of plasma and thus can be readily detected by molecular assays, shortening the window period from 20–30 days to 10–15 days.⁴ The viral p24 antigens

encapsulating the viral RNA can also be detected but with less sensitivity. With the use of fourth generation enzyme-linked immunosorbent assays (ELISA) that detect p24 antigen and IgG and IgM antibodies, the window period between HIV entry and serologic positivity has been narrowed from approximately 1 month or more to 15–20 days.^{5,6}

A CD4 T-cell count is used as the principal ART eligibility criterion for infected patients in most countries, whereas plasma viral load assays are used to monitor ART efficacy. Qualitative molecular assays have also been used to detect HIV vertical transmission from infected pregnant mothers to infants. HIV-1 drug resistance tests, which include genotyping assays for detecting nucleotide mutations in viral genomes, and phenotype assays for detecting viral replication in the presence of antiretroviral drugs, have become routine in the management of patients receiving ART in most developed countries. Furthermore, an assessment of host gene polymorphisms and immune responses has developed in the clinic for the better assessment and monitoring of HIV-1 infections. To facilitate the linkage of testing and subsequent patient care and treatment and in order to prevent loss of follow-up, molecular testing that can be used at point-of-care (POC) locations has been under vigorous development and may find utility in both diagnosis and ART monitoring in the near future (Table 1). This review summarizes the development and the potential utility of all molecular assays currently used to diagnose and characterize HIV infections.

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Received 2 March 2012; revised 8 May 2012; accepted 21 May 2012

Table 1 Current molecular methods for laboratory diagnosis and characterization of HIV infections

Method	Applicability ^a	Turnaround time	Main devices (manufacturer)	Applications	Comments
DNA/RNA qualitative assays	B	1–2 days	APTIMA HIV-1 RNA Qualitative Assay or Procleix HIV-1/HCV Assay (Gen-Probe)	Blood donor screening and aid in acute or primary HIV infection diagnosis	For blood donor screening in pooled specimens; test of choice for HIV infections in newborns and infants
RNA viral load testing	A	1–2 days	COBAS AmpliPrep/TaqMan HIV-1 (Roche); VERSANT HIV-1 RNA (Siemens); NucliSens HIV-1 RNA QT (bioMérieux) RealTime m2000 HIV-1 (Abbott Molecular)	ART monitoring	To guide HAART initiation and monitor the treatment efficacy in conjunction with CD4 counting
Genotypic drug resistance testing	A	1–3 days	TruGene HIV-1 Genotyping (Siemens); ViroSeq HIV-1 Genotyping (Abbott Molecular); HIV PRT GeneChip assay (Affymetrix); HIV RT Line Probe Assay (Innogenetics)	Antiretroviral drug resistance determination	Indirect determination by detecting drug resistance-related HIV gene mutations; may miss low level mutations
Phenotypic drug resistance testing	B	3–6 weeks	AntiVirogram assay (VIRCO Lab; PhenoSense HIV, Trofile (MonoGram Biosciences); SensiTrop II HIV (Pathway Diagnostics)	Antiretroviral drug resistance determination	Direct determination by measuring HIV ability to grow in presence of drugs; time consuming and expensive
Molecular POC testing	C	1–4 h	IsoAmp HIV-1 Assay (BioHelix); ExaVir Load Assay (Cavidi); Liat HIV Quant Assay (IQuum); SAMBA system (Diagnostics for the Real World)	POC diagnosis and monitoring POC	Still in research and development; include HIV RNA detection and quantification
Host polymorphism testing	D	1–3 days	None	Determine host susceptibility to HIV infection	To determine HIV infection susceptibility and monitoring treatment efficacy and side effects
Host response and transcriptome testing	D	1–3 days	Human Genome U133 Array Strip (Affymetrix)	Assess disease progression and outcome; monitor ART	To determine HIV infection susceptibility and monitoring treatment efficacy and side effects

Abbreviation: ART, antiretroviral therapy; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; POC, point of care.

^a A, molecular assay is widely used for clinical diagnosis and/or monitoring of HIV-1 infections; B, molecular assay is useful under certain circumstances or for the diagnosis of particular populations; C, molecular assay is seldom useful for general diagnostic purposes but may be useful in specific researches and trials; D, molecular assay is not available or not used for laboratory diagnosis and monitoring.

HIV QUALITATIVE NUCLEIC ACID ASSAYS

The qualitative detection of HIV nucleic acids is used in three major areas: the identification of acute infection, assurance of blood safety, and in early infant diagnosis. A revised testing algorithm employing qualitative nucleic acid testing was recently proposed to address the shortcomings of western blot analysis to confirm HIV infection.⁷ The APTIMA HIV-1 RNA Qualitative Assay and the Procleix HIV-1/HCV assay (Gen-Probe, San Diego, CA, USA) are both Food and Drug Administration (FDA)-approved for blood-donor screening to exclude blood from donors with acute HIV infection.⁸ These two assays use transcription-mediated amplification technology.⁹ The persistence of maternal antibodies directed against HIV in exposed infants up to 18 months of age prevents the use of antibody-based assays for the early diagnosis of HIV infection. Infected infants have a high morbidity and mortality in the first 2 years of life; thus, it is important to promptly establish the infection status of the exposed infant in order to employ appropriate ART sufficiently early.^{10,11} Qualitative nucleic acid assays for the detection of HIV pro-viral DNA,¹² viral RNA^{13,14} and total nucleic acids^{13,15} have become the methods of choice for diagnosis in infants born to HIV-1-infected mothers.^{12,16}

HIV RNA VIRAL LOAD ASSAYS

HIV-1 infection results in lifelong viral persistence. In chronically infected patients, the HIV RNA viral load in the plasma together with CD4 T-cell numbers are the two routine laboratory markers used to guide ART initiation, monitor treatment effectiveness, determine

clinical progression^{17,18} and determine treatment regimens.¹⁹ An HIV RNA level below the detection limit is indicative of excellent drug intake adherence by patients and ART efficacy.¹⁷ HIV-1 viral load determination is typically performed with HIV RNA amplification by reverse transcription-polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA) or branched chain DNA tests.²⁰ Typically, viral load falls by at least 10-fold 1 month post-ART, and below the detection limit by 4–6 months post-ART, usually to less than 50–75 copies/ml.¹⁹

An ideal viral load assay should have a broad dynamic detection range and be able to quantitate HIV-1 Group M subtypes A–G and N and O with equal efficiency. While HIV-1 subtype B predominates in Western countries, non-B subtypes predominate in resource-poor parts of the world.²¹ The ability of a test to detect a broad range of these genetically diverse viruses with comparable sensitivity is therefore important to global HIV patient care. In the United States, five commercial plasma RNA assays from four companies are FDA-approved for HIV-1 monitoring in infected patients. Although these assays are not licensed for use in HIV screening or for confirmatory HIV tests, they have often been used in this context.^{13,20,22}

Plasma is the main specimen for HIV-1 viral load testing. Non-plasma specimens, such as peripheral blood mononuclear cells, saliva, cerebrospinal fluid, seminal plasma, dried plasma and dried blood spots (DBS), can also be readily tested.^{16,23,24} Plasma from whole blood collected by plasma preparation tubes should be separated from red blood cells within 6 h and transferred to a new vessel prior to freezing and transportation.^{25,26} Compared with HIV DNA, viral

RNA is relatively unstable, thus, it is recommended that plasma is quickly separated and stored in a $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$ freezer prior to testing. Recently, DBS specimens have drawn increased attention because of their usefulness in remote rural areas²⁷ where there is difficulty in plasma transportation and also concerns as to biosafety. HIV when present in dried blood is not considered infectious. When protected from RNA degradation in conditions of high humidity, DBS can serve as a viable approach for preservation and transportation. DBS-based viral load assays have been developed for several main diagnostic platforms including the Roche Cobas, Abbott *m2000* and bioMerieux NucliSens platforms.^{16,23}

The Cobas AmpliCor HIV-1 Monitor Assay (Roche Diagnostics, Indianapolis, IN, USA) is based on back-to-back RT and PCR.^{28,29} There are two basic assay platforms: (i) the AmpliCor HIV-1 Monitor assay, a manual test performed in 96-microwell plates; and (ii) the Cobas AmpliPrep/Cobas TaqMan HIV-1 assay with full automation of nucleic acid preparation and real-time RT-PCR amplification. The latter was the first FDA-approved HIV viral load test in 1996 with a low detection limit of 400 RNA copies/ml using 140 μl of plasma. AmpliCor Ultra-Sensitive test is a variation using 1 ml of plasma in order to improve the detection limit to 50 copies/ml. The new fully automated version of the COBAS AmpliPrep/COBAS TaqMan HIV-1 system (version 1.0) has a detection limit that ranges from 50 to 1 000 000 copies/ml.³⁰ The version 1.0 system targets the HIV-1 *gag* gene and can quantify all group M and N viruses and many circulating recombinant forms.^{20,30,31} The newer 2.0 version test targets the *gag* and conserved LTR regions of the viral genome and extends subtype coverage to Group O.^{20,32,33}

The branched chain DNA-based VERSANT HIV-1 RNA 3.0 Assay (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) incorporates a unique signal amplification technology and provides good reproducibility at the lower end of the detection range.^{20,28,31} This assay is also less affected by the presence of inhibitory substances and product carryover contamination problems associated other methods. The disadvantages of branched chain DNA technique include the requirement for larger plasma volumes.^{20,31}

The NucliSens HIV-1 RNA QT assay (bioMerieux, Inc., Durham, NC, USA) incorporates three key technologies: (i) silica-based nucleic acid extraction; (ii) NASBA; and (iii) electrochemiluminescence detection.²⁹ The NASBA technology is a sensitive and rapid amplification method that does not require a thermocycler and heat-stable enzymes. This assay can be used for measuring viral loads in other body fluids, because the RNA extraction procedure consistently generates RNA products that are free of interfering substances.^{28,30} The current second generation assay is reported to quantify subtypes A and G less reliably than other subtypes.^{20,31}

The real-time HIV-1 assay in the *m2000* system (Abbott Molecular, Des Plaines, IL, USA) consists of two components: the *m2000sp* component that allows automatic nucleic acid extraction and loading of sample and reagent, and the *m2000rt* component that performs nucleic acid amplification and detection^{30,31} using a single partially double-stranded linear DNA probe.³⁴ This assay offers a broad dynamic detection range and improved sequence variation tolerance. The manual operation is kept to a minimum and the amplification and detection steps are both carried out in a closed system in order to greatly reduce the risk of carryover contamination. The system can detect a wide coverage of HIV-1 including groups M, circulating recombinant forms, and groups N and O viruses.^{20,31}

Performance may vary significantly between HIV-1 viral load assay platforms depending on the HIV-1 subtypes present in the sample.³⁵

A Canadian study compared the RealTime HIV-1 *m2000rt*, COBAS TaqMan 48 v1.0, Easy Q v1.2 and Versant v3.0 assays for the determination of HIV-1 viral loads in a cohort of patients with diverse HIV subtype infections.²⁰ Discordant viral load results were obtained in 26.4% of samples due to missing values, false negatives, and a significant under-quantification, the extent of which varied according to HIV-1 subtype. False-negative results occurred for all platforms including 2.0% for *m2000rt*, 2.5% for COBAS, 6.0% for Easy Q and 2.0% for Versant. Easy Q and Versant had the highest rates of under-quantification by $\geq 1.0\text{ log}(10)$ copies/ml, mainly for HIV non-B subtypes.²⁰ HIV viral diversity in the population being tested must be considered in selecting the most appropriate detection platform and, once selected, the platform must be used consistently.³² The development of quantitative HIV-1 viral load tests with increasing sensitivity has recently been reviewed.³⁶ In contrast, the development of HIV-2 specific tests has not been accomplished. The small number of HIV-2 cases restricted to West Africa provides little incentive for commercial manufacturers to develop assays specific for HIV-2.

HIV-1 GENOTYPIC DRUG RESISTANCE TESTING

The ultimate goal of highly active antiretroviral therapy is to completely suppress viral replication, the emergence of drug resistant virus mutants and clinical progression.^{17,37} Antiretroviral drug resistance is defined as the ability of HIV-1 to multiply in the presence of antiretroviral drugs. In the clinical setting, if a viral load fails to fall adequately, or if it rebounds to 1000 copies/ml or higher, tests for drug resistance are recommended.³⁸ Two types of laboratory methods are available to determine drug resistance. Phenotypic drug resistance method directly measures viral replication capability in a recombinant viral form in the presence of antiretroviral drugs, whereas genotypic drug resistance methods detect viral genome mutations known to confer a decreased drug sensitivity. Both methods are commercially available. Phenotypic assay is much more expensive and is usually reserved for patients with prior viral resistance. The clinical utility of HIV drug resistance testing has been evaluated in a number of prospective randomized clinical trials.^{39–41}

Genotypic drug resistance testing has been recommended by the International AIDS Society-USA panel as an important tool to guide therapy changes, the overall direction of therapy, and more recently, the initiation of therapy.^{39,42,43} There are two currently FDA approved commercial drug resistance assays: (i) the TruGene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA); and (ii) the ViroSeq HIV-1 Genotyping System (Abbott Molecular). Both systems generate reverse transcriptase and protease gene sequences following RT-PCR amplification. One major difference between the two systems is the sequencing chemistry. ViroSeq uses a four-dye termination system, while the TrueGene uses a dye primer system. In addition, six sequencing reactions are needed to analyze one patient for ViroSeq, whereas 12 are needed for TruGene. The ViroSeq system requires an additional purification step to remove dye terminators.⁴⁴ While the TrueGene system is limited in throughput, it provides an interpretative report containing information as to the drug resistance mutations present in the sample.⁴⁵ Both systems work well for the HIV-1 B subtype circulating in North America. One recent study used the ViroSeq HIV-1 genotyping system to determine drug resistance on a panel of diverse HIV-1 group M isolates circulating in Cameroon.⁴⁶ The data and others performed on user-developed assays revealed that the performance of genotyping assays can be greatly influenced by the sequence variations of non-B HIV-1 strains that predominate in Africa.^{46,47}

The two above-mentioned assays are mostly useful for B-subtype viruses. They detect mutations in the reverse transcriptase and protease genes. They do not detect mutations associated with fusion, integrase and C-C chemokine receptor type 5 (CCR5) inhibitors. Furthermore, the proportion of drug resistance variants have to be at least 25% of the patient's total viral population in the patient.⁴² Mutant viruses existing at a level lower than 25% would escape detection. These viruses can lead to subsequent treatment failure.⁴⁸ Two other genotyping formats, the HIV PRT GeneChip assay (Affymetrix, Santa Clara, CA, USA) and the HIV-1 RT Line Probe Assay (Innogenetics, Ghent, Belgium), have shown to detect rapidly any drug resistance. Allele-specific PCR,^{49,50} single-genome sequencing^{48,49,51} and next generation sequencing (NGS)^{49,52,53} have also been reported to increase the detection sensitivity for minor mutants. However, due to the innate error rate of reaction enzymes, it has been reported that NGS had limited sensitivity to about 0.5% and therefore may have less utility among those treatment-experienced patients with a persistent viremia. The majority of low-frequency drug resistance-related mutations detected using NGS are most likely due to errors inherent in the NGS method or as a consequence of error-prone HIV-1 replication,⁵⁴ thus clinicians prescribing ART should interpret such findings with considerable caution.

HIV PHENOTYPIC ANTIRETROVIRAL SUSCEPTIBILITY TESTING

Phenotype assays measure the ability of HIV-1 to grow in the presence of various concentrations of antiretroviral agents. Phenotyping uses clinical cutoffs associated with the treatment outcome data and estimates the net effect of multiple mutations directly.⁵⁵ Phenotype assays are considered as molecular methods, since these assays require the generation of recombinant viruses using sophisticated molecular techniques. HIV-1 RNA from a patient's plasma is extracted and protease and reverse transcriptase genes are amplified by RT-PCR. The amplified gene fragments are then inserted into HIV-1 vectors in order to generate a replication competent recombinant. The replication of the new resultant HIV-1 recombinant is measured by a reporter gene system.^{56,57} Data from these assays are expressed as either 50% or 90% inhibitory concentrations (IC₅₀ or IC₉₀), values familiar to clinicians and thus easily understood. Due to the fact that the test is labor-intensive and technically complicated, the testing turn-around time can be as long as 6 weeks. As with genotype testing, phenotype assays can only detect mutant variants that comprise more than 25% of the viral population in any individual patient. Currently, two HIV-1 phenotyping assays are available: the AntiVirogram assay at VIRCO Lab (Bridgewater, NJ, USA)⁵⁶ and the PhenoSense HIV assay at MonoGram Biosciences (South San Francisco, CA, USA).⁵⁷ More data are needed to demonstrate the clinical advantage of phenotyping over genotyping.⁵⁸ A virtual phenotyping procedure has also been described that provides an estimation of the phenotype by averaging viruses with similar genotypes.⁵⁹

As integrase and entry/fusion inhibitors become available for ART, HIV-1 genotyping assays have been extended to cover drug resistance mutations related to these inhibitors.⁶⁰ Since inhibition by Enfuvirtide, an entry/fusion inhibitor, blocks HIV-1 entry to an immune cell, genotyping alone cannot provide sufficient information as to resistance. The PhenoSense Entry assay (MonoGram) has been developed to assess resistance to entry inhibitors.⁶¹ Maraviroc, a CCR5 inhibitor drug, is only effective against viruses that use CCR5 as a co-receptor for entry.^{62,63} Prior to Maraviroc administration, a tropism assay must be performed to determine whether the virus is

CCR5-tropic.^{64,65} Two commercial tropism assays are available at the MonoGram Biosciences and Pathway Diagnostics^{63,66} (Table 1).

POC TESTING

POC testing can be performed at home, in the workplace, at pharmacies and physician's offices, outpatient clinics, emergency rooms, and at the bedside of patients. The need of POC testing has been driven by the clinical need to make a rapid, evidence-based, decision as to the most appropriate therapeutic regimen at or near the site of patient care.⁶⁷ This form of diagnosis has also been available for several years for other infectious diseases such as group A *Streptococcus* detection in throat swab. Numerous products are commercially available for POC diagnosis of viral, bacterial and parasitic infections.⁶⁸ Considerable efforts have been spent in developing POC devices including HIV antibody, nucleic acid detection, CD4 T-cell quantitation and viral load for the use in resource-limited settings in order to facilitate patient identification and delivery of care.⁶⁹

Currently, there are no FDA-approved molecular assays that can be used in a POC setting. Some simple nucleic acid detection devices have the potential for HIV nucleic acid amplification and detection. A SAMBA system (Diagnostics for the Real World, Cambridge, UK) performs HIV detection through an isothermal nucleic acid amplification method in an integrated cartridge combined with a small bench-top instrument.⁷⁰ A helicase-dependent amplification was developed to separate DNA strands at 37 °C rather than the typical 95–97 °C used in PCR.^{71,72} This modification greatly simplifies the enzymology involved in the amplification process whilst retaining the advantage common to all isothermal amplification techniques. Another helicase-dependent amplification-based IsoAmp HIV-1 assay (BioHelix Corp., Beverly, MA, USA) has been developed using a small containment device. It targets the HIV-1 *gag* for amplification and uses an embedded vertical-flow DNA detection strip to detect amplicons.⁷³ Similar to the serologic rapid tests, this vertical-flow DNA detection strip has an internal control line to validate the proper performance of the reactions and has a test line to detect the amplicons. The preliminary limit of detection of the IsoAmp HIV assay is 50 copies of the HIV-1 Armored RNA (Assuragen, Austin, TX, USA) introduced into the IsoAmp HIV reaction.⁷⁴

Molecular diagnostic methods can be subdivided into the three steps nucleic acid extraction, amplification and detection components.⁷⁵ A rapid POC extraction of HIV-1 proviral DNA from whole blood and its detection using real-time PCR was recently reported.⁷⁶ Simple and inexpensive molecular assays based on dipstick and zipper technology have also been described.^{77,78} The Cepheid GeneXpert System (Sunnyvale, CA, USA) is a single-use sample processing cartridge system with an integrated multicolor real-time PCR capacity.⁷⁹ Microarrays have also been incorporated with nucleic acid probes and peptides to detect and quantify HIV-1.^{80,81} Miniaturized PCR devices have been reported for microbial agent detection and identification. Integration of microfluidics and lensless imaging for POC testing has also been reported.⁸² With the incorporation of micro/nano fabrications/crystals (e.g., quantum dots), microfluidics and array based systems, the development of more feasible immunological and molecular tests for HIV POC testing in resource-limited settings is expected.⁸³

There is a crucial need for low-cost, simple and accurate HIV-1 viral load monitoring technologies in resource-limited settings, particularly when first- and second-line treatment regimens are scaled up.^{84–86} The Liat HIV Quant Assay (IQuum, Marlborough, MA, USA) is comprised of two components, the Liat Analyzer and the Liat Tube. This method can yield automated sample-to-result in a near-patient

setting within 1.5 h. It can detect HIV-1 group M (subtypes A–H), group O, and HIV-2 with a detection linearity of 6 logs and a limit of detection of 57 copies/ml.⁸⁷ In contrast to the use of nucleic acid amplification, the ExaVir Load assay (Cavidi, Sweden) measures viral reverse transcriptase activity using simple equipment and a modified ELISA procedure. This method can be performed in a relatively simple laboratory environment. The measured viral reverse transcriptase activity correlates with plasma RNA levels.^{88–90} In addition to the simple ExaVir Load start-up equipment, only that ELISA equipment which commonly found in an HIV laboratory is required for performing the tests. The integrated equipment used in the current Version 3 makes the procedure less time-consuming, more efficient and even easier to handle. This viral reverse transcriptase measurement system has been extended to HIV phenotypic susceptibility testing. This new ExaVir drug assay has been reported to work well for efavirenz but not for nevirapine.⁹⁰

HOST GENOME POLYMORPHISM TESTING

Enhanced by the success of human genome programs, diagnostic virologists envisioned the utilization of genetics beyond HIV-1 genomes to help manage HIV infections.^{91,92} Chronic and persistent HIV-1 infections can be viewed as ‘horizontally acquired’ genetic diseases. An HIV pathogen and its host can be viewed and treated as an integrated biological system.⁹² There is ample evidence to indicate that the outcome of HIV infections is greatly influenced by the host’s genetic background of the host.^{92,93} Polymorphisms in AIDS restriction genes, e.g., CCR5, CCR2 and C-X-C chemokine receptor type 4, influence the risk of HIV infection and the rate of AIDS progression. Pine *et al.*⁹⁴ revealed that polymorphisms in Toll-like receptors 4 and 9 influenced the level of viral load in a sero-incident cohort of HIV-1-infected individuals. A recent study of high-risk South African black women indicated that the risk of acquiring HIV infection was 3-fold greater in those with the trait of Duffy-null-associated low neutrophil counts when compared with all other study participants.⁹⁵ Because of the high prevalence of this trait among persons of African ancestry, it may contribute to the dynamics of the HIV epidemic in Africa.⁹⁵

Hence, the detection of host polymorphisms in the HIV diagnostic field can help identify those who are at risk of rapid disease progression and thus the need to initiate early treatment. Allele frequencies and relative hazard values of CCR5-Δ32, CCR2 64I, CCR5 P1, interleukin (IL)-10 5’A, human leukocyte antigen (HLA)-B*35 and HLA homozygosity were determined to generate a composite relative hazard of progression to AIDS.⁹⁶ Possession of a chemokine (C-C motif) ligand 3-like 1 (CCL3L1) copy number lower than the population average is associated with markedly enhanced HIV/AIDS susceptibility; which is enhanced in individuals with the CCR5-Δ32 genotype.⁹⁷ Retaining CCR5-Δ32, CCR2 64I, CCR5 59029AA, CCL3 495TT, stromal cell-derived factor-1 (SDF1) 3’A, progressive multifocal leukoencephalopathy (PML) –225TT, peptidylprolyl isomerase A (PPIA) 1650G, and tumor susceptibility gene 101 (TSG101) –183C genetic variants may translate into lengthening or shortening the time from 500 CD4 T cells/μl to <200 CD4 T cells/μl by up to 2.8 years.⁹⁸ Genetic polymorphic profiles in cytochrome P450s and transporters facilitate optimal chemotherapy for HIV infections. An association between cytochrome P450 2D6 (*CYP2D6*) genetic variants and plasma levels of efavirenz and nevirapine in treatment-naïve individuals with HIV infection was observed. Patients carrying a loss-of-function *CYP2D6* allele had higher median plasma levels of both drugs compared to those without the mutation.⁹⁹ Polymorphisms in *CYP2B6* correlate with high efavirenz concentrations in the plasma and the central

nervous system.^{100,101} A recent study identified more than 300 genome-wide significant single-nucleotide polymorphisms within the major histocompatibility complex, and the data implied that HLA–viral peptide interaction was a major factor in the modulation of HIV infections.¹⁰²

Microarray has become a powerful technique to screen genes for multiple polymorphisms on hundreds of samples.⁸¹ With recent technological advances, it is now possible to genotype over one million polymorphisms at high throughput by using Illumina or Affymetrix system.^{103,104} While increasing HIV resistance and disease progression-related host gene polymorphisms, simple and user-friendly techniques for the detection of such known mutations will soon be adapted and correctly interpreted by those responsible for a diagnosis. Currently available techniques include allele-specific nucleotide amplification,^{105,106} single nucleotide primer extension,¹⁰⁷ and oligonucleotide ligation assay.^{108,109} Real-time PCR assays based on TaqMan hydrolysis probes play an important role as a pathogen sequence-specific probe to confirm the authenticity of the detection target. These methods are very robust but less cost-effective for large-scaled studies.^{110,111} DNA sequencing remains the gold standard and is enhanced by recent high-throughput processing and deep production scaling.¹¹² It is now considered the most powerful procedure for polymorphism detection.

HOST RESPONSE AND TRANSCRIPTOME ANALYSIS

In addition to measuring the levels of CD4 T cells, other host responses can be used for monitoring therapy efficacies and side effects in HIV-infected patients receiving ART. T-cell-receptor-chain rearrangement excision circles (TREC) are episomal double-stranded DNA circles, which are generated during T-cell maturation in the thymus.¹¹³ TREC are stable and persist in newly matured T cells but are replication-deficient. After the entry of TREC-bearing T cells into the peripheral blood circulation, with time TREC molecules are progressively diluted out during T-cell mitosis. Quantification of TREC present in naïve T cells is thus a measure of thymic function.¹¹⁴ Although thymic function declines with age, substantial output is still maintained into late adulthood. HIV infection leads to a decrease in thymic function that can be measured in the peripheral blood and lymphoid tissues. In most treated adults, there is a rapid and sustained increase in thymic output, indicating that the adult thymus can contribute to immune reconstitution following ART.^{114,115} Thus, in addition to CD4 and viral load, TREC has been considered as another biomarker to monitor treatment efficacy, as well as to evaluate clinical disease progression.^{115,116}

The mitochondrial toxicity of antiretroviral drugs, particularly that of the nucleoside reverse transcriptase inhibitor, has been postulated to be responsible for the pathogenesis of many secondary treatment side effects, including hyperlactatemia.^{117,118} During ART, clinically symptomatic mitochondrial dysfunction has been associated with mitochondrial DNA depletion. A real-time PCR was developed to determine a mitochondrial DNA versus nuclear DNA ratio as a biomarker of nucleoside reverse transcriptase inhibitor toxicity.¹¹⁹ The observed increase in mitochondrial DNA and RNA contents during the first year of treatment may represent a restorative trend resulting from suppression of HIV-1 infection, independent of the treatment used. The mitochondrial DNA and RNA contents in individual cell subtypes, rather than in peripheral leukocytes, may be better markers of toxicity and worthy of further investigation.¹²⁰ Other assays, which include mitochondrial RNA quantification by real-time PCR¹²¹ and mitochondrial protein synthesis by western blot immunoanalysis,¹²² have been described to measure mitochondrial toxicity-related

functional changes. Recently, a flow cytometric assay was developed to gauge mitochondrial function. Flow cytometry of a mitochondrial DNA-encoded mitochondrial protein together with a nuclear DNA-encoded mitochondrial protein was optimized and validated, allowing for the simultaneous detection of mitochondrial DNA and nuclear DNA encoded proteins at the single-cell level; this approach offers a method whereby mitochondrial function can be monitored.¹²³

New microarray techniques including those from Affymetrix (Santa Clara, CA, USA), have recently allowed host transcriptome analyses in individuals infected with HIV-1.^{81,124} A comprehensive review of the 34 studies involving HIV-1 and microarrays from 2000 to 2006 concluded that these studies yielded important data on HIV-1-mediated effects on gene expression and provided new insights into the intricate interactions occurring during infection.¹²⁵ Several recent studies have demonstrated progress in expanding the pool of target genes and understanding the functional correlates of gene modulation to HIV-1 pathogenesis *in vivo*.^{91,126} It is predicted that such host transcriptome profiles will be used for the assessment of disease progression and prognosis. The precision of transcriptome analyses will be greatly improved through the added resolution of the RNA-Seq approach using deep-sequencing technologies for transcriptome profiling.^{127,128}

CONCLUDING REMARKS

The development and application of molecular techniques has initiated a revolution in the diagnosis and monitoring of many infectious diseases. Molecular techniques have quickly become the mainstay for laboratory diagnosis and assessment of HIV-1 infections. Qualitative molecular assays have been used as the test of choice to diagnose perinatal and acute HIV-1 infections. HIV-1 plasma viral load assays are routinely used in combination with CD4 T-cell counts in order to determine when to initiate therapy and when a treatment regimen is failing. HIV-1 antiretroviral susceptibility tests, which include a genotyping assay to detect mutations known to confer resistance together with phenotyping measuring recombinant viral replication in the presence of antiretroviral drugs, have both become routine in the management of HIV patients undergoing antiretroviral treatment. POC or near-the-patient molecular assays are being developed with the potential to make rapid, evidence-based therapeutic intervention at or near the site of patient care. The initiation of alternative tools for improved diagnosis and the monitoring of HIV infections have led to a better understanding of the association between host gene polymorphism and viral immune responses.

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